Worldwide Research Efforts in the Fighting Against Microbial Pathogens

From Basic Research to Technological Developments

This book aims to disseminate recent findings in the fight against microbial pathogens which were presented at the second edition of the ICAR Conference Series (ICAR2012) on Antimicrobial Research, held in Lisbon, Portugal, November 2012, which attracted about 425 scientists from 55 countries. This forum was the natural continuation of this new series of conferences: the first edition, held in Valladolid, Spain in 2010, gathered more than 500 researchers from nearly 60 countries. ICAR aims at establishing itself as a key forum in Europe for the presentation, exchange, and dissemination of information and experiences on anti-microbe strategies. "Anti" is here taken in the broadest sense as "against cell cycle, adhesion, or communication," when harmful for the human health, industry or economy (e.g. infectious diseases, chemotherapy, food, biomedicine, agriculture, livestock, biotechnology, water systems). Topics on antimicrobial natural products, antimicrobial resistance, antimicrobial surfaces, as well as methods and techniques, are included.

This volume is a compilation of chapters written by active researchers that will provide readers with an up-to-date information about the current knowledge on antimicrobials in a worldwide context marked by the threat posed by the increasing antimicrobial resistance of microbial pathogens.

ISBN 978-1-61233-636-7
53295
9 781612 336367

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BrownWalker Press
Boca Raton

Worldwide Research Efforts in the Fighting Against Microbial Pathogens: From Basic Research to Technological Developments

BrownWalker Press
Boca Raton, Florida • USA
2013

ISBN-10: 1-61233-636-1
ISBN-13: 978-1-61233-636-7

www.brownwalker.com

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INTRODUCTION

This book contains a selection of papers presented at the II International Conference on Antimicrobial Research (ICAR2012), which was held at the University of Lisbon-Portugal (Faculty of Dentistry and ISCTE-Lisbon University Institute) during November 21st-23rd 2012, and which attended by more than 425 researchers from around 60 countries (http://www.formatex.org/icar2012)

The conference provided a forum for the presentation, exchange and dissemination of information and experiences on the following topics:

Antimicrobial chemistry (experimental and computational). Analytical detection of antibiotics in complex samples.
Synthesis and screening of novel chemical compounds for antimicrobial action. Natural, synthetic and semi-synthetic antibiotics. Analogs. Structural determination. *In-silico/ab-initio/de-novo* antimicrobials discovery. New targets for antimicrobials. Rational design of antimicrobials. Bioinformatics and comparative genomics for the identification of antimicrobial targets…

Antimicrobial natural products.
Antimicrobial substances from terrestrial and marine organisms. Antimicrobial peptides. Antimicrobial enzymes. Essential oils. Bioactive phytochemicals. Plant/Herbal extracts. Purification. Structural determination…

Antimicrobials mechanisms of action.
Methods and Techniques.

Antimicrobial resistance. Superbugs. Multi-resistant strains. Emerging and re-emerging pathogens.
Microbial resistance to antibiotics and biocides. Molecular mechanisms. Resistance genes. Prevention of resistance. Surveillance & statistics. Genetics and Proteomics. Emerging and re-emerging bacteria and fungi in humans, animals, and plants. Methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin Intermediate/Resistant *Staphylococcus aureus* (VISA/VRSA), *Clostridium difficile*, *Mycobacterium tuberculosis*, Vancomycin-resistant *enterococcus* (VRE), *Cryptosporidium*, *Plasmodium parasite*, *Plasmodium falciparum*, *Leishmania* species, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, *Cryptococcus*, *Escherichia coli* O157:H7, *Helicobacter* spp., *Enterobacter sakazakii*, *Serratia* spp., Fluoroquinolone-Resistant *Pseudomonas aeruginosa* (FQRP)...

Antimicrobial microbes.
Microbial-derived toxins. Bacteriocins (colicins, microcins, lantibiotics…). Archaeocins. Biocontrol approach to microbial invasions (probiotics, lactic acid bacteria…). Biosynthesis of antibiotics. Genetic and metabolic engineering. Gene regulation…

Antimicrobial viruses.
Bacteriophages. Phage therapy and biocontrol in humans, animals (agriculture-farm animals, aquaculture), plants, food industry… Materials functionalization with bacteriophages. Using bacteriophages for microbiological detection…

Antimicrobial materials science and surface chemistry. Biofilms.
Antimicrobial, anti-adhesive surfaces & coatings. Microbial adhesion to surfaces. Biofouling. Biofilm formation, control and eradication. Novel characterization techniques. Physical and chemical (inorganic (e.g. silver, copper compounds) and organic) surface modification. Cationic surfaces. Functionalization strategies for polymers, metals, metal oxides, ceramics. Drug-eluting concepts. Biofilms susceptibility to antimicrobials. Antibiotic resistance of microorganisms in biofilms. Genomics and Proteomics...

Antimicrobials in consumer products.
Textiles (hygienic clothing, activewear, medical textiles…), paper industry, active packaging (food industry…), public buildings (hospitals, schools, restaurants, day care centers, nursing homes…). Safety and toxicological aspects…

Antimicrobial physics.
Exploitation of physical properties for killing/inactivating microbes: surface tension (nano-emulsions),

radiation, ultrasounds, temperature, specific properties of nano-materials (nano-particles, nano-tubes/wires, nano-crystals, nano-grained materials…). Resistance to physical agents…

Non-antibiotic biocides. Hygiene and Sterilizing.
Disinfectants, antiseptics, preservatives… Mechanism of action. Resistance to non-antibiotic biocides. Combination of physical and chemical treatments. Hygiene and Sterilizing. Sanitizers. Regulatory issues. Good practices…

Techniques and Methods.
Susceptibility Testing. Rapid microbial and resistance detection. Detection of antibiotics in environmental samples. Microscopy, microanalysis & spectroscopy, single-cell studies, high-throughout studies, nanomechanical studies, microfluidics, lab-on-a-chip concepts, miniaturized science, analysis of microbial surfaces, heterogeneity, statistics. Interaction of antimicrobial drugs with model membranes. Analytical techniques…

The Intelligent war.
Interfering microbe-microbe communication (quorum sensing) as antimicrobial strategy.

Strengthening of innate immune system as antimicrobial strategy.
Immunotherapy, immunomodulating agents, cytokines (interleukins, colony-stimulating factors, interferons…), hormones… Novel vaccines for preventing or treating disease…

Antimicrobials evaluation. Pre-clinical and clinical trials.

Public awareness, learning & teaching, influence on policy-makers. Regional regulatory frameworks and experiences on antimicrobials.

The following researchers accepted to be part of the Scientific Advisory Committee of the conference. Their work was essential to fulfill the requirements of quality, relevance and interest planned for the scientific program of the conference. Our sincere gratitude for their work:

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We would like to thank the company TITK for its support as sponsor at this conference.

This book serves as formal proceedings of the meeting (in addition to the abstracts book that every attendant received at the conference). We hope readers will find this set of papers inspiring and stimulating in their current research work and look forward to seeing another fruitful edition in 2014.

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Worldwide Research Efforts in the Fighting against Microbial Pathogens: From Basic Research to Technological Developments

Action of chitosan, nisin and sodium lactate on the inhibition and cell membrane damage of *Listeria innocua* and *Shewanella putrefaciens*

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The effect of chitosan, nisin, sodium lactate and binary combinations of chitosan with nisin or sodium lactate on the growth of *Listeria innocua* and *Shewanella putrefaciens* was study. Their mode of action through the evaluation of cell surface damage was also investigated. Minimal inhibitory concentrations, antimicrobial interactions, cell constituents´ release and cell hydrophobicity were determinated. Although studied antimicrobials were able to inhibit the growth of *L. innocua* and *S. putrefaciens*, their mode of action may be different according to the structural characteristics of the microorganisms. *L. innocua,* was sensitive to nisin, and the action of the mentioned antimicrobial on the cell membrane was evident when the cell constituents' release was evaluated. Regarding to chitosan, it also seems to act on the cell surface, but in a smaller magnitude than nisin, since cell constituents' were not release but hydrophobicity was increase. Sodium lactate would pass the cell membrane and acts in the cytoplasm. When it comes to *S. putrefaciens*, nisin and sodium lactate would be able to disrupt the outer membrane, while chitosan may cause the inhibition through other mechanisms, or higher concentrations would be necessary to detect its action.

Keywords antimicrobials; cell damage

1. Introduction

Chitosan, nisin and sodium lactate show different properties which make them useful in food preservation. All of them are obtained from natural sources, and are generally recognized as safe compounds. Chitosan is a biopolymer obtained from the deacetylation of chitin. It has numerous properties, but, its antimicrobial action and its ability to form edible films are the most outstanding when it comes to the extension of food shelf life [1]. Nisin effectively inhibits Gram-positive bacteria [2]. Its antilisterial effect has been widely reported [3]. Sodium lactate has shown to exert antibacterial effects against many spoilage and pathogenic microorganisms [4]. It is used to extend the shelf life of many products, including fish [4].

Listeria monocytogenes and *Shewanella putrefaciens* are usually isolated from fishery products. *L. monocytogenes* is a Gram positive bacterium which represents one of the most important foodborne pathogens, since once it enters to an animal or human host, it can cause severe problems [5]. It is able to survive a wide spectrum of adverse conditions, such as acidic pH, low temperatures, and high concentrations of sodium chloride [6]. As a result, the growth of this microorganism is difficult to control in foods. *S. putrefaciens* is a Gram negative microorganisms commonly associated with fish spoilage, especially when it comes to marine cold or temperate water fish, stored under aerobic and iced conditions [7]. *S. putrefaciens* acts on the fish muscle and produces trimethylamine, hydrogen sulphide, hypoxanthine and other metabolites that produce intensive off-odours and cause the rejection of the product [7].

Use of mentioned antimicrobials in fishery products may reduce the occurrence of foodborne disease and extend their shelf life [8]. Moreover, the knowledge of the effect of chitosan, nisin and sodium lactate against bacteria and their mode of action would optimize their use.

Base on the topics commented, the aims of this study were to evaluate the inhibitory effect of chitosan, nisin, sodium lactate, and the binary combinations of chitosan with nisin or sodium lactate, on the growth of *L. innocua* and *S. putrefaciens*. In addition, antimicrobials mode of action was investigated through the evaluation of cell surface damage.

2. Materials and methods

2.1 Bacterial strains and antimicrobial agents

In order to emulate *L. monocytogenes* development, *L. innocua* was used in this study, because of its similar response to stress factors [9]. *L. innocua* 6a ATCC 33090 and *S. putrefaciens* ATCC 8071 were stored at -30ºC

in Mueller Hinton broth (Biokar Diagnostics, Beauvais, France) plus 10%w/w glycerol (Sintorgan S.A., Buenos Aires, Argentina) and 10%w/w skim milk. Before the use, they were grown twice in Mueller Hinton and Tryptone Soy broth (Biokar Diagnostics, Beauvais, France), respectively, at 30ºC during 18 hours.

Chitosan, (Sigma, U.S.A.) with a deacetylated degree of 85%, was dispersed in 1.0%w/w acetic acid solution (Anedra S.A., Buenos Aires, Argentina) and pH was adjusted to 5.5 using 0.4M NaOH. Solution of sodium lactate (55.3%w/w) (Parafarm, Buenos Aires, Argentina) was adjusted to pH 5.5 using 10%w/w citric acid (Parafarm, Buenos Aires, Argentina). Nisin was added in the form of Nisaplin (Danisco A/S DK, Denmark), it contains 10^6 IU/g. Nisaplin was dissolved in distilled water acidified to pH 2.0 with HCl plus 0.75%w/w NaCl. All solutions were autoclaved at 121ºC for 15 minutes. Before using the solution of nisin, its pH was adjusted to 5.5 using sterilized 0.4M NaOH.

2.2 Determination of minimum inhibitory concentrations and antimicrobial interactions

The minimum inhibitory concentration (MIC) was determined by a microdilution method in 96 well-round bottomed sterilized microtitre plates (Kartell S.p.a., Italy). Serial dilutions of each antimicrobial were prepared in Mueller Hinton broth at pH 5.5. Portions of 90 µl of the diluted antimicrobials were pipetted into the wells of the microtitre plates, together with 10 µl of a 10^6 CFU/ml culture of each microorganism, once a time. The ranges of concentrations tested were from 7.0 to 2000 ppm for chitosan, from 16.0 to 4300 IU/g for nisin, and from 281 to 22500 ppm for sodium lactate. The microtitre plates were incubated at 30ºC for 24 hours. The visual detection of turbidity in the wells, as compared with the negative and positive controls, was considered as the absence of inhibition. Negative and positive controls were tested in parallel, being the former no inoculated Mueller Hinton broth, and the latter inoculated Mueller Hinton broth free of antimicrobials. The MIC was defined as the highest dilution showing inhibition after 24 hours of incubation according to the NCCLS (1999) [10] recommendations.

To rule out the effect of acetic acid (used to dissolve chitosan) on the development of the studied microorganisms, a similar trial was conducted. For this purpose, serial dilutions of acetic acid, from 1.00 to 0.25% (w/w) were prepared in Mueller Hinton broth, and pH was adjusted to 5.5 using sterilized 0.4 M NaOH.

In order to evaluate antimicrobial interactions, serial dilutions of two antimicrobials were mixed in a microtitre plate so that each row or column contained a fixed amount of the first antimicrobial and increasing amounts of the second one. Each plate also contained a row and a column in which each antimicrobial was present alone [11]. The microtitre plates were inoculated with 10 µl of a 10^6 CFU/ml culture of each microorganism.

The MIC of each antimicrobial, alone and in combination with the others was used to calculate the fractional inhibitory concentrations (FIC). They are defined as the relationship of the MIC of an antimicrobial when combined (MIC_{A-B} or MIC_{B-A}) divided by the MIC of this antimicrobial when used alone (MIC_A or MIC_B). Fractional inhibitory concentrations of each pair of antimicrobials were added to obtain the FIC index: $FIC_I = (MIC_{A-B} / MIC_A) + (MIC_{B-A} / MIC_B)$. Considering the FIC index value, the type of interaction between the antimicrobials can be determined. A FIC index value near to 1 indicates an additive effect; if less than 1 it indicates synergism; and if greater than 1, the interaction is antagonic [12]. All the experiments were made in duplicate and replicated at least twice.

2.3 Cell constituents´ release

L. innocua and *S. putrefaciens* were grown for 18 hours at 30ºC in Mueller Hinton and Tryptone Soy broth, respectively. After incubation populations were higher than 10^9 CFU/ml. Aliquots of bacterial suspensions were centrifuged (10000 rpm, 15 minutes) in order to separate the growth medium. Microorganisms were washed twice and resuspended in Ringer´s solution (Biokar Diagnostics, Beauvais, France) at pH 5.5. Antimicrobials were added, alone or in the binary combinations (chitosan-nisin and chitosan-sodium lactate). The concentrations of each antimicrobial used were equivalent to twice the minimal inhibitory concentration determinated in the previous item (chitosan: 200 ppm; nisin: 2000 IU/g; sodium lactate: 36000 ppm). Systems were incubated at 30ºC for 24 hours under agitation. Samples were withdrawn at 0, 3 and 24 hours. Cells were pelleted twice at 10000 rpm for 15 minutes. Absorbance of cell-free supernatant was measured at 260 nm. Ringer´s solutions with the respective antimicrobials concentrations were taken as blanks.

2.4 Cell surface hydrophobicity

Cell surface hydrophobicity was determined using the bacterial adhesion to hydrocarbon (BATH) test [13]. *L. innocua* and *S. putrefaciens* were grown for 18 hours at 30ºC in Mueller Hinton and Tryptone Soy broth, respectively. They were washed twice and resuspended in Ringer´s solution at pH 5.5. Systems were prepared using the concentrations mentioned in the previous item. Six tubes containing four ml of each system with 10^8

CFU/ml of *L. innocua* or *S. putrefaciens* were prepared for this test. All of them were incubated at 30ºC for 3 hours. One ml of xylene was added to three tubes of each system (A_b). The tubes without xylene were used as the bacterial control (A_c). After 10 minutes of incubation, tubes were vortexed for 30 seconds, and equilibrated at 30ºC for 20 minutes. Two ml of aqueous phase was removed and transferred to an empty tube. The optical density at 600 nm was determined using a spectrophotometer, which was zeroed using Ringer´s solution. The absorbance of the bacteria assay tubes (A_b) and the absorbance of the bacteria control (A_c) were used to calculate the adhesion to hydrocarbon (%) = $(A_c - A_b)/A_c$ x 100.

2.5 Experimental Design and Data Analysis

Data obtained from the study of cell constituents´ release were analyzed by two-way repeated measures ANOVA, while data obtained from BATH test were analyzed by one-way ANOVA. Both of them were followed by Tukey's multiple comparison test.

Experiments were conducted by triplicate. Data were processed using the statistical program Statgraphics (Statgraphics Plus for Windows, version 5.1, 2001, Manugistics, Inc., Rockville, Maryland, USA). The significance level was 0.05%.

3. Results and discussion

3.1 Minimum inhibitory concentrations and antimicrobial interactions

Antimicrobials tested were able to inhibit the growth of both microorganisms. Acetic acid at the level used to dissolve chitosan did not influence the development of microorganisms demonstrating that the inhibition of the growth observed in the presence of chitosan can be attributed to its addition.

Table 1 shows MIC values. The highest MIC values were found for sodium lactate. Lactates inhibit microbial growth by depressing a_w and by the action of their undissociated form [14]. Probably, in the studied systems a combination of both mechanisms would be present. As expected, nisin inhibited the growth of *L. innocua*. It is well known the ability of nisin for inhibiting Gram positive bacteria, especially *Listeria* [15]. Although it is widely reported that nisin is not effective against Gram-negative bacteria, it is able to inhibit their growth if the outer membrane is disrupted [15]. The presence of citric acid used to adjust pH may have caused this effect, and allowed nisin to act against *S. putrefaciens*. The smallest MIC values corresponded to chitosan. The range of chitosan MIC obtained was in accordance with the MIC values previously reported [16].

According to FIC index, which are shown in Table 1, chitosan and nisin exerted and additive effect against *L. innocua* and a synergistic one against *S. putrefaciens*. Regarding the effect on the latter one, chitosan acted as chelating agent which causes the disruption of the outer membrane cooperating with the action of nisin. The mixture of chitosan and sodium lactate showed to be synergistic against both microorganisms. At pH 5.5, chitosan is a polycationic compound and can interact with the anionic components of the microbial surface [1], which may facilitate the action of sodium lactate. No interaction was found between nisin and sodium lactate.

Table 1 Minimum inhibitory concentrations (MIC) and interactions between antimicrobials.

Microorganism	**MIC**			**Interactions**		
	Chitosan (ppm)	Nisin (IU g^{-1})	Sodium lactate (ppm)	Chitosan Nisin	Chitosan Sodium lactate	Nisin Sodium lactate
L. innocua	96	1183	18000	A (1.05)	S (0.80)	NI
S. putrefaciens	125	1075	19600	S (0.47)	S (0.56)	NI

S: synergistic interaction; A: additive interaction; NI: no interaction. Numbers between brackets are FIC indexes.

3.2 Cell constituents´ release

In order to dilucidate the effect of chitosan, nisin, sodium lactate and mixtures of chitosan with nisin or sodium lactate on the cell membrane of *L. innocua* and *S. putrefaciens*, the study of cell constituents´ release and cell surface hydrophobicity was carried out.

When it comes to cell constituents' release, a high absorbance value implies an increase of intracellular constituents' loss as a result of the action of antimicrobials on cell membrane. At the beginning, no differences

in absorbance values between treatments were found. Throughout incubation, the treatments with nisin, alone or in combination with chitosan, showed a significant increase of the cell constituents´ release, as it is shown in Fig. 1. In the case of *L. innocua* the highest increase was found in presence of nisin and chitosan, while there was no significant difference between the effects of nisin alone or in combination with chitosan in the case of *S. putrefaciens*. Nisin forms pores and destroys the membrane integrity leading to the leakage of intracellular material [17]. As it was mentioned previously, nisin is able to act against Gram-negative bacteria when the outer membrane is disrupted, which is the case of *S. putrefaciens* at pH 5.5 or in the presence of chitosan.

It have been reported that interactions between chitosan molecules and microbial cell membranes produce a change in permeability leading to the leakage of proteinaceous and other intracellular constituents [1]. However, this effect was not observed since no differences were found between the absorbance of the control samples and those where chitosan have been added (data not shown). It is possible that the concentration studied was not enough to cause damage in *L. innocua* and *S. putrefaciens* cell membrane. It have been reported that 250 ppm of chitosan caused no release of lipids from the outer membrane of Gram negative bacteria [18].

Regarding to *L. innocua*, treatments with sodium lactate, alone or in combination with chitosan, did not exerted any effect on cell constituents´ release (data not shown), which is in concordance with the mechanisms of inhibition previously mentioned. On the other hand, an increase in the cellular constituents´ release was observed when *S. putrefaciens* was treated with sodium lactate alone, as it can be seen in Fig. 1. This fact may be explained by the disruption of the outer membrane caused by lactate.

No increase in absorbance values were observed when sodium lactate was used in combination with chitosan (data not shown). The latter trend would be linked with the fact that Gram negative cell treated with chitosan showed an additional layer of material and a thickened cell envelope [18]. Thickening of cell envelope by chitosan may cause masking of results when it comes to the quantification of the loss of intracellular material.

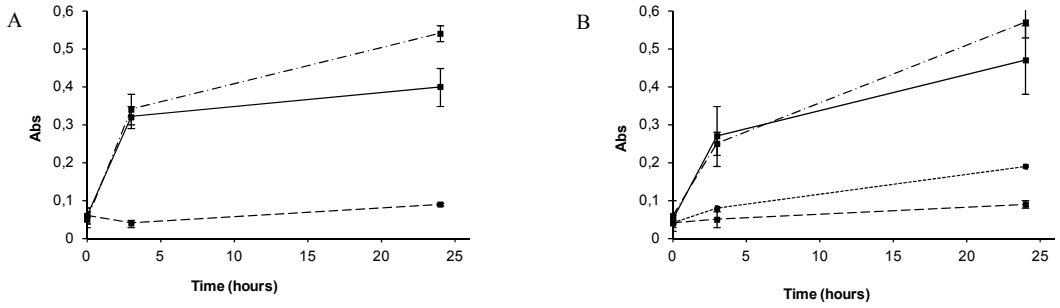

Fig. 1 Cell constituents´ release. Panel A: *L. innocua*. Panel B: *S. putrefaciens*. --- control; … sodium lactate; __ nisin; .-.- chitosan and nisin. Treatments which are not shown were not significantly different from control samples.

3.3 Cell surface hydrophobicity

Although BATH test was carried out with all antimicrobials and mixtures studied, those systems containing nisin were discarded, since it was not possible to separate the phase due to the emulsifier action of nisin.

Bacterial cell surface hydrophobicity is determined by its composition [19]. A change on cell hydrophobicity implies that the structure has been altered. When bacteria were incubated in presence of sodium lactate, hydrophobicity values were approximately 40%, being similar to those values found in control samples (incubated in Ringer´s solution). However, significant increases were observed when sodium lactate was used together with chitosan. *L. innocua* showed a hydrophobicity value of 74%, while it was 60% for *S. putrefaciens*. When it comes to the effect of chitosan, two different trends were obtained. In the case of *L. innocua* a similar increase to the one produced by the mixture was found. On the other hand, chitosan did not cause any increase in *S. putrefaciens* hydrophobicity when sodium lactate was absent. This trend could be explained by the fact that Gram positive bacteria are more sensitive to chitosan than Gram negative ones [20].

4. Conclusion

Data obtained suggest that although studied antimicrobials were able to inhibit the growth of *L. innocua* and *S. putrefaciens*, their mode of action may be different according to the structural characteristics of the microorganisms. *L. innocua,* was sensitive to nisin, and the action of the mentioned antimicrobial in the cell membrane was evident when the cell constituents' release was evaluated. Regarding to chitosan, it also seems to act on the cell surface, but in a smaller magnitude than nisin, since cell constituents' release was not observed but hydrophobicity was increase. Sodium lactate would pass the cell membrane and act in the cytoplasm. When

it comes to *S. putrefaciens*, nisin and sodium lactate would be able to disrupt the outer membrane, while chitosan may cause the inhibition through other mechanisms, or higher concentrations would be necessary to detect its action on the outer membrane.

Acknowledgements The support of Universidad de Buenos Aires and Consejo Nacional de Investigaciones Científicas y Técnicas of Argentina is gratefully acknowledged.

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Activity of allylisothiocyanate and 2-phenylethylisothiocyanate on motility and biofilm prevention of pathogenic bacteria

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Isothiocyanates (ITCs) are plant secondary metabolites with a range of biological effects including antimicrobial activity. This study reports the activity of two ITCs [allylisothiocyanate (AITC) and 2-phenylethylisothiocyanate (PEITC)] on bacterial motility and prevention of biofilm formation by *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Listeria monocytogenes*. AITC caused total inhibition of swimming (*P. aeruginosa*) and swarming (*E. coli*, *P. aeruginosa*) motilities. PEITC caused total inhibition of swimming (*E. coli*, *P. aeruginosa* and *L. monocytogenes*) and swarming (*E. coli* and *P. aeruginosa*) motilities. Colony spreading of *S. aureus* was completely inhibited with PEITC. Total biofilm prevention was observed for *E. coli* with AITC. AITC and PEITC had no preventive effects in biofilm formation by *S. aureus* and *L. monocytogenes*, respectively. Significant preventive action with AITC on biofilm formation by *P. aeruginosa* (90%) and by *L. monocytogenes* (61%), and with PEITC on biofilm formation by *S. aureus* (75%) was verified. In terms of viability, AITC and PEITC promoted reductions higher than 87% for all the biofilms tested. In conclusion, these molecules demonstrated potential to inhibit bacterial motility and to prevent biofilm formation of pathogenic bacteria.

Keywords isothiocyanates; pathogenic bacteria; motility; biofilm prevention

1. Introduction

Biofilms comprise sessile microbial communities surrounded by a matrix of extracellular polymeric substances (EPS). This phenotype represents the prevalent mode of microbial life in nature, industrial process and infections [1]. Bacteria in biofilms can cause serious problems in biomedical systems [2, 3]. This attached mode of growth protects the bacteria from environmental stresses [4]. One serious problem is the faster establishment of resistance to antimicrobial agents than in planktonic state [5]. The best strategy to control or eradicate biofilms is to prevent their development [6]. In general, the transition from free-living cells to a sessile form of life begins with the transportation and attachment of microorganisms to a particular substratum. It has been shown that cell surface motility structures, such as pili, fimbrae, flagella and curli play an important role in the early attachment processes. These are structural components that serve as sensory systems for dislocation of bacteria and adhesion to a particular substrate, i.e. for the initial biofilm formation [7]. Therefore, the inhibition of bacterial motility can represent an interesting approach to prevent biofilm formation.

The emergence of resistant bacteria to conventional antimicrobials clearly shows that new biofilm control strategies are required [8]. Natural antibacterial compounds which restrict the ability of bacteria to adhere, communicate, and form biofilm complexes can represent a source of lead biofilm control molecules [9]. In this context, glucosinolates and their hydrolysis products, particularly isothiocyanates (ITCs), a group of plant secondary metabolites belonging to the *Brassicaceae* family (i.e. cabbage, broccoli, mustard, horseradish and wasabi) have long been recognized for their antimicrobial activity against clinical important microorganisms (e.g. *E. coli*, *C. albicans*, *B. subtilis*, *C. jejuni*, *H. pylori* and *V. parahaemolyticus*) [10, 11]. In addition, these compounds have other benefits for human nutrition, such as anticarcinogenic and antioxidant properties [12, 13]. In this work the activity of two selected ITCs (allylisothiocyanate and 2-phenylethylisotiocyanates) was evaluated on the prevention of biofilm formation by selected pathogenic bacteria. The assessment of ITCs on the inhibition of bacterial motility was also performed.

2. Material and methods

2.1 Bacteria and culture conditions

Escherichia coli CECT 434, *Pseudomonas aeruginosa* ATCC 10145, *Staphylococcus aureus* CECT 976 and *Listeria monocytogenes* ATCC 15313 were used in this study.

2.2 Phytochemicals

A stock solution of allylisothiocyanate (AITC) and 2-phenylethylisothiocyanate (PEITC) (Sigma-Aldrich,) at 10000 μg/mL was prepared in dimethyl sulfoxide (DMSO, Sigma) and was stored at -20 ºC until use. Phytochemicals are routinely classified as antimicrobials on the basis of susceptibility tests that produce inhibitory concentrations in the range of 100 to 1000 μg/mL [14]. In this work, each product was tested at a concentration of 1000 μg/mL. Negative controls were performed with DMSO.

2.3 Motility assays

Overnight cultures grown on Luria-Bertani broth (LBB) (Merck, Germany) were applied (15 μL of a suspension with 1×10^8 cells/mL) in the center of plates containing 1% tryptone, 0.25% NaCl, and 0.3%, 0.7% or 1.5% (w/v) agar for swimming/colony spreading, swarming and twitching motilities, respectively [15, 16]. Colony spreading was assessed for *S. aureus* and twitching motility was only assessed for *P. aeruginosa*. AITC and PEITC at 1000 μg/mL were incorporated in the growth medium (tempered at 45 ºC). Plates were incubated at 30 °C and the diameter (mm) of the bacterial motility halos were measured at 24 h.

2.4 Biofilm formation

Biofilms were developed according to the modified microtiter plate test proposed by Stepanović et al. [17]. A sterile 96-wells flat-bottomed PS tissue culture plates with a lid were filled with 200 μL of bacterial suspension with a density of 1×10^8 cells/mL. Negative control wells contained Mueller-Hinton broth (MHB) without bacterial cells. The plates were incubated for 24 h at 30 ºC and agitated at 150 rpm.

2.4.1 Biofilm prevention

Overnight batch cultures in MHB supplemented with AITC and PEITC at 1000 μg/mL were grown at 30 ºC and 150 rpm. Those cells were used to assess their ability to form biofilms in microtiter plates, as previously described. Biofilms (24 h aged) were characterized in terms of biomass formation and metabolic activity. Final results are presented as percentage of biofilm mass reduction and inactivation.

2.4.2 Biofilm mass quantification by crystal violet staining

The biofilm mass was quantified using crystal violet (Merck) staining, according to Simões et al. [18]. The absorbance was measured at 570 nm using a Microplate reader (Spectramax M2e, Molecular Devices, Inc.). Biofilm removal was given by Eq. (1), where %BR is the percentage of biofilm removal, OD_C is the OD_{570nm} value of biofilms non-exposed to ITCs and OD_W is the OD_{570nm} value for biofilm exposed to AITC or PEITC.

$$\%BR = \frac{OD_C - OD_W}{OD_C} \times 100 \quad (1)$$

2.4.3 Biofilm metabolic activity quantification by alamar blue assay

The modified alamar blue (7-hydroxy-3H-phenoxazin-3-one-10-oxide) (Sigma-Aldrich) microtiter plate assay was applied to determine the bacterial activity of the cells as reported by Sarker et al. [19]. For the staining procedure, fresh MHB (190 μL) was added to the plates. To each well 10 μL of alamar blue (400 μM) indicator solution was added. Plates were incubated during 20 min in darkness and room temperature (RT). Fluorescence was measured at $\lambda_{excitation}$ = 570 nm and $\lambda_{emission}$ = 590 nm with a Microplate reader. The percentage of biofilm inactivation was given by Eq. (2), where %BI is the percentage of biofilm inactivation, FI_C is the fluorescence intensity of biofilms non exposed to ITCs and FI_W is the fluorescence intensity value for biofilms exposed to AITC or PEITC.

$$\%BI = \frac{FI_C - FI_W}{FI_C} \times 100 \quad (2)$$

2.4 Statistical analysis

The data were analysed using the statistical program SPSS version 17.0 (Statistical Package for the Social Sciences). The mean and standard deviation within samples were calculated for all cases. At least three

independent experiments were performed for each condition tested. All data were analysed by the application of the non-parametric Wilcoxon test (confidence level ≥ 95%).

3. Results and discussion

In the last years microbial infections have become difficult to control using conventional antimicrobial therapy, particularly those biofilm-related [5]. This has encouraged the search for new therapeutic alternatives. Due to the safe status and history of use in traditional medicine, plant compounds are widely accepted as a source of therapeutic molecules [20]. Research on natural products as antimicrobial agents, has almost been exclusively focused on the effects against planktonic microorganisms. However, the effects of phytochemicals on biofilms remain largely unexplored [20]. In this study the effects of AITC and PEITC at 1000 μg/mL was assessed on motility inhibition and biofilm prevention of four bacteria with biomedical importance. Motility is amongst the first steps for pathogenesis and biofilm development. Three forms of surface motility, swimming, twitching and swarming, are documented for *P. aeruginosa* [21]. *P. aeruginosa* swims by means of flagella, and during biofilm formation, swimming motility is involved in initial location and adherence to solid surfaces [7]. After surface attachment, *P. aeruginosa* moves by surface motility known as twitching [21]. *E. coli* and *L. monocytogenes* has two flagella-driven motility types, swimming and swarming [22, 23]. *S. aureus* is a non-flagellated Gram-positive bacterium with a motility phenomenon defined as colony spreading [24]. Therefore, in this work, the ability of AITC and PEITC to interfere with swimming, swarming and twitching motilities of *P. aeruginosa*, swimming and swarming of *E. coli* and *L. monocytogenes* and colony spreading of *S. aureus* was investigated (Table 1).

Table 1 Motility (swimming, swarming, twitching and colony spreading) (mm) of *E. coli*, *P. aeruginosa*, *S. aureus* and *L. monocytogenes* in the absence (control) and presence of AITC and PEITC.

		E. coli	*P. aeruginosa*	*S. aureus*	*L. monocytogenes*
Control	Swim	41 ± 1.0	17 ± 0.0	-	19 ± 0.6
	Swarm	9.0 ± 0.0	9.0 ± 0.0	-	8.0 ± 0.0
	Twitch	-	9.7 ± 0.6	-	-
	Colony spreading	-	-	20 ± 0.0	-
AITC	Swim	20 ± 1.0	8.0 ± 0.0	-	10 ± 0.6
	Swarm	8.0 ± 0.0	8.0 ± 0.0	-	8.0 ± 0.0
	Twitch	-	8.0 ± 0.0	-	-
	Colony spreading	-	-	13 ± 1.1	-
PEITC	Swim	8.0 ± 0.0	8.0 ± 0.0	-	8.0 ± 0.0
	Swarm	8.0 ± 0.0	8.0 ± 0.0	-	8.0 ± 0.0
	Twitch	-	8.0 ± 0.0	-	-
	Colony spreading	-	-	8.0 ± 0.0	-

Results are shown as mean ± standard deviation of at least three independent experiments. The 15 μL of bacterial culture produced an 8 mm (baseline) spot on the agar.

The application of AITC and PEITC promoted total inhibition in swimming, swarming and twitching motilities for *P. aeruginosa* ($P < 0.05$). The same result was verified for swarming of *E. coli* with both ITCs and for swimming with PEITC ($P < 0.05$). For this bacterium, swimming motility was significantly reduced by the addition of AITC ($P < 0.05$). The swimming motility of *L. monocytogenes* was completely inhibited with PEITC ($P < 0.05$); however, AITC promoted a significant reduction ($P < 0.05$). *S. aureus* colony spreading was reduced by AITC and completely inhibited with PEITC ($P < 0.05$). For most of the cases, PEITC was more efficient in motility reduction than AITC ($P < 0.05$). The inhibition of bacterial motility can represent an important strategy to control biofilms. Bacteria in a motile state undergo alterations in their morphology which distinguishes them from their planktonic state. Lai et al. [25] found increased resistance of swarming bacteria compared with their planktonic counterparts. These results may be important as changes in motility can be correlated with a decreased ability of bacteria to form biofilms. In fact, motility plays a major role in the transition from planktonic to surface-associated life-style [7]. Other reports described that many mutants with altered swarming motility were also defective in biofilm formation, indicating that it may play a key role in early biofilm development [26]. Shrout et al. [26] demonstrated that differences in surface motility could explain differences in biofilm structure at early stages of development. In order to ascertain the potential of AITC and

PEITC on biofilm prevention, planktonic bacteria were grown in the presence of ITCs and used to form biofilms on polystyrene microtiter plates (Fig. 1a).

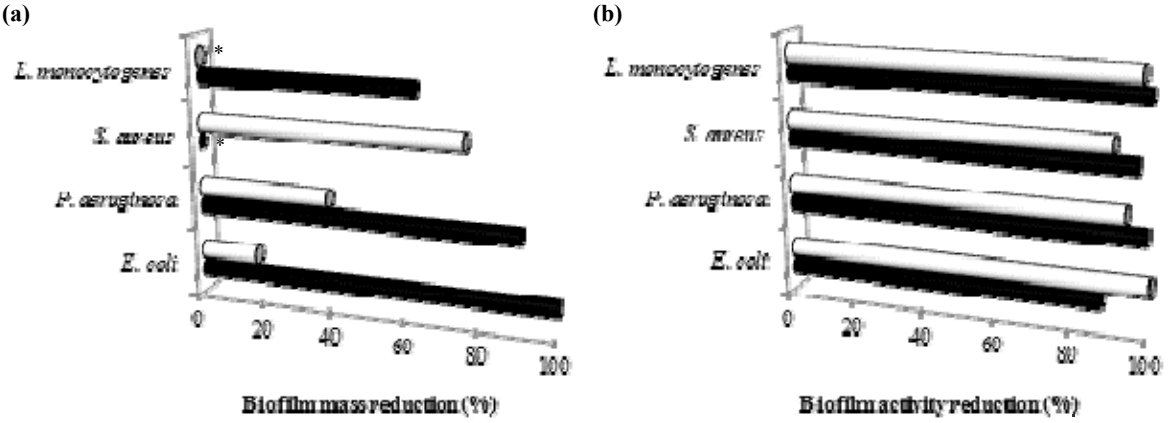

Fig. 1 Preventive action (24 h aged biofilms formed in the presence of ITCs) of AITC (■) and PEITC (□) on biomass formation (a) and metabolic activity (b) of *E. coli*, *P. aeruginosa*, *S. aureus* and *L. monocytogenes*. * - no prevention on biomass formation or metabolic activity reduction was found. Mean values ± standard deviation for at least three replicates are illustrated.

PEITC had no preventive effects in biofilm formation by *L. monocytogenes* ($P > 0.05$). However, significant prevention in biofilm formation was verified for this bacterium with AITC (61%) ($P < 0.05$). The opposite effect was demonstrated for *S. aureus* with AITC (no prevention in biomass formation), and PEITC (75%) had significant preventive action in *S. aureus* biofilm formation ($P < 0.05$). Total biofilm prevention was observed only for *E. coli* with AITC. , In general, AITC had higher preventive effects on biofilm formation than PEITC ($P < 0.05$) (*E. coli* (AITC - 100%; PEITC - 16%) and *P. aeruginosa* (AITC - 90%; PEITC - 37%)). In terms of metabolic activity, the analysis of biofilms formed by planktonic bacteria grown in the presence of ITCs (Fig. 1b) shows that AITC and PEITC promoted reductions higher than 90% for all the biofilms tested, except for *E. coli* with AITC, where the biofilm activity reduction was approximately 87%. Note that, AITC reduced biofilm activity of *S. aureus*, although the chemical had no effects ($P < 0.05$) on the biomass reduction (0% biofilm mass reduction). A similar result was obtained with PEITC for *L. monocytogenes*, biofilm activity reduction was observed for this bacterium, while the ITC had no effects ($P < 0.05$) on the biomass reduction (0% biofilm mass reduction). These results are in agreement with previous study where no correlation between the biomass and metabolic activity was found [20]. In this study the comparison between the results of motility inhibition and biofilm prevention (AITC - *E. coli*, *P. aeruginosa*, *L. monocytogenes* and PEITC - *S. aureus*) suggest that the inhibition of motility can interfere with ability to form biofilms. In a study performed by Sandasi et al. [20], extracts of culinary herbs and medicinal plants had antibiofilm activity against strains of *L. monocytogenes*. Moreover, these authors also found that although most extracts were able to inhibit cell attachment and the growth inhibition of a preformed biofilm was hard to achieve. In fact, inhibit the growth of an already established biofilm (control) is more difficult to achieve than inhibit the initial stage of biofilm formation, namely cell attachment (prevention) [20].

4. Conclusions

AITC and PEITC seem to be promising products for anti-biofouling strategies. These compounds demonstrated potential to inhibit bacterial motility and to prevent biofilm formation of important pathogenic bacteria. This study also emphasizes the potential of phytochemicals as an emergent source of biofilm prevention products. Further studies are in progress to assess the mechanisms of antibacterial action of AITC and PEITC and their cytotoxicity to mammalian cells.

Acknowledgements This work was supported by Operational Programme for Competitiveness Factors – COMPETE and by FCT – Portuguese Foundation for Science and Technology through Project Bioresist - PTDC/EBB-EBI/105085/2008 and the PhD grant awarded to Anabela Borges (SFRH/BD/63398/2009) and Lúcia C. Simões (SFRH/BPD/81982/2011).

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Addition of Resveratrol to Smoked Sea Bass (*Dicentrarchus labrax*) Fillets in Order to Achieve a Longer Shelf Life

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Keywords: sea bass; liquid smoke; resveratrol; shelf life.

1. Introduction

Liquid smoked fishes are lightly preserved products which have to be protected against spoilage with several additional barriers such as vacuum packaging and/or refrigeration. Even though, these products usually show poor quality even during their commercial life.

In this experiment resveratrol was added to liquid smoked sea bass fillets. It is a phytoalexin with high well-known antioxidant potential that can protect the fish flesh from oxidation. Moreover, it has been probed to cause changes in microbial ecology, as certain species can metabolize it. Thus, the effect of the surface addition of reveratrol in the shelf-life of liquid smoked sea bass fillets was studied.

2. Material and methods

2.1. Sample preparation

Fishes (sea bass from aquaculture) were cleaned (skin and bones) and filleted. A control batch of raw fillets (C) and both, a salted (S) and a salted and smoked (H) group were prepared. Salting was performed by inmersion in saturated brine (210 g/L) for 12 h at 4ºC and smoking by inmersion of the salted fillets in liquid smoke (Scansmoke, Broste) during 30 s. From the H batch, half of the fillets were treated with resveratrol from *Polygonum cuspidatum* (98%). It was added homogeneously (1:1000 w/w) on the surface of sea bass (R samples). All the fillets were vacuum-packed and stored at 4ºC for 5 weeks.

2.2. Microbiological analysis

In all cases, 10 g samples of fish muscle were transferred aseptically into a stomacher bag containing 90 ml of 0,1% peptone water and were homogenized (Masticator, IUL Instrument) for 2 min at room temperature. Serial dilutions were prepared and 1 ml samples of the corresponding dilutions were planted by the pour plate method. Different growth media and incubation times and temperatures are specified in Table 1. Microbiological (mesophilic or TVC, psychrophilic and anaerobic bacteria, *Enterobacteriaceae*, *Pseudomonas* sp. and moulds and yeasts), sensory assessment by atrained panel was performed weekly in order to determine the acceptability of the fillets and define a rejection point. Thrimethylamine content (TMA) was also determined [1].

Table 1. Culture media and conditions used for the different microbial groups.

	Culture medium	**Incubation temperature (°C)**	**Incubation time**
Total viable counts	Plate count agar (PCA)	32	72 h
Anaerobic mesophilic bacteria	PCA	32 (anaerobic atmosphere)	72 h
Psycrophilic bacteria	PCA	7	7 days
Pseudomonas spp.	Pseudomonas agar base + CFC supplement	24	72 h
Enterobacteriaceae	Violet Red Bile Glucose Agar (VRBG)	37	48 h
Moulds and Yeast	Oxytetracycline-Glucose-Yeast Extract Agar (OGYE) + Oxytetracycline selective supplement	24	5 days

3. Results and discussion

Looking at the growth curves for TVC, anaerobic and psychrophilic (Figure 1), there is a considerable difference between the sample C and the rest of the samples (S, M, R). Therefore, it seems that salt limits mainly the growth of microorganisms.

For all groups of microorganisms analyzed the highest counts were obtained for the sample C, and the lowest for the samples R.

The acceptable maximum contaminant level for TVC is 7 log cfu / g [2]. R are the only samples that are kept within the permissible range (Figure 1). The sample C exceeded 7 log cfu/g around the day 10 which agrees with other studies such as Papadopoulos *et al*. [3] and Paleologos *et al*. [4] describing a count around 7.9 ± 0.4 cfu/g for sea bass fillets by the 9th day of ice storage.

Pseudomonas spp. and *Enterobacteriaceae* are part of the sea bass own microflora [4-5]. Therefore, *Pseudomonas spp.* count at t = 0 in the sample H and *Enterobacteriaceae* count at t = 0 in the sample C may result from contamination by the fish's own microflora in the filleting time.

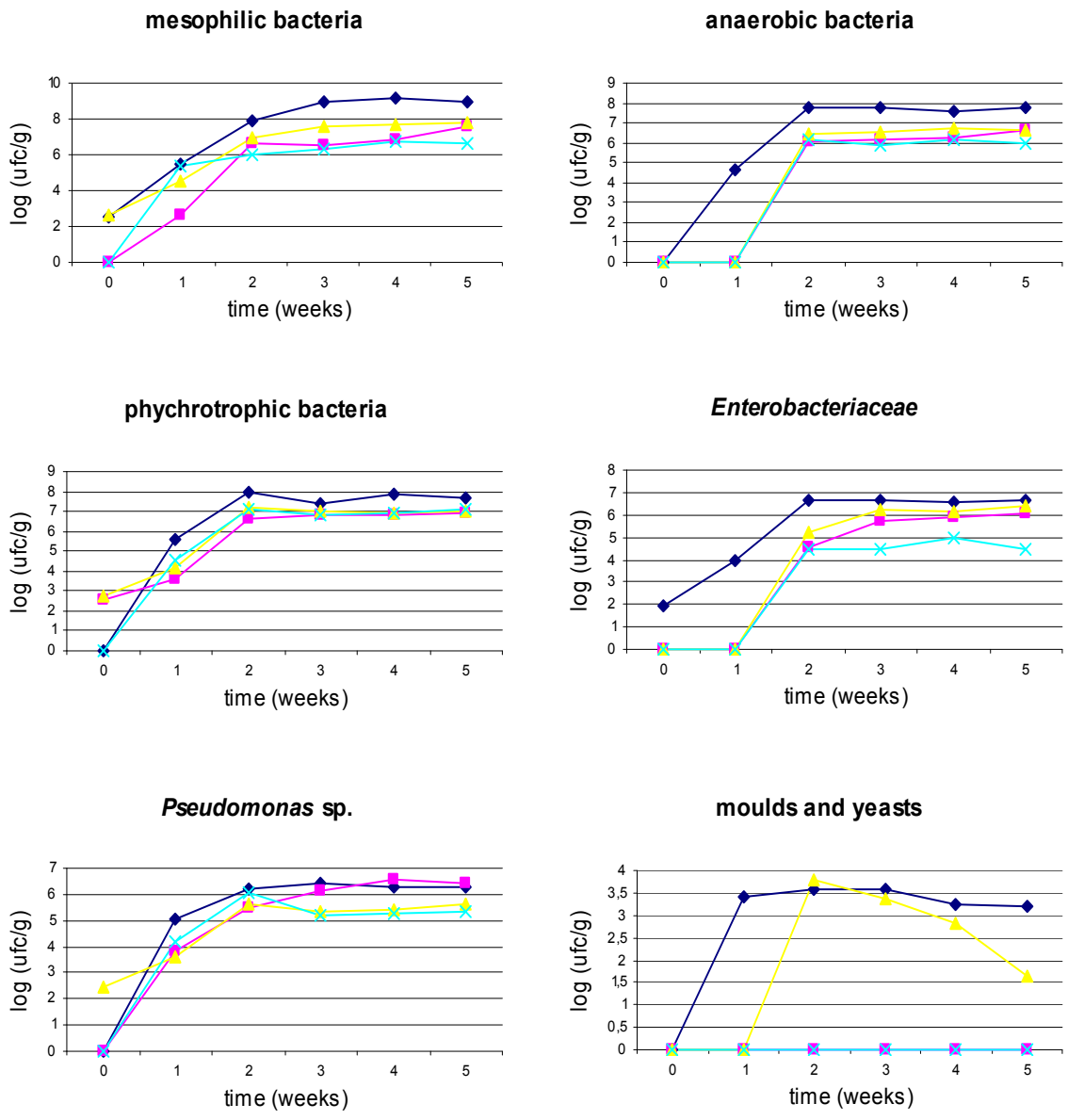

Figure 1. Changes in total counts. Batches are identified as follows:

Authors such as Filip *et al*. [6] concluded that resveratrol doesn´t have any antibacterial effect. However, it has been probed to cause changes in microbial ecology as certain species can metabolize it [7] and some authors have defined minimun inhibitory concentrations for *P. Cuspidatum* extracts and resveratrol [8]. In any case, it is noteworthy that it has not led to increased microbial growth in this experiment. On the other hand, it didn´t have any bad affect on the sensory acceptance of the fillets (that were rejected by the panel in the same moment that the batches S and H. Two weeks) and contributed to delay oxidation processes (data not shown). These aspects are very important to consider this compound as useful to improve the quality and extend the life of the sea bass. TMA content, responsible of the fish off-odour which made the panel reject the samples the second week, showed a similar evolution pattern to the microbial growth with highest values for C samples (Figure 2).

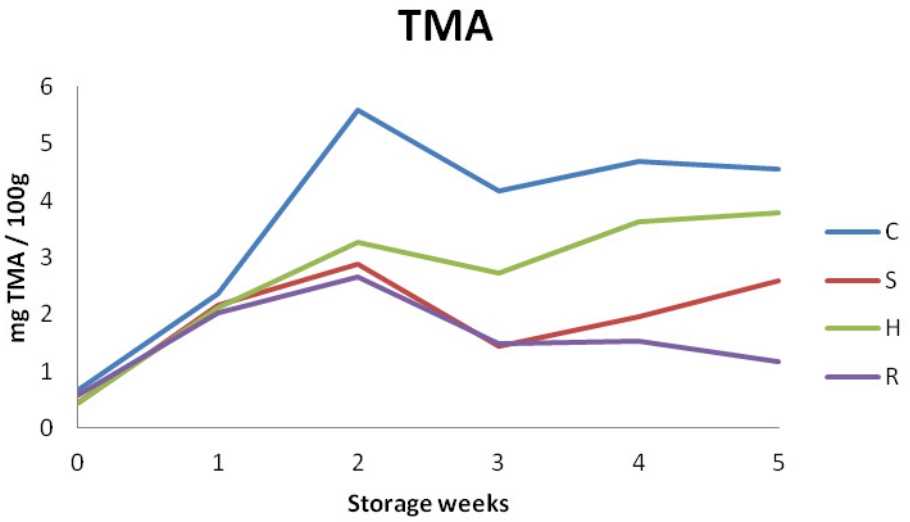

Figure 2. Evolution of the thrimethylamine content in sea bass fillets from each batch tested along a 5 week period.

4. Conclusions

All the samples showed values near the maximum acceptable limit after 15 days of refrigerated storage. C, S and H samples showed even higher counts on later analysis. R batch did not exceed this limit (was just under it) during the experiment. Sensory and physicochemical measurements supported these results also suggesting a shelf life period of 15 days for liquid smoked sea bass fillets under the conditions described. Resveratrol seems to help keeping the fish quality under acceptable limits but does not seem to act as a definitive factor. Further studies (regarding dosage or addition method) are needed to understand the roll of this antioxidant as possible food preservative.

Acknowlwdgements. The support of the *Eusko Jaurlaritza* (Basque Gobernment) (SAIOTEK2011) is gratefully acknowledged.

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Antibacterial activity of biogenic silver nanoparticles against isolates from *Enterobacteriaceae* producers of beta-lactamases (KPC and ESBL)

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Many efforts in these last years have dedicated in the development of new drugs or materials due to the emergence and increase of microbial organisms resistant to multiple antibiotics. The most promising nanomaterials with antibacterial properties are the metallic nanoparticles such as silver nanoparticles (AgNP) produced by different microorganisms like fungi. The study of biogenic nanoparticles efficacy against microorganism resistant a antibiotics is very important due to increase of resistant bacteria.

Keywords Antibacterial; eterobacteriae, beta-lactamase, KPC

1. Introduction

The enzymes beta-lactamases are distributed in all the planet and have the capacity of degrade the beta-lactamic antibiotics, a class of pharmaceutical widely used in a severe bacterial infection. Amonsg these enzymes is the ESBL (beta lactamase of extended spectron) andj a KPC (*Klebsiella pneumoniae* carbapenemase) [1] that exhibits diferente action spectra on beta-lactamic antibiotics [2,3]. Many efforts in these last years have dedicated in the development of new drugs or materials due to the emergence and increase of microbial organisms resistant to multiple antibiotics. The most promising nanomaterials with antibacterial properties are the metallic nanoparticles such as silver nanoparticles (AgNP)[4]. The aim of this work was to evaluate the antibacterial activities of silver nanoparticles combined with imipenem against bacteria with resistance mechanism for beta-lactamase.

2. Experimental part

2.1. Biogenic synthesis of silver nanoparticles

The biogenic synthesis of the silver nanoparticles was carrying out using the *Fusarium oxysporum* as describe in the Fig.1. [4][6]

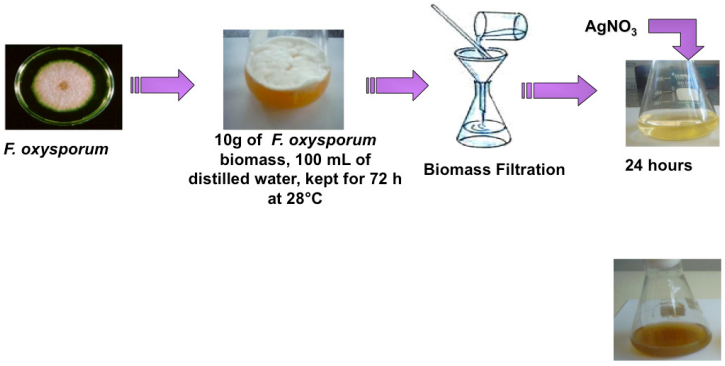


Figure 1. Preparation of biogenic silver nanoparticles

The biogenic silver nanoparticles (AgNP) was characterized by UV-Vis spectroscopy (plasmon band at 450 nm). The particles morphology was carrying out by Transmission Electron Microscopy (TEM) using a Carl Zeiss (Libra) transmission electron microscope (120 KeV). The zeta potential and diameter was analyzed by and by photon correlation spectroscopy (PCS) (Zetasizer, NanoZS, Malvern).

2.2. Antibacterial assays

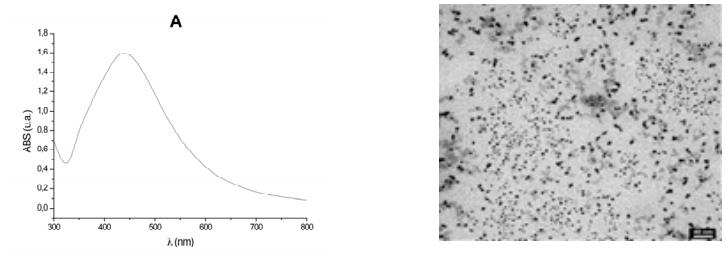
It was studied 5 isolated with resistance mechanisms for beta-lactamases and 1 negative control (*E. coli* ATCC 25922), and the antimicrobial activity of silver nanoparticles with or without imipenem was evaluated by disc diffusion assay.

Initially, it was determined the minimum inhibitory concentration (MIC) by macrodilution [5] de AgNP (from 84.5 to 1.32 µg/mL). The MIC value was used in the diffusion disc assay. In the diffusion disc assay, the agar Mueller Hinton (Oxoid, United Kingdom) plates were inoculated with each bacteria in test adjusted to standard equivalent to 0.5 McFarland (1.5 x 10^8 UFC/mL). It was used four discs of imipenem (IPM) and on two of these discs were applied 6.2 µL (10.52 µg/disco) of silver nanoparticles solution of 1690 µg/mL. In parallel, two discs of filter paper received 6.2 µL (10.52 µg/disco) of silver nananoparticles solution of 1690 µg/mL (control). As the plates were incubated for 18 h at 35°C ±2°C and the average diameter of inhibition was registered. Al the tests were carried out in duplicate.

3. Results and Discussions

3.1. Biogenic synthesis of silver nanoparticles

Biogenic silver nanoparticles (AgNP) exhibited a plasmon band around 450 nm confirming the nanoparticles formation (Figure 2A). Spherical and homogenous AgNP with 7.3 nm of diameter was observed by TEM as shown the Figure 2B. The surface charge of these particles was negative (δ = - 20 mV) and the diameter measured by PCS was around 130 nm due to protein capping around the particles as described in the literature [4,6] and showing excellent antibacterial activities [7].



(A) (B)

Figure 2. (A) UV-visible spectrum of silver nanoparticles, (B) TEM micrography.

3.2. Antibacterial assay

Table. 1. Evaluation of synergistic effect at MIC values of silver nanoparticles (AgNP) and imipenem (IPM)(duplicated)

Strain	IPM Average (mm)	IPM/AgNP Average (mm)	AgNP Average(mm)	Increase in Fold area ($b^2 - a^2$)/a^2 Antibiotics Zone of Inhibition mm
SER PCR	17.0	18.5	7.5	0.19
KPN 710	19.0	20.0	7.0	0.11
KPN PCR	18.0	19.0	6.7	0.12
KPN HC PCR	18.0	18.5	6.0	0.06
ESBL 700603	28.0	29.0	7.5	0.07
***E. coli* 25922**	30.0	30.0	7.0	NSE

SER: *Serratia mascescens*; KPN: *Klebsiella pneumoniae*; ESBL: *K. pneumoniae* producer of Extended Spectrum Beta-Lactamase; NSE: Non synergistic effect; IPM: imipenem; AgNP: silver nanoparticles

All the bacterial that produced beta-lactamases (ESBL or KPC) demonstrated an increase of inhibition zone diameter with the presence of IPM/AgNP when compared to the diameter produced only with IPM. This behavior suggests a synergism between the IPM antibiotic and silver nanoparticles. The results found with *E. coli* 25922 reinforced the potentiality of silver nanoparticles on beta-lactamase enzymes, since these bacteria are free of any protected enzymatic mechanism on beta-lactamases, non-underwent any alteration in the IPM halos with the silver nanoparticles.

4. Conclusion

Silver nanoparticles alone or their formulations in combination with commonly used antibiotics can be used as effective bactericidal agents showing to be an interesting material against resistant bacteria.

Acknowledgements The support from FAPESP, CNPq, INOMAT (MCTI/CNPq), Brazilian Network of Nanotoxicology (MCTI/CNPq), NANOBIOSS (MCTI/CNPq) is gratefully acknowledged.

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Antibacterial activity of Swietenia mahagoni, Jacq and Carapa procera, DC, against methicillin-resistant coagulase negative staphylococci

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Methanolic leaf extracts of S. mahagoni, Jacq and C. procera, DC, were screened for antibacterial activities against seven methicillin-resistant and one methicillin-sensitive staphylococcal strains. Air-dried leaves of each plant material were pulverized into fine powder and extracted using 70% cold methanol. The extracts were evaporated to dryness at 40^0C and their yields determined. The extracts were screened for antibacterial activity using the agar well diffusion method by dispensing 50 ul of 20 mg/ml of each test extract into wells bored into Mueller-Hinton agar inoculated with the test organisms. The minimum inhibition concentrations (MICs) and the minimum bactericidal concentrations (MBC) of the plant extracts against the test bacteria were determined using broth dilution technique. The mean inhibition zone diameter for both extracts ranged from 13mm – 18.5mm while the MIC ranged from 0.625 – 2.5 mg/ml. The MBC for S. mahagoni ranged from 2.5 – 5 mg/ml while it ranged from 5 – 10 mg/ml for C. procera. Swietenia mahagoni and Carapa procera could be a veritable source of antibacterial substances against methicillin-resistant staphylococci.

Key words: Antibacterial; Swietenia mahagoni; Carapa procera; methicillin- resistant staphylococci.

Methicillin-resistant staphylococci have become a serious problem in many parts of the world. The incidence of methicillin resistant strains of staphylococci varies from country to country and from hospital to hospital and has been increasing steadily worldwide in the last decade [1; 2]. Methicillin-resistance is an alarming condition for treatment because it implies resistance not only to all beta-lactam antibiotics but also to a wide range of other antibiotics [3; 4]. Transmission of methicillin resistant staphylococci among humans and animals has been reported [6; 7].

Carapa procera is a tall tree which attains a height of about 50feet; mature stems fairly straight, usually fluted, small buttresses and 2-3 feet in diameter. It has wide spreading branches and large leaves up to 2m long with 8 pairs of opposite and alternate leaflets that are clustered at the ends of the twigs. The bark is pale brown; thin and smooth with pink or red slash [7]. The bark and seed oil are used in the treatment of rheumatism, cutaneous and subcutaneous parasitic infections, emetics, fabrifuges, leprosy, pulmonary trouble, venereal diseases. The seeds are used as an antidote for venomous stings and bites and painkillers [8]. Carapa procera has been shown to have antibacterial activities (9)

Swietenia mahagoni is an evergreen to semi-evergreen hardwood timber species of the family *Meliaceace* having seeds chestnut brown in colour, 4-5 cm long, compressed, crested and extended into a wing at the attachment end [10; 11]. This plant species has ethnomedical uses [12] and *in vitro* antibacterial and antifungal property of its extract has been reported [12].

Several efforts aimed at discovering new antimicrobial compounds from various sources such as soil, microorganisms, animals and plants have been documented. Systemic screening of plants used in traditional medicine may result in the discovery of novel effective compounds [13]. The specific objective of this study was to screen the methanolic extracts of *Swietenia mahagoni* and *Carapa procera* leaves for activity against methicillin-resistant staphylococci.

Materials and methods

Plant collection and identification

Fresh leaves of *Swietenia mahagoni* and *Carapa procera* were collected in the month of June, 2008 from a forest in Abak Itenge Village in Abak Local Government Area, Akwa Ibom State, Nigeria. They were all identified by a botanist in the Botany Department, University of Nigeria, Nsukka. The leaves were air-dried, after which they were pulverized into fine powder in the Crop Science Laboratory, University of Nigeria, Nsukka.

Extraction

One kilogram of *C. procera* and one kilogram of *S. mahagoni* ground materials were extracted with 500ml and 1000ml of 70% cold methanol respectively in separate bottles. The bottles were allowed to stand for 24hours, being shaken vigorously at regular intervals. The extracts were filtered using Whatman filter paper. The filtrates were evaporated to dryness at 40ºC. After evaporation, the extracts were recovered and stored in air tight bottles at 4ºC in the refrigerator till use.

The percentage yields of the extracts were 8.4%w/w and 11.76%w/w for*, S. mahagoni* and *C. procera* respectively.

Bacteria cultures

Stock cultures of seven methicillin-resistant and one methicillin-sensitive staphylococcal strains isolated from dogs and maintained on nutrient agar slants at 4ºC in the Microbiology Diagnostic Unit, Department of Veterinary Pathology and Microbiology, University of Nigeria, Nsukka were used in this study. The methicillin-resistant strains used were: *Staphylococcus sciuri subspecies rodentium* (5 strains), *S. haemolyticum* (1 strain) and *S. lentus* (1 strain). The methicillin-sensitive strain used was *S. pseudintermedius*.

These stocked cultures were sub-cultured on nutrient agar plates and incubated at 37ºC for 24 hours to check for their purity and re-identification. The colonial morphology of the different bacterial species were observed and identified accordingly. Pure colonies were stained for microscopic identification before re-stocking from which subcultures were made before use.

Evaluation of plant extracts for antibacterial activity

The assay was conducted using agar well diffusion method [15]. A 20mg/ml concentration of each plant extract was constituted by dissolving 0.04g of each extract in 2ml of dimethyl sulfoxide (DMSO). A single colony of each test isolate was suspended in 2ml of sterile normal saline. The suspension of each isolate was standardized and used to inoculate the surface of the Mueller-Hinton agar. The excess fluid was drained into a disinfectant jar. The inoculated agar surface was allowed to dry and the plates were appropriately labelled.

Using a cock borer of 7mm diameter, wells were bored in the inoculated nutrient agar, 1 for each of the extract and 1 for DMSO (control). With a micropipette, 50 μl of each test extract was delivered into each well and 50 μl of DMSO into the control well. The plates were left on the bench for 30minutes to allow the extract to diffuse into the agar. Thereafter, the plates were incubated at 37ºC for 24 hours. After incubation, the plates were observed for inhibition zones around the wells. The inhibition zones were measured with a meter rule and recorded. Each test was performed in triplicates and the mean IZD recorded to the nearest millimeter.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Minimum inhibitory concentration (MIC) of both extracts were determined by broth dilution method. Extract concentrations used ranged from 10 mg/ml – 0.15625 mg/ml. A 10µl volume of each standardized inoculum was inoculated into each extract dilution. Inoculated tubes were incubated aerobically at 37^0C for 18 – 24hr. The lowest concentration of the extract that produced no visible growth (no turbidity) when compared with the control tubes were regarded as MIC.

Minimum bactericidal concentration (MBC) was determined by sub-culturing the test dilution that did not produce visible growth (i.e no turbidity) on fresh nutrient agar. Inoculated plates were incubated for 18 – 24 hr. The highest dilution that yielded no bacterial growth on the agar was taken as MBC.

Results

The mean inhibition zone diameters produced by *S. mahagoni* extract ranged from 13.5 mm – 18.5mm while those produced by *C. procera* ranged from 13.0 mm – 18.5 mm (Table 1). The MIC values ranged from 0.217mg/ml to 1.052mg/ml for *S. mahagoni* extract and 0.079mg/ml to 8.680mg/ml for *C. procera* extract (Table 2). The MBC ranged from 2.5 to 5 mg/ml for *S. mahagoni* and 5 to 10 mg/ml for *C. procera* extract (Table 3).

Table 1: Antibacterial activity of methanolic leaf extracts of *S. mahagoni* and *C. procera*

S/N	**Staphylococcus strains**	***Mean inhibition zone diameters (mm) produced by plant extracts (20mg/ml)***	
		S. mahagoni	*C. procera*
1	*S. scuiri rodentium*	17.5	16
2	*S. scuiri rodentium*	16.5	15
3	*S. scuiri rodentium*	13.5	13
4	*S. scuiri rodentium*	14.5	14
5	*S. scuiri rodentium*	18.5	15.5
6	*S. haemolyticum*	18.5	18.5
7	*S. lentus*	16.5	17.5
8	*S. pseudintermedius*	14.5	14

Table 2: Minimum inhibition concentrations (mg/ml) of methanolic plant extracts against the test bacteria

S/N	***Staphylococcus strains***	**Minimum Inhibitory Concentration (mg/ml)**	
		S. mahagoni	*C. procera*
1	*S. scuiri rodentium*	0.217	0.811
2	*S. scuiri rodentium*	1.052	1.991
3	*S. scuiri rodentium*	0.586	0.125
4	*S. scuiri rodentium*	0.667	8.680
5	*S. scuiri rodentium*	0.594	0.141
6	*S. haemolyticum*	0.530	0.079
7	*S. lentus*	0.355	0.682
8	*S. pseudintermedius*	0.676	0.412

Table 3: Minimum bacteriocidal concentration (mg/ml) of methanolic plant extracts against the test bacteria

S/N	*Staphylococcus strains*	**Minimum Bactericidal Concentration (mg/ml)**	
		S. mahagoni	*C. procera*
1	*S. scuiri rodentium*	2.5	5
2	*S. scuiri rodentium*	2.5	5
3	*S. scuiri rodentium*	5	5
4	*S. scuiri rodentium*	5	5
5	*S. scuiri rodentium*	5	5
6	*S. haemolyticum*	2.5	5
7	*S. lentus*	5	10
8	*S. pseudintermedius*	5	5

Discussion

Over the years, World Health Organization (WHO) advocated that countries should interact with traditional medicine with a view to identifying and exploiting aspects that provide safe and effective remedies for ailments of both microbial and non-microbial origins [15]. The results of this study indicate that the methanolic extracts of *S. mahagoni* and *C. procera* leaves are effective against the test bacteria. This current finding has shown that *S. mahagoni* and *C. procera* have remarkable antibacterial activities against methicillin-resistant staphylococci.

The antibacterial activity of a plant extract depends on factors such as type of soil where it is grown, its mode of extraction, temperature and mode of storage, mechanisms of action, strain of the bacterium, fractionation, concentration used and the phytochemical properties of the plant amongst others [16]. The varied zones of inhibition produced by the two plant extracts could be attributed to one or more of the afore-mentioned factors.

It is interesting that growth of methicillin resistant staphylococci, which are Gram-positive bacteria experienced every day, were appreciably inhibited by the methanolic plant extracts of *S. mahagoni* and *C. procera* leaves.

Generally, the mean inhibition zone diameter (IZD) decreased with decreased plant extracts concentrations showing a concentration dependent activity of *S. mahagoni* and *C. procera.*

The results of this study have shown that *S. mahagoni* and *C. procera* have very promising medicinal (antibacterial) properties. The results also offer a scientific basis for the forkloric use of parts of these against staphylococci-associated diseases. Other studies on the extracts of these plants are necessary and should seek to determine the toxicity, serum-attainable levels, pharmacokinetic properties and diffusion in different body sites. The antimicrobial activities could be enhanced if the active components are purified and adequate dosage determined for proper administration.

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Antibacterial agents from some Indonesian dammar and bamboo

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Dammar is a common name for resin excreted from *Dipterocarpaceae* plants, such as stone dammar from *Shorea eximia*, flesh dammar from *S. leprosula*, and cat eye dammar from *S. javanica*. Both stone and flesh dammars had antibacterial activity against *Chromobacter violaceum, Streptococcus* sp*., Staphylococcus aureus, S. epidermidis,* and *Bacillus cereus* but cat eye dammar did not have. The bioactive compounds in stone dammar and flesh dammar belong to sesquiterpenes, but their percentage varied upon the species of dammar and the extracting solvents.

Bamboo is another group of abundant plant in Indonesia forests. Local people know the bioactivity of bamboo, especially its root and leaf. Our study showed that bamboo leaf contained fatty acids, esters, aldehydes and alcohols, and it had antidiarrheal activity. It is potential as remedy against diarrhea in poultry and piglets because it can inhibit the growth of diarrheagenic *Escherichia coli*.

Keywords antibacterial activity; dammar; sesquiterpenes; bamboo leaf; fatty acid

1. Introduction

Indonesia is a tropical country and it has over 120 million ha of natural forests. These represent a tenth of the world's remaining tropical rain forests and are among the most biologically diverse in the world, which is mainly dominated by family members of Dipterocarpaceae. Distribution of Dipterocarpaceae is mainly in Sumatera, Kalimantan, Sulawesi, and Papua. Dipterocarpaceae forest has high ecological, economical, and social functions. However, forest exploitation, forest conversion to other land-use, forest fire, and illegal logging drastically decreased the area of Dipterocarpaceae forest. This paper aims to review antibacterial activity from dammar resin and bamboo leaf, both are nontimber forest products and abundant in Indonesia.

Dammar resins are semisolid exudates produced as secondary metabolites from tropical forest plants, such as stone dammar (*Shorea eximia*), flesh dammar (*S. leprosula*), and cat eye dammar (*S. javanica*). Their woods are favorable for plywood, furniture, boat decking and many other purposes. The resin of stone dammar has been used as adhesive for wooden boat and film forming material for transdermal application [1], while that of flesh dammar has been used by local people to heal leprosy [2]. The resin of cat eye dammar is known as the best dammar in the world and has been used in paint and food industries [3]. Additionally, cat eye dammar also had antitermite and antifungal activities [4].

Bamboo is a group of large woody grasses belonging to the family *Poaceae* and subfamily *Bambusoideae*. It has been used extensively, especially its shoot for food and wood for housing and furniture. In Bali, bamboo root is used to cure such as cough, hepatitis, hypertension, breast cancer, and diabetes mellitus [5]. On the other hand, it was reported that bamboo leaf is the most utilized as traditional medicine among all parts [6].

As much as 50 avian pathogenic *E. coli* (APEC) from broiler chickens showed their high resistance (≥ 80%) against common antibiotics, such as nalidixic acid (100%), lincomycin (100%), erythromycin (97%), oxytetracycline (95%), chlortetracycline (95%), tetracycline (94%), flumequine (94%), tiamulin (91%), doxycycline (88%), difloxacin (83%), neomycin (81%), streptomycin (81%), and trimethoprim-sulphamethoxazole (80%) and 56-100% of *E. coli* isolates from human fecal were resistant to tetracycline, ampicillin, chloramphenicol, and streptomycin [7, 8]. In addition, using oxytetracycline, tetracycline, and chlortetracycline as antibiotics for poultries will be deposited in meat products [9].

2. Antibacterial activity of dammar

Our study indicated that either flesh or stone dammar extract could inhibit all Gram-positive bacteria tested, i.e: *Streptococcus* sp*., S. aureus, S. epidermidis,* and *B. cereus,* but stone dammar seemed to be more effective than flesh dammar (Table 1) [10, 11]. Those Gram-positive bacteria had one layer cell wall and its major component was peptidoglycan which was easily to be penetrated with antibacterial agent [12]. Other bacteria, such as *E. coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, and *Proteus mirabilis* could not be inhibited by the extract. These Gram-negative bacteria had multilayer cell wall and its outer membrane contained lipopolysaccharide. This complex macromolecule had polar glycoside group on the surface which hindered lipophilic compounds to

enter the cell. Gram-positive bacteria were more sensitive towards natural antimicrobial agents from some spices compared to Gram-negative bacteria [13].

The exceptional data was shown by *C. violaceum*, which was inhibited by the extract. In addition, its inhibition zone was larger than that of the Gram-positive bacteria tested. This activity probably was caused by the interaction between the extract and the unique metabolite from *C. violaceum,* namely violacein pigment. The other possibility was that these bacteria possessed similar mesosome to Gram-positive bacteria [14].

Table 1 Antibacterial activity and chemical constituents in dammar

	Flesh dammar	Stone dammar	Cat eye dammar
The best solvent	Ethyl acetate	Ethanol	-
Soluble matter (% w/w)	58.79	45.35	-
Effectivity compared to tetracycline (%)	0.006-0.013	0.007-0.015	-
% of bioactive compounds in extract	36.2	33.2	-

Our study showed that the effectivity of either flesh or stone dammar extracts was lower than that of tetracycline because the extracts had not been purified yet. Chemical constituents in the selected extracts have been identified, some of them were present in other medicinal plants and had been proved about their antibacterial activity [15–18] (Table 2).

Table 2 Some physical properties of chemical constituents in ethyl acetate soluble extract of flesh dammar and ethanol soluble extract of stone dammar

Chemical constituents	Flesh dammar		Stone dammar		Formula Weight	Refractive index	Surface Tension (dyne/cm)
	%[1)]	SI[2)]	%[1)]	SI[2)]			
α-copaene ($C_{15}H_{24}$)	1.40	94	5.51	95	204.35	1.509 ± 0.02	30.7 ± 3.0
β-elemene ($C_{15}H_{24}$)	0.92	97	-	-	204.35	1.501 ± 0.02	29.8 ± 3.0
δ-cadinene ($C_{15}H_{24}$)	-	-	4.69	95	204.35	1.498 ± 0.03	29.8 ± 5.0
Aromadendrene ($C_{15}H_{24}$)	-	-	1.13	89	204.35	1.505 ± 0.03	30.6 ± 5.0
Valencene ($C_{15}H_{24}$)	-	-	3.53	88	204.35	1.494 ± 0.03	29.7 ± 5.0
α-Gurjunene ($C_{15}H_{24}$)	-	-	5.09	78	204.35	1.512 ± 0.03	31.4 ± 5.0
caryophyllene oxide ($C_{15}H_{24}O$)	-	-	4.50	91	220.35	1.507 ± 0.03	32.5 ± 5.0

[1)] relative peak area, [2)] similarity index (%), -: absent

3. Antibacterial activity of bamboo leaf extract

In this part of research, our focus was the inhibition activity of some Indonesia bamboo leaf toward *E. coli* O157:H7 isolated from well water and river water and *E. coli* isolated from diarrheal poultry and piglets. Petung bamboo (*Dendrocalamus asper*) from Mataram, apus bamboo (*Gigantochloa apus*) from Central Java, and golden bamboo (*Bambusa vulgaris*) from West Java were evaluated for their antidiarrheal activity *in vitro*. The best solvent to obtain antidiarrheal agent from bamboo leaf was ethanol, but the yield and effectivity in inhibiting *E. coli* compared to tetracycline depended on the bamboo species and the tested bacteria (Table 3). The antibacterial activity of bamboo leaf extract from *Phyllostachys* sp. in China has been reported [19–21]. Other group with antibacterial activity against *E. coli* O157:H7 was saponin [22].

Table 3 Antidiarrheal activity of some bamboo leaf extracts

	D. asper	*G. apus*	*B. vulgaris*
Yield of extract (%)	28.5	18.7	21.9
Effectivity (%)			
E. coli from diarrheal poultry	0.54	1.34	1.33
E. coli from diarrheal piglets	0.39	0.08	0.22
E. coli O157:H7 from well water (JB4)	3.30	0.27	2.61
E. coli O157:H7 from river water (AT9)	0.58	0.07	0.51
Average	1.20	0.44	1.17

Our study showed that bioactive compounds in bamboo leaf extracts were fatty acids, esters, aldehydes, and alcohols. This result was in accordance with other papers [23–28].

Table 4 Bioactive compounds in some bamboo leaf extracts

Groups	Bioactive compounds	Formula	*D. asper*		*G. apus*		*B. vulgaris*	
			%	SI	%	SI	%	SI
Fatty acids and esters	Lauric acid	$C_{12}H_{24}O_2$	0.45	93	48.76	96	0.98	97
	Ethyl laurate	$C_{14}H_{28}O_2$	-	-	7.03	92	-	-
	Myristic acid	$C_{14}H_{28}O_2$	-	-	4.53	95	1.07	96
	Capric acid	$C_{10}H_{20}O_2$	-	-	6.45	93	-	-
	Caprylic acid	$C_8H_{16}O_2$	-	-	1.35	97	0.35	94
	Citronellyl acetate	$C_{12}H_{22}O_2$	-	-	1.01	87	-	-
	Menthol acetate	$C_{12}H_{22}O_2$	0.60	79	-	-	-	-
	Methyl palmitate	$C_{17}H_{34}O_2$	2.82	97	-	-	1.28	97
	Palmitic acid	$C_{16}H_{32}O_2$	7.69	96	6.72	95	18.12	95
	Methyl hexadecadienoate	$C_{17}H_{30}O_2$	-	-	-	-	0.79	84
	Methyl eicosanoate	$C_{21}H_{42}O_2$	-	-	1.71	86-94	-	-
	8,11,14-Eicosatrienoic acid	$C_{20}H_{34}O_2$	-	-	3.57	88	-	-
	Methyl heptadecanoate	$C_{18}H_{36}O_2$	-	-	-	-	0.41	92
	Allyl stearate	$C_{21}H_{40}O_2$	0.44	86	-	-	-	-
	Methyl linoleate	$C_{19}H_{34}O_2$	4.37	93	-	-	1.76	93
	Methyl behenate	$C_{23}H_{46}O_2$	-	-	-	-	0.19	95
	Methyl lignocerate	$C_{25}H_{50}O_2$	-	-	-	-	0.25	95
	Methyl stearate	$C_{19}H_{38}O_2$	1.95	89	-	-	-	-
	Stearic acid	$C_{18}H_{36}O_2$	-	-	0.81	79	-	-
	Oleic acid	$C_{18}H_{34}O_2$	-	-	-	-	0.66	89
	Stigmast-5-en-3-ol, oleat	$C_{47}H_{82}O_2$	0.99	85	-	-	-	-
	Methyl linolenate	$C_{19}H_{32}O_2$	29.44	92-94	1.90	95	17.90	91-93
	Ethyl linoleate	$C_{20}H_{36}O_2$	0.58	90	-	-	-	-
	Linoleic acid	$C_{18}H_{32}O_2$	-	-	1.64	89	-	-
	Dioctyl adipate	$C_{22}H_{42}O_4$	24.88	84	1.16	92	28.62	84
Long chain aldehydes	9,12,15-Octadecatrienal	$C_{18}H_{30}O$	0.52	88	-	-	-	-
	4,8,12-Tetradecatrienal, 5,9,13-trimethyl-	$C_{14}H_{24}O$	-	-	-	-	0.44	89
	Myristaldehyde	$C_{14}H_{28}O$	0.37	82	-	-	-	-
Long chain alcohols	Dihydrophytol	$C_{20}H_{42}O$	-	-	-	-	0.97	89-92
	Phytol	$C_{20}H_{40}O$	1.77	93	5.30	90	4.29	84-92
	4,8-Dimethyl-1-nonanol	$C_{11}H_{24}O$	0.45	90	-	-	-	-
	Hexahydrofarnesol	$C_{15}H_{32}O$	0.28	93	-	-	-	-

[1)] relative peak area, [2)] similarity index (%)

Acknowledgements The support of Ministry of Agriculture Republic of Indonesia and Atma Jaya Research Center is gratefully acknowledged.

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Antibacterial and fungicidal plastics by dendritic hyperbranched polymer-metal-hybrids

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Plastics are widely used in medical applications. However, because their use involves the crossing of natural biological barriers (e.g., wound dressings, catheters and implants) – there is always the risk that pathogens will be transferred to patients, despite all care. To solve this problem and to minimize the risk of infections as well as unwanted colonization and spreading of pathogenic germs / pathogens on material surfaces, the polymeric materials can be finished with antimicrobial properties. This can be achieved by addition of various antimicrobial additives. Considering the progress in nanoparticle technology especially silver has become prominent. Here we report about the design of antimicrobial nano-hybrids with silver, copper or zinc utilizing an amphiphilic hyperbranched polymer core-shell-carrier and their application as surface finish additives.

Keywords hyperbranched polymers; silver; copper; zinc; antimicrobial; surface finishes

1. Introduction

The antimicrobial properties of silver and copper are well known for a long time [1]. But the ions of other metals like gold or zinc may also have similar effects, if they interact with bacterial cells in a dosage above a critical concentration [2, 3]. This toxicity of particularly heavy metal ions towards bacterial cells, molds, spores or fungi is known as the so called oligodynamic effect [4, 5]. The progress on the field of nanotechnology has enabled the production of appropriate metal particles with only few nanometers in size. In interworking research it could be shown that nanoparticles have a significantly higher biological activity than commonly used particles with dimensions in the micro-scale [6-8]. This is, amongst others, due to the increasing specific surface area of the particles with decreasing particle size. The ion release properties are direct related to the specific surface area of the particles (number of lattice ions situated on the particle surface). Therefore, metal nanoparticles are potential antimicrobials and in these context especially silver has become very prominent [8, 9]. However, the processing and incorporation of such antimicrobial metal nanoparticles into polymers (e.g. coating or bulk doping) is still a challenging issue. Considering that the antimicrobial activity of the particulate addition agents depends on the particle size [10, 11], a poor dispersion or agglomeration of the primary particulate additive could cause that the antimicrobial finish fails. To overcome this drawback, we have developed metal-hybrids utilizing hyperbranched polymers as uni-molecular carriers (nano-capsules). This approach enables both, prevention of agglomeration of the individual particles and provision of a high dispersity by commonly used additive blending techniques and thus ensures a high efficiency of the antimicrobial material finishes.

2. Material and Finishing Methods

2.1 Preparation of the Metal-Hybrids

The synthesis of the hyperbranched polymer carriers and the corresponding metal-hybrids with silver or zinc was carried out as previously described by us in detail [12, 13]. The copper-hybrids, based on a same dendritic hyperbranched polymer with amphiphilic core-shell-architecture (polyethylenimine partially modified with palmitic acid), were produced as followed: In a first step an appropriate amount of the copper precursor was added to a solution of the hyperbranched amphphilic carrier polymer in 2-propanol (Sol.A). The solution turns deep blue by dissolving of the copper precursor and coordination of the copper ions to nitrogen atoms of the carrier polymer. In a second step the coordinated copper ions were reduced to copper atoms by chemical reduction. Therefore the reduction agent (HP) was solved in ethylene-glycol (Sol.B). Afterwards the mixture of both solutions (Sol.A + Sol.B = 1 : 1 (v/v)) was heated in a microwave oven under nitrogen atmosphere for up to 5 minutes. The obtained copper dispersion was of deep red color. Finally the copper-hybrid particles were isolated by centrifugation or by distilling of residue solvent in a vacuum oven.

The molar loading of the carrier polymer with the metal ions / atoms was maximized but is limited by the intra-molecular uptake capacity of the hyperbranched polymer core (Table 1).

2.2 Antimicrobial Material Finishes

Antimicrobial surface finishes were prepared by adding the metal-hybrids into coating formulations or by incorporation in the polymer bulk.

For coating purposes a co-polyamide (Ultramid 1C, BASF) was solved in methanol and a dispersion of the metal-hybrid in chloroform was added in an appropriate amount. These formulations were applied to a PET-foil by doctor blade technique and the wet films were subsequently dried in a vacuum oven.

For polymer bulk modification the pre-dried metal-hybrid-powder was incorporated into polyamide 6 (Ravamid R200S, Ravago) by melt mixing utilizing a synchronous twin screw extruder (ZSK-25, Coperion, Werner&Pfeiderer). The fabricated granules were then shaped into plates by utilizing an injection molding machine (Battenfeld H110).

Table 1 Constitution of the antimicrobial metal-hybrids

Material	Embedded metal form	Molar loading ratio	Hydrodynamic radius* (nm)
Ag-hybrid	Nanoparticles	1 : 8	6
Cu-hybrid	Nanoparticles	1 : 8	25
Zn-hybrid	Metal ions	1 : 6	7

* as received

3. Hyperbranched polymer-metal-hybrids as antimicrobial additives

3.1 Characterization of the metal-hybrids

In the proper case, the maximum size of the synthesized metal nanoparticles is determined by the hyperbranched amphiphilic carrier polymer (template effect). Therefore the hybrids are to a large extent of a monomodale spherical habitus and the embedded particles have a narrow size distribution (Figure 1). However, the Cu-hybrids form spontaneous supramolecular structures opposite to the Ag-hybrids or Zn-hybrids (latter one not shown) as evident by the DLS-spectra in Figure 1. Nevertheless, only few larger copper-nanoparticles with rod or polygonal shape were observed by TEM (inset: Figure 1c). This indicates that the supramolecular assemblages are mainly aggregates of primary hybrids which are loosely attached to each other.

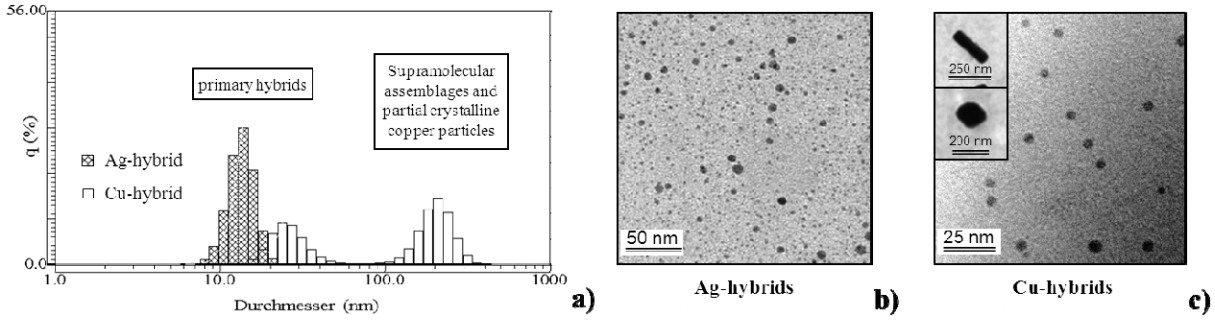

Fig. 1 (a) DLS-spectra of a dispersion of Ag-hybrids in chloroform and Cu-hybrids in ethylene-glycol; (b, c) TEM images of the corresponding metal-hybrids.

As illustrated by the data in Table 1 and in Figure 1, the described approach does allow the synthesis of very small, uniform and nanosized metal particle-hybrids having a high specific surface area.

3.2 Effect of the polymeric shell on the particle distribution

The effect of the polymeric outer shell of the hybrid-structure in reducing the tendency to form large particle agglomerates in the final application is demonstrated by the SEM- and TEM-pictures in Figure 2. In comparison with both, hydrophilic adjusted silver nanoparticles (d_{90} < 10 nm) as well as with micron-sized silver particles

(d_{50} ≈ 8 µm), it could clearly be seen that the Ag-hybrids are better dispersible under identical processing (mixing / distribution) conditions. Only few single agglomerates are still existent (Figure 2 a, c).

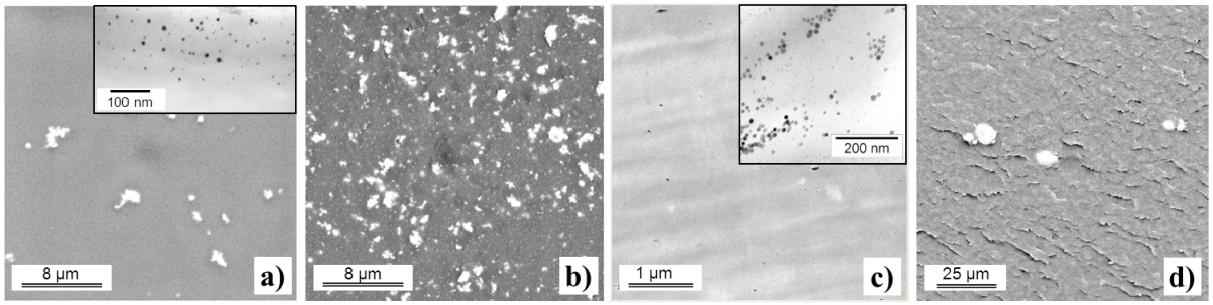

Fig. 2 (a) SEM (BSE) pictures of the distribution of Ag-hybrid and (b) of water dispersible Ag-nanoparticles in a surface coating application produced by solution blending; (c) distribution of Ag-hybrid and (d) micron-sized silver in the bulk of polyamide 6 after dispersion by melt extrusion (insets in (a) and (c): TEM-pictures of the same sample).

The presented results demonstrate the advantage in dispersibility due the higher compatibility of the hybrids opposite to "naked" metal particles or hydrophilic surface modified one. A direct outcome of these is a achievable higher grade of distribution under commonly applied processing procedures. This means a high specific surface area to volume ratio of the antimicrobial additive in the coating formulation or compound, which directly influences the biological efficacy.

3.3 Antimicrobial assessment

The modification of surface properties of plastics can be performed by incorporation of the active hybrid additives into coating formulations or into the bulk of a thermoplastic matrix material as described above. Table 2 gives few examples of the antimicrobial efficacy of polyamide 6 compounds, finally shaped into plates by injection molding, and polyamide-based surface coatings. Moreover, the comparison of compounds with even potential antimicrobial additives having particle sizes with a dimension in the micro meter range clearly reveals the advantage of the nanodisperse metal hybrids (cf. Figure 2). To achieve an equal level of activity it would be need more of the micro-size additives. But with increasing additive concentration further issues arises, e.g. influencing of mechanical properties.

Table 2 Antimicrobial activity R of modified polyamide compounds and coatings according to ISO 22196 and ISO 20743 respectively

Material	Function-alization	Metal content (wt-%)	Test germ	U_t	R (control)	R (sample)
PA / Ag-hybrid	Compound	0.13 Ag	*S.aureus*	1.3	0 ± 0.13	4.98 [3]
	Compound	0.33 Ag	*C.albicans*	1.5	0 ± 0.10	4.79 [3]
PA / Ag (micro), Ref.[1]	Compound	2.00 Ag	*S.aureus*	0.6	0 ± 0.10	3.80 ± 1.30
	Compound	2.00 Ag	*C.albicans*	---*	---*	---*
PA / Cu-hybrid	Coating	0.63 Cu	*S.aureus*	1.2	0 ± 0.19	5,45 ± 0.10
	Coating	1.89 Cu	*C.albicans*	1.3	0 ± 0.02	4.79 [3]
PA / Zn-hybrid	Compound	0.20 Zn	*S.aureus*	2.4	-0.03 ± 0.19	5.77 [3]
	Compound	0.40 Zn	*C.albicans*	2.0	0.03 ± 0.08	3.01 ± 0.97
PA / ZnB (micro), Ref.[2]	Compound	1.50 Zn	*S.aureus*	2.4	-0.03 ± 0.19	2.07 ± 0.42
	Compound	1.50 Zn	*C.albicans*	2.0	0.03 ± 0.08	0.85 ± 0.09

[1] Ag (micro) particle size d_{50} ≈ 8 µm, compound as shown in Figure 2 d; [2] Zinc borate particle size d_m ≈ 6 µm; [3] 100% mortality; * not tested

It is evident by the values in Table 2 that the activity of the nano-hybrids is noticeably higher than the efficacy of the reference compounds utilizing micron-sized antimicrobials. Independent thereof, it is also evident that usually a higher additive content is required to achieve a pronounced fungicidal activity (e,g, *C.albicans*) than just an antibacterial surface effect.

3.4 Biocompatibility assessment

For functionalization of medical devices further requirements must are met. Among others, the biocompatibility of the surface-active materials is an important issue.. Due to research in close collaboration with the University Medical Center in Jena regarding to biocompatibility properties of various polymeric materials functionalized by the metal-hybrids with silver, copper or zinc, there was none to only a slight effect on cell proliferation found in comprehensive tests according to DIN 10993-5. Particular material finishes with zinc-hybrid leads to an outstanding combination of antimicrobial surface activity and well biocompatible properties including compatibility with blood contact. Latter one is illustrated by the results in Figure 3. Usually, biomaterial surface properties can influence their coating with plasma proteins and are therefore known to strongly affect the blood clot formation [14]. The antimicrobial material functionalization by the zinc-hybrid does not show an effect on blood clotting over time with respect to the reference (neat polymer) and the control (empty test tube). Moreover, synthetic materials like polymers (e.g. implants) are also known to generate an immediate and complex object-related inflammatory reaction of the immune system indicated by the release of PMN-elastase. The in-vitro test results do not indicate such an effect for zinc-hybrid finishes in direct contact with human whole blood.

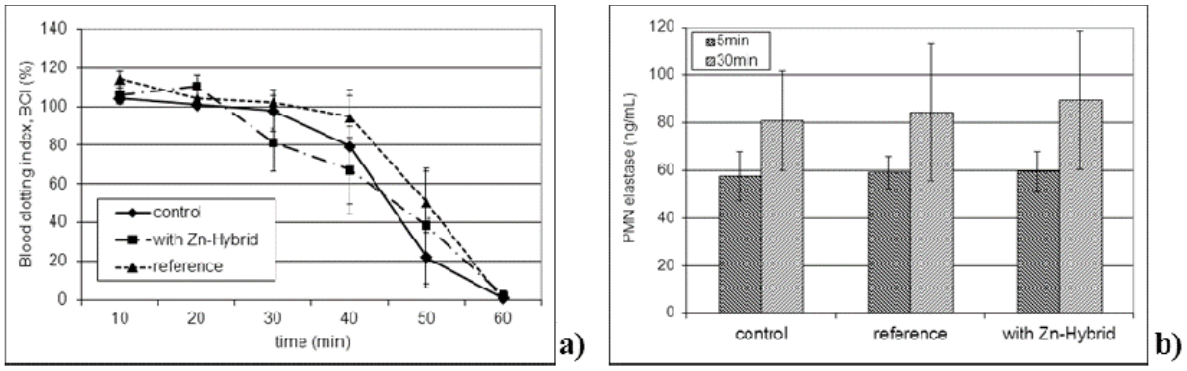

Fig. 3 (a) Influence of material extracts (72h-extracts) of antimicrobial polyamide 6 material functionalized with Zn-hybrid on blood clotting and (b) immune response by direct contact with human whole blood.

4. Conclusions

In this field of ongoing research, our investigation results demonstrate that the use of dendritic hyperbranched polymers as macromolecular carriers enable the design of a novel class of antimicrobial active release addition agents based on silver, copper or zinc and the respective bioactivity of their ions. These organic-inorganic-hybrids can be used as dispersions for surface coatings or deployed as addition agents in polymer bulk, which render the material surfaces with high germ-killing efficiency. The distribution and the specific surface / volume ration are crucial to achieve the highest possible inhibitor efficiency of the antimicrobials. Furthermore, especially a material functionalization with zinc-hybrid makes it possible to design compositions with antimicrobial surface properties in combination with extraordinary high cell- and hemocompatibility. Such functionalized polymeric materials are promising for many medical applications, e.g. wound dressings.

Acknowledgements The support of the projects InnoWatt, No. IW091073 and InnoKom, No. MF100065 is gratefully acknowledged.

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Antibiotic resistance patterns among common Gram positive and Gram negative pathogens in clinical isolates from major hospitals in Cairo, Egypt

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Antibiotic misuse and overuse in developing countries are leading causes of the spread of resistance worldwide. Studying the patterns of antibiotics resistance among patients admitted to local hospitals in populated areas as Cairo, Egypt can be used as an indicator for mechanisms behind the spread of resistance. Four major groups of microorganisms representing common pathogenic Gram positive bacteria: Methicillin-resistant *Staphylococcus aureus* and *Enterococcus* spp. as well as Gram negative bacteria: *Pseudomonas* spp. and *Enterobacteriaceae* were selected for this study. Clinical isolates were collected from hundreds of patients admitted to several hospitals in the greater Cairo area. Microscopic, cultural and biochemical tests were used to identify the isolates and confirm their proper classification. Antibiograms were carried out for the isolates against several antibiotics belonging to different classes. Minimum inhibitory concentrations were determined for the resistant isolates. Molecular approaches were followed to determine the presence of antibiotic resistance as well as virulence genes.

Keywords Antibiotic misuse; Resistance genes; MRSA; VRE; FQRP; ESBLs

1. Introduction

1.1 Background

The alarming rise of antibiotic resistance among leading pathogens is a global problem with the devastating consequence of depleting the antibiotic arsenal. Antibiotic misuse and overuse is a common problem in developing countries enhancing the spread of resistance to other regions as well. The greater Cairo metropolitan area in Egypt is one of the most densely populated regions in the world with a population reaching over 18 million inhabitants. A large proportion of the population in lower socioeconomic classes relies on general public and university hospitals spread in various locations in Cairo, for their medical needs. Studying the patterns of antibiotics resistance among patients admitted to these hospitals can be used as a measure for mechanisms behind the spread of resistance. Four major groups of microorganisms representing common pathogenic Gram positive bacteria: Methicillin-resistant *Staphylococcus aureus* and *Enterococcus* spp. as well as Gram negative bacteria: *Pseudomonas* spp. and *Enterobacteriaceae*, were selected for this study.

1.2 Methicillin resistant *Staphylococcus aureus* (MRSA)

Methicillin was developed in the late 1950's after penicillin failed to control staphylococcal infections [1]. However, by the early 1960's methicillin-resistant, (also known as multidrug resistant), *S. aureus* (MRSA) was isolated in a British hospital and in the following ten years, it became widespread in Europe, Australia and the United States [2]. Resistance to antibiotics is often acquired by the horizontal gene transfer from outside sources, although chromosomal mutations and antibiotic selection are also reported [3]. Vancomycin is the drug of choice for MRSA isolates. Patients unable to tolerate vancomycin have been treated with fluoroquinolones (the subset of quinolone-antibiotics in clinical use) [5].

1.3 Enterococcus

Enterococci are considered as a major causative agent of nosocomial infection. They can cause a group of serious diseases such as bacteremia, endocarditis, and urinary tract infections. The ability of enterococci to cause such diseases is due to acquisition of certain virulence factors such as heamolysin, gelatinase and

enterococcus surface protein [6]. Enterococci became a clinical concern due to emergence of strains resistant to many antibiotics specially vancomycin.

1.4 Pseudomonas

Pseudomonas aeruginosa is a major opportunistic pathogen that severely affects immunocompromised patients and burn victims [7]. As a result of its high resistance to antimicrobial agents, infections caused by *Pseudomonas aeruginosa* are associated with significant morbidity and mortality. The organism exhibits a high level of intrinsic resistance and only a limited number of antimicrobial agents are effective. Treatment with a combination of antibiotics is preferred to provide a wider antimicrobial spectrum and to prevent the rapid emergence of resistance.

1.5 Quinolones

Quinolones have been used to treat both nosocomial and community acquired infections. Quinolones act by binding to complexes that form between DNA and gyrase or topoisomerase IV inducing a conformational change in the enzyme. After the enzyme breaks the DNA, the quinolone prevents re-ligation of the broken DNA trapping the enzyme on the DNA and resulting in the formation of a quinolone-enzyme-DNA complex which inhibits replication [8]. Mutations in gyrase or topoisomerase IV enzymes produce changes that cause resistance to fluoroquinolones [9]. The mutations responsible for resistance occur in certain regions of each enzyme subunit called the quinolone-resistance-determining-region (QRDR), particularly in the *gyrA* and *gyrB* genes of topoisomerse II making the enzyme less sensitive to inhibition by fluoroquinolones or reducing the affinity between the enzyme-DNA complex and the drug [10].

1.6 AmpC β-lactamses

AmpC β-lactamases are enzymes that hydrolyze all β-lactam antibiotics except cefepime and imipenem. They are chromosomally encoded in the majority of *Enterobacteriacea* [11] where they are inducible except in *Escherichia coli* and *Shigella* spp. where they are constitutively expressed at very low level that do not cause resistance. Recently, plasmid-encoded AmpC β-lactamases have been detected even in species known not to have chromosomally encoded enzymes [12].

1.7 Extended spectrum beta lactamases

Extended-spectrum β-lactamases (ESBLs) emerged in numerous *Enterobacteriaceae* in many hospitals worldwide after the introduction of the extended spectrum cephalosporins in the 1980s [13]. These enzymes confer resistance to extended-spectrum cephalosporins and related oxyimino-β-lactams (ceftazidime, ceftriaxone and aztreonam). Beta-lactamase producers are typically gram negative organisms, mainly *Escherichia coli* and *Klebsiella pneumoniae*. They were also observed in *Proteus, Pseudomonas, Serratia, Enterobacter, Salmonella, Acinetobactor and Citrobacter* spp. [14]. Class A enzymes are further classified into the TEM and SHV variants commonly detected in *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates from the urinary tract, hospital-acquired respiratory tract, and bloodstream infections [15].

2. Methodology

2.1 Statement of Ethical Approval

All experiments, involving any samples taken from personnel, were done in accordance and approval of the ethical committee at Cairo University. All personnel who contributed any samples did this according to their written informed consent. In case of children, written consent from the guardian or parent was obtained.

2.2 General isolation and identification scheme

In all cases, clinical isolates were obtained from University hospitals affiliated either with Cairo University or Ain Shames University, in addition to their specialized hospitals as the National Cancer Institute or Children's hospitals. The isolates were identified according to flow charts of Bergey's Manual of Systemic Bacteriology beginning with microscopic examination and Gram staining, culture characters on selective and differential cultural media, biochemical tests characteristics of each group of microorganisms and final identification using API identification kits (BioMerieux, France). Antibiograms were carried using various groups of antibiotics

followed by MIC determination following CLSI, 2007 guidelines. PCR detection of virulence and resistance genes was carried followed by sequencing the amplified fragments.

3. Results and Discussion

3.1 Methicillin-resistant *Staphylococcus aureus*

A total of 94 clinical *S. aureus* isolates were collected and were identified morphologically as Gram-positive cocci and confirmed to be *S. aureus* by typical growth on mannitol salt agar medium and conventional biochemical tests (coagulase positive, oxidation/ fermentation positive, DNase positive). The findings of those conventional tests were confirmed by the API KB004 Histaph identification system. Isolates were confirmed as MRSA by positive agglutination using the Dryspot Staphytect Plus Kit, positive PBP2a test, blue colonies on ORSAB confirmatory media and antibiotic resistance to methicillin and oxacillin on susceptibility testing.

Among the 94 isolates, 49 isolates were sensitive to methicillin and oxacillin and 45 isolates were resistant and identified as MRSA. A total of 26 isolates from the MRSA collection were resistant to fluoroquinolones (ciprofloxacin and levofloxacin) as well. The antibiotic susceptibility pattern of the 26 MRSA isolates is shown in Table-1. For fluoroquinolone resistant MRSA, the MIC values for ciprofloxacin and levofloxacin in the presence and the absence of efflux pump inhibitors, omeprazole and piperine were determined. The reduction in MIC results of the tested fluoroquinolones in the presence of efflux pump inhibitors showed a much more significant effect in the majority of the isolates when ciprofloxacin was combined with omeprazole (81% of isolates) and piperine (96% of isolates) compared to levofloxacin when combined with omeprazole (23% of isolates) and piperine (58% of isolates). This might indicate the involvement of an additional mechanism of resistance in case of levofloxacin.

For the isolates that showed a decrease in ciprofloxacin MIC, in the presence of the efflux pump inhibitor omeprazole, ciprofloxacin uptake was measured in presence and absence of omeprazole. Five isolates showed an obvious increase in ciprofloxacin accumulation in presence of omeprazole, while seven others showed only a slight increase in accumulation, indicating the involvement of other mechanism of resistance, in addition to the activity of efflux pumps.

The QRDR regions of *gyrA* and *gyrB* genes, in the 12 quinolone resistant MRSA isolates tested for ciprofloxacin uptake, were amplified by PCR. Products of sizes 222 bp and 250 bp were obtained for *gyrA* and *gyrB* respectively. PCR products were sequenced in three of the twelve isolates to detect possible mutations. Results showed that for *gyrA* gene, the three isolates showed a C2402T mutation, which led to S84I substitution in GyrA. Two isolates showed a T2409C mutation which led to silent mutation at I86 in GyrA. One isolate showed a T2460G mutation leading to silent mutation at L103 in GyrA. For GyrB, the same three isolates showed a change in nucleotide T1497C and two isolates showed A1578G mutation leading to silent mutations at A439 and K465 in GyrB, respectively.

Table 1 Antibiotic resistance patterns of MRSA isolates

Antibiotic	Resistance
Ofloxacin	58%
Ampicillin/Sulbactam	55%
Amoxacillin/Clavulanic acid	100%
Cefepime	94%
Gentamicin	43%
Tetracycline	67%
Chloramphenicol	20%
Vancomycin	2%

Table 2 Antibiotic resistance patterns of *Enterococcus* spp. isolates

Antibiotic	Resistance
Penicillin	24%
Ampicillin	52%
Ciprofloxacin	30%
Gentamicin	56%
Erythromycin	41%
Teicoplanin	2%
Cefotaxime	100%
Streptomycin	100%
Chloramphenicol	16%
Tetracycline	68%
Vancomycin	2%

3.2 Enterococcus

A total of 73 isolates were identified as belonging to *Enterococcus* spp. By Gram stain; the isolates showed a Gram positive cocci or coccobacilli arranged in pairs and short chains. The identification of positive *Enterococcus* spp. was confirmed by other biochemical tests including catalase (catalase negative). Cultivation of the isolates on Enterococcosel Agar showed brownish-black colonies surrounded by a black zone. Isolates appeared as yellow colonies on CLED agar. Cultivation on MacConkey no.2 showed small intensely red colonies. Identification to species level was done by cultivation on HiCrome *Enterococcus faecium* Agar. A

total number of 26 isolate were identified as *Enterococcus faecium,* producing green coloured colonies along with yellow coloration of the medium. Meanwhile, 47 isolate were identified as *Enterococcus faecalis,* producing blue colonies without changing the color of the media.

Cytolysin production was screened by cultivation on blood agar, 9 isolates produced complete (β) haemolysis, 8 isolates produced partial (α) haemolysis and 56 isolate did not produce haemolysis on blood agar (ɣ) haemolysis. The *Enterococcus* spp. isolates were tested for resistance to different classes of antibiotics, the results are shown in Table-2. The MIC performed illustrated that two isolates were highly resistant to vancomycin. Virulence genes (*gelE, esp, cyl, agg*) and vancomycin resistance gene (*vanA*) were detected in several isolates by PCR (Figure-1). No mutations were detected in the sequenced fragments of virulence or resistance genes.

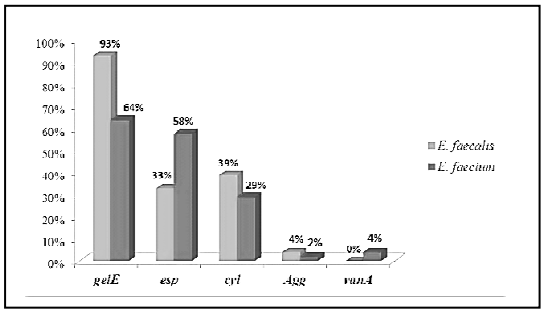

Fig. 1 Presence of virulence genes in *Enterococcus* spp. as detected by PCR

Table 3 Antibiotic resistance patterns of *Pseudomonas* spp. isolates

Antibiotic	Resistance
Amoxicillin	100%
Spiramycin	100%
Amoxicillin/ Clavulanic acid	79%
Azithromycin	81%
Clarithromycin	89%
Piperacillin	10%
Gentamicin	13%
Tobramycin	7%
Ciprofloxacin	9%
Levofloxacin	9%
Gatifloxacin	8%

3.3 Pseudomonas

A total of 103 isolates were identified as *Pseudomonas aeruginosa* out of clinical specimens collected. Isolates were confirmed by culture (green colonies on cetrimide agar, fluorescein and pyocyanin production on F and P agar) and oxidase test (positive). Antibiotic resistance patterns are shown in Table-3. Antibiotic sensitivity and MIC determination indicated the presence of nine isolates resistant to fluoroquinolnes (ciprofloxacin, levofloxacin and gatifloxacin). No effect on MIC was observed when the fluoroquinolens were combined with efflux pump inhibitors (omeprazole and piperine) suggesting that resistance of *Pseudomonas aeruginosa* towards FQs did not depend only on efflux pump.

When protein synthesis inhibitors chloramphenicol and tetracycline, reported to be substrates for *Pseudomonas aeruginosa* poly-specific efflux pumps (Mex-Opr), were added, the MIC to fluoroquinolones was raised, as the simultaneous expression of two or three Mex pumps is expected to have additive effects on the MICs of common effluxed substrates, while with gentamicin, the MIC was lowered which needs further investigation. Ciprofloxacin uptake by the fluoroquinolone resistant *Pseudomonas aeruginosa* isolates was measured, however, no significant difference was observed. The QRDR region of *gyrA* and *parC* was PCR amplified and sequenced. Mutations in QRDR region were (GyrA: T83I and ParC: S87L and E91K) and they were in agreement with previous studies on fluoroquinolone resistant *Pseudomonas aeruginosa*.

3.4 AmpC β-lactamase

A total of 258 clinical isolates were screened for AmpC β-lactamase production. A number of 139 isolates were screened positive and they were identified as *Escherichia coli*, *Pseudomonas* spp. *Klebsiella* spp. and *Acinetobacter* spp. From this collection, five isolates carried plasmid mediated AmpC β-lactamse as detected by multiplex PCR. Sequencing the PCR products showed that the AmpC β-lactamase genes of these isolates belonged to: CIT group genes (CMY-4 genes) in case of *Serratia marcescens* and one *Klebsiella* spp. isolate, CIT (CMY-6 gene) in one *E. coli* isolate and DHA group genes (DHA-1 genes) in one *Klebsiella* spp. and one *E. coli* isolates. This is the first report of detecting plasmid mediated AmpC β-lactamase in *Serratia marcescens*.

3.5 Extended spectrum beta-lactamases in Gram-negative rods

A total of 130 isolates were recovered from urine specimens from infected patients and subjected to proper identification schemes. The most predominant isolate was *E. coli* (27%), followed by *Proteus mirabilis* (19 %),

Pseudomonas aeruginosa (11.5%) and *Klebsiella pneumoniae* (11%). Initial and then phenotypic confirmatory tests revealed the presence of 38 isolates to have ESBLs, their prevalence is indicated in Figure-2 and antibiotic susceptibility patterns compared to the non ESBLs isolates in Figure-3. No ESBLs confirmed isolate was resistant to imipenem or meropenem which may indicate that these antibiotics are drugs of choice in treatment of infections caused by ESBL bacteria.

The sixteen ESBL positive *E. coli* isolates were further characterized revealing that they all had $bla_{\text{CTX-M}}$ gene but not the bla_{SHV} gene, while 69% of the isolates carried bla_{TEM} gene. Sequencing representative clones showed complete homology with the common region of $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-3}}$ and $bla_{\text{CTX-M-28}}$ which all belong to $bla_{\text{CTX-M-1}}$ group. The sequenced PCR product of bla_{TEM} gene showed complete homology with β-lactamase TEM precursor carried on *E. coli* plasmid pEK499, $bla_{\text{TEM-1b}}$ carried on *E. coli* plasmid pUMB-9 and inhibitor-resistant TEM beta-lactamase gene.

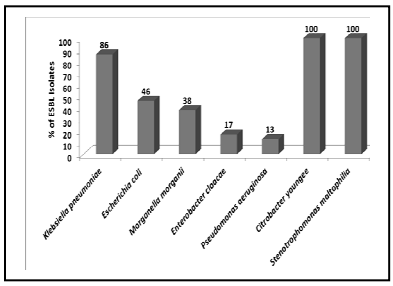

Fig. 2 Prevalence of ESBLs Isolates

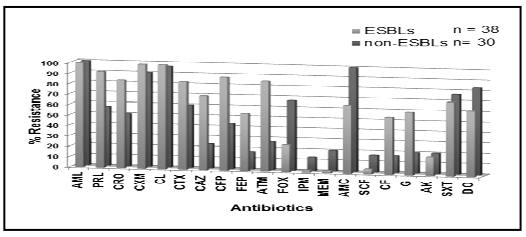

Fig. 3 Resistance pattern of ESBLs in comparison with non-ESBLs isolates.

Amikacin (AK), Amoxicillin (AML), Amoxicillin/clavulanic acid (AMC), Aztreonam (ATM), Cefepime (FEP), Cefoperazone (CFP), Cefoperazone/sulbactam (SCF), Cefotaxime (CTX), Cefoxitin (FOX), Ceftazidime (CAZ), Ceftriaxone (CRO), Cefuroxime *sodium (CXM), Cephalexin (CL), Ciprofloxacin (CF), Doxycycline (DO), Gentamicin (G), Imipenem (IPM), Meropenem (MEM), Piperacillin (PRL), Sulphamethoxazole/trimethoprim (SXT*).

4. Conclusions

The observed patterns of virulence and resistance in these collections of clinical isolates indicate that different mechanisms of resistance to antibiotics can exist in different isolates or even "coexist" within the same organism. Resistance to fluoroquinolones does not depend only on mutation in target genes (*gyrA, gyrB* and *parC*), other mechanisms of resistance are involved (permeability effect, efflux pumps and decreased availability of quinolones at target site). The highest prevalence of ESBLs was found in the *Klebsiella pneumoniae* (86%) followed by *E. coli* (46%). Clinical microbiology laboratories should routinely test and confirm the ESBLs production in all *Enterobacteriaceae*. Due to the diversity of resistance mechanisms and the constant appearance of new patterns, antibiotic utilization in developing countries should be under strict control and should be monitored to avoid the exhaustion of the antibiotic arsenal that is under intense use.

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Antifungal prophylaxis and treatment with micafungin in critically ill patients

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This was a retrospective single-centre observational study conducted in the Intensive Care Unit (ICU) including patients who received micafungin between 2010 and 2012. It was aimed to describe the epidemiologic data and variables of patients treated with micafungin and to compare if the finding of fungal colonization or fungal invasive infection was a predictive factor of poor clinical result.

Keywords: invasive candidiasis, antifungal treatment, echinocandins, micafungin, critically ill patients.

Introduction

The incidence of invasive fungal disease (IFI) in critically ill patients is difficult to know because of the lack of clear definitions and inaccurate diagnostic procedures. Moreover, there has been a steady increase in the rate of nosocomial fungal infections. Risk factors are very common in most hospitalized patients; therefore, it is often difficult to determine which patients are at greatest risk for developing it. These diseases are known to cause significant mobimortality in critically ill patients in spite of the development of new antifungal agents [1]. Micafungin, a new echinocandin, has very good antifungal activity with a favorable pharmacokinetic profile, few drug-drug interactions, and rare reports of resistance. Invasive fungal infections, especially in the critical care setting, have become an excellent target for prophylactic, empiric, and pre-emptive therapy interventions due to their associated high morbidity, mortality rate, increased incidence, and healthcare costs [2].

Patients and Methods

Design and setting

This was a retrospective single-centre observational study conducted in the ICU setting in routine clinical practice including every patient older than 18 years who received at least two doses of micafungin between 2010 and 2012. It was aimed to describe the epidemiologic data and variables of patients treated with micafungin and to compare if the finding of fungal colonization or fungal invasive infection was a predictive factor of poor clinical result.

Screening, microbiological cultures, and Candida score

Surveillance cultures for *Candida* spp. were performed using samples obtained from rectal smear, urine, axillary smear, tracheal aspirates, pharyngeal smear, catheter tip, drainage, peritoneal liquid and peripheral blood. Results were considered positive in the presence of *Candida* growth in the culture medium. *Candida* score (CS) was calculated as soon as the results of surveillance cultures were available at the beginning of antifungal therapy [3].

Definitions

Candida colonization was considered unifocal when *Candida* spp. was isolated from one site and multifocal when Candida spp. was simultaneously isolated from various noncontiguous sites, even if two different *Candida* spp. were isolated [14, 15]. The diagnosis of IFI required one of the following criteria: presence of candidemia, i.e., documentation of one or more blood culture(s) that yielded a Candida spp. in a patient with consistent clinical manifestations, isolation of *Candida* spp. from a normally sterile body fluids (e.g., pleural fluid,

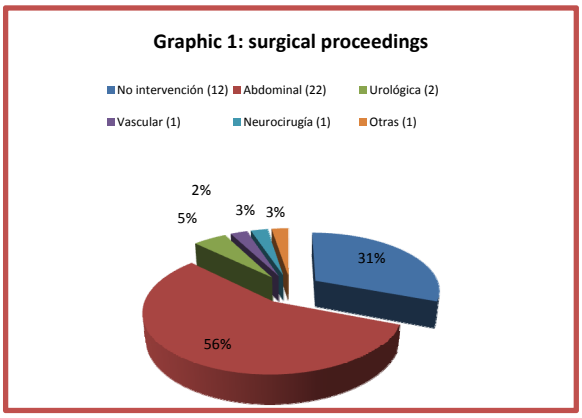
pericardial fluid) or candidal peritonitis, ophthalmic examination consistent with candidal endophthalmitis in a patient with clinical sepsis, or histologically documented candidiasis. Patients were classified in 3 groups: i) patients no colonized nor infected (NCNI), ii) Candida infected patients (IFI) y iii) Candida colonized patients but no infection confirmed [4].

Study protocol and collection data

The following data were recorded on the day of admission to ICU: age, sex, APACHE II score, SOFA score, Candida score, cause of admission, number and type of surgical proceedings, surveillance cultures, and the presence or absence of sepsis, severe sepsis or septic shock at the beginning of antifungal therapy. Patients were followed until ICU discharge or death.
Categorical variables are expressed as frequencies and percentages, and continuous variables as mean and standard deviation (SD).The data analysis was carried out using SPSS 11.5 program.

Results

A total of 39 patients were analysed (28 males (52%) and 11 females (21%)), with an average age of 59±14 years, APACHE II on ICU admission 21 ± 9, SOFA 8,5 ± 4 and CS 3,5±1 at the beginning of antifungal therapy. The cause of ICU admission was classified in medical origin: 16 patients (41%) or surgical 22 patients (59%). 19 patients (48,7%) were reoperated. The average of surgical proceedings was 1,4 ± 1,3 per patient. Abdominal surgery was the main cause of surgical proceedings and perforation of small bowel (SB) the most frequent among them. Graphic 1 summarizes the surgical proceedings.



Microbiologic results: Patients were classified in three groups depending on microbiologic results as: i) non colonized patients nor infected (NCNI): 14 patients (36%), ii) colonized patients with Candida without confirmed infection: 16 patients (41%) (abridged results in table 1 and graphic 2) y iii) patients infected with Candida (IFI): 9 patients (23%) (abridged results in table 2 and graphic 3).

Most frequently colonized samples were tracheal aspiration with 22 isolations (56,4%) and rectal smear with 7 isolations (17,9%). The specie more frequently isolated was *C. albicans* followed by *C. parasilopsis*. Most of colonized patients presented multifocal colonization (62,5% vs 37%). A high rate of infected patients presented simultaneously multifocal fungical colonization (6 out of 9).

All patients with IFI and colonization had a CS >= 3 at the begining of treatment. The cause of using Micafungin was: 46% CS >= 3, inmunosupression 15,4%, rescue (bad evolution) 30,8%, multifocal isolation 7,7%. Treatment was hold for 10±6 days. De-escalation was only used in 2 patients.
In order to show if the finding of fungal colonization or IFI was a predictive factor of poor clinical result we compared the three groups of patients. There were 14 patients in the neither colonized nor infected group, 16 in the colonization group, and 9 in the IFI group. Table 4 provides the clinical characteristics of the participants and table 5 summarizes the result variables (length of stay (LOS), days of mechanical ventilation, days of renal replacement therapy (RRT) and mortality).

100% of patients needed mechanical ventilation for an average of 20±14 days. RRT was used in 16 patients (41%) (mean 8±16 days). ICU average length of stay: 22±15 days. Mortality 27 patients (69,2%).

Table 1 and graphic 2: colonization results

TABLE 1: colonization results		
Number	Samples	Species
7 (17,9%)	Rectal smear	C. albicans (1) C. parasilopsis (1) C. Krusei (1) C. glabrata (1) C. spp (3)
1 (2,6%)	Axilar smea	C. parasilopsis (1)
2 (5,1%)	Urine	C. parasilopsis (1) C. spp (1)
22 (56,4%)	Tracheal Aspirated	C. albicans (12) C. parasilopsis (3) C. krusei (3) C. glabrata (2) C. tropicalis (1) C. spp (1)
3 (7,7%)	Faríngeal smear	C. parasilopsis (1) C. krusei (1) C. spp (1)
6 (15,4%)	Catheter tip	C. albicans (1) C. parasilopsis (2) C. krusei (1) C. glabrata (1) C. spp (1)
6 (15,4%)	Drainage	C. albicans (3) C. parasilopsis (1) C. spp (2)

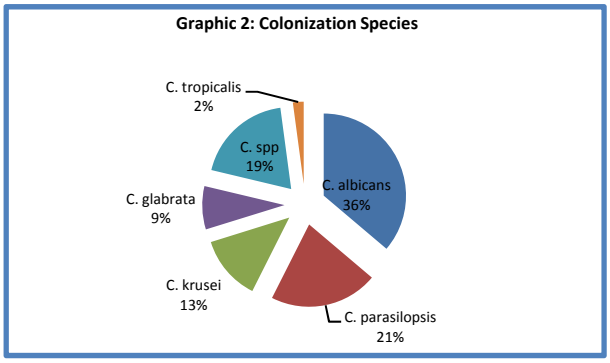


Table 2 and graphic 3: invasive candidiasis result

TABLE 2: IFI results		
Number (9)	Samples	Especies
4	Peritoneal Líquid	C. albicans (1) C. parasilopsis (1) C. glabrata (1) C. famata (1)
5	Blood	C. albicans (3) C. parasilopsis (1) C. glabrata (1)

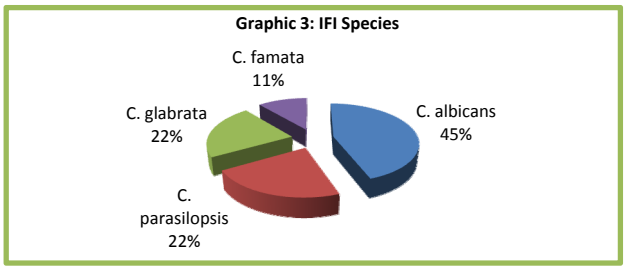


Table 4: characteristics of the study patients according to colonization and infection status

Table 4: Characteristics of the study patients according to colonization and infection status	IFI (N=9)	COL (N=16)	NCNI (N=14)
DEMOGRAPHIC VARIABLES			
Age	60,11 ± 14,5	56 ± 12,9	61,43 ± 14,5
Men/women %	66,7/33,3	68,8/31,2	78,6/21,4
APACHE II on admision	20,8 ± 6,1	21,9 ± 10,9	20,86 ± 9
SOFA on la admision	7,9 ± 4,5	9 ± 4,3	8,3 ±3,7
Type of patient (%)		1 (6,3%)	1 (7,1%)
Medical	3 (33,3%)	7 (43,8%)	6 (42,9%)
Surgical	6 (66,7%)	9 (56,3%)	8 (57,1%)
Mean of surgery proceedings	2,11 ± 1,76	1,31 ± 1,19	1 ± 0,99
Surgery (%)	7 (25,9%)	11 (40,7%)	9 (33,3%)
CS at begining of ATF	4,22 ± 0,97	3,75 ± 0,86	2,8 ± 0,89
ICU CAUSE OF ADMISSION.			
Septic Shock	0 (0%)	3 (18,8%)	3 (21,4%)
Emergency abdominal surgery	5 (55,5%)	3 (18,8%)	7 (50%)
Peritonitis	1 (11,1%)	2 (12,5%)	0 (0%)
Pancreatitis	2 (22,2%)	3 (18,8%)	0 (0%)
Sepsis	1 (11,1%)	2 (12,5%)	1 (7,1%)
Non septico Shock	0 (0%)	1 (6,3%)	1 (7,1%)
ACS	0 (0%)	1 (6,3%)	0 (0%)
Trasplant	0 (0%)	1 (6,3%)	0 (0%)
Neurological Problems	0 (0%)	0 (0%)	2 (14,3%)
RISKS FACTORS ASSOCIATED TO IFI			
ATB wide range	9 (100%)	15 (93,8%)	13 (92,9%)
Central Access	9 (100%)	16 (100%)	14 (100%)
Artery	9 (100%)	16 (100%)	14 (100%)
Urinary catheter	9 (100%)	16 (100%)	14 (100%)
MV	9 (100%)	16 (100%)	14 (100%)
TNP	7 (77,8%)	12 (75%)	6 (42,9%)
RRT	4 (44,4%)	7 (43,8%)	5 (35,7%)
Corticosteroids	0 (0%)	3 (18,8%)	3 (21,4%)

APACHE: Acute Physiology and Chronic Health Evaluation score. SOFA: Sepsis-related Organ Failure Assessment score. CS: candida score. ATF: antifungal. ATB: antibiotic. MV: mechanical ventilation. TNP: total parenteral nutrition. RRT: renal replacement therapy.

Table 5: result variables according to colonization and infection status

TABLE 5: Results variables	IFI (N=9)	Colonizated (N=16)	NCNI (N=14)
Hospital LOS prior to ICU	2,78 ± 4,5	4,25 ± 6,2	6,3 ± 8,3
UCI LOS	30,22 ± 20	23,5 ± 15	15,6 ± 8,7
Hospital days post UCI	4,22 ± 7,5	7,38 ± 19	11,6 ±15,8
MV days	23,7 ± 16,9	23,9 ± 14,9	13,4 ± 8,12
RRT Days	11,56 ± 26	9,6 ± 14,6	3,43 ± 6,1
	MORTALITY		
UCI	6 (66,7%)	13 (81,3%)	8 (57,1%)
Hospital	0 (0%)	1 (6,3%)	1 (7,1%)

LOS: lengh of stay. ICU: intensive care unit. MV: mechanical ventilation. RRT: renal replacement therapy.

Discussion

The diagnosis of invasive candidiasis (IC) in patients admitted to the ICU still poses a challenge [4]. Delay in diagnosis and the beginning of therapy increase significantly the mortality. We observed a mortality rate (69,2%) higher than expected according to the average APACHE II (38,9%), but similar to mortality rates reported in other studies associated with this pathology.

Although there were no differences in severity scores on admission to ICU, we observed a higher CS in patients that afterwards developed IFI. Most frequently colonized samples were tracheal aspiration and rectal smear, being *C. albicans* the most prevalent specie. The average of surgical proceedings in IFI patients was also higher. The most prevalent cause of admission for colonized and infected patients was emergency abdominal surgery, being small bowel (SB) perforation the one associated with more IFI. The percentage of patients that needed total parenteral nutrition (TPN) was higher in those with IFI and colonization than in NCNI. The amount of patients that needed RRT was higher in those with IFI as well as in days of use. The same occurred for LOS in ICU as well as days of MV. Mortality was higher in colonized patients and with IFI although it was also high in NCNI probably due to be very severely ill patients.

41% of patients were treated with micafungin presenting neither colonization nor fungal infection. Obtaining fungal marks will avoid many not well justified treatments and will cut down costs. We observed a scarce de-escalation strategy in spite of being effective as demonstrated in many studies. High rate of severity illness could have influenced this finding.

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Antimicrobial activities of spices and herbs

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Spices and herbs have been long used for thousands of centuries by many cultures to enhance the flavor and aroma of foods. Ancient societies recognized the value of using spices and herbs in preserving foods and for their medicinal value. During the 19th century, scientific experiments have documented the antimicrobial properties of some spices, herbs and their components. In this review, we pointed out some of the effects of different spices and herbs on microorganisms; particularly, that cause food poisoning.

Keywords antimicrobial effect, spices, herbs, food poisoning.

1. Introduction

Spices and herbs have been long used for thousands of centuries by many cultures to enhance the flavor and aroma of foods. Early cultures also recognized the value of using spices and herbs in preserving foods and for their medicinal value. Scientific experiments since the last 19th century have documented the antimicrobial properties of some spices, herbs and their components [1, 2]. Plant-derived antimicrobial compounds have been recognized for hundreds of years as a means of inhibiting undesirable bacteria. Numerous reviews and research papers have described the antimicrobial properties of plant and plant extracts, such as oregano, thyme and their essential oils (EO) [3, 4**].** garlic and its extract [5, 6], and black cumin seed and its volatile oil [7]. So, plant essential oils are receiving particular attention and widely studied feature as antimicrobial, which is important both for food preservation and the control of human and plant diseases of microbial origin. This aspect assumes a particular relevance, since an increased resistance of some very dangerous bacterial strains to the most common antibiotics has recently been observed consequently, the use of these natural substances in both pharmaceutical and food fields may be valuable for food storage free from contamination as well as controlling plant and human diseases of microbial origin [8, 9, 10]. As well as, plant derived products have been used for medicinal purposes for centuries. At present, it is estimated that about 80% of the world population rely on botanical preparations as medicines to meet their health needs. Herbs and spices are generally considered safe and proved to be effective against certain ailments. They are also extensively used, particularly, in many Asian, African and other countries. In recent years, in view of their beneficial effects, use of spices/herbs has been gradually increasing in developed countries also [11]. Naturally occurring antimicrobial compounds could be applied as food preservatives to protect food quality and extend the shelf life of foods and beverages. These compounds are naturally produced and isolated from various sources, including plants, animals and microorganisms, in which they constitute part of host defence systems. Many naturally occurring compounds, such as nisin, plant essential oils, and natamycin, have been widely studied and are reported to be effective in their potential role as antimicrobial agents against spoilage and pathogenic microorganisms. Although some of these natural antimicrobials are commercially available and applied in food processing, their efficacy, consumer acceptance and regulation are not well defined [12]. Therefore, there has been an increasing interest in the use of natural substances such as spices, herbs and their extracts which cause no health problem to the handlers and consumers due to their availability, fewer side effects, fewer toxicity as well as better biodegrability compared to the available antibiotics and chemical preservatives which are corrosive and carcinogenic [13].

2. Antimicrobial compounds in spices and herbs

Essential oils extracted from spices and herbs are generally recognized as containing the active antimicrobial compounds. Table 1 is outlined a list of the proximate essential oil content of some spices and herbs and their antimicrobial components.

Spices and herbal plant species have been recognized to possess a broad spectrum of active constituents that exhibit antimicrobial (AM) activity. These active compounds are produced as secondary metabolites associated with the volatile essential oil (EO) fraction of these plants. A wide range of AM agents derived from EOs have the potential to be used in AM packaging systems which is one of the promising forms of active packaging systems aimed at protecting food products from microbial contamination. Many studies have evaluated the AM activity of synthetic AM and/or natural AM agents incorporated into packaging materials and have demonstrated effective AM activity by controlling the growth of microorganisms [14]. Allicin and allyl

isothocyanate are sulfur-containing compounds. Allicin, isolated from garlic oil, inhibits the growth of both Gram-negative and Gram-positive bacteria. Sulfur-containing compounds are also present in onions, leeks, and chives. Eugenol, carvacrol, and thymol are phenol compounds and found in cinnamon, cloves, sage, and oregano as indicated in Table 1. The essential oil fraction is particularly high in cloves, and eugenol comprises 95% of the fraction. The presence of these compounds in cinnamon and cloves, when added to bakery items, function as mold inhibitors in addition to adding flavor and aroma to baked products.

Table 1 Antimicrobial components of spices and herbs*

Spice / herb	Proximate essential oil content (%)	Antimicrobial component(s)
Garlic	0.3 - 0.5	Allicin
Mustard	0.5 - 1.0	Allyl isothiocyanate
Cinnamon	0.5 - 2.0	Cinnamaldehyde, Eugenol
Sage	0.7 - 2.0	Thymol, Eugenol
Oregano	0.8 - 0.9	Thymol, Carvacrol

* Adapted from [1]

3. Antimicrobial effectiveness of spices and herbs

Due to recent outbreaks of contaminations associated with food products, there have been growing concerns regarding the negative environmental impact of packaging materials of antimicrobial biofilms, which have been studied. Chitosan has a great potential for a wide range of applications due to its biodegradability, biocompatibility, antimicrobial activity, nontoxicity and versatile chemical and physical properties [15]. Table 2 is showed the relative antimicrobial effectiveness of some spices and herbs.

Table 2 Antimicrobial effectiveness of spices and herbs*

Spices and herbs	Inhibitory effect
Cinnamon, cloves, mustard	Strong
Allspice, bay leaf, caraway, coriander, cumin, oregano, rosemary, sage, thyme	Medium
Black pepper, red pepper, ginger	Weak

* Adapted from [2]

Studies in the past decade confirm that the growth of both gram-positive and gram-negative foodborne bacteria, yeast and mold can be inhibited by garlic, onion, cinnamon, cloves, thyme, sage, and other spices. Effects of the presence of these spices / herbs can be seen in food products such as pickles, bread, rice, and meat products. The fat, protein, water, and salt contents of food influence microbial resistance. Thus, it is observed that higher levels of spices are necessary to inhibit growth in food than in culture media [1]. Antimicrobial investigations on extracts are presented where the most active plants are identified from screening publications. A summary of some bioactive compounds are given with data restricted to papers reporting quantitative antimicrobial activity equivalent to or below 200 microg/ml. Antimicrobial activities on the essential oils of indigenous medicinal aromatic plants are also reviewed [16]. The antibacterial activity of two varieties of garlic (ophioscordon and sativum) against enteric pathogens such as *Escherichia coli, Proteus mirabilis, Salmonella typhi, Shigella flexineri and Enterobacter aerogenes* was studied. Aqueous extract of both the garlic varieties inhibited the growth of enteric pathogens at the concentrations of 200,300,400 and 500mg. However *Enterobacter aerogenes* was not susceptible to the aqueous extract of both the garlic varieties. Ethanolic extract of sativum was found to be highly effective against all the bacteria tested. HPTLC analyses of garlic varieties confirm the presence of allicin in various concentrations. Further analysis using GC-MS identified other compounds such as n-hexadecanoic acid, 3-deoxy-d-mannoic lactone, thymine and hexanedioic, bis (2-ethylhexyl) ester [17]. Table 3 is described a list of various spices and herbs and their inhibitory effect on various microorganisms. As well as, many scientific citations support antimicrobial properties of spices and herbs and their extracts. Fat'hi [18] investigated the effect of two concentrations of both potassium sorbate (0.1 and 0.2%) and grind black cummin (*Nigella sativa*) (1% and 3%) on the survival of 4 strains of *Yersinia enterocolitica* (two standard strains, American serotype 0:8 and European serotype 0:9, and other two locally isolated strains from sausage) inoculated into minced meat. They found that 0.1 and 0.2% potassium sorbate and 1% and 3% *Nigella sativa* induced a decrease in numbers of *Yersinia enterocolitica* and higher concentrations of them exhibited more inhibition of counts this organism than low concentrations. Garlic appears to satisfy all of

the criteria for antibacterial agents, being cheap and safe. The historical view that garlic can 'cure all' may not be unjustified. Since the introduction of antibiotics there has been tremendous increase in the resistance of many bacterial pathogens. Because garlic is known to act synergistically with antibiotics, and resistance has not been reported for garlic, more dose-response preclinical studies and eventually clinical studies should be done to assess the use of an antibiotic/garlic combination for bacteria that are difficult to eradicate [29].

Table 3 Inhibitory Effects of Spices and Herbs

Spice / herb	Microorganisms	Reference
Garlic	*Salmonella typhymurium, E. coli*, *E. coli* O_{157}:H_7, *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*, mycotoxigenic *Aspergillus*, *Candida albicans*, *Aeromonas hydrophila*	[11, 19, 20, 21]
Onion	*Aspergillus flavis, Aspergillus parasiticus*	[6]
Cinnamon	*Mycooxigtenic Aspergillus, Aspergillus parasiticus, Aeromonas hydrophila*	[20, 22, 23, 24]
Cloves	*Mycotoxigenic Aspergillus, Aeromonas hydrophila*	[7, 20, 24]
Mustard	Mycotoxigenic *Aspergillus*	[20]
Allspice	Mycotoxigenic *Aspergillus*	[7, 20]
Oregano	*Mycotoxigenic Aspergillus, Salmonella spp., Vibrio parahaemolyticus, E. coli* O_{157}:H_7	[19, 20, 23, 25, 26]
Rosemary	*Bacillus cereus, Staphylococcus aureus, Vibrio parahaemolyticus*	[27]
Bay leaf	*Clostridium botulinum*	[28]
Sage	*Bacillus cereus, Staphylococcus aureus, Vibrio parahaemolyticus*	[18, 27]
Thyme	*Vibrio parahaemolyticus, Aeromonas hydrophila, E. coli* O_{157}:H_7	[11, 24, 25, 26]
Black cummin (*Nigella sativa*)	*Yersinia enterocolitica*	[8]

Antimicrobial activity was observed for a number of the plant samples against at least one or more pathogen. Tarchonanthus camphoratus (solvent extract) showed the most significant broad spectrum activity with minimum inhibitory concentration (MIC) values ranging between 0.50 and 0.70 mg/ml against five of the six pathogens tested. The most noteworthy susceptibility for the aqueous extracts was noted with the plant extract Syzygium cordatum (MIC value 0.1 mg/ml against *Candida albicans*). While, the most noteworthy activity for the essential oils was observed for *Tarchonanthus camphoratus* (0.8 mg/ml) against *Oligella ureolytica* [16]. Antimicrobial activity of garlic (*Allium sativum*) and its extract has been recognized for many years in all parts of the world. Scientific studies made on garlic in 20th century revealed that it was effective against a wide variety of microbial pathogens [6]. Antibacterial activity of extracts of *Allium sativum* (garlic), *Myristica fragrans* (nutmeg), *Zingiber officinale* (ginger), *Allium cepa* (onion) and Piper nigrum (pepper) has been evaluated against 20 different serogroups of *Escherichia coli*, 8 serotypes of *Salmonella, L. monocytogenes* and *Aeromonas hydrophila*. Garlic extract showed excellent antibacterial activity against all the test organisms, except *L. monocytogenes*. Nutmeg showed good anti-listerial activity, although activity against E. coli and Salmonella were serotype dependent. Both garlic and nutmeg extracts were effective against A. hydrophila. Extracts of ginger showed inhibitory activity against two serogroups of *E. coli*: as O8 (enterotoxigenic *E. coli*) and O88 only. Extracts of onion and pepper did not show any antibacterial activity against the test organisms [23]. Four spices (garlic, oregano, thyme and black cumin seed) with different concentrations (0, 0.5, 1.0, 3.0, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5%) were tested for activity against *E. coli* O_{157}:H_7 in MacConkey sorbitol agar (MSA). The obtained results showed that garlic had the strongest inhibitory action, followed by oregano and thyme, which had medium inhibition, while black cumin seed had the weak inhibitory activity. Aqueous extract of garlic and essential oils of oregano, thyme and black cumin seed were tested for their inhibitory activity against *E. coli* O_{157}:H_7. The MIC of garlic extract against *E. coli* O_{157}:H_7 was 1.56% (w/v), while the MLC was 3.12% (w/v). The inhibitory effect of various EO concentrations indicated that oregano EO followed by thyme EO had the highly inhibitory effects. Spices and spice extracts which had the best inhibitory effects against *E. coli* O_{157}:H_7 in the laboratory medium and in-vitro study, should be chosen and tested for the food model study. The obtained results showed that garlic 3% has the highest inhibitory effect against *E. coli* O_{157}:H_7 at the 3rd day of storage with reduction rate of 100% [19]. Effectiveness of selected essential oils for the control of growth and

survival of pathogenic microorganisms of significant importance in food hygiene were examined. MIC and MBC were determined by the tube dilution method. Essential oils from Thymus vulgaris from Spain and France, *Salvia sclarea*, *Salvia officinalis*, *Salvia lavandulifolia, Lavandula latifolia, Lavandula angustifolia*, three hybrids of *Lavandula latifolia* x *Lavandula angustifolia* (Lavandin 'Super', Lavandin 'Abrialis', and Lavandin 'Grosso'), *Rosmarinus officinalis, Hissopus officinalis*, and *Satureja montana* were evaluated. Inhibition ranged from the strong activity of *Satureja montana* and *Thymus vulgaris* (France) to no inhibition with *Salvia sclarea* and *Hissopus officinalis* for each of the test strains: *Salmonella enteritidis, Salmonella typhimurium, E. coli* O_{157}:H_7, *Yersinia enterocolitica, Shigella flexneri, L. monocytogenes* serovar 4b, and *Staph. aureus*. Because some of the essential oils were highly inhibitory in small quantities to selected pathogenic microorganisms, they may provide alternatives to conventional antimicrobial additives in foods [30].

Table 4 Antibacterial activity of different concentrations of aqueous garlic extract (AGE) by agar well method

Test organisms	Diameter of Inhibition zone mm) of different concentration			
	100%	50%	25%	10%
Aeromonas caviae	26	14	8	0
A. hydrophila	27	15	11	0
A. sobria	25	14	7	0
Chromobacterium violaceum	40	22	12	0
Escheichia coli	33	16	10	0
Enterobacter faecalis	42	23	12	0
Klebsiella pneumonia	39	21	10	0
Proteus mirabilis	22	11	6	0
Pseudomonas aeruginosa	25	12	7	0
Salmonella mgulani	36	18	10	0
S. typhi	44	21	10	0
S. roan	42	20	9	0
S. senflenberg	45	23	12	0
S. typhimurium	38	18	8	0
S. weltevreden	40	20	9	0
Bacillus subtilis	54	28	15	0
Staphylococcus aureus	33	16	8	0

Source [29]

Indeed, allicin and garlic extract have been shown to have a wide spectrum of antibacterial activity, including effects on *Escherichia, Salmonella, Staphylococcus, Streptococcus, Klebsiella, Proteus, Clostridium, Mycobacterium and Helicobacter* species [26, 31]. A 1% solution of garlic from fresh cloves has been shown to have antibacterial activity against E. coli and antibiotic-resistant (methicillin) *Staph aureus*, *Salmonella*, a common cause of food poisoning, and *Candida albicans*, the most common yeast infection [27]. Garlic exhibits strong activity against bacteria, even multi-drug resistant strains, as well as against fungi (yeast) and viruses [32]. Animals could be given natural antibiotics like garlic and oil of oregano and these germ-killers can also be placed in foods to kill off any pathogens and to reduce spoilage. Oil of oregano has been shown to exhibit the greatest inhibition of bacteria among food spices in a recent test against pathogens such as *E. coli, Staph aureus*, *Pseudomonas* and others germs [24]. A study conducted at Cornell University found that spices such as garlic, oil of oregano, onion, and allspice kill every bacterium tested, even anthrax [33]. On the other hand, [34] mentioned that spices have been shown to possess medicinal value, in particular, antimicrobial activity. This study compares the sensitivity of some human pathogenic bacteria and yeasts to various spice extracts and commonly employed chemotherapeutic substances. Of the different spices tested only garlic and clove were found to possess antimicrobial activity. The bactericidal effect of garlic extract was apparent within 1 h of incubation and 93% killing of *Staph. epidermidis* and *Salmonella typhi* was achieved within 3 h. Yeasts were totally killed in 1 h by garlic extract but in 5 h with clove. Some bacteria showing resistance to certain antibiotics were sensitive to extracts of both garlic and clove. Greater anti-candidal activity was shown by garlic than by nystatin. Spices might have a great potential to be used as antimicrobial agents. Zaika [2] has given an excellent summary of the antimicrobial effectiveness of spices and herbs. This summary can be rephrased as follows; microorganisms differ in their resistance to a given spice or herb, bacteria are more resistant than fungi, spores might be more affected, Gram-negatives are more resistant, spice or herbal effects could be germicide or germistatic, they may contain microbes and promote growth and toxins, their concentrations added as food preservatives are too low in order to be preventive, and other factors; e.g. NaCl may contribute synergistically. Chemical and biochemical antimicrobial compounds derived from natural sources and their activity against a

range of pathogenic and spoilage microorganisms pertinent to food, together with their effects on food organoleptic properties, are outlined. Factors influencing the antimicrobial activity of such agents are discussed including extraction methods, molecular weight, and agent origin. These issues are considered in conjunction with the latest developments in the quantification of the minimum inhibitory (and noninhibitory) concentration of antimicrobials and/or their components. Natural antimicrobials can be used alone or in combination with other novel preservation technologies to facilitate the replacement of traditional approaches [35]. Thus, food product safety and shelf life depend in some part on the type, quantity, and character of spices and herbs added to the products.

Conclusion

Although the antimicrobial activity of some spices and herbs is documented, the normal amounts added to foods for flavour is not sufficient to completely inhibit microbial growth. The antimicrobial activity of many spices and herbs varies widely, depending on the type of spice or herb, test medium, and microorganism. For these reasons, spice antimicrobials should not be considered as a primary preservative method.

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Antimicrobial activities of triterpenoids against Methicillin-resistant *Staphylococcus aureus* (MRSA)

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In this study the attempt has been made to advance our understanding of the nature and the mode of the action/s of a range of triterpenoid isolated from natural products. Some of these triterpenoids mimic a well known antibiotic, Fusidic acid. Fusidic acid is a narrow spectrum antibiotic, used primarily against Gram-positive bacteria. Most wild-type strains of *Staphylococcus, Streptococcus, Neisseria, Corynebacterium, Clostridium* and some mycobacteria are sensitive to Fusidic acid. It is also used to treat topical skin infections and infected wounds and burns caused by sensitive strains of *Staphylococcus aureus, Streptococcus* species and *Clostridium. minutissimum*. Reports of the resistance to Fusidic acid have been recently increased, mainly in hospitals where cross infections are common. The rate of resistance is higher in methicillin-resistant strains of *S. aureus* (MRSA) and it occurred by a number of mechanisms that includes alterations in EF-G and drug permeability. Fusidic acid had shown the capacity to stabilize the ribosome-translocation factor-GDP complex. It was shown that the presence of a carboxyl group at C-20 and a 17, 20-double bond was critical in forming the complex. The positions of carboxylic group, the double bond of the side chain of Fusidic acid and the configuration are different with these triterpenoids. This class of compounds showed a wide range of Minimum Inhibitory Concentration (MIC) from 0.01-100 mg/mL against MRSA, which is a very promising data.

Keywords; MRSA, Triterpenoids, SRA

1. Introduction

The emergence of antimicrobial resistance in hospitals and the community has compromised the physicians' ability to treat serious infections [1], in particular methicillin-resistant *Staphylococcus aureus* (MRSA) which causes surgical-site infections. The treatment and the management of MRSA has a big social and financial burden, particularly on developing and undeveloped countries whereas the antibiotics are readily available to patients.

Antimicrobial resistance is specifically a concern in the management of post-operative complications of cardiac disease and infections [2]. Antibiotic prophylaxis has been routinely practiced to prevent surgical-site cardiac infections, such as mediastinitis and endocarditis [3]. The cardiac surgical procedures are different from other surgeries due to use of a cardiopulmonary bypass, thus significantly compromise immunity and reduce phagocytosis, predisposing cardiac patients to infectious complications [4]. The new classes of antibiotic are needed to tackle these problems.

Triterpenoids are extracted from plants are shown some antimicrobial activities against variety of Gram-positive and Gram-negative bacteria [5-8]. These plants are traditionally used for medicinal purposes around the globe [9, 10].

Two most commonly occurring genera; Combretum and Terminalia, from Combretaceae family, each with almost 250 species [11], are widely used in African traditional medicine. Pentacyclic tritepenes isolated from Combretum imberbe have shown antimicrobial activities against *Mycobacterium fortuitum* (*M. fortuitum*) and *Staphylococcus aureus* (*S. aureus*).

It has also been reported that oleanolic acid and ursolic acid had antimicrobial activities against vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) with the minimum inhibitory concentrations (MICs) 8 and 4mg/ml, respectively [12]. In this study the isolated/purchased triterpenoids were screened against MRSA.

2. Material and Methods

MRSA strains OM481was obtained from our institute [7]. Moronic acid, Oleanonic acid, Ursonic acid, Oleanolic acid, Isomasticadienonic acid, 3-epi-isomasticadienolic acid, Masticadienonic acid, Dihydromasticadienonic acid, 3-O-acetyl-3epi(iso)masticadienolic acid, Masticadienolic acid, Dihydromasticadienolic acid, 3-acetoxy-3- epiisomasticadienolic acid and 3-acetoxy-3-epimasticadienolic acid were isolated as described previously [7]. The MICs of these isolated triterpenoids were determined as described previously [7] with cells of MRSA.

3. Results and Discussion

The MICs for the tested triterpenoids are shown in table 1.

Table 1. The MIC values of the triterpenoids against the MRSA strain OM481 (μg/mL)

Isolated triterpenoids	MRSA OM481
Moronic acid	100
Oleanonic acid	10
Ursonic acid	10
Oleanolic acid	10
Isomasticadienonic acid	5
3-epi-isomasticadienolic acid	10
Masticadienonic acid	5
Dihydromasticadienonic acid	5
3-O-acetyl-3epi(iso)masticadienolic acid	0.01
Masticadienolic acid	5
Dihydromasticadienolic acid	1
3-acetoxy-3- epiisomasticadienolic acid	0.1
3-acetoxy-3-epimasticadienolic acid	0.5

As it is shown the MICs ranged from 0.01-100 μg/mL, with the exception of Moranic acid, these MICs are within the range of conventional antibiotics and it is a very promising data. There are two major groups within these triterpenoids; Olean base skeleton and Lanosta base skeleton. The latter mimics Fusidc acid and have the same mode of the action as it has been reported elsewhere [7]. The MICs are dramatically increased with methylated of both types, indicating the significance of the polarity. The presence of a carboxyl group at C-20 and a 17, 20-double bond are critical in forming the complex [7]. The positions of carboxylic group, the double bond of the side chain of Fusidic acid and the configuration are different with these triterpenoids, however, it does not seem to alter the activities significantly.

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Antimicrobial activity and chemical composition of the essential oil of *Satureja thymbra* L. from Lebanon and comparison with its major components

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The antimicrobial activity and essential oil composition of *Satureja thymbra* L., a native plant from Lebanon, were studied. The essential oil was obtained from the aerial parts before the flowering stage by hydrodistillation for 3h, and analyzed by GC/MS. Twenty seven compounds representing 97.44% of the total oil were identified. The major components were phenolic monoterpene: thymol (28.43%) followed by ρ-cymene (19.89%), γ-terpinene (20.64%) and carvacrol (5.53 %) then acyclic monoterpene alcohol linalool (2.16%) and terpinolene (0.19%). The whole oil and each main component alone were assayed by applying the broth dilution and disc diffusion methods for antibacterial activity against 2 strains of ATCC certified bacteria "*Escherichia coli* and *Pseudomonas aeruginosa*". The whole oil of *S. thymbra* and carvacrol were found to be highly bactericidal, as it has shown lowest MBC values and high growth inhibition zone diameter in comparison to certain antibiotics.

Keywords: Essential oil; antimicrobial activity; *Satureja thymbra;* thymol; carvacrol; ρ-cymene; γ-terpinene.

1. Introduction

Accessibility and affordability of the medicinal herbs have made them as fundamental part of many people's life all over the world. Considering the failure to acquire new molecules with antimicrobial properties from microorganisms, the identification of antimicrobials from other natural sources is of great importance.

The genus *Satureja* L. (savory) belonging to the Lamiaceae family contains about 200 species of aromatic herbs and shrubs that are endemic in the Mediterranean region and characterized by a similar oregano-like smell. Over 30 species of this genus are distributed in eastern parts of Mediterranean area [1]. It is frequently used as a spice and herbal tea in the Lebanese traditional medicine where it is used to relieve minor digestive discomfort and bronchial congestion as well as being used in the food industry for flavour, aroma and preservation. The leaves have recently gained much popularity as a remedy to combat hypercholesterolemia [2].

It is well known that, the chemical composition of essential oils (EO) depends on climatic, seasonal and geographic conditions, harvest period, and distillation technique. In addition, their antibacterial activity depends on the type, composition and concentration of the essential oils, the type and concentration of the target microorganism, the composition of the substrate, the processing and the storage conditions [3, 4].

In the present study, *Escherichia coli* and *Pseudomonas aeruginosa* were screened for their susceptibility to essential oil extracted from *Satureja thymbra* through hydrodistillation by broth dilution and disk diffusion methods with the aim to evaluate both methods for their influence on the antimicrobial activity and to determine to what extent the antibacterial activity is related to the content of carvacrol, thymol, *ρ*-cymene or γ-terpinene.

2. Material and Methods

2.1 Plant material

The sample of *Satureja thymbra* L. has been harvested before the flowering period, during the second week of October, a prairie at Nahr Brahim 200 m altitude. Only the aerial parts (leaves and stems) were collected in the early morning so that the plant material is as fresh as possible.

The identity and the systematics of the plant have been confirmed by Dr Marc El Beyrouthy according to the keys of the new flora of Lebanon and Syria and voucher specimens were deposited at the Herbarium of the Faculty of Agricultural and Food Sciences (USEK).

2.2 Isolation of essential oil

The essential oils of the species were obtained by the Clevenger hydrodistillation method. 50 grams of fresh aerial parts were mixed with distilled water in a glass bulb connected to a Clevenger-type apparatus and subjected to hydrodistillation for 3 hours according to the method described in the European Pharmacopoeia (1997). The Condensed oils were collected and dried using anhydrous sodium sulfate and after filtration stored at 4°C until analysis by GC/MS.

2.3 Identification and quantification of the oil components

S. thymbra E.O. composition was analyzed by GC using a Varian gas chromatograph CP3800 coupled with Mass Detector 1200 MS/MS. The split/splitless injector model 1177 was at 280 °C in split 1:100. The CP 8400 Autosampler was injecting each time 1 µl of each sample. The carrier gas was helium, at a working constant flow rate of 1 ml/min. GC/MS analysis were done using a fused silica capillary column Factor Four VF-5 ms, measuring 30 m x 0.25 mm internal diameter, film thickener of 0.25 µm. The temperature was programmed at 40^{o}C (5min) with an increase of 5^{o}C per minute until 310^{o}C (1 min). Mass spectra were recorded at 70 eV, Manifold 40°C, Ion Source temperature 280°C, Transfer line 320°C, Acquisition: Full Scan 40 – 800 amu.

Kovat's indices (KI) of all constituents were determined by Kovat's method by co-injection of the samples with a solution containing the homologous series of n-alkanes (C8–C20) (Fluka, Buchs/sg, Switzerland) and mass spectra with those from co-injected mixtures of standard individual compounds (purchased from Aldrich and Alfa Aesar) available in our laboratories and those reported in literature32. Standards of some EOs of known composition (such as EO of *Rosmarinus officinalis* L. - Phytosun' Aroms, Plelo, France) have been injected in similar conditions to check the retention times and the mass spectra. The percentage of each compound was determined using GC-FID.

2.4 Determination of antibacterial activity

The following Gram-negative bacteria were used: *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027) obtained from Epower™.

Antimicrobial activities of the Essential oil and its four major compounds were evaluated by broth and disc diffusion methods using MacConkey and Pseudomonas agars at 6 different volumes: 1, 2, 3, 5, 10 and 50 µl. Standard reference antibiotics were used in order to control the sensitivity of the tested bacteria. The incubation conditions used were 24h at 37°C. All the experiments were carried out in duplicate and averages were calculated for the inhibition zone diameters. Note that carvacrol, γ-terpinene and ρ-cymene are present as oil whereas thymol has been diluted with DMSO and water.

2.4.1 Determination of antibacterial activity by broth dilution method

The culture of bacteria was held under laminar flow, sterilized directly before each manipulation by UV rays for 15 minutes, and alcohol 70%. Suspension of bacteria in a nutrient broth was prepared and incubated for 24h, then a Serial dilution were done and 1ml from tubes containing 10^{-3}, 10^{-4} and 10^{-5} of the original inoculums was paid in six eppendorf tubes. Six concentrations of the essential oil and its major components were added to each tube after 10min, 1h and 24h. Finally, 0.1 mL of each eppendorf tube is distributed on a corresponding Petri dish by micro-pipette. After 24h of incubation, the survival colonies are counted and the minimum bactericidal concentration (MBC) can be determined.

2.4.2 Determination of antibacterial activity by disc diffusion method

The tested bacteria incubated in a nutrient broth at 37°C for 24h, after adjusting the turbidity of the inoculum suspension, 100µl poured over base plates containing 10 ml Nutrient agar in sterile 9 cm Petri dishes, then distributed using disposable pipette over the entire surface of the medium three times. Six volume of the essential oil and its major components were pipetted on sterile filter paper discs (Whatman No.1. 5 mm in diameter), which were allowed to dry in an open sterile Petri dish in a biological safety cabinet with vertical laminar flow (Nuaire Laminar Flow Products, USA). Discs were placed on the surface of the inoculated plates and incubated for 18 h. Standard reference antibiotics were used in order to control the sensitivity of the tested bacteria and size of inhibition zone diameters surrounding filter paper disc was measured.

3. Results and Discussion

3.1 Chemical composition of the essential oil

Twenty seven components, representing 97.44% of *Satureja thymbra* EO were found and summarized in Table 1. The essential oil isolated from flowering vegetative cycle was obtained in 1.8% yield. Therefore, this species can be assigned to oil-rich species of the Lamiaceae, in which the oil presents a large diversity of volatile constituents. Our results showed that the major compounds in the essential oil of *S. thymbra* were phenolic monoterpene: thymol (28.43%), p-cymene (19.89%), γ-terpinene (20.64%) and carvacrol (5.53 %), then acyclic monoterpene alcohol linalool (2.16%) and Terpinolene (0.19%), and thus the sum of the four compounds constitute the bulk of the essential oil equal to 74.4%.

Table 1 Composition of essential oil and their relatives MBC.

Bacteria		EO	Thymol	Carvacrol	ρ-cymene	γ-terpinene
	Fresh %	Yield:1.8%	28.43	5.53	19.89	20.64
E. coli	MBC	1	1	1	50	-
P. aeruginosa	MBC	3	50	1	-	-

3.2 Determination of antibacterial activity by broth dilution method

The essential oil of *S. thymbra*, thymol and carvacrol were proved highly lethal to *E. coli* with a lowest MBC equal to 1µL/mL. A percentage of inhibition of 100% is maintained for the 24h and 48h of incubation, giving a bactericide property to these components with MBC equal to 1µL/mL. In case of ρ-cymene, it shows an intermediate effect against *E. coli* with MBC equal to 5µL/mL, however the *E. coli* growth again and spread on the whole dishes after 24h(same result obtained at 10µL/mL) but it achieves the 100% of inhibition only at high concentration equal to 50µL/mL and last for 24h and thus the MBC of ρ-cymene is equal to 50µL/mL (48h test was done to confirm this result, data not shown). In addition γ-terpinene shows no measurable antimicrobial activity against *E. coli* even at high concentration (50µL/mL).

The effect on *P. aeruginosa* highlights the difference between EO, thymol and carvacrol. Although ρ-cymene doesn't show any antimicrobial activity even at high concentration, same as on *E. coli*, no antibacterial activity was detected for γ-terpinene. The EO and carvacrol still exhibited relatively strong antibacterial activity against *P. aeruginosa* which is not the case in thymol that needed more than 10 µL/mL to begin its inhibitor activity against *P. aeruginosa* and thus its effect is highly correlated to concentration and time of incubation. On the other hand, the EO posses its MBC at 3µL/mL and among these components carvacrol was found to be highly bactericidal, as it has shown lowest MBC (1µL) values. Table 1 is a summary table of the MBC of the essential oil as well as of the studied compounds and their percentage.

3.3 Determination of antibacterial activity by disc diffusion method

The essential oil from *S. thymbra* and carvacrol were inhibitory to the growth of both bacteria under test compared to antibiotics, and these finding are summarized in Fig 2.

Among these components carvacrol was found to be highly bactericidal, as it has shown lowest MBC values in broth dilution method and high growth inhibition zone diameter in comparison to antibiotics 31mm on *E. coli* with an intermediate effect on *P. aeruginosa* (12mm).

The essential oil of *S. thymbra* was more effective than the commercial drug penicillin and erythromycin, and have approximately same effect of amikacin on *E. coli* (16mm) and same effect of sulfamoxazol + trimethoprime on *Pseudomonas aeruginosa* (14mm at 10µL/mL), as we expect no antimicrobial activity was detected for ρ-cymene and γ-terpinene in this method according to previous results, however both bacteria are resistant against thymol (<9 mm) against what we should expect.

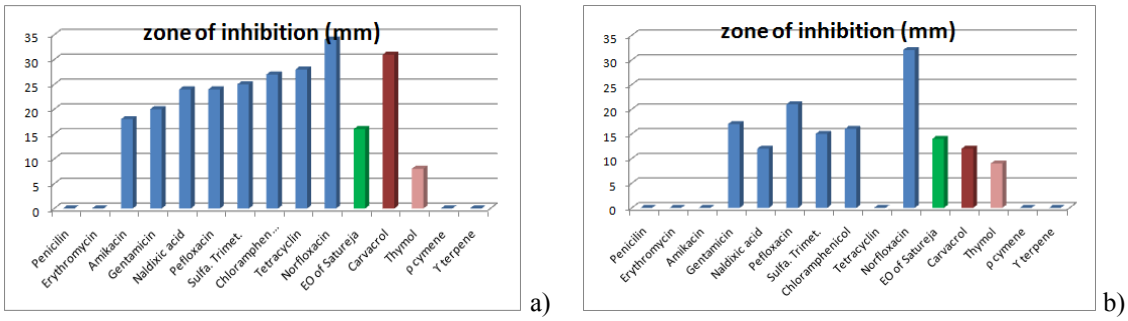

Figure 2 Disc diffusion methods: zone of inhibition in mm on *E. coli* (a), zone of inhibition in mm on *P. aeruginosa* (b).

4. Discussion

These results confirm the synergistic antibacterial effect of carvacrol which consist only 5.53% of the total oil and the null effect of both ρ-cymene and γ-terpinene which could be related to the cell membrane of gram negative bacteria. Carvacrol and thymol have a very similar chemical structure consisting of a system of delocalized electrons and a hydroxyl group, which makes it likely that they have a similar mechanism of antimicrobial activity. Both are able to disintegrate the outer membrane of gram-negative bacteria, releasing lipopolysaccharides (LPS) and increasing the permeability of the cytoplasmic membrane to ATP [5].

Further effectiveness of each compound was determined by agar disc diffusion method. Among the studied compounds, carvacrol was found to be highly bactericidal, as it shows high growth inhibition zone diameter in comparison to antibiotics 31 mm on *E. coli*. But, disk diffusion method is generally used as a preliminary check for antibacterial activity prior to more detailed studies. Factors such as the volume of EO placed on the paper disks, the thickness of the agar layer and whether a solvent is used vary considerably between studies. This means that this method is useful for selection between EOs but comparison of published data is not feasible.

Presumably the antibacterial activity is not only derived from the presence of monoterpene phenols, but also from the synergistic presence of their active constituents, such as γ-terpinene and ρ-cymene. ρ-cymene (1-methyl- 4-(1-methylethyl)-benzene) and γ-terpinene (1- methyl-4-(1-methylethyl)-1,4-cyclohexadiene) are the biological precursors of carvacrol (2-methyl-5-(1-methylethyl)- phenol) and thymol (5-methyl-2-(1-methylethyl)phenol) [6, 7]. This could explain the difference of EO composition between harvesting seasons and between geographical sources and has been found to be the case for *S. thymbra*. In addition, it appears that ρ-cymene, a very weak antibacterial, swells bacterial cell membranes to a greater extent than carvacrol does. By this mechanism ρ-cymene probably enables carvacrol to be more easily transported into the cell so that a synergistic effect is achieved when the two are present in the essential oils [8].

Up to date, limited investigations have noticed the safety and toxicity of Satureja's oil in humans and animals. It is necessary to differ between the cytotoxic effects of the EOs on microorganism and its toxic effects in eukaryotic cells. Thus, it is recommended that more pharmacological and safety studies must be carried out before the EOs can be reliably used in commercial applications. Further studies are needed to confirm the novel pharmacological and toxicological aspects of this plant in human and animal.

5. Conclusion

Clearly, herbal-based pharmaceuticals, cosmetics and flavoring are more welcoming every day. It is evident that there is a strong relationship between the high activity of *Satureja*'s oil and their high phenolic content. In addition, the minor components are critical to the antimicrobial activity and may have a synergistic effect or potentiating influence.

MBC determination performed as agar dilution is regarded as the gold standard for susceptibility testing because disc diffusion method is strongly influenced by agar depth, the volume of EO placed on the paper disks diffusion rate of the antimicrobial agent and growth rate of the specific bacteria.

More than 90% of the present investigations only emphasized on the antibacterial activities of *Satureja* sp. [9]. While the other biological effects such as analgesic, antiviral, anti-diabetic, antioxidant and vasodilator activities also seem promising. Monitoring and standardization is needed to direct studies passing through clinical trials. Various molecular, cellular and whole animal models should be used to demonstrate more valuable significance of *Satureja thymbra*. Future explorations are critical to assess the synergistic effects, efficacy and edibility of these essential oils.

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Antimicrobial effect of microencapsulated essential oil mixtures in footwear

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In this study a series of three mixtures of essential oils with antibacterial properties were microencapsulated and incorporated to footwear insoles to evaluate their antimicrobial effect against the feet microbiota. The essential oil mixtures were microencapsulated by two different technologies. Microcapsules with a melamine-formaldehyde shell were obtained by *in situ* polymerisation and gelatine-carboxymethylcellulose shell microcapsules were obtained by a complex coacervation process. In-shoe performance wear trials using the shoes incorporating different microencapsulated essential oil mixtures were undertaken by a panel constituted with 10 men and 10 women. After a week, the shoes were collected and the samples were analysed in order to determine the antibacterial effect of the microcapsules during wear. This was analysed by measuring the growing of the microorganisms in agar plates for each used shoe after contact between the plates and the used insole. Then the plates were incubated for 48-72 h at 32ºC. The results showed that there are differences between the antimicrobial activities for the microencapsulated oil mixtures that result in different inhibition grades along the wearing tests.

Keywords: antibacterial; *S.aureus*; *in situ* polymerisation, complex coacervation, melamine, gelatine.

1. Introduction

Nowadays, the antimicrobial properties of different materials are a significant feature in a wide range of products, not only in the healthcare sector, but also in sports, housekeeping, etc... In the case of footwear, microorganisms, such as bacteria, fungi and viruses, are likely to proliferate in the shoe environment under appropriate temperature and humidity conditions. The microbial overgrowth on feet can cause bad odours in areas close to the skin and also the outbreak of infections in the case of pathogen organisms [1-3]. Consequently, the demand for new and different coatings for footwear materials that offer good antibacterial protection has grown.

In recent years, the interest in natural extracts has increased as an alternative to the control of pathogen microorganisms [4, 5]. Essential oils such as Melaleuca alternifolia, lemon oil, clove oil, eucalyptus oil among others, have been successfully employed for their germicidal activity to control wound infections and feet infections caused by dermatophytes, as well as in traditional medicine for severe atopic and bacterial dermatitis caused by human skin bacteria such as Staphylococcus or Streptococcus [6-11]. On the other hand, microencapsulation technology allows the protection of active substances from the environment and also their controlled release exactly when needed [12-15].

In this study, three different oil mixtures were prepared from six essential oils with known antimicrobial properties to study their effect against the feet microbiota for footwear applications. The resultant mixtures were microencapsulated by *in situ* polymerisation and complex coacervation methods and the microcapsules obtained were incorporated to the shoe insoles to analyse the in-shoe performance related to their antimicrobial properties during shoe wear.

2. Materials and Methods

2.1. Essential oil mixtures.

The essential oils employed in this study were *Tea tree* oil, Lemon oil, Chamomile oil and clove oil and were purchased from Guinama S.L.U. (Alboraya, Spain). Neem oil was obtained from Laboratorios Martínez Nieto S.A., (Cartagena, Spain).

Three mixtures containing different essential oils were prepared. Table 1 shows the composition of the oil mixtures. The oil mixtures were prepared by mixing them until they reach homogenisation.

Table 1. Composition of the different antimicrobial essential oil mixtures.

Essential oil	Base mixture	Lemon mixture	Tea mixture
Clove oil	20%	-	-
Chamomile oil	20%	20%	-
Tea tree oil	20%	20%	50%
Lemon oil	20%	40%	-
Eucalyptus oil	20%	20%	-
Neem oil	-	-	50%

2.2. Microcapsule synthesis.

2.2.1. Synthesis of melamine-formaldehyde microcapsules containing antimicrobial essential oil mixtures.

Melamine (99.9 % purity) and formaldehyde were obtained from Quimidroga S.A. (Barcelona, Spain) and used as received. Formaldehyde was a 36.5% solution containing around 4.8% of methanol. Sodium dodecylsulphate (SDS) from Sigma-Aldrich was used as surfactant. Water was purified with a Millipore automatic Sanitization Module (Molsheim, France).

The first step of the synthesis was the preparation of a melamine-formaldehyde (MF) resin to be used as the microcapsules' shell. Melamine, water and formaldehyde were added to a round bottom glass flask. The vessel was immersed in a water bath heated to 70ºC and the mixture was magnetically stirred for 1 h. Finally, a clear solution was obtained.

After that, an O/W emulsion was prepared. The oil phase was composed of one of the different essential oil mixtures described in Table 1 and the aqueous phase was constituted of SDS as asurfactant and distilled water. Both phases were mixed and then emulsified by a Branson sonifier (Danbury, USA) for 30 s at 50% amplitude. The result was a milky emulsion. Subsequently, the emulsion was maintained at 45 ºC and magnetically stirred, while the MF resin prepolymer obtained in the previous section was added drop by drop. After this addition, the pH of the mixture was adjusted to 4 and the emulsion was left stirring for 1 h. After this time the temperature was increased to 85 °C and kept stirring for 2 h. Finally, it was allowed to cool to room temperature and the pH was adjusted to 10.

2.2.2. Synthesis of gelatine-sodium carboxymethylcellulose microcapsules containing antimicrobial essential oil mixtures.

Type B gelatine (225 g Bloom) and sodium carboxymethylcellulose (of medium viscosity) supplied by Sigma-Aldrich (Madrid, Spain) were used as shell-forming materials. Formaldehyde and gluteraldehyde, supplied by Quimidroga (Barcelona, Spain), were used as crosslinking agents for the polymeric shell. All reagents were used as they were received without further purification.

Microencapsulation through the complex coacervation (CC) method consisted of several fundamental stages. Firstly, the emulsification of the essential oil mixture was completed in an aqueous gelatine solution at 50ºC, where a 5 wt% sodium carboxymethylcellulose solution was added. Then the formation of the coacervate was induced through the reduction of the pH with 10 wt% acetic acid. Following this, the system was cooled to 5-10ºC and the coacervated gelated capsule was hardened by adding a 1:1 (v:v) mix of formaldehyde/glutaraldehyde.

2.3. Antimicrobial assays.

Antimicrobial assays were performed with two different media, Tryptone Soy Agar (TSA) and Mannitol Salt Agar (MSA), both from Cultimed Panreac (Barcelona, Spain). Both media were prepared according to the supplier instructions. TSA medium supports the growth of many microorganisms. MSA medium is selective for bacteria that tolerate high NaCl concentrations, and differential for those microorganisms that ferment mannitol (a sugar alcohol). It is used for the selective and differential isolation of *Staphylococcus aureus*.

Sampling was performed by pressing and rubbing the surface of the Rodac® contact plate filled with the different culture media against the treated and untreated insole, with the samples being taken as close as possible to the metatarsal area. The plates were immediately incubated at 32 °C for 48 h.

Finally, the grown microorganisms present on the plate were identified by their morphological differences, and determined by colony counting means.

3. Experimental

3.1. Characterisation of microcapsules.

In order to analyse the morphology of the synthesised microcapsules, the microcapsules obtained by the previously mentioned processes were analysed by Scanning Electronic Microscopy (SEM). The analysis was carried out by leaving a drop of the synthesised microcapsules dispersion in a sample tube and leaving it to dry overnight. The samples were coated by sputter-coating.

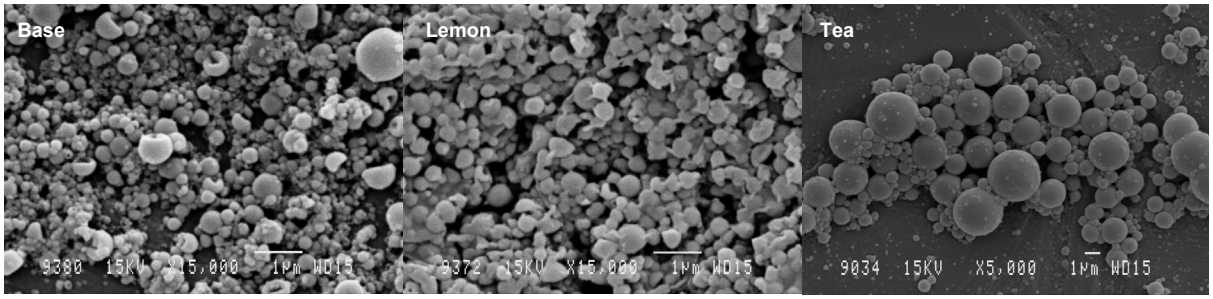
3.2. In-shoe performance.

Two different kinds of shoes, men's and women's styles, were selected to carry out wear trials (Table 2). Both models were sandals. Insoles made of split leather were coated with the microcapsules containing the different essential oils mixtures emulsions and left to dry at room temperature. The antimicrobial activity wear trial was carried out as follows: Firstly, the selected shoes were sterilised in an ozone chamber. Then, the microencapsulated antimicrobial formulations were applied as a coating of the insole material, only in the left foot, by brush, and left to dry at room temperature. The shoes were worn by the users for an average total of 50 h over 7 days. The selected users were healthy men and women without any recognised foot pathologies.

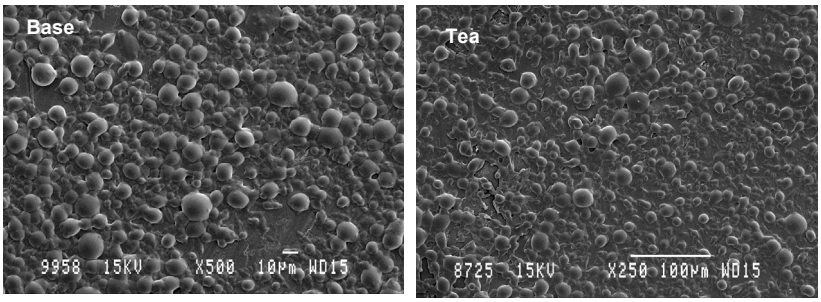
4. Results and Discussion

The morphology of the microcapsules synthesised by both methods was characterised by SEM. Figure 1.a shows the images obtained for the microcapsules containing the three different essential oil mixtures synthesised by *in situ* polymerisation. Figure 1.b shows the microcapsules synthesised by complex coacervation (CC). The microcapsules obtained by *in situ* polymerisation show a spherical morphology with a smooth shell. The melamine-formaldehyde microcapsules showed sizes of around 1 μm for the Tea oil mixture and sizes of less than 1 μm for the base and lemon mixtures.

In the case of complex coacervation, the morphology of the microcapsules was also spherical. The mean size of the CC microcapsules is higher than that of the melamine microcapsules, at around 3-20 μm.



a)

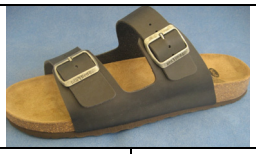
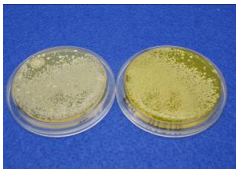
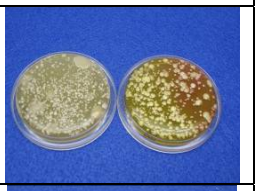


b)

Fig. 1. SEM images corresponding to: a) *In situ* polymerisation microcapsules, and b) complex coacervation microcapsules.

Table 2 includes images corresponding to microorganism growth in two different media after wear trials.

Table 2. Wear trials results, the images show the different growing plates for the antimicrobial evaluation.

	Samples			
				
	Untreated shoe	**Treated shoe**	**Untreated shoe**	**Treated shoe**
Base mixture MF				
Base mixture CC				
Tea mixture MF				
Tea mixture CC				
Lemon mixture MF				

A different inhibition effect of the microorganisms was observed depending on the kind of essential oil mixture and the microencapsulation process used. For the majority of the samples studied, in the plates corresponding to the untreated shoe, the growth of the microorganisms was higher than that of the shoe treated with microcapsules. Lemon and tea base mixtures seemed to be the most effective formulations, as they provided higher inhibition results.

Regarding the microencapsulation technique, the microcapsules obtained by *in situ* polymerisation showed better inhibition results than those of complex coacervation. This could be due to the fact that the melamine-formaldehyde microcapsules synthesised by *in situ* polymerisation have a higher strength shell that allows their application without breaking easily [15, 16] in contrast with the coacervation microcapsules. Furthermore, their smaller size makes their release rate slower than that of the complex coacervation microcapsules, which allows a long lasting effect and could be another reason for the higher inhibition level of this kind of microcapsules.

5. Conclusions

Melamine-formaldehyde resin and gelatine-sodium carboxymethylcelullose microcapsules containing five different essential oil mixtures, as a natural biocide, were synthesised by *in situ* polymerisation and complex coacervation, respectively. According to the results, the microencapsulation techniques and conditions determined the microcapsules' properties, especially the release behaviour which would affect the lasting of the antimicrobial effect during use.

The essential oil mixtures were released from the microcapsules incorporated in footwear materials by mechanical rupture of the shell during the time in which the shoe was worn, so the effect starts and is prolonged during use.

The antimicrobial effect of the microencapsulated essential oil mixtures was demonstrated in most of the samples studied. Tea and lemon mixtures showed the best results for inhibition assays.

Acknowledgements The partial support of the Spanish Ministry of Economy and Competitiveness, project no. CTQ 2010-16551 is gratefully acknowledged.

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Antimicrobial peptides in the tunic of *Ciona intestinalis* (Tunicata)

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Screening the expressed sequence tag database of the tunicate *Ciona intestinalis* two putative antimicrobial peptide genes were identified, and the natural peptides molecules were recently demonstrated to be synthesized and stored in distinct hemocyte types. The natural peptides were also localized in the tunic of *C. intestinalis.* The tunic represents a body surface barrier exposed to constant microbial assault. Using the antibodies generated against the corresponding synthetic peptides we investigated whether the distribution of AMPs in the tunic cell population is different during the defence reaction naturally occurring in the tunic of naive *C. intestinalis* in comparison to the inflammatory-like reactions experimentally induced.

Keywords AMPs; innate immunity; tunicates; *Ciona intestinalis*

1. Introduction

The marine organisms are probably the major source of bioactive molecules, and antimicrobial compounds have been described in them including tunicate species. Tunicates are marine invertebrate organisms considered to be a sister group of vertebrates - subphylum Urochordata, Tunicata [1]. Most tunicates are characterized by the presence of the tunic, an outer protective specialized tissue, covering the mantle epithelium or epidermis. Apart from its role as a support and an adhesive to the substratum, the tunic is considered as a protective barrier of the soft body against mechanical damage and infection, and a site of self/non-self recognition because free cells randomly distributed in it take part in various immune functions [2, and references therein].

Tunicates lacking an adaptive immune system rely on innate immunity [3,4] which consists of both cellular and humoral component. Humoral responses include the production of various killing factors such as antimicrobial peptides (AMPs) [5-7]. All antimicrobial peptides described from tunicates so far have been isolated from circulating hemocytes that are considered to be responsible for most of the defense reactions in these organisms [8].

In the solitary ascidian *Ciona intestinalis* (Tunicata, Ascidiacea) a cosmopolitan reference species, two novel gene families coding for putative antimicrobial peptides were identified in the EST data base. The corresponding antimicrobial natural peptides (Ci-MAM-A24 and Ci-PAP-A22) were shown to be synthesized and stored in a subpopulation of hemocytes [9-10]. The synthetic peptides (Ci-MAM-A24 and Ci-PAP-A22) display potent antimicrobial activity against bacteria and pathogenic bacteria of humans [11].

By performing immunoelectron microscopy we have tested specific antibodies generated from the synthetic peptides Ci-PAP-A22 and Ci-MAM-A24 as antigens on tunic samples from the *C. intestinalis* and we found the natural peptides to distinct tunic cells within granulocyte subtype [12].

Here we report and compare the presence of natural peptides monitored in the tunic samples from adult naive *C. intestinalis* when damages caused by encrusting organisms or mechanical assaults can produce a defence reaction and during local inflammatory-like reactions experimentally induced by the application of an elicitor.

2. Material and methods

2.1 Animals

C. intestinalis adult specimens were collected from the sicilian coast (Italy) and maintained at 15-18°C in aerated sea water. To provoke an inflammatory reaction, sheep erythrocytes (1 x 107 suspended in 0.2 ml phosphate buffered saline, PBS, pH 7.4) were injected into the tunic tissue. Four days later, the specimens showing an immune reaction in the tunic were chosen for further analyses. *Ciona* specimens injected with 0.2 ml PBS served as a control.

2.2 Microscopy

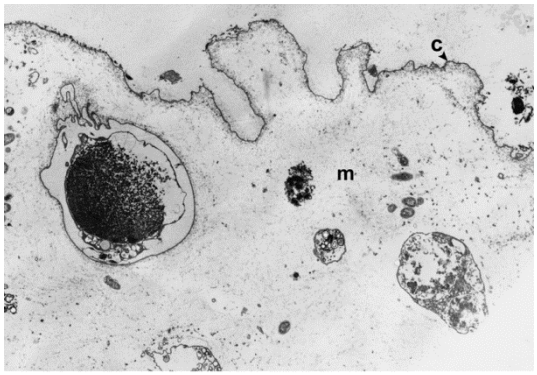
For routine microscopy, tunic fragments, 1-3 mm3 in size, cut off from different regions of the animal body and from the oral siphon, as well as excised from the injection site were processed by standard techniques: fixed with 1.5% glutaraldehyde, buffered in 0.05 M sodium cacodylate, pH 7.3, post-fixed in 1% OsO4, and dehydrated in a graded series of ethanol solutions, and subsequently embedded in epoxy resin. Ultrathin sections (50-70 nm thick) stained with uranyl acetate and lead citrate solution were examined using a Hitachi S7000 transmission electron microscope (80 kV). Immunostaining was carried out by placing thin sections on drops of the primary antisera, either anti-Ci-PAP-A22 or anti-Ci-MAM-A24. After washing, the sections were exposed to protein A-conjugated colloidal gold particles of either 10 or 5 nm diameter, counterstained with uranyl acetate prior to examination in the electron microscope.

As for the production of antisera against Ci-MAM-A and Ci-PAP-A the synthetic peptides were coupled to keyhole limpet hemocyanin (KLH) and these conjugates were used as antigens to immunize rabbits [9,10], antisera were preincubated with KLH prior to their use to exclude the possibility that the staining was due to anti-KLH antibodies with cross-reactivity to *C. intestinalis* hemocyanin-like proteins.

3. Results

The tunic of *C. intestinalis* consists of a thin outer sheet, the cuticle, and an underlying gelatinous layer (the matrix). Both are composed of fibrous material that in the cuticle results in a closely interwoven network, while as seen in the Figure 1 the inner layer is composed of loose fibrous components embedded in an amorphous matrix where free living cells are randomly distributed.

Fig. 1 Electron micrograph of *Ciona* tunic. The tunic is in close contact with the environment of the seawater, and it is the outpost for the interaction with parasites and pathogens. In the cuticle (**c**) sheet fibrils are tightly compressed. 2,650x



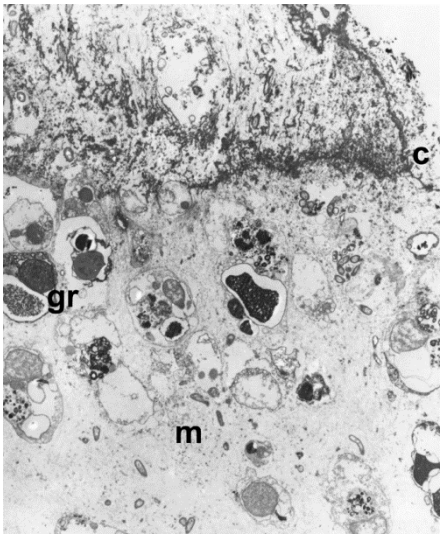


Fig. 2 Electron micrograph surveying a portion of *Ciona* damaged tunic. In the damaged areas the architecture of the cuticle (**c**) and its compactness is lost. The outermost layer appears very thin and the subcuticle consists of loose fibrillar material. Encrusting organisms that are entrapped into the tunic matrix (**m**) produce a simple defence reaction. In the healing area aggregation of numerous granulocytes (**gr**) can be observed; these cells contain fibrogranular material with different density. Many cells often undergo to drastic changes releasing dense materials, fibrils; clear vacuoles, a lot of vesicles, and lysosomic figures can be observed. 1,650x

Following the damages produced in the cuticle by sand grains or by other agents, openings enable encrusting organisms to reach the matrix of the tunic. Figure 2 shows aspects of the simple defence reaction induced by the massive presence of foreign agents in the subcuticular areas (here reported as damaged tunic). As in the acute inflammation reaction experimentally induced, tunic cell number is massively increased as hemocytes migrate by diapedesis from the hemolymphatic lacunae. In Figure 3 most of granulocyte cells show phagocytic activity, presence of phagosomes, debris and remains of disintegrating agents. The cells are often in close contact to one another and appear frequently to be in a degranulating active state, releasing vesicles and showing drastic structural changes so that many cellular ghosts are observed.

AMPs seem to be involved in these inflammation-like states. Using the anti-Ci-PAP-A and anti-Ci-MAM-A antibodies immunolocalization was performed on samples of injected tunic. Figure 4 shows gold particles in the

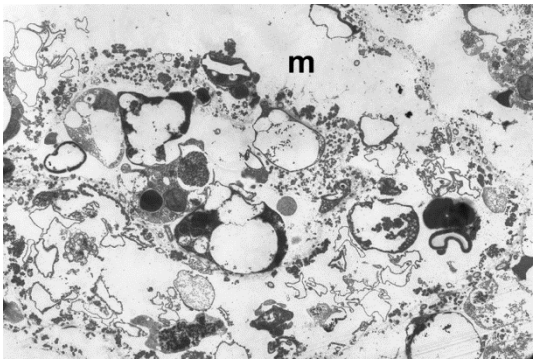


Fig. 3 Section of inflamed tunic area after the experimentally inoculation of foreign materials. Different cells in a degranulating state; cell ghosts and inflammatory features can be seen. 1,450x

infiltrating granulocytes identified as "globular granulocytes" cell type including compartment/morula cells, and "tunic large granule cells" or unilocular granulocytes". Immunostaining is seen in the cytoplasmic small granules adjacent to the globules of tunic compartment/morula cells, and inside the single electron-dense large inclusion of homogeneous fibrogranular material occupying entirely the cytoplasm of tunic large granule cell.

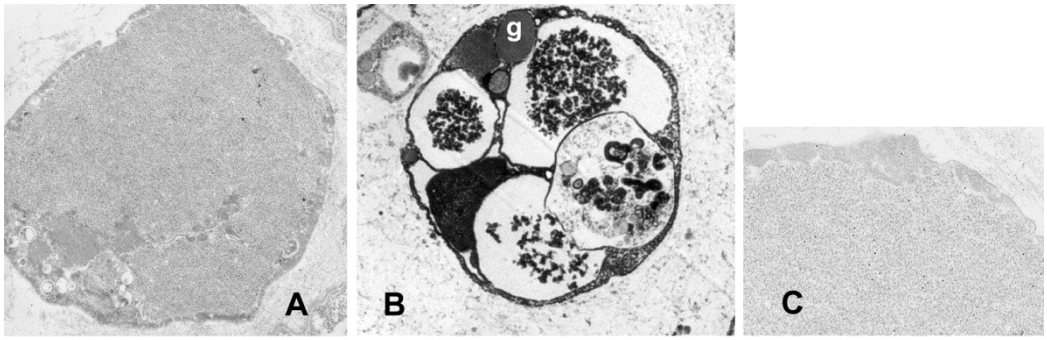


Fig. 4 Immunolocalization of peptides in tunic cells **(A)** Tunic large granule cell possessing a single electron-dense large granule surrounded by a thin peripheral rim of cytoplasm with some vesicles and free ribosomes. **(B)** Morula cell with a berry-like appearance and possessing several tightly packed globular vacuoles partially or filled with masses of granular dense material. The peptide Ci-MAM–A is associated with small granules (**g**) scattered among the globules. **(C)** enlargement of the unilocular granulocyte shown in **A** Ci-PAP-A positive inside the large inclusion that occupies the entire cell. A=6,400x; B= 6,550x; C=16,000x

Immunostaining is seen inside degranulating cells. Many cells often undergo to drastic changes releasing dense materials, fibrils; in Figure 3 clear vacuoles, a lot of vesicles, and lysosomic figures can be observed. Discharging their contents cells are contributing to the neutralization of foreign agents and the repair of the tunic architecture as filaments appear around in the matrix.

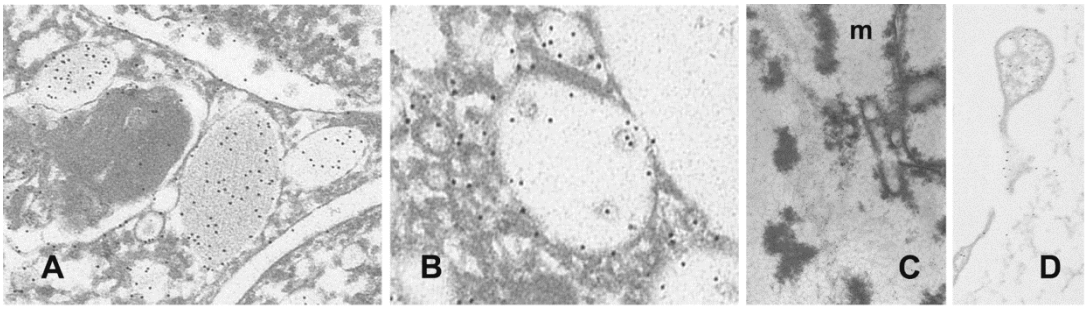


Fig. 5 (A,B): Details of small granules from tunic compartment/morula cells showing colloidal gold particles representing Ci-MAM-A or Ci-PAP-A peptide associated to the content inside or extruded by granule discharge. **(C)** Gold particles representing CI-PAP-A are seen associated with electron dense particles, membrane debris, and fine fibrils in the tunic matrix after the inoculation of foreign materials. **D**: Labeling for Ci-MAM-A localized within the tunic matrix among the remnants of cells in a damaged area of the tunic. A=29,000x; B=49,000x; C= 14,000x; D=16,500x

As seen in Figure 5 when the material inside the little granules is lost owing to the discharging process, the gold particles decorated the remnant of the content or appeared attached at the granule margin.

Figure 5C,D evidences labeling in the vesicles released by cells and gold particles are seen associated to the material extruded after the membrane dissolution in the surrounding matrix area.

Immunoreactivity performed on samples of naturally damaged tunic, is more consistently present in the cells when the anti-Ci-PAP-A antibody is used.

Immunostaining was still observed in the tunic sections when antisera were pretreated with KLH, confirming the specificity of the staining, whereas no positive staining was observed in negative controls (data not shown).

4. Discussion

Using the antibodies generated against the synthetic peptides Ci-MAM-A24 and Ci-PAP-A22, corresponding to the transcripts of two putative antimicrobial peptide genes of the *Ci-mam* and *Ci-pap* gene families, the natural peptide molecules have been localized to distinct hemocyte types [9,10], and in the granulocyte population resident in the tunic of *C. intestinalis* adults [12].

As described by De Leo et al., [13,14] upon injection of sheep erythrocytes into the tunic of *C. intestinalis* the cell number is massively increased in the injected inflamed areas as hemocytes migrate by diapedesis from the hemolymphatic lacunae [15]. Most of tunic cells are in a degenerative state and undergo drastic changes so that sometimes it is difficult to identify all cell types on the basis of their ultrastructural aspects.

Infiltration of hemocytes is also observed during inflammation reactions produced in the tunic after interruptions or damages of the cuticle continuity that enable foreign agents to reach the tunic matrix. Sometimes a massive presence of foreign agents is seen in the subcuticular areas, a simple defence reaction leading to wound healing, neutralization of foreign bacteria and algae, and maintenance of tunic tightness is performed: cellular and chemical defences as the production of AMPs, should be available in the tunic. The majority of cells shows phagocytic aspects and entrap bacteria, and appears in a degenerative and release their contents.

These features are very similar to those observed during the induced acute inflammation in *Ciona* when the production of AMPs in the tunic cells is significantly increased [12]. The observations disclose an involvement of particular cell types in the production of AMPs in the inflamed tunic. The present findings show immunoreactivity present in the tunic large granule cells from both naive and immune-stimulated ascidians, and labeling is seen in the sole large inclusion. These cells appear to be particularly immune competent as they have been shown to be involved in different defense reactions. AMPs are also observed within other granulocyte subtypes residing in the tunic, stored in the cytoplasm of tunic morula/compartment cells exclusively in the small granules found among the globules or vacuoles.

Immunoreactivity is significantly positive to Ci-PAP-A in damaged tunic from naive *C. intestinalis* samples where a defence reaction is naturally occurring, while both Ci-MAM-A and Ci-PAP-A peptides are localized in the injured animals where an inflammatory reaction is experimentally induced. As evidenced by electron microscopy Ci-MAM-A and Ci-PAP-A are consistently present in the vesicles released by cells and gold particles are associated to the material extruded in the surrounding matrix area after the membrane dissolution upon the activation of degranulation processes. The release of cellular content could take part in the destruction of the foreign cells; cell migration and aggregation in the wound sites participate to destroy foreign organisms. Although it is not know how initiates the defence reactions, how the antimicrobial factors are released and which molecules are involved in the whole healing phenomenon, the increased presence of these AMPs in the damaged or injured *C. intestinalis* tunic give further interest in the potential development of AMPs as therapeutics.

Complex interactions occur between the expression of antimicrobial peptides and the involvement of cell components in host defence, therefore the mechanisms leading from recognition of invaders via signal transduction to induction of AMP genes needs further investigation.

The expression of AMPs is usually high in primary barrier tissues of the organism, to prevent colonization by pathogens but actually the knowledge of their mechanisms of action has not been fully understood.

The resistance of pathogens to antibiotics is an increasing threat to public health, leading to a demand of new classes of antibiotics that may overcame this problem. Many efforts in these last years have dedicated in development of new drug and the purification of the bioactive compounds, and efforts are now concerned on studies about mechanisms of action. It could be a strategy to search for antibiotic substances in environments where the ecology of microbial interactions are markedly different.

Acknowledgements The support from the Italian Ministero della Università e della Ricerca (MIUR) and the University of Palermo research grant to M.A.D. and G.DL., is gratefully acknowledged.

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Antimicrobial property of the hydro-alcoholic extract from purple basil (*Ocimum basilicum* var. *purpurascens*)

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Antibacterial and antifungal activity of the hydro-alcoholic extract of purple basil (*Ocimum basilicum* var. *purpurescens*) were investigated. The extract, containing an amount of polyphenols of 0.78 mg gallic acid equivalent/ml of extract, was tested against a certain number of Gram positive (*Bacillus cereus*) and Gram negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Cronobacter sakazakii*), as well as against some moulds (*Aspergillus niger*, *Aspergillus versicolor*, *Penicillium citrinum*, *Penicillum digitatum*, *Penicillium expansum)*. *B. cereus* and *E.coli* were the most resistant to the activity of the extract*;* on the other hand, *C. sakazakii* was the most sensitive, and the extract produced an inhibition halo of 15 mm. The extract was effective against almost the moulds, except *P. citrinum and A. niger*, giving halos until 29 mm (against *A. versicolor*). Thus, the extract could be taken into consideration as natural antibiotic and preservative agent in agro-food industry.

Keywords basil; antimicrobial; antifungal

1. Introduction

Plant extracts are used for a wide variety of purposes since many thousands of years [1]. These purposes vary from the use in perfumery, to flavoring drinks and for the preservation of stored food crops [2]. In particular, the antimicrobial activity of plant extracts has formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies [3-4]. Basil (*Ocimum basilicum*) is one of the oldest spices belonging to the *Ocimum* genus and to the *Lamiaceae* family, widely diffused in tropical, subtropical and temperate areas in the world (Fig. 1). Basil is a popular culinary herb (Fig.1), used extensively also in the flavoring of confectionary and baked goods, condiments (tomato processed products, chili sauces, pickles, and vinegars), sausages, meats, etc. Purple basil (*O. basilicum* var. *purpurascens*) is an aromatic annual plant with erect growth habit. It has strongly scented purple leaves and pink flowers (Fig.2).

Fig. 1 Ocimum basilicum

Fig. 2 Ocimum basilicum var. purpurascens

Due to its high content of polyphenols and anthocyanins, purple basil can represent a potential new source of such important biomolecules to be used as antioxidant and antimicrobials in different fields of food science and medicine [5-6]. Nowadays, due to the preference by the consumer natural over synthetic direct or indirect food additives, naturally derived antimicrobial agents such as basil are becoming increasingly more important in several fields of agro-food industry and processes, as they present a perceived lower risk to the consumers. Aim of our work was to evaluate the potentiality of a hydro-alcoholic extract of purple basil as antibacterial and antifungal agent.

2. Materials and methods

2.1 Extraction

The aerial parts of *Ocimum basilicum* var *pupurascens* were extracted (1:10 w:v) with ethanol:deionised water (50:50 v/v) for 2 days at 4°C avoiding the exposure to the light. The crude extract was concentrated to eliminate the presence of ethanol, then subjected to the analyses.

2.2 Colorimetric analysis of total phenolic compounds

Total phenolics were spectrophotometrically determined with the Folin–Ciocalteu reagent [7]. The absorbance was determined at room temperature at λ= 760 nm by using a Cary Uv/Vis spectrophotometer (Varian Cary 50 MPR, USA). Quantification was based on a standard curve generated with gallic acid. The results were expressed as μg gallic acid equivalents (GAE)/ml of extract.

2.3 Antimicrobial assays

To screen the inhibitory activity of the *O. basilicum purpurascens* hydro-alcoholic extract against microorganisms, a filter paper disc method was used [8]. The bacteria used in this study included the Gram-positive *Bacillus cereus* and the Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa* as well as the emergent pathogen *Chronobacter sakazakii*. The moulds included *Aspergillus niger*, *Aspergillus versicolor*, *Penicillium citrinum*, *Penicillum digitatum*, and *Penicillium expansum*. All strains were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ Germany). Bacteria were incubated in Nutrient Broth (Oxoid, Milano, Italy) at 37°C for 18 h. Moulds were incubated in Potato Dextrose Broth at 28°C for 24-48 h. Cultures were spread onto Nutrient agar (bacteria) and Potato dextrose agar (moulds) plates in sterile conditions. Sterile filter paper disc (5mm) were impregnated with 1.56 μg, 3.13 μg, 6.26 μg, 12.52 μg and 15μg GAE/disc of the extract. Plates were left 30 min at room temperature in sterile conditions, and incubated at 37 °C and 28 °C (for bacteria and moulds, respectively) until 72 hours, depending on the tester strain. The results (mean value, n = 3) were recorded by measuring the zones of growth inhibition surrounding the discs.

3. Results

Plant metabolites and plant based pesticides appear to be one of the better alternatives as they are known to have minimal environmental impact and danger to consumers conversely to chemicals. Extracts and essential oils extracted from spices and other herbs, as well as their biologically active components, have attracted of attention many authors to investigate their antimicrobial property. In food production and processing, they are important as food preserving agents against microorganisms, especially of short shelf-life products, such as bread, bakery products, fresh fruit and vegetable, which are the most sensitive to microbiologic spoilage. Their use in the food industry decreases the use of synthetic preservers and additives, and, at the same time, improves the freshness and sensory of the product quality. The antimicrobial activity exhibited by numerous spices and herbs is also used in folk medicine to combat different pathogenic fungi and bacteria. Different species of the family *Lamiaceae*, belonging to genus *Ocimum*, have been extensively studied for the biological activity both of their alcoholic extracts and for their essential oils, which exhibit antibacterial and antifungal properties against a wide spectrum of microorganisms [8].

Table 1 shows the antimicrobial activity exhibited by the hydro-alcoholic extract of purple basil against different strains of bacteria (**1.a**) and moulds (**1.b**) of agro-food and health interest.

Table 1.a	1.565 µg GAE (M± SD)	3.13 µg GAE M± SD)	6.26 µg GAE (M± SD)	12.52 µg GAE (M± SD)	15 µg GAE (M± SD)	gentamycin 8µg (M± SD)	DMSO
Ps. aeruginosa	**4.0± 0**	**5.0 ± 0**	**7.33 ± 0.58**	**9.33 ± 0.58**	**9.33 ± 0.58**	**15.3 ± 0.6**	**nd**
E.coli	**nd**	**nd**	**nd**	**nd**	**nd**	**15.7 ± 1.20**	**nd**
B.cereus	**nd**	**nd**	**nd**	**nd**	**nd**	**10.33 ± 0.6**	**nd**
C. sakazakii	**4.66 ± 0.58**	**5.0 ± 0**	**8.33 ± 1.52**	**9.67 ± 0.58**	**13.67 ± 1.52**	**15.3 ± 1.2**	**nd**

Table 1.b	1.565 µg GAE (M± SD)	3.13 µg GAE M± SD)	6.26 µg GAE (M± SD)	12.52 µg GAE (M± SD)	15 µg GAE (M± SD)
A.niger	**nd**	**nd**	**nd**	**nd**	**nd**
A.versicolor	**9.33 ± 1.15**	**10.0 ±0**	**11.33 ±1.52**	**20.33 ±0.58**	**29.67 ±1.58**
P.citrinum	**nd**	**nd**	**nd**	**nd**	**nd**
P.digitatum	**5.66 ±1.15**	**9.66 ±0.58**	**9.67± 0.58**	**19.67 ±0.58**	**22.33 ±1.52**
P.expansum	**3.33 ±0.58**	**5.33 ±0.58**	**9.33 ±1.15**	**15.33 ±0.58**	**19.0 ±1.73**

Table 1. Antimicrobial activity exhibited by the hydroalcoholic extract of *Ocimum basilicum var. purpurascens* against different bacteria (1.a) and moulds (1.b). The activity was evaluated by the inhibition halo test, using different concentration of extract, ranging from 1.56 to 15 mg GAE of polyphenols. DMSO and gentamycin (8 mg) were used as negative and positive control, respectively (Table 1.a). Data are shown as mean (M) ± Standard Deviation (SD) of the inhibition halo (in mm) (n=3).

The extract was tested using different amount, ranging from 1.56 µg GAE to 15 µg of crude extract. Among bacteria, the extract was effective against *Pseudomonas aeruginosa*, giving inhibition halos until 9.33 mm (with the highest concentration of polyphenols used) but mainly against *C.sakazakii,* herein reported for the first time: in this last case, in fact, the halo measured 13.7 mm. *C. sakazakii* is a member of the family *Enterobacteriaceae*, and an emerging pathogen of neonates with mortality rates until 80%. It has implicated in severe forms of neonatal infections, such as meningitis, bacteremia, sepsis, and necrotizing enterocolitis. More than 70 cases of *C. sakazakii* infection were reported and more than 1.5 million cans of dry infant formula recalled because of contamination with *C. sakazakii* worldwide. Moreover, it was found in several types of foods, food-processing plants, and the environment [9], thereby representing a potential risk of causing human infections [10]. *C. sakazakii* possess the characteristic of resistance or tolerance against several stresses that enable it to survive in foods and the environment and cause foodborne illness. Hence, the use of natural organic compounds like extracts of *O. basilicum purpurascens* as food additives could be one possible way to control *C. sakazakii* in various types of foods. The activity against *Pseudomonas* herein reported for the ethanolic extract of *O. basilicum purpurascens* is in agreement with Opalchenova and Obreshkova [11], which studied the activity of sweet basil against multi-drug resistant isolates from the genera *Staphyloccus* and *Pseudomonas*, reporting a strong inhibitory effect of basil on the test bacteria. The extract was ineffective against the toxigenic strain of *E.coli* used in the experiments. Such results are in contrast with previous works reporting an effective activity of ethanol extract of *O.basilicum* against *E.coli* [12]. Probably, the different behaviour might be caused also both by the different species used of *O.basilicum* used and from the different method of extraction applied.

Numerous investigations established the significant antifungal properties of from many vegetal species and the increasing frequency of serious fungal infections. This last aspect is attributed to different reasons, such as the extensive use of newer and more powerful antifungal agents, a growing use of cytotoxic and immunosuppressive drugs to treat malignant and non-malignant diseases, an increasing occurrence of infection due to human immuno-deficiency virus [13]. All these factors are reinforced by the fact that antifungal treatments are always too long because of fungus resistance to standard treatments. In addition, exploitation of plant metabolites in crop protection and prevention of bio-deterioration caused by fungi appear to be promising. In Table 1.b is shown the antifungal activity exhibited by the hydro-alcoholic extract of purple basil. The most sensitive strains resulted *Aspergillus versicolor* and *Penicillium digitatum*, with inhibition halos ranging from 5.6 mm to almost 30 mm (observed using 15 µg of GAE against *A. versicolor*). The good antifungal activity was also shown against *P. expansum,* the other species of *Penicillium* tested, confirming the antifungal activity of the hydro-alcoholic extract against different *Penicillium* spp, frequent contaminants of food. *Aspergillus*

species are frequent contaminants of medium and low moisture food. Their metabolic activity is cause of food spoilage and of an enormous economic loss. Besides that, toxin producing species biosynthesize toxic secondary metabolites-mycotoxins, such as ochratoxin A (i.e. produced by *A. niger*), and sterigmatocystin (i.e. produced by *A. versicolor)* [14]. Intake of fungal toxins through food consumption provokes intoxications in animals and humans, so called mycotoxicoses which can occur on a large-scale [15]. The hydroalcoholic extract obtained from *O. basilicum purpurascens* was completely ineffective against *A. niger* but exhibited a noticeable activity against *A. versicolor*: this last result could be used as basis for a large scale study for food protection against the *A. versicolor* growth. Indeed, it might represent a starting point also to evaluate the suitability of use such extract also to inhibit or limit the *A. versicolor* sterigmatocystin production, enlarging the classes of natural antifungal compounds useful as substitutes of synthetic preservatives in food safety to control fungal contamination and mycotoxin production.

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Antimicrobial prowess of a soil isolate *Bacillus subtilis*

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1. Abstract

An ever escalating antibiotic resistance accentuates the compelling need to recover novel molecules that are insensitive to resistance processes in order to combat resistant pathogens. Through primary and secondary screening, a wild-type soil *Bacillus isolate* RLID 12.1 showed wide-spectrum antimicrobial activity against pathogenic *Streptococcus pyogenes*, *Staphylococcus aureus*, *Escherichia coli, Proteus vulgaris*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Candida albicans* and *Cryptococcus neoformans*. By morphological, 16s RNA sequencing and FAME analysis, the active strain was identified as *Bacillus subtilis.*

Keywords: Antibiotic resistance; broad spectrum; *B. subtilis*

2. Introduction

Most of the antimicrobial production by the producer strains are due to the defensive action to maintain their ecological niche in an established microbial community structure. The genus *Bacillus*, one of the most abundant bacterial strains found in soil, is able to produce several dozens of antibiotic compounds with various chemical properties, among which peptide derivatives are elaborately studied [1,2]. In a natural environment these soil bacteria generate antimicrobial compounds, including peptide antibiotics responsible for their survival in hostile environment. When two species of peptide-antibiotic producing bacilli coexist, they inactivate or shield the peptide antibiotics of the other as a survival strategy [3].

Bacillus isolates are rather well known for the production of a vast array of structurally unrelated antimicrobial compounds, which include lipopeptides like iturin, surfactin, fenzycin etc. and bacteriocins [1, 4]. The production of lipopeptides allows certain *Bacillus subtilis* strain to modify their outer surface which permits them to regroup together in a biofilm in order to proliferate and spread in the territory. Biofilm formation also contributes to the defense and resistance mechanism of *B. subtilis* species towards other peptide or antibiotic producing organisms [5]. The aim of this work was to investigate the broad-spectrum antimicrobial potential of the soil derived *Bacillus* sp. against pathogenic strains of gram positive, gram negative bacteria, and some yeasts like *Candida* sp. and *Cryptococcus neoformans* followed by its characterization studies.

3. Materials and methods

3.1 Isolation of antagonistic Bacillus sp. from soil

Soil samples were collected from the forests of Goa and garden soil of East Dehradun. 1g of each soil samples were dissolved in sterile saline solution and heated up to 70˚C for 10 min to kill the vegetative cells of other bacteria. Subsequently, 100 µl was used for pour plating on nutrient agar and incubated at 37˚C for 48-72 h. The colonies that inhibited the surrounding colonies in vicinity were picked up, purified and were subjected to microscopic examination with reference to gram and endospore staining [6]. Freshly grown *Bacillus* isolates in Tryptic soy broth with additional 0.5% yeast extract at 37˚C for 48 h were subjected to spot agar assay. All the isolates were maintained as 20 % glycerol stock at -80˚C. The indicator organisms used in this investigation were obtained from Microbial Type Culture Collection (MTCC) Chandigarh, National Collection of Industrial Microorganisms (NCIM) Pune, India and clinical isolates from hospitals. All are listed in (Table 1).

Table 1 Antimicrobial activity of RLID 12.1 by Spot agar method

S.No	Indicator organism	Temperature	Media	RLID 12.1
Gram negative bacteria				
1	*Pseudomonas aeruginosa* MTCC 741	37 °C	BHI	+++
2	*P.aeruginosa* MTCC 2582	37 °C	BHI	+
3	*P.aeruginosa* MTCC 424	37 °C	BHI	+
4	*P.aeruginosa* (clinical isolate)	37 °C	BHI	++
5	*Klebsiella pneumoniae*	37 °C	BHI	+
6	*Klebsiella pneumoniae* MTCC 109	37 °C	BHI	+
7	*Salmonella infantis* MTCC 1167	37 °C	BHI	+
8	*Escherichia coli* MTCC 723	37 °C	BHI	+
9	*E.coli* MTCC 729	37 °C	BHI	+
10	*Serratia* sp. (soil isolate)	37 °C	BHI	+
11	*Acinetobacter baumanii* MTCC 1425	37 °C	BHI	+
12	Water isolate-CH3 hostel BITS*	37 °C	BHI	+
13	Water isolate-AH5 hostel BITS$^{\$}$	37 °C	BHI	-
14	Water isolate-4FT3 hostel BITS$^{\#}$	37 °C	BHI	-
15	*Proteus vulgaris* MTCC 1771	37 °C	BHI	++
16	*Yersinia aldovae* (arctic isolate)	37 °C	BHI	++
Gram positive bacteria				
17	*Staphylococcus aureus* MTCC 737	37 °C	BHI	+
18	*S. aureus* MTCC 96	37 °C	BHI	+
19	*S. aureus* NCIM 5021	37 °C	BHI	+
20	*Staphylococcus* sp. coagulase (+) (clinical isolate)	37 °C	BHI	-
21	*Staphylococcus* sp. coagulase (-) (clinical isolate)	37 °C	BHI	-
22	*Nocardia sp.* (clinical isolate)	37 °C	BHI	+
23	*Bacillus subtilis* (clinical isolate)	37 °C	BHI	+
24	*B. subtilis* MTCC 2195	37 °C	BHI	+
25	*B. brevis*	37 °C	BHI	++
26	*B. cereus*	37 °C	BHI	-
27	*Micrococcus luteus*	37 °C	BHI	+
28	*Lactococcus lactis*	37 °C	BHI	+++
29	*Streptococcus pyogenes* MTCC 442	37 °C	BHI	++
30	*Streptococcus* sp. (clinical isolate)	37 °C	BHI	-
31	*S. pyogenes* MTCC 1928	37 °C	BHI	+
32	*S. pyogenes* NCIM 2608	37 °C	BHI	+
33	*Staphylococcus epidermidis* ATCC 12228	37 °C	BHI	-
34	*Carnobacterium maltaromaticum* (arctic isolate)	37 °C	BHI	++
35	*Enterococcus faecalis* APR 210 (arctic isolate)	37 °C	BHI	++
Yeasts				
36	*Candida albicans* DI (clinical isolate)	30°C	MGYP	+++
37	*C. albicans* WT (clinical isolate)	30°C	MGYP	++
38	*C. albicans* MTCC 227	30°C	MGYP	+
39	*C. albicans* NCIM 3471	30 °C	MGYP	+
40	*C. albicans* MTCC 854	30°C	MGYP	+
41	*C. glabrata* NCIM 3019	30°C	MGYP	+
42	*C. glabrata* MTCC 3814	30°C	MGYP	++
43	*C. krusei* NCIM 3129	30°C	MGYP	++
44	*Cryptococcus neoformans* NCIM 3541	30°C	MGYP	++
45	*Cryptococcus neoformans* NCIM 3378	30°C	MGYP	+

+++ indicates very strong activity, ++ indicates strong activity, + indicates presence of activity and – indicates no activity and *, $^{\$}$ and $^{\#}$ indicate pink mucoid colonies isolated using MacConkey agar media from water sample.

3.2 Primary in vitro screening for antimicrobial properties against indicator organisms

The wild type bacterial isolates of *Bacillus* sp. were screened using spot agar method. Serially diluted indicator organism was uniformly spread over the Brain Heart Infusion plate and after 5 min, 5µl of freshly grown test strain were spotted and incubated for 24 h at the respective temperatures mentioned in Table 1.

3.3 Identification of Bacillus isolates RLID 12.1 down to a level of species

3.3.1 Biochemical studies

The antibiotic producing *Bacillus* strains RLID 12.1 was identified by various biochemical tests like Methyl Red and Voges-Proskauer tests, production of indole and catalase, growth at different temperatures and different concentrations of NaCl according to Bergey's Manual of Determinative Bacteriology [6] (Table 2). Carbohydrate fermentation tests were done using KB009 Kit from HiMedia Pdt. Ltd., India.

Table 2 Biochemical characterization of *Bacillus* strain RLID 12.1

Tests	***Bacillus* sp. RLID 12.1**	**Tests**	***Bacillus* sp. RLID 12.1**	**Tests**	***Bacillus* sp. RLID 12.1**
Morphology					
Colony morphology	Wrinkled	Sorbitol	+	Xylose	+
Colour	White	Mannitol	+	Ribose	+
Gram staining	positive	Adonitol	-	Maltose	-
Spores	+	Arabitol	-	Fructose	+
Pigment production on glutamate glycerol agar media	No pigment	Erythritol	-	Dextrose	+
NaCl		Methyl-D-glucoside	-	Galactose	+
2%	+	Rhamnose	-	Raffinose	+
5%	+	Cellobiose	+	Trehalose	-
7%	+	Melezitose	-	Melibiose	-
10%	+	Methyl-D-mannoside	-	Sucrose	+
Biochemical		Xylitol	-	L-Arabinose	-
Catalase	+	ONPG	-	Mannose	+
Indole	-	Esculin hydrolysis	+	Inulin	+
Starch	+	D-Arabinose	-	Sodium gluconate	-
Production of mixed acid (MR test)	-	Citrate utilization	-	Glycerol	+
Acetoin production (VP test)	+	Malonate utilization	-	Salicin	-
Lactose	-	Sorbose	-	Dulcitol	-
Tween 80	-	Tween 20	+	Inositol	-

3.3.2 16S rRNA analysis

Total genomic DNA of the selected soil isolates were extracted from 24 h trypticase soy broth cultures. PCR with 50 µl reaction volume was performed using about 100 ng of genomic DNA, 10X reaction buffer, 10 mM (each) deoxynucleoside triphosphates, 1.5 mM $MgCl_2$ and 1.0 U of Taq polymerase (Bangalore GeneI, India). The universal eubacterial primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) (Lane 1991) were used at the rate of 10 picomoles to amplify small-subunit rRNA (16S rRNA) gene sequences of the wild-type soil isolates. PCR was carried out under the following cycling conditions: initial denaturation at 95 ºC for 5 min, followed by 30 cycles of 94 ºC for 1 min, 55 ºC for 1 min and 72 ºC for 1 min, with a final extension at 72 ºC for 1 min. The expected PCR product of around 1.5 Kb was checked by gel electrophoresis using 5 µl of the PCR product on 1% agarose gel in 1X TBE buffer. The sequencing of the target gene (amplicon) was done using BigDye Chemistry, and performed as per the

manufacturer's protocols (Applied Biosystems 3730xl DNA Analyzer) in Royal Life Sciences Pvt. Ltd., Secunderabad, India.

3.3.3 Computational analysis and phylogenetic tree construction

Sequence data analysis was done using ChromasPro and Sequencing analysis software like BLAST to compare the sequences in the GenBank nucleotide sequence database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [7]. The 16S rRNA sequence of RLID 12.1 strain were aligned using the CLUSTALX 2.1 program [8] against corresponding nucleotide sequences of representatives of the genus *Bacillus* retrieved from GenBank. Phylogenetic tree was inferred using PHYLIP (PHYLogeny Inference Package) and the neighbour-joining method with a bootstrap value of 1000 replicates [9] for inferring evolutionary trees (phylogenies). All 16S rRNA gene sequences of the isolate described in this study have been deposited in the GenBank nucleotide sequence database.

3.3.4 FAME analysis

The analysis of the cellular fatty acid methyl esters (FAMEs) was performed at Royal Life Sciences Pvt. Ltd., Secunderabad, India using MIDI Sherlock software. The FAME extracts were prepared by a procedure that consisted of cell harvesting, saponification, methylation, and extraction from the aqueous phase and finally analysis was done by gas chromatography [10]. FAME profiles achieved were analysed by comparing with Sherlock MIS RTSBA6 identification library. Further cluster analysis was carried using dendrogram tool with the FAME profile data for confirmation of species.

4. Results and discussion

A consequence of tight relationships and increasing antibiotic resistance is the urgent requirement to develop novel molecules that are insensitive to resistance processes in order to combat resistant pathogens. The most abundant bacterial strain of soil from *Bacillus* genus are able to produce more than two dozens of antibiotic compounds with various structural and functional properties based on different mechanisms such as competition for an ecological niche or a substrate and induced systemic resistance [11]. The aim of this study was to isolate antagonistic *Bacillus* sp. with potent and broad inhibitory spectral antimicrobial compounds from various sources of soil.

One of the main representatives, *Bacillus subtilis* had been investigated elaborately for its antagonistic property where 4-5% of its genome was devoted to antibiotic production which is of peptide nature [1]. Although, *B. subtilis* is commonly investigated, the production of antimicrobial compounds varies with the strain based on its ecological niche, thereby tempting us to speculate novel compounds with broad-spectrum potential.

4.1 Isolation and primary screening of antibiotic producing genus *Bacillus* from soil

63 single colonies of genus *Bacillus* were isolated as pure cultures based on standard phenotypic (morphological) characterization. During the primary screening by spot agar method against 19 gram-positive, 16 gram-negative bacteria and 10 yeasts, soil isolate RLID 12.1 from East Dehradun, India showed clear zones of inhibitions around most of the indicator organisms used (Table 1).

4.2 Identification of *Bacillus* isolates RLID 12.1

The complete identification of RLID 12.1 were done using polyphasic approach because environmental soil isolates, particularly aerobic endospore producers share common characteristics for their survival which cause a problem in species level identification [6]. Based on morphological and biochemical characterization (Table 2) and BLAST analysis of partial sequence of the 16S rRNA gene of RLID 12.1, its taxonomic identity was identified as *B. subtilis*. Since many *B. subtilis* sub sp. also share the same characteristics, further confirmation was done by FAME analysis. The partial 16S rRNA sequence of RLID 12.1 was found 99 % similar to the *B. subtilis* strain 407D3 accession number HM099655.1. Phylogenetic tree was also constructed by neighbor joining method (Fig. 1). The accession number of RLID 12.1 submitted to the GenBank nucleotide sequence database is JX089317.

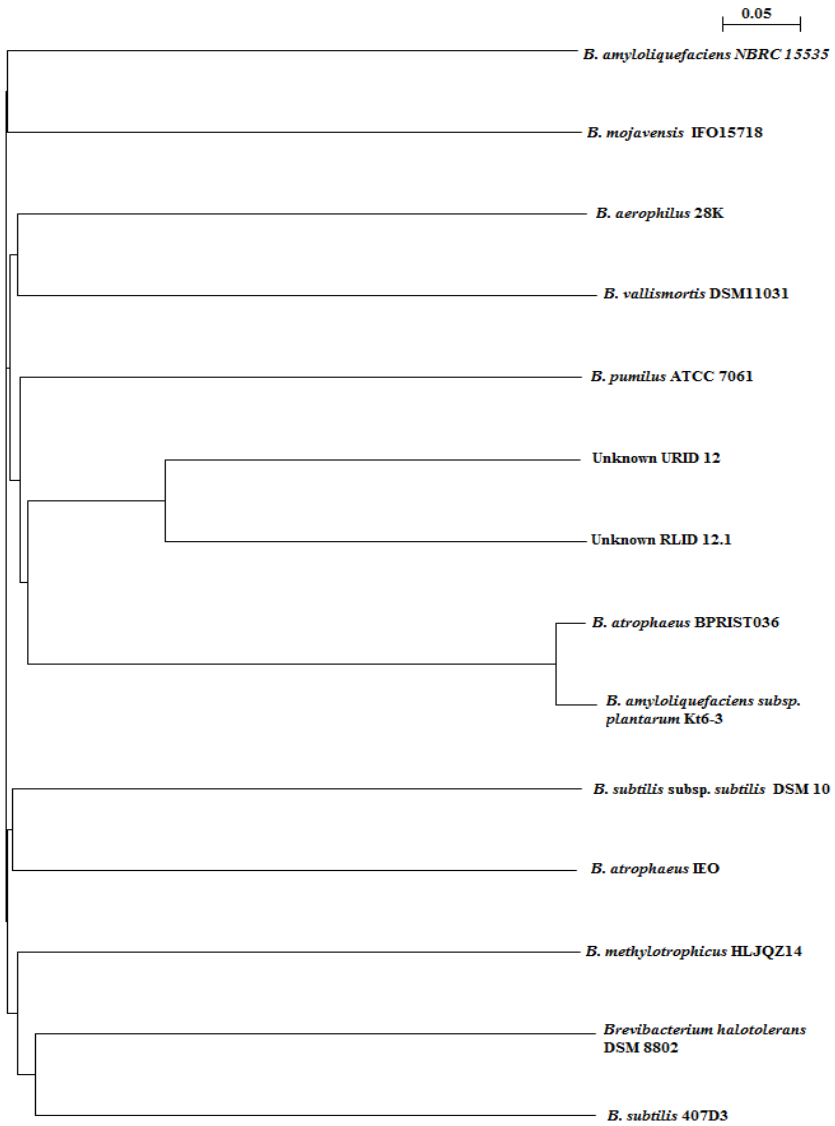

Fig.1 Distance tree of unknown *Bacillus* sp. RLID 12.1 constructed using CLUSTAL X2.1 by the neighbor joining method.

Antimicrobial compound produced by *Bacillus subtilis* RLID 12.1 have broad inhibitory spectrum against famous hospital acquired infections like "ESKAPE" (*Enterococcus faecium*, *Staphylococcus aureus, Klebsiella pneumoniae*, *Acinetobacter* sp., *Pseudomonas aeruginosa*, and *Enterobacter* species) [12] and yeasts like *Cryptococcus neoformans* and candidiasis causing *Candida* sp. Broad inhibitory spectral property had been reported in many *B.subtilis* LFB 112 [13], *B.amyloliquefaciens* MBL27 [14]. Further molecular characterization of partially purified or purified substance is underway to determine its actual molecular nature and classification.

Acknowledgements This work was financially supported by the Department of Science & Technology (DST) New Delhi and DST (FIST), New Delhi. The facility provided by BITS Pilani KK Birla Goa Campus is thankfully acknowledged.

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Antimicrobial suceptibility of *Campylobacter jejuni and Campylobacter coli* strains isolated from turkeys slaughterhouses located in the area of Algiers (Algeria)

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One of the most important objective of our research was to study the antimicrobial susceptibility of *Campylobacter jejuni and Campylobacter coli* strains after their isolation from samples of algerian turkey slaughterhouses (neck skins and caecums). 200 samples were collected in 3 turkey slaughterhouses. After research and identification of the thermotolerant *Campylobacter* (ISO 10272, 1995; WHO, 2003; OIE, 2005), an antibiogram was realized with the gel diffusion method in culture media (CA-SFM, 2007). The tested antibiotics were ampicillin (AM), gentamicin (GM), erythromycin (E), ciprofloxacin (CIP), tetracycline (TE), chloramphenicol (C) and nalidixic acid (NA). Our results showed that the isolated strains were resistant to the majority of the tested antibiotics: ampicillin (66%), erythromycin (25%), ciprofloxacin (75%), tetracycline (81%) and nalidixic acid (88%). No resistance was recorded for gentamicin (0%) and chloramphenicol (0%). Furthermore, 20 different resistance profiles were established. In conclusion, turkey participates wildly in the emergence of antibiotic resistance phenomenon.

Keywords turkey; thermotolerant *Campylobacter*; antimicrobial resistance.

1. Introduction

Thermotolerant *Campylobacter* are slender, spiral or curved, microaerophilic gram-negative rods that represent actually one of the most commonly reported bacterial causes of enteritis in the developing and developed world (Allos, 2009). Transmission to humans usually occurs through ingestion of contaminated poultry meat, as avian species (chicken and turkey) are the main reservoir of *Campylobacter*. In addition, a low infectious dose is enough to cause an ordinary *Campylobacter* enteritis which may progress to hemorrhagic enteritis and sometimes even to a Guillain-Barre syndrome (Dromigny, 2007).
In general, the patient eventually heals without resorting to antibiotic treatment but in severe cases, antibiotic treatment is needed. The thermotolerant *Campylobacter* both in animals and humans, have acquired over time resistance to various antibiotics, including erythromycin and ciprofloxacin; which are the major molecules for the treatment of *Campylobacter* infection (Mégraud, 2007). Thus, the presence of *Campylobacter* strains resistant to antibiotics in foodstuffs of animal origin represents a significant threat to public health.

One of the most important objective of our research was to study the antimicrobial susceptibility of *Campylobacter jejuni and Campylobacter coli* strains after their isolation from samples of Algerian turkey slaughterhouses (neck skins and caecums).

2. Materials and methods

2.1 Sample collection

200 samples were randomly and aseptically collected in the region of Algiers. A total of 100 samples of neck skins and 100 others of caeca were carried out just after the evisceration of turkey carcasses in 3 poultry slaughterhouses located in Boumerdès between the month of Jun and august 2010. Thereafter, all the samples were placed in a cool box and transported rapidly to the laboratory, where they were processed the same day. Once arrived to the laboratory, all the *Campylobacter* cultures (isolation, identification and antimicrobial susceptibility testing) were obtained with the microaerophilic generators GENbag microaer (bioMérieux) (5% O_2, 10% CO_2 and 85% N_2). Negative and positive controls were realized with ATCC strains (*C. jejuni* ATCC 396, *C. coli* ATCC 0321 and *C. fetus* ATCC 443).

2.2 Microbiological methods

Isolation, identification and antimicrobial susceptibility testing of thermotolerant *Campylobacter* were performed in the microbiology laboratory of the Central Hospital of Army, following the WHO (2003), the OIE (2005) recommendations and the ISO 10272 (1995) standard. Briefly, from each sample of caecal content, 1 g was aseptically taken, inoculated in 9 ml of sterile saline (0,9% NaCl, w/v) and homogenized. For neck skins, 10 g of each sample were aseptically collected and added to 90 ml of Preston broth (Oxoïd) supplemented with 5% full horse blood (IPA :Institute Pasteur of Algeria) and incubated microaerobically at 42°C for 24 hours (WHO, 2003). Each loopful of culture was taken and streaked onto Butzler selective agar plates supplemented with 5% full horse blood (Columbia BIO-RAD ; supplement OXOID). The selective media were then incubated microaerobically at 42°C for 48 hours.

All the presumptive Campylobacter colonies were subcultured on Columbia agar (BIO-RAD) plates supplemented with 5% full horse blood (Institut Pasteur d'Algérie: IPA) and incubated microaerobically at 42°C for 24h. Only one strain per sample was kept to confirm the suspected *Campylobacter* colonies by the following tests: Gram staining, motility, oxydase production, sugar fermentation on TSI media and growth at 25°C (OIE, 2005). Additional tests represented by: H_2S production on TSI media, catalase production and susceptibility to nalidixic acid (NA) (30µg) and cephalothin (CF) (30µg) were also done (ISO 10272, 1995).

API-Campy system (Biomerieux, Marcy l'Etoile, France) was also used to identify the *Campylobacter* species. After confirmation of the colonies, antimicrobial susceptibility testing for the isolated strains of thermotolerant *Campylobacter* was realized by the agar diffusion method (disk diffusion test) as recommended by the antibiogram committee of the "French Society of Microbiology" (Anonymous, 2008). In addition to nalidixic acid, the tested antibiotics were: ampicillin (AM) (10 µg), gentamicin (GM) (15 µg), erythromycin (E) (15 UI), ciprofloxacin (CIP) (5 µg), tetracycline (TE) (30 UI) and chloramphenicol (C) (30 µg). From each subculture of 18 to 24 hours of incubation, a suspension having a turbidity of 0,5 McFarland was prepared in sterile physiological saline (0,9% NaCl, w/v) and diluted (1/10). After that, a sterile cotton swab was dipped into each suspension and streaked on the entire surface of a Mueller Hinton agar (BIO-RAD) supplemented with 5% full horse blood (IPA). Finally, the antibiotic discs (bioMérieux) were placed on each plate and after 24 hours of microaerobic incubation at 37°C, the diameter of the inhibition zone was measured with metal calipers. The ATCC strains *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were plated as controls. All the strains were inoculated into brain heart infusion broth containing 20% glycerin and stored in a freezer (Mégraud, 2007).

The comparison test of χ^2 was performed to analyze the significance of the difference. The difference was considered significant when the probability (P) was less than or equal to the risk α (0.05) ($P \leq 0.05$).

3. Results and discussion

From the 200 samples, we detected 145 thermotolerant *Campylobacter* (*Campylobacter jejuni* or *Campylobacter coli*) strains, which correspond to an overall prevalence of 72.5%. They were isolated in 90.0% (90/100) of the caecal contents samples and 55.0% (55/100) of the neck skins samples. Figure 1 shows the prevalence of thermotolerant *Campylobacter* in the analyzed samples. The difference among these results was statistically significant ($P \leq 0.05$).

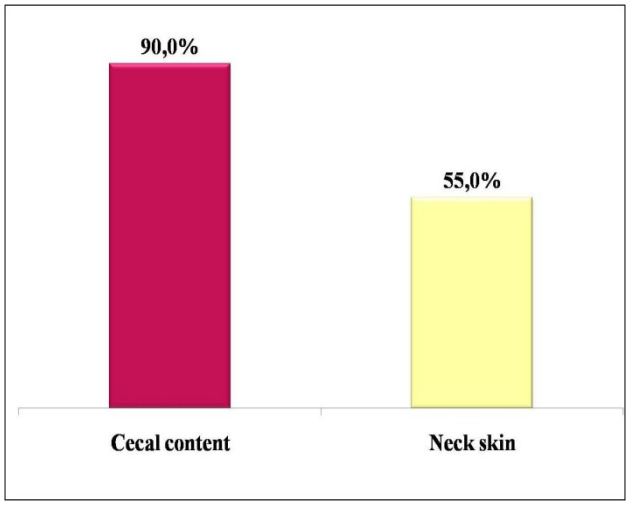
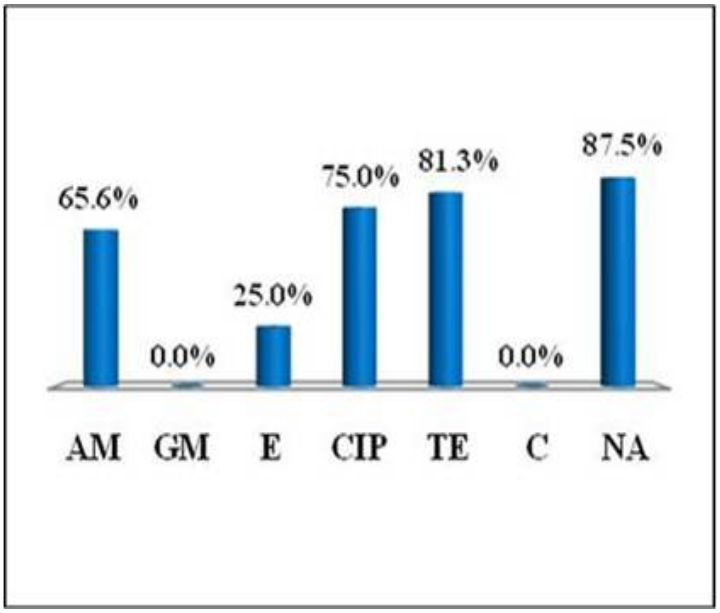


Fig. 1 Prevalence of thermotolerant *Campylobacter* in the analyzed samples.

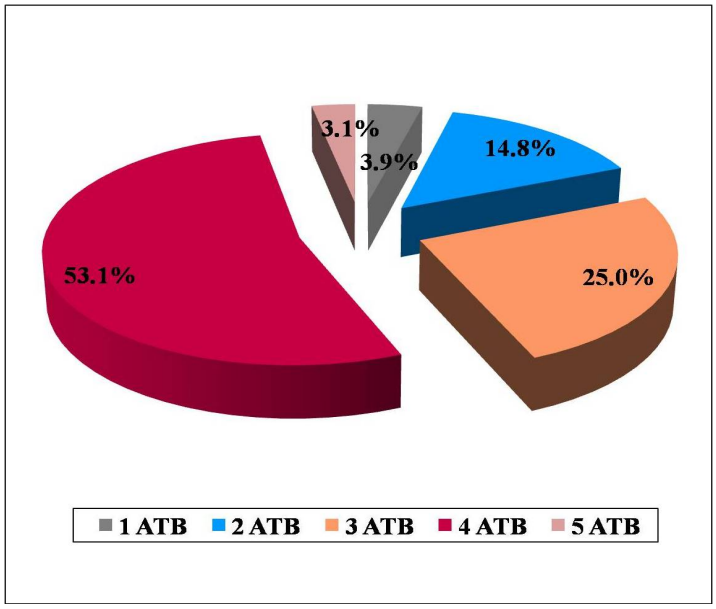
17 strains were impossible to re-streak. Consequently, the antimicrobial susceptibility testing was realized only for 128 out of 145 strains isolated from the poultry slaughterhouses. 87.5% (n = 112) strains were resistant to nalidixic acid, 81.3% (n = 104) to tetracycline, 75.0% (n = 96) to ciprofloxacin, 65.6% to ampicillin (n = 84), 25.0% (n = 32) to erythromycin and no resistance was recorded for gentamicin and chloramphenicol (0.0%). Figure 2 shows the rates of resistance to the tested antibiotics for the 128 isolates.



Ampicillin (AM), gentamicin (GM), erythromycin (E), ciprofloxacin (CIP), tetracycline (TE), chloramphenicol (C) and nalidixic acid (NA).

Fig. 2 Rates of resistance to the tested antibiotics for the 128 isolates.

All the tested strains were resistant to at least one antibiotic (100%) and 104 (81.2%) were multiresistant (resistant to at least three antibiotics). 3.9% (n = 5) of the strains were resistant to only one antibiotic, 14.8% (n = 19) to two antibiotics, 25.0% (n = 32) to three antibiotics, 53.1% (n = 68) to four antibiotics and 3.1% (n = 4) to five antibiotics. Figure 3 shows the rates of multiresistance to the tested antibiotics for the 128 isolates.



ATB: antibiotic.

Fig. 3 Rates of multiresistance to the tested antibiotics for the 128 isolates.

20 different resistance profiles were established. The most common resistance profile was observed 55 times (43.0%) and included four antibiotics: ampicillin, nalidixic acid, ciprofloxacin and tetracycline. The resistance to ciprofloxacin and / or to erythromycin has characterized most of the phenotypes of multiresistant bacteria. Figure 4 shows the antimicrobial resistance profile for the 128 isolates.

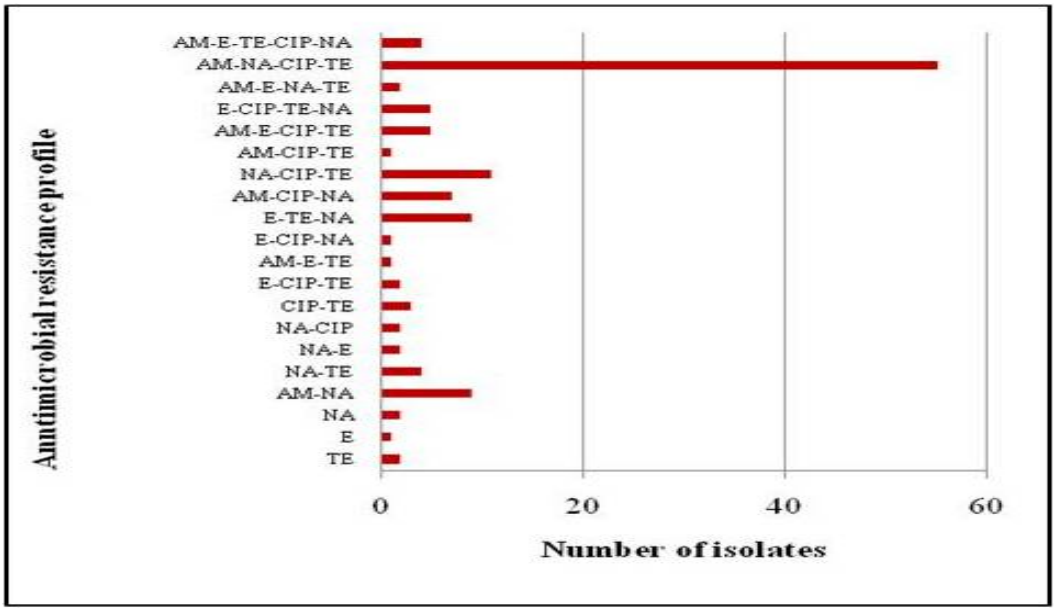


Fig. 4 Antimicrobial resistance profile for the 128 isolates.

Since turkey is one of the main reservoirs of thermotolerant *Campylobacter* (Corry and Atabay, 2001), the frequency of these bacteria is naturally higher in the caecal content. The contamination of the carcass, in general, and the neck skin, in particular, can be done from the intestinal contents during the evisceration step (AFSSA, 2003); which would explain the observed prevalence (55.0%).

The high rates of resistance and multiresistance, and the numerous observed resistance patterns, may be related not only to the uncontrolled administration of certain antibiotics, but also to the extended use (16 to 20 weeks) of those molecules in turkey flocks. Indeed, Nayak *et al*. (2006) found that the duration of the breeding played a significant role in increasing the number of resistant *Campylobacter* strains to antibiotics.

4. Conclusion

Our results suggest that the turkey industry in Algeria could be the cause of a major public health problem through the spread of pathogenic strains of *Campylobacter* and the spread of the phenomenon of antibiotic resistance in particular to those used in the treatment of human campylobacteriosis (erythromycin and ciprofloxacin).

Acknowledgements This work was supported by the High National Veterinary School and the Central Hospital of Army of Algiers. Thanks to all the collaborators.

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Antioxidant and antimicrobial activity of quinoa seeds (*Chenopodium quinoa* Willd.) from three geographical zones of Chile

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Six different cultivars of quinoa seeds (Chenopodium quinoa Willd.) cultivated in three distinctive geographical zones of Chile were evaluated as potential new sources of antimicrobial and antioxidant activity. Ethanol extracts of seeds were tested for antioxidant levels using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant assay and for antimicrobial activity using a disk diffusion assay against *Staphylococcus aureus* and *Escherichia coli*. Antioxidant levels ranged from 3773.37 to 461.89 µg/mL. The cultivars that showed the highest zone of inhibition were those of North (Ancovinto and Cansosa) with % inhibition >62% for *E. coli* and >51% for *S. aureus*. For both bacteria studied Cancosa cultivar had the highest antimicrobial action. The correlation between six cultivars with antioxidant levels and those with antimicrobial activity was quite low. In this study, we identified cultivars of quinoa seeds as potential sources of antioxidant and antimicrobial compounds.

Keywords quinoa; antimicrobial; antioxidant; cultivars

1. Introduction

Recently, there are a lot of reports on the antioxidant and antimicrobial activities of plant-based extract, which has a potential application both in the food and in medicinal industries [1]. Spices and herbs have been added to food since ancient times, not only as flavoring agents, but also as folk medicine and food preservatives [2]. Antimicrobial compounds present in foods can extend the shelf life of unprocessed or processed foods by reducing the microbial growth rate or viability. Extensive research has investigated the potential application of natural antimicrobial agents in food preservation. Because of these reasons, plant extracts are promising natural antityrosinase, antioxidant, or antimicrobial agents with potential applications for the preservation of foods [1].
Quinoa seeds (Chenopodium quinoa) were originated some seven thousand years ago from South America, in today's countries such as Peru, Bolivia, Chile, Ecuador, Colombia and Argentina. This crop constitute a great potential for agronomic demands because it can adapt to produce high grain yields under adverse or stressing conditions [3,4]. In Chile, the quinoa has been cultivated in three different zones. Its conservation reveals its rich biodiversity, as the quinoa landraces can be suitable for very different environments. Six different cultivars of quinoa seeds cultivated in three distinctive geographical zones of Chile (North Highlands, Center and Southern) were evaluated as potential new sources of antimicrobial and antioxidant activity.

Fig. 1 Quinoa cultivated in three different zones of Chile.

2. Materials and Methods

2.1 Origin of quinoa seeds and preparation of samples.

The quinoa seeds were harvested from the three ancestral production areas of Chile (Fig. 1)(North Highlands (N) at 19°S, Center (C) of th country at 34°S and Southern (S) Chile at 39°S). A total of 6 cultivars of quinoa were chosen (seeds were of the harvest season of 2010). The two north Highlands cultivars were Ancovinto and Cancosa (around 19 ºS). The two centre cultivars were Cáhuil and Faro (around 34 ºS) and the two southern cultivars were La Regalona (official variety) and Villarrica (around 39 ºS). The samples were analyzed without a dehusking treatment, so that they were only inspected visually to discard contaminant particles or impurities. Analytical determinations were carried out on ground quinoa seeds using a grinder (MC0360, UFESA, Zhejiang, Chine).

2.2 Antimicrobial activity

Extracts from seeds were tested for antimicrobial activity against two microorganisms, *Staphylococcus aureus* (ATCC 25923) (Gram-positive) and *Escherichia coli* (ATCC 25922) (Gram-negative), using the disk diffusion assay technique [5,6]. All the bacterial strains were provided by the Chilean Institute of Public Health (Instituto de Salud Pública de Chile, ISP).

2.2.1 Preparation of extracts

Ten-gram quinoa seeds of finely-powdered of each cultivars with 50 mL of absolute ethanol (99.0%, Sigma–Aldrich, Steinheim, Germany) were mixed. The mixtures were then left, in the dark, at room temperatures for 24 h with orbital shaker (Boeco OS-20, Hamburg, Germany) at 200 rpm, prior to filtration (Whatman no. 1) and centrifugation (Eppendorf 5804R, Hamburg, Germany) at 5000 rpm for 10 min at 5 °C. The clear extracts were filtered by Millex-GV Filter Units (0.22 µm, Merck KGaA, Darmstadt, Germany) followed by concentration under reduced pressure at 40 °C (Buchi, model RE-121, Flawil, Switzerland). Dried quinoa extracts were dissolved in 15 mL sterile distilled water to a final concentration of approx 30.0 mg of extract/mL and refrigerated until antimicrobial assay.

2.2.2 Antibacterial assay procedure

All of the tested bacteria were cultured over night at 37 ºC in the Muller-Hinton Broth (MHB, Merck KGaA, Darmstadt, Germany) and used as inoculums. The turbidimetry of the suspension was adjusted to the McFarland 0.5 turbidity standard (10^8 cfu/mL) [6]. At first, a total of 0.1 mL of bacterial suspension was poured on each plate containing Muller-Hinton Agar (MHA, Merck KGaA, Darmstadt, Germany). The lawn culture was prepared by sterile cotton swab and allowed to remain in contact for 1 min. The sterile filter paper discs (6 mm

diameter) were saturated by 60 µL of different extracts of each cultivars and then were placed on lawn cultures. The Petri dishes were subsequently incubated at 37 ºC for 24 h and the inhibition zone around each disc was measured in mm. This experiment was carried out in duplicate. As positive control, disc containing Amoxicillin 100 µg/mL was used. Disc impregnated with absolute ethanol was also included to test if they had an inhibitory effect on the test bacteria [5,6].

2.3 Determination of antioxidant activity

Free radical scavenging activity of the samples was determined using the 2,2,-diphenyl-2-picryl-hydrazyl (DPPH) method with some modifications [7]. Different dilutions of the extracts were prepared in triplicate. An aliquot of 2 mL of 0.15 mM DPPH radical in ethanol was added to a test tube with 1mL of the sample extract. The reaction mixture was vortex-mixed for 30 s and left to stand at ambient temperature in the dark for 20 min. The absorbance was measured at 517 nm, using a spectrophotometer (Spectronic® 20 GenesysTM, Illinois, USA). 80% (V/V) ethanol was used to calibrate the spectrophotometer. Control sample was prepared without adding extract. All solvents and reagents were purchased from Sigma (Sigma Chemical CO., St. Louis, MO, USA). All the analyses were made in triplicate and total antioxidant activity (TAA) was expressed as the percentage inhibition of the DPPH radicals scavenging rate and was determined by Eq. (1):

$$\%TAA = \left(1 - \left(\frac{Abs_{sample}}{Abs_{control}}\right)\right) \times 100 \quad (1)$$

where Abs_{sample} was the absorbance with sample and $Abs_{control}$ was the absorbance without sample. IC_{50}, which is the concentration required to obtain a 50% antioxidant capacity, is typically employed to express the antioxidant activity and to compare the antioxidant capacity of various samples. IC_{50} was determined from a graph of antioxidant capacity (%) against extract concentration (µg/mL sample).

2.1 Statistical analysis

All data were expressed as mean ± standard deviation (SD). Analysis of data was performed using Statgraphics Plus 5 (Statistical Graphics Corp., Herndon, Virginia, USA). Significance testing was performed using Fisher's least significant difference (LSD) test; differences were taken as statistically significant when $p<0.05$. The Multiple Range Test (MRT) included in the statistical program was used to prove the existence of homogeneous groups within each of the parameters analyzed. Excel statistical software was used to compare the level of antioxidant activity (IC_{50}) to antimicrobial activity (inhibition zones in mm). A regression line was generated and R^2 calculated (Fig. 2).

3. Results and Discussion

3.1 Antimicrobial activity

Results obtained for the antimicrobial tests performed on ethanol extracts of six different cultivars of quinoa seeds are presented in the Table 1. Results showed that all ethanol extracts of the quinoa seeds showed broad spectrum of activity, being active almost to both the Gram-positive and Gram-negative organisms in the antimicrobial assays. The inhibition zone ranged from 8.29 to 14.79 mm and 8.53 to 15.03 mm for *E. coli* and *S. aureus*, respectively. Similar inhibition zones for *E. coli* and *S. aureus* were reported by Borchardt et al. [5]: 7-20 mm (*S. aureus*) and 7-14 mm (*E. coli*) working with crude seed extracts from plants found in the Mississippi River Basin; Mahomoodally et al. [8]: 3-15 mm (*E. coli*) and 4-17 mm (*S. aureus*) working with aqueous and methanolic extracts of endemic plants of Mauritius; Zeng et al. [1]: 19.5 mm (*E. coli*) and 24.7 mm (*S. aureus*) working with water-soluble extract from pine needles of *Cedrus deodara* and Keskin et al. [2]: 7-12 mm (S. aureus) and 7-14 mm (E. coli) working with extract of different species. Difference in the activity of quinoa extracts among studies could be partially explained by variations in bioactive agents of extract, strains sensitivity and antimicrobial procedures adopted in tests. The cultivars that showed the highest bacterial inhibition were those of the Northern localities (Ancovinto and Cansosa) with perceptual inhibition higher than 62% for *E. coli* and higher than 51% for *S. aureus*, being Cancosa cultivar had the highest antimicrobial action. The Gram-positive bacteria (*S. aureus*) were more sensitive to the quinoa antimicrobial action than the Gram-negative bacteria (*E. Coli*) as observed in Table 1.

Table 1 Antimicrobial activity and DPPH free radical scavenging activity (IC_{50}) of six quinoa cultivars.

	Diameter of zone of inhibition (mm)								
Cultivars	*Escherichia coli*			*Staphylococcus aureus*			IC_{50}-Concentration (ug/mL)		
Ancovinto	12.60	±	0.62^{b}	12.89	±	0.51^{b}	3773.37	±	204.46^{a}
Cancosa	14.75	±	0.12^{c}	15.03	±	0.64^{c}	1311.32	±	98.86^{b}
Cáhuil	9.95	±	0.45^{d}	11.57	±	0.73^{bd}	567.96	±	85.00^{c}
Faro	9.64	±	0.77^{d}	9.73	±	0.91^{ef}	461.89	±	104.92^{c}
Regalona	8.30	±	0.32^{e}	8.53	±	0.87^{f}	1043.82	±	75.34^{d}
Villarrica	9.45	±	0.54^{d}	11.09	±	0.84^{de}	791.02	±	211.64^{e}
Control*	20.57	±	0.21^{a}	28.03	±	1.19^{a}			

Values are expressed as mean ± standard deviation. Different letters denotes significant differences among cultivars ($p<0.05$). *Reference antibiotics Amoxicillin (100 ug/mL).

3.2 Antioxidant activity

We attempted to evaluate the relationship between seed antioxidant and antimicrobial activities because antioxidants are known to exhibit antimicrobial activity [1,5,8]. Antioxidant levels ranged from 3773.37 to 461.89 µg/mL. Faro (461.89 µg/mL) and Cáhuil (567.96 µg/mL) had the highest levels of antioxidant activity (Table 1). Nsimba et al. [9] working with different ecotypes of quinoa presented IC_{50} values between 100-15800 µg/mL. Figure 2 compares the antioxidant level (IC_{50}) to the antimicrobial activity (inhibitions zones) of the six quinoa extracts against the two tested microorganisms. Antioxidant activity, with an R^2 of 0.22, explained only 22% of the variation in antimicrobial activity (Fig. 2). Correlation coefficients between antioxidant and antimicrobial activity for individual microorganisms were: S. aureus (0.17) and E. coli (0.28). Similar behaviour has been reported by Borchardt et al.[5].

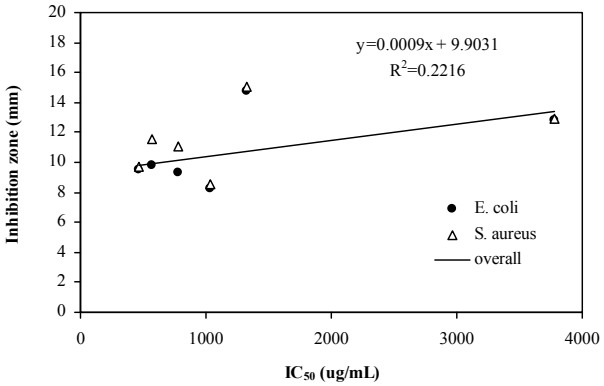

Fig. 2 Correlation between antioxidant activity (IC50) versus antimicrobial activity (inhibition zone) of six quinoa cultivars.

4. Conclusion

It is interesting that there are differences in the antibacterial effects of seed groups, due to the phytochemical differences between cultivars and collection site. The screening of seeds with high antioxidant values may not identify ecotypes with effective antimicrobial activity because the two parameters are not highly correlated. According to our results, we identified cultivars of quinoa seeds as potential sources of antioxidant and antimicrobial activity.

Acknowledgements The authors are grateful to project FONDECYT 1100638 for financial support of this work.

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Application of nisin and chitosan as antimicrobial agents in traditional cured and smoked "Alheira"

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Keywords: Antimicrobial agents, nisin; chitosan; smoked and cured meat products; "Alheira"

1. Abstract

In order to evaluate the influence of antimicrobial agents on the microbiological safety and stability of traditional smoked and cured meat products, it was investigated i) the effect of nisin and chitosan application in controlling *Listeria monocytogenes* growth in "Alheira"; and ii) the organoleptic properties of "Alheira" processed and packed under vacuum or under modified atmosphere (MA: 20 % CO_2 and 80 % N_2) during storage at 4 ºC.

A significant reduction in the level of *L. monocytogenes* was observed in the presence of chitosan (2 and 3 log cycles reduction during the first 15 and 30 days of storage, respectively). On the contrary no inhibition was detected in the presence of nisin. No significant differences were observed in the microbiological quality of "Alheira" packed under vacuum or under modified atmosphere conditions.

Further studies are significantly required to obtain an effective antilisterial agent that simultaneously does not affect consumer's perception and consequently acceptability for these traditional cured meat products.

2. Introduction

The actual consumers interest on traditional food products as well as on natural ones, with no synthetic chemicals addition, are recognized as key market trends that play an important role in new product development. New strategies that take in consideration the quality and sensory characteristics of Portuguese traditional smoked and cured pork products, maintaining their safety and enlarging their shelf life are under investigation. Biopreservation or biocontrol, through introduction of natural substances, is an interesting technology that creates adverse conditions in situ to pathogenic development and consequently assures microbiological safety, and simultaneously allows chemical additives reduction, maintaining nutritional and sensorial product characteristics.

In last decade, a large variety of bacteriocins (lactic acid bacteria peptides) have already been identified and characterized, which allowed a considerable research progress in biocontrol. Several studies focused on antimicrobial ability of different bacteriocins isolated or in mixtures [1, 2, 3, 4, 5].

Nisin is the only antimicrobial agent allowed to be introduced directly in food products. United States Food and Drug Administration recognized nisin in 1988 with a GRAS label and European Union in 1995 authorized the use of nisin (E234) in food products by directive 95/2/EC. Chitosan, extracted from crustacean shells, is another antimicrobial agent with application potential.

In order to evaluate the influence of antimicrobial agents on the microbiological safety and stability of traditional smoked and cured meat products, it was investigated i) the effect of nisin and chitosan application in controlling Listeria monocytogenes growth in "Alheira", a traditional Portuguese cooked, cured and lightly smoked mixture of pork, cow and chicken with bread sausage; and ii) the organoleptic properties of "Alheira" processed and packed under vacuum or under modified atmosphere (MA: 20 % CO_2 and 80 % N_2) during storage at 4 ºC.

3. Materials and Methods

3.1. Microbiological Analysis

"Alheiras" from Ponte de Lima were produced at industrial scale. During the operation of mixing ingredients, the following treatments were considered: i) addition of 50 g of chitosan (dissolved in 1.5 L vinegar) per 5 kg; ii) addition of nisin (final concentration 6.3 ppm) and iii) no addition (control). In order to evaluate the antimicrobial effect of nisin and chitosan in "Alheira", 0.2 ml of a 10^6 CFU/mL suspension of *Listeria monocytogenes* (previously isolated from "Alheira") in Ringer's solution were inoculated with a syringe into 5 different points of each "Alheira". *L. monocytogenes* was enumerated at 0, 2, 4, 7, 15, 30, 45 and 60 days of storage at 4 °C.

3.2. Sensory Analysis

In order to characterize the sensory profile of "Alheira", a quantitative descriptive sensory test was performed, involving previous sessions for main descriptors definition, their scale limits as well as verbal anchors by panel consensus. A final sheet with 16 descriptors, each one with a 13-point scale was validated. Then, all treatments of "Alheira", packed under vacuum or under MA, were evaluated by a semi-trained panel after 5, 40 and 60 days storage at 4 °C.

3.3. Statistical analysis

An analysis of variance (one-way ANOVA) was carried out to assess the effects of storage time and antimicrobial addition treatments on panel results using STATISTICA 7 and Microsoft® Office Excel 2007 tools.

4. Results and Discussion

Comparing Control with Chitosan addition in Fig.1, a significant reduction in the level of *L. monocytogenes* was observed in the presence of chitosan (2 and 3 log cycles reduction during the first 15 and 30 days of storage, respectively); thus the use of chitosan is an alternative to start cultures and may be used in the preparation of sausages to reduce the growth of pathogens. No other bacteria or fungi growth in general medium (TSAYE) in "alheiras" with chitosan addition was observed, confirming the antimicrobial activity of this natural compound against other microorganisms. Despite the positive microbiological results, the use of chitosan did not present good sensory results; at the 5th day the panel considered the product below the threshold of acceptability.

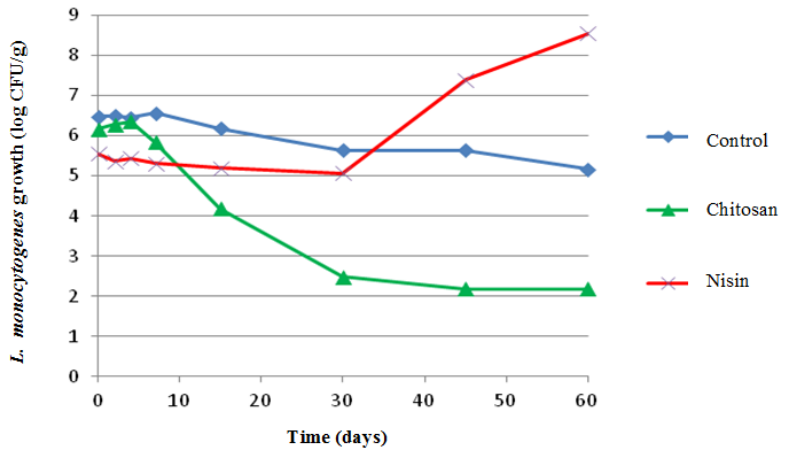
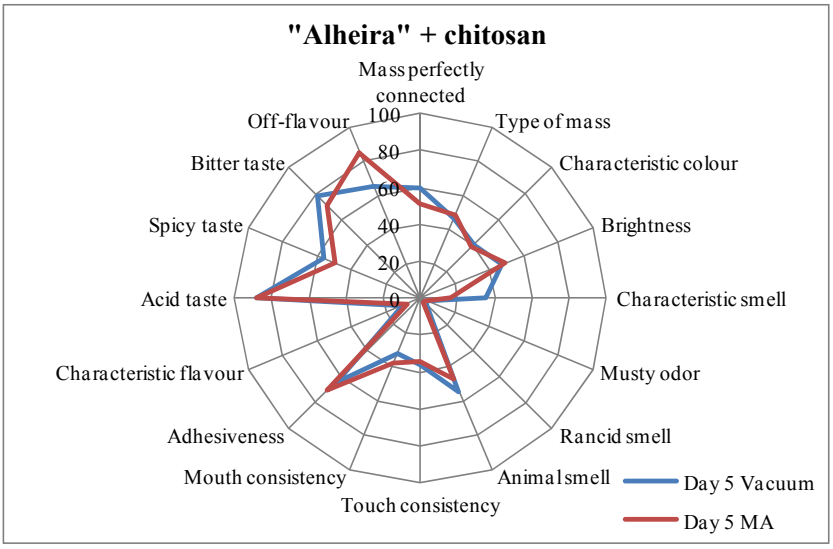


Fig. 1. Growth of *L. monocytogenes* with chitosan and nisin incorporation (Control – pathogen growth in sausage only; Chitosan – pathogen growth with chitosan addition in sausage; Nisin – pathogen growth with nisin addition in sausage).

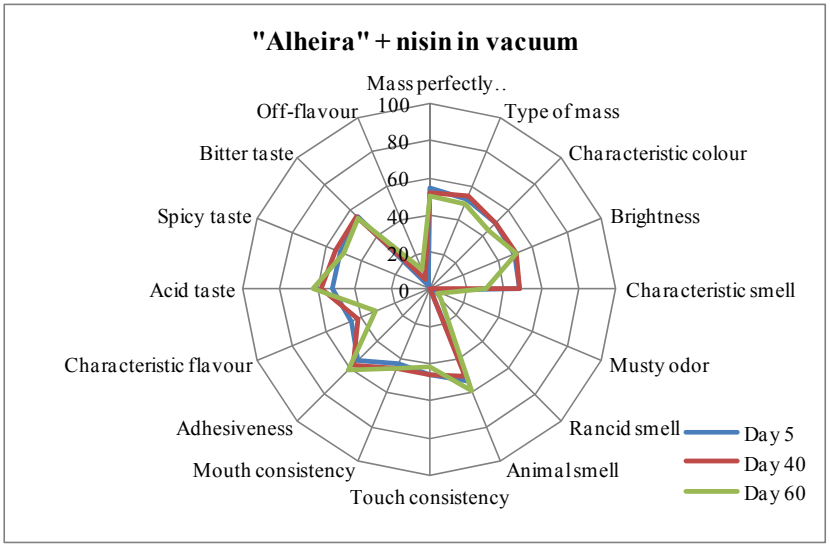
Comparing Control with Nisin Addition in Fig.1, this compound did not prove to be a good option for pathogen reduction. Additionally, no significant differences were observed in the microbiological quality of "Alheira" packed under vacuum or under modified atmosphere conditions.

Figure 2 a) shows the sensory profile of the sausage with chitosan addition in the 5th day of storage for both packaging conditions. It is visible an increase in the perception of the attribute "Off-flavour" packed under MA. A very high intensity of the attribute "Acid taste" was also perceived. Due to the unsatisfactory results in the first day of analysis no more sensory analysis were performed in the following days for chitosan addition. Figure 2b) and 2c) show the sensory profile of the sausage with nisin addition for vacuum and MA over storage life. The acid taste intensified over storage for the nisin-added samples, yet panellists did not find significant differences between them (Fig 2 b) and c)).

a)



b)



c)

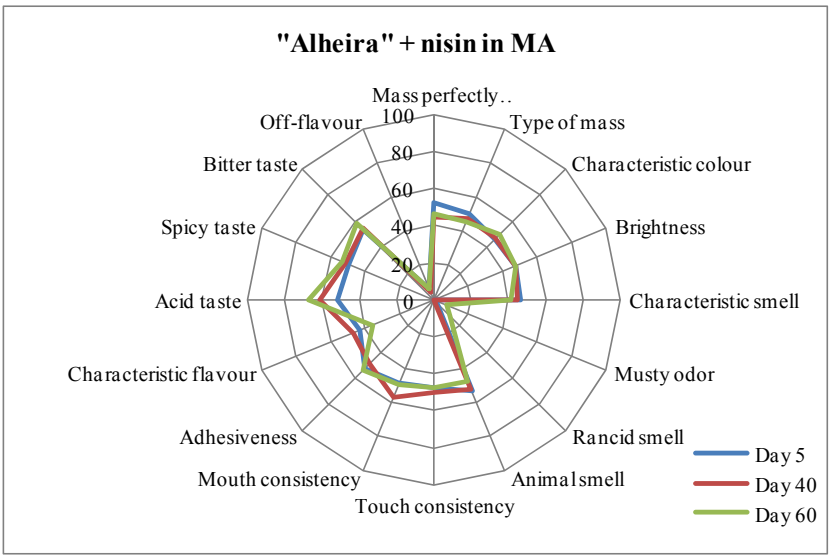


Fig. 2. Sensory profiles of "Alheira" over storage life for (a) chitosan addition in vacuum and MA only for day 5, (b) with nisin addition under vacuum packaging and (c) with nisin addition under MA.

5. Conclusion

The condition with nisin addition, despite having had a good acceptability by the panel, the level of microbial reduction was not as effective. Rather, the condition with chitosan addition had an excellent performance in terms of bacterial reduction, but did not get good results in sensory analysis. Further studies are importantly required to obtain an effective antilisterial agent that simultaneously does not affect consumer's perception and consequently acceptability for these traditional cured meat products.

Acknowledgements The accomplishment of this work was also supported by QREN project - ADI 13338 "Biofumados: Tradição vs Qualidade". Authors are grateful to Minhofumeiro, Enchidos Artesanais, Ponte de Lima, Portugal.

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Are we using the appropriate dose of antimicrobials for the treatment of urine tract infections in primary care patients?

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Keywords: Antimicrobials; Urine Infections; Liquid Chromatography.

Introduction

Urinary tract infections (UTIs) in primary care (PC) are the most common infections requiring antimicrobial stewardship [1]. Typically, standard doses are used with little individualization of drug regimens. The antimicrobials used in each country or clinic will depend among other things on bacterial susceptibility profiles prevailing in the local epidemiology of each health area [2]. But common to them is that they must have a high rate of urinary excretion so that they can be effective. However, few studies have been conducted on the actual removal of antimicrobials in patients treated in primary care.

Taking advantage of the synergies found between the Andalusian Health Service and the University of Almería, clinical practice and innovative modern analytical methodologies based UPLC-MS/MS (Ultra Performance Liquid Chromatography) will be placed at the service of the hospital. This will help to determine whether they reach the optimum concentrations of antimicrobial agents in the different origins infectious diseases of the primary care patients who have a poor clinical outcome despite adequate medical therapy.

We think that is necessary to prescribe individualized dose of antimicrobials for each patient, adapting it to its pharmacological characteristics depending on the pharmacokinetics (PK) and pharmacodynamics (PD) in order to ensure the patient's safety and to encourage the antibacterial activity, thanks to the fast and reliable measurement of the concentrations of several families of antimicrobials (aminoglycosides, cephalosporin, macrolides, sulphonamides and quinolones) simultaneously in different matrices biological fluids as serum and urine fluids samples [3-7].

Objetives

The objectives of our study were:

1) Application of liquid chromatography techniques for the measurement of antimicrobials in biological samples.
2) Measurement of antibiotic levels in blood and urine of patients treated as outpatients in primary care.
3) To evaluate antibiotic concentrations *in biological fluids* regarding the minimum inhibitory concentration (MIC) bacteria.

Methods and Results

We have obtained a total of 21 samples (13 urine and 8 blood) from patients seen in primary care with a diagnosis of UTI from March to September 2012.

We have developed an innovative methodology based on UHPLC-MS/MS technology (Ultra High Performance Liquid Chromatography) with clinical application for determining the concentrations of 22 different antimicrobial agents (doxycycline, josamycin, erythromycin, cloxacillin, amoxicillin, sulfamethoxazole, norfloxacin , ciprofloxacin, nitrofurantoin, levofloxacin, moxifloxacin, clavulanate, clarithromycin, trimethoprim, fosfomycin, ofloxacin, pipemidic acid, cefaclor, cefixime, azithromycin, cefuroxime axetil, and clindamycin) in less than 10 minutes.

We showed the antimicrobials concentration levels of cefuroxime, ciprofloxacin, cefixime and amoxicillin which have been detected in blood and urine samples to primary care patients (table 1).

Then, we compared the levels measured with bacterial MIC to see the adequate concentration of these antibiotics to kill the bacteria (fig. 1).

Table 1. Antimicrobials concentrations in blood and urine samples.

ANTIBIOTIC	BLOOD	URINE
CEFUROXIME	-	No detection
	21,3 µg/mL	400,7 µg/mL
	0,05 µg/mL	0,3 µg/mL
	<0,05 µg/mL	177,9 µg/mL
	2,19 µg/mL	110,6 µg/mL
	0,7 µg/mL	95,8 µg/mL
	-	14,3 µg/mL
CIPROFLOXACIN	5,50 µg/mL	54,5 µg/mL
	-	<0,02 µg/mL
	0,35 µg/mL	<0,02 µg/mL
CEFIXIME	12 µg/mL	83,5 µg/mL
	-	<0,3 ug/ml
	<0,5 µg/mL	<0,3 µg/mL
AMOXICILLIN	5,24 µg/mL	<0,4 µg/mL

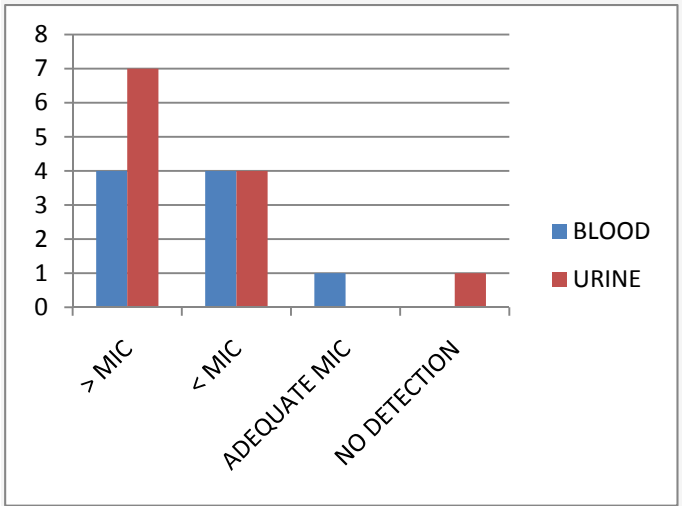

Fig. 1. Antimicrobials concentrations compared MIC bacteria.

Conclusions

1. In 52% of the samples much higher levels of antibiotics to the bacterial MIC (between 10 and 200 times) were found, and in 38% of cases, concentrations were below the MIC bacteria.
2. According to the initial results, we should consider the individualization of dose, and further research on this field.

Lastly, verify the effectiveness of this analytical model (UPLC-MS/MS) because it offers the advantage, being easily exportable and adaptable to other laboratories could provide a useful analytical tool to support antimicrobial therapy in patients with deficient response to treatment, and with potential to map the distribution, dissemination and disposal of antibiotics in humans, without the need of using models to extrapolate animal experimental results.

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Bacterial inactivation by non-thermal plasma treatment

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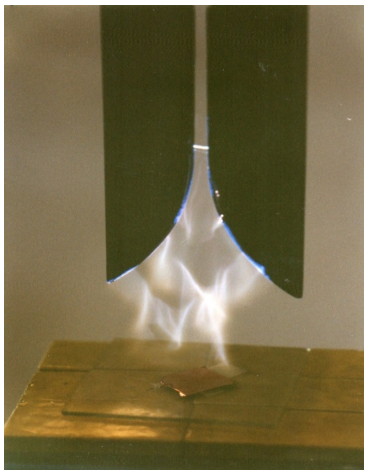
Electrical discharges at atmospheric pressure are a developing source of inactivating species efficient for sterilization. A rapid analysis of the active species formed in a gliding arc in humid air ("glidarc"), such as °NO and °OH radicals, shows that these Reactive Oxygen and Nitrogen Species are directly or indirectly (by means of derivatives) responsible for the oxidizing and acidifying chemical effects in aqueous solutions exposed to the discharge. The standard oxidation potentials of °OH/H_2O and its associated dimer H_2O_2/H_2O are respectively 2.85 and 1.78 V/SHE. °NO is implied in a set of reactions leading to the formation of Reactive Nitrogen Species, e.g., peroxynitrite, as another water soluble intermediate and a precursor for nitric acid. The standard oxidation potential of $ONOO^-/NO_2$ is higher than $E°(H_2O_2/H_2O)$, so that this system may be actually considered as a determining agent in inactivating bacteria processes.
Matching kinetic studies on Gram positive and negative bacteria directly exposed to the plasma show that the inactivation kinetics involve a pseudo zero order step followed by a diffusion controlled pseudo first order step. Temporal Post-Discharge Reactions also take place after switching off the discharge, which confirms the occurrence of active plasma species in the liquid, probably H_2O_2 and $ONOOH/ONOO^-$, in agreement with Biochemistry studies on the peroxynitrite degradation of bacterial wall components (e.g., lipoproteins or teichoic acid, after splitting into °OH and ONO^-). Peroxynitrite was also found responsible for the degradation of nucleic acid, and therefore for the lethal effect of plasma treatment of bacteria.

Keywords: bacterial inactivation; non-thermal plasma; gliding electric discharge in humid air; reactive nitrogen species; reactive oxygen species; peroxynitrite; kinetic study; post-discharge.

1. Introduction

Electrical discharges, e.g., corona, dielectric barrier and gliding discharges are known sources of non-thermal plasma at atmospheric pressure. When they burn in humid air, i.e., a mixture of N_2, O_2 and H_2O molecules, these discharges generate active species such as °NO and °OH radicals, which are identified and quantified by emission spectroscopy. These key species are responsible for most physico-chemical effects of the plasma treatments, i.e., the oxidizing and acidifying effects, when they are in contact with (non-buffered) aqueous solutions. The gliding arc ("glidarc") discharge is probably the most illustrative example of plasma-chemical application, due to the large amount of energy carried away and transferred to the ambient gas, and the matching production of active species.

The glidarc forms at the minimum gap between two diverging electrodes connected with a suitable High Voltage source, e.g., a HV transformer (50 Hz; 6000V; 160 mA in usual working conditions). The arc which is a thermal plasma is pushed along the electrodes to their tips by a humid air flow directed along the reactor axis. When the arc is short-circuited by a new one, a quenched non-thermal plasma cloud forms, with a composition close to the arc but a temperature by few degrees higher than room. The aqueous target solution is disposed in front of the gas flow in a cooled vessel to limit evaporation (Fig. 1), so that the plasma species are in contact with the organic species present at the liquid surface or dispersed in the bulk liquid.



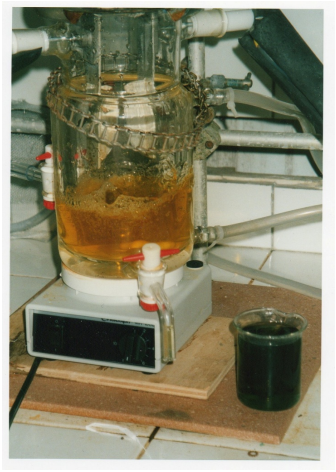


Fig. 1. Glidarc reactor:

Left: Detail of the electrodes for the treatment of Cu foils.
Right: View of the reactor for batch treatments of liquids.

The energy source (HV Transformer) is not shown.
Specific energy : 500-700 JL^{-1}

1.1. The plasma species and their chemical properties

The high energy concentration associated with discharges induces strong interaction with the surrounding gas and leads to excitation, bond breaking and ionization of the molecules. Hydroxyl radical mainly results from electron impact at excited H_2O molecules, with the matching formation of O atoms which are also yielded by breaking the O-O bonds [1].

The electron channel of the arc passing through humid air generates excited O atoms that are able to dissociate N_2 molecules by direct impact, according to the set of endothermal reactions governing the Birkeland process: $O_2 + e^- \rightarrow 2\,O + e^-$; $N_2 + O \rightarrow {}^\circ NO + N$; $N + O \rightarrow {}^\circ NO$
°NO is then in position to fix an O atom provided by any O-donor moiety (e.g., $^\circ O_2H$, O_3, H_2O_2…) and yields the linear °ONO radical. Nitrogen dioxide then enters a set of complex reactions developed elsewhere [1-3]. °NO is thus the source of linear and transient nitrous ONOH (pK_a: 3.3) and peroxynitrous ONOOH (pK_a: 6.8) acids, before isomerization of ONOOH takes place and yields stable, trigonal and strong nitric acid. Such feature accounts for the observed pH lowering of non-buffered solutions.

The standard oxidation potentials of the systems $^\circ OH/H_2O$ and its associated dimer H_2O_2/H_2O with water are respectively 2.85 and 1.78 V/SHE. °NO is thus implied in a file of complex reactions [1,2] which leads to the formation of Reactive Nitrogen Specie (RNS) such as peroxynitrite as another water soluble intermediate and a precursor for nitric acid [1,3,4]. The standard oxidation potentials of the systems peroxynitrite and its matching acid with NO_2 are higher than that of the H_2O_2/H_2O system: such a feature shows that peroxynitrite may be actually considered as a determining agent in inactivating bacteria processes. Peroxynitrite is claimed to oxidize lipids, proteins and lipoproteins, a feature which induces the breaking of molecular bondings and therefore serious damage on bacterial membranes [2,5,6]. Many Biology studies admit that the compound is implied in molecular stress and able to react with components of the bacterial membranes; works also claim that it is directly concerned with the development of deseases, such as Alsheimer's. Biologists' works also show that peroxynitrite is able to split into ONO^- and °OH and participate to hydroxylation, nitration and nitrosation reactions, as illustrated by Figures 2 and 3, additionally to the nucleophilic character evidenced by the reaction with carbon dioxide [6-9].

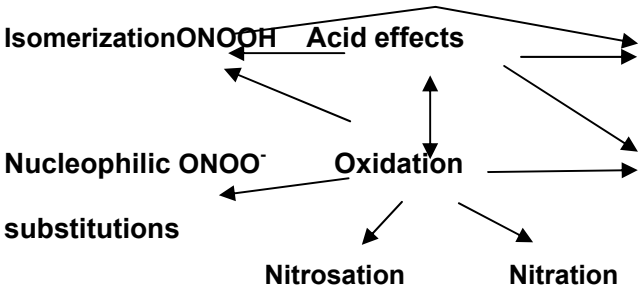


Fig. 2. Synoptic scheme of the ONOOH /$ONOO^-$ reactivity

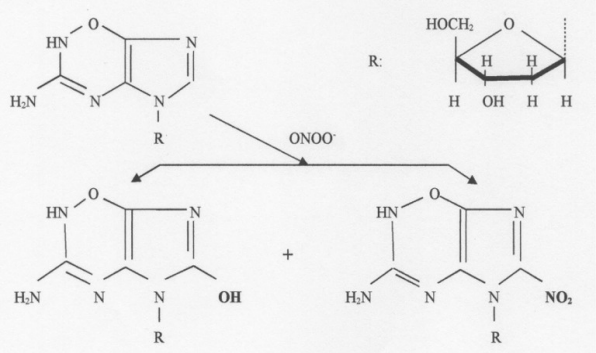


Fig. 3. Action of ONOOH on deoxyguanosine resulting of the splitting of the RNS reagent (adapted from [9]).

1.2. The space distribution of active species in a gliding arc discharge

Figure 4 illustrates the distribution of the main species formed in gliding discharges [1]. The "parent species" refer to the the gas molecules at the reactor input, i.e., O_2, N_2 and H_2O. The hydroxyl radicals result from electron impact at the H-OH molecules and implies electrons in the core of the electron channel, as well as O atoms, ions and various excited species. In particular, the largely endothermic dissociation of N_2 probably takes place in the arc or at its immediate surrounding involving then slower electrons that tend to escape from the electron channel. These "primary species" then react both among them and with parent species in the plasma cloud and yield "secondary species" (e.g., NO_2, or the dimer H_2O_2). The resulting primary and secondary species impinge at the liquid surface and react with the target molecules present. They can also solubilize in water (H_2O_2, ONOOH are water soluble) and diffuse in the liquid phase before reacting with the solutes or the dispersed species.

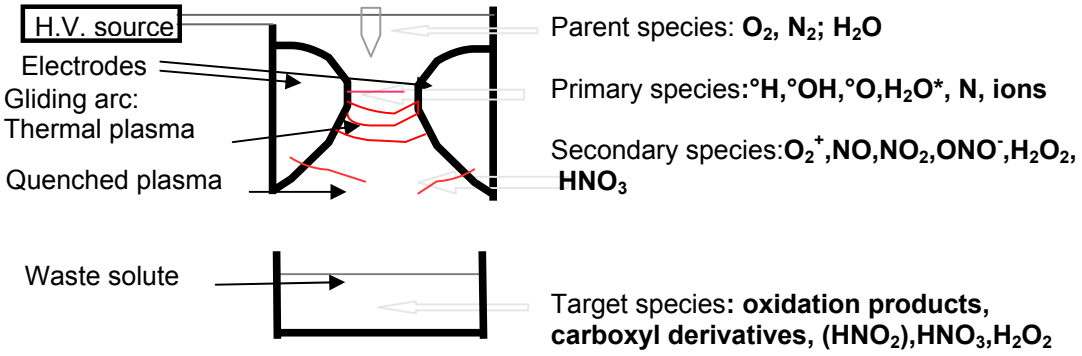


Fig. 4. Schematic distribution of the main reactive species in a glidarc reactor (adapted from [1])

2. Bacterial inactivation

The large and diversified experience acquired in the glidarc treatment of organic wastes [3] led us to consider bacteria, yeasts, moistures and mushrooms as particular forms of organic materials. Hence the organic pollutant abatement observed enven for recalcitrant molecules let us think that plasma-chemical treatments might be successful in case of living matter. Since these targets are scarcely "soluble" in water, we considered that they were actual aggregates dispersed in water. Hence, the plasma species should intact with bacteria both at the liquid surface, in the same way as for surfactants, and in the bulk solution. For the second case we guessed that the plasma active species (soluble ROS/RNS) and the bacteria diffuse in the solution, and that the inactivation reactions take place at the external membrane of the bacteria by mere chemical effect.

2.1. Lethal effects

Bacterial colonies exposed to a gliding discharge burning in humid air are severely damaged up to the death, whatever may be the physiological state of the treated bacteria (planktonic, adherent, detached states or even spores).

The examined bacteria are selected among Gram positive and Gram negative bacteria, e.g., *St. epidermidis, Leuconostoc mesenteroides,* as well as *E. coli, Erwinia carotovara, Hafnia alvei* respectively or *fecal coliforms* and *streptococci* present in industrial effluents of African tanning workshops. Bacterial inactivation resulted also of the plasmachemical treatment of sulfato-reductive and thiosulfato-reductive bacteria. Same preliminary results were observed for mushrooms and moistures.

Yeasts (e.g., *Saccharomyces cerevisiae*) were also exposed to the discharge and confirmed the inactivating effect of the plasma treatments.

2.2. Analytical methods

The analytical methods used for analyses were those employed in Microbiology laboratories. They mainly consist in plating, i.e., in counting the number of colony forming units (CFU) per mL on gelose plates, according to standard procedures. The CFU results were the mean values of three sets of independent experiments.

Scanning Electron Microscopy of 5 min treated *Erwinia* (Fig.5) shows serious damage at the external surface of the bacteria.

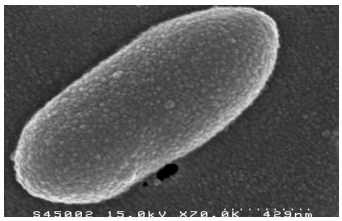
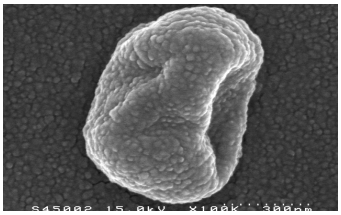


Fig. 5 . *Erwinia carotovora atroseptica* 1526. [10] directly exposed to the gliding arc for t*= 0 min (left) and for t* = 5 min (right). *Pictures by courtesy of Institut Micalis – BHM.*



Chemical analyses of the membrane components, in particular proteins, were titrated by the Bradford's method and confirmed by biuret tests: they evidenced a maximum for increasing exposure time t*. Also, chemical analyses of the lipopolysaccharide (LPS) molecules released in the plasma treatment performed by KDO (keto2-deoxy3-octonate) titration showed a continuous decrease for increasing t*.

Life/Death tests were also achieved on various bacteria but were found not reliable, and did not agree with plating numeration.

2.3. Inactivation kinetics

Bacterial inactivation kinetics performed by glidarc technique with *direct* exposure of the bacteria to the discharge usually present two steps, i.e., a pseudo zero-order step followed by a pseudo first order step, as presented in Figure 6. *Hafnia alvei* was selected as a typical example to illustrate the kinetics of the plasma treatments which confirm the observed behaviour of organic wastes under plasma conditions

Delayed inactivation also takes place with plasma-chemical inactivation and fits with previously observed "Temporal Post-Discharge Reactions" (TPDR) [3] which develop after having switched off the discharge and account for the action of plasma species dissolved in the solution. Evolution of *H. alvei* CFU is illustrated by Figure 6 in case of TPDR conditions.

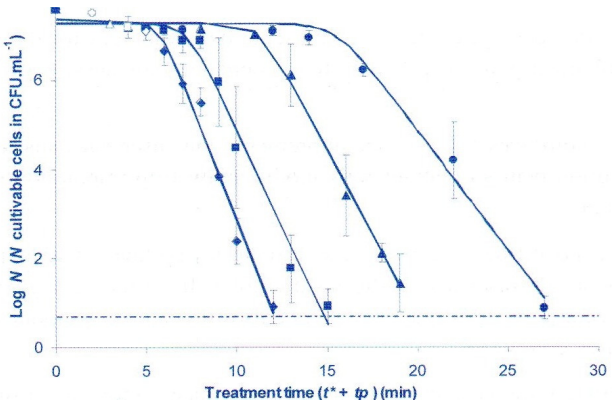


Fig. 6. Temporal Post-Discharge evolution of *H. alvei* [11] for various exposure times t* and relevant pseudo 1st order kinetic constants k, min^{-1}: *t**= 2min (black dots, k=1.2)*; t*= 3 min triangles, k*= 1.8*); t* = 4 min (squares, k*= 2.0*); t*= 5 min (diamonds, k*= 2.4*).* Note that the k values linearly depend on *t**.

3. Concluding remarks

This study confirms that living materials are inactivated by exposure to gliding discharges in a two-step process, as organic wastes are degraded. Temporal Post-Discharge Reactions (TPDR) also take place and involve the diffusion of water soluble plasma species, i.e., Peroxynitrite and H_2O_2. The occurrence of TPDR is of key

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Bacteriophage cocktail in prophylaxis against food-borne infections

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The risk of infection caused by food products contaminated with Salmonella, Escherichia, Shigella, Listeria, Staphylococci or other pathogenic organisms is very high, especially when ready-to-eat products are used. We developed formulas for phage-based probiotic dietary supplement containing effective concentrations of bacteriophages, active against these food-borne pathogens. Phages were isolated and selected, focusing on their host range activity, titre, productivity, ability to retain their characteristics in storage, their sensitivity to adverse factors as well as ensuring safety for lab animals and humans. No phage included in the bacteriophage cocktail carries known toxin-encoding or other unwelcome genes. The bacteriophage cocktail was subsequently purified of endotoxins generated during phage production. These phages culture well, in certain conditions we can reproduce 10^{10-13} pfu/ml of each phages. Peroral form of the phage cocktail will reduce the risks of outbreaks and sporadic cases of food-borne infections.

Keywords bacteriophage cocktail; food-borne infections.

1. Background and aims

In the XXI century the microbiological safety of foodstuffs continues to be the major problem of nutrition hygiene. In order to ensure food safety for consumers, systems that protect human health from microbial risks are being developed and improved. Nevertheless the risk of infection caused by food products contaminated with Salmonella, Escherichia, Shigella, Listeria, Staphylococci or other pathogens is very high, especially when ready-to-eat products are used [1]. The use of antibiotics in agriculture cannot solve this problem because it decreases the ecological quality of the production and gives rise to a new type of pathogen, that is, strains of opportunistic, antibiotic-resistant bacteria. The analysis of the vast global knowledge in this subject shows that it is logical to suggest that bacteriophages should be used to create a new class of probiotic dietary supplements.

2. Methods

Using the clinical material obtained from ambulant cases a collection of strains of Salmonella, Escherichia, Shigella, Listeria and Staphylococci was created. Bacteriophages effective against these pathogens as well as well-documented bacterial strains (i.e. reference strains), were isolated and selected, focusing on their host range activity, titre, ability to retain their characteristics in storage, sensitivity to adverse factors as well as ensuring safety for lab animals and humans (Fig. 1). Phage stocks were prepared using the solid agar media in mattress flasks: 4.5 ml of bacteriophage sensitive bacterial suspension cultured for 18 hours (end concentration of 10^8-10^9 cfu/ml) was poured over 1.5% solid agar and was incubated at 37°C for 3 to 6 hours, after that 2 ml of bacteriophage suspension that had a titre of 10^4-10^5 pfu/ml [2] was introduced into the mattress and incubated at the same temperature for 15-18 hours (depending on the phage species). The viral particles were washed out from the surface of the medium with physiological solution. After the chloroform was added the suspension was centrifuged at 6000 revolutions per minute. The resulting 4 ml of phage suspension had a titre of between 10^{10} and 10^{13} pfu/ml [2]. It was sterilized through disposable filtration attachments with pores 0.22 microns in diameter. Endotoxin purification was done by means of a commercially available column endotoxin puri kit, based on affinity chromatography principles. To confirm the absence of temperate bacteriophages, originating from the bacterial hosts used to grow the bacteriophages, a standard technique for bacteriophage induction using the DNA-damaging antimicrobial agent mitomycin C was carried out [3]. Quality control of end product (phage-based probiotic dietary supplement) also included following parameters: pH, sterility, absence of shiga-like and other toxins, and minimal level of endotoxins (not more than 50 EU/ml).

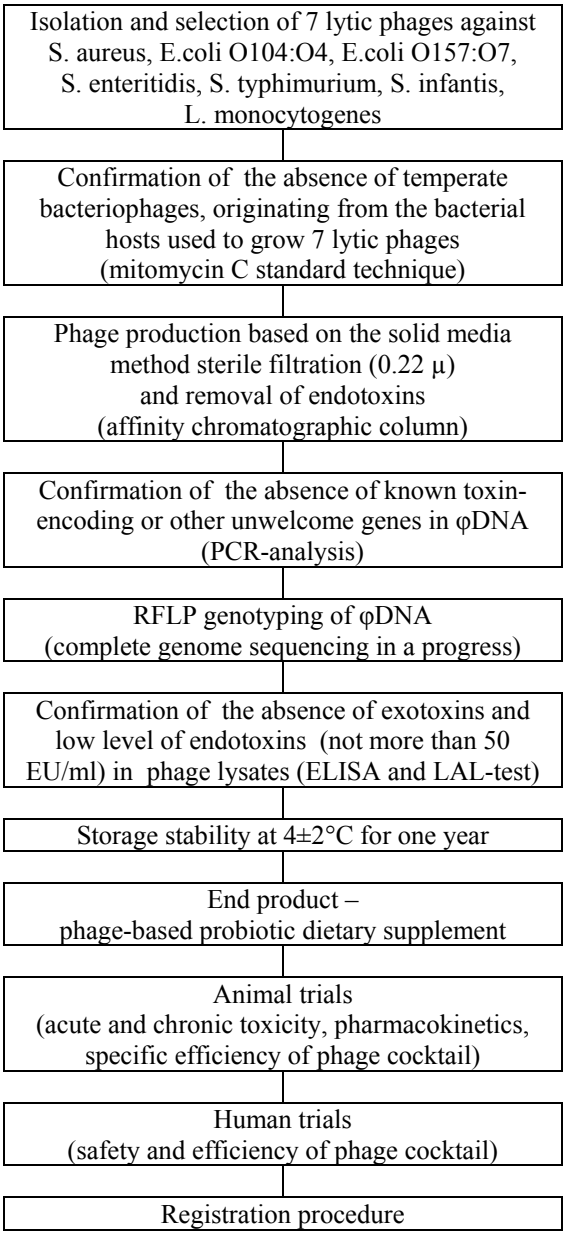

Fig. 1 Phage cocktail pipeline.

3. Results

Detailed discription of phages included in the cocktail are presented at the Table 1. Using polymerase chain reaction with specific primers we showed that all phages do not carry known toxin-encoding or other unwelcome genes. The susceptibility of the host species strains to infection by the tested phages (i.e. host range activity) was determined by the "spot test" method [2]. We used not less than 50 strains of each host species.
Restriction fragment length polymorphism (RFLP) analysis was used for detecting the approximate length of φDNA of Escherichia, Salmonella, Staphylococcus, Listeria phages and for tentative genotyping before complete sequencing (Fig. 2).

The acute toxicity of bacteriophage cocktail (BPhC) was estimated in compliance with Russian State Pharmacopoeia [4] using two species of laboratory animals: outbred mice and guinea pigs (Table 2). The Institute's Ethical Committee approved all animal experiments. Throughout the experiments, mice and guinea pigs were given food and water ad libidum. After examining all ways of BPhC administration we found no intoxication symptoms.

The chronic toxicity assessment [5] of BPhC was carried out on 30 healthy white outbred mice. The BPhC in a dose of 0.5 ml was injected intragastrically once a day for 14 days. The 15 mice from the control group were injected intragastrically with 0.5 ml of physiological solution using the same technique. The BPhC injection did not cause the animals' death and had no influence on their physiological condition, weight nor on morphology

of organs (liver, lungs, heart, thymus, spleen, base of tongue, lymph nodes, stomach, small intestine and colon, mesenterium and brain).

Table 1 Characteristics of the BPhC.

Phage	CH1	ECD7	V32	SE40	STO	SI3	Lm1
Host species	S. aureus	E. coli O104:H4	E. coli O157:H7	S. enteritidis	S. typhimurium	S. infantis	L. monocytogenes
Initial source & place of isolation	patient's wound, Chelya-binsk	chicken feces, Moscow Region	cowshed sewage, Moscow Region	soil, Moscow Region	chicken feces, Kaluga Region	cattle feces, Moscow Region	sheep feces, Astrakhan Region
Host range activity (%/bacterial cultures)	92/ MRSA strains	100/ E. coli O104:H4 O104:H12 O157:H7 etc., 70/ Sh. sonnei & flexneri	100/ E. coli O157:H7 $O157:H^-$	85/ S. enteritidis	100/ S. typhimurium	64/ S. infantis	92/ L. monocytogenes
Presence of known toxins-related genes	No α-toxin gene	No rfb, stx1, stx2, eae & fliC genes	No rfb, stx1, stx2, eae & fliC genes	No stx A gene	No stx A gene	No stx A gene	No LLO-toxin gene
Activity (titre), pfu/ml	10^{10}	5×10^{10}	5×10^{10}	10^{13}	10^{10}	5×10^{12}	10^{10}

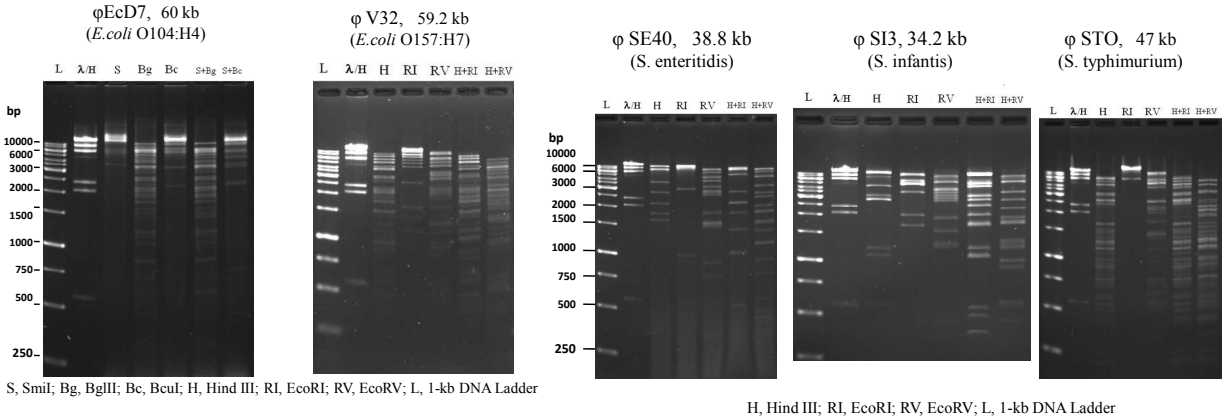

Fig. 2 RFLP genotyping of φDNA.

The pharmacokinetics of BPhC was studied on outbred mice model (10 individuals) after single intragastric injection of the phage-based probiotic dietary supplement (Table 3). The performed experiments showed that the Salmonella phages are present in the highest proportion in mice feces. The Listeria, Escherichia (V32) and Staphylococcus bacteriophages are in smaller proportion in mice feces. In animal intestines, the time (T_{max}) needed for all phages to reach maximum concentration was 9 hours, with the exception of Staphylococcus phage. Subsequently the number of phage particles decreased and after 48 hours only isolated viral particles of Salmonella phages remained in mice feces.

Table 2 Results of acute toxicity experiments.

Animals	N	Method of administration	BPhC dose, ml	Period of observation, days	Intoxication symptoms	
					Deaths	Weight reduction
Outbred mice	15	Intraperitoneally	1	7	0	0
Outbred mice	15	Subcutaneously	1	7	0	0
Outbred mice	15	Per os	0,5	7	0	0
Guinea pigs	10	Intraperitoneally	5	7	0	0

Table 3 Dynamics of phages persistence in mice intestines after single intragastric injection of BPhC [lg(pfu)/g].

Name of phage	Phage titre in BPhC, pfu/ml	Phage titre after injection of BPhC, lg (pfu)/g							$C_{max,}$ lg(pfu)/g	$T_{max,}$ hour	$T_{0,}$ hour
		0 h	3 h	6 h	9 h	12 h	24 h	48 h			
Lm1	0.5×10^{10}	0	0	4.0	4.5	3.8	0	0	4.5	9	< 24
EcD7	1.1×10^{10}	0	1.6	5.7	5.9	5.3	3.0	0	5.9	9	< 48
SI3	0.3×10^{10}	0	2.9	7.7	8.1	6.2	4.1	2.3	8.1	9	> 48
STO	1.0×10^{10}	0	2.9	7.2	8.0	6.4	5.3	2.8	8.0	9	> 48
SE40	9.6×10^{10}	0	0	2.8	7.6	7.1	5.0	3.1	7.6	9	> 48
V32	1.0×10^{10}	0	0	3.7	4.1	3.0	1.6	0	4.1	9	> 24
CH1	1.3×10^{10}	0	n/d	5.8	5.6	5.1	0	0	5.8	6	< 24

Specific efficiency of BPhC was studied during experimental salmonellosis infection of 20 outbred white mice. The rifampicin-resistent strain 92Rif^r of Salmonella enteritidis was used. The experimental salmonellosis was caused by intragastric injection of night agar-medium culture in 10^9 cfu/ml dose ($100LD_{50}$). The BPhC was injected intragastrically (study group) 24 hours before introducing infection and then for 5 days in a dose of 0.5 ml/day. The 10 mice from the control group were injected intragastrically with 0.5 ml of physiological solution using the same technique. The average death term of individuals from the control group was 8 days after intragastric injection of infection. The S. enteritidis 92Rif^r culture was cultivated from liver, spleen and blood of dead animals that is the evidence of the infection generalization. If the BPhC was used, 70% of mice survived, the average death term of diseased mice was 11.4 days. Bacteriological analysis of bodies and feces of the survived animals on the 14th day after finishing the treatment confirmed the 100 % sanitation.

Complex safety and efficiency assessments of the probiotic dietary supplement, based on bacteriophages, was carried out within the medical rehabilitation program of 46 men with chronic diseases of the digestive tract between the ages of 18 and 65. The institute's and the hospital's (respective) ethical committees approved human trials. The typical rehabilitation program included: diet-, phyto-, balneo- and kinesitherapy, training, gastroschool, etc. As a result of bacteriological research of patients' feces Staphylococcus aureus, enteropathogenic Escherichia coli and other representatives of opportunistic microflora typical for intestinal dysbiosis were identified. All patients were divided into two groups: for patients of the study group (30 persons) the BPhC was prescribed additionally to a typical rehabilitation program in a dose of 50 ml 3 times a day with food for 10 days. The 16 patients of the control group were rehabilitated according to the standard program. It was shown that those patients who took the BPhC demonstrated more positive dynamics: they stopped complaining sooner (p-value< 0.05), dyspepsia and meteorism ceased, their stool normalized, tongue plaque and bowel sounds disappeared; the number of patients with dysbacteriosis stages 2 and 3 decreased by 33%, whereas 37% of the patients showed complete normalization of microbiocenosis due to elimination of Staphylococcus aureus and enteropathogenic Escherichia coli (Fig. 3).

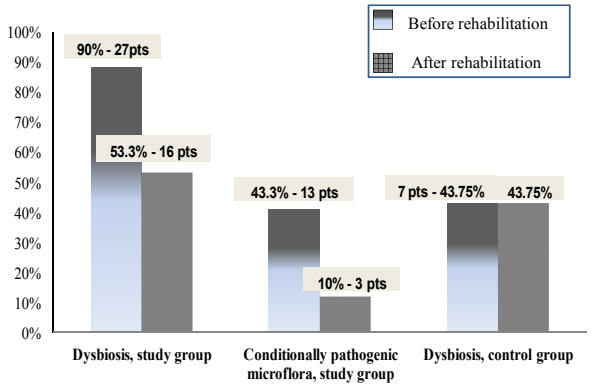

Fig. 3 Dynamics of microbial indicators (according to the study of feces).

4. Conclusions

The data obtained prove the safety and efficiency of designed phage-based probiotic dietary supplement for humans and animals. At the present time we are comleting the sequencing of the genomes of all phages included in the cocktail. After that phage-based probiotic dietary supplement will be presented for registration and we hope that in the near future it may be used effectively as a means of phage prophylaxis for food industry workers and other population groups in order to decrease the risk of sporadic cases and outbreaks of food-borne infections.

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Bioactive and biocompatible cellulose-chitosan composite for medical applications

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The increase of multi-resistant strains and associated hospital acquired infections long for solutions to limit the spreading of pathogenic bacteria in order to decrease infection rates. One approach is the finishing of implants and other medical devices with antibacterial properties. For example wound dressings with such antimicrobial activity can reduce infections with pathogenic bacteria. Therefore, we developed an antimicrobial and haemostatic material for such medical applications based on the two biopolymers cellulose and chitosan. The two polymers were dissolved in an ionic liquid and then moulded into non-wovens by melt-blown spinning technique or the chitosan was used for coating cellulose melt-blown mat. The obtained non-wovens are antibacterial active against the pathogenic bacteria *Staphylococcus aureus* and *Klebsiella pneumoniae* as well as against the human pathogenic yeast *Candida albicans*. Additionally, the melt blown non-wovens show haemostatic properties and are not cytotoxic against L929 mouse fibroblast.

Keywords wound dressing; cellulose; chitosan; antibacterial; melt blow spinning

1. Introduction

The number of multi-resistant strains like methicillin-resistant *Staphylococcus aureus* (MRSA) or vancomycin-resistant enterococci (VRE) is steadily increasing. During hospitalization these bacteria can cause serious infections with a rate of up to 700.000 cases each year in Germany and a death rate of about 30.000 patients[1]. To decrease the bacterial infection rate medical devices can be finished with antibacterial properties. For example, antibacterial wound dressings are able to reduce wound infection rates[2]. Often silver or silver-nanoparticles are used as antibacterial additives in such wound dressings, but in case of nanoparticles or high concentrations of silver toxic effects can occur[3]. As alternatives to silver, antibiotics or disinfectants serve as antibacterial agents in wound dressings. Despite their high efficacy the use of such substances can induce bacterial resistance or are even ineffective[4].

An alternative approach is the development of antibacterial non-wovens for wound dressings based on the natural polysaccharides cellulose and chitosan. Cellulose is widely used for wound applications such as cloths, membrane dressings and gauze because of its high hydrophilicity and both biodegradability and biocompatibility. Chitosan is a biopolymer with antibacterial and haemostatic properties based on its polycationic structure and therefore depend on its molecular weight and degree of deacetylation as well the amount of active ammonium groups. The polymer promotes wound healing and stimulates the immune response[5]. It is biodegradable and biocompatible which makes it an ideal raw material for medical applications.

Here, we used these two polysaccharides to develop a non-woven with antibacterial and haemostatic properties. Therefore, the polymers were dissolved in an ionic liquid and moulded into non-wovens by melt-blown spinning technique and the chitosan was used to coat cellulose melt blown spun non-wovens. After several washing steps the antibacterial and haemostatic properties as well as biocompatibility were determined.

2. Materials and methods

2.1 Production of non-wovens by melt-blow spinning

Two different approaches were used to establish melt-blow non-woven consisting of the polysaccharides cellulose and chitosan. First, cellulose (spruce sulfit pulp, DP 468) non-wovens were produced by melt-blow spinning from a cellulose solution in N-methylmorpholine N-oxide (NMMO) and and then coated with chitosan dissolved in aqueous acetic acid. In the second approach cellulose and chitosan (85/120/A1, Biolog, Halle, Germany) were dissolved together in ethylmethylimidazolium acetate (EMIMAc) in different concentration ratios. The rheological behaviour of the solutions was measured at 85°C in the oszillation mode with a HAAKE MARS measuring system. The obtained polymer solutions in EMIMAc were moulded into non-wovens by melt-blowing and precipitated with a fine 0,01% NaOH solution spray on a PP-matrix. The melt-blown non-wovens were then washed and analysed by raster-electron microscopy (REM).

2.2 Washing procedure

For stabilizing the chitosan and to activate the amino groups of the polymer the cellulose-chitosan non-wovens were washed in 1% NaOH solution for 24h, the NaOH was removed by pressing the non-wovens and a second washing step in destilled water for 24h. Finally, they were washed with phosphate buffer pH 6.0.

2.3 Determination of the antibacterial activity

The antibacterial activity of the cellulose-chitosan non-wovens was determined according to the industrial standard method ISO 20743:2007 “Textiles - Determination of antibacterial activity of antibacterial finished products”. Prior testing the non-wovens were steam sterilised at 120°C for 15 min. A log-phase growing bacteria culture in TSB (15g/l tryptone, 5g/l soya peptone, 5g/l sodium chloride) with a cell density of $1 \cdot 10^5$ cfu/ml was used for inoculating the non-wovens. Test strains were *Staphylococcus aureus* ATCC 6538, *Klebsiella pneumoniae* ATCC 4352 and *Candida albicans* ATCC 10321.

2.4 Haemocompatibility and haemostatic activity testing

The haemolysis quotient of erythrocytes was determined by optical density measurements of fresh blood at 540 nm compared to distilled water as positive control and physiologic salt solution as negative control. To calculate the haemostatic activity the cellulose-chitosan non-wovens were incubated with activated blood for at least 5 minutes and then the blood clotting index was calculated by absorption measurement.

2.5 In vitro cytotoxicity

The in vitro cytotoxicity was identified according to the industrial standard method ISO 10993-5:2009 “Biological evaluation of medical devices -- Part 5: Tests for in vitro cytotoxicity”. The materials were extracted at 1g in 5ml RPMI-1640 with 10% fetal calf serum and then the extracts were incubated with L929 mouse fibroblasts for 24hours at 37°C.

3. Results and discussion

3.1 Antibacterial activity

The antibacterial activity of chitosan coated non-wovens was tested for antibacterial activity against *Staphylococcus aureus* ATCC 6338 according to the industrial standard method DIN EN ISO 20743 “Textiles - Determination of antibacterial activity of textile products” (Fig. 1).

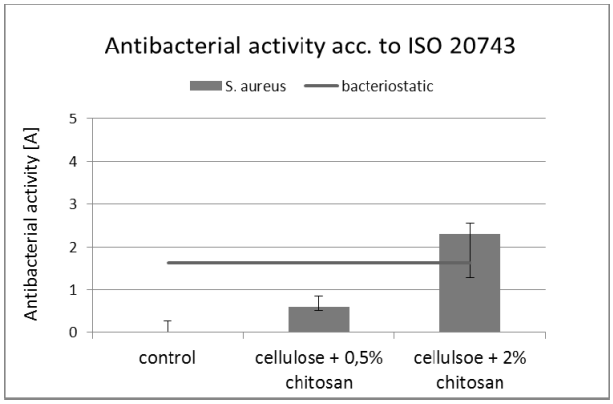

Fig. 1 *Antibacterial activity of chitosan-coated cellulose non-wovens*. Cellulose non-wovens coated with 0,5% and 2% chitosan solution in aqueous acetic acid show weak antibacterial to bacteriastatic activity compared to a control material (lyocell fibres).

To compare the two techniques concerning antibacterial activity of the non-wovens the bacterial growth on melt-blown cellulose-chitosan non-wovens with a content of 25% to 40% chitosan was determined. All tested chitosan concentrations show bacteriostatic behaviour compared to a control material (Fig. 2).

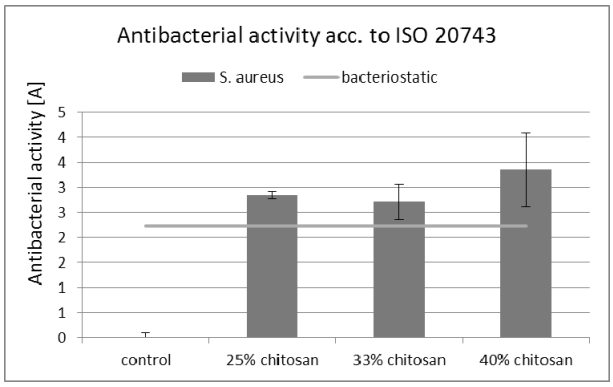

Fig. 2 *Antibacterial activity of cellulose-chitosan non-wovens*. Cellulose-chitosan non-wovens were tested for antibacterial activity acc. to ISO standard 20743 against the gram-positive bacterium *Staphylococcus aureus* ATCC 6538. Non-wovens with a content of 25% to 40% chitosan show bacteriostatic properties compared to lyocell fibres (control).

In comparison, chitosan-coated cellulose with a 2% chitosan solution and cellulose-chitosan non-wovens show comparable bacteriostatic effects.

a. Haemocompatibility and haemostatic activity

The haemolysis quotient of chitosan coated non-wovens was determined by absorption measurement of blood after incubation with the material and the control. A quotient of 5% haemolysis of erythrocytes is indicated as assessment criteria for haemocompatible materials.

Table 1. Haemolysis quotient. The haemolysis of erythrocytes is not influenced by chitosan, except chitopharm M. Chitosan containing non-wovens (coated) show a slight haemolyis effect of 0,27% to 0,35% (Table 1).

Probe	**Hemolysis quotient [%]**	
phys. NaCl [neg. control]	**0**	± 0,007
H_2O [pos. control]	**100**	± 3,3
Chitosan 85/30/A1	**0**	± 0,02
Chitosan 85/120/A1	**0**	± 0,02
Chitopharm M	**0,046**	± 0,09
Chitosan 90/120/A1	**0**	± 0,02
Non-woven 0% Chitosan	**0**	± 0,04
Non-woven 1% Chitosan	**0,35**	± 0,02
Non-woven 2% Chitosan	**0,27**	± 0,07

In contrast to the haemolysis effect the chitosan coated cellulose non-wovens should show a haemostatic behaviour to support blood clotting in open wounds when used as wound dressing. Hence, the blood clotting

index of the cellulose-chitosan non-wovens was determined. The non-wovens showed an increased adhesion of thrombocytes after incubation with activated citrated human blood (Fig. 2).

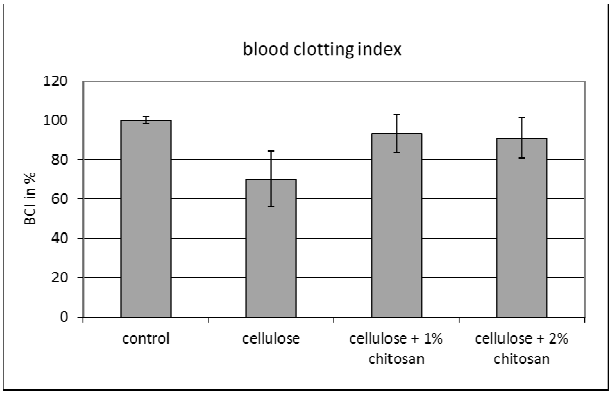

Figure 2. *Blood clotting index*. The cellulose-chitosan non-wovens show similar thrombocyte adhesion as $CaCl_2$-activated blood and the blood clotting is increased compared to pure cellulose.

b. In vitro cytotoxicity

The *in vitro* cytotoxicity is the first test method for a biocompatibility assessment. Material extracts were incubated with L929 mouse fibroblasts for 24 hours and then the cytotoxicity was determined by calculating cell viability compared to an untreated control. The chitosan-coated cellulose non-wovens have no toxic effect on L929 mouse fibroblast cells when 70% to 100% cell growth is assessed as not cytotoxic.

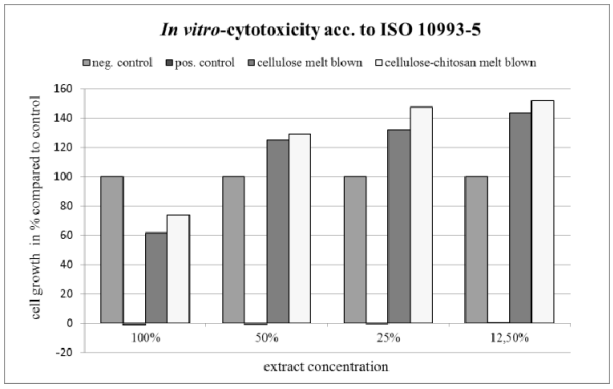

Figure 3. *In vitro-cytotoxicity*. The pure cellulose melt-blown non-wovens show a weak cytotoxic effect of 61% whereas chitosan coated non-wovens show a reduced cell growth of 74% compared to an untreated control which is assessed as not cytotoxic.

4. Conclusion

The melt blown technology is a new method for moulding polymer solutions into non-wovens with fine fibre diameters by blowing the polymer solution with high streaming air. We used this technique to develop an antibacterial and haemostatic non-woven for wound dressing applications. The two polysaccharides cellulose and chitosan were used as raw materials due to their biocompatibility and sustainability and additionally, chitosan has antibacterial and haemostatic properties.

Here, we developed a non-woven consisting of these two polymers. The basis for this was the dissolving of both polysaccharides in one solvent, an ionic liquid. Prior spinning the cellulose-chitosan solutions were characterised for rheology in dependence of the molecular weight and the concentration of the chitosan to obtain an optimal viscosity of the spinning solution. Since the antibacterial activity of chitosan depends on its molecular weight and the degree of deacetylation we had chosen the optimal chitosan with the highest antibacterial activity for our approach.

After spinning the cellulose-chitosan solutions to non-wovens we established a washing procedure to stabilize the chitosan and to increase the antibacterial activity of the non-wovens.

We could demonstrate that the material is antibacterial active against *Staphylococcus aureus* and other human pathogenic strains (data not shown). Chitosan-coated cellulose as well as directly moulded melt blown cellulose-chitosan non-wovens have bacteriostatic activities. The materials are biocompatible concerning *in vitro*-cytotoxicity and haemolytic activity. In addition to this, the cellulose-chitosan non-wovens have haemostatic properties.

Acknowledgements The support of the project no. MF0990038 by the Federal Ministry of Economics and Technology is gratefully acknowledged.

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Cold plasma - a new antimicrobial treatment tool against multidrug resistant pathogens

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Keywords Atmospheric low pressure plasma jet (APPJ) dielectric barrier discharge plasma (DBD); antimicrobial effects; plasma susceptibility; wound pathogens; plasma medicine

1. Introduction

Cold Plasma (CP) has been introduced in medical and biologic applications since it has demonstrated well characterized antimicrobial in vitro efficacy as well as other medically important biochemical effects [1-3]. In the last years first results of clinical plasma applications were undertaken to treat diverse skin and soft tissue infections like bacterial dermatitis, chronic ulcer wounds and eye lid infections [4, 5].
In our previous work we were able to show that two plasma sources, the APPJ and the DBD, were highly effective in reducing bacterial and fungal species [3, 6]. CP killed most species on agar after 3 s up to 30 s exposure time without exception and in combination with non-critical data from risk assessment [7- 9] it can be deduced that CP could also be effective in a) hospital hygiene b) wound medicine to disinfect skin and contaminated, colonized and infected skin and wounds and to prevent nosocomial infections. Up to now systematic susceptibility data of CP are lacking therefore we systematically tested the plasma susceptibility of different sets of clinical relevant bacterial strains in vitro.

2. Methods

2.1 Plasma sources

In vitro susceptibility was tested using two well referenced plasma sources. The first was the atmospheric pressure plasma jet (APPJ, kINPen 09) developed by the INP (Institute for Plasma Science and Technology) Greifswald e.V. in Greifswald, Germany, with Argon as feeding gas and a beam diameter of around 3 mm. The APPJ was used in two modifications, one pulsed and one non-pulsed mode. For a detailed characterization of the APPJ see [10]. The second plasma source was a dielectric barrier discharge (DBD) plasma device developed by the CINOGY GmbH (Duderstadt, Germany). The dielectric barrier electrode measures 20 mm in diameter. For detailed technical characterization see [11].

2.2 Microbiology

In total we collected 194 clinical wound pathogens belonging to 13 different species: *Escherichia coli* (EC), *Pseudomonas aeruginosa* (PA), Extended spectrum β-lactamase (ESBL), *Staphylococcus epidermidis* (SE), *Staphylococcus aureus* (SA), Methicillin resistant *Staphylococcus aureus* (MRSA), Methicillin resistant *Staphylococcus epidermidis* (MRSE), *Acinetobacter spp*. (ACI), *Klebsiella group* (*K. pneumoniae ssp. pneumoniae, K. oxytoca*) (KLEBS), *Enterococcus faecalis* (Efaecalis), *Enterococcus faecium* (Efaecium), Vancomycin resistant *enterococci* (VRE) and High level gentamycin resistant *enterococci* (HLGR). All except VRE strains were isolated from acute or chronic wounds of patients of our dermatologic clinic during routine microbiology diagnostics or in the course of MRSA surveillance (admission screen for multidrug resistant strains), each strain was isolated from different patients (no double testing). Samples were processed following the national guidelines for microbiologic diagnostics.
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2.3 In-vitro susceptibility test model

Directly after plating the suspensions of fresh overnight grown colonies on agar, CP treatment was initiated. Cultures were radiated by distance of 2 mm over 3 s. Directly after each plasma treatment the agar plates were incubated at 36 °C, the growth of bacteria visualized after 48 h. For determining the susceptibility of isolates, the inhibition zones obtained after radiation were calculated using the formula $A=\pi r^2$. The calculation of the inhibition zone allows direct comparison of different plasma sources and the susceptibility of different targets (e.g. bacterial species) and permits an estimation of the antimicrobial potency of a CP device.

2.4 Statistical analysis

Calculations were performed using the free statistical software R (R Development Core Team, 2011). The one-sided p-values were calculated using the t-test. The plasma sources were additionally tested by a one-way analysis of variance and by the Tukey range test (for determining adjusted p-values). A p-value of < 0.05 was considered statistically significant.

3. Results

All plasma irradiations using both sources resulted in circular areas without growth on test agar 24 h after plasma treatment. The obtained inhibition zones (mm²) treating the different species after 3 s treatment are given in table 1. As expected, treatment with the largest plasma beam diameter (20 mm by DBD) resulted in the largest circular inhibition zones ranging from 100.0 mm² (KLEBS) to 479.7 mm² (*E. faecalis* and *E. faecium*), however, not all species (and isolates) showed inhibition zones ≥ the area corresponding to the zone irradiated by the visible beam geometry. The zones caused by non-pulsed APPJ ranged from 10.7 mm² (ACI) to 47.3 mm² (PA), with the pulsed device from 4.8 mm² (SE) to 27.2 mm² (PA) (Table 1). Furthermore, in 10 of 13 species (not so for *E. faecium*, MRSE and ESBL) the zones obtained by the non pulsed APPJ were larger than by the pulsed APPJ (Table 1). Treated by DBD, largest zones were found with gram positive germs compared with gram negative ones. After treatment with APPJ (non-pulsed and pulsed), gram-negative germs were high significantly more susceptible than gram-positive germs ($p< 0.001$) but in contrary they were less susceptible compared with gram-positive germs after treatment with DBD ($p<0.001$), as shown in Figure 1. The pulsed APPJ showed significantly less pronounced efficacy compared with the non pulsed APPJ reaching only 62 % (data not shown) of the non-pulsed APPJ efficacy ($p<0.001$).

Table 1 Backward ranking of germ free surface areas (mean mm^2 and SD) per bacterial species obtained after treatment with three different plasma sources DBD, APPJ pulsed and APPJ non-pulsed, gram–negative (bold), gram-positive (italic)

species (resistant to n classes of antibiotics)	DBD Inhibition zone, mean mm^2 (SD)	species (resistant to n classes of antibiotics)	non-pulsed APPJ Inhibition zone, mean mm^2 (SD)	species (resistant to n classes of antibiotics)	pulsed APPJ Inhibition zone, mean mm^2 (SD)
Efaecalis	479.7 (37.3)	PA	47.3 (8.2)	PA	27.2 (6.8)
Efaecium	479.1 (67.1)	*MSSA*	24.2 (4.0)	*E. faecium*	14.7 (2.2)
MSSA	461.9 (44.8)	*MRSA*	23.4 (3.8)	*MSSA*	14.0 (1.7)
MRSA	403.3 (52.4)	*Efaecium*	20.0 (1.7)	ESBL EC	13.6 (2.9)
HLGR	312.0 (25.3)	ESBL EC	18.6 (2.6)	*MRSA*	13.4 (2.7)
VRE	285.4 (39.0)	EC	17.7 (2.4)	*E. faecalis*	9.8 (1.6)
SE	242.7 (20.3)	KLEBS	12.8 (3.2)	KLEBS	9.3 (2.1)
MRSE	226.5 (19.7)	*E. faecalis*	12.0 (2.6)	EC	9.3 (2.2)
ACI	192.2 (17.2)	*VRE*	12.0 (2.8)	ACI	8.8 (2.0)
EC	121.0 (19.9)	*HLGR*	11.9 (2.7)	*VRE*	7.8 (1.6)

PA	114.9 (17.9)	*MRSE*	11.4 (1.6)	*HLGR*	6.2 (2.4)
ESBL EC	109.7 (25.0)	*SE*	11.3 (2.9)	*MRSE*	5.3 (1.6)
KLEBS	100.0 (19.2)	ACI	10.7 (2.7)	*SE*	4.8 (1.5)

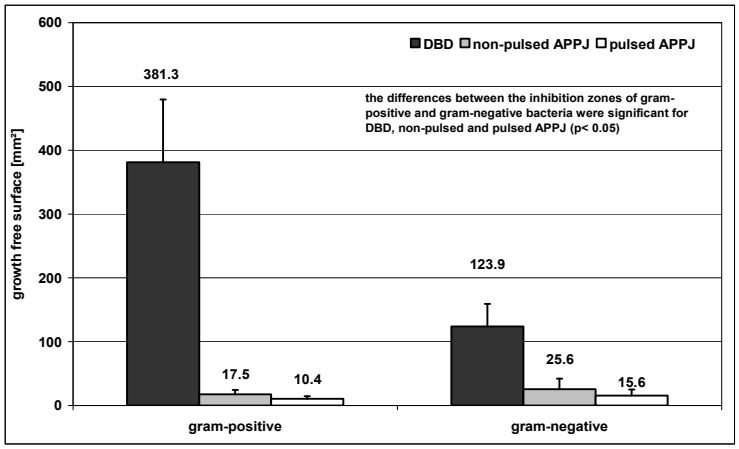

Fig. 1 Mean (SD) inhibition zones obtained after 3 s plasma treatment (DBD, non-pulsed APPJ, pulsed-APPJ) for gram-positive and gram-negative bacteria.

4. Discussion

In our previous work we were able to show that two CP sources, the APPJ and the DBD, were highly effective in reducing bacterial and fungal load in vitro [3, 6] and it can be deduced that plasma could also be effective in the antimicrobial treatment of focal superficial skin colonization and skin infections and also in hospital hygiene to eradicate *MRSA* with plasma as "first physical *MRSA* antiseptic". (Daeschlein *et al*., submitted 2012).

The prediction of antimicrobial susceptibility on the base of statistical data represents an imperative necessity in modern antibiotic treatment. Thus, when plasma is intended for local antiseptic use this application has to be based on validated reproducible and standardized susceptibility testing. Therefore we investigated the antimicrobial CP efficacy in vitro against a set of different clinical strains. CP treatment of all isolates including the multiresistant ones resulted in inhibition zones. The multiresistance did not influence the inhibition zone. In conclusion no resistant isolates or species were found and the inhibition zones did not differ between isolates with more or less pronounced resistance against antimicrobials. Another "atmospheric plasma dispenser", a plasma device currently under investigation based on a corona plasma discharge technology was recently presented by the group of Morfill and coworkers [12] and proved convenient rapid and safe plasma disinfection of agar plates in vitro. The same group recently could demonstrate significant antimicrobial CP properties treating highly colonized chronic dermal wounds [13]. Fridman et al. [2, 14] already demonstrated skin decontamination on dead and living animals (without any relevant harm). Furthermore, a clinical trial to treat chronic venous leg ulcers with DBA plasma has just been initiated at the Department of Dermatology in Göttingen (see clinical trials.gov).

In conclusion CP treatment proved high efficacy in killing clinical skin and wound pathogens. Cold plasma treatment can be a suitable alternative option to conventional antibiotics and antiseptics in the antimicrobial strategy in wound management including multi drug resistant strains.

Acknowledgements: This study was realized within the joint research project "Campus PlasmaMed" supported by the *German Federal Ministry of Education and Research* (grant no. 13N9773 and 13N9779).

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Comparative analysis of the mechanism of action of Furvina® and GE81112, two translation initiation inhibitors

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Although approximately half of all known antibiotics interferes with the bacterial translation apparatus, the translation initiation pathway represents an underexploited target for anti-infectives; Furvina® (or G1), and GE81112 are among the few antibiotics which selectively inhibit this step of protein synthesis. Both molecules are effective against a wide range of Gram-positive and Gram-negative bacteria and inhibit protein synthesis both *in vivo* and *in vitro*. Both antibiotics target the small ribosomal subunit at or near the P-decoding site and inhibit fMet-tRNA binding during formation of the 30S initiation complex. Aside from these similarities, the mechanism of inhibition is different for the two antibiotics. In fact, while G1 interferes with the formation of the 30S pre-initiation complex, GE81112 inhibits the first order isomerization of the 30S pre-initiation complex which yields the 30S initiation complex.

Keywords: Translation initiation; P-site inhibition; fMet-tRNA binding; initiation triplet bias; initiation factors

1. Introduction

New anti-infectives endowed with novel chemical structures and novel modes of action are urgently needed to overcome the health threats posed by the rapid diffusion of multidrug-resistant pathogens [1-3]. Initiation of protein synthesis is the phase of translation displaying the greatest evolutionary divergence displaying kingdom-specific characteristics and therefore represents a potentially ideal, selective target of anti- bacterial drugs. Nevertheless, although the translational apparatus is the target of about half of all known antibiotics [1-4], very few inhibitors of the initiation phase of protein synthesis are known. In this article we describe the main features of Furvina® (G1) and GE81112 (Fig.1), two molecules which represent the most effective inhibitors of translation initiation known so far.

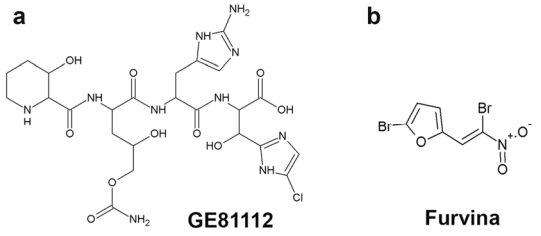

Fig. 1 Chemical structures of a) GE81112 and b) Furvina® (G1).

2. Results

2.1 GE81112

GE81112 [5,6] is a non-cyclic, tetrapeptide constituted by non-proteinogenic L-amino acids and endowed with a novel chemical structure (Fig. 1a) . GE81112 is produced by *Streptomyces* sp. L-49973 and its non-ribosomal synthesis occurs through the action of enzymes encoded by a partially characterized biosynthetic gene cluster [7]. GE81112 is effective (MIC ≤ 0.2 μg/ml) in minimal medium on almost all Gram-positive and Gram-negative bacteria tested, including clinical isolates of *Pseudomonas aeruginosa*; in rich medium the MIC is ~ 3 orders of magnitude higher due to competition for the OPP (oligopeptide permease) by other oligopeptides [5,8]. Chemically modified molecules with increased lipophilicity seem to be able to bypass this problem showing potential for further improvement of this molecule.

GE81112 is the most specific inhibitor of bacterial translation initiation found so far (Table 1) [6]. Its mechanism of action consists in the inhibition of the P-site binding of fMet-tRNA. Fast kinetics analyses reveal

that this antibiotic does not inhibit the initial interaction of the initiator tRNA with the 30S ribosomal subunit which gives rise to the formation of the 30S pre-IC, but instead interferes with the first order isomerization of the 30S pre-IC which yields the *bona fide* 30S IC (Fig. 2). In agreement with its mechanism of action, probing by chemical modifications and by hydroxyl radical cleavage indicate that GE81112 affects (i.e. protects or exposes) a number of 16S rRNA bases located at or near the P-decoding site and affects the position of both mRNA and fMet-tRNA on the small ribosomal subunit, underlying the premise that this molecule causes a generalized perturbation of the 16S rRNA structure around the P-site and interferes with and prevents codon-anticodon interaction at the P-site [9]. Furthermore, the structure of the 30S IC formed in the presence of GE81112 is "non-canonical" and therefore unfit for optimal docking by the 50S subunit. Consequently, in the presence of GE81112 the transition 30S IC → 70S IC and the consequent dissociation of the initiation factors IF1, IF2 and IF3are slowed down [9].

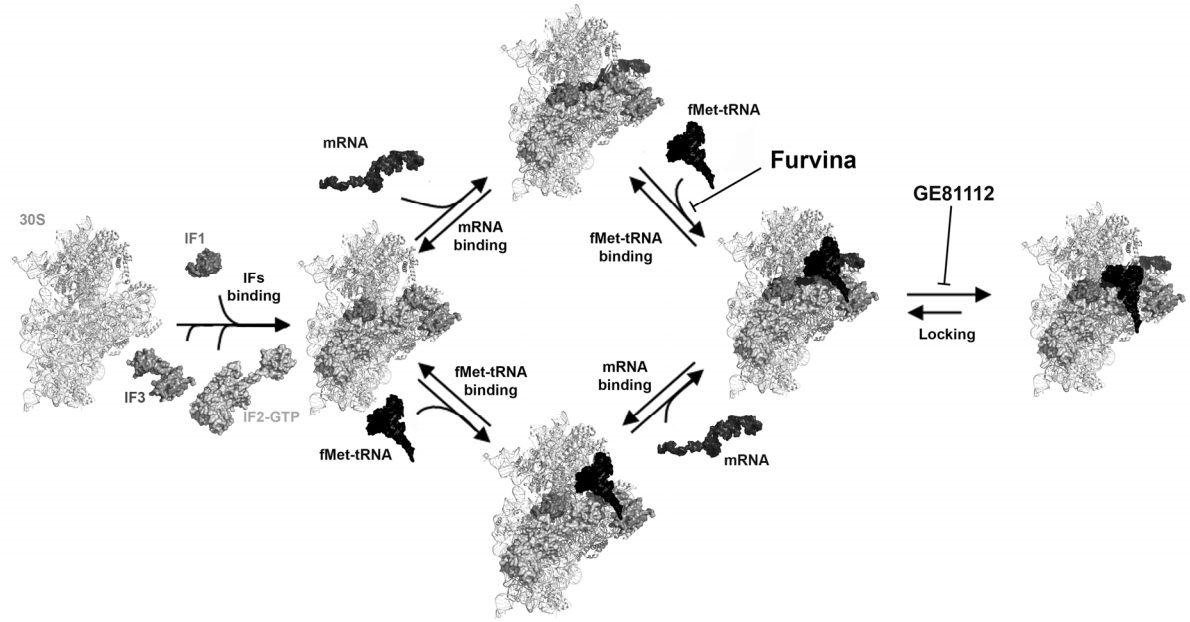

Fig. 2 Schematic representation of the early events occurring during initiation of bacterial protein synthesis. The individual translational steps inhibited by GE81112 and Furvina® (G1) are indicated.

2.2 Furvina®

Furvina®, also known as G1 (MW 297), is a nitrovinylfuran [2-bromo-5-(2-bromo-2-nitrovinyl)-furan] (Fig. 1b) developed in Cuba starting from sugar cane bagasse. Although microbiologically active against Gram positive and Gram negative bacteria and against pathogenic yeasts and filamentous fungi [10,11] unfortunately it is inactive against biofilms. Furvina® is used as an ointment (Dermofural®) for the topical therapy of dermatological infections without causing any relevant side effect.

In bacteria (but not in yeast) Furvina® was shown to inhibit preferentially protein synthesis both *in vivo* and *in vitro*. Like GE81112, Furvina® inhibits the binding of fMet-tRNA to the 30S ribosomal subunit but, unlike the case of GE81112, this antibiotic interferes with the initial interaction of the initiator tRNA with the ribosomal subunit, interfering with the formation of 30S pre-IC (Fig. 2) [12]. It is particularly interesting that the inhibition by Furvina® displays an initiation codon bias. In fact, efficient inhibition occurs when the mRNA directing 30S IC formation and protein synthesis has a start codon with a purine at the 3' end (AUG, AUA) whereas in the presence of a codon ending with a pyrimidine (AUU, AUC) the inhibition by Furvina® is much less pronounced [12]. This codon discrimination seems to be of opposite sign compared to that displayed by IF3 in its "initiation fidelity" function [13,14] and it is perhaps not by chance that codon-dependent inhibition bias by Furvina® requires the presence of IF3 and that probing by *in situ* cleavage experiments demonstrate that the accessibility of nearly all 16S rRNA bases implicated in P-site decoding and in IF3-dependent discrimination against non-canonical start codons are affected by Furvina® binding [12 and references therein] .

A comparative list of the main properties (similarities and differences) of GE81112 and Furvina® is presented in Table 1.

Table 1

Properties/Activities	**Furvina®**	**GE81112**
MW	297	658 (factor B)*
Hydrophilic	-	+
Hydrophobic	+	-
Halogen atom(s) present	2 x Br	Cl
Bacteriostatic activity (Gram positive)	+	+
Bacteriostatic activity (Gram negative)	+	+
Bactericide activity (Gram positive)	+	-
Bactericide activity (Gram negative)	+	-
Antifungal activity	+	-
Translation inhibition *in vivo* (yeast)	-	-
Translation inhibition *in vivo* (bacteria)	+	+
Translation inhibition *in vitro* (HeLa)	-	-
Translation inhibition *in vitro* (yeast)	-	-
Translation inhibition *in vitro* (bacteria)	+	+
Induction of miscoding	-	-
Binding to 30S ribosomal subunit	+	+
Binding to 50S ribosomal subunit	-	-
Binding to 70S ribosomal monomers	±	-
Inhibition fMet-tRNA binding	+	+
Inhibition 30S pre-IC formation	+	-
Inhibition 30SIC "locking"	-	+
Inhibition displaying codon bias	+	-

* Three variants (A, B and B1) of GE81112 are present, B the most active

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Control of Mozzarella spoilage bacteria by using bovine lactoferrin pepsin-digested hydrolysate

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In this work, bovine lactoferrin (BLF) and its pepsin-digested hydrolysate (LFH) were evaluated as potential control agents of High Moisture Mozzarella cheese spoilage. Five bacterial strains (*Pseudomonas fragi* 23A, *P. gessardii* 2A, *Serratia proteamaculans* 19A, *Aeromonas salmonicida* 22DB and *Rahnella aquatilis* 19B), isolated from cold-stored Mozzarella cheese and able to grow and promote caseinolytic activities in the governing liquid at 4 °C, were inoculated at 3 and 6 log cfu mL^{-1} in the governing liquid-based medium amended with BLF or LFH and incubated at 4 °C or 30 °C for two weeks. Interestingly, *R. aquatilis* 19B, inoculated at 3 log cfu mL^{-1}, resulted completely inhibited up to day 14 of cold storage. The remaining strains were partially inhibited, depending on the strain, under the same conditions. Differently from LFH, BLF showed a very low antimicrobial activity. On the other hand, no antimicrobial activity was observed when strains were inoculated at 6 log cfu mL^{-1}. This work demonstrates the efficacy of LFH to control some psychrotrophic proteolytic bacteria during the first days of cold storage and at a low initial microbial concentration.

Keywords: psychrotrophic bacteria; *Pseudomonas* spp.; cheese shelf-life; antimicrobial peptides

1. Introduction

Mozzarella cheese can be considered by far the world's most popular Italian dairy product. Based on its moisture content (US-FDA, 1989), “low moisture” (LM) and “high moisture” (HM) Mozzarella cheese are produced and used as a recipe ingredient or consumed fresh as a table cheese, respectively. HM Mozzarella, traditionally manufactured in Southern Italy by fermenting raw whole cow’s milk with natural whey cultures [1-3], is packaged in a governing liquid mainly composed of tap water, brine and whey. This preserves the soft-springy texture and high amounts of expressible serum during HM Mozzarella storage at 4 °C.
During 10-12 days of cold storage, HM Mozzarella loses proteins, calcium and sugars (lactose, glucose and galactose) that enrich the composition of the governing liquid and favours the growth of microbial contaminants [4]. Lately, Baruzzi et al. [5] isolated 66 potential spoilage strains from cold-stored HM Mozzarella cheese, mainly belonging to *Pseudomonas*, *Acinetobacter* and *Rahnella* species. Some of them expressed high proteolytic activity in milk and also caused partial hydrolysis of α, β and γ caseins on HM Mozzarella outer surface where a concomitant wrinkling and successive exfoliation became visible.
Antimicrobial peptides (<10kDa; 3–50 amino acid residues) have been extensively investigated for promising applications in food preservation [6]. Among milk proteins, bovine lactoferrin (BLF) has gained much interest for its antimicrobial activity against fungi, yeasts and gram-negative and positive bacteria [7]. Some authors have demonstrated that pepsin digested BLF hydrolysate (LFH) releases the peptide lactoferricin B, largely responsible for BLF antibacterial activity [8].

The aim of this work was to control HM Mozzarella spoilage bacteria by the use of BLF and LFH in the governing liquid under refrigerate condition. Besides, growth and proteolytic activities of target strains were evaluated.

2. Material and Methods

2.1 Bacterial strains and culture conditions

The strains *Pseudomonas fragi* 23A, *P. gessardii* 2A, *Serratia proteamaculans* 19A, *Aeromonas salmonicida* 22DB and *Rahnella aquatilis* 19B, previously isolated from HM Mozzarella cheese [5], were used as target microorganisms for antimicrobial assays. *Escherichia coli* K12 (ATCC 29425) was included in the tests as reference strain. Microbiological media and supplements, if not differently reported, were purchased from Biolife (Biolife Italiana S.r.l., Milan, Italy). Each experiment was carried out using fresh growing bacterial cells obtained inoculating 10 mL of Plate Count Broth (PCB; composition in g/L: yeast extract, 2,5 g; glucose, 1 g;

tryptone, 5 g) with 50 µL of a frozen (-80 °C) culture of each microorganism grown under shaking conditions (140 rpm) for 16 h at 30 °C.

2.2 Assessment of microbial growth and proteolytic activity in governing liquid-based medium

Microbial growth kinetics and antimicrobial assays were carried out in one single stock of governing liquid-based medium (glbm) drained from 18h old commercial packs of refrigerated HM Mozzarella cheese. Briefly, two litres of governing liquid was centrifuged (10.000 rpm, 10 min, at 4 °C); the supernatant was autoclaved (121 °C for 5 min), cooled at room temperature, and centrifuged again. Sterility of the glbm was checked by plate count [9] and stored at 4 °C until used.

Fresh bacterial cell cultures, with an average of OD_{600} of 0.325±0.05 (corresponding to 7-8 log cfu mL^{-1}, depending on the strain), were centrifuged (7.000 rpm, 10 min, at 4 °C) and diluted 10 times in sterile saline solution. Then, 100 µl of each bacterial suspension was used to inoculate 10 mL of glbm that was incubated at 4 °C for 14 days under static conditions. Cell viability was monitored applying the UNI EN ISO 4833:2004 method [9]. During the same period, the strains were evaluated for their ability to hydrolyze caseins released by HM Mozzarella cheese. Caseins were extracted from glbm [10] and quantified for protein concentration [11]. Ten µg of proteins were run in SDS-PAGE (12% T, 3% C) together with α-, β-, and κ- casein standards (cat. number C6780, C6905, C0406, Sigma-Aldrich), as previously reported [12].

2.3 Lactoferrin hydrolysis and antibacterial assays in the governing liquid-based medium

A 5% (w/v) bovine lactoferrin (BLF; NZMP lactoferrin 7100, Fonterra, Boulogne- Billancourt, France) solution was hydrolyzed with 3% (w/v) pepsin from porcine gastric mucosa (250 units mg^{-1} solid, Sigma-Aldrich, St Louis, MO, US) as previously described [8]. Bovine lactoferrin hydrolysate (LFH) was freeze-dried and stored at -20 °C in order to be used in the subsequent experiments.

Target strains, cultured as described in the 2.2 paragraph, were inoculated at 3 and 6 log cfu mL^{-1} in glbm supplemented with 10 mg mL^{-1} of BLF or LFH. Microbial growth kinetics were evaluated by plate counts [9], after 0, 1, 2, 3, 6, 7, 10 and 14 days of static incubation at 4 °C and 30 °C.

Proteolytic activities of strains incubated at 4 °C were checked by SDS-PAGE (as described in the 2.2 paragraph) after 0, 3 and 7 days of cold storage.

3. Results

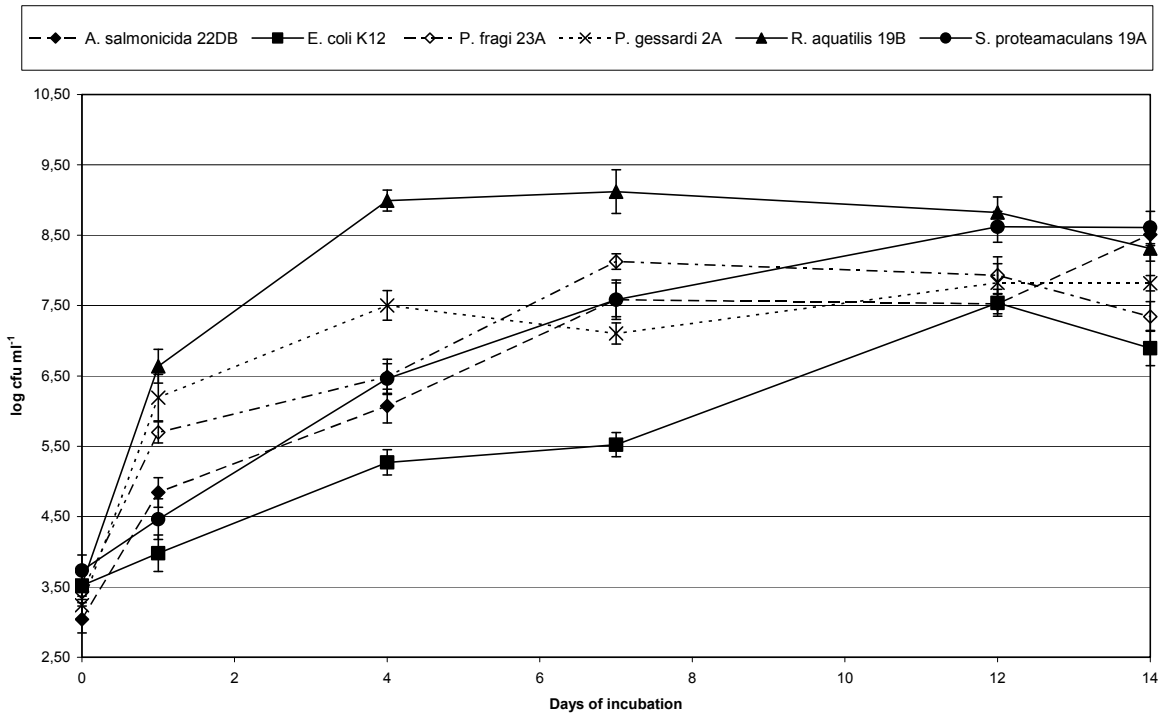
3.1 Assessment of microbial growth and proteolytic activity in governing liquid-based medium

HM Mozzarella cheese releases proteins, sugars and mineral salts in the governing liquid during cold storage, allowing the growth of psychrotrophic bacteria. The catabolism of caseins and amino acids, occurring in the governing liquid, can reduce quality of HM Mozzarella cheese. Thus, in the present work we tested the viability and the caseinolytic activity of the selected bacterial strains in one single stock of governing liquid-based medium at 4 °C.

R. aquatilis 19B resulted, among the tested strains, the best adapted to the experimental conditions, reaching the stationary growth phase on the fourth day with a microbial load of 9 log cfu mL^{-1} (Fig. 1). *R. aquatilis* 19B and *P. fragi* 23A showed an increase in microbial load of about 4 and 3 log cfu mL^{-1}, respectively, after 24 h of cold incubation (Fig. 1). The remaining spoilage strains reached, on average, 7 log cfu mL^{-1} after seven days at 4 °C. As expected, *E. coli* K12, whose optimal growth temperature is 37 °C, needed 12 days to reach the same cell load.

The proteolytic activity towards casein fractions was evaluated on SDS-PAGE after 0, 4, 7 and 12 days of incubation at 4 °C (Fig. 2). None of the target strains was able to hydrolyze caseins until the fourth day of incubation. After additional three days, the complete absence of casein bands was observed in *P. gessardii* 23A , whereas a weak κ-casein band, which disappeared at day 12, was found in *P. fragi* 2A (Fig. 2).

Fig. 1 Growth of spoilage bacteria in the governing liquid-based medium (glbm) incubated at 4 °C.



S. proteamaculans 19A and *A. salmonicida* 22DB proteolytic activities resulted only after 12 days of incubation, when all casein bands disappeared from SDS-Page patterns (Fig. 2). Same results were obtained for the reference strain *E. coli* K12 (Fig. 2). Differently from the other target strains, *R. aquatilis* 19B did not exhibit a strong proteolytic activity as it completely hydrolyzed only κ-casein after 12 days (Fig. 2).

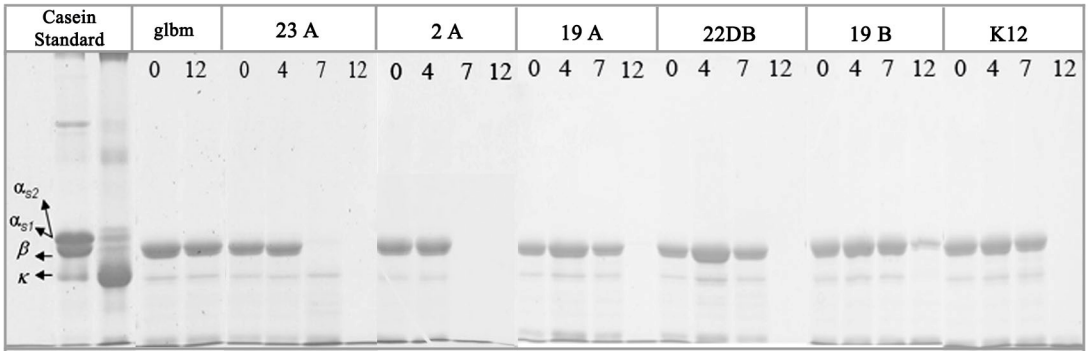


Fig. 2 SDS-PAGE casein profiles from governing liquid-based medium and the glbm inoculated with *P. fragi* 23A, *P. gessardii* 2A, *S. proteamaculans* 19A, *A. salmonicida* 22DB, *R. aquatilis* 19B and *E. coli* K12, incubated at 4 °C up to day 12.

It is well known that chymosin, used in cheesemaking, coagulates milk by hydrolyzing the Phe105-Met106 bond of κ-casein, releasing the glycomacropeptide (residues 106-169). The hydrophobic para κ-casein (residues 1-105) in cheese and part of intact κ-casein remain entrapped in the curd, as suggested by the adhesive hard sphere theory [13]. The finding of a specific microbial metabolism towards κ-casein, expressed by *R. aquatilis* 19B, the best adapted target strain to conditions occurring during HM Mozzarella cheese storage, could shed light about the role played by para κ-casein in the stability of casein network of this cheese.
However, the finding of high viable cell counts (usually higher than 7 log cfu mL^{-1}) and the hydrolysis of casein fractions within the 12th day of incubation sustain the need to control psychrotrophic proteolytic bacteria to preserve the freshness of HM Mozzarella cheese. Our results are in accordance with the data obtained by Sørhaug et al. [14] on the role of proteolysis in the spoilage of other cheeses.

3.2 Antibacterial assays in the governing liquid-based medium

In order to check the efficacy of BLF and LFH in the control of spoilage bacteria, the target strains were inoculated in the glbm (amended with 10 mg mL^{-1} BLF or LFH) at two levels of initial inoculum (3 and 6 log cfu mL^{-1}), and incubated at 4 °C or 30 °C for two weeks.

Interestingly, *R. aquatilis* 19B inoculated at 3 log cfu mL^{-1} and incubated at 4 °C resulted completely inhibited up to day 14 by LFH. On the other hand, LFH reduced the growth of *R. aquatilis* 19B and *P. fragi* 23A within 24h and 48h of incubation at 30 °C, respectively (Fig. 3). Besides, *P. fragi* 23A and *P. gessardii* 2A viable cell counts were lower in glbm amended with LFH in comparison with the un-supplemented glbm until the 6th and 7th day of cold storage (Fig. 3). As concerns 3 log cfu mL^{-1} initial inoculum level, BLF showed a low antimicrobial activity against *P. fragi* 23A and *P. gessardii* 2A within 24h of incubation at 30 °C (Fig. 3). *A. salmonicida* 22DB and *S. proteamaculans* 19A were resistant to BLF and LFH independently from the temperature of incubation. BLF and LFH were unable to control target strains when the initial inoculum was 6 log cfu mL^{-1}. Our results related to the inhibition of *E. coli* K12 are accordance with those previously reported [15]. All the target strains, except *R. aquatilis* 19B, were no longer controlled in any of the assays after seven days of incubation (data not shown).

As regards proteolytic activities performed by strains subjected to BLF or LFH control, casein band intensities on SDS-PAGE was maintained only in experiments showing the lowest microbial viable cell concentration (data not shown)

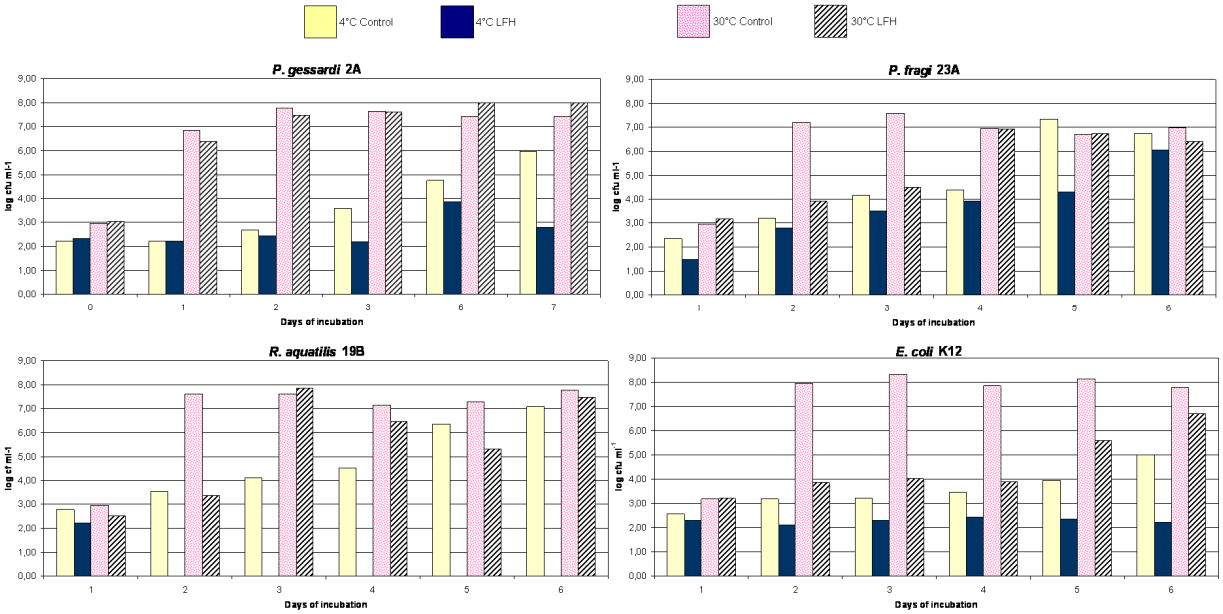


Fig. 3 Microbial counts of target strains grown for seven days at 4 °C and 30 °C in the glbm supplemented with 10 mg mL^{-1} of pepsin-digested lactoferrin hydrolysate (LFH).

In conclusion, this work demonstrates that strains spoiling HM Mozzarella cheese can grow in the governing liquid during cold storage, causing the hydrolysis of caseins. Thus, the control of their growth could be useful to reduce HM Mozzarella cheese spoilage. Furthermore, the addition of LFH to the governing liquid efficiently controlled viable loads of some cheese-spoiling strains under cold condition. Based on these results, the use of LFH makes it feasible to extend the shelf-life of traditional Mozzarella cheese when other factors as microbial milk quality, hygienic procedures and maintenance of refrigeration temperature, are met.

Acknowledgements. This work was supported by the Italian Ministry of Education, University and Research through the project: "AgroBioPack: Technologies for bioactive packaging of fresh dairy Apulian foods".

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Dandruff - A management strategy for one of the most chronic & commercially exploited skin conditions.

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Dandruff is one of the commonest conditions affecting the scalp and is very often embarrassing for both, the person suffering from it and the observer. Dandruff, caused by the commensal lipophilic yeast belonging to the genus *Malassezia,* cannot be cured completely but can be managed effectively. The effective management of dandruff poses a global challenge and drives the billion dollar cosmetic industry with the primary objective of achieving a zero dandruff state.
The present study involved testing the efficacy of different plant based extracts in anti-dandruff formulations like oil, shampoo and cream on three different *Malassezia* spp. The efficacies of the various polyherbal formulations were compared to popular brands of products in the market and tests such as Minimum Inhibitory Concentration, Zone of Inhibition Assay, Methylene Blue Reductase Test, Half-head Assay Method, Consumer Evaluation Studies were employed for a comparative study. The paper attempts to devise a key to effective management of dandruff using synergy studies and selection of the appropriate ingredients in the formulation to increase the efficacy of the functional anti-fungal agent. It also addresses other intricate factors to be considered while formulating an effective anti-dandruff product.

Keywords Dandruff; Malassezia; anti-dandruff

1. Introduction

Dandruff is a chronic condition that usually occurs when the scalp becomes dry or greasy and produces white flakes of dead skin. It is a major cosmetic problem globally. It is neither contagious nor serious, but can be a source of embarrassment in public, with people constantly scratching their head and trying to brush off the flakes/white powder from their collar or shoulders. There is no complete cure to this common problem till date, although it can be effectively managed. Dandruff can be related to several predisposing host conditions; however it is widely associated with the hyperproliferation of a commensal lipophilic fungus belonging to the genus, *Malassezia*. *M. globosa* and other *Malassezia* species have different types of lipases, phospholipases and acid sphingomylinases [1] which help in breakdown of the sebaceous secretions (oils) in the scalp, thereby providing suitable ecological niche for their rapid multiplication. Therefore, most anti-dandruff (AD) products are designed to exert fungicidal action on *Malassezia* species, the primary target in the management of dandruff. However, being a commensal flora, the recurrence in growth of these fungi is inevitable. Hence, the challenges in management of dandruff should focus on use of synergistic combination of antifungals (a) to minimize development of resistance, (b) to prevent recurrence and (c) to make the AD product skin friendly.

2. Objectives

1. To look for natural plant based compounds that can be combined with selective synthetic AD compounds with anti-Malassezia activity to deliver the desired 'functional' activity in different AD formulations such as shampoo, oil and cream. This synergistic combination should confer a lasting anti-dandruff effect.

2. To compare these AD formulations with popular brands available in the market containing the same synthetic AD ingredients (Zinc Pyrithione, Ketoconazole, Climbazole, Octopirox, etc) for their efficacy through *in vitro* and *in vivo* studies.

3. Materials and Methods

3.1 Herbs and Medicinal plants used.

Over 150 herbs with anti-dandruff activity were identified based on traditional knowledge and literature search and were screened for their syngergistic anti-Malassezial activity with and without synthetic AD ingredients on

as is basis and in a range of cosmetic formulations. Some of these herbal extracts were selected based on their activity to be used in the study formulations (Table 1)

Table 1: Herbal extracts used in the test cosmetic/personal care formulations

Name of the plant	Common name	Parts used	Solvent
Albizia amara	Silk plant	Leaf	Ethanol
Andrographis paniculata	King of bitters	Whole plant	Ethanol
Azadirachta indica	Neem	Leaf	Ethanol
Cinnamomum cassia	Chinese Cinnamon	Bark	Ethanol
Cinnamomum tamala	Malabar leaf	Leaf	Ethanol
Cinnamomum zeylanicum	Ceylon cinnamon	Bark	Ethanol
Datura metal	Angels trumpet	Leaf	Ethanol
Melaleuca leucadendron	Tea tree	Leaf	Ethanol
Premna serratifolia	Headache tree	Leaf	Ethanol
Rosmarinus officinalis	Rosemary	Leaf	Ethanol
Wrightia tinctoria	Sweet Indrajao	Leaf	Ethanol and Ethyl acetate

3.2 Formulations

Anti-dandruff study formulations (shampoo, oil and creams) were prepared with selective herbal and synthetic AD ingredients with the help of a cosmeticologist. To evaluate the activities of the test formulations, corresponding popular brands (names not disclosed to avoid any commercial implications) of AD formulations were procured from the market and used for comparative studies.

3.3 Cultures used

Ten isolates each of *M. globosa, M. restricta* and *M. furfur* of clinical origin were used for the study. All the isolates were collected from patients attending Dermatology Department, Madras Medical College, Chennai, India. (Year of study: 1998 -2008)

3.4 Different methods of evaluation & Comparison with popular brands

3.4.1 Minimum Inhibitory Concentration (MIC) assay was done for all test AD formulations (shampoo, oil and cream) in comparison with the popular brands in the market. In addition, Zone of Inhibition (ZOI) was determined for the shampoo using standard procedures.

3.4.2 Methylene Blue Reductase Test

The culture of *Candida albicans* was allowed to grow in a medium containing the test formulation. After incubation, the organism was scooped from the surface of medium and stained with Methylene Blue stain & examined under microscope. Dead cell takes up the stain and as a result cells appear blue in color, while live cells appear colorless as these organisms have an enzyme methylene blue reductase which reduces the dye. In a microscopic field, the total number of stained vs unstained cells would reflect killing effect of formulations. The percentage death was calculated by the above method. (*Candida albicans* was used for the assay of AD efficacy of test formulations and the data was extrapolated to *Malassezia* species as both the genera are phylogenetically related [1] and this method was standardized in our earlier study [2].

3.4.3 Half-head method

Three female volunteers of the age group between 18-28 who had 'very severe' complaints of dandruff were chosen for the study. Informed consent for participation in the trial was obtained from the volunteers after explaining the test procedure in detail. The three volunteers were chosen such that each of them were representative users of different competitor brands. The total scalp area of each of the volunteer was divided through the center to get two equal halves (Left and Right). In all the three volunteers, Market shampoo (PS) with Zinc Pyrinthione was applied on the right half/left halves. The other half in all the above volunteers were applied with test formulation 1(TS). Utmost care was taken to prevent mixing of the market shampoo and test formulation during application and rinse off. Uniformity in the quantity of shampoo used, shampoo retention time before rinse off and adequacy in washing was practiced to avoid bias in the results. This could be achieved

because the study was conducted in the house hold and the hair washes were given by the study investigator. A series of 5 washes were given to all the volunteers at an time interval of 3 days between each wash.

A. Clinical evaluation

Extensive clinical examination was done by grading symptomatic reduction in the severity of dandruff on the scale of "Very severe-Severe-Moderate-Mild-Traces-Nil". Symptoms like scaling (adherent scales and powdery deposit), itching, hair fall and dryness of scalp were assessed before use and after each wash with the shampoo and the scores of grading were recorded.

0	1	2	3	4
Nil	Mild	Moderate	Severe	Very severe
No clinical symptoms /itching	Presence of mild powdery material	Presence of white powdery material & itching	Presence of flakes, powder and itching	Presence of signs of inflammation with flakes, powder and severe itching

B. Microbiological evaluation

The scrapings from the left and right halves of the scalp of each volunteer were taken using a sterile blunt scalpel separately and aseptically collected before the start of the experiment (Pre use) and after each wash. The scrapings were microbiologically examined for the presence of *Malassezia* spp.

(i) Direct examination: One part of the scrapings were treated with potassium hydroxide and were examined under the microscope to assess the presence or absence of the organism and the physical appearance of the cells after each wash.

(ii) Culture study: The other part of the scarping was cultured on Sabouraud's dextrose agar overlaid with olive oil to determine the bio burden (total count) and the viability of *Malassezia* spp.

3.5 Consumer evaluation

Questionnaires were distributed to the volunteers. They were requested to give the comparative ratings in the questionnaires with respect to the application and functional attributes including anti-dandruff efficacy of the test AD formulations after using for 4 weeks. The results, as perceived by the consumers, were collated and tabulated for comparison.

Poor	Satisfactory	Good	Very good	Excellent
0-2	2-4	4-6	6-8	8-10

4. Results

The test formulations viz, shampoo, oil and cream made with syngegistic combinations of synthetic and herbal ingredients were found to be more effective in demonstrating AD activity than the popular brands that used either herbal or synthetic AD ingredient individually (Tables 2-4, Fig 2). Further, Half-head and Consumer Evaluation Studies show that the test formulations were superior in AD activity and was rated high for their functional anti-dandruff activity in comparison to the market brands by the microbiological assessment (direct microscopy of scales and culture), clinical examination and grading (by a dermatologist) and the consumer's perception (by observation) (Tables 5a, 5b, Graphs 1-3). These results were also confirmed by Methyle blue reductase test (Fig 1)

Table 2: Anti-dandruff Test Formulation vs Popular Brands of Anti-dandruff Shampoos

S. No.	Brand	Active Ingredient	Level in %	ZOI (mm)	MIC Value (mg/ml)		
					M.globosa	*M. restricta*	*M.furfur*
1	Brand 1	Tea Tree oil	0.15	20	30	30	30
2	Brand 2	ZnPTO Benzyl alcohol	1.05 0.02	26	10	10	5
3	Brand 3	ZnPTO Benzyl alcohol	0.92 0.02	26	10	10	5
4	Brand 4	Climbazole	0.23	25	10	10	10
5	Brand 5	Ketoconazole	0.10	26	5	5	5
6	Test Formulation 1	ZnPTO Herbal Extract (*Datura metal*)	0.02 0.02	28	5	2.5	2.5

		Herbal Extract *(Albizia amara*)	0.02				
7	Test Formulation 2	Ketoconazole Herbal Extract (*Datura metal*) Herbal Extract (*Albizia amara*)	0. 02 0.02 0.02	28	5	5	5
8	Test Formulation 3	ZnPTO Herbal Extract (*C.zeylanicum*) Herbal Extract (*C.cassiia*)	0.02 0.02 0.02	28	5	2.5	5

Table 3: Anti-dandruff test formulation vs popular brands of Anti-dandruff Oil

S. No	Brand	Active Ingredient	Level in %	MIC Value (mg/ml)		
				M. globosa	*M. restricta*	*M.furfur*
1	Brand 1	Climbazole	0.3	30	20	20
2	Brand 2	Tea Tree oil	0.5	30	25	25
3	Brand 3	Octopirox	0.3	20	20	20
4	Brand 4	*Wrightia tinctoria* *Rosmarinus officinalis* *Melaleuca leucadendron* *Azadirachta indica*	ND	20	20	15
5	Test Formulation 1	Clove oil	1.0	30	20	20
6	Test Formulation 2	Basil oil	1.0	30	20	20
7	Test Formulation 3	Coleus oil	1.0			
8	Test Formulation 4	Rosemary oil	1.0	30	20	15
9	Test Formulation 5	Octopirox C*innamomum zeylanicum* *C.tamala* *C.cassia*	0.02 0.05 0.25 0.25	10	10	10

Table 4: Anti-dandruff test formulation v/s popular brands of Anti-dandruff Cream

S. No	Brand	Active Ingredient	Level in %	MIC Value (mg/ml)		
				M. globosa	*M. restricta*	*M.furfur*
01	Brand 1	Climbazole	0.3	20	15	15
02	Test Formulation 1	Climbazole *Andrographis paniculata* *Premna serratifolia*	0.05 0.2 0.2	20	15	15
03	Test Formulation 2	Climbazole *C.zeylanicum* *C.tamala* *C.cassia*	0.05 0.1 0.05 0.05	10	5	5

Table 5A : Direct examination of *P.ovale* cells - Half Head Method - Popular Shampoo vs Test Shampoo

No. of washes	Volunteer A		Volunteer B		Volunteer C	
	PS	TS	PS	TS	PS	TS
Pre use	Present	Present	Present	Present	Present	Present
I wash	Present	Present	Present	Present	Present	Present
II wash	Present	Present	Present	Present	Present	Present
III wash	Present	Present	Present	Present	Present	Present
IV wash	Present	Absent	Present	Absent	Present	Absent
V wash	Absent	Absent	Absent	Absent	Absent	Absent

Table 5b Reduction in *P.ovale* burden in culture - Half Head Method Popular Shampoo vs Test Shampoo

No. of washes	Volunteer A	Volunteer B	Volunteer C

	PS	TS	PS	TS	PS	TS
Pre use	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
I wash	355	326	380	392	289	271
II wash	211	200	246	233	204	197
III wash	65	48	58	39	83	41
IV wash	26	2	22	NIL	33	3
V wash	NIL	NIL	NIL	NIL	NIL	NIL

TNTC : Too Numerous To Count
PS – Brand 3 (with ZPTO), TS – Test formulation 1

Methelene blue reductase test to demonstrate the efficasy of AD shampoo test formulation 1 (TF1)

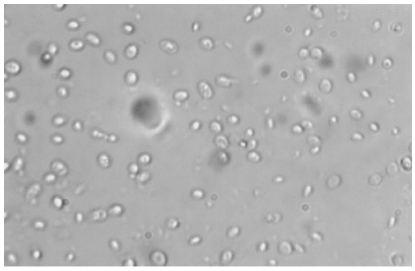


Fig 1a

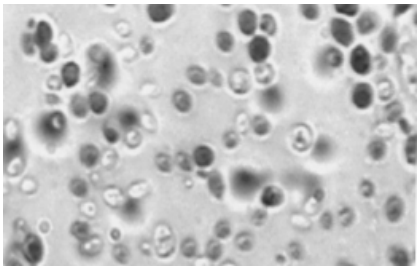
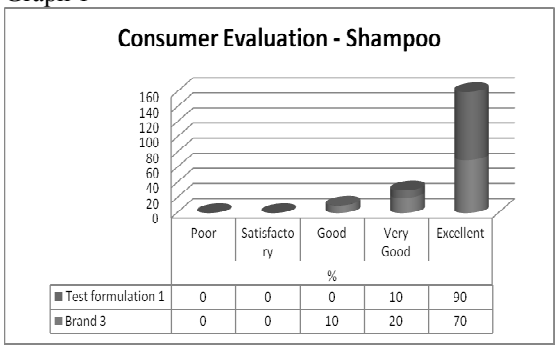


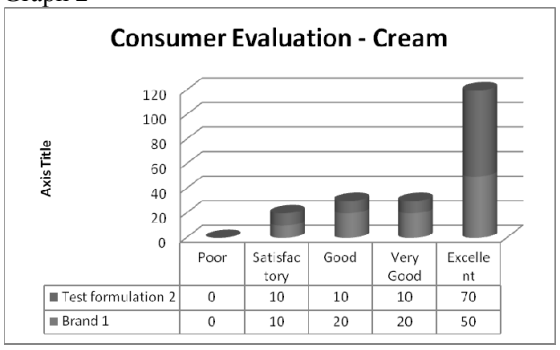
Fig 1b

Fig 1a Live cells (no uptake of dye) under high power objective (45x magnification) – Untreated control
Fig 1b Dead cells (darkly stained with methylene blue) – Treated with TF 1

Graph 1



Graph 2



Graph 3

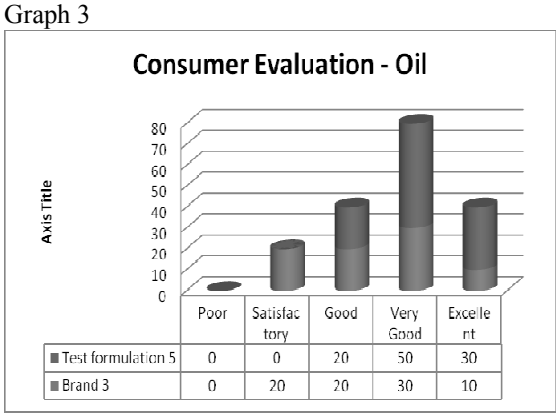


Fig 2

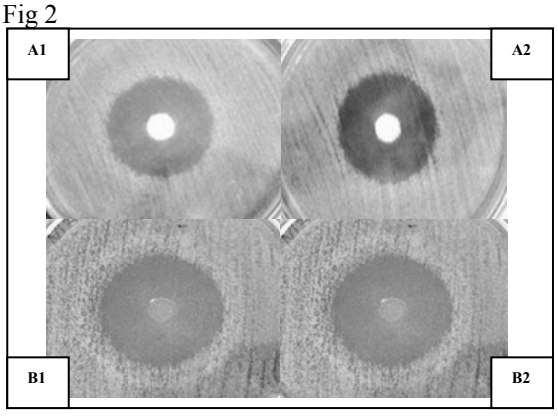


Fig 2 : Zone of Inhibition of Shampoos – Popular brands vs test formulations

A1 : Brand 3 (with ZPTO), A2 : Test formulation 1(ZPTO + Herbal Extract)
B1 : Brand 5 with Ketoconazole , B2 : Test formulation (with Ketoconazole + Herbal Extract)

5. Discussion

Malassezia is a monophyletic genus of fungi found on the skin of approximately 7 billion humans and associated with a variety of conditions, including dandruff, atopic eczema (AE)/dermatitis, pityriasis versicolor,

seborrheic dermatitis, and folliculitis [3]. Anti-fungal mechanism of action is the common strategy that has been established for management of dandruff and hence anti-dandruff formulations employ active ingredients like zinc pyrithione (ZPTO), ketoconazole, climbazole, octopirox etc. to target the causative organism. Since *Malassezia* spp had mastered their commensal life on humans with obligate anthropophilic nature, it is often impossible to completely eradicate their population in the scalp, which poses the problem of recurrence in dandruff despite treatment with topical antifungal / AD formulations. Recent studies indicate the role of copper in ZPT-mediated growth inhibition of *M. globosa* making ZPTO an ideal candidate for AD shampoos. This also vouches for its global popularity as an AD ingredient. In our study, we have demonstrated that addition of herbal extracts not only enhances the anti-*Malassezia* activity of ZPTO (both in vivo and in vitro conditions) but also further helps to lower the level of active ingredient required in AD formulations. A similar finding was recorded for synergistic combinations of herbal extracts and other AD ingredients like Climbazole and Octopirox. We have demonstrated this unique synergistic antimicrobial phenomenon in our earlier studies using cosmetically significant skin microflora [4]. Although we are not sure of the mechanisms of these herbal extracts in enhancing the antifungal activity, the results of the present study clearly indicate their synergistic activity.

6. Conclusion

The chronic and recurrence nature of Dandruff, makes its management difficult. Individuals with dandruff have a skin barrier defect that is enhanced by *Malassezia* to produce increased levels of irritating fatty acids due to lipase-mediated hydrolysis of sebum triglycerides [1]. The released fatty acid acting as chemotactic substance attracts neutrophils which release reactive oxygen species and cytokines causing dermal inflammation and tissue damage [5]. Ingredients like ZPTO, Climbazole, Octopirox and Ketaconazole used in AD formulations are effective in keeping the growth of *Malassezia* spp under control. Repeated and extensive use of AD products may pave way for the emergence of resistance among *Malassezia* spp. The other major concern is the possibility that resistance to antifungals used in AD formulations may also contribute to reduced susceptibility to clinically important antifungals, due to either cross-resistance or co-resistance mechanisms [4]. In the era of immune-suppression/compromisation and opportunistic infections, it would be a disaster if organisms develop resistance to available anti-fungals. To combat the development of resistance and deliver the anti-dandruff benefit, a combination of synergistic antimicrobials can be used. In our current study, we have shown that the addition of one or more herbal extracts, not only helps to reduce the level of synthetic AD ingredients like ZPTO and Ketoconazole in the formulations, but also make them more efficacious than those popular brands of shampoos, creams and oil that use stand alone synthetic/herbal AD ingredients. Further, the study provides a new insight in the development of future AD formulations that are not only efficacious but also eco-friendly (reducing environmental accumulation of anti-fungals) and skin- friendly.

Acknowledgements The support of the Managements of UniKL RCMP, AIMST University, QUIP, Malaysia and Cholayil Private Limited, India are gratefully acknowledged.

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Diffusivity of antimicrobial agents through biofilms of *Bacillus cereus* and *Pseudomonas fluorescens*

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The proliferation of biofilms in industrial settings, especially in the food industry, can result in serious operation and maintenance costs. Biofilm cells are known to be highly resistant to antimicrobial agents. This is in part due to the diffusional limitations promoted by the extracellular polymeric (EPS) matrix produced by the biofilm cells. The purpose of this work was to study the diffusional limitations of selected antimicrobial agents on colony biofilms of *B. cereus* and *P. fluorescens.* The biofilms displayed different characteristics (cell density, thickness and EPS content). Different diffusional limitations were found according to the antimicrobial agent and the biofilm type. No significant mass transfer limitations were found for benzalkonium chloride and ciprofloxacin. Ethanol, benzyl dimethyl dodecyl ammonium chloride, tetracycline, erythromycin and sodium hypochlorite had modest hindrance; cetyltrimethyl ammonium bromide and streptomycin were completely retarded by the presence of a biofilm. These results highlight the importance of antimicrobial agent selection as a key step in the design of a disinfection process for biofilm control.

Keywords antimicrobial resistance; *Bacillus cereus*; biofilms; diffusional limitations; *Pseudomonas fluorescens*

1. Introduction

Bacteria within biofilms produce extracellular polymeric substances (EPS) [1, 2] and these are mainly composed of polysaccharides, proteins, extracellular DNA, and lipids, which are typically dense and highly hydrated [3, 4]. One of the functions of EPS is to contribute to the mechanical stability of the biofilms, enabling them to withstand shear forces, dehydration and chemical attack [3, 5]. Moreover, it is where the convective and diffusional transport of oxygen and nutrients to the biofilm takes place [6].

Biofilms can be 10-1000 fold more resistant to antimicrobials when compared to their planktonic equivalents [7]. The way how microorganisms develop resistance is not totally understood. However, the presence of EPS in a biofilm is considered a microbial survival strategy, including the resistance to antimicrobial agents. In industrial systems, the main objective of a disinfection plan is to eliminate, or reduce the microorganisms and their activity to acceptable levels, as well as the prevention and control of the formation of biological deposits on process equipment [8].

Biofilms, when exposed to antimicrobial agents, present specific survival strategies, *i.e.* expression of specific resistance genes, decreased growth rates, restricted diffusion of antimicrobial agents due to the presence of an EPS matrix, quorum sensing specific effects and the existence of persister cells [2, 6]. In order to optimize a disinfection plan, it is important to understand the interaction of a specific antimicrobial agent with the contaminant. The objective of this study was to assess diffusional limitations of *B. cereus* and *P. fluorescens* biofilms, two bacteria commonly associated with food contaminations [9], to selected antimicrobials currently used in clinical settings, households and food industry.

2. Experimental procedures

2.1 Microorganisms and culture conditions

The bacteria used in this work were *Pseudomonas fluorescens* ATCC 13525^{T} and a *Bacillus cereus* strain isolated from a disinfectant solution and identified by 16S rRNA gene sequencing [9]. Bacterial strains were incubated at a temperature of 27 ± 2 ºC, using glucose as the main carbon source. Culture media consisted of 5 g.L^{-1} glucose, 2.5 g.L^{-1} peptone and 1.25 g.L^{-1} yeast extract, in phosphate buffer (PB) (pH 7, 5 mM) [10]. Bacterial suspensions were incubated overnight at the given temperature with agitation (120 min^{-1}). After the growth period, the suspension was washed in two consecutive steps of centrifugation (3202 *g*, 10 min), and resuspended in PB, in order to obtain a final concentration of 1 x 10^{9} cells.mL^{-1}.

2.2 Antimicrobials

The antimicrobials used throughout the experiments were cetyltrimethyl ammonium bromide (CTAB) (350 µg), benzalkonium chloride (BAC) (350 µg), sodium hypochlorite (350 µg), ethanol (70%), streptomycin (10 µg), and tetracycline (30 µg) obtained from Sigma-Aldrich. Benzyl dimethyl dodecyl ammonium chloride (BDMDAC) (350 µg) was obtained from Merck, ciprofloxacin (5 µg) was acquired from Fluka and iodine (15 µg) purchased from Prolabo Normapur. Isopropanol (100%) and erythromycin (15 µg) were obtained from Applichem. Some masses were previously optimized to obtain a detectable inhibition halo and others were used at the reported minimum inhibitory concentration [11, 12]. These compounds were used in aqueous solutions, prepared with sterile distilled water.

2.3 Diffusion test apparatus

The tests were performed using an adapted technique that was previously described by Anderl et al. [13] and later by Singh et al. [14]. Biofilms were grown inverted in sterile Mueller-Hinton agar plates (24 h, 27 ± 2 ºC), from a 40 µL drop of washed cell suspension placed on top of a 13 mm polycarbonate membrane (pore size 0.2 µm) originating colony biofilms. Afterwards, the biofilms were transferred to a fresh plate containing the same growth medium seeded with *Staphylococcus aureus* CECT 976 at a McFarland standard of 0.5 [13, 14]. Another polycarbonate membrane was placed on the top of the biofilm so that the antimicrobial discs were not in direct contact with the biofilms. The antimicrobial discs were impregnated with a 15 µL drop containing the desired concentration of the different antimicrobial agents tested (Figure 1). The negative controls contained a 15 µL drop of sterile distilled water; positive controls were composed by the same apparatus with no biofilm. The plates were incubated for 24 h at 27 ± 2 ºC before measurement of inhibition halos. The positive controls were taken as 100% penetration and used to calculate the penetration ratios when biofilms were present.

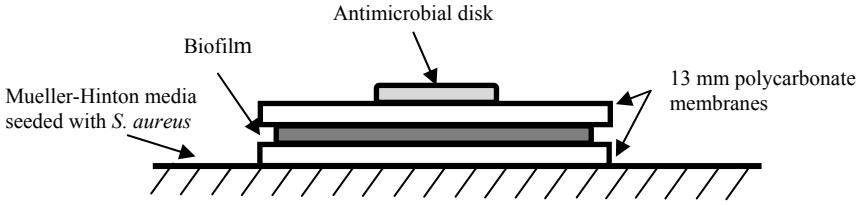

Figure 1 Array of polycarbonate membranes and biofilms for the study of the diffusion of antimicrobials through biofilms (adapted from the work of Anderl et al. [13] and Singh et al. [14]).

2.4 Biofilm characterization

The biofilms were characterized in terms of diameter, thickness and colony forming units (CFUs), according to Simões et al. [9, 15]. Biofilms were also characterized in terms of total and extracellular proteins and polysaccharides. The biofilms were scraped and diluted in extraction buffer (0.760 $g.L^{-1}$ $Na_3PO_4 \bullet 12H_2O$, 0.356 $g.L^{-1}$ $Na_2HPO_4 \bullet H_2O$, 0.526 $g.L^{-1}$ NaCl and 0.075 $g.L^{-1}$ KCl). Total proteins and polysaccharides quantification required the use of an extraction resin Dowex® sodium form (Na^+-form, strongly acidic, 20-50 mesh), according to Frølund et al. [16]. After extracellular proteins and polysaccharides extraction, proteins were quantified with the Total Protein Kit, micro Lowry, according Peterson's modification [17] purchased from Sigma-Aldrich, and using bovine serum albumin as standard. The polysaccharides were assessed by the phenol-sulphuric acid method [18], using glucose as standard.

2.5 Statistical analysis

For each parameter tested, the average and the standard deviation were calculated. The statistical significance of the results was evaluated using the Student's paired t-test (confidence level of 95%) to investigate whether the differences between the controls and the actual tests could be considered significant.

3. Results and discussion

Microorganisms in biofilms are more resistant to environmental stress conditions than their planktonic counterparts [19]. Cells embed themselves in EPS so that they can be protected from environmental adversities. EPS is seen as a barrier for cell protection by hindering diffusion of antimicrobial compounds. However, antimicrobial resistance is multi-factorial and usually does not only depend on one specific mechanism [20, 21].

In order to ascertain possible factors involved in biofilm resistance/susceptibility to the selected antimicrobials, *B. cereus* and *P. fluorescens* biofilms were characterized in terms of their diameter, thickness, CFUs, in addition to proteins and polysaccharides of the cells and the EPS matrix (Table 1).

Table 1 Characteristics of *B. cereus* and *P. fluorescens* biofilms. The mean ± SD is presented.

	B. cereus	***P. fluorescens***
Diameter / (mm)	8.3 ± 0.71	9.0 ± 0.31
Thickness / (mm)	0.34 ± 0.10	0.28 ± 0.0
Log_{10} CFU per biofilm area / (cells)	7.2 ± 1.3	8.6 ± 0.50
Matrix proteins / (μg per membrane)	76 ± 8.2	131 ± 4.9
Total proteins / (μg per membrane)	273 ± 13	204 ± 17
Matrix polysaccharides / (mg per membrane)	27 ± 5.9	45 ± 3.6
Total polysaccharides / (mg per membrane)	79 ± 9.1	68 ± 2.4

Biofilms of *B. cereus* and *P. fluorescens* had similar sizes ($P > 0.05$) but *B. cereus* biofilms were thicker than those of *P. fluorescens* ($P < 0.05$). Cell density in *P. fluorescens* was significantly higher than in *B. cereus* ($P < 0.05$) and *P. fluorescens* biofilms had higher amounts of extracellular proteins and polysaccharides ($P < 0.05$). This fact is in accordance with a previous report that demonstrated the lower amounts of extracellular proteins and polysaccharides of *B. cereus* biofilms compared with *P. fluorescens* [22].

EPS are often shown to bind to antibiotics and biocides [23]. Anderl et al. [13] suggested that EPS can either bind to antimicrobials, delaying their diffusion, or chemically react with them, causing their inactivation. This will drastically reduce the levels of antimicrobial available to react with the biofilm cells.

In order to ascertain the diffusional limitations of a selected antimicrobial agent, a colony biofilm method was used. The presence of inhibition halos on the *S. aureus* culture is indicative of the efficacy of the antimicrobial against the biofilms. Additionally, diffusion can be correlated with the antimicrobial potency of each antimicrobial.

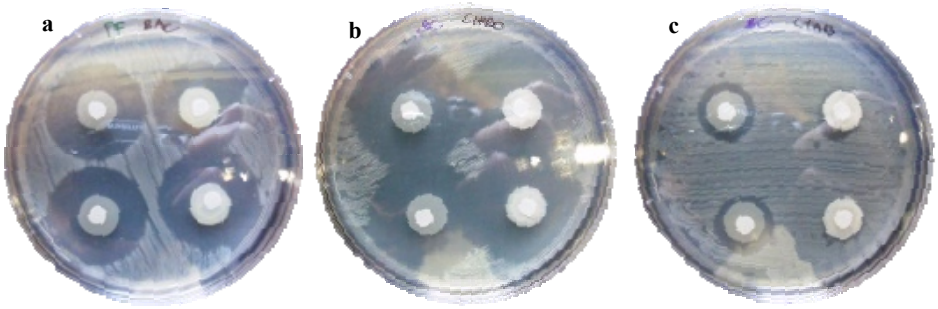

Figure 2. Inhibition halos on *S. aureus* when exposed to: (a) BAC in the presence of a *P. fluorescens* biofilm; (b) ciprofloxacin in the presence of a *B. cereus* biofilm; (c) CTAB in the presence of a *B. cereus* biofilm.

Some antimicrobials were almost not retarded. This is the case of ciprofloxacin and BAC for both bacteria (Figure 2) and sodium hypochlorite for *P. fluorescens* biofilms (Table 2). Retardation values comprised between 5% and 20% were observed for ethanol, BDMDAC and tetracycline for both biofilms and erythromycin and sodium hypochlorite for *B. cereus* biofilms. Iodine and erythromycin were retarded by 30% in the presence of *P. fluorescens* biofilms. *P. fluorescens* biofilms retarded streptomycin diffusion by 40% and isopropanol by 50%. Isopropanol and iodine were retarded more than 70% by *B. cereus* biofilms. Total antimicrobial retardation was verified for CTAB by both biofilms (Figure 2), and streptomycin by *B. cereus* biofilms. These results clearly show that biofilms significantly affected the diffusion of some antimicrobials. CTAB, BDMDAC, tetracycline and streptomycin for both biofilms; ethanol for *P. fluorescens* biofilms; iodine, isopropanol and erythromycin for *B. cereus* biofilms, were the cases where retardation was significant ($P < 0.05$). The diffusional limitations found in this study are apparently related with the effects of EPS in mass transfer through the biofilm [24]. The EPS may also degrade antimicrobials, particularly antibiotics, due to the presence of enzymes [23]. The polyanionic nature of the biofilm matrix may hinder the effects of antimicrobial agents. The charged or hydrophobic polysaccharides may work as a sorption mechanism of defence [6, 25, 26]. Furthermore, cells in biofilms are also known to exchange genetic information that provides resistance to certain disinfectants [27].

Table 2 Percentage retardation of antimicrobials in the presence of *B. cereus* and *P. fluorescens* biofilms.

Antimicrobials	*B. cereus*	*P. fluorescens*
ethanol	12 ± 7.0	9.3 ± 2.5
isopropanol	70 ± 8.8	52 ± 22
sodium hypochlorite	21 ± 0.45	1.9 ± 3.1
BAC	0.11 ± 0.75	0.0 ± 0.0
BDMDAC	15 ± 1.1	13 ± 0.54
CTAB	100 ± 0.0	99 ± 1.6
iodine	73 ± 20	29 ± 11
ciprofloxacin	0.0 ± 0.0	0.0 ± 0.0
erythromycin	14 ± 0.32	28 ± 3.1
streptomycin	100 ± 0.0	40 ± 0.89
tetracycline	6.9 ± 3.93	12 ± 1.8

This study also showed that the type of biofilm affects the antimicrobial hindrance: isopropanol, sodium hypochlorite, iodine and streptomycin diffused differently through the biofilms of both species. *B. cereus* formed biofilms with the lowest amount of extracellular proteins and polysaccharides; however, it caused the highest retardation rates. This result suggests that other resistance factors, excluding the EPS effects, may promote biofilm resistance to antimicrobials.

5. Conclusions

The biofilm colony technique is suitable to detect mass transfer limitations of antimicrobials in the presence of *B. cereus* and *P. fluorescens* biofilms. CTAB, isopropanol and iodine were severely hindered by the presence of the biofilms. Sodium hypochlorite, BAC, BDMDAC, ciprofloxacin, ethanol and tetracycline were the antimicrobial agents that better diffused through the biofilms (less than 15% retardation).

The overall results demonstrate that the choice of a suitable antimicrobial agent, with high penetration rates through a biofilm contamination, is of utmost importance when developing disinfection plans.

Acknowledgements The authors acknowledge the financial support provided by Operational Programme for Competitiveness Factors – COMPETE, European Fund for Regional Development – FEDER and by Portuguese Foundation for Science and Technology – FCT through the project Bioresist PTDC/EBB-EBI/105085/2008.

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Does the *bee* locus encode an additional or alternative pilus in *Enterococcus faecalis*?

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Enterococcal biofilm infections represent a severe problem in nosocomial infections. Treatments with antibiotics are often problematic because cells become resistant upon changes from planktonic to sessile life-styles. Besides other factors involved in the first step of biofilm formation, pili particularly seem to play a crucial role in this process. The clinical isolate of *Enterococcus faecalis* 1.10 harbours two pilus loci with one chromosomal locus being functionally inactive. This is due to insertional inactivation by IS elements. The second pilus is located on a large conjugative plasmid and confers strong biofilm formation ability to isolate 1.10. An isogenic mutant of this isolate with a defect in the second pilus locus displayed a significant reduction in biofilm formation. In this study, we show that at least one intact pilus locus is necessary for strong biofilm formation.

Keywords *E. faecalis*; *bee*; *ebp*; biofilm formation; pilus

1. Introduction

Microbial biofilm formation is a wide-spread life-style of bacteria in nature beneficial for cell growth and survival, e.g. due to conferment of antibiotic resistance [1]. Especially the occurrence in human infections and the difficult treatment of such biofilm based diseases represent a severe problem [2]. In the multi-stage biofilm formation process, we are particularly interested in the first step, which in addition to other factors is mediated by pili. In *Enterococcus faecalis* (*E. fc*), the predominant pilus is encoded by the chromosomal *ebp*-locus (endocarditis and biofilm-associated pilus) [3, 4]. However, in a small percentage of *E. fc* isolates, a second gene cluster, called *bee*-locus (biofilm enhancer in enterococci) and is encoded by conjugative plasmid pBEE99 [5, 6, 7], has been identified. This pilus also confers high biofilm formation strength to those isolates.

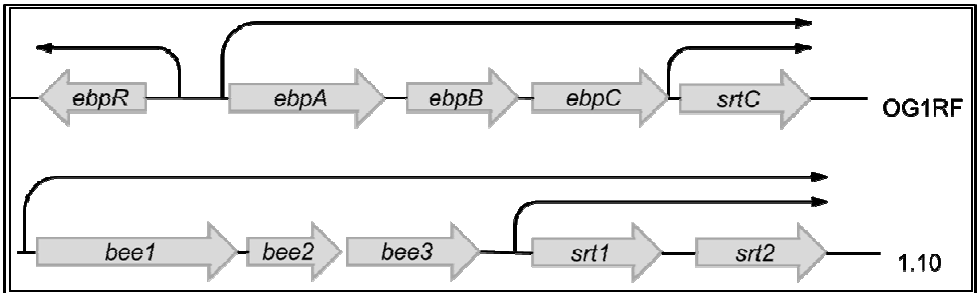

Fig. 1. Arrangement of the two pilus loci, *ebp* and *bee*, in *Enterococcus faecalis* strain OG1RF and clinical isolate 1.10, respectively. Both of them are composed of pilus structural genes and associated sortase (*srt*) genes. Arrows indicate individual promoters and their directionality.

Sequence analysis of the clinical isolate *E. fc* 1.10 revealed a new arrangement of the *ebp*-locus, resulting in the loss of the respective pilus. Simultaneously, this isolate harbours the second pilus locus, which is responsible for its ability to form biofilms [5]. An isogenic biofilm negative mutant *E. fc* 1.10.16 lost this capacity due to a point mutation in the *srt1* gene of the *bee* locus.

2. Materials and Methods

For biofilm assays, 96 well microtitre plates were filled with TSBG (tryptic soy broth + glucose 1%) bouillon and inoculated 1:10 with cells to be analyzed. Following incubation, washing and drying steps the adhering cells were stained with crystal violet which was dissolved in 100 % ethanol before OD_{595} was measured [8]. Whole cell protein extraction was performed using standard methods for Gram positive microorganisms. Proteins were separated by SDS-PAGE (12%), the resulting banding patterns and corresponding proteins were excised, trypsinised and identified in LC-ESI-MS/MS analysis. Western blot analysis was done using polyclonal anti-EbpA, EbpB and EbpC antibodies (a kind gift of Dr. Barbara Murray) and HRP-conjugated goat anti-rabbit IgG antibodies (secondary antibody). Development was done using the ECL detection reagents [3]. Gene sequencing

was performed by LGC-Genomics and resulting data were analyzed by NCBI blast online tool. To complement the *srt1* mutant in the biofilm negative strain *E. fc* 1.10.16, wild type *srt1* was fused to the *aph*A-3 promoter of pTCV-*lac* [9] and ligated into the shuttle vector pAT28 [10]. PCR fragments were generated using specific primers [6]. Using electroporation, *E. fc* 1.10.16 cells were transformed and grown on selective media containing 500 µg/ml spectinomycine. For raster electron microscopy (REM), single colonies of solid media grown cells were stamped out, prepared according to standard protocols and analyzed on a HITACHI S-4000.

3. Results and Discussion

Using SDS-PAGE combined with mass spectrometry and Western blot analysis, we found out, that the *ebp*-pilus was not expressed in isolate *E. fc* 1.10 (data not shown). Therefore, we amplified and sequenced parts of the *ebp* locus. This analysis revealed two insertion sequences, IS1062 and IS6770, within the *ebpR* and *ebpC* genes, respectively, and an *srtC* gene copy that acquired a 400 base pair extension in relation to the type strain *E. fc* OG1RF (Fig. 2). The assumption, that this may have led to a reduced biofilm formation, could not be phenotypically verified, since the isolate 1.10 still formed strong biofilms based upon OD measurements of crystal violet stained cell masses [5]. Biofilm formation in isolate *E. fc* 1.10 is due to a second plasmid encoded pilus gene locus called *bee* [5] (Fig. 1).

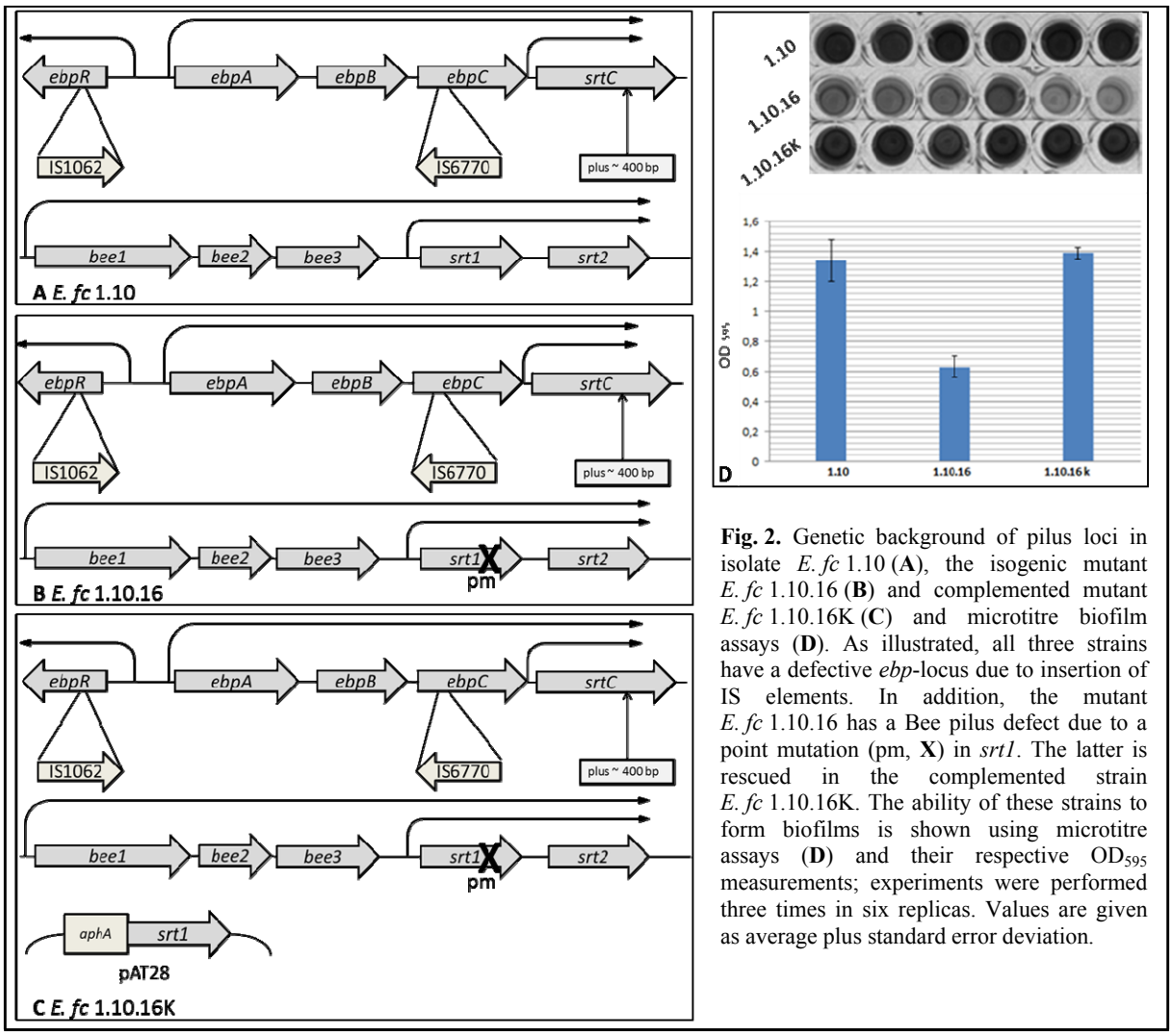

Fig. 2. Genetic background of pilus loci in isolate *E. fc* 1.10 (**A**), the isogenic mutant *E. fc* 1.10.16 (**B**) and complemented mutant *E. fc* 1.10.16K (**C**) and microtitre biofilm assays (**D**). As illustrated, all three strains have a defective *ebp*-locus due to insertion of IS elements. In addition, the mutant *E. fc* 1.10.16 has a Bee pilus defect due to a point mutation (pm, **X**) in *srt1*. The latter is rescued in the complemented strain *E. fc* 1.10.16K. The ability of these strains to form biofilms is shown using microtitre assays (**D**) and their respective OD_{595} measurements; experiments were performed three times in six replicas. Values are given as average plus standard error deviation.

As one can see in Fig. 2 A-C, the Ebp pilus is defective due to insertion of two IS Elements in all three strains. For mutant *E. fc* 1.10.16, the second pilus locus is additionally inactivated by a point mutation in the *srt1* gene [5]. Complemented *E. fc* 1.10.16K cells were transformed with wild type *srt1* gene under control of constitutive *aphA3* promoter on vector pAT28.

The insertion element IS1062, which interrupts the *ebpR* gene, and IS6770, which is inserted in the *ebpC* gene are indicated with arrows and show the new arrangement of the *ebp* locus in these strains (Fig. 2 A-C).

Due to these insertions correct Ebp pilus assembly is prevented. The decisive defect lies with the *ebpC* gene insertion, because it no longer encodes for a functional EbpC, the major shaft protein [3]. Therefore, there will be no more substrate EbpC for the sortase C which usually links EbpC to the Gram positive cell wall during pilus assembly. As a result, the remaining pilus subunits, encoded by *ebpA* and *ebpB,* will not be assembled into the pilus structure as a whole. With regards to the inactivated regulatory *ebpR* gene, we expect no further influence on pilus assembly since even in its active form, the transcription regulator, EbpR, is not likely to overcome a defective EbpC subunit or bypass the pilus assembly defect.

Fig. 2 A shows the genetic background of isolate *E. fc* 1.10. In addition to defective chromosomal *ebp* locus, the isolate harbours the second plasmid encoded intact *bee* locus. The double pilus defective mutant *E. fc* 1.10.16 is illustrated in Fig. 2 B. Investigation of this isogenic biofilm defective mutant (Fig. 2 D) revealed a point mutation at position 751 (G→T) in the bee-locus associated *srt1* gene product, which leads to a stop codon and hence abrogated enzyme activity [5]. Mass spectrometry analysis of areas corresponding to the HMW (high molecular weight) pilus complex of isolate *E. fc* 1.10 showed loss of pilus subunit Bee2 in mutant *E. fc* 1.10.16. This defect has led Schlüter *et al*. (2009) to postulate that inactive sortase 1 is responsible for incorrect assembly of the Bee pilus and consequently, for reduced biofilm formation strength [5]. To prove and verify this assumption, mutant strain *E. fc* 1.10.16 was complemented with the intact wild type gene *srt1* under control of the *aphA3* promoter [9] using shuttle-vector pAT28 [10]. The phenotypic loss of biofilm formation strength could be rescued by this complementation. Resulting strain *E. fc* 1.10.16K showed wild type-like (isolate 1.10) characteristic values of crystal violet stained cells, whereas mutant *E. fc* 1.10.16 values are reduced by more than 50 percent. In addition, the rescue of mutant *E. fc* 1.10.16 was confirmed by REM and by mass spectrometry analysis of the HMW pilus complex from whole cell extracts (Fig. 3). The loss of Bee2 in HMW corresponding area of whole cell protein extract of mutant *E. fc* 1.10.16 could be rescued. In complemented mutant *E. fc* 1.10.16K all subunits of correct Bee pilus have been reidentified. In this study we were able to verify the postulation of Schlüter *et al*. (2009) by complementation of the *srt1* biofilm mutant *E. fc* 1.10.16.

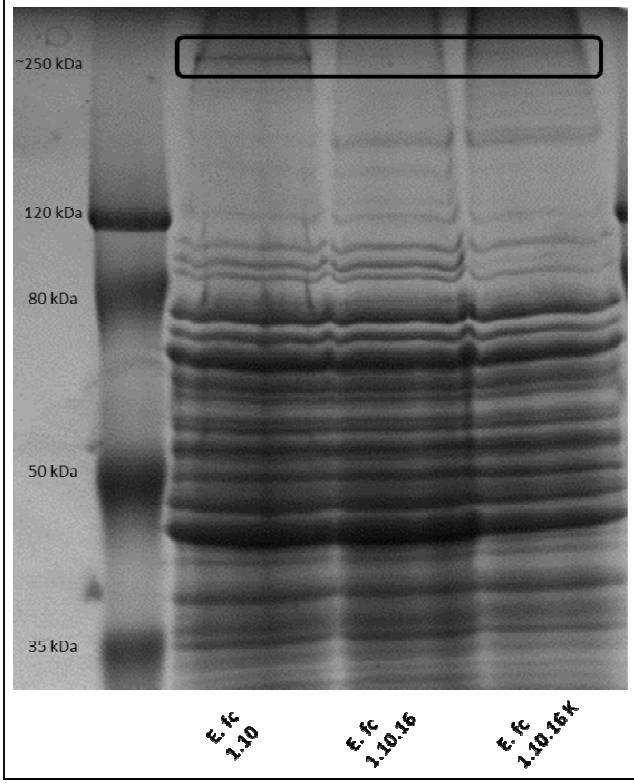

Fig. 3. Whole cell protein extracts of *Enterococcus faecalis* 1.10, 1.10.16 and rescued 1.10.16K were separated on a 12% SDS polyacrylamide gel. The high molecular weight complex HMW (black box) with a size of approximately 250 kDA is absent in biofilm negative mutant *E. fc* 1.10.16 but detectable in wild type and rescued biofilm positive strains *E. fc* 1.10 and *E. fc* 1.10.16K, respectively. Using mass spectrometry, all three pili subunits could be verified in the HMW of isolate 1.10 and complemented mutant 1.10.16K. In the corresponding area of the mutant strain 1.10.16 pili subunit Bee2 is lacking.

Upon analyzing REM pictures of all three strains, the complemented cells of *E. fc* 1.10.16K showed the same pili-like structures on the cell surface as isolate *E. fc* 1.10 (Fig. 4 A & C). These pili connect the cells not only to the surface, but also to each other. In contrast, the surface of mutant *E. fc* 1.10.16 cells showed no structures at all, which indicates the complete loss of the Bee pilus (Fig. 4B). Therefore, an additional function of sortase 1 for the assembly of major subunit Bee1 appears likely. Because of detectable subunits Bee1 and Bee3 in HMW complex of mutant *E. fc* 1.10.16, the sortase activity of Srt2 may account for Bee1 and Bee3 pilus subunit

integration. A putative involvement of the housekeeping sortase SrtA in pilus assembly can also not be excluded for the time being.

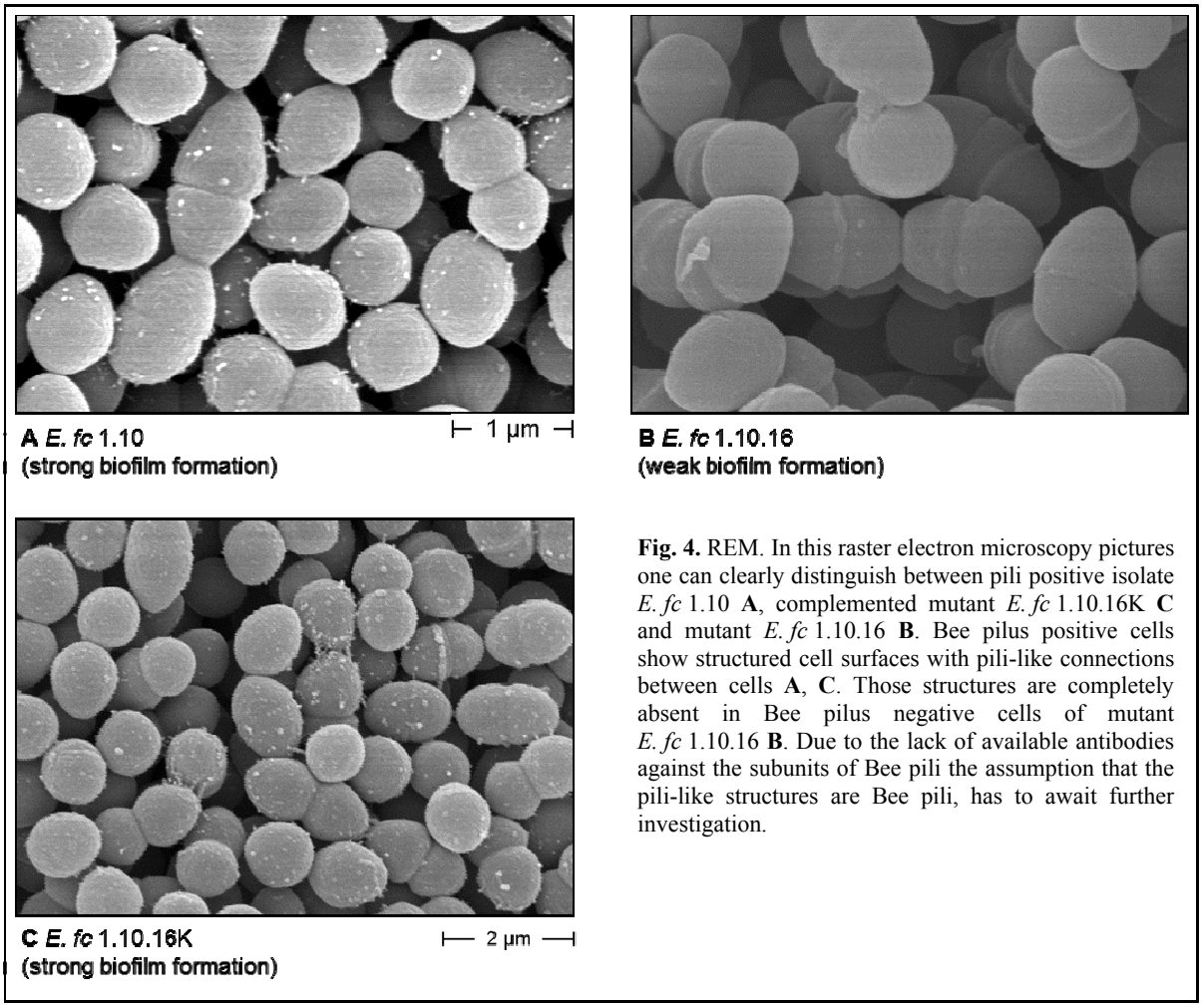

Fig. 4. REM. In this raster electron microscopy pictures one can clearly distinguish between pili positive isolate *E. fc* 1.10 **A**, complemented mutant *E. fc* 1.10.16K **C** and mutant *E. fc* 1.10.16 **B**. Bee pilus positive cells show structured cell surfaces with pili-like connections between cells **A**, **C**. Those structures are completely absent in Bee pilus negative cells of mutant *E. fc* 1.10.16 **B**. Due to the lack of available antibodies against the subunits of Bee pili the assumption that the pili-like structures are Bee pili, has to await further investigation.

Employing several different approaches including sequencing, Western blot analysis, mass spectrometry and phenotypic assays, we were able to show the necessity of at least one pilus species at the surface of the Gram positive *E. fc* cell to induce biofilm formation by initial adherence (Fig. 2). Insertion of IS elements IS1062 and IS6770 into the *ebp*-locus caused the loss of the Ebp pilus. This is mainly due to the defective major subunit EbpC, since this renders the cells without a backbone for an incorporation of the other subunits. The disrupted EbpR regulator has no further impact on the expression of the defective Ebp pilus.
Guiton *et al*. (2009) have shown in their experiments that the inactivation of Ebp pilus by a sortase C gene knock out resulted in great reduction of biofilm formation [11]. Our experiments did not confirm this result, because of the emergence of the second Bee pilus [5, 6, 7]. We have demonstrated that an Ebp pilus defect can be bypassed by expression of the additional Bee pilus. A double negative pilus mutant *E. fc* 1.10.16K showed weak biofilm formation, indicating the importance of pilus-directed cell attachment to surfaces and subsequent biofilm formation. This biofilm-minus phenotype could be rescued by complementation of the defective *srt1* gene of the *bee* locus, resulting in a correct pilus and hence strong biofilm formation ability.
The answer to the title question whether or not the pili play alternative or additional roles in biofilm formation, still remains open since our study provides solely evidence in favour of an alternative pathway. To study any additional effects, a particular strain harbouring both intact Ebp and Bee pili has to be developed and analyzed.

Acknowledgements: We want to thank Barbara Murray (The University of Texas Health Science Centre at Houston, Medical School, Department of Infectious Diseases) for providing us the antibodies against pilus subunits EbpA, EbpB and EbpC.
A special thanks goes to the Department of Biochemistry, University of Kassel, particularly Oliver Bertinetti for mass spectrometry analysis.

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Drug resistance preventive antivirals based on nano-responsible poly-ligands

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An effectiveness of low-molecular-mass anti-viral/microbial agents is crucially limited by their small size inadequacy to the scale of virus/microbe relevant biopolymers (nucleic acids, proteins, etc.) representing key macromolecular and nano-targets for preventive and therapeutic interventions. This inadequacy facilitates the progression of mutation-caused drug resistance. We developed effective approaches to overcoming this problem trough integration of such small molecular agents on basis of rationally designed water-soluble synthetic polymers. This article demonstrates the discovered possibilities by example of adamantane derivatives.

Keywords drug resistance prevention, antivirals, HIV, influenza, nano-responsible poly-ligands, adamantan

Introduction

Drug resistance becomes a central problem of modern antimicrobial prevention/therapy, especially in focus of highly mutable viruses. The mutability is only one side of the drug resistance source, while the second side is an inadequate effectiveness of antimicrobial drugs. Widely used small molecules (**SM**) are capable of only small-locus binding targets relevant for viral (or bacteria) life cycle. These targets represent, as a rule, macromolecules (nucleic acids/ proteins/ polysaccharides ...) forming nano-scale structures. And no any single small molecule can cover the full-size biopolymeric target to cardinally prevent a drug resistance progression. Antibodies (**Ab**), as polymeric (protein) type macromolecules, can be more effective. But Ab-related therapy/vaccination is also limited (within an antigen determinant sub-regions), becoming insufficient in the case of intensively mutating viruses, such as human immunodeficiency virus (**HIV**), the causative agent of AIDS. To create the more effective blockers of the biopolymer targets, the size-adequate polymer-type substances for novel nano-responsible and poly-functional drug redesign [1] is urgent need. In this report we demonstrate some possibilities by example of adamantane (**AD**) related derivatives within the scope of rational drug redesign from small-molecular (**1**) toward polymeric (**2-4**) compounds (Fig. 1).

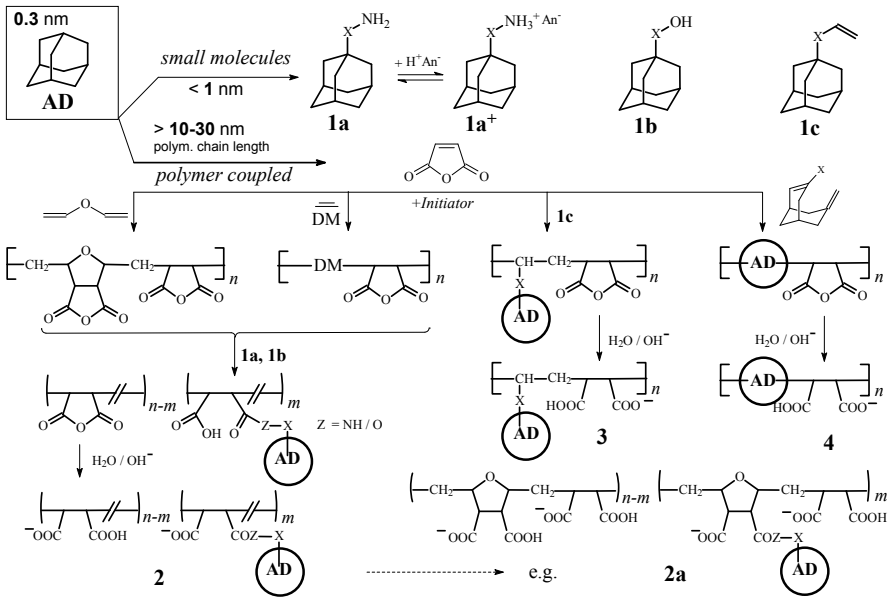


Fig. 1 The adamantane (*tricyclo[3.3.1.1^{3,7}]decene*) (**AD**) is a chemical core for anti-influenza A active small molecules (**1a**), such as amantadine (-X- = -) and rimantadine (-X- = -$CH(CH_3)$-). These and many other **AD** derivatives can be developed toward **AD**-containing polymeric compounds (e.g. **2**, **3**, and **4**) rational design of which opens more possibilities to enhanced anti-microbial effects, for instance to block the HIV entry, see below.

1. Adamantane-derived small molecules

AD-derived small molecules are widely investigated source for development of antiviral agents. The well known *rimantadine* and *amantadine* (Fig. 1: **1a**, -X- = -CH(CH_3)- and -X- = -, respectively) inhibit influenza type A viruses infection due to disruption of the viral proteins M2 self-assembly toward transmembrane proton channels (Fig. 2) that are required to uncoating of the virus's protective shells (lipid membrane-like envelope and capsid).

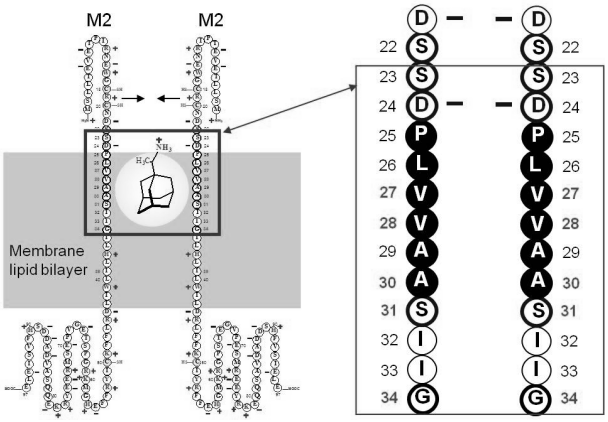


Fig. 2 The main macromolecular target and their sub-molecular locus sensitive to rimantadine/amantadine anti-influenza A2 inhibition. The black background marked amino acids sequence is the crucial section for the drug resistance mutations [2].

However, molecular size (~ 0.3 nm) of these **AD**-derived compounds is too small in comparison with macromolecular scale of the viral M2 proteins. This is why the **AD** family small molecule is capable of blocking only local part(s) of the biopolymer chains (Fig. 2 [2] and alternatively near the Trp43-Arg45 [3]). As the result, even any single mutation crucially within the 25-30 amino acids sequence can lead to the drug resistance [2]. Many currently circulating sub-types of influenza viruses type A possess resistance to rimantadine. Other viruses (not using the M2 protein), e.g. influenza virus type B and HIV [4,5] are insensitive to the rimantadine/amantadine as well.

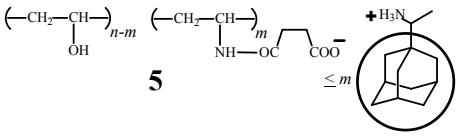
This example illustrates the fundamental principle that small molecules cannot be fully effective blockers of macromolecular targets because of the drug-target size inadequacy facilitating the viral drug resistance caused by single or few mutations. To overcome this limitation of anti-microbial potency of **AD**-derived (and other) small molecules, we involved these species into novel macromolecular drug design.

2. Adamantane-containing polymeric systems

2.1. Strategy and principles for the polymeric drugs design

Today many polymeric compounds are used as carriers or systems for delivery/release of known small molecular drugs. In this scope the small molecule remains the role of main active agent keeping the above mentioned problems in view of a drug resistance.

Rarely a polymeric component of such drug-delivery systems can play an additional bioactive role independently on small molecular component. Thus, ionic (non covalent) complex **5** of rimantadine cations with polyanionic carrier ("polyrem") releases rimantadine molecules (inhibiting influenza virus A), and the polymeric carrier possesses own immune-mediated anti-herpetic efficiency [6]. Inherently, the compound **5** is binary composition of two non-covalently combined drugs: the small molecules of rimantadine plus separable macromolecule of the copolymer (of vinyl alcohol with vinyl amid of succinic acid).



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In contrast with such commonly used approaches to drug design we consider a polymeric compound not as a separable carrier for small molecules, but as integrative basis that covalently links together small molecular precursors to cooperate their antiviral potentials within new entire polymeric molecule. This molecule should be size-adequate to a virus relevant biopolymeric target and capable of a selective binding not small-size locus (one-point) but majority or full-size of the target's connectable interface (multi-point binding network). An alternative requirement may be a possibility of the designed macromolecule affects not one target but various targets within viral life cycle. In these cases to become resistant to such multi-functional inhibitors the virus needs not one-point mutation, but multiple (multi-point/multi-target) mutations. A probability of many mutative changes (simultaneously for all polymer-sensitive viral structures) should be *a priory* much less than probability of one-point (small molecule adequate) mutation. This theoretical prediction is basis of our strategy for development of novel macromolecular drugs/agents having polymer-cooperative anti-microbial effects [1,5]. Taking into account an evolution of natural biopolymers we orient this drug design toward similarity and/or complementarity with virus relevant biopolymers [1]. Thus, a poly-acidic nature of synthetic polyanionic chains is similar to nature of nucleic acids (**NA**), or of heparan sulfate cellular receptors, negative charge of which is complementary vector to excess of positive charge on surface of viral particles (virions). In view of mimicry to

the natural prototypes we focus on synthetic poly-anions in search for water-solubility, biocompatibility, non-toxicity, NA-imitative antiviral immunogenicity, and heparin-like electrostatic selectivity to virions. Hypothetically suitable for these tasks copolymers of maleic acid (e.g. the **2**, **3**, and **4**, Fig. 1) were starting objects for synthesis and bio-evaluations. And expected mission of the hydrophobic (lipophilic) **AD** components was to concentrate interactions toward viral/cell membranes and in hydrophobic sites of viral targets. The shown in Fig. 1 various modes of **AD**-derived species insertion in the polymeric structures **2**, **3**, and **4** were estimated to be factors for regulation of the purposed properties.

2.2. Synthesis of the polymers

Synthesis of polymers of formula **2** (Fig.1) was carried out in the following three stages. The first stage provided poly- anhydrides as precursors suitable for graft-modifications. The alternating copolymers of maleic anhydride with divinyl ether (the precursor for the **2a** series), furan (for **2b** series), 2,3-dihydrofuran (for **2c** series), 2,5-dihydrofuran (for **2d** series), etc. were obtained via free-radical copolymerisation controlled by chain-transfer agents. The second stage was a step-by-step partial (to a desired degree of modification) aminolysis or etherification of the polymeric chains incorporated anhydride units with HZ-X-**AD** reagents (Z = NH or O, respectively). The final stage was a hydrolysis of the unused anhydride units preferably to carboxylic acid semi-sodium salt derivatives (-COOM ↔ $-COO^- + M^+$, M = H/Na). The obtained water-soluble products were purified trough a multi-cyclic ultrafiltration and isolated as lyophilised substances. The products were characterised by an element analysis, UV, FTIR, NMR, viscometry, and GPC. Detailed descriptions can be find in patent [4] and by references reviewed in [1,5,7]. The compounds **3** and **4** have been described as well [5].

2.3. Biocompatibility and toxicity

The variable ways of synthesis resulted in diverse types of the **AD**-containing compounds **2**, **3**, and **4** (Fig. 1) with different properties. The compounds **3** and **4** having high content of hydrophobic **AD** were generally insoluble in aqua (physiological) medium or poorly soluble possessing enhanced toxicity. Analogical behaviour was typical for the compounds of **2** series if degree of **AD**-modification $\boldsymbol{m} \rightarrow 100\%$ of $\boldsymbol{n}$ [5]. However, moderately modified compounds **2** became safer.

The lowest toxicity was observed in case of flexible chain polymers having high concentration of hydrophilic carboxy-groups per monomer unit. For instance, every repeated unit of the compound **2a** unmodified by **AD** ($\boldsymbol{m} = 0$) have four –COOH ↔ $-COO^-$ side groups, that can be step-by-step converted to the **AD**-linked –COZ-X-**AD** groups (Fig. 1). Particularly, the polymer-coupled **AD**-derivatives of **2a** series (Z-X- = NH-$(CH_2)_2$-, $\boldsymbol{n}$ = 40-50) at the modification degree of $\boldsymbol{m/n}$ = 0.21- 0.86 (5-21 % of all side groups) were 5-15 times less toxic *in vitro* than small molecular rimantadine itself (Table 1). At the $\boldsymbol{m}/\boldsymbol{n} \leq 0.2$ the series **2a** polymers possessed high safety CC_{50} = 800-2000 μg/ml for many cell cultures (MDCK, L-989, L-41, M-19, MT-4, etc.). The coupling of **AD** into the **2** (**2a**) polymeric macromolecules leaded also to prevention of the rimantadine-induced electric imbalances on epithelial membranes [8], and significantly reduced toxicity *in vivo* was recorded as well [5]. Therefore, under criteria of solubility and safety the **2** (**2a**)-series compounds have a priority.

Table 1 Toxicity (CC_{50}, μg/ml, in MDCK cell culture) of the compounds **2a** and rimantadine

Compounds of **2a** series (Z = NH, -X- = -$(CH_2)_2$-)				Riman-tadine	Ref.
$\boldsymbol{m}/\boldsymbol{n}$ = 0	0.21	0.43	0.86		
> 500	> 500	> 500	> 500	100	[7]
> 1000	750	500	300	50	[8]

2.4. Antiviral activity

In contrast to small molecule **AD**-derived drugs, the polymeric-coupled compounds **2** possessed much more broaden activity against both rimantadine-sensitive and rimantadine-resistant viruses (Fig. 3, Tables 2,3).

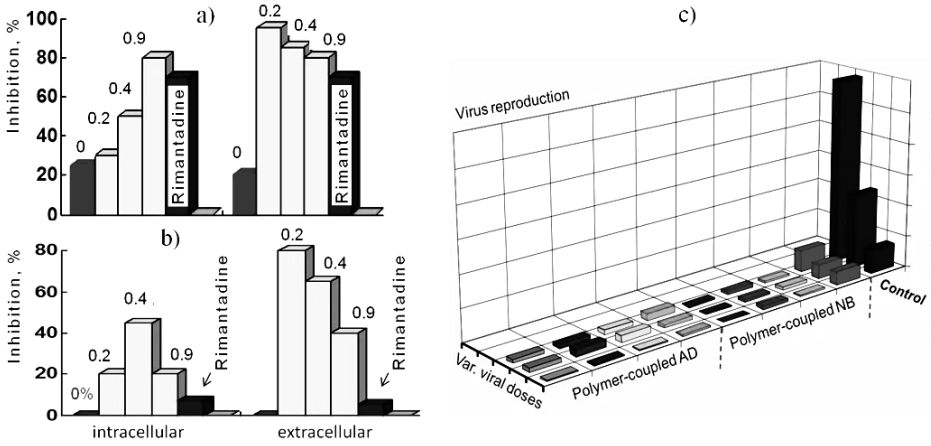

Fig. 3 Antiviral effects *in vitro:* a) and b) – inhibition of influenza viruses A/Victoria/35/72(H3N2) and B/USSR/100/84, respectively, by the **2a** compounds with variable degree of modification ***m/n*** = 0, 0.2, 0.4, and 0.9 vs. rimantadine [8]; c) – inhibition of HIV-1 by polymer-coupled **AD** derivatives and analogous norbornan (**NB**) derivatives [5], whereas the rimantadine is inactive [4].

Table 2 Degree of various viruses inhibition by rimantadine (Rim) and polymeric compounds of series **2a** *in vitro* [9]

Virus	Cell	Rim	**2a**
Influenza viruses:			
A/Victoria/35/72(H3N2)	HAO	≤ 1000	≤ 3000
A/PR/8/34(H1N1)	HAO	≤ 6	≤ 600
B/Sankt-Petersburg/71/97	HAO	0	≤ 300
Parainfluenza viruses:			
type I, Senday strain	HAO	≤ 3	≤ 300
type III, V2932 strain	MA-104	≤ 3	≤ 300
Respiratory syncytial virus.			
Long	MA-104	0	≤ 40
Herpes simplex virus	Hep-2	0	≤ 300
type 1, WIP-2 strain	FHEL	0	≤ 1000
HIV-1 more than 15 strains	MT-4; MAGI...	0	up to 100%

Table 3 Inhibition of various HIV strains by polymeric compounds of series **2a** *in vitro*

HIV strain	Cells	Inhibiting concentration μg/ml		ref *
		IC_{50}	IC_{90}	
HIV-1, IIIB	MT4	6. - 25	-	B
HIV-1, IIIB	MT4	0.7 - 11.2	1.2 - 24.9	C
HIV-1, 899	Magi	10 - 25	-	B
HIV-1, X794 LAI	MT4	0.1 - 6.7	-	R
HIV-1, J450 HXBZ	MT4	4.5	-	R
HIV-1, X165-11 (492/24)	MT4	28	-	R
HIV-1, LAV	PBMC	3.6	-	T
HIV-1, LAV.04	PBMC	26	80	M
HIV-1, H112-2 (10; AZT^s)	PBMC	2.8	-	T
HIV-1, G910-6 (9F; AZT^s)	PBMC	2.8	-	T
HIV-1, EVK	MT4	0.4 – 4.0	2.8 – 9.4	P
HIV-1, EVK	PBMC	5.0 – 7.3	-	P
HIV-1, AZT^R EVK	MT4	0.8 – 3.8	2.4 – 9.0	P
HIV-1, AZT^R	MT4	1.5 – 3.8	5.9 – 9.5	P
HIV-1, AZT^R	PBMS	9.7	-	P
HIV-1, SF162	PBMS	31 – 40	91 – 160	M
HIV-2, ROD	MT4	93.4	-	C

* The experimental data from: B – A.G. Bukrinskaya (Russia); C – E. De Clercq (Belgium); R - D.D. Richman (USA); T - S.R. Turk (USA); M - L. Margolis (USA); P – N.G. Perminova and I.V. Timofeyev (Russia)

Besides the anti-influenza A/Victoria/35/72(H3N2) activity comparable with similar effect of rimantadine (Fig. 3 a), Table 2), the polymeric compounds **2a** efficiently inhibit many other viruses which are resistant to rimantadine: influenza A/PR/8/34(H1N1) (Table 2), influenza B/USSR/100/84 (Fig. 3 b)), influenza B/Sankt-Petersburg/71/97 (Table 2), parainfluenza viruses type I/Senday and type III/V2932, herpes virus type I/WIP-2 (Table 2), as well as many HIV-1/2 strains (Fig. 3 c), Tables 2, 3).

These experimental data evidently indicate a significant amplification of antiviral potency of the **AD**-derived substances due to integration of many **AD** species in side-positions of rationally designed polymeric acid chains (**2a**, at least).

A possible explanation for this fact can be addressed to role of polymeric carrier (similarly the additional activity of polyacid component in the above mentioned "polyrem" complex). However, in our case the polyacid **2a** non modified by **AD** (***m*** = 0) was notably less active *in vitro* than **AD**-modified derivatives (***m/n*** ≥ 0.2), see, for example, Fig. 3 a) and b), as well as publications [1,4,5,7-9].

A considerable contribution of polyacid component in antiviral activity was observed only *in vivo* – on the level of immune competent organisms of experimental animals (mice/rats). *In vivo* (not *in vitro*) the polymeric basis of **2a** compounds and relative polymeric acids of **2** series are capable of possessing significant interferon inducing and other immune stimulating effects similar to effects of viral nucleic acids or their synthetic analogous [5,7]. This activity of the polyacid itself appears independently

on the **AD** presence (***m/n*** = 0.0 - ≤ 0.4) resulting in additional immune mediated preventive protection of animals against lethal doses of many viruses, e.g.: rabies virus, tick-born virus, eastern equine encephalomyelitis virus [5,7]. But as we noted, this effect appears *in vivo* without any significant manifestation *in vivo*. Therefore, *in vitro* the amplified and broad antiviral activity can be related only with entire **AD**-containing polymeric molecules **2** but not with separate components (acidic polymeric chain and **AD** groups). Just macromolecular cooperation of the both components provides a nano-responsible potentiality to strongly bind many sequences of viral target(s), preventing viral reproduction and drug resistance. This aspect can be illustrated more clearly through a consideration of recently explored mechanisms of HIV-1 entry inhibition by the compounds of series **2.**

2.5. Molecular mechanisms of enhanced anti-HIV effectiveness and drug resistance prevention

To clarify concrete molecular mechanisms of an amplified antiviral potency of the series **2** compounds, a comparative investigation of the anti-HIV activity of variable structures of **2** series were performed in correlation with computational modelling of their interactions with most probable HIV-specific targets, in part, the viral envelope glycoproteins gp120 and gp41.

Docking of single **AD** small molecules models revealed multiple sites on the gp120 [5] and more on the gp41 surface [7,10] accessible for the **AD** binding. But estimated binding energies for the each single-point contact was too week (≤20 kcal/mol) to be stable at physiology temperatures, predicting no significant anti-HIV efficacy for small molecules of **AD**. This prediction well correlated with experimental data *in vitro*.

On the contrary, docking and molecular dynamics of the polymer-chain coupled models of **2** series [7,10] evidently demonstrated possibilities to many fold increasing the binding energies (>>100 kcal/mol) due to multi-point cooperative contacts of repeated along polymeric chain **AD** anchors and anionic groups, with the gp41 [**HRI**]$_3$ nano-complex (native and mutant). This complex is a key mediator of the HIV-1 fusion for the virus entry into cell, and binding this target means prevention of the virus entry. Multiplicity and mode of this binding by polymers **2** can be selectively regulated through the polymeric backbone and **AD** anchors parameters. The modelling-based optimal design predicts a possibility for combined axial-co-belting blockage of the viral nano-target, occupying at least the L1-L3 levels of the target triplet pockets (Fig. 4).

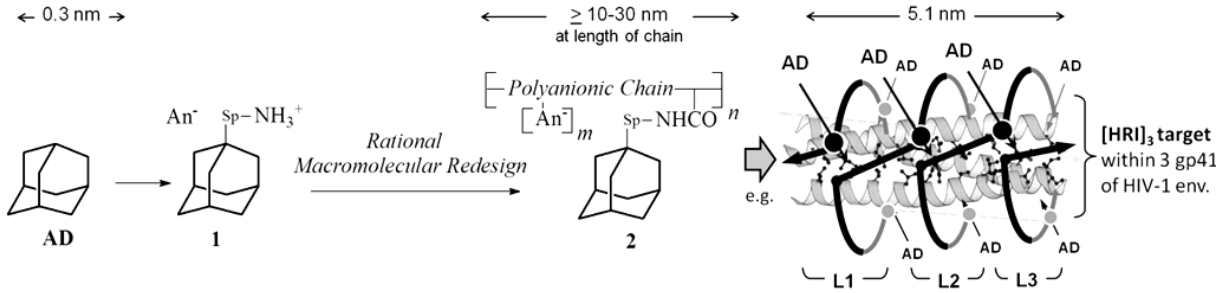

Fig. 4 The **AD**-derived small molecule (**1**) antivirals are small-size ligands capable of only narrow-specific (one-point) blocking viral/microbial targets, and therefore, such antiviral agents cannot prevent the drug resistance. But **AD** derivatives can be linked via polymeric nano-scale chains creating the novel **AD**-containing polymeric compounds (e.g. **2**), rational design of which opens more opportunities to multi-point blocking viral targets, for instance the HIV fusion mediator ([NHR]$_3$ tri-helix core of gp41).

Under such blockage, the virus needs at least the three levels (L1-L3, ~ 36 amino acids sequence) many-point mutations to become resistant. The probability of three-level mutations is many times less than that of a single one-point mutation. This property apparently explains why the test-sample of **2a** series effectively prevented HIV-1 resistance in a long-term high-cycle experiment in spite of multiple mutative transformations [5].

Conclusion

The adamantane is well known chemical base for small molecule (**1**) antivirals, that are capable of narrow-specific (one-point) blocking only certain viral targets (e.g., the M2 proteins of influenza A virus) but cannot prevent drug resistance. These derivatives and other small molecules (**SM**) can be developed toward SM-cooperating polymeric compounds (e.g. **2**) rational design of which leads to strongly amplified potency for multi-point (and nano-competent) blocking a broad spectrum of viral targets, preventing the drug resistance.

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Effect of the combined use of cinnamon oil and stevia on the growth rate of *Zygosaccharomyces bailii*

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The aim of this study was to evaluate the effect of cinnamon oil and stevia on the growth rate of *Z. bailii* in acidified aqueous systems resembling low sugar products. A factorial design in three blocks and a central point was performed. The levels of cinnamon oil and stevia were the factors studied. A microtitre plate growth assay was used. The systems were formulated in Sabouraud broth at pH 3.50 and inoculated with *Z. bailii* reaching a population of 1.10^6 CFU/ml. The microplate was incubated at 30ºC. Absorbance readings were automatically recorded during the incubation. Growth rates were calculated by using the Logistic model. Cinnamon oil was able to decrease significantly the growth of *Z. bailii* whereas stevia did not affect it. An antagonistic interaction between both additives was observed. These results suggest that stevia protects the yeast against oil antimicrobial action. A level of cinnamon oil of 0.0056% w/w would be necessary to minimize the growth rate of *Z. bailii*.

Keywords cinnamon oil; stevia

1. Introduction

Over the past decades, obesity has reached epidemic rates worldwide becoming a health problem. It led to the development of foods with low sugar content, such juices, jellies and jams.

The main cause of spoilage of these foods is the microbiological instability due yeasts and moulds growth [1]. Particularly, *Zygosaccharomyces bailii* is a spoilage yeast, resistant to the hurdles commonly applied to preserve these foods. It is an osmophilic, acid tolerant and preservative resistant yeast. It can grow under anaerobic conditions and with minimum nutritional requirements. It is also resistant to cleaning agents and industrial sterilization causing significant economic losses [2, 3, 4, 5, 6]. Therefore, the knowledge of the effects of additives on growth of *Z. bailli* is of interest for manufacturers of acidic products.

Current consumers demand for products free of preservatives or with reduced levels of them highlights the importance of studying the effect of other additives on antimicrobial action. In this context are the essential oils, oily liquid mixtures of volatile compounds which are synthesized by plants as secondary metabolites. They are mainly obtained by steam distillation or extraction with supercritical fluids [7, 8]. Essential oils may include between 20 and 60 compounds depending on the material source and the extraction method used. They are mainly terpenes and terpenoids, and as minority group, aromatic and aliphatic compounds of low molecular weight [8]. The antimicrobial action of essential oils is widely reported and it is attributed to the penetration of lipophilic components through the membrane to the site of action within the cell [9]. Being "generally recognized as safe" additives (GRAS), together with its wide acceptance by consumers and the increasing demand on reducing intake of synthetic additives reemerged the study of the use of these preservatives in foods. In particular, cinnamon oil presents inhibitory action on growth of pathogens and spoilage yeasts *in vitro* and *in vivo* [10, 11].

Stevia is a natural sweetener extracted from the leaves of *Stevia rebaudiana* (Bertoni), a plant native from Paraguay. It is 300 times sweeter than sucrose and has a low glycaemic index making it attractive for diabetic people. It has GRAS status and use as sweetener in beverages and foods [12]. It is approved for use in Brazil, Argentina, Paraguay, China, Korea and Japan.

Essential oils and stevia might be suitable to be used in the formulation of low sugar content foods.

There is little information about the antimicrobial action of cinnamon oil on the growth of yeasts [13, 11]. Therefore, the aim of this study was to evaluate the effect of cinnamon oil and stevia on the growth rate of *Z. bailii* in acidified aqueous systems resembling low sugar products.

2. Materials and methods

2.1 Systems formulations

The composition of systems is given in Table 1. The systems were formulated in Sabouraud broth with 0.15% w/w of agar (Biokar Diagnostics, Beauvais, France) at pH 3.50, adjusted by citric acid (Parafarm, Buenos Aires, Argentina) addition. Before adding cinnamon oil (*Cinnamomum zeylanicum*) (Sigma, U.S.A.), systems were autoclaved 15 min at 121ºC excepting those containing stevia (Inmobal Nutrer, Argentina), which were autoclaved 30 min at 100ºC. After that, oil was added aseptically.

2.2 Experimental design

To assess the effect of cinnamon oil and stevia on *Z. bailii* growth, a factorial design (2^2) in three blocks and a central point was performed. The factors studied were the concentrations of cinnamon oil and stevia at the levels indicated in Table 1. The complete design consisted of five experimental trials (Table 1). Each of these experiments was conducted and evaluated in triplicate resulting in a total of 15 experimental runs. The design was created with Statgraphics Plus for Windows, version 5.1 (Manugistics, Inc., Rockville, Maryland, U.S.A.).

Table 1 Factorial design used to evaluate the effects of cinnamon oil and stevia on the growth rate of *Z. bailii*.

Cinnamon oil concentration		Stevia concentration	
Uncoded value (% w/w)	Coded value	Uncoded value (% w/w)	Coded value
0.0000	-1	0.0000	-1
0.0000	-1	0.5000	+1
0.0056	+1	0.5000	+1
0.0056	+1	0.0000	-1
0.0028	0	0.2500	0

2.3 Preparation of inoculum

Zygosaccharomyces bailii NRRL 7256 was stored at -30ºC in Sabouraud broth (Biokar Diagnostics, Beauvais, France) plus 10% w/w glycerol (Sintorgan S.A., Buenos Aires, Argentina). Before using they were grown twice in Sabouraud broth at 30 ± 1 ºC during 24 hours.

2.4 Inoculation and growth assay

One hundred µl volumes of each system were dispensed into the wells of a 96-well-round bottomed sterilized microtitre plate.

The inoculum was appropriately diluted in Sabouraud broth (pH = 3.5) and 100 µl volumes were added to each well reaching a population of 1.10^6 CFU/ml. Negative and positive controls were tested in parallel, being the former no inoculated Sabouraud broth, and the latter inoculated Sabouraud broth free of additives.

The microtitre plate was incubated at 30ºC for 45 h, for system containing oil, and 30 h, for the rest of systems, in a plate reader driven by Gen 5 reader control and data analysis software (BioTek Instruments, ELx808, U.S.A.). Absorbance readings were automatically recorded at 600 nm every 30 min during the incubation shaking at slow intensity for 30 s before each reading. Absorbance readings of controls were used as blank.

Growth curves were obtained from absorbance readings and they were replicated five times.

2.5 Data analysis

Growth curves were modeled using the Logistic model [14]:

$$Abs_t = Abs_{min} + \frac{Abs_{max} - Abs_{min}}{1 + \exp[-\mu(t - t_i)]} \quad (1)$$

where Abs_t is the absorbance at time t; Abs_{min} and Abs_{max} are the asymptotic minimum and maximum absorbance; μ is the specific growth rate, and t_i is the time at inflection point.

Growth rate was calculated for each curve and the average was subjected to a multiple regression analysis to fit the following first-order regression model and predict the impact of the factors on *Z. bailii* growth rate:

$$\mu = \alpha_0 + \alpha_1[CO] + \alpha_2[S] + \alpha_3[CO][S] + \varepsilon \quad (2)$$

where $\alpha_{0,1,2,3}$ are the regression coefficients for the intercept, linear and interaction, respectively; [*CO*] and [*S*] are the levels of cinnamon oil and stevia in coded units; ε is the error term.

The adequacy of the regression model was examined by analysis of variance (ANOVA) at 5% significance level and correlation coefficient (R^2). Also, ANOVA and p-value were used to evaluate the significance of the linear and interaction terms of model. The analyses were performed using Statgraphics Plus for Windows, version 5.1 (Manugistics, Inc., Rockville, Maryland, U.S.A.).

3. Results and discussion

Yeast growth curves were satisfactory modeled by the Logistic model as it was proved throughout the ANOVA and by the correlation coefficient (R^2) which showed values of 0.98–0.99 for all systems. Experimental data and modeled curves for the system free of additives and the system containing 0.0028% w/w of cinnamon oil and 0.25% w/w of stevia are shown in Fig. 1 as an example. For the rest of the systems analyzed, modeled curves showed a similar pattern.

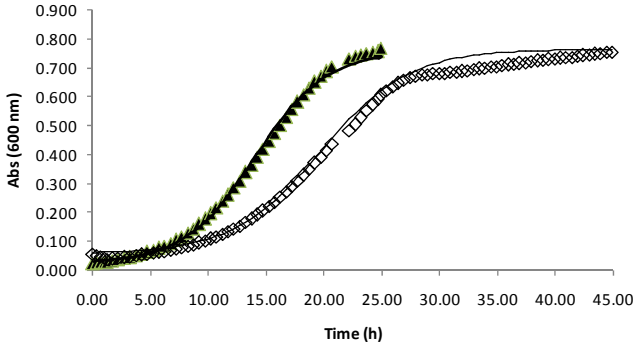

Fig. 1 *Z. bailii* growth. Experimental data and modeled curves (—) for the system free of additives (▲) and the system containing 0.0028% w/w of cinnamon oil and 0.25% w/w of stevia (◊).

The first-order regression model fitted was:

$$\mu = 0.24 - 0.034[CO] + 0.005[S] + 0.032[CO][S] \quad (3)$$

The correlation coefficient was 0.85 indicating a good correlation between the observed and the predicted values.

Cinnamon oil was able to significantly decrease the growth rate of *Z. bailii* as it was demonstrated by the negative sign of regression coefficient and in the Pareto chart (Fig. 2). Stevia did not significantly affect growth rate (Fig. 2).

An antagonistic interaction between both additives on *Z. bailii* growth rate was observed (Fig. 2).

The antagonistic interaction between cinnamon and stevia may be explained by Figure 3. In the absence of stevia, oil addition decreased growth rate from 0.30 h^{-1} to 0.17 h^{-1}. However, subsequent addition of stevia produced the increase in growth rate to 0.25 h^{-1} (Fig. 3).

These results suggest that stevia protects the yeast against oil antimicrobial action through a mechanism not studied yet. Probably, it might involve the interaction with the cell membrane.

Based on the regression model, it was estimated that a concentration of 0.0056% w/w of cinnamon oil would be necessary to minimize the growth rate from 0.302 h^{-1}, in the absence of additives, to a value of 0.172 h^{-1}.

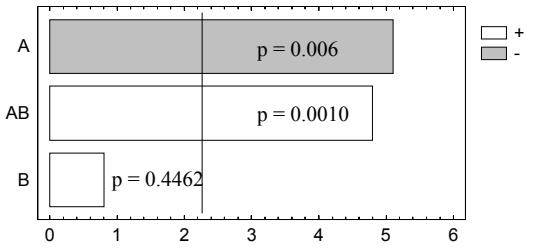

Fig. 2 Pareto chart showing the effect of cinnamon oil (A) and stevia (B) on growth rate.

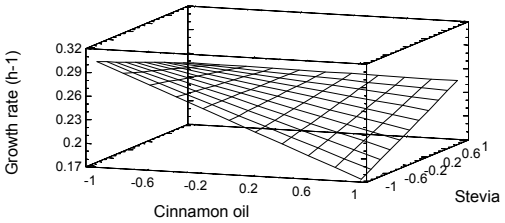

Fig. 3 Estimated response surface graph for the combined effect of cinnamon oil and stevia on growth rate of *Z. bailii.* (In coded units, showed in Table 1).

4. Conclusion

Data obtained showed that cinnamon oil was able to decrease the growth rate of *Z. bailii* in broth at pH 3.5 while an antagonistic interaction between stevia and cinnamon oil was observed.

Exposed trends highlight the importance of understanding the effect exerted by interactions between additives on the development of *Z. bailii.* The knowledge of these interactions may allow establishing suitable concentrations to optimize the microbiological stability of the system. Thus, mentioned results would contribute to the development of safe and healthy foods.

Acknowledgements The support of Universidad de Buenos Aires, Agencia Nacional de Investigaciones Científicas y Tecnológicas of Argentina and Consejo Nacional de Investigaciones Científicas y Técnicas of Argentina is gratefully acknowledged.

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Effects of benzyl isothiocyanate and (*E,Z*)-4-methylthio-3-butenyl isothiocyanate on *in vitro* growth of human intestinal bacteria and on *in vivo* formation of short-chain fatty acids in the rat intestine

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In this study, we investigated the *in vitro* antibacterial effects of benzyl isothiocyanate (BITC) and (*E,Z*)-4-methylthio-3-butenyl (MTBITC) isothiocyanate on the growth of 19 bacterial strains isolated from the human intestine. We also studied the effects of BITC on the production of short-chain fatty acids in the rat intestine *in vivo*. Among the bacterial strains tested, *Clostridium* spp., *Anaerococcus* sp., and *Fusobacterium* sp. showed the highest susceptibility to BITC; the growth of all these strains was virtually inhibited at over 20 ppm. In contrast, most strains of *Lactobacillus* spp., *Bifidobacterium* sp., and *Bacteroides* spp. showed much stronger resistance to BITC at even 100 ppm. These differences were seen when the bacterial strains were treated with BITC or MTBITC for a short time, such as 30 or 60 min. The effect of BITC on the *in vivo* formation of short chain fatty acids was minor. However, a dose-dependent increase occurred in the formation of hexanoic acid following the administration of BITC, suggesting that BITC has the potential to alter the intestinal microflora in rats.

Keywords intestinal bacteria; susceptibility; isothiocyanate; rat microflora; short chain fatty acid

1. Introduction

Isothiocyanates (ITCs) are widely known phytochemicals that are derived from the hydrolysis of glucosinolates by the enzyme myrosinase (thioglucohydrolase; E.C. 3.2.1.147). Benzyl isothiocyanate (BITC) and (*E,Z*)-4-methylthio-3-butenyl isothiocyanate (MTBITC) are characteristic pungent components found in garden cress (*Lepidium sativum* L.) and radish (*Raphanus sativus* L.), respectively [1, 2]. ITCs, including BITC and MTBITC, have been recently recognized as naturally occurring cancer-protective agents [3-5]. Therefore, considerable research has focused on their activity in the human intestine along with that of their parent glucosinolates, and it has been reported that the intestinal microflora can degrade glucosinolates to form isothiocyanates and nitriles. By virtue of their reactivity with thiol and/or amino groups of proteins, ITCs also act as antimicrobial agents against a variety of fungi, yeasts, and bacteria [6, 7]. However, little information is available on the effects of ITCs on the microflora present in the mammalian intestine. In this study, we examined the *in vitro* antibacterial effects of both BITC and MTBITC on the growth of 19 bacterial strains found in the human intestine. In addition, we investigated the effects of benzyl isothiocyanate on the *in vivo* formation of short-chain fatty acids in the rat intestine.

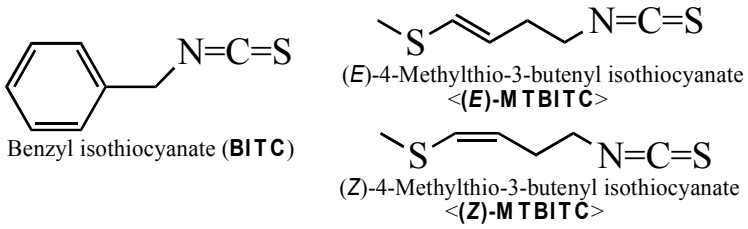

Fig. 1 Chemical structures of BITC and (*E*)- and (*Z*)-MTBITC.

2. Materials and Methods

2.1 Chemicals and bacterial strains BITC was purchased from a commercial source, and MTBITC was prepared from radish sprouts by extraction using diethyl ether and subsequent silica gel column chromatography. The 19 bacterial strains used (Table 1) were obtained from RIKEN Bioresource Center (Tukuba, Japan). The *Lactobacilli* strains were cultured in MRS broth (Kanto Chemical Co. Inc., Tokyo, Japan) and the other strains were cultured in GAM broth (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan).
2.2 In vitro *experiments* The test compounds dissolved in dimethyl sulphoxide were added to achieve final concentrations of 0, 20, 40, or 100 ppm in bacterial cultures, which were anaerobically (H_2:N_2:CO_2 = 10:10:80)

incubated for 12 h at 36°C, and the bacterial growth was then evaluated by measuring the turbidity at 660 nm. For another experiment, some bacterial strains were treated with 60 ppm BITC or MTBITC for 30 or 60 min. After rinsing twice with 0.1 M phosphate buffer (pH 7.2), the bacteria were cultured anaerobically in MRS broth or GAM broth for an additional 8 h, and the O.D. values at 660 nm were recorded.

2.3 In vivo *experiments* All *in vivo* experiments were performed by using 15 Donryu male rats (age, 6.5 weeks) according to the Guidelines for Animal Experiments (protocol #A07-0034) and were approved by the Utsunomiya University Animal Research Committee. The rats were grouped into control and treated groups, with 5 rats in each group. The rats in the treated group were dosed with 1 mL of corn oil containing 0.4 mg or 0.8 mg BITC, thrice on alternate days by using a gastric tube, and the control group was administered 1 mL of corn oil without BITC at the same frequency. On the day after completion of the third dose, each rat was decapitated and the contents of its intestinal tract were immediately collected and mixed with 25 mL of sterilized MilliQ water. We added 3 mg of 2-hexenoic acid to the mixture as the internal standard, and the mixture was extracted with 25 mL of diethyl ether. After centrifugation at 3500 rpm for 6 min, the ether layer was collected and dehydrated with anhydrous sodium sulphate and concentrated under a nitrogen stream to ca. 0.1 mL. The concentrate was analysed by gas chromatography (GC) (column: 5% Thermon-1000; Shinwa Chemical Industries, Ltd., Tokyo, Japan; 3 mm [i. d.] × 2 m); the temperature was programmed to rise from 80°C to 180°C with a 5°C/min increase, and the injection port temperature was maintained at 200°C. Gas chromatography-mass spectrometry (GC-MS) analysis was also performed to identify the short-chain fatty acids under conditions similar to those of the GC analysis.

3. Results and Discussion

3.1 In vitro growth-inhibitory effects of BITC and MTBITC on the 19 bacterial strains Evaluation of the growth-inhibitory effects was performed considering the ratios of the turbidity of the test compound-containing media to the control media. The results are shown in Table 1.

Table 1 Inhibitory effects of BITC and MTBITC on the 19 bacterial strains from the human intestine

Bacterial strain	Conc. of BITC (ppm)			Conc. of MTBITC (ppm)		
	20	40	100	20	40	100
Eggerthella lenta JCM 9979	VW	VW	W	-	-	-
Lactobacillus reuteri JCM 1112	VW	VW	W	-	-	-
Lactobacillus fermentum JCM 1137	VW	VW	W	VW	VW	VW
Bacteroides vulgatus JCM 5826	VW	W	W	VW	VW	W
Bacteroides distasonis JCM 5825	VW	W	S	-	-	-
Collinsella aerofaciens JCM 7790	VW	W	S	-	-	-
Bifidobacterium pseudocatenulatum JCM 7040	VW	W	S	-	-	-
Bifidobacterium longum JCM 7050	VW	S	S	VW	S	VS
Blautia producta JCM1471	VW	S	S	-	-	-
Lactobacillus acidophilus JCM 1028	VW	S	S	VW	W	S
Lactobacillus brevis JCM 1059	VW	S	S	VW	W	S
Lactobacillus gasseri JCM 1025	W	S	S	-	-	-
Lactobacillus salivarius JCM 1045	W	S	S	-	-	-
Bifidobacterium adolescentis JCM 7045	S	VS	VS	S	S	VS
Anaerococcus hydrogenalis JCM 7635	VS	VS	VS	-	-	-
Bacteroides fragilis JCM 11019	VS	VS	VS	W	W	S
Clostridium innocuum JCM 1292	VS	VS	VS	VS	VS	VS
Clostridium perfringens JCM 3816	VS	VS	VS	VS	VS	VS
Fusobacterium varium JCM 3722	VS	VS	VS	S	VS	VS

Inhibition percentage: VS, 80–100%; S, 61–79%; W, 31–59%; VW, 0–30%; -, Not examined.

The bacterial strains tested were broadly divided into 3 groups on the basis of susceptibility to BITC, that is the group with the highest susceptibility, the group with the strongest resistance, and an intermediate group.

Among the bacterial strains tested, *Clostridium* spp., *Anaerococcus* (*Peptostreptococcus*) sp., and *Fusobacterium* sp. showed the highest susceptibility to BITC; the growth of all these test strains was virtually negligible at over 20 ppm. In contrast, most strains of *Lactobacillus* spp., *Bifidobacterium* sp., and *Bacteroides* spp. showed much stronger resistance to BITC at even 100 ppm. The characteristic behaviours of these particular bacterial strains were observed in the case of MTBITC treatments as well.
In addition, 2 bacterial strains, ITC-susceptible *Clostridium perfringens* JCM 3816 and ITC-resistant *Lactobacillus fermentum* JCM 1137, were treated with 60 ppm BITC and MTBITC for 60 min. After rinsing twice with 0.1 M phosphate buffer (pH 7.2), the bacteria were cultured anaerobically in GAM (*Clostridium perfringens* JCM 3816) and MRS (*Lactobacillus fermentum* JCM 1137) broth for an additional 8 h, and their OD values at 660 nm were recorded. The results have been shown in Fig. 2. The growth of *Clostridium perfringens* JCM 3816 was seemingly suppressed by treatment with BITC or MTBITC for a relatively shorter period such as 60 min, while the growth of *Lactobacillus fermentum* JCM 1137 was only slightly affected. These results show that the lactic acid bacteria in the human intestine may have a low ITC susceptibility, whereas some strains of *Clostridium* spp. are highly susceptible to ITCs.

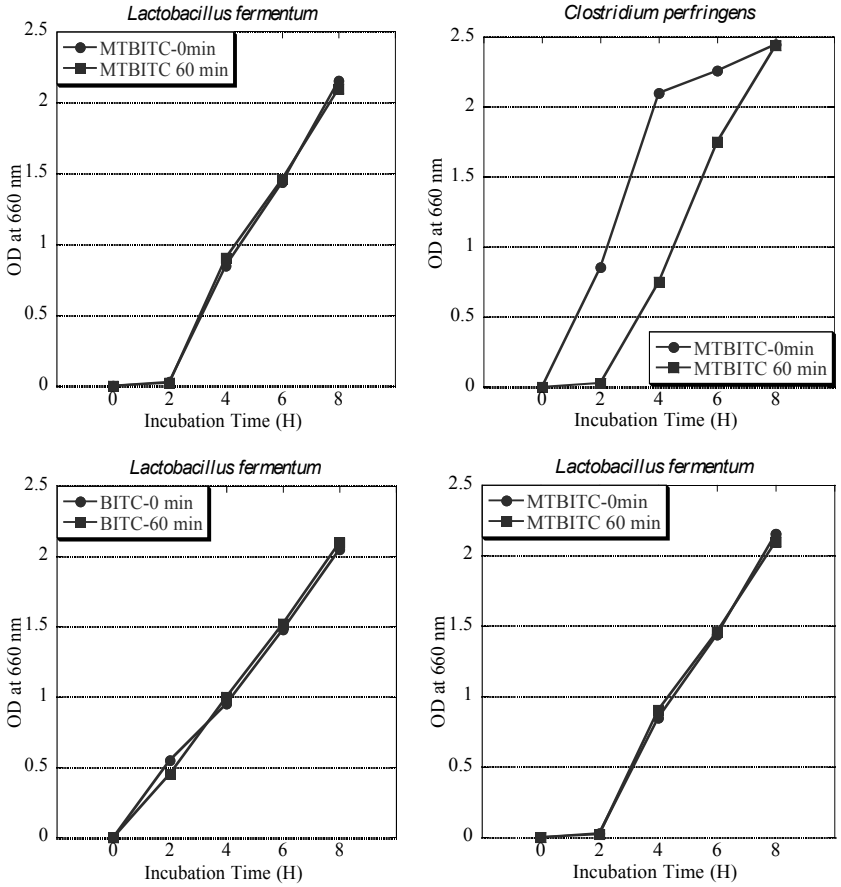

Fig. 2 Effect of shorter-duration treatment with 60 ppm BITC or MTBITC on the growth of 2 bacterial strains, *Clostridium perfringens* JCM 3816 and *Lactobacillus fermentum* JCM 1137.

3.2. In vivo *effects of BITC on the formation of short chain fatty acids in the rat intestine* We examined the effect of BITC on the *in vivo* formation of short chain fatty acids in the intestines of rats administered BITC at a dose of 0.4 mg/day (Group A) or 0.8 mg/day (Group B) for 3 consecutive days. On the third day, the intestinal contents of each rat were collected, and the fatty acids therein were extracted and analysed by GC and GC-MS. The typical GC profile is shown in Fig.3, indicating that the intestinal contents had 7 types of short-chain fatty acids, namely acetic, propionic, butyric, isobutyric, isopentanoic, pentanoic, and hexanoic acids, of which butyric acid was the most abundant.

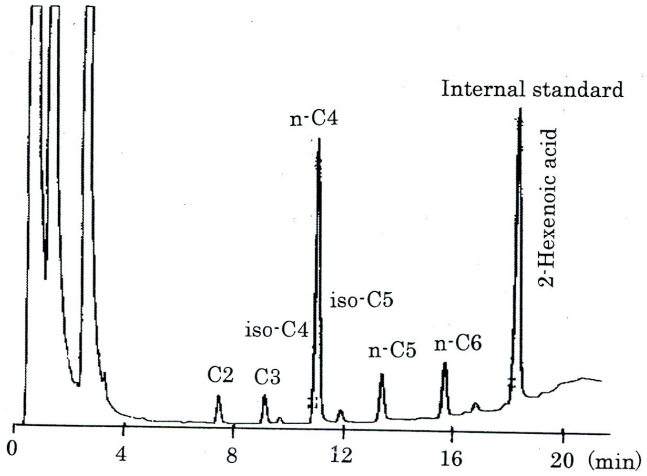

Fig. 3 GC profile of fatty acids extracted from the intestines of rats dosed with BITC at 0.8 mg/day for 3 consecutive days. C2, acetic acid; C3, propionic acid, iso-C4, isobutyric acid; n-C4, butyric acid; iso-C5, isopentanoic acid; n-C5, pentanoic acid; n-C6, hexanoic acid.

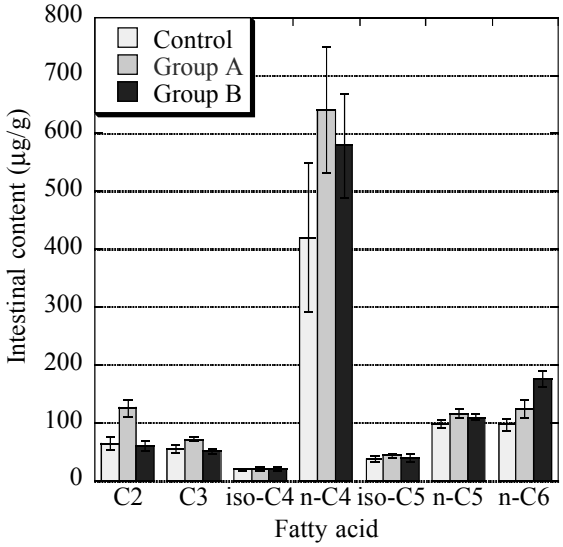

Fig. 4 Fatty acid composition of the intestinal contents of rats treated thrice on alternate days with BITC at 0.4 mg/day (Group A) or 0.8 mg/day (Group B). Control rats were treated with corn oil instead of BITC.

As shown in Fig. 4, the effect of BITC on the *in vivo* formation of such short chain fatty acids as propionic, isobutyric, isopentanoic, and pentanoic acids was minor. However, the intestinal formation of acetic, n-butyric, and hexanoic (caproic) acids increased after the administration of BITC at a dose of 0.4 mg/day, suggesting that BITC alters the microflora in the rat intestine. Presuming that the average volume of bowel contents of a rat may be *ca*. 10 mL, dose amounts such as 0.4 mg and 0.8 mg should be equivalent to 40 and 80 ppm, respectively. When BITC was administered to rats at a dose of 0.8 mg/day, the rats developed diarrhoea. We think that the diarrhoea might be responsible for a decrease in the intestinal formation of fatty acids such as acetic and n-butyric acids.

4. Conclusion

The results of our study suggest that the lactic acid-producing bacterial strains such as those of *Lactobacillus* spp. and *Bifidobacterium* spp. in the human intestine have a low susceptibility to isothiocyanates, whereas some strains of *Clostridium* spp., *Anaerococcus* sp., and *Fusobacterium* sp. are highly susceptible to isothiocyanates. The increased levels of certain short-chain fatty acids seen in the rat bowel also suggest that the isothiocyanates alter the intestinal microflora.

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Farnesol antimicrobial role as biofilm cell detachment inducer in *S. epidermidis* biofilms

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Keywords biofilms; antimicrobial resistant; cell detachment; gene expression;

1. General remarks

Staphylococcus epidermidis is a Gram-positive bacterium that normally colonizes the human skin and mucous membranes. Previously regarded as an innocuous commensal microorganism it is now seen as an important opportunistic pathogen [1]. In the past few decades*, S. epidermidis* became the most frequent causative agent of nosocomial infections [2]. This mainly resulted from the increasing use of medical devices, such as peripheral and central intravenous catheters, prosthetic joins, vascular graft or cardiac devices [3], that provides a source of colonization and biofilm formation [4]. Microbial biofilms are communities of bacteria that live adhered to a surface and are surrounded by an extracellular polymeric matrix [5]. In this environment, bacteria have demonstrated an increased antibiotic resistance [6] and tolerance to the immune system [7]. The increase in the tolerance to conventional antibiotic therapy has lead to the search for new antimicrobial therapeutic agents such as bacteriophages [8] or essential oils from plants [9]. Farnesol is a naturally-occurring sesquiterpene that was originally isolated from essential oils found in many plants [10]. We recently described that while farnesol was not efficient at killing biofilm bacteria, a strong reduction on biofilm biomass was detected, and we hypothesize that farnesol could be inducing biofilm detachment [11]. Here we describe the experiments that tested this hypothesis.

2. Material and Methods

2.1 Bacterial strains

S. epidermidis 9142 and 1457 were used as positive biofilm forming controls. Additionally, 25 clinical strains previously characterized by several different molecular typing techniques, namely staphylococcal chromosome cassette *mec* (SCC*mec*) and multilocus sequence typing [12], were selected from a total of 217 nosocomial isolates collected between 1996 and 2001 in 17 different countries from disease (107 isolates) and from carriage (87 isolates). Isolates were selected to include the highest diversity as possible in terms of genetic backgrounds, geographic and clinical origins. All strains are listed in the results section.

2.2 Biofilm formation

Biofilms were grown in Tryptic Soy Broth supplemented with 1% (w/v) of glucose (TSBG), and incubated at 37 °C in a shaker at 120 rpm in 96 well culture plates for 24 h. Biofilms were then washed twice with 0.9% NaCl and fresh TSBG was added with or without 300 μM of farnesol and allowed to incubate in the same conditions for further 24 h. For biofilm biomass determination, the standard crystal violet staining method was used [13]. To determine cell viability, biofilms were washed twice with 0.9% NaCl and resuspended in 0.9% NaCl followed by gentle sonication. CFUs were determined by the standard plating method, using Tryptic Soy Agar plates. Finally, to determine the percentage of growth of bacteria inside the biofilm after 48 h, we quantified the relative population density of biofilms and the respective suspension formed during the last 24 h of growth, on each 96 well, as described before [14].

2.3 Molecular and phenotypic characterization of the clinical strains

All strains were characterized by multilocus sequence typing (MLST) following the scheme proposed by Thomas *et al.* [15]. The MLST data were analysed using the goeBURST algorithm. Isolates were considered as belonging to the same clonal complex (CC) if sharing six out of seven loci. The SCC*mec* type was determined by the combination of the class of *mec* complex and the type of *ccr* complex as previously suggested [16]. SCC*mec* was considered non-typable when either *mec* complex or *ccr* complex, or both, were non-typable by the methods used or when the isolate carried more than one *ccr* type. SCC*mec* was considered to be new if a new combination of *mec* complex and *ccr* complex was found. The presence of the genes associated to biofilm formation, namely *icaA*, *aap* and *bhp* was detected by PCR, using DyNAzyme II PCR mix (Finnzymes, Vantaa, Finland), in the following thermal conditions: initial denaturation at 94ºC for 5 min followed by 35 cycles of 94ºC at 30 s, 54ºC at 30 s and 72ºC at 45 s. A final extension step was performed for 10 min at 72 ºC. Negative results were re-checked with a second set of primers. Oligonucleotide primers were designed using the Primer3 software, having *S. epidermidis* RP62A genome as template. The primers used were the following: 16S fw gggctacacacgtgctacaa, 16S rev gtacaagacccgggaacgta, *icaA* fw tgcactcaatgagggaatca, *icaA* rev taactgcgcctaattttggatt, *aap* fw gcaccagctgttgttgtacc, aap rev gcatgcctgctgatagttca, bhp fw tggactcgtagcttcgtcct, *bhp* rev tctgcagatacccagacaacc, *RNAIII* fw ggagagactcacggcttgataa, *RNAIII* rev gcatgtaagctatcgtaaacaaca, *rsbU* fw taacgtgttttgggactcacac, *rsbU* rev tgttgaaaagaacgttaaccaaa, *agrB* fw tacgcacctgcagcaactaa, *agrB* rev agggaaaaagatgggtagtaatg. All strains were tested for biofilm formation, during 24 h, following the above described procedures and biofilm biomass quantified using the standard crystal violet staining method [13].

2.4 Gene expression quantification

Total RNA from the biofilms was isolated as described before [17]. The final RNA fraction was obtained by suspending the pellet in 45 µL nuclease-free water (Fermentas, Burlington, Canada). To digest contaminating DNA, DNase (Fermentas) treatment was performed. RNA yield and purity (A_{260}/A_{280} and A_{260}/A_{230}) were verified spectrophotometrically by NanoDrop 1000™ (Thermo Scientific, MA, USA). RNA integrity was assessed by loading the samples in a 1 % agarose gel run at 80 V for 60 min and stained with ethidium bromide. cDNA of each sample was synthesized by the iScript™cDNA Synthesis Kit (Bio-Rad, CA, US) using of 1 µg of total RNA as template and following the manufacturer instructions. qPCR reactions were performed on a CFX96™ Real-Time PCR Detection System (Bio-Rad). Thermal cycling conditions were as follows: 3 min initial denaturation at 95ºC, followed by 40 cycles of 5 s denaturation at 95ºC, 10 s annealing/extension at 60ºC. Melting curves were performed at the end of each run, with readings from 65ºC to 95ºC every 1ºC for 5 s, in order to confirm that only the desired product was amplified. Data were analysed using the CFX Manager™ version 1.6 (Bio-Rad) and the relative quantification method $2^{\Delta Ct}$ [18], using 16S ribosomal RNA as the internal control. The cDNA was validated by comparing with a RNA sample not submitted to the reverse transcriptase reaction (no-RT).

2.5 Statistical analysis

All the assays were compared using one-way analysis of variance (ANOVA) by applying Levene's test of homogeneity of variances and Tukey multiple comparisons test, and also the paired sample t-test, using SPSS. All tests were performed with a confidence level of 95%.

3. Results and discussion

3.1 Effect of farnesol on established biofilms

To better characterize the effect of farnesol on biofilm physiology, we characterized three different parameters on the biofilm formation ability of *S. epidermidis* 9142 exposed to farnesol: biofilms were quantified regarding biomass accumulation, viable cell concentration and also by the percentage of bacteria that grew in the biofilm mode.

Table 1 Characterization of mature biofilms formed in the presence or absence of farnesol.

	S. epidermidis 9142		*S. epidermidis* 1457	
Parameter	Control	Farnesol	Control	Farnesol
Total biomass	1,17 ± 0,07	0,95 ± 0,09*	0,95 ± 0,09	0,95 ± 0,12
CFU/mL	3,70 ± 2,12E9	3,38 ± 1,88E9	3,00 ± 1,03E9	3,50 ± 0,71E9
% cells in biofilms	83,2 ± 2,6	69,9 ± 5,9*	83,2 ± 4,9	81,1 ± 5,2

* significant different as compared to control.

This last method was originally described by Harwich *et al.* [19] to analyse the relationship between bacteria growing inside the biofilm and bacteria living in suspension, in the supernatant of the biofilm ecosystem. As can be seen in Table 1, only *S. epidermidis* 9142 was affected by farnesol. Furthermore, while there was a significant reduction in the biofilm biomass (paired samples t-test, $p<0.05$), the total number of viable cells was not affected. Interestingly, in the presence of farnesol, there was a significant (paired-samples t-test, $p<0.05$) increase of 44.1% in the number of cells living outside the *S. epidermidis* 9142 biofilm, similar to our first report on this topic [11]. This data suggested that while farnesol was reducing the biofilm biomass, a higher number of total cells were detected in the supernatant of the biofilms as the result of biofilm detachment. However, this was only true in one of the tested strains.

3.2 Farnesol induced detachment in clinical isolates

Table 2 Phenotypic and molecular characterization of the *S. epidermidis* strains used, and farnesol-mediated cell release.

Strain	SCC*mec* typing	ST	CC	Biofilm genes	Biofilm formation	Farnesol induced cell release
1457	MSSE	86	2	*ica, aap, bhp*	+++	-
9142	II	10	2	*ica, aap, bhp*	+++	+
RP62A	II	10	2	*ica, aap, bhp*	++	-
COB17	33	33	33	*n/d*	-	n/a
CV45	IV	79	2	*ica, bhp*	-	n/a
DEN110	IV	68	66	*aap*	-	n/a
DEN116	I	42	2	*n/d*	-	n/a
DEN120	A/C	40	2	*n/d*	-	n/a
DEN185	IV	21	2	*aap, bhp*	++	+
DEN19	IV	1	2	*aap*	-	n/a
DEN69	V	56	S56	*ica*	+++	+
DEN94	IV	49	247	*n/d*	-	n/a
FJ6	MSSE	10	2	*ica, aap bhp*	+	-
GRE26	IV	11	11	*n/d*	-	n/a
HUR51	B/3	47	33	*n/d*	-	n/a
ICE102	IV	52	2	*aap, bhp*	++	-
ICE192	IV	5	2	*aap, bhp*	-	n/a
ICE20	IV	89	2	*ica, aap*	++	-
ICE21	I	36	2	*ica, aap*	++	+
ICE24	IV	38	2	*aap*	-	n/a
ICE5	IV	23	2	*ica, bhp*	++	+
ICE9	III	6	2	*ica, bhp*	+++	-
IE186	IV	367*	S367	*ica, aap*	+++	-
IE214	NT	10*	2	*ica, aap*	++	+
IE75	IV	1	2	*ica, bhp*	+	+
ITL34	IV	66	66	*n/d*	-	n/a
JI6	III	366*	2	*ica, aap, bhp*	+++	+
LE7	III	9	2	*ica, aap*	+	-
M129	III	366	2	*ica, aap*	++	-
M187	IV	367	S367	*ica, bhp*	++	-
MCO150	IV	46	2	*aap, bhp*	-	n/a

MEX37	II	71	11	*n/d*	-	n/a
MEX60	NT/2	61	2	*ica*	++	+
PE9	II	10*	2	*ica, aap bhp*	+++	-
PLN64	NT/2	64	247	*n/d*	-	n/a
TAW113	MS	85	2	*aap*	++	+
URU23	IV	86	2	*ica, aap*	+++	-

*inferred due to similarity of PFGE macrorestriction pattern. n/a – non applied; n/d – none detected) (-/+/++/+++ for biofilm formation qualitative scale comparison)

In order to understand if the different responses to farnesol were associated with different strain genetic backgrounds, we decided to further test this phenomenon in a collection of well characterized nosocomial *S. epidermidis* isolates, the most diverse as possible in terms of genetic backgrounds, geographic and clinical origins. All strains were screened by MLST, SSCmec, the presence of biofilm formation associated genes (*icaA*, *aap*, *bhp*) and the ability to form biofilms (Table 2). Of the 27 strains used in this study, 12 were biofilm positive (44.4%) and *icaA* and aap were detected in 11 strains (30.7% incidence), and *bhp* in 9 strains (33.3 % incidence). All the strongest biofilm-forming strains presented at least the *icaA* gene. A similar result was described by Rodhe *et al.* [20]. Farnesol-induced cell detachment was detected in 7 of the 12 biofilm-forming strains (58.3% incidence). However, no correlation was found between cell detachment and biofilm formation ability or molecular typing. The lack of correlation between the effect of farnesol and the genetic typing suggests that other phenomena, independent of the genetic background, such as the overall metabolism, or the type of biofilm formed may influence the outcome of farnesol effect in several clinical *S. epidermidis* isolates.

3.3 Farnesol induced detachment is influenced by molecular mechanisms

Despite no correlation found between the effect of farnesol and genetic typing of the tested strains, previous reports related to biofilm detachment have shown to have a correlation to specific genes expression. In a CLSM experiment by Wang *et al.*, addressing the regulated detachment of *S. epidermidis* biofilms, it was demonstrated that a localized induced over-expression of phenol-soluble modulins (psm) was followed by a void space in that specific region of the biofilm [21]. In a previous report we showed that *S. epidermidis* biofilms exposed to several antibiotics over-expressed biofilm associated genes, namely *icaA* and *rsbU*. In the current study we addressed the effect of farnesol in both *S. epidermidis* 9142 and 1457 gene expression. We selected genes involved in biofilm formation, such as *icaA*, *aap* and *bhp*, biofilm regulation, such as *rsbU* and *agrB*, and biofilm detachment, such as *RNAIII, in which the* phenol soluble moduline gamma psmγ) is embedded. As can be seen in Table 3, there was some strain variability, comparable with the phenotypic results. Interestingly, our results show that farnesol strongly affects *rsbU* expression, either by increased expression in strain 9142 or decreased expression in strain 1457. It has also been described that in the absence of the accessory gene regulator quorum sensing system (*agrB*), biofilm formation is enhanced [22]. Farnesol also interfered with *agrB* expression in all the tested strains but it only induced *aap*, *bap* and *icaA* expression in strain 9142. In concordance with our phenotypic experiments, *RNAIII* was up regulated in the strain 9142, providing evidence that biofilm detachment had a genetic basis. However, the exact mechanism of how farnesol induces *RNAIII* expression was not determined.

Table 3 Gene expression of biofilms exposed to farnesol expressed as fold difference related to the control.

Target gene	9142	1457
icaA	2,61 ± 0,09*	0,35 ± 0,02*
aap	2,95 ± 0,63*	0,37 ± 0,02*
bhp	2,87 ± 0,24*	0,72 ± 0,04*
rsbU	15,43 ± 0,83*	0,23 ± 0,03*
agrB	0,57 ± 0,02*	0,68 ± 0,05*
RNAIII	1,93 ± 0,18*	0,57 ± 0,02*

* significant different as compared to control.

4. Conclusions

We previously reported that farnesol had the potential to be used as an adjuvant in antimicrobial chemotherapy [23]. Taking in consideration the results shown here, the role of farnesol as an adjuvant can now be better explained. We hypothesized that despite the inability of farnesol to kill the bacteria inside biofilms, by inducing cell detachment from biofilms, via a yet unknown mechanism, the suspended bacteria will be potentially more prone to antibiotic attack, taking into consideration that no diffusion barrier would be present in such cases. While we did not address this issue, it would be interesting to determine the role of farnesol over longer periods of time, in order to access its effect on mature biofilms. Furthermore, to better understand the molecular mechanisms underlying this effect, it would be important to test different concentrations of farnesol, including sub-MIC concentrations.

Acknowledgements The following authors were supported by individual fellowships from Fundação para a Ciência e Tecnologia (FCT), Portugal: FG: SFRH/BD/32126/2006, AF: SFRH/BD/62359/2009, JCB: SFRH/BD/66250/2009, JR: SFRH/BD/72675/2010 and PT: SFRH/BPD/ 26803/2006. The work performed at ITQB was supported with grant #Pest-OE/EQB/LAO004/2011 from FCT and CONCORD–HEALTH-F3-2008-222718 from the European Commission.

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Garlic antimicrobial susceptibility in *Enterococcus sp* strains

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1. Introduction:

Folk medicine is characterized by the use of medicinal herbs based on popular knowledge that are usually transmitted orally. It's hard to find someone who has never heard about the calming effects of chamomile tea or lemon balm, or even about the stimulating effects of guarana[1].

The use of folk medicine in some foods is also very common. These are classified as functional foods because they contain substances with biological activity, which may be involved in disease prevention and health promotion, beyond their basic nutritional aspects. For instance, among others, tomatoes, soybeans, peas, flax, grape, onion, cold-water fish with high fat, ginger, and garlic, which will be part of this study[2].

Garlic (*Allium sativum*) is an edible bulb, used in different culinary cultures, and has been used as a medicine since before Christ's birth [3]. Scientific studies have identified the presence of therapeutic substances for the treatment of parasitic infections, gastrointestinal discomfort, dyslipidemia, intestinal worms, hypertension and cancer, in addition it has antimicrobial activity mainly by action of a component called allicin. In 1944, Cavalitto and Bailey tested the bactericidal action of allicin, with positive effects on growth inhibition of several bacteria, both Gram-positive and Gram-negative [4]. Studies have shown that garlic has an excellent antibacterial activity against *Escherichia coli*, *Salmonella sp* and *Aeromonas hydrophila* [3].

Enterococci are Gram-positive, catalase negative, typically arranged in pairs and short chains. The species most clinically important and frequently isolated are *Enterococcus faecalis* and *Enterococcus faecium*. They are part of the microbiota, which can be found in the intestines and genitourinary tract[5,6,7].

Enterococci are commensal organisms that do not have a potent toxin or other virulence factors well defined, but they are naturally resistant to many antibiotics. So mainly cause diseases in hospitalized patients or those treated with broad-spectrum antibiotic. The majority of human infections by this organism originates from the intestinal microflora of the patient, although organisms may also be transferred from patient to patient or acquired through consumption of contaminated food or water. They are a major cause of nosocomial infections and a serious consequence of enterococcal bacteremia is endocarditis, a disease with high mortality rate[5,6,7].

2. Objetive:

- Evaluate the utilization of natural products used in popular medicine and prove this effects scientifically.
- Evaluate the anti-microbial effects of *Allium sativum* in *Enterococcus sp* strains.

3. Methodology:

This study had been conducted in the microbiology discipline of pathology medical science of Santa Casa of São Paulo Medical School during the 2011 year.

Garlic samples were obtained from the local market and its principles were made by grinding and obtaining a concentrated product, which was impregnated into blank discs that were placed on Muller-Hinton ágar plates previously seeded with strains of enterococci. We compared the antimicrobial effect of garlic with antibiotics such as vancomycin, gentamicin and ciprofloxacin, through treated disk in a total of 167 strains of *Enterococcus sp*.

The concentrated, 15µL of product was impregnated in filter paper discs with an approximated diameter of 7mm and arranged in Agar Muller–Hinton plates previously seeded with 1,5 x 10^8 UFC/mL of *Enterococcus sp*. serotypes. Antibiotics of various groups were placed in discs, like vancomicyn (role in the cell wall), gentamicin (role in the protein synthesis) and ciprofloxacin (role in the bacteria genetic material) to evaluate and compare the results obtained. All samples were seeded twice.

The *Enterococcus sp* kept, originating from the laboratory bacterial, were tested using the Kirby-Bauer technique following the standards of releasing of CLSI current effective[6,8,9]. The viability of strains, kept in glycerol, were evaluated through the previously seeding in TSB, being only seeded if there has been turbidity in the environment in which it is grown, in other the log growth phase. In the plates containing *Enterococcus* sp we put disc of ciprofloxacin, gentamicin, vancomicyn and garlic and incubated during 24 hours at 37±2°C and then were performed the diameter read of inhibition to compare the results obtained.

We used 2 strain of *Enterococcus sp* ATCC to compare and reported the results. It was considered resistant to garlic the ones that did not present the evidence of inhibition zone throughout the read, and sensible the ones that presented the evidence of inhibition zone regardless of its size while the results in respect to vancomicyn, gentamicin and ciproflozaxin were analyzed against the Clinical and Laboratory Standards Institute *(CLSI)* standards.

It was performed a chi-squared test to compare the sensibility of *Enterococcus* sp. serotypes against garlic in relation to vancomicyn, gentamicin and ciprofloxacin, individually, significance level adopted was $p < 0.05$.

4. Resultd and Discussion:

The inhibition zone diameter of the disc impregnated with garlic ranged from 10mm to 30mm for the sensible *Enterococcus* sp ATCC. The tests were performed twice to confirm the results, and did not present different results in both tests. Regarding the 26 serotypes resistant to the 3 antibiotics tested and also sensible to garlic, the inhibition zone diameter ranged from 12mm to 23mm.

The total of 167 strains of *Enterococcus sp*, 81 (48,50%) strains were found resistant to vancomycin, 37 (22,15%) resistant to gentamicin and 98 (58,68%) resistant to ciprofloxacin and only 11 (6,58%) resistant to garlic. In addition, 26 (15,57%) of the strains were VRE (entecorocci resistant to vancomycin), but sensitive to garlic, while only 03 (1,80%) strains were resistant to both antibiotics as garlic. We conducted the chi-square test to compare the susceptibility of strains of enterococci garlic compared to vancomycin, gentamicin, and ciprofloxacin, alone. The comparing of the susceptibility of strains garlic in sensitivity to vancomycin obtained $p=1.0 \times 10^{-17}$ in relation to gentamicin obtained $p=2.97 \times 10^{-5}$, and compared to ciprofloxacin, $p=3.22 \times 10^{-24}$. Thus, in these three comparisons "p" proved be much less than 0.05, and may be concluded that the sensitivity of strains of enterococci "in vitro" of garlic is significantly higher compared to antibiotics tested in this study, table1 and table2.

The susceptibility analysis demonstrated that 93,4% of the serotypes were sensible to garlic, 77,8% were sensible to gentamicin while only 51,5% were sensible to vancomicyn. According to this information, the garlic presented an important therapeutic alternative to be studied for treatment in patients with infection caused by *Enterococcus* sp, since the use of vancomicyn, in this analysis, demonstrated inferior to garlic and also this can prevent the inadequate use of antibiotics and the induction of multi- resistant serotypes.

Table 1: Profile for susceptibility of 167 *Enterococcus sp* strains and their percentages compared to the antibiotics: vancomycin (bacterial action on the wall), gentamicin (acting in protein synthesis) and ciprofloxacin (acting on bacterial genetic material)

	Resistant	**%**	**Sensible**	**%**	Total	**%**
Vancomicyn	81	48,5	86	51,5	167	100
Gentamicin	37	21,2	130	77,8	167	100
Ciprofloxacin	98	58,7	69	41,3	167	100
Garlic	11	6,6	156	93,4	167	100

Source: Microbiology Laboratory FCMSCSP, 2011.

Table 2: Comparison of the susceptibility of garlic in relation to antibiotics vancomycin. gentamicin and ciprofloxacin, to *Enterococcus sp* strains, through the Qui square statistic test, significance level adopted was p <0.05.

Garlic susceptibility	X^2, $p \leq 0,05$
Vancomicyn	$p = 1,00\ 10^{-17}$
Gentamicin	$p = 2,97\ 10^{-5}$
Ciprofloxacin	$p = 3,22\ 10^{-24}$

Source: Microbiology Laboratory FCMSCSP, 2011.

5. Conclusion:

We observed that the popular say about the garlic anti-microbial activity was confirmed in our tests (see table 1 and table 2).

The susceptibility of the garlic profile against *Enterococcus* sp. serotypes (156 sensible serotypes, 93.4%).

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SUPPORTED BY
PIBIC/CNPq – Scientific Initiation of National Counsel of Technological and Scientific Development

Immunomodulating properties of the antimicrobial preparation composed of incapsulated rifampicin and interferon inducer

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The immunomodulating activity of composition preparation containing rifampicin encapsulated in polysaccharide capsules with double-stranded RNA was studied in experiments in vivo and in vitro. It was shown that antibiotic accumulation in mouse peritoneal exudate cells significantly increased after administration of the composition rifampicin-dsRNA preparation compared to rifampicin. The evident increase of IFN-α in blood and enhancement of the oxidation-reduction activity of phagocytizing peritoneal macrophages was observed in composition-administered group. Both rifampicin and the composition of rifampicin and dsRNA possessed the ability to increase the metabolic activity of macrophages *in vitro,* though the stimulating effect of the composition appeared at lower doses as compared to rifampicin and was more pronounced. The data obtained suggest that the studies of the encapsulated rifampicin-dsRNA preparation as antibacterial agent are perspective.

Keywords incapsulated rifampicin; double-stranded RNA; macrophage accumulation; oxidation-reduction activity; interferon-α; peritoneal macrophages; mice

1. Background

Elaboration of the methods of tuberculosis therapy continues to be one of the urgent problems of modern medicine. The low efficacy of treatment of the disease is conditioned by various factors, including toxicity of many anti-tuberculosis drugs, the rapid spread of multidrug-resistant forms of tuberculosis (TB) that poorly responds to therapy and proceeds with evident depression of the immune system. As the literature data suggest, therapeutic properties of anti-TB drugs such as specificity and duration of pharmacological action, severity of side effects may be improved by incorporating preparations into the nano- or microparticles formed by various polymers [1-4]. Targeted delivery of anti-TB drugs to the sites of *M. tuberculosis* infection is able to increase the concentration of drugs in the target organ and decrease their content in the systemic circulation, which can result in ten-fold increase of the drug efficacy and side effect reduction of encapsulated antibiotics in comparison to their free forms.

It is known that a key component of the host's response to M. tuberculosis infection is the complex of reactions of T-cell immunity and mononuclear phagocyte system. By present the evidence has been accumulated about the necessity to use drugs for TB treatment that induce macrophage activation [5,6]. A number of studies demonstrated the efficacy of interferon and its inducers in the treatment of tuberculosis [7,8]. It is shown that interferon inducers based on double-stranded RNA (dsRNA) have a wide range of immunomodulating activity, increase the efficacy of phagocytosis and destruction of mycobacteria by macrophages [9]. These data suggest the possibility to enhance the therapeutic properties of anti-TB drugs by incorporating them in micro-and nanocapsules and combining with dsRNA. Therefore, the objective of this study was to evaluate the biological activity of the drug composed of encapsulated anti-TB antibiotic rifampicin (Rif) and dsRNA.

2. Materials and Methods

In experiments we used composition preparations of rifampicin encapsulated in alginate-chitosan capsules (Rif(encap)), without and with dsRNA (Rif(encap) + dsRNA), produced by method developed in the IMBT. The reference substances were rifampicin (medicinal drug, "Ferane", Russia) and dsRNA from the yeast *Saccharomyces cerevisiae* (substance of ridostin, medicinal drug, containing dsRNA, 16.8% of total, "Diafarm", Russia).

Oxygen-dependent bactericidal activity of macrophages in vitro was evaluated by spectrophotometric HCT-test [10]. The preparations in concentrations of 1, 50, 100 and 200 μg / ml (by rifampicin) were added to the monolayer of cells of peritoneal exudate, followed by culturing for an hour.

In vivo experiments were carried out in 100 white outbread male mice ICR, weighing 22-25 g. The animals were divided into 8 experimental groups of 10 mice in each. The mice of experimental groups (groups 1-4) were treated with the preparations of Rif (encap) with and without dsRNA, in a dose of 5 or 50 mg / kg (rifampicin) and 5 mg / kg (dsRNA). The mice of reference groups were administered with rifampicin in a dose of 5 or 50 mg / kg (groups 5 and 6) and dsRNA in a dose of 5 mg / kg (group 7). A single dose of all preparations was orally administered at a volume of 0.5 ml per mouse. The mice injected with an equivalent volume of saline served as controls (group 8). We determined the level of macrophage functional activity and IFN-α concentration in blood 3 and 24 h after administration to evaluate the immunomodulating activity of Rif preparations. Macrophage activity was assessed by HCT-test [10], IFN-α concentration in mice blood serum was measured by Mouse IFN Alpha ELISA Kit (PBL Biomedical Laboratories).

To evaluate the accumulation of Rif in peritoneal macrophages the experimental group of mice was treated with Rif (encap), containing dsRNA, in a dose of 50 mg / kg (rifampicin) and 5 mg / kg (dsRNA), the reference group was administered with rifampicin in a dose of 50 mg / kg. Accumulation of Rif was estimated in peritoneal exudate which was obtained 1 and 3 hours after administration of the preparations. Exudate cells, after separation of supernatant by centrifugation, were lysed with distilled water and exposed to a freeze / thaw followed by centrifugation. Rif content in the supernatant and cell lysate was estimated by spectrophotometry at a wavelength of 475 nm.

Statistical processing of the data of all the experiments was performed with the use of the statistical software package “Statgraphics, Version 5.0” (Statistical Graphics Corp., USA). The significance of differences was evaluated by the Student t-criterion.

3. Results

The study has demonstrated that the composition of encapsulated rifampicin and dsRNA evoked more evident increase of functional activity of peritoneal macrophages compared to free rifampicin. An increase in HBT-reduction level 24 h after administration of the composition of encapsulated rifampicin (5 mg/kg) and dsRNA (5 mg/kg) was 39% higher than that in the reference group (free rifampicin, 5 mg/kg) and 24% higher than that in the reference group (encapsulated rifampicin, 5 mg/kg).

Similar results were obtained when we compared stimulating effect of free, encapsulated rifampicin and the composition of encapsulated rifampicin and dsRNA on peritoneal exudate cells *in vitro*. It was shown that the preparations of either free rifampicin or encapsulated rifampicin possessed the ability to increase the metabolic activity of macrophages. However, the stimulating effect of the composition containing dsRNA appeared at lower doses as compared with the preparations of rifampicin and encapsulated rifampicin, and the level of stimulating at equivalent doses increased by 38 and 40% respectively (Fig.1).

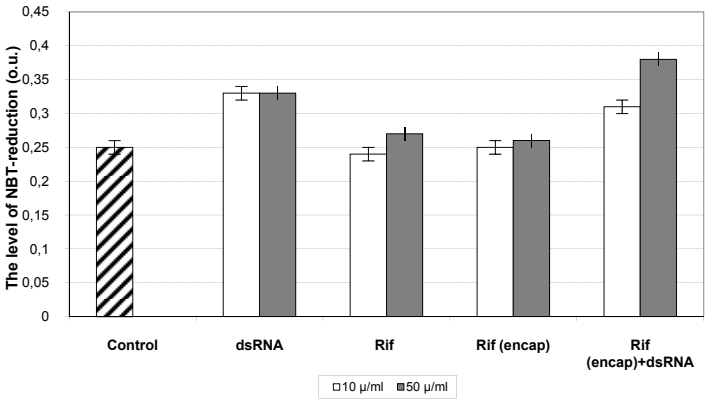

Fig. 1 The effect of rifampicin and dsRNA preparations in concentrations of 10 and 50 μg/ml on the functional activity of mouse peritoneal macrophages after 1 h of cultivation. *- The differences are significant as compared to the parameters in the wells with intact macrophages, $p<0,05$.

The quantitative analysis of IFN-α in mice blood testified that neither free rifampicin nor the preparation of dsRNA significantly changed the level of IFN-α in serum. Rifampicin incorporated in alginate-chitosan capsules did not increase or decrease IFN-α concentrations. On the contrary, the composition of encapsulated rifampicin and dsRNA caused a significant increase of IFN-α. The level of IFN-α in serum of mice after administering Rif(encap)+dsRNA in doses of 5 mg / kg and 50 mg / kg exceeded the control level by 67 and 43% (Fig.2).

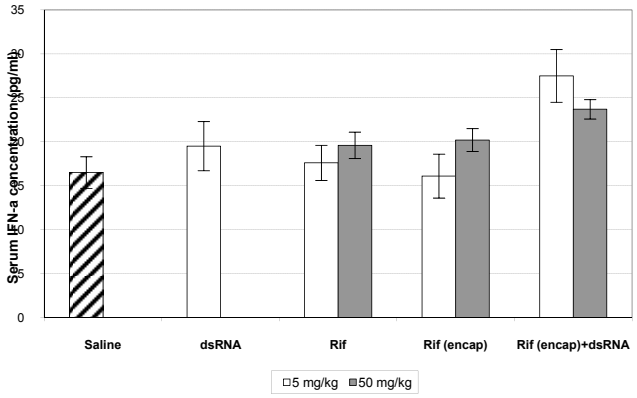

Fig. 2 The effect of rifampicin and dsRNA preparations (5 or 50 mg/kg, oral administration) on the IFN-α content in mouse blood 3 h after administration. *- The differences are significant as compared to the parameters of control group, $p<0,05$.

One of the possible reasons for a more significant stimulation of the oxidation-reduction activity of macrophages and their ability to synthesize IFN-α under the effect of encapsulated rifampicin with dsRNA may be an intense accumulation of the active components in peritoneal cells after administration of the composition preparation.

Our study in mice showed that the accumulation of rifampicin in the supernatant of mice peritoneal exudates one hour after administration of Rif(encap) + dsRNA was 3-fold lower than its rate after the administration of free rifampicin (Table). Three hours after administration the difference in rates between the groups disappeared. At the same time, the composition rifampicin preparation excelled by its ability to be accumulated by phagocytes more intensively as compared with the free antibiotic. The concentration of rifampicin in macrophages of Rif(encap) + dsRNA-administered mice after 3 hours was 12.9 times higher than that observed in the control group (Table).

Table Rifampicin accumulation in the supernatant and peritoneal exudate cells after single oral administration of the preparations to mice

Preparation	Dose (mg/kg)	Concentration of rifampicin (ng/ml)			
		Supernatant		PE cell lysate	
		1 h	3 h	1 h	3 h
Rif	50	13.6±1.4	23.6±2.3	8.0±4.2	8.3±2.1
Rif(encap) +dsRNA	50 5	5.4±2.4*	24.0±7.0	7.0±1.1	107.8±10.6*

Note. * - the differences are significant, as compared to Rif-administered mice, $p\leq0,05$

Intensive accumulation of rifampicin in peritoneal cells, from our point of view, may be associated both with more pronounced ability of macrophages to phagocytize corpuscular objects, which is encapsulated rifampicin [11,12], and the ability of dsRNA, the drug component, to increase phagocytic activity of macrophages [13].

According to the data obtained we have concluded that the preparation of encapsulated rifampicin containing in its composition dsRNA, excels by its ability to accumulate in macrophages more intensively and stimulate the oxygen-dependent bactericidal activity of phagocytic cells compared with the free rifampicin, which is important in terms of strengthening their antibacterial activity. Moreover, incorporating dsRNA into the composition ensures its ability to induce endogenous interferon which is significant for modulating immunosuppressive effects of the antibiotic observed with the free rifampicin.

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In Vitro antimicrobial properties of coriander (*Coriandrum sativum*) and parsley (*Petroselinum crispum*) essential oils encapsulated in β-cyclodextrin.

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The aim of this study was determine the antimicrobial activity against several bacteria and yeasts of coriander (*Coriander sativum*) and parsley (*Petroselinum crispum*) essential oils (EOs) encapsulated in β-cyclodextrin. The encapsulated EOs were individually tested against several bacteria and yeast using colorimetric broth microdilution method. All species were supplied by the Spanish Type Culture Collection (CECT) of the University of Valencia. Both coriander and parsley encapsulated EO had a very similar antibacterial activity against all the tested strains, with MIC values ranged between 10 and 20 mg/mL. *Y. lipolytica* showed lower MIC values (5 mg/mL) for both encapsulated EOs than *S. cerevisiae* and *C. Zeylanoides* (10 mg/mL).This study showed that both coriander and parsley encapsulates EOs might be a good alternative, as natural antimicrobial agents, to the actual synthetic compounds used in Food industry. However more studies must be done in order to have a better evaluation on the antimicrobial activity.

Keywords: essential oils, encapsulated, antibacterial

1. Introduction

Food and food products can suffer degradation, which is essentially due to the lipid oxidation and the growth of spoilage microorganisms and food-borne pathogens[1]. In the food industry, a range of different chemical and synthetic compounds have been used as antibacterial and antifungal agents to inhibit microbial food spoilage. Antimicrobial substances used include chemical substances (added or already present naturally in foods), which are used in the food industry for two main reasons: (i) to control natural spoilage processes (food preservation), and (ii) to prevent/control growth of micro-organisms, including pathogenic microorganisms (food safety)[2].

At present, the consumer demands for less use of synthetic food preservatives have increased throughout the world. So, the substitution of traditional food preservations and synthetic one by natural plants materials have caused great interest in nutrition research[3]. One of these alternative additives are the essential oils (EOs) extracted from several plants. Coriander (*Coriandrum sativum* L.) and parsley (*Petroselinum crispum* L.) are examples of plants from which the essential oil can be extracted.

Due to the susceptibility of the EOs to air, light, humidity and their insolubility in water, the industries have begun to develop the encapsulation process in order to protect these EOs from the effects of these factors as well as allowing a better dissolution of the compound in water and also allow it to be released in the necessary tissue/organ [4]. The main advantages of the encapsulation are minimized evaporation, increased shelf-live and controlled release, which may increase the biological efficiency.

There are many encapsulation agents that range from synthetic compounds to natural ones, although the synthetic ones are being discarded due to its cost and the growing awareness on environmental concerns. The natural encapsulation agents are mainly polymers, like chitosan, hydroxypropyl methylcellulose, arabic gum and/or cyclodextrins [5,6]. The cyclodextrins are polymers made of D-glucopyranose linked by α-(1,4) bound These compounds are obtained by the enzymatic digestion of starch by the action of some bacteria, e.g. *Bacillus macerans*[5,7]. There are 3 natural forms of cyclodextrin; α, β and γ cyclodextrins that have 6, 7 and 8 units of glucopyranose, respectively[5]. The cyclodextrin have a hydrophobic cavity and a hydrophilic exterior, this property make them suitable for the encapsulation of several compounds since the cavity have a dimension that allows the entrance of compounds with a reasonable dimension[7]. Thus, the aim of this study was determine the antimicrobial activity against several bacteria and yeasts of coriander (*Coriander sativum*) and parsley (*Petroselinum crispum*) essential oils encapsulated in β-cyclodextrin.

2. Material and methods

2.1 Plant material

Coriander (*Coriander sativum*) and parsley (*Petroselinum crispum*) were collected from Escola Superior Agrária de Coimbra (Instituto Politécnico de Coimbra, Bencanta, Coimbra, Portugal) during the flowering period.

2.2 Essential oil extraction

The EOs of coriander and parsley were extracted from entire plant (stems, leaves and flowers) by hydro-distillation assisted by microwaves using a Clevenger-type apparatus for 3 h. The oily layer obtained on top of the aqueous distillate was separated and dried with anhydrous sodium sulphate (0.5 g). The extracted EOs was kept in sealed air-tight glass vials and covered with aluminium foil at 4 ºC until further analysis.

2.3 Encapsulation process

The encapsulated EOs were made as following; 4 g of β-cyclodextrin was dissolved in 100 mL of water at 45 ºC. Afterwards 0.5 mL of coriander or parsley EOs were added and the reaction occurred for 30 minutes while the solution was stirred. Then the solution was left to cold and afterwards was putted at 4 ºC for 48h. When the time had passed the supernatant was rejected and the β-cyclodextrin was dried at 35 ºC.

2.4 Microbial strains

The encapsulate EOs were individually tested against several bacteria (*Listeria innocua* CECT 910, *Shewanella putrefaciens* CECT 5346, *Achromobacter denitrificans* CECT 449, *Pseudomonas fragi* CECT 446, *Enterobacter amnigenus* CECT 4078) and yeast (*Yarrowia lipolytica* CECT 1240 *Saccharomyces cerevisiae* CECT 1383, *Candida zeylanoides* CECT 10048). All species were supplied by the Spanish Type Culture Collection (CECT) of the University of Valencia (Valencia, Spain).

2.5 Antimicrobial assay

Antimicrobial activity was determined based on a colorimetric broth microdilution method proposed by Abate, et al[8]. Bacterial strains *A. hydrophila, P. fragi, A. denitrificans, S. marcenscens* and *S. putrefaciens* were cultured 24 h at 26 ºC and *A. faecalis, E. amnigenus* and *E. gergoviae* were cultured 24 h at 37 ºC, both in the Nutrient Broth No. 2 (NB No2) (Oxoid Ltd, England) and *L. innocua* was cultured 24 h at 37 ºC on Brain Heart Infusion Broth (BHI) (Scharlau S.L., Spain) and adjusted to a final density of 10^6 CFU/mL and used as inoculum. The antimicrobial activity was also tested against yeasts using the same assay. In this case the strains used were *Y. lipolytica, S. cerevisiae, C. zeylanoides,* which were cultured 24h at 26 ºC in the LM broth.

The encapsulated EOs were dissolved in dimethyl sulfoxide (DMSO) to reach a final concentration of 40 µL/mL. Serial twofold dilutions were made in a concentration ranged from 0.02 to 40 µL/mL in sterile test tubes containing Muller Hinton broth. The 96-well microplates (Iwaki, Japan) were prepared by dispensing into each well 90 µL of Muller Hinton broth and 10 µL of the bacterial inoculums. A 100 µL from each EOs initially prepared was added into the first wells. Then, 100 µL from their serial dilutions was transferred into ten consecutive wells. The last well containing 190 µL of Muller Hinton broth without compound and 10 µL of the inoculums on each strip was used as negative control. The final volume in each well was 200 µL. Nisin at the concentration range of 0.01 to 10 µL/mL was prepared in Muller Hinton broth and used as positive control. DMSO was used as negative control.

Contents of each well were mixed on a plate shaker at 150 rpm for 2 min prior to incubation during 24 h at the optimal temperature depending of microbial inoculums. After incubation, 25 µL of 3-{4.5-dimethylthiazol-2-yl}-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Life Science), dissolved in DMSO (0.8 mg/mL) was added to each of the wells and subjected to an incubation process for 1 h, in order to allow the viable microorganisms to metabolize the yellow dye MTT into formazan (purple crystals). The minimum inhibitory concentration (MIC) value was considered as the concentration of the first well that did not undergo color change (from yellow to purple) and confirmed by plating 10 µL samples from clear wells on Mueller Hinton agar medium. The procedure was repeated three times for each microorganism.

3. Results and discussion

The broth microdilution method was used to determine the antimicrobial activity of coriander and parsley encapsulated EOs against Gram-positive and Gram-negative bacteria and yeast.

Table 1 showed minimum inhibitory concentration (MIC) of encapsulated EOs against *L. innocua*, *A. denitrificans*, *S. putrefaciens*, *E. amnigenus* and *P. fragi*. Both, coriander and parsley encapsulated EOs had a very similar antibacterial activity against all these strains. For *L. Innocua*, encapsulated parsley EO showed lower antibacterial activity than encapsulated coriander EO. However for *P. fragi,* encapsulated parsley EO showed higher antibacterial activity than encapsulated coriander EO. As regards, *A. denitrificans*, *S. putrefaciens*, *E. amnigenus,* both, coriander and parsley encapsulated EOs had the same antibacterial activity with values of 10 mg/mL for *A. denitrificans* and *S. putrefaciens* and 20 mg/mL for *E. amnigenus*. The β-cyclodextrin complexes improved the water solubility of the compounds and made the EOs more effective antimicrobial. The β-cyclodextrin seems to enhance the antimicrobial capabilities of the essential oils in several ways, but the primary site of action of EOs is at the membrane and inside the cytoplasm of bacteria and β-cyclodextrin may have enhanced essential oil access to these regions by increasing aqueous solubility[9,10]

Nisin used as positive control showed the lowest MIC values against all bacteria tested with values ranged between 0.62 and 2.5 mg/mL. The high MIC of the encapsulated EOs when compared to nisin might be explained by the fact that β-cyclodextrin is a starch-derived polymer and due to that it might have a stimulatory effect on the microbial growth since it is a carbon source[11].

Table 1 Minimum Inhibitory Concentration (MIC) of encapsulated essential oils against several bacteria

Encapsulated EO	**Minimum Inhibitory Concentration (mg/mL)**				
	L. innocua	*A. denitrificans*	*S. putrefaciens*	*E. amnigenus*	*P. fragi*
Parsley	20	10	10	20	10
Coriander	10	10	10	20	20
Nisin	2.5	2.5	0.625	1.25	1.25

Besides the antibacterial activity, the encapsulated EOs were tested against yeasts in order to determine the MIC values.Table 2 showed the minimum inhibitory concentration (MIC) of encapsulated EOs against several yeast. *Y. lipolytica* showed lower MIC values (5 mg/mL) for both encapsulated EOs than *S. cerevisiae* and *C. zeylanoides*. As occur with bacterial strains, the yeast can used the β-cyclodextrin as a carbon source.

Table 2 Minimum Inhibitory Concentration (MIC) of encapsulated essential oils against several yeast

Encapsulated EO	**Minimum Inhibitory Concentration (mg/mL)**		
	Y. lipolytica	*S. cerevisiae*	*C. zeylanoides*
Parsley	5	10	10
Coriander	5	10	20

There are some studies regarding the antimicrobial activity of encapsulated EOs, in different matrix such as zein or β-cyclodextrin[12,13]. Although the EOs in general and the encapsulated EOs en particular showed antimicrobial activity, the reason behind this capacity are not well documented. This antimicrobial activity could be provoked by the major compounds of the EOs or due to a synergistic effect between the major compounds and the minor ones[14]. The antimicrobial mechanisms of EOs may be an attack on the cell membrane's phospholipid bilayer, the disruption of the enzymatic systems, the compromising of the genetic material of the bacteria/yeast, the formation of fatty acid hydroperoxidase by the oxygenation of unsaturated fatty acids, the coagulation of the cytoplasm, the damage of lipids and proteins, the distortion of the proton motive force (PMF), the electron flow and/or the active transport[1,15,16]. Thus, the EO components can provoke an increase in K^+ and often cytoplasmic content effluxes from cells in response to antimicrobial challenge. These effects may develop as result of (i) membrane depolarization by altered ion transport or through changes in membrane structure; (ii) inhibition of energy (ATP) generation by interference with glucose uptake; or (iii) inhibition of enzymes involved in oxidative or substrate level phosphorylation[17].

Conclusions

This study showed that both coriander and parsley encapsulates EOs might be a good alternative, as natural antimicrobial agents, to the actual synthetic compounds used in Food industry. The benefits of BCD encapsulation extend beyond masking sensory attributes of antimicrobial compounds. Encapsulation can also enhance the mechanism of antimicrobial action and decrease the concentration of antimicrobial compound needed for inhibition via increased EO delivery to the membranes of microorganisms in aqueous environments. However more studies must be done in order to have a better evaluation on the antimicrobial activity.

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Influence of cutting shape on microbiological quality of fresh-cut summer squash washed with sodium hypochlorite solution

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Washing and disinfection steps directed to remove organic matter liberated in cutting operations are critical in fresh-cut vegetables industry. It is advisable to add a disinfectant agent to the washing solution to slow down microbial growth and to avoid cross contamination in order to minimize microbiological food hazards associated to these products. A matter of special concern is knowledge about which substances are able to give a good performance in this type of processes. Sodium hypochlorite solutions have been broadly used in this kind of operations, but its antimicrobial efficacy is hampered by some technological factors as organic matter concentration or pH of washing solution, due to chemical behaviour of this substance in water solution. Furthermore, because of the mechanism of action of this chemical, factors as type of produce, surface morphology or cutting shape can influence the overall performance of this well-known disinfectant.
The objective of this survey was to determine the possible influence of cutting shape in the microbial load of two different fresh-cut summer squash produce (slices and sticks), washed with a sodium hypochlorite solution and packed in a passive modified atmosphere. For this purpose, summer squash (*Cucurbita pepo* subsp. *pepo*) fruits were cut and washed in 150 ppm chlorine solutions. Afterwards, water excess was removed by centrifugation and produce was packed in polyethylene trays and sealed with semi-permeable plastic film. Microbial load of fresh-cut produces was investigated immediately after centrifugation step and along conservation after packaging under cooling conditions. Mesophilic and psychrotrophic aerobic bacteria, enterobacteria, yeasts and moulds were counted following standard microbiological procedures. Results showed that microbiological quality of fresh-cut produce is influenced by cutting shape for equal washing and storage conditions.

Keywords: sodium hypochlorite, disinfection, chlorine, fresh-cut, summer squash, microbiological quality

1. Introduction

Summer-squash (*Cucurbita pepo* L.) is a relevant intensive farming produce in Spain and particularly in Almeria, first Spanish region producing this fruit vegetable in plastic green-houses.

Ready to eat fresh-cut vegetables constitute a still growing agro-business. Consumers in developed countries demand these kind of products as a consequence of their actual way of life. Though fresh-cut leafy vegetables represent the main production of these type of products, new market trends lead vegetal growers and marketers to look towards new fresh-cut vegetables as bell-pepper, tomato or squash. Different characteristics of these produce with regard to lettuce, baby leafs or another leaf vegetables result in somewhat different processing facts that have been found amongst fruit vegetables.

Fresh-cut products undergo a severe reduction in their sensorial and microbiological quality during processing operations and along their shelf life, due to their inherent properties and to the effects of cutting and slicing operations, which provide optimal conditions for microbial survival and growth not only of spoilage organisms but also of human pathogenic bacteria. So, it's necessary to include washing and disinfection steps in the fresh-cut plant in order to slow down microbial proliferation and reduce microbiological food hazards associated to these products. Special care must be taken about the use of disinfectants in vegetable washing operations. Sodium hypochlorite solutions in concentration of 50-200 ppm have been broadly used in this kind of operations (Sanz et al., 2002), but its antimicrobial efficacy is hampered by some technological factors as organic matter concentration or pH in washing solution (Sanz et al., 2002; Gil et al., 2009). In addition, several authors have demosntrated that microorganisms tend to attach to groves or cavities of fruit tissues, which provides protection to the cells against washing treatments (Wang et al., 2009). There are still few data available about chlorine efficacy when it is used in whashing fresh-cut summer squash under MAP conditions. The aim of this study was to evaluate changes in the microflora of two different fresh-cut summer squash products in order to determine their shelf-life after washing with chlorine solution and storage under passive Modified Atmosphere Packaging (MAP) conditions.

2. Material and Methods

2.1 Vegetal material

Cucurbita pepo L. fruits were obtained by growing following usual regional culture technics on synthetic perlite substrate in a plastic green-house located in La Mojonera (Almeria, Spain) and selected to commercial standard sizes (18 – 21 cm). Immediately after harvest fruit were cold stored (6ºC temperatura and 95% relative humidity) until processing in no more than four hours.

2.2 Processing

In a cold room (6ºC) fruits were previously rinsed in cold tap water and non-edible parts were removed. Then fruits were cut in two batches of different formats, slices and sticks, 1 min. washed with 150 ppm sodium hypochlorite solution and finally rinsed in cold tap water. Each batch was centrifuged to remove excess water and weight. Portions of 150 g of product were placed in plastic trays and packaged into semipermeable plastic bags to achieve passive modified atmosphere conditions (MAP). Products were stored during 14 days at 6ºC and 95% relative humidity.

2.3 Microbiological analysis

Microbiological analysis were performed immediately before packing and along storage time. 10 g of product were homogenized in Buffered Peptone Water in a smasher (*Smasher*, *AES Laboratoire, Combourg, France*) and decimal dilutions were prepared for plate counting. Mesophilic and psychrophilic aerobic microorganisms were counted using Plate Count Agar (*Plate Count Agar, Oxoid Limited*) and incubation at 30 2ºC for 72 h. or at7ºC for ten days respectively. Chromogenic specific agar (*Biolife Italia Srl.*) were used for enterobacteria counting by incubation at 37 ºC for 24 h. Yeasts and moulds were investigated using Rose Bengal Chloranphenicol (*Biolife Italia Srl.*) and incubation at 25ºC for five days. Experiments and sample analysis were performed in duplicate and results were expressed as decimal logarithmic units of bacteria per gram (log cfu/g).

3. Results and discussion

Microbial cell concentration found in freshly prepared product were similar to those published by other authors in cucurbits (Erkan et al., 2001; Jacxsens et el., 2002). Fig. 1 illustrates the growth curves of total psychrotrophic, total mesophilic, enterobacteriaceae, yeasts and moulds counts in slice squash product along 14 days of storage at 6ºC under MAP conditions. These two groups of microorganisms reached a cell concentration of 6 log cfu/g on 9th day of storage. Enterobacteria only reached that level on 14th day. Yeasts did not reach that level even on last day (5 log cfu/g) and moulds were not found in any sample tested.

Several authors recognize that even though product quality of ready to eat foods can be acceptable despite of high microbial counts, there are several groups of pathogenic psychrotrophic microorganisms, as *Listeria monocytogenes* or *Yersinia enterocolitica*, capable of growing or mantaining an infectious potential under usual MAP conditions (Szabo et al., 2000; Jacxsens et al., 2002). So, if we consider 6 log cfu/g as acceptability level on the basis of microbial load, mesophilic and psychrotrophic organisms load was the limitant factor in slice product, results that are concordant with these authors. On this basis we can establish 9 days as shelf-life of sliced squash product on the basis of spoilage microorganisms load.

Fig. 2 show the growth curves of total psychrotrophic, total mesophilic, enterobacteriaceae, yeasts and moulds counts in sticks squash product along 14 days of storage at 6ºC under passive modified atmosphere (MAP) conditions. Unlike slice product, in sticks enterobacteria followed a pattern similar to generic microorganisms. All groups of microorganisms tested in this product showed higher cell concentrations than in slice product, reaching 6 log cfu/g level all of them before 5 days of storage, so we consider this conservation period as shelf-life of sticked squash product on the basis of spoilage microorganisms load.

The different evolution of microbial spoilage on these two products could be explained by the greater rate of surface of cut tissue per volume in stick fragments, so as the main direction of cut, which is cross sectional to the main vessels in slices but lengthwise in sticks. Cut tissues release vascular fluids rich in nutrients that allow microorganisms to grow easily. As much exposed cut tissue we achieve in squash pieces, quicker spoilage is produced by microbial growth.

The use of sodium hypochlorite solutions in washing steps of fresh-cut produce has been broadly recommended because of its good efficacy as disinfectant. However, this product has some disadvantages as it is inactivated in high concentrations of organic matter.

Another factor to consider is surface roughness, as microorganisms tend to attach to or be entrapped in the grooves or cavities of fruit tissues which provide protection to the cells against washing treatments (Wang et al. 2009). In squash slices surface appearance seems to be smoother than sticks surface. This fact can be due to the main direction of the cut which in the core of fruit produces sticks with placental tissue exposed to air in four longer sides of the fragment. As chlorine effect is achieved by contact with microbial cells, this couldn be an obstacle to its performance.

4. Conclusions

In general, squash sticks showed shorter shelf-life than squash slices in similar processing and preserving conditions, so we can say than morphology of pieces in fresh-cut squash can influence shelf-life of the product. So, when processing squash fruits cutting format should be considered in order to determine the optimum processing conditions, particularly disinfectant concentration in washing operations. Special care should be taken about *Enterobacteriaceae* related bacteria when roughly surface fragments are produced. Disinfectant efficacy should be checked and shelf-life tests should be developed in order to guarantee food safety. In addition, more research is needed to test alternative disinfectant products which could be more effective than chlorine in washing ready-to-eat squash products.

5. Figures

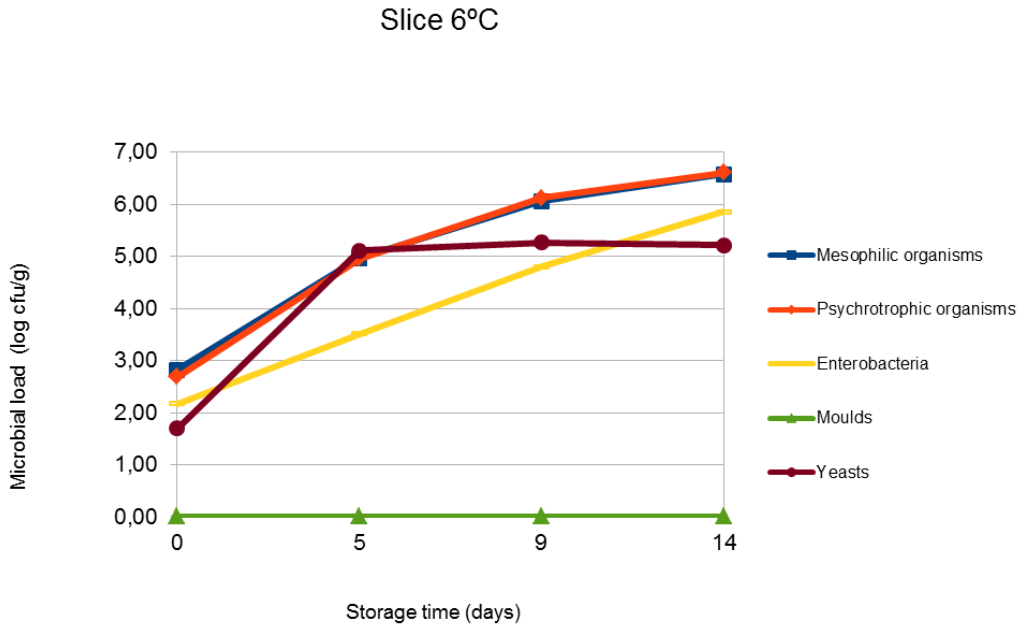

Fig. 1. Growth curve of different microbial groups in slice squash product stored in MAP conditions at 6ºC.

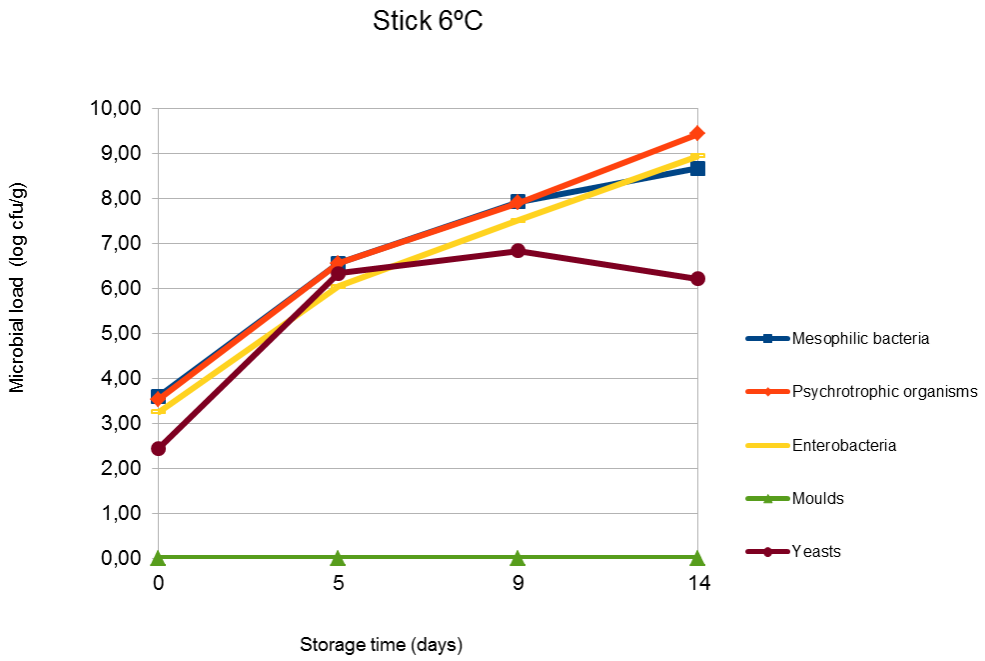

Fig. 2. Growth curve of different microbial groups in stick squash product stored in MAP conditions at 6ºC.

Aknowledgements: This research was funded by INIA and the European Union (FEDER Funds) under the project RTA2009-00036-00-00.

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Inhibitory effect of garlic oil against *Aspergillus niger* on rubberwood (*Hevea brasiliensis*)

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The antifungal activity of garlic oil at 10-100 µl/m against major mold found on the surface of rubberwood (*Hevea brasiliensis*) was investigated using the agar dilution method. The minimum inhibitory concentration (MIC) of the garlic oil was determined to be 70 µl/mL for *Aspergillus niger*. Antifungal activity of the garlic oil at the concentration of 70 µl/mL was further examined on the rubberwood surface under storage conditions of 25°C and 100% RH. It was found that *Aspergillus niger* on the rubberwood was completely inhibited for at least 12 weeks under the storage conditions. The main components of the garlic oil were determined by gas chromatography-mass spectrometry (GC–MS) analysis. Three constituents representing 77.55% of the garlic oil were identified, the major ones being diallyl disulfide (38.86%), diallyl sulfide (19.81%), and 2-vinyl-1,3-dithiane (18.88%)

Keywords antifungal, *Aspergillus niger*, rubberwood

1. Introduction

Rubberwood is used in children's toys, kitchenware and furniture. It's the main raw material in Thailand's furniture production [1]. Today rubberwood is cultivated not only for these industries but also to produce natural latex. Thailand possesses excellent soil and climate conditions for rubberwood trees [2] and latex production was estimated to be at 3 million tons with an average yield of 5.64 tons per hectacre during the 2007 harvests [3] with Southern Thailand being the greatest producer in the world. The latex yields from rubberwood trees decline significantly 25 to 30 years later after which the trees are cut down and replanted. Rubberwood material will then be taken to wood industries. Unfortunately, mold contamination with some mycotoxin is one of the main factors that compromise the quality of the rubberwood after production. *Aspergillus niger* and *Penicillium spp.* were the major mold frequently isolated from the rubberwood surface after cutting [4]. In addition, *Aspergillus niger* could produce Ochratoxin A [5]. Ochratoxin A commands attention as it has been implicated in a diverse range of toxicological effects, including renal toxicity, mutagenicity, teratogenicity and immunotoxicity in both animals and humans [6].

Garlic oil (*Ocimum basilicum* L., Lamiaceae) is a common herb used for culinary and medicinal purposes. Garlic contains various types of polyphenol and sulphur compound [7]. It may contain polar products able to lower plasma lipid concentrations and might be beneficial in preventing hyperlipidemia and related cardiovascular diseases [8-9]. The effectiveness of garlic oil against microbes was reported by [10-11]. However, the effect of garlic oil of aromatic plants has not been specifically investigated on rubberwood. Therefore, the objective of this work was to study the inhibitory effects of garlic oil on the growth of *Aspergillus niger* on both a medium and rubberwood.

2. Materials and methods

2.1 Essential oil

The food-grade garlic oil derived by steam distillation was provided by the Thai China Flavors & Fragrances Industry Co., Ltd. of Bangkok.

2.2 GC-MS analysis

This analysis was carried out on a gas chromatograph (Hewlett-Packard Model 7890A, USA) equipped with a DB-5 column (J&W Scientific, USA) at dimensions of 30 cm × 0.25 mm ID and 0.25 µm film thickness. The

average helium carrier gas flow rate was 1ml/min; the split ratio of the column was 50:1 and the injector and detector temperatures were set at 250°C and 260°C, respectively. The column oven temperature was held at 60°C for 30 sec, then programmed to 150°C at 40°C/min and then to 260 °C at 2°C/min. Citronella oil (1.0 µl) was injected manually. Identification of the constituents was based on comparison of the retention times with those of authentic samples comparing their Kovats indices, and on computer matching with the *NIST 08.L* (database/ chem- station data system)

2.3 Preparation of inoculum

*Aspergillus nige*r WU 0701 was identified from rubberwood surfaces. Codes refer to strains held in the culture collection of the Wood Science and Engineering Research Unit of the Center for Scientific and Technological Equipmnts at Walailak University. Spores were obtained from mycelium grown on a malt extract agar (MEA; Merck Ltd, Thailand) medium at 25°C for 7 days and were collected by flooding the surface of the plates with ~5 ml sterile saline solution (NaCl, 8.5 g/l water) containing Tween 80 (0.1% v/v). After counting the spores using a haemocytometer, the suspension was standardized to concentrations of 10^7 spore/ml by dilution with sterile water before using. The viability of all strains checked using quantitative colony counts were at 10^7 CFU/ml.

2.4 Minimal inhibitory concentration (MIC)

The antifungal analysis and determination of minimal inhibitory concentration (MIC) of the garlic oil was performed by the agar dilution method in a Petri dish. The essential oil of 10 to 100 µL/mL was added to the malt extract agar (MEA). One hundred µL of spore suspension was then inoculated on the MEA. The vegetable oil was used as a control at the same concentration. The Petri dish was then incubated at 25°C for 3 days. Tests were performed in triplicate. The highest dilution (lowest concentration) showing no visible growth was regarded as the MIC.

2.5 Mold test on rubberwood

Rubberwood specimens (7mm x 20mm cross section by 70 mm long) were prepared from freshly cut rubberwood lumber obtained from a plantation site in the Nakhon Si Thammarat province of Thailand. The average moisture content of the rubberwood specimens before testing was 49±2 % (n=10). Sets of five random replicate specimens were dip treated according to ASTM test methods D4445-91 [12] for 15 seconds with individual garlic oil at various concentrations ranging from 10 to 100 µL/mL. Vegetable oil was used as the control. Different dilutions of the oils were made with methanol. Dip treated specimens were held in a closed container overnight at room temperature before inoculation with the spores of the test mold.

The oil treated specimens were inoculated with 1 mL of *Aspergillus niger* spore inoculum (10^7 CFU/ml) and were incubated at 25°C with 100% RH in an environmental chamber (Contherm, Petone, New Zealand) for 12 weeks. The specimens were then individually rated for mold growth on a scale of 0 to 5, with 0 denoting a clean specimen and 5 representing heavy mold growth (0=clean, 1=20%, 2=40%, 3=60%, 4=80%, 5=100% of mold growth). The percent of stain and mold (based on control) for the garlic oil concentration was calculated the following way: Percent of stain and mold (based on control) = [(Total score for each mold at each concentration of garlic oil / Total score for each mold at control) x100].

3. Results and discussion

3.1 Chemical compositions

The GC-MS analysis of the garlic oil led to the identification of 7 components (Table 1) accounting for 77.55%. Of the components present, diallyl disulfide (38.86%), diallyl sulfide (19.81%), and 2-vinyl-1,3-dithiane (18.88%) were the three most abundant components. The GC-MS result obtained in this study was significantly different from previously published results [13-14]. From other results, diallyl disulfide was the greatest compound to be found in garlic oil. However, both components diallyl disulfide and diallyl sulfide (in this study) are classified as sulphur compound. Compositional differences may be explained in terms of genetic variability, geographic localization, harvest time and climate conditions.

Table 1 Chemical composition of garlic oil

No.	Compound	Composition (%)
1	diallyl disulfide	38.86
2	diallyl sulfide	19.81
3	2-vinyl-1,3-dithiane	18.88

3.2 Antimicrobial activity

The result of the antimicrobial activity of garlic oil against *Aspergillus niger* is summarized in Table 2. In the agar diffusion assay, 70 µl/ mL of garlic oil exhibited a significant effect against *Aspergillus niger* with no growth on MEA. The concentration of garlic oil was the same with Benkeblia in 2004 [15] which reported concentration of garlic oil (50-100 µl/mL) could against *Aspergillus niger*. Avato et al. (2000) [16] reported the antimicrobial activity of essential oils from garlic oil and its main component, may be in due part to the presence of high content of allylsulfide (sulphur component). The essential oils of this plant show that it has sulphur compounds such as diallyl disulfide and diallyl sulfide as the major components. These compounds possess strong antimicrobial activities [16]. In addition, Matan et al. 2012 [16] reported diallyl disulfide and diallyl sulfide, main components of garlic oil in this test, could inhibit growth of *Aspergillus niger*. Another important characteristic of the essential oils is their hydrophobicity which enables them to penetrate lipid components of microbial cell membrane and mitochondria, disrupting the cell structure and rendering them more permeable and resulting in leakage of critical molecules from within the cell and eventual death of the cells [17].

Table 2 Minimal inhibitory concentrations (MIC) of garlic oil against *Aspergillus niger*

Concentration (µL/mL)	Garlic oil	Vegetable oil
0	+	+
10	+	+
20	+	+
30	+	+
40	+	+
50	+	+
60	+	+
70	-	+
80	-	+
90	-	+
100	-	+

+ =Growth
- = Non-growth

Mold test on rubberwood

Mold resistance levels of the treated rubberwood specimens inoculated with the test *Aspergillus niger* are shown in Figure 1.

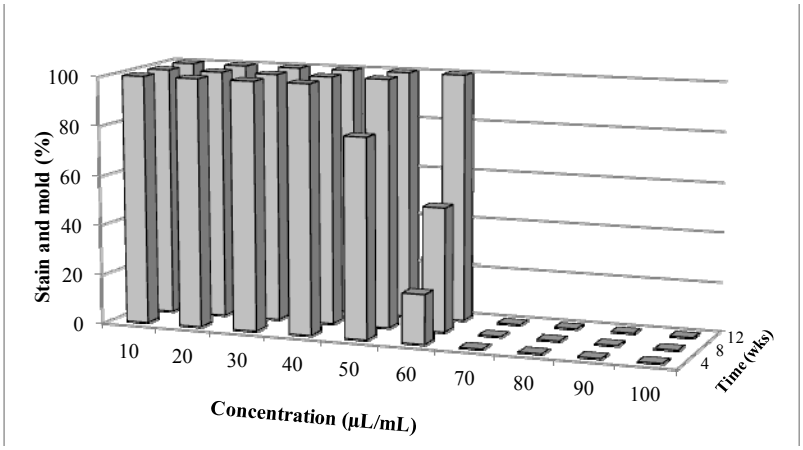
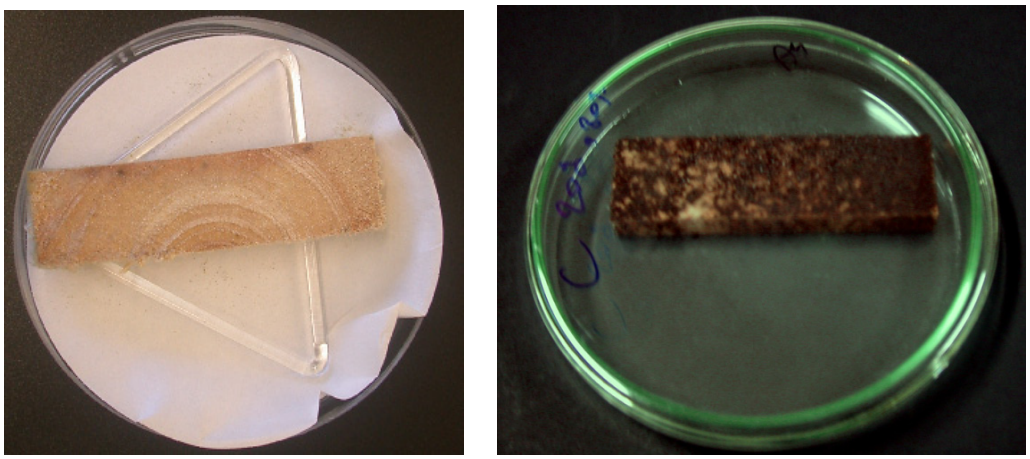


Figure 1 Percentages of stain and mold growth of garlic oil after 4, 8 and 12 weeks.

The results are presented as the average rating of five specimens. Oil treatment might have some effects on moisture exclusion from the test specimens but the controls (treated with vegetable oil) reached a value of 100% mold coverage within four weeks. This suggests that garlic oil at the MIC (70 µL/mL) was capable of inhibiting *A. niger* for at least 12 weeks (Figure 2).



(a) (b)

Figure 2 Growth of *Aspergillus niger* after 12 weeks at 25 °C on (a) rubberwood with garlic oil at 70 µl/mL (b) rubberwood with vegetable oil at 70 µl/mL

4. Conculsions

The garlic oil contained mainly sulphur compound and showed good potential to inhibit the growth of *Aspergillus niger* both on the agar and on the rubberwood with the minimum concentration of 70 µL/mL. Garlic oil at those concentrations was capable to give protection from mold growth on rubberwood for at least 12 weeks at the storage condition of 25°C with 100%RH.

Acknowledgements The authors gratefully acknowledge the support from a research grant provided by the Office of the Higher Education Commission, the Thailand Research Fund (TRF), and Walailak University through the new researchers grant MRG5480002.

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Isolation of actinomycetes producing antimicrobial substances from Bechar region (southwestern Algeria)

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The constant evolution of the bacterial resistance to antibiotics makes necessary the research for new antimicrobial substances. The objective of the study is the isolation of actinomycètes from the soil of the Algerian southwest (Bechar) and the evaluation of their antimicrobial activity.

Keywords: Actinomycetes, Isolation, southwestern Algeria, Antimicrobial activity, Identification.

Introduction

With the increasing problem of drug resistance there is a dire need to isolate, identify and utilize newer molecules of biomedical importance **(Kamat. K. T).**

Soil microorganisms provide an excellent resource for the isolation and identification of therapeutically important products. Among them, actinomycetes, Gram-positive filamentous bacteria with true aerial hyphae, are an important group producing antibiotics of agricultural and medicinal importance**.** In fact two-thirds of known antibiotics have been isolated from actinomycetes **(Hozzein & al. 2011).**

However, this group of microbes has been tapped to such an extent that the chances of isolating a novel strain with a unique metabolite producing capability are slim, a useful approach to address this problem is to explore unusual ecosystems such as the soils of Algerian sahara **(Khanna & al. 2011).**

The objective of the study is the isolation of actinomycètes from the soil of the Algerian southwest (Bechar) and the evaluation of their antimicrobial activity.

1. Material and Methods

a) Soil Sample collection and pre-treatment:

Different soil samples were collected from three stations of Bechar (southwestern of Algeria) : Lahmar, Kenadsa, Béni-Abbes, by the method **of Ponchon and Tradieux (1962).** After the separation of the first five centimeters of soil, the sample is taken a depth of 15 to 20 cm below the surface.

The samples were packed in sterile polyethylene bags and aseptically transported to the laboratory for further analysis. The collected samples were air dried for seven days, mixed and homogenised manually by removing roots. Each soil sample was pre-treated with 0.1 g of calcium carbonate (CaCO3) and incubated at 25°C for two weeks (**El-Nakeeb & Lechevalier. 1963).**

b) Isolation of actinomycetes:

10 g of soil samples is diluted in 100 ml of physiological water (NaCl 9 g/l). 1 ml of each suspension are sown on the surface on four different cultures mediums **(Kumar & al. 2010):** Bennett, starch casein agar, Kuster starch agar and glycerol yeast extract agar. All the four agar media were supplemented with 80 µg/mL of oxytétracycline to minimize the other bacterial growth . The dishes are incubated at 28 ° C and observed daily for a period of 4 weeks. After incubation the actinomycete colonies were purified and sub-cultured on starch casein plates and stored for further assay.

c) Screening for antimicrobial activity:

Antibacterial activity of actinomycetes were tested in vitro against bacterial pathogens that included*:* Two Gram positives bacteria *(Enterococcus faecalis (ATCC25212), Staphylococcus aureus (ATCC6538))*, and three Gram négatives bacteria *(Salmonella heinderlberg, Klebsiella pneumoniae (ATCC 70603), Pseudomonas aeruginosae (ATCC10145)).* While the antifungal activity was achieved against yeast *Candida albicans* and the mold *Fusarium oxysporum f.sp. albedinis.*

The antibacterial activity is carried out by the agar piece method**:** The strains of actinomycetes were grown on casein streach agar medium during 14 days. Cylinders (3 mm in diameter) are cut and placed on Muller-Hinton medium, previously inoculated by the test germs. Plates were kept at 4 °C during 4 hours for a good diffusion of the antibacterial metabolite, then incubated at 37°C. The diameters of inhibition are determined after 24 h.

Antifungal activity against growth of *Fusarium oxysporum f.sp. Albedinis* was carried out according to the method of **Crawford and al. 1993:** Spores (10^6 spores/ml) of actinomycetes was spotted on one side of a PDA plate and incubated at 28 °C for 9-10 days. A mycelial plug of 6 -mm diameter from 3-days-old of mold *Fusarium oxysporum f.sp. Albedinis* was cut and transferred to an actinomycetes-pregrown PDA plate. The fungal plug was additionally placed on uninoculated PDA plates separately as control treatment. The radial fungal growth in the direction of the antagonist in both the control and the dual culture plates was measured after 6 days of incubation. The levels of inhibition were calculated by using the equation**:**

$$\boldsymbol{\Delta Y = Y0 - Y}$$

Y_0= Diameters of the radial fungal growth of a control culture.
Y= Radial fungal growth of paired-cultures in the direction of actinomycetes.

d) Preliminary identification of the isolated actinomycetes:

The potent Actinomycetes isolates selected from primary screening were characterized by morphological tests. The spore surface morphology of the mycelium of actinomycetes isolats was observed after 14 days old, using cover slip culture technique(1000X) (George & al. 2010). The following characteristics were checked: Spore chains, Sporangia, Single spores, Fragmentation of aerial or substrate mycelium, Spore chain morphology: Recti flexibilis, Retinaculumapertum, Spira, Verticillus).The observed morphology of the isolates was compared with the Actinomycetes morphology provided in Bergey's Manual of determinative Bacteriology, 9th edition (2000) for the presumptive identification of the isolates.

2. Résultats and discussion

a) Resultats Isolation of actinomycetes :

22 different types of Actinomycetes were isolated, The comparison between the number of isolates isolated from each culture medium is shown in figure 2.

The majority of the isolats were collected from Bennette and Kuster agar (7 isolats for each), followed by the medium casein starch agar (5 isolats), the glycerol yeast extract agar medium does not allow the isolation of many actinomycetes (only 3 isolats).

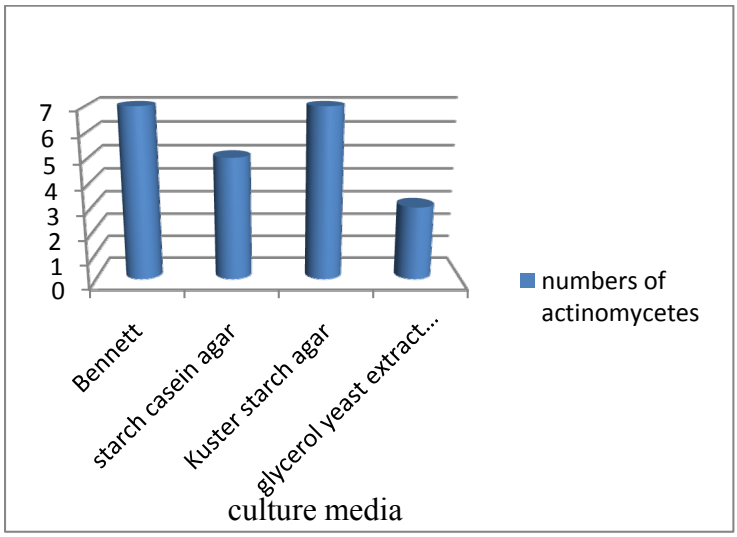


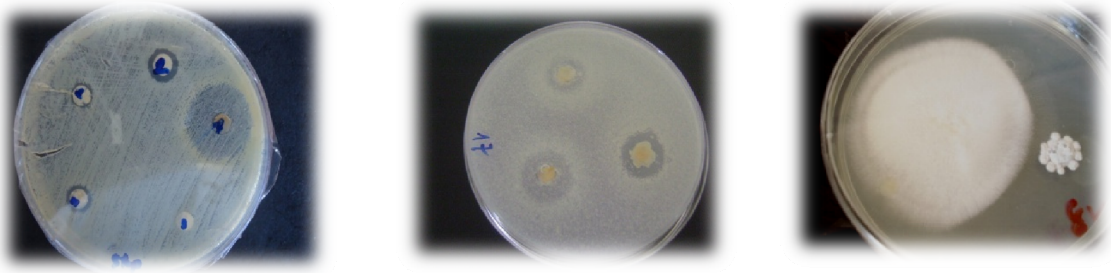
Figure 1: Number of actinomycetes isolated from each culture medium

b) Antibacterial activity:

The results presented in the table indicate that out of 22 isolats, 16 isolates (73 %) had antibacterial activity against one or more test bacteria, the antibacterial activities of the active isolats could be divided according to the spectrum of their activity into three groups as follow:

- Group I: includes the actinomycete isolates capable of producing antimicrobial activities against Gram-positive bacteria only. This group contains 2 isolates: ACT10 and ACT16.
- Group II: includes the actinomycete isolates capable of producing antimicrobial activities against Gram-negative bacteria only. This group includes3 isolates: ACT14 ACT20 and ACT22.
- Group III: includes the isolates that have the ability of producing antimicrobial activities against both Gram-positive and Gram-negative bacteria. This group includes 11 isolates: Act15, Act5, Act6, Act7, Act11, Act12, Act13, Act30, Act17, Act23 and Act2.

The lowest zone of inhibition was observed in the strain ACT2 against the *S. heinderlberg* (6.33 mm), maximum zone of inhibition was observed against *Salmonella heinderlberg (22 mm)* (ACT11). Among the test organisms, *Staphylococcus aureus* was found to be affected by most of the actinomycetes isolated (13 isolats actifs). *Pseudomonas aeruginosae* is the most resistante (6 isolats actifs only).



A B C

Figure 2: Antibacterial activity, A : Against S. aureus, B: against C. albicans and C: against *Fusarium oxysporum f.sp. Albedinis*

antifungal activity results indicate that isolates ACT16, ACT5 and ACT17 have weak antifungal activity against FAO ($\Delta\gamma \geq 5$ - 9 mm), while the isolate act13 has a antifungal activity moderate against the FAO ($\Delta\gamma \geq 10$ - 19 mm).

c) Preliminary identification of the isolated actinomycetes:

A B

Figure 3: A: Microscopic observation of vegetative mycelium of the isolat Act15; B: Microscopic observation of aerial mycelium of the isolat Act15 (G × 1000).

The isolate Act15 is characterized by an aerial mycelium abundant, gray on ISP2 medium, Branched substrate mycelium and unfragmented. chains of spores is flexibilis, Based on morphological data, the isolate ACT15 can be classified in the genus *Streptomyces* according to Bergey's manual of systematic (1989).

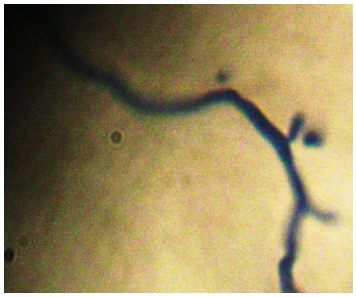


Figure 4: Microscopic observation of vegetative mycelium of the isolat Act11

The isolate Act11 is characterized by: No aerial mycelium, the vegetative mycelium is little branched and not fragmented. Single spores formed on substrate mycelium. Based on morphological data, the isolate ACT11 can be classified in the genus *Micromonospora* according to Bergey's manual of systematic (1989)

Acknowledgements: This work confirms that the soil Algerian sahara is rich in actinomycetes producing antibiotic.

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MIC determination of new antimicrobial agents in coagulase-negative staphylococci resistant to oxacillin isolated from patients in a Brazilian hospital

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Coagulase–negative staphylococci (CoNS) are the most frequently isolated organisms from clinical materials, and their increasing resistant to antimicrobials over the last years is of great relevance for nosocomial infection. Resistance to oxacillin is mediated by the *mecA* gene; this gene is highly frequent in CoNS samples, highlighting the need to test new antimicrobials for the treatment of infection related to these organisms. The aim of this study was therefore to identify CoNS species isolated from blood culture specimens from inpatients at the Botucatu School of Medicine, University Hospital, Brazil, in order to determine minimal inhibitory concentrations (MIC) for new antimicrobials used for treatment of infection related to these organisms.

Keywords oxacillin; vancomycin; linezolid; daptomycin; quinupristin/dalfopristin; tigecycline

1. Introduction

Coagulase–negative staphylococci (CoNS) are normal resident microbes of human skin, often isolated from clinical specimens [1]. They are recognized as opportunistic organisms which may take advantage of situations such as a skin barrier rupture from trauma or foreign bodies to reach other tissues, proliferate, and develop pathogenic behavior [2]. CoNS have been emerging during the last few decades as important nosocomial pathogens, due chiefly to increasing resistance to antimicrobials used for treatment of infection related to these organisms [3].

Increasing antimicrobial resistance over the last few years is of high significance for nosocomial infection. Studies show that 60 to 85% of staphylococci from clinical specimens are resistant to methicillin [4]. Oxacillin is the drug selected as a sensitivity tester for determination of β-lactam antimicrobial use in staphylococcal infection; CoNS intrinsic resistance to oxacillin is mediated by production of a supplementary penicillin binding protein (PBP 2a) determined by the *mecA* gene [5]. This gene is carried by a specific mobile genetic element identified as staphylococcal chromosomal cassette *mec* (SCC*mec*), which comprises the *mecA* gene complex, *ccr* gene complex, and J region. The *mec* gene complex comprises the *mecA* gene and its regulatory genes, *mecI* and *mecRI*, and the *ccr* gene complex is responsible for SCC*mec* integration to, and excision from, chromosome and phenotypic resistance to β-lactam antimicrobials. The J region, although not essential to the chromosomal cassette, may carry genes coding for resistance to non-β-lactam antimicrobial agents and heavy metals [6]. Eleven SCC*mec* types have been described so far, defined by combination of *ccr* gene complex types and *mec* gene complex classes, whereas subtypes are defined by J region polymorphisms in the same combination of *mec* and *ccr* complexes [6].

CoNS containing types I, II, and III are classically of nosocomial origin, whereas the other types are of communitarian origin. Type III SCC*mec* codes for the largest number of resistance genes, and is a major pathogen causing severe infection in hospital settings [7]. Type IV is of smaller size and metabolic cost, which makes it a selectively favored element for transfer among staphylococci [8]. Moreover, type IV SCC*mec* does not impose adaptive cost to its host and can spread in the absence of selective antibiotic pressure. Some *S. epidermidis* strains may carry different SCC*mec* types, indicating that these organisms often lose and gain SCC*mec* elements [6].

The increasing number of methicillin–resistant strains (MRS) among CoNS leaves little alternative for the treatment of staphylococcal infection, which highlights the need to test new antimicrobials. The aim of this study was therefore to identify CoNS species isolated from blood culture specimens from inpatients at the Botucatu School of Medicine, University Hospital, Brazil, in order to determine minimal inhibitory concentrations (MIC) for new antimicrobials used for treatment of infection related to these organisms.

2. Material and Methods

2.1 Specimens

One hundred and eighty-eight blood culture specimens of CoNS isolated from inpatients at the Botucatu School of Medicine, University Hospital, Brazil between 1990 and 2009, were examined. Specimen isolation procedures followed the directives established by Koneman *et al* [2].

Phenotypic identification of CoNS species was carried out by means of biochemical tests using the simplified method proposed by Cunha *et al* [9]. Confirmation of phenotypic identification by genotypic identification was achieved through the internal transcribed spacer technique (ITS–PCR) using primers of conserved sequences adjacent to genes 16S and 23S [10].

2.2 Detection of *mecA* gene and characterization of SCC*mec*

PCR procedures for *mecA* gene detection were according to parameters described by Murakami *et al* [11]. SCC*mec* type determination was carried out using the PCR–Multiplex method, as described by Oliveira and de Lencastre [12] and modified by Machado et al [13].

2.3 Determination of minimal inhibitory concentration (MIC)

Determination of minimal inhibitory concentration (MIC) for CoNS specimens was carried out by E-test, and the antimicrobial tested were oxacillin, vancomycin, daptomycin, linezolid, quinopristin/dalfopristin, and tigecycline.

3. Results

3.1 Specimens

One hundred and eighty-eight CoNS were identified: 148 *S. epidermidis* (78.7%), 17 *S. haemolyticus* (9.0%), 15 *S. hominis* (7.9%), four *S. capitis* (2.1%), three *S. warneri* (1.6%), and one *S. lugdunensis* (0.5%).

3.2 Detection of *mecA* gene and characterization of SCC*mec*

The *mecA* gene was detected in 80.9% of all CoNS, in 82.4% of *S. epidermidis* specimens, 94.1% of *S. haemolyticus* specimens, 66.7% of *S. hominis* specimens, 75.0% of *S. capitis* specimens, and in the sole *S. lugdunensis* specimen isolated. SCC*mec* was characterized in CoNS specimens presenting *mecA* gene, with SCC*mec* Type I being found in 32 specimens (21.1%), Type II in nine specimens (5.9%), Type III in 92 specimens (60.5%), and Type IV in 19 specimens (12.5%). Distribution rates for different SCC*mec* types varied among CoNS species. For total SCC*mec* Type I, 59.3% were detected in *S. epidermidis*, 18.7% in *S. haemolyticus*, 18.7% in *S. hominis*, and 3.1% in *S. capitis*; for SCC*mec* Type II, 66.6% were detected in *S. haemolyticus* and 33.3% in *S. epidermidis*; for SCC*mec* Type III, 88.0% were detected in *S. epidermidis*, 4.3% in *S. haemolyticus*, 4.3% in *S. hominis*, 2.2% in *S. capitis*, and 1.0% in *S. lugdunensis*; whereas SCC*mec* Type IV was detected in *S. epidermidis* only.

Table 1 Determination of *mecA* gene and SCC*mec* detection in CoNS specimens from blood culture isolates.

CoNS species	*mecA* (%)	SCC*mec* types			
		Type I *n*=32	Type II *n*=9	Type III *n*=92	Type IV *n*=19
S. epidermidis (n=148)	82.4	59.3%	33.3%	88.0%	100.0%
S. haemolyticus (*n*=17)	94.1	18.7%	66.6%	4.3%	0.0%
S. hominis (*n*=15)	66.7	18.7%	0.0%	4.3%	0.0%
S. capitis (*n*=4)	75.0	3.1%	0.0%	2.2%	0.0%
S. warneri (*n*=3)	0.0	0.0%	0.0%	0.0%	0.0%
S. lugdunensis (*n*=1)	100.0	0.0%	0.0%	1.1%	0.0%

n= number of CoNS specimens

3.3 Determination of minimal inhibitory concentration (MIC)

Minimal inhibitory concentration (MIC) was determined for all CoNS species, as Fig. 1 shows. MIC_{50} (concentration inhibiting 50% of CoNS specimens) and MIC_{90} (concentration inhibiting 90% of CoNS specimens) were determined for antimicrobials tested. MIC_{50} and MIC_{90}, as shown on Table 2, were respectively 2 and >256 μg/mL for oxacillin, 1.5 and 2 μg/mL for vancomycin, 0.25 and 0.5 μg/mL for linezolid, 0.094 and 0.19 μg/mL for daptomycin, 0.19 and 0.38 μg/mL for quinopristin/dalfopristin, and 0.125 and 0.38 μg/mL for tigecycline.

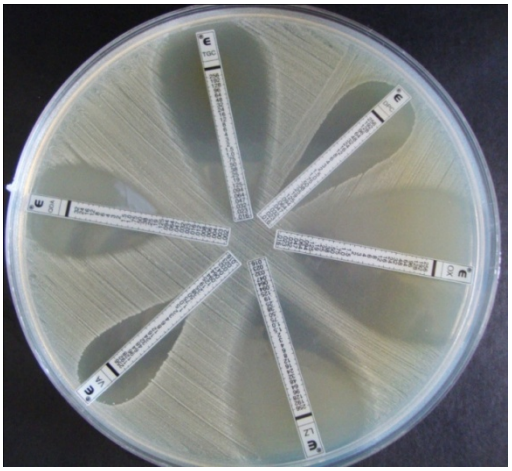

Fig. 1 Determination of minimal inhibitory concentration in CoNS specimens from blood culture isolates for oxacillin, vancomycin, linezolid, daptomycin, quinopristin/dalfopristin, and tigecycline.

Table 2 Determination of minimal inhibitory concentration in specimens of coagulase–negative staphylococci from blood culture isolates.

ANTIMICROBIAL	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)
Oxacillin	2	>256
Vancomycin	1.5	2
Linezolid	0.25	0.5
Daptomycin	0.094	0.19
Quinopristin/Dalfopristin	0.19	0.38
Tigecycline	0.125	0.38

MIC_{50}: Antimicrobial concentration inhibiting 50% of bacterial growth
MIC_{90}: Antimicrobial concentration inhibiting 90% of bacterial growth

3. Discussion

The relevance of CoNS in hospital settings has increased over the last years, due chiefly to their increasing resistance rates to antimicrobials used for treatment of infection related to these organisms. In this study the *mecA* gene was detected in 80.9% of CoNS, with species examined showing high oxacillin–resistance rates, except for *S. warneri*, shown to be negative for *mecA* gene. Similar results were found by Michelin *et al* [14], who observed resistance to oxacillin among 80% of CoNS isolated from clinical materials. Krediet *et al* [15] reported an oxacillin–resistance rate of 97% among *S. epidermidis* specimens from a neonatal intensive care unit between 1999 and 2001. According to Ferreira *et al* [16] approximately 96% of *S. haemolyticus* specimens isolated in Brazil are resistant to oxacillin, and high resistance rates have been reported for *S. hominis*: 100% in 21 specimens isolated in a Spanish neonatal ICU [17].

S. capitis has shown high resistance rates to oxacillin, with three of four specimens isolated showing positive for *mecA* gene, unlike Caierão *et al* [18], who detected in their study the gene *mecA* in 33.3% of *S. capitis* specimens. The *mecA* gene was first described for *S. lugdunensis* in a study conducted by Kawaguchi *et al* [19] who detected this gene in one of two specimens isolated in a neonatal unit. According to Tee *et al* [20],

identification of *S. lugdunensis* is of importance not only because of its clinical implications, since it is the most aggressive CoNS species, but also for determination of susceptibility to oxacillin in order to ensure early treatment with proper antibiotics and good clinical results.

SCC*mec* characterization enabled distinction of Types I, II, III, and IV for oxacillin–resistant CoNS specimens studied. SCC*mec* Types I, II, and III, the most commonly found in hospital settings, were also the most frequently characterized, with Type III being the leading one (60.5%), identified in all species presenting *mecA* gene. Whilst SCC*mec* Type I carries no resistance genes except for *mecA*, Type II does carry multiple resistance genes, and is related to staphylococcal strains that have begun to prevail in the 1980s [7]. SCC*mec* Type III is the largest of the remaining types (66,896 bp) and codes for the highest number of resistance genes; it is a major nosocomial pathogen and causes serious infection [8].

SCC*mec* Type IV was characterized in 12.5% of oxacillin–resistant CoNS, detected in *S. epidermidis* only. Ibrahem *et al* [21] have described SCC*mec* Type IV for 37% of *S. epidermidis* specimens in their study, with this type being the most prevalent amongst *S. epidermidis* strains. According to Wisplinghoff *et al* [22], SCC*mec* Type IV was first described for *S. epidermidis*, suggesting transfer of SCC*mec* Type IV from *S. epidermidis* to *S. aureus*. Its smaller size as compared to nosocomial types probably increases its mobility and ability to transfer among specimens [23], suggesting that conditions related to clones containing SCC*mec* Type IV tend to become more frequent [13].

Minimal inhibitory concentrations for antimicrobials were analyzed in the specimens studied. A high oxacillin–resistance rate was found for CoNS specimens (82.5%), with MIC_{50} and MIC_{90} as high as 2.0 and >256 μg/mL, respectively. All specimens showed sensitivity to vancomycin, however MIC_{90} values were higher than those for the remaining antimicrobials tested, except for oxacillin. The remaining antimicrobials tested showed satisfactory activity on CoNS specimens, therefore they might be alternatives for treatment of infection related to these organisms, including oxacillin–resistant CoNS.

In a recent study, linezolid was compared to vancomycin for the treatment of patients with pneumonia caused by methicillin–resistant *S. aureus*, and shown to be safer and more effective than vancomycin [24]. *S. epidermidis*, according to Otto [25], is seldom resistant to linezolid and tigecycline. Linezolid has turned out to be important for the treatment of protracted CoNS infection, whereas tigecycline, a tetracycline derivative, shows excellent activity against oxacillin–resistant, as well as susceptible, CoNS [26]. Genes coding for resistance to these antimicrobials are mediated by plasmids, and more frequently found in methicillin–resistant strains, often occurring in nosocomial strains undergoing higher selective pressure [25].

Daptomycin is an antimicrobial drug that has been studied for decades and used since 2003 for the treatment of skin and soft tissue infection, showing activity similar to that of vancomycin for the treatment of staphylococcal bacteremia [27]. In a study by Critchley *et al* [28], encompassing 1126 CoNS, daptomycin was shown to be active within a MIC range of 0.015–2.0 μg/mL and MIC_{90} of 0.5 μg/mL . According to the authors, daptomycin and quinopristin/dalfopristin have been the most active antimicrobial agents in this analysis, which highlights the importance of these drugs for the treatment of CoNS–related conditions.

High antimicrobial resistance rates of CoNS are a reflection of excessive use of antibiotics and the human commensal status of these organisms, making them ideal carriers and reservoirs for resistance genes, especially those not imposing high adaptive cost to bacteria, such as SCC*mec* elements [25]. According to the authors, *S. epidermidis* provides a reservoir for genetic element transfer to *S. aureus*, thereby increasing the pathogenic potential of these organisms and playing a major role in human clinical conditions related to *S. aureus*.

Acknowledgements The support São Paulo Research Foundation (FAPESP) and National Council for Scientific and Technological Development (CNPq) is gratefully acknowledged .

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Microbiological effect of repeated antimicrobial photodynamic therapy on subgingival periodontal pathogens in chronic periodontitis

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Periodontitis is a chronic inflammatory disease of the supporting structures of the teeth. The main approach to treating periodontitis involves the removal of supragingival and subgingival plaque biofilm by means of mechanical debridement. The aim of our study was to evaluate the microbiological effect of repeated antimicrobial photodynamic therapy (aPDT), which followed mechanical debridement, on subgingival biofilm composition in chronic periodontitis. Fourteen patients with chronic periodontitis were included in our preliminary study. All patients were free of systemic disease, non-smokers and with plaque index < 20%. At the baseline the following periodontal parameters were evaluated at six sites per tooth: plaque index (PI), pocket probing depth (PPD), bleeding on probing (BOP), and clinical attachment level (CAL). Control measurements were performed 3 months after the treatment. Ultrasonic scaling was done on all teeth supra- and sub-gingivally. Patients were divided in 2 groups. The control group was composed of patients receiving only ultrasonic scaling. In the treated group, ultrasonic scaling was followed by 3 aPDT sessions (day 1, 3 and 7 after mechanical debridement) using diode laser HelboTeralite 660nm with Helbo 3-D pocket probe and Helbo-Blue photosensitizer. In each patient, 4 samples of subgingival biofilm were collected, 1 from each quadrant. The first 2 out of 4 samples were from moderate pockets (4-5 mm) and the second 2 samples from deep pockets (≥6 mm). Biofilm samples were collected with sterile paper points at the baseline, 1 week (after the last aPDT session), and 3 months after the treatment. Microbiological analysis of *A. actinomycetemcomitans (A.a.), P. gingivalis (P.g.), P. intermedia (P.i.), T. forsythia (T.f.) and T. denticola (T.d.)* was performed with the Micro-Ident ® Plus test. The results were statistically analysed using ANOVA and $\chi 2$ tests. No differences in any of the investigated parameters were observed at the baseline between the two groups. On day 7 after the treatment, the microbiological analysis showed a statistically significant reduction in all 5 periodontal pathogens in moderate and deep pockets ($p<0.05$) in both groups. However, 3 months after the treatment, the reduction of *A.a.* and *P.i.* in the control group and the reduction of *P.i., T.f.,* and *T.d.* were observed in the treated group. The additional application of aPDT to ultrasonic scaling failed to result in further improvement in terms of PPD reduction and CAL gain, however, it resulted in a higher reduction of BOP compared to ultrasonic scaling alone.

Keywords chronic periodontitis; antimicrobial photodynamic therapy; periodontal pathogens

1. Introduction

Periodontal diseases are infections that are caused by microorganisms that colonize the tooth surface at or below the gingival margin. G-negative anaerobes, bacteria such as *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis and Tannerella forsythia* play a major role in periodontal tissues destruction [1]. The main approach to treating periodontitis involves the removal of supra- and subgingival plaque biofilm by means of mechanical debridement. Studies have shown comparable clinical outcomes between the use of hand instruments or ultrasonic scalers for mechanical debridement [2]. Despite the improvement in terms of clinical parameters, neither of these techniques removes bacterial plaque entirely [3]. In the last decade antimicrobial photodynamic therapy (aPDT) with diode laser as an adjunctive to mechanical debridement demonstrated additional clinical benefits. Effectiveness of aPDT has been evaluated in several studies. This method contributes to improvement of clinical periodontal parameters, especially reduction of bleeding on probing [4-8]. Study of Theodora and co-workers [9] has not been able to approve effect on clinical periodontal parameters, however, there was a significant reduction in the number of periodontal pathogenic bacteria (*Aa, Pg, Pi, Pn* and *Tf*). The aim of our preliminary study was to evaluate the antimicrobial effect of repeated aPDT, which was performed after ultrasonic removal of supra-and subgingival plaque, on periodontal clinical parameters and microbiological composition of subgingival microbial flora in patients with chronic periodontitis.

2. Materials and methods

The study was approved by the Commission for medical ethics Republic of Slovenia (No.: 144/02/11). Our preliminary study included 14 patients of both genders, mean age of 51 years, with untreated chronic periodontitis. Only systemically healthy non-smokers, that were not taking antibiotics in the last six months before the examination and had plaque index < 20% were included in the study. At the baseline, periodontal clinical parameters (PI, PPD, CAL, BOP) were evaluated at 6 sites on each tooth. Control measurements were performed 3 months after treatment. All measurements were carried out by one experienced person (KP), intra-examiner calibration score was 0.80. Patients were divided into 2 groups. In control group (US) which included 6 subjects, supra-and subgingival plaque removal was performed using an ultrasonic scaler. Test group (aPDT) included 8 subjects, and these received combined treatment with an ultrasonic scaler, followed by 3 episodes of aPDT, 1st, 3rd and 7th day after the ultrasonic debridement. We used phenothiazinchloride as a photosensitizer (HELBO-Blue), and diode laser (Helbo TeraLite, 660nm, 60mW/cm2) with the probe, which was inserted into a periodontal pocket (HELBO-3D Pocket Probe). aPDT was performed on all teeth. In each of the 4 quadrants, subgingival plaque sample from 1 periodontal pocket was collected for microbiological analysis at the baseline, 1 week, and 3 months after the treatment. In each subject 2 samples from moderate (4-5 mm) and 2 samples from deep periodontal pockets (≥ 6 mm) were collected. The total number of samples was 72 in the control group and 96 in the test group. Microbiological analysis of subgingival plaque samples for 5 periodontal pathogenic bacteria *A. actinomycetemcomitans (A.a.), P. gingivalis (P.g.), P. intermedia (P.i.), T. forsythia (T.f.)* and *T. denticola (T.d.)* was performed with Micro-IDent ® plus test, which is based on the method of polymerase chain reaction (PCR). Statistical analysis of the data was performed using ANOVA and χ^2 test. Differences were considered statistically significant when the p value was less than 0.05.

3. Results

In both groups of subjects the improvement of all clinical parameters was found. The average probing depth (PPD) in the control group decreased from 3.41 +/- 0.76 mm at the baseline to 3.01 +/- 0.68 mm at 3 months after the treatment ($p < 0.05$). In the test group PPD improved from 3.55 +/- 0.54 mm at the baseline to 2.96 +/- 0.57 mm at 3 months after the treatment ($p < 0.05$). Among the groups there were no statistical differences. The average clinical attachment level (CAL) in the control group improved, from 4.13 +/- 1.07 mm at the baseline to 3.49 +/- 0.87 mm at 3 months after the treatment ($p < 0.05$). In the test group average CAL 3 months after the treatment improved from 4.19 +/- 0.88 mm to 3.69 +/- 0.91 mm ($p < 0.05$). Among the groups there were no statistical differences. In both groups we found reduction in bleeding on probing (BOP). In the control group, the percentage of bleeding sites decreased from 21.3% to 15.7%, the difference was 5.6%. In the test group, this percentage decreased from 24.9% to 13.1%, with the difference of 11.4%. In the same group before and 3 months after the treatment, and between the 2 groups after the treatment there was a statistically significant difference ($p < 0.05$).

In the control and test group of patients, 1 week after treatment, the presence of all 5 periodontal pathogenic bacteria statistically significantly decreased ($p < 0.05$), both in moderate (4-5 mm) and deep (≥ 6 mm) periodontal pockets. Three months after treatment, in moderate pockets of the control group statistically significant reduction of *A.a.* and *P.i.* ($p < 0.05$) was found, however the presence of *P.g., T.f.* and *T.d*. increased in comparison with the baseline value. In deep pockets of the control group, 3 months after the treatment, a significant reduction in the presence of *A.a.* and *P.i.* ($p < 0.05$) was found; *P.g.,* and *T.f*. returned to the baseline values, while the presence of *T.d* increased. In the test group, 3months after the treatment in moderate pockets statistically significant reduction of *T.f.* and *T.d.* ($p < 0.05$) was determined. Presence of *P.g.* returned to the baseline value, while *A.a.* and *P.i.* increased. In deep pockets of the test group 3 months after the treatment, the presence of *P.i., T.f.* and *T.d.* significantly decreased ($p < 0.05$). Presence of *A.a.* and *P.g.* returned to the baseline values (Table 1).

Table 1. Presence of bacteria in moderate (4-5 mm) and deep (≥6 mm) periodontal pockets at baseline and 3 months after the treatment.

	US				aPDT			
	Baseline		3 months		Baseline		3 months	
PD (mm)	4-5	≥6	4-5	≥6	4-5	≥6	4-5	≥6
A.a.	16.6	25.0	8.3*	12.5*	19.1	26.7	23.8	26.7
P.g.	58.3	62.5	66.7	62.5	33.3	53.3	33.3	53.3
P.i.	33.3	62.5	25.0*	50.0*	14.3	33.3	19.1	26.7*
T.f.	66.7	100.0	91.7	100.0	71.4	86.7	52.4*	73.3*
T.d.	53.3	87.5	75.0	100.0	52.4	80.0	42.9*	66.6*

Numbers represent percentage of the sites positive for each tested bacteria.

*p<0.05

4. Discussion

This preliminary study showed that treatment with US as well as the combined treatment with US and aPDT leads to improvement of all observed clinical parameters. Combined treatment, if compared to US alone, has not been proven superior in reducing PPD or increasing CAL, but statistically significant reduction in BOP was confirmed. Studies from Wainwright [10] and Konopka [11] demonstrated bactericidal effect of diode laser and thereby reduced inflammation and accelerated healing of periodontal tissue after the initial periodontal treatment. Contemporary periodontist is focused on minimally invasive treatment methods to replace invasive surgical treatment. These alternative approaches, compared to surgical periodontal treatment, allow minimal manipulation of the periodontal tissues and, consequently, less post operative gingival recession. In addition, the recovery is faster after the treatment, and the treatment costs reduced [12, 13]. In our study we compared two minimally invasive methods of treatment of periodontal disease, namely US and combined US with repeated aPDT. Repeated aPDT was carried out in three sessions, 1st, 3rd and 7th day after ultrasonic debridement was completed. This protocol was selected to prevent bacterial recolonization at three critical stages. After removal of the supra-and subgingival plaque, G-positive coccus *S. oralis* reached the baseline level in one single day. *S. oralis* is a numerically important member of the commensal oral microbiota, isolated from all intra-oral surfaces and a pioneer organism involved in the colonization of the primary dentition [14]. Surface receptors on the deposited G-positive cocci and rods allow subsequent adherence of G-negative organisms, which otherwise express only poor ability to attach directly to the pellicle. Therefore *F. nucleatum* and *P. intermedia* also reach the baseline values third to fourth day after removal of plaque. On the seventh day, the baseline values are again exceeded [15]. Three months after aPDT, we found significant reduction in presence of three periodontal pathogenic bacteria (*P.i., T.f.* and *T.d.*) in moderate and deep periodontal pockets. US debridement 3 months after the treatment was observed to reduce two different periodontal pathogenic bacteria (*A.a.* and *P.i*). Other studies have shown that the effect of aPDT on microbial flora is contradictory. Some studies did not show any impact of aPDT on microbial flora [16-18]. Positive effect of a single aPDT has been approved by Theodoro and co-workers, who found significant reduction of five periodontal pathogenic bacteria (*A.a., P.g., P.i., T.f.* and *P.n.*) 6 months after the treatment [9]. Cappuyns and co-workers also described significant reduction of three periodontal pathogenic bacteria (*P.g., T.f.* and *T.d.*) 6 months after a single aPDT treatment [8].

Further research is planned with a greater number of patients included and maintenance treatments of periodontal disease scheduled for every 3 months for 1 year. At each visit, periodontal parameters will be recorded. Subgingival plaque samples will be collected also at 6 months after the treatment.

Acknowledgment The diode laser, photosensitizer, and fiber optic applicator used in this study were donated by Bredent – Medical, Germany.

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Molecular dynamics of synthetic polymers interference with viral biopolymer targets pretested via docking

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Antiviral design of alicyclic modified synthetic polyelectrolytes (**ASP**) resulted in effective inhibitors of human immunodeficiency virus type 1 (**HIV-1**) entry. Recently, the ASP interactions with key mediator of HIV-1 entry by fusion (tri-helix core of heptade repeat regions [**NHR**]$_3$ of viral envelope protein gp 41) were pre-studied via docking. In this work the docking results are verified under molecular dynamics (**MD**) modelling. In comparison with docking MD allowed of using more larger models of the polymeric ligands (approximating to real size of the synthetic polymers) and considering flexibility not only of ligands but of the [NHR]$_3$ target as well. For verification of probability of binding of the polymer with the ligand in the sites, predicted by doking, various starting positions of polymer chains were tried. The MD simulations confirmed the main docking-based conclusions. Newly discovered dynamics aspects of the ligands-target interfere clarify mechanisms of the ASP anti-HIV activity and drug resistance prevention. The obtained knowledge may be useful basis for novel anti-(microbial/viral) drug design and development.

Keywords drug resistance prevention; antivirals, HIV, influenza, nano-responcible poly-ligands, adamantan

Introduction

The first heptade repeat region (HRI, the **NHR**) tri-helix core, [NHR]$_3$, of human immunodeficiency virus type 1 (**HIV-1**) envelope glycoprotein gp41 is a key mediator of the virus entry into a cell via fusion (Fig. 1).

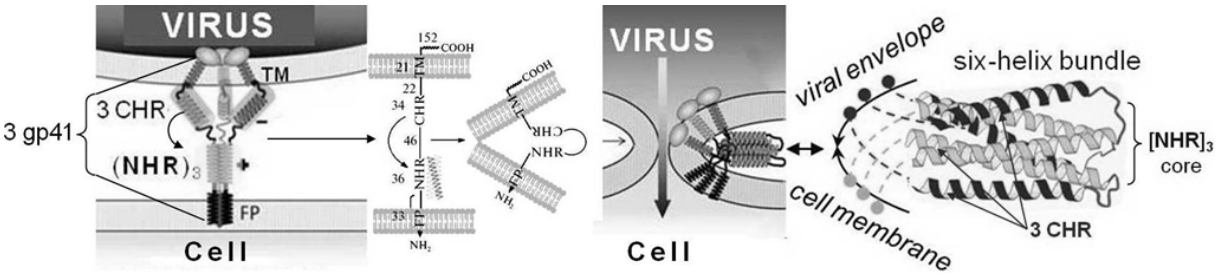
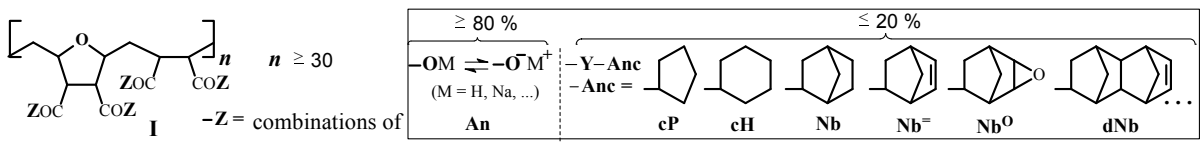


Fig. 1 Pre-fusion adsorption of HIV-1 virion on a permissive cellular membrane (with CD4 and CCR5/CXCR4 receptors) exposing the three gp41 molecules. Their hydrophobic N-terminus (FP)$_3$ anchored in lipid matrix of cell membrane, while the C-tails (3TM) are bounded in the virus envelope. Ectodomains between the FP and TM came into self-aggregation forming tri-helix intermediate (3 NHR → [NHR]$_3$) that becomes a core for consecutive hairpin-like folding with contra-charged 3 CHR. The resulted collapsing leads to the fusion, which can be prevented via therapeutic blocking the [NHR]$_3$ + 3 CHR contacts.

This fusion mediator represents itself a nano-scale (5.1 × 2.5 nm) protein-type object. It is intensively studied as one of the most promising targets for a therapeutic intervention against HIV-infection to prevent the HIV-1 caused acquired immunodeficiency syndrome (**AIDS**). Recently, applying a novel algorithm of step-by-step flexible docking of synthetic polymers to biopolymers [1,2], we modelled interactions between the [NHR]$_3$ and polymeric compounds of the following generic formula **I**, earlier discovered as a high-effective inhibitors of the HIV-1entry [3].



Several levels of multi-point binding with the [NHR]$_3$ were determined via the docking of models (ligands) of the polyanions **I** modified by various side-group anchors (**Anc**). However, the docking procedure used for

polymer-polymer interaction modelling mainly takes into account conformational flexibility of ligands only, while the target is considered as a crystal-like rigid structure. The docking also limits the possible size of ligand models by relatively short fragments of the real polymeric chains. Thus, a suitable for simulation via Dock-6.4 program degree of polymerisation was limited by oligomer scale of n ≤ 6. Molecular dynamics (**MD**) allows of overcoming this restriction. In the current article we briefly resume the docking result, and demonstrate how the docking data can be applied for consequent deeper polymer-polymer interaction modelling with the help of MD computational experiments. The obtained from MD results are compared with the docking ones.

Experimental

The suggested and applied docking methodology was described in detail on our previous reports [1-3]. The MD was carried out using Amber 8 software. To calculate interatomic interaction energy required the atoms parameters were taken in the GAFF (general AMBER force field) for polymers, and for the target - in the ff03 force field. Influence of the solvent media was taken into consideration by two ways: 1. Implicitly for folding into coil, with application of Hawkins-Cramer-Truhlar (HCT) model within GB/SA(Generalized Born/Solvent-Accessible Surface Area) formalism with the addition of 0.1 M NaCl (dielectric constants of 1(interior) and 80 (exterior)). 2. Explicitly, using the TIP3P model of water with periodic boundary conditions and calculation of coulomb interactions of atoms in cells with self-images by Ewald method. The SHAKE algorithm was applied to constrain the bonds to hydrogen atoms, that allowed to use a 2 fs step. To control the temperature Langevin thermostat was used with the collision frequency of 1 ps-1. For trajectory analysis (of both full polymer macromolecule and its sub-molecular fragments) at every computation step the following parameters were determined: (1) quantity of contacts of the side groups and backbone with the target, (2) functionally active elements of the side groups-target distance, (3) contributions in to the total binding energy of the partial energies of: van der Waals, coulomb, solvation and the solvent accessible hydrophobic surface, as well as quantity of H-bonds. Additionally a statistic analysis of the target amino acids contacts with the ligand models (and their fragments) was performed.

Results and Discussion

1. Docking

Step-by-step docking of ligands **I** from separate fragments of backbone and side-groups (step 1) to oligomeric motifs of polymeric chain (***n*** → 6, step 2) with theoretical extrapolation toward the real synthetic polymers degree of polymerization (***n*** → 30-50) in interaction with the $[NHR]_3$ target lead to following results (Fig. 2).

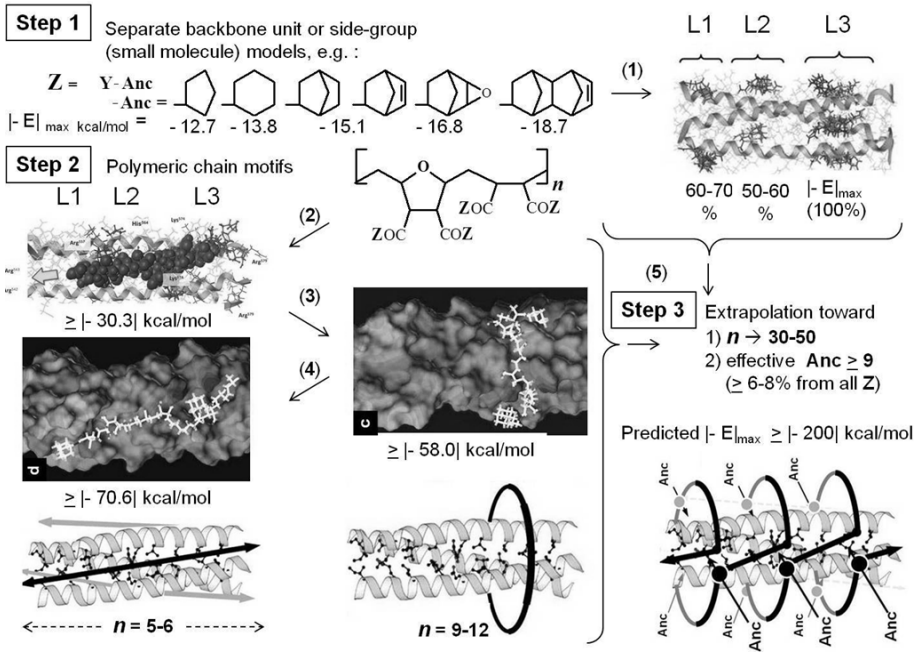
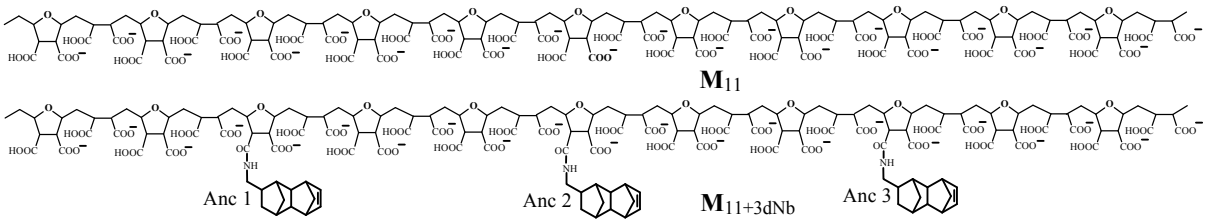


Fig. 2 **Step 1** models, e.g. (1), bind the target within the tree levels (**L1-L3**) of triplet cavities around target, preferably the deepest cavities (pockets) at the L1, where the small molecular models of Anc possess maximal binding energies |-12.7| - |-18.7| kcal/mol (the dNb is the most active Anc). **Step 2** models provides more effective binding with capacity of both axial and belting orientation depending on absents (2) or presents (3)-(4) of active Anc and distances between the Anc along the polymeric chain. **Step 3** extrapolates the steps 1-2 results toward the real size of polymers **I** chains predicting possibility for significant combined binding, E ≥ |-200| kcal/mol.

2. Molecular dynamics

Taking into account the previous docking results, the following models $\mathbf{M_{11}}$ and $\mathbf{M_{11+3dNb}}$, a model of the most anti-HIV active compound among the copolymers **I** [1,2]:



were used as representative fragments of the Anc-free and Anc-containing polymeric chains, respectively. In accordance with the docking result eleven monomers should be enough for both axial (***n*** = 5-6) and one-level belting (***n*** = 9-12) modes of the target binding. In case of $\mathbf{M_{11+3dNb}}$ existence of only three active Anc(s) (we modelled the most active Anc = dNb) allows of them to occupy the three-level (L1-L3) cavities along the target α-helix (for axial connection) and triplet cavities around the every level (for the belting) be occupied. To verify the binding sites and orientations defined via docking, the MD modelling was performed using various starting positions of the polymeric chain: (1) the $\mathbf{M_{11}}$ (1a) and $\mathbf{M_{11+3dNb}}$ (1b) linearly unfolded along the target; (2) the $\mathbf{M_{11+3dNb}}$ oriented transversely to the target; and (3) the $\mathbf{M_{11+3dNb}}$, preliminary folded in coil, contacting the target nearly the L1 pocket (3a) or similar position (3b), where the Anc 1 was buried in the target pocket. The positions (1b), (2), and (3a), illustrated by MD snapshots at the following points in time within 80 ns, are shown in Fig. 3 a), b), and c), respectively.

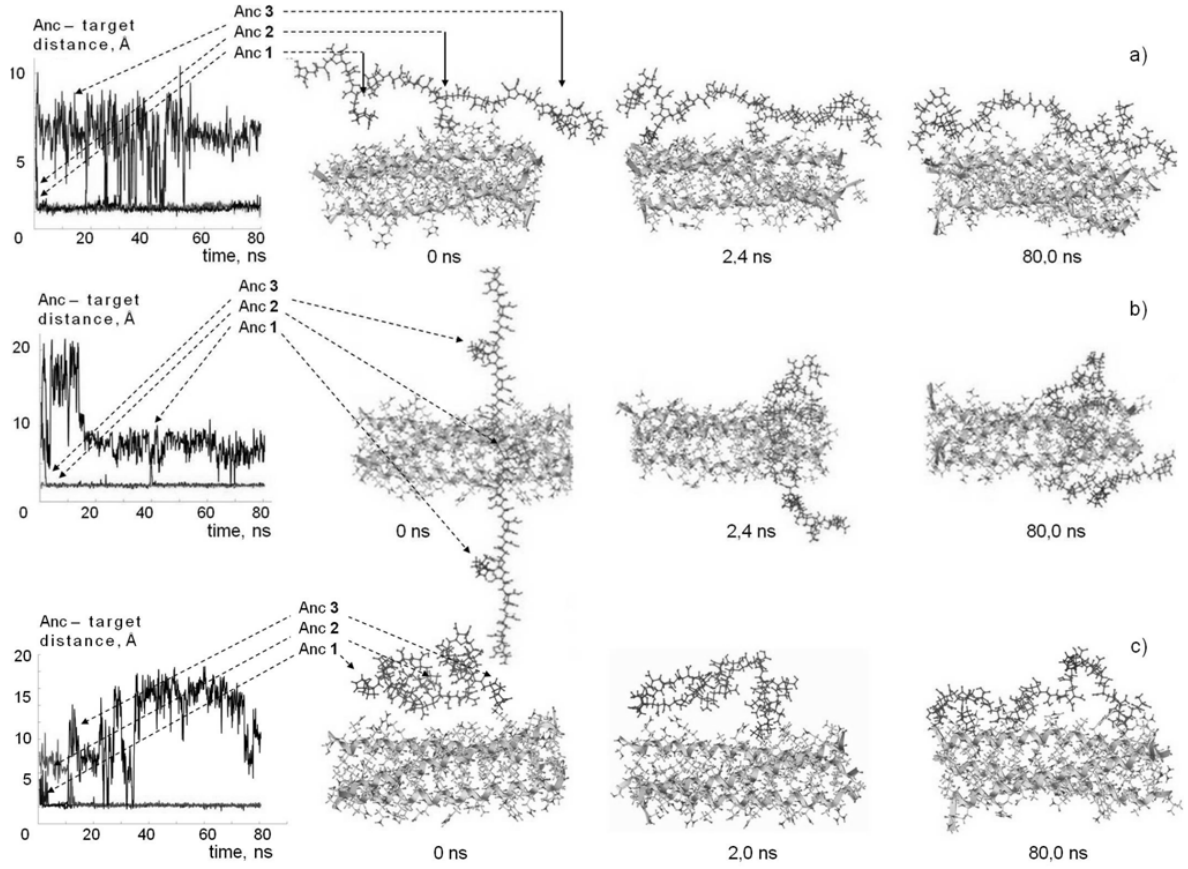


Fig. 3 MD-simulated interactions between the $\mathbf{M_{11+3dNb}}$ and the $[NHR]_3$ target for the different starting positions: a), b), and c). On the left - a dynamics of every Anc – target distances, and on the right - snapshots.

The MD evaluation of the cases (1a) and (1b) (Fig. 3a)) from axial starting position evidently confirmed the docking-predicted capacity of both anchor-free and anchor-containing polymeric ligands **I** to be effective binding agents in axial direction along the target helix within all three levels (L1-L3) of cavities. An analogous result was observed if instead of the unfolded chain $\mathbf{M_{11+3dNb}}$ its coil-type conformation was tested, see Fig. 3c. On the contrary, a transversely oriented starting position (2) of unfolded $\mathbf{M_{11+3dNb}}$ chain located near L1 pockets of target possessed tendency to belt the target intensively covering at least two helices exactly at the L1 pockets region (Fig. 3b)). This result also correlates with docking-predicted belting mode of interaction if the polymeric chains **I** are equipped by active anchors (Fig. 2). More detailed understanding of the ligand-target interaction

under various starting conditions can be extracted from a statistical analysis of the MD-simulated multipoint contacts between structural components of ligand and amino acids of target. The corresponding examples are represented in Fig. 4 and Table 1.

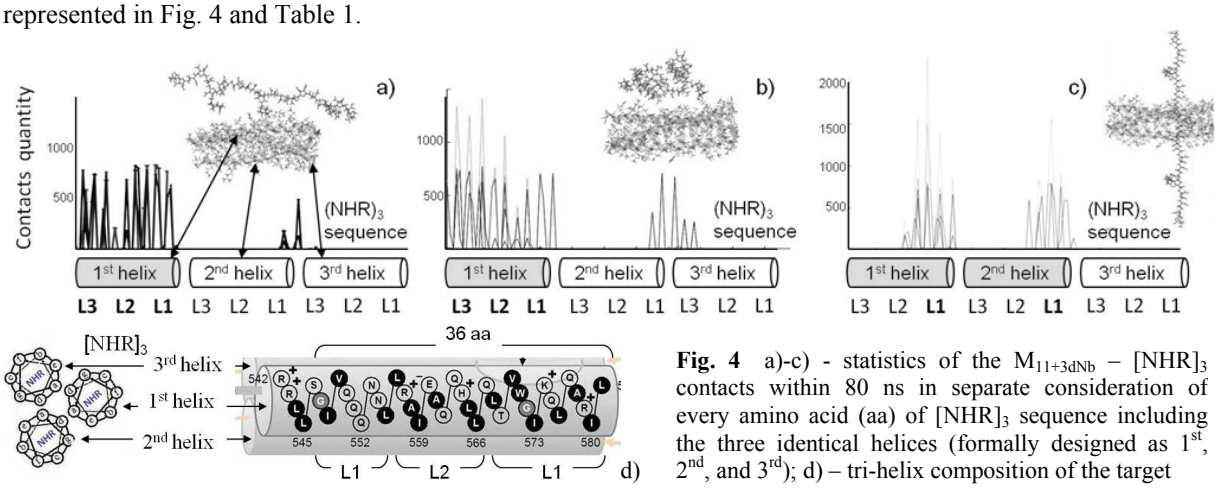


Fig. 4 a)-c) - statistics of the $M_{11+3dNb}$ – $[NHR]_3$ contacts within 80 ns in separate consideration of every amino acid (aa) of $[NHR]_3$ sequence including the three identical helices (formally designed as 1st, 2nd, and 3rd); d) – tri-helix composition of the target

The Fig. 4 a) and b) visually demonstrate that starting cases (1b) and (3a) gave the similar results: majority of the ligand-target contacts are concentrated along full-length (L1-L3) single (1st) helix. From the Table 1 data we can explain this situation in terms of the contacts distribution for the ligand components (backbone units (**BU**) and Anc(s)) with the target helices and L1, L2, L3 sub-helical levels of cavities. At the (1b) and (3a) starts of $\mathbf{M_{11+3dNb}}$, as well as at the (1a) start of anchor-free $\mathbf{M_{11}}$, the most intensive contacts are located mainly along the 1st helix L1-L3 levels, where the L1plays a leading role (% of total contacts): 63.0 (1a), 55.1 (1b), and 37.1 (3a).

Table 1 Statistic data of the ligand – target contacts quantity for partial contributions of $[\text{-BU-}]_n$ and every Anc(s)1-3 of the ligands to connections with the target amino acids of the levels (L1-L2) of every NHR helixes.

Target			Ligands												
			M_{11}	M_{11+dNb}											
NHR helix	level	amino acids	(1a)*	(1b)*				(2)*				(3a)*			
			BU	BU	Anc1	Anc2	Anc3	BU	Anc1	Anc2	Anc3	BU	Anc1	Anc2	Anc3
1	L3	4-9	1059	1441	2104	0	0	0	0	0	0	2257	2177	0	0
	L2	11-20	1051	896	387	659	0	408	0	3	174	1604	597	2468	70
	L1	22-36	4039	4975	0	2279	50	3002	0	2999	2167	2792	0	0	283
	sub-total		6149	7312	2491	2938	50	3410	0	3002	2341	6653	2774	2468	353
2	L3	4-9	0	0	0	0	0	0	0	0	0	0	0	0	0
	L2	11-20	0	0	0	0	0	1	0	0	0	0	0	0	0
	L1	22-36	0	30	0	0	0	3925	0	2798	0	1862	0	0	0
	sub-total		0	30	0	0	0	3926	0	2798	0	1862	0	0	0
3	L3	4-9	213	10	481	0	0	0	0	0	0	0	536	0	0
	L2	11-20	54	0	0	0	0	0	0	0	0	0	0	0	0
	L1	22-36	16	0	0	0	0	10	0	0	0	0	0	0	0
	sub-total		283	10	481	0	0	10	0	0	0	0	536	0	0
		total	6432	13312				15487				14646			

* - various starting ligand – target positions, see above.

Moreover the L1 pockets of target became fully dominant region for contacts with $\mathbf{M_{11+3dNb}}$, providing 96.2 % of total contacts (Table 1) if this ligand model started from the transversal position (3a) located near the L1. Therefore, the MD confirms the docking-predicted role of the L1 pockets of target as a general locus sensitive to the ligands **I** attacks. In addition to the docking findings the MD simulations discovered that the contacts distribution between the L1-L3 levels depended on the starting positioning of ligand toward the target. The MD-based statistics demonstrated also important peculiarities determined by the acidic (hydrophilic and H-bond capable) backbone units (BU) and side-grafted hydrophobic Anc units, estimating a relative contributions of the both structural species into the ligand potency to bind the target. As we can see in Table 1, the fully acidic model $\mathbf{M_{11}}$ (containing 44 acidic groups, 100%) is capable of 6432 contacts (start (1a), 80 ns) while the derived model $\mathbf{M_{11+3dNb}}$, where only 3 from 44 (7%) side groups are substituted by Anc = dNb, demonstrates twice more intensive contacting with the target (up to 13312 contacts under start (1b)). The same enhanced contacting potency of the Anc-containing $\mathbf{M_{11+3dNb}}$ was recorded for other starting conditions: (2) and (3a) (Table 1). These MD data are in high correlation with docking data and results of *in vitro* evaluation of anti-HIV efficiency of

real polymeric samples (where **ASP** containing the dNb anchor was 12 folds more anti-HIV-1 active, than it's anchor-free analogues [1,2]). Moreover, both docking and MD defined identical main sites of binding: Arg^{579}, Lys^{574}, and Arg^{557} – ionisable points for the acidic BU binding; Ala^{578}, Trp^{571}, Val^{570}, ... – hydrophobic sensors for optimal contacts with Anc(s). Together these results support a conclusion about multipoint binding capacity of the copolymers **I** derivatives due to cooperation of acidic BU with additional side-chain Anc(s) in connection with the target. Besides the above suggested statistics even more interesting aspect (undetectable via docking) is evolution of the various contacts in dynamics. The diagrams of ligand Anc(s) – target distances dynamics of the $\mathbf{M_{11+3dNb}}$ model (Fig. 3) show that at least two from of three Anc(s) achieved stable contacts with target by first 1-3 ns (from the varied starts), keeping these contacts mainly over whole period of MD-simulation (80 ns). A comparative role of Anc and BU components in cooperative interaction with target become more clear from analysis of their contributions to the energy of the target binding (see, for instance, Fig. 5).

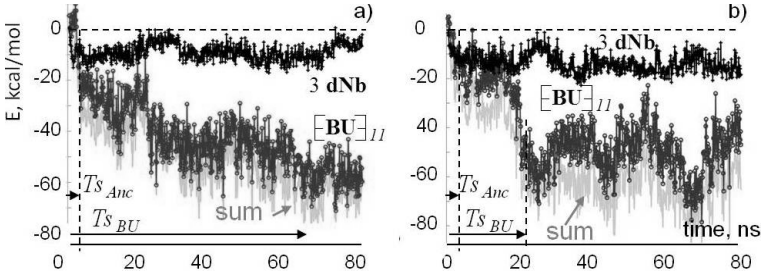


Fig. 5 $\mathbf{M_{11+3dNb}}$ – $[\mathbf{NHR}]_3$ binding energy dynamics for separated contributions of backbone ($[\text{-BU-}]_{11}$) and anchors (3 dNb), as well as integrated energy, for starts from: a) – axial (1b) and b) - transversal (2) positions.

The Anc(s) linked through flexible bridges to backbone are more mobile (than BU) agents for initial contacts with the targets. Their contributions to energy of binding with the target achieved the maxima during very short time ($T_s \leq 5$ns) while the same for the $[\text{-BU-}]_{11}$ required longer period of time ($T_s \geq 20$ns) (Fig. 5). Therefore, the Anc(s), being less energetically power than full polymeric backbone, nevertheless, play very important role providing starting contacts with the target, and involving BU(s) in subsequent contacts. On the other part multiple BU(s) stabilisation on the target surface leads to more stable Anc(s) – target contacts too. As result even those local contacts, which were estimated as week (unstable) in the docking of small-molecular fragments of polymers **I**, in MD of high-molecular models became more stable Pdue to the cooperation effects of polymeric chain. Locally lost contacts of any single Anc/BU with the target were reversibly restored in dynamics due to other (neighbouring) BU(s)/Anc(s) connection with target. The integrated binding energies were estimated via MD (depending on start position) as E_{min} (kcal/mol) = |-50|-|-70| (1b), |-50|-|-80| (2), and |-50|-|-70| (3a). Partial contributions in total binding energies of different forces (van-der-Waals, solvent accessible surface, hydration, and electrostatic with H-bonds recording) in relation with components of ligand molecule were analysed as well, and obtained results will be reported in the next publications.

Conclusions

1) The MD data confirmed the docking predicted role of the "main" triplet of hydrophobic pockets of the $[NHR]_3$ L1 as general sites for contacts with the studied synthetic polymers**.** 2) Similar to docking the MD demonstrated a significant affinity of the modelled polymeric structures to not only the L1 level pockets but to other cavities of the target L2 and L3 levels too. 3) The mutual cooperation of the multiple contacting with target side-anchors and the carboxylic chain backbone appeares in MD more clearly than in docking. The MD provides more opportunities for analysis of these contacts in dynamics. 4) Combination of the docking and MD methodology is the most efficient for study of features of macromolecular (in contrast with small molecular) mechanisms of blocking the protein-type mediators of HIV-1 entry (fusion step).

Acknowledgements The authors are thankful to Lilia Alkhanova for the reviewing this paper and helpful discussion.

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Nitric oxide releasing-iron oxide magnetic nanoparticle acting on isolated Bovine Mastitis *Staphylococcus aureus* and *Escherichia coli*

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The pharmacological uses of nanosystems as drug delivery platform have greatly increased in recent years due to the possibility to improve therapeutic efficacy, as well as to provide physical stability of the drug, and to reduce possible side effects. Nitric oxide (NO) is an important biomolecule involved in several physiological and pathophysiological processes. Our group has already reported the efficacy of NO donors as antibacterial and antileishmanicidal agents. In this present work, iron oxide magnetic nanoparticles were synthesized and used as vehicle to carry and to delivery NO in antibacterial applications, in particular against Bovine mastitis. The aim of this work was to evaluate the antibacterial activity NO-releasing magnetic nanoparticles on *Staphylococcus aureus and Escherichia coli* survivals.

Keywords: Magnetic iron nanoparticles; Mastitis; Nitric oxide

1. Introduction

The endogenous molecule nitric oxide (NO) is a free radical and it is involved in several physiological and pathophysiological processes [1]. Indeed, NO is a key molecule synthesized by the immunological system, and it is released by macrophages upon infection, acting as a biocidal agent [2]. The antibacterial efficacy of NO-releasing biomaterials was already reported [3]. However, as free radical, NO is ready inactivated in biological system. In this scenario, there is a great interest on the use of vehicles that can be used as NO carriers and donors in several biomedical applications [4]. Among these vehicles, nanostructured materials, such as magnetic nanoparticles appears as good candidates for drug delivery [5]. In particular, iron oxide magnetic nanoparticles comprised by magnetite (Fe_3O_4) are known to be biocompatible, super-paramagnetic at room temperature, with a great surface area that can be ready functionalized to carry and to delivery NO [5].

In this work, magnetic iron oxide nanoparticles comprised by Fe_3O_4 were synthesized by co-precipitation technique. The surface of nanoparticles was coated with the hydrophilic thiol-containing ligand, mercaptosuccinic acid (MSA) (Fig. 1).

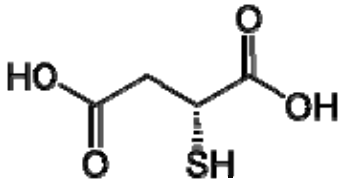

Fig. 1 Chemical representation of MSA used as coating for magnetic iron oxide nanoparticles.

Free thiols groups on the surface of nanoparticles were nitrosated leading to the formation of S-nitroso-magnetic nanoparticles. S-nitroso groups (S-NO) are known as spontaneous NO donors, due to the homolytic S-NO bound cleavage with free NO release [6].

The aim of this work was to evaluate the antibacterial activity of NO-releasing magnetic nanoparticles against two bacterial strains: *Staphylococcus aureus*(*S. aureus*) and *Escherichia coli* (*E. coli*) of Bovine mastitis infection. Bovine mastitis is an important disease of dairy farms typically caused by bacteria as *S. aureus* and *E. coli* strains [7]. This disease constitutes a serious problem in dairy herds with great economic losses, mainly due to reduced milk production [8].

2. Experimental part

2.1 Preparation of MSA-coating nanoparticles

Iron oxide magnetic nanoparticles were prepared through co-precipitation method by using ferrous and ferric chlorides in acid solution, and further coated with MSA (Fig.2)

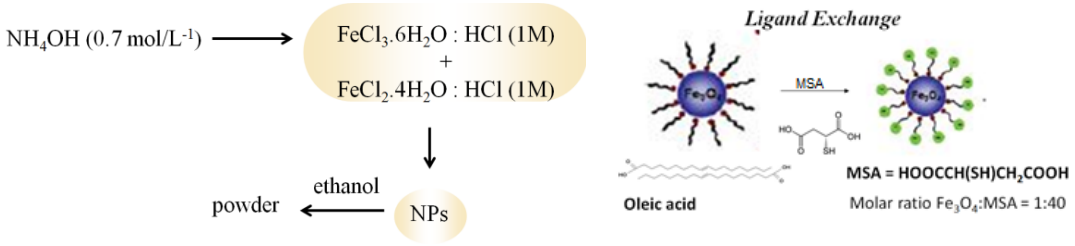

Fig.2 Synthesis of iron oxide magnetic nanoparticles and their functionalization with mercapto succinic acid (MSA).

Briefly, 4.0 mL of $FeCl_3 \cdot 6H_2O$ solution and 1.0 mL of $FeCl_2 \cdot 4H_2O$ solution (molar ratio 2:1), in 1.0 mol/L HCl, were mixed and stirred, while a volume of 50 mL of NH_4OH (0.7 mol/L) was added as precipitator. The solution was centrifuged and the precipitate was decanted, followed by the addition of 6.0 mL of oleic acid. This mixture was stirred for 20 minutes. The solution was centrifuged several times and the new precipitate was washed several times with ethanol and acetone, leading to nanoparticles covered with oleic acid. Oleic acid coated-nanoparticles (~10.0 mg) were dissolved in 1.0 mL of toluene while mercapto succinic acid (MSA) (molar ratio Fe_3O_4:MSA = 1:40) was dissolved in dimethyl sulfoxide (DMSO). A black powder was isolated by centrifugation. This procedure led to ligand exchange and, hence, to the formation of water stable thiol-containing iron oxide nanoparticles.

2.2 Nitrosation of thiol groups (SH) on the surface of MSA-iron oxide nanoparticles

Thiol groups (SH) on the surface of MSA-coated iron oxide nanoparticles were nitrosated by the addition of sodium nitrite ($NaNO_2$) leading to the formation of S-nitroso (SNO) nanoparticles, which are spontaneous NO donors. In this step, filtered MSA nanoparticles was suspended in deionised water. A volume of 200 μL of aqueous sodium nitrite (60 mmol/L) was added to the nanoparticle suspension. After 15 min of incubation at room temperature, the nanoparticle suspension was filtered by centrifugal ultrafiltration by using a Microcon centrifugal filter device containing ultrafiltration membranes (MWCO 10-kDa molar mass cut-off filter, Millipore, Billerica, MA, USA) and washed with deionised water to remove excess of unreacted nitrite.

2.3 Culture conditions.Bacterial strains

The two bacterial strains that were used were isolated from Bovine mastinis infection: *S. aureus* from Bovine mastitis (MBSA 4 and MBSA 19); and *E. coli* from Mastitis bovine (MBEC 01 and MBEC 10). All strains were cultured in Muller Hinton broth (37°C, 150 rpm, 18 h).

2.4 Antibacterial assay. Determination of Minimum Inhibition Concentration (MIC)

Both bacterial strains (*S.aureus and E. coli*) were incubated with thiol-coated iron oxide nanoparticles (MSA-NPs) and with S-nitroso iron oxide nanoparticles (SNO-MSA-NPs). The MIC values were obtained by CLSI, 2010 – microdilution method, and growth and dead curves assays were carried out by counting of CFU/mL for time (0, 2, 4 and 6 hours) at the concentrations of compounds (500 and 1000 μg/mL).

3. Results and Discussion

3.1 Synthesis of nanoparticles

MSA-coated iron oxide nanoparticles (Fig. 3) were prepared by co-precipitation method.

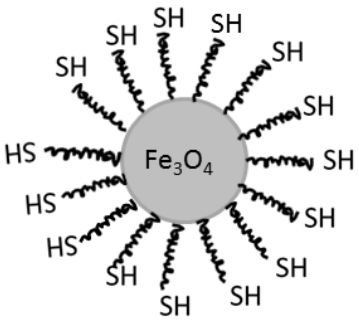

Fig. 3 MSA-coated magnetic iron oxide nanoparticles.

The presence of thiol-containing ligand (MSA) on nanoparticle surface is responsible to increase water-solubility of the nanoparticles, to provide thermal stabilization, and to avoid nanoparticles aggregation, moreover, it represents an important site for bioconjugation. Indeed, in this work, free thiol groups (SH) on the nanoparticle surfaces were used to load NO to the nanoparticle.

3.2 Nitrosation of thiol groups on MSA coated MNPs

Thiol groups on MSA - coated nanoparticles were nitrosated by the addition of sodium nitrite, leading to the formation of S-nitroso-magnetic nanoparticles (Fig. 4). In acidified aqueous solution, nitrite (NO_2^-) is in equilibrium with nitrous acid (HNO_2), which is considered the nitrosating agent of -SH groups leading to the formation of -SNO [3]. SNO moieties act as spontaneous NO donor, due to the homolytic S-N bond cleavage, with free NO release [3], as represented in Fig. 4.

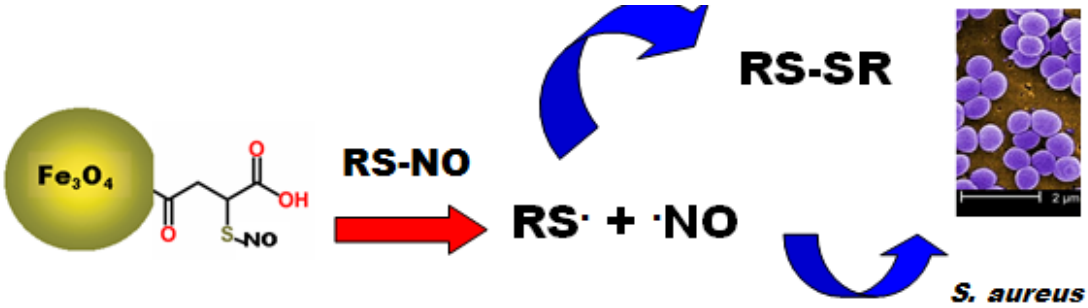

Fig. 4 NO-releasing from S-nitro iron oxide magnetic nanoparticles

3.3 Determination of Minimum Inhibition Concentration (MIC)

The range of concentrations of thiol-coated (Fe_3O_4-SH) and S-nitroso-coated (Fe_3O_4-S-NO) iron oxide nanoparticles incubated with bacterial strains were: MBSA 19 = 2500 µg/mL; MBSA 04 = 2500 µg/mL; MBEC 01 = >2500 µg/mL; MBEC 10 = >2500 µg/mL, where MBSA (*S. aureus*) and MBEC (*E.coli*).MBSA 4 is a subclinical mastitis and resistant to penicillin. MBSA19 is a subclinical mastitis and resistant to penicillin/erythromycin. MBEC 01 is a clinical mastitis and resistance not tested. MBEC 10 is a clinical mastitis and resistance not tested.

The MIC value were found to be the similar for both Fe_3O_4-SH and Fe_3O_4-S-NO nanoparticles, however growth and dead curve monitored at 2, 4 and 6 h of incubation indicate a bacteriostatic effect, in a shorter time measured for MIC values (i.e. 24 h) (see Table 1 and Fig.5).

Table.1. Growth and dead curves (CFU/mL) of MBSA 19 and MBEC 01 at different times of incubation (0, 2, 4 and 6 h) and nanoparticle concentrations (500 and 1000 µg/mL).

Bacterial strains and nanosystem	Incubation time			
	0 h	2 h	4 h	6 h
MBSA 19 NP (1000 µg/mL)	2.8×10^6	3.0×10^6	3.8×10^7	
MBSA 19 NP+NO (1000 µg/mL)	1.7×10^6	1.7×10^6	1.6×10^5	
MBSA 04 NP (500 µg/mL)	1.3×10^6	2.7×10^5	2.2×10^7	1.8×10^8
MBSA 04 NP+NO (500 µg/mL)	1.3×10^6	8.3×10^5	2.4×10^7	1.0×10^8
MBEC 01 NP (1000 µg/mL)	1.6×10^6	1.4×10^7	1.8×10^8	
MBEC 01 NP+NO (1000 µg/mL)	2.0×10^6	9.0×10^6	1.6×10^8	

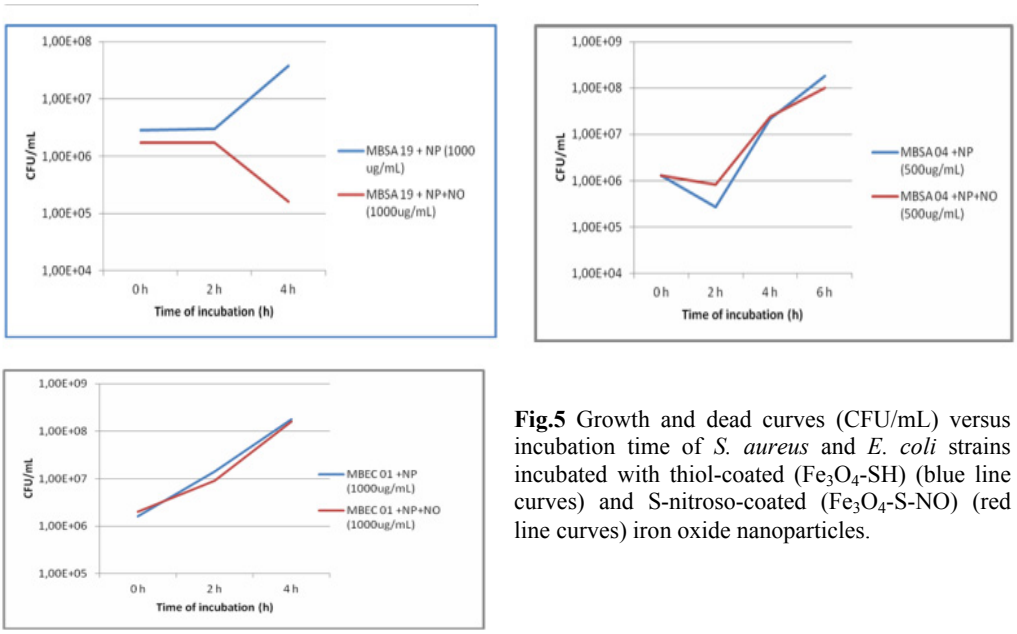

Fig.5 Growth and dead curves (CFU/mL) versus incubation time of *S. aureus* and *E. coli* strains incubated with thiol-coated (Fe_3O_4-SH) (blue line curves) and S-nitroso-coated (Fe_3O_4-S-NO) (red line curves) iron oxide nanoparticles.

Despite the observed high values of MIC for *S. aureus* and *E. coli* isolated from Bovine mastitis incubated with both Fe_3O_4-SH and Fe_3O_4-S-NO nanoparticles, the growth and dead curves for Fe_3O_4-S-NO nanoparticles explained the antibacterial effects of these compounds. In fact, the growth and dead curves showed that Fe_3O_4-S-NO iron oxide nanoparticles had anti-staphylococci effect, at the high tested concentration. After 4 hours of incubation of MBSA 19 strain at concentration of 1000 µg/mL of Fe_3O_4-S-NO, the number of CFU decreased 94% and it was found to be 200 fold lower than the control group (Fe_3O_4-SH). This difference in the number of UFC indicates that NO-releasing nanosystem can be an alternative approach for staphylococci control. However, antibacterial effect was not observed for *E. coli* strain (MBEC 01) for all tested nanoparticle concentrations. Taken together, these results showed that the concentration of NO-releasing nanoparticles and bacterial species are important factors on antibacterial actions. The biological activity of NO-releasing iron oxide nanoparticles on *S. aureus* reinforces the therapeutic potential of this NO-nanomaterial and opens new perspectives for further studies based on the use of NO-releasing nanoparticles as antimicrobial agents for the treatment of bovine mastitis caused by *S. aureus*.

4. Conclusions

The results are indicative that NO-releasing iron oxide magnetic nanoparticles (Fe_3O_4-S-NO) can be an alternative for the treatment of Bovine mastitis caused by *S. aureus*.

Acknowledgements The support from FAPESP, CNPq, INOMAT (MCTI/CNPq), Brazilian Network of Nanotoxicology (MCTI/CNPq), NANOBIOSS (MCTI/CNPq) are gratefully acknowledged.

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Phytochemical and antiinfective studies of *Alchornea cordifolia* leaf extracts and sub-fractions against bacteria isolates from wound infection.

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Wound Infection is an important cause of morbidity and mortality especially in surgical patients. Phytochemical analysis of the water and ethanol extracts, ethyl acetate and n-butanol sub-fractions of the ethanol extract was carried out. The antibacterial activity of the leaf extract and sub-fractions of *Alchornea cordifolia* against bacteria isolates from 150 patients with wound sites was investigated using disc diffusion and agar dilution methods. Phytochemical screening of the extracts/fractions revealed the presence of carbohydrates, cardiac glycosides, saponins, tannins and flavonoids. The diameter zones of inhibition showed by the extracts ranged between 24.8 mm and 17. 2 mm. Minimum Inhibitory Concentration (M. I. C.) of the extracts/fractions against organisms ranged from 3.125mg/ml to 100mg/ml. Studies on the rate of death/survival of the isolates in the ethyl acetate sub-fraction indicated that it was bacteriostatic at M. I. C. but bactericidal at higher concentrations. The results have therefore authenticated the claims of traditional healers in the use of the plant leaf in the treatment of wound infections.

Keywords *Alchornea cordifolia*; Wound infection; Antibacterial activity; Ethyl acetate Fraction.

1. Introduction

A wound is a breach in the skin and the exposure of subcutaneous tissue following loss of skin integrity which provides a moist, warm, and nutritive environment that is conducive to microbial colonization and proliferation. In spite of technological advances that have been made in wound management, wound infection has been regarded as one of the common nosocomial infections. Organisms commonly found in infected wounds include Gram positive cocci such as *S. aureus, Streptococcus spp,* Gram negative bacilli mostly Acinetobacter, Enterobacter, *E. coli*, *Proteus spp*, *Ps. aeruginosa* and anaerobic bacteria such as *Propionibacterium spp* and *Klebsiella spp*. as reported by Taiwo *et al*.[1]

Alchornea cordifolia (Schumach. & Thonn) Müll. Arg belongs to the Family Euphorbiaceae. The plant is geographically distributed in secondary forests usually near water, moist or marshy places. *Alcornea cordifolia* is commonly used as a medicinal plant throughout its area of distribution. The leaves are mostly used, but also the stem bark, stem pith, leafy stems, root bark, roots and fruits are used in local medicine. According to Muanza *et al*. [2], the plant is used traditionally for treating infected wounds, diarrhorea, gonorrhea, urinary tract infections, conjunctivitis, fever, malaria.

2. Materials and methods

2.1. Collection of samples, culturing and identification of bacteria isolates

A total of 150 wound swabs collected from different wards of the hospital and submitted at the general culture bench of the Microbiology department were used. The specimens were cultured and the bacteria isolated were identified using standard microbiological standards.

2.2. Collection, extraction, fractionation and phytochemical screening of the plant material:

Alchornea cordifolia leaves were collected from Idu village, Abuja. The plant was collected from February 2010 - June 2010 and identified at the herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria. The sample of the plant has been deposited in the herbarium for reference purpose with Voucher No.401. Extraction and fractionation of the leaves were carried out using the method of Aliyu *et al*., [3]. Phytochemical Screening of the extracts and fractions were carried out using the methods of Evans, [4] and Sofowora, [5].

3. Results

3.1 Bacteria isolate from the samples of the wound sites infections.

Staphylococcus aureus (31), *Pseudomonas aeruginosa* (28), *Klebsiella* (9), *Enterobacter* spp. (7), *Citrobacter* spp. (21), *Escherichia coli* (18), *Proteus mirabilis* (19) and *Proteus vulgaris* (5) were isolated from the wound swab samples.

3.2 Phytochemical Screening of the extracts and fractions

The phytochemical screening of the extracts and fractions revealed the presence of glycosides, cardiac glycosides, saponin, tannins, triterpenes, flavonoids, alkaloids, anthraquinones and carbohydrates.

3.3 Susceptibility of the bacteria isolates to the extracts and fractions of the plant leaves.

Table 1 The mean diameter of zones of inhibition of extracts/fractions at 50 mg/ml and gentamicin against 99 % of the isolates (IZD_{99}).

	Mean diameter of zone of inhibition (mm)				
Bacteria	WE	EE	EAF	NBF	Gentamicin
S. aureus (n=12)	28.8±4.8	24.8±4.3	32.8±2.8	22.2±5.8	15.6±6.4
Ps. aeruginosa (n=10)	22.0±3.6	19.4±2.3	27.9±1.7	19.3±3.1	14.0±8.8
Klebsiella spp (n=5)	3.0±4.8	4.2±6.7	22.2±2.0	7.4±8.9	10.0±4.0
Enterobacter spp (n=5)	2.6±4.2	2.6±4.2	18.2±1.0	3.0±4.8	12.0±3.2
Citrobacter spp(n=8)	9.6±9.6	0	19.3±2.8	4.3±6.4	7.6±7.6
Pr. mirabilis (n=7)	8.1±7.0	7.6±6.5	24.6±2.4	16.4±4.7	13.9±5.1
Pr. vulgaris (n=3)	10.0±6.6	10.0±6.2	24.6±3.1	19.3±0.4	16.3±2.9
E. coli (n=16)	0	1.9±3.4	24.9±2.1	1.6±2.8	11.0±1.25

Key: WE = Water Extract; EE = Ethanol Extract; EAF = Ethyl acetate Fraction; NBF = N-butanol Fraction; n = number of organisms

Table 2 Percentage distribution of bacteria isolates according to the M. I. C. values of ethanol extract and ethyl acetate fraction.

	M. I. C. values (mg/ml)													
Bacteria	3.125		6.25		12.5		25.0		50.0		100.0		>100.0	
	EE	EAF	EE	EAF	EE	EAF	EE	EAF	EE	EAF	EE	EAF	EE	EAF
S. aureus (n=12)	0.0	8.3	8.3	83.3	75.0	0.0	8.3	8.3	8.3	0.0	0.0	0.0	0.0	0.0
Ps. aeruginosa (n=10)	0.0	20.0	0.0	0.0	0.0	20.0	20.0	60.0	60.0	0.0	20.0	0.0	0.0	0.0
Klebsiella spp (n=5)	0.0	0.0	0.0	0.0	20.0	0.0	0.0	80.0	0.0	0.0	20.0	0.0	60.0	0.0
Enterobacter spp (n=5)	0.0	0.0	0.0	0.0	0.0	20.0	0.0	80.0	20.0	0.0	0.0	0.0	80.0	0.0
Citrobacter spp(n=8)	0.0	0.0	0.0	25.0	0.0	25.0	0.0	50.0	0.0	0.0	62.5	0.0	37.5	0.0
Pr. mirabilis (n=7)	0.0	0.0	0.0	42.9	0.0	57.1	0.0	0.0	0.0	0.0	28.6	0.0	71.4	0.0
Pr. vulgaris (n=3)	0.0	0.0	0.0	33.3	0.0	66.7	0.0	0.0	0.0	0.0	33.3	0.0	66.7	0.0
E. coli (n=16)	0.0	0.0	0.0	12.5	0.0	75.0	12.5	12.5	0.0	0.0	0.0	0.0	87.5	0.0

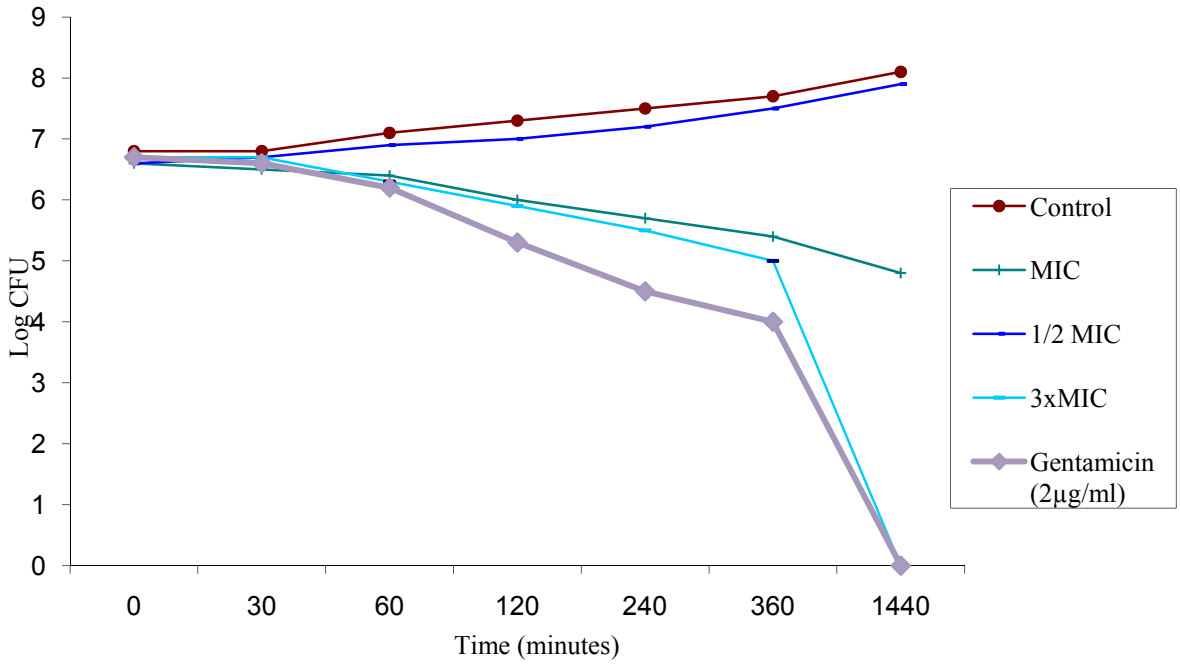

Fig. 1 Death/survival of *S. aureus* (most resistant) on exposure to concentrations of the ethyl acetate fraction and Gentamicin

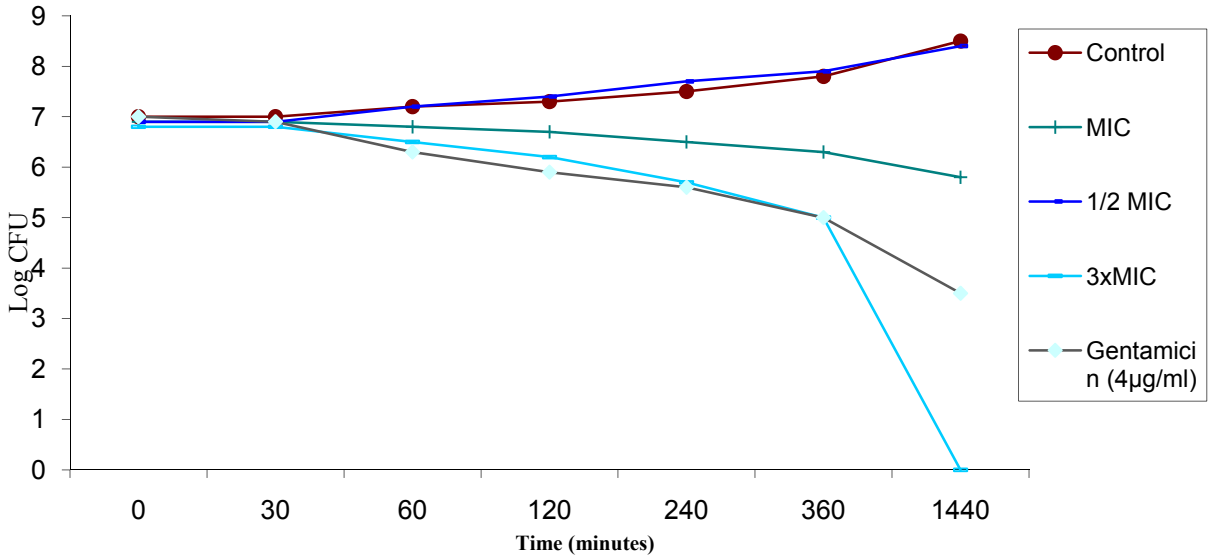

Fig. 2 Death/survival of *Ps. aeruginosa* (Most Resistant) on exposure to different concentrations of the ethyl acetate fraction and Gentamicin

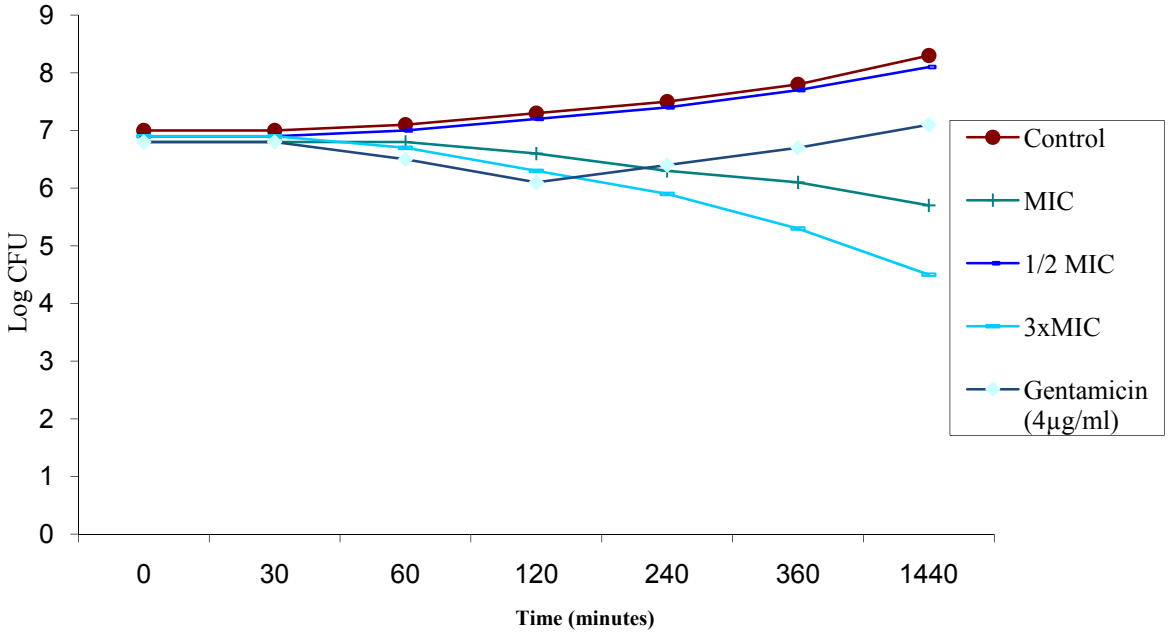

Fig. 3 Death/survival of *E. coli* (most resistant) on exposure to different concentrations of the ethyl acetate fraction and Gentamicin

4. Discussion

The type of organisms isolated from the infected wounds such as *Ps. aeruginosa*, *S. aureus*, *Citrobacter spp* and *E. coli* confirm the report of Schultz et al., [6] that all wounds, whether acute or chronic are contaminated with microorganisms from environment or the patient.

Irrespective of the type of solvent, all the extracts and fractions contained secondary metabolites: carbohydrates, cardiac glycosides, saponins, tannins and flavonoids and alkaloids which are generally found as components of plants. It also confirms findings made by Abdullahi *et al*., [7]. The secondary metabolites have been variously reported to possess appreciable inhibitory activities against various organisms. Tannins have been reported [8] to hasten the healing of wounds and inflamed mucous membrane. The higher efficacy of the

ethyl acetate fraction might be attributed to the higher proportion of some of these secondary metabolites in this extract, as judged by the higher percentage of yield.

The study demonstrated that different extracts exhibited different levels of antibacterial activities which were also dependent on the nature of the test organisms. Generally, Gram positive bacteria showed more susceptibility to the extracts and fractions than the Gram negative bacteria. The water extract and the ethyl acetate fraction exerted highest activity against *S. aureus* isolates at a concentration as low as 6.25mg/ml. Similar level of activity has been reported by Adeshina *et al*., [9]. While good inhibitory activities were exerted against *Ps. aeruginosa* especially by the ethyl acetate fraction, the Enterobacteriaceae isolates were more resistant to the activities of the water and ethanol extracts. The differences in the susceptibilities of the isolates to the plant extracts can be related to the cell wall composition of the organisms. Gram positive bacteria have a cell wall of peptidoglycan with techoic acid in between; therefore they are more sensitive than Gram negative bacteria which have their cell wall surrounded by lipopolysaccharides and lipoproteins, which prevent penetration of antibiotics through their cell wall.

This variation in level of the activity among the extracts could be due to the difference in solubility of the active ingredient in each solvent on one hand and to the constitutional or structural variability of the tested organisms on the other hand. Generally, the ethyl acetate fraction had a lower M. I. C. value (6.25 mg/ml-12.5 mg/ml) and M. B. C. values (12.5mg/ml-100 mg/ml) against *E. coli* isolates compared with other fractions, which were lower than the figures reported by Tona *et al*., [10].

The rate of survival/death studies showed two discernible patterns: initial low kill rate in the first 120 minutes of contact between the organisms and the ethyl acetate extract, followed by a more rapid decrease in bacteria cell population. This can be due to the action of tannin preceding the flavonoids action i.e. coagulation of the cell wall protein and then the release of cytoplasmic poison. This pattern of kill indicates that the ethyl acetate fraction exerts bactericidal activity on susceptible organisms.

In conclusion, bacteria isolates associated with wound infections in this study, which were mostly *S. aureus,* enteric bacteria and *Ps. aeruginosa* are consistent with reports of similar studies conducted globally and in various parts of the country. Ethyl acetate fraction of the ethanol extract of *A. cordifolia*, particularly possess useful antimicrobial activities that can be used in the therapy and management of infections of various wound sites. This study, therefore, justifies and authenticates the use of extract of *A. cordifolia* by herbalists in the treatment of wound infections.

Acknowledgements The support of the technical staff of the Department of Pharmaceutics and Pharmaceutical Microbiology is gratefully acknowledged.

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Plant bioactive metabolites for crop protection: the barley-powdery mildew-essential oils model of study

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Biotic stresses are responsible for severe yield losses in the major crops. In particular, fungal pathogens associated with plant diseases account for relevant reductions of agro-food production and quality. Crop protection is therefore of outstanding relevance in ensuring food security worldwide. However, the use of synthetic pesticides is now subjected to more stringent rules and increasing interest is focused on the development of alternative "green" strategies in the view of integrate pest management systems. In this frame, our work has been focused on powdery mildews control with essential oils and with their single compounds, alone or in mixtures. A survey of available knowledge on this topic is reported, together with new results on the control of *Blumeria graminis* with Tea Tree Oil.

Keywords barley; crop protection; tea tree oil; *Blumeria graminis*

1. Introduction

Recent studies have shown the importance of natural plant products as fungicidal agents for crop protection [1] . Crop protection plays a key role in ensuring food security to a human population that is projected to 10 billion peoples after 2020. Weeds, animal pests, pathogens and viruses are responsible for competition and destruction of cultivated plants; with the overall result that crop yield can be strongly threatened. In particular, fungal pathogens associated with plant diseases account for great losses in crop production. For their control, it has been estimated that over 23 million kg of synthetic fungicides are used annually worldwide. The benefits of pesticides to mankind and to the environment have been reviewed by evaluating the complex matrix of benefit interactions in the social, economic and environmental domains. However, the use of synthetic fungicides in crops can result in problems linked to water and environmental pollution, phytotoxicity and selection of resistant pathogen populations. Consequently, in recent years, the use of chemicals has increased consumer concern and their use is becoming more restrictive and alternative "green" measures have been proposed for crop protection, including mineral salts, biological agents and plant extracts, that are very promising because expected to have a narrow target range and a highly-specific mode of action, to show a brief field persistence and also to have a shorter shelf life [2,3].

In our work the effects of an essential oil (Tea Tree Oil, extracted from *Melaleuca alternifolia*) has been evaluated for the control of powdery mildew infection on barley. The rationale behind this model of study has been suggested by the relevance of both the plant and the fungus and by the availability of the essential oil.
Barley (*Hordeum vulgare*), considered the earliest plant domesticated, is in fact the fourth cereal both in terms of worldwide production and cultivation area and, according to 2010 FAO statistics, is the sixth more important crop. Moreover, barley can be considered from a genetic and genomic point of view a model plant for *Triticeae*, thanks to its diploid genome, the strictly autogamous mating system, the wide adaptability and availability of genetic resources belonging to the primary, secondary and tertiary gene pools. The complete genome sequence of barley has been recently published [4], providing therefore a platform for genome-assisted research applied to several aims.

Blumeria graminis is an important barley pathogen responsible for yield reduction as high as 40%. It belongs to the extremely successful powdery mildews parasites, consisting of more than 700 species able to colonize several plant species, many of which are cultivated crops. *Blumeria graminis* is an obligate biotrophic fungus that grows epiphitically on all aerial parts of barley, but in particular on leaf surfaces, and obtains the nutrients from the host using haustoria, specialized intracellular feeding structures. In 2010 it has been released the whole sequence of *Blumeria* genome, that displays massive retrotransposon proliferation, genome-size expansion, and gene losses, probably in relation to the exclusively biotrofic life-style [5].

Tea Tree Oil (TTO) is the essential oil extracted from Melaleuca alternifolia, a plant belonging to *Myrtacee* family extensively and almost exclusively cultivated in the South Wales region of Australia continent. TTO has a long history of use as topical microbicide in human pharmacology [6]. Although there is a natural variation in the TTO essential oil content the composition of oil sold as TTO is standardized (ISO4730:2004) and

characterised, as major components, by terpinen-4-ol (40% in a typical composition), gamma-terpinen (23%), and 1,8-cineole (5.1%). TTO is commercially available at costs that are compatible with applications in crop protection.

2. Material and methods

To test the efficacy of TTO and oil components for Blumeria control, barley leaf segments were inoculated with powdery mildew under controlled conditions and treated with TTO and oil component at different percentages. Five 10-day-old leaves segments (40 mm length) of barley cultivar Golden Promise (susceptible to mildew) were laid out in a petri dish over a medium containing 0.5% agar (Fig. 1). For each experimental condition, a set of four independent petri dishes was prepared. All the plates were placed on a rotating platform under an inoculation tower (diameter 50 cm, height 100 cm) and inoculated with K1 isolate. The leaf segments were then incubated for 72 h at 20_C in the dark and then sprayed with a control solution containing 0.05% Tween or a solution containing 0.05% Tween 20% and 0.5% TTO or 1% TTO. After 7 days of incubation at 20°C, the mildew colonies on the leaf segments were counted.

Fig. 1 Powdery mildew is an obligate biotrophic fungus, therefore the *in vitro* assay for TTO treatment evaluations have been done on detached barley leaves, cultivar Golden Promise, infected with standardized inoculum of a very aggressive *Blumeria graminis* strain. After the inoculum, the leaves were subjected to treatments with solutions at different TTO percentages or at different single compounds percentages.

3. Results

In figure 2 is reported the mean growth inhibition of powdery mildew colonies after treatments. Percentages of 0.1% TTO, eugenol, thymol and terpinen-ol completely inhibited the formation of mildew colonies on leaf surface. From the results obtained, TTO is therefore very effective against Blumeria.

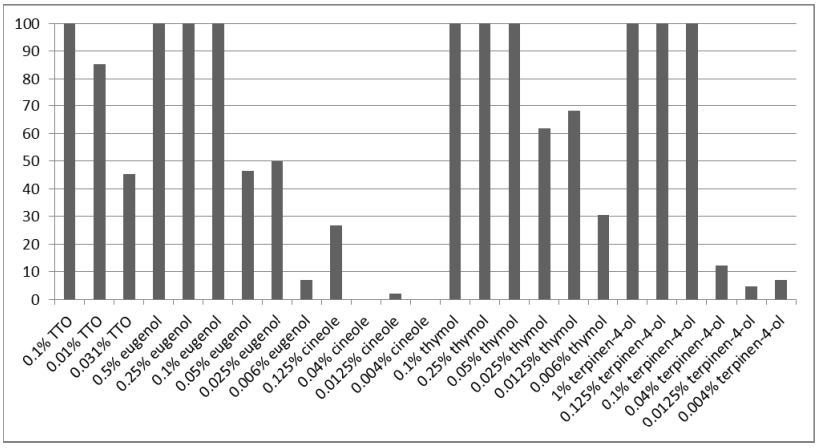

Fig. 2 Mean mycelium growth inhibition (expressed as %) after indicated treatments.

3. Conclusions

In our work, the antifungal activity of TTO and of essential oil components has been demonstrated against *Blumeria graminis*. These results offer new opportunities for powdery mildews control. The near perspectives are focused on the better characterization of molecular tagets of TTO in the model of study barley-*Blumeria graminis*. A great body of literature is in fact available on EOs antifungal activities, however, no obvious linkage is apparent between their chemical composition and their molecular effects. However, the finding of new strategies for crop protection and for post-harvest control based on natural products is now moving toward a more integrated approach involving disciplines such as molecular and cellular biology, proteomics, trascriptomics and bioinformatics. The rationale behind this innovative approach is that a better knowledge of

the molecular targets and mechanisms of actions of plant metabolites at cellular level can optimize their utilization, alone or in combination, and maximize the antifungal efficacy.

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Polyclonal KPC-3-producing *Enterobacteriaceae* in Portugal

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All 6 KPC-3-producing *Enterobacteriaceae* isolates (5 *K. pneumoniae* and 1 *Enterobacter cloacae*), identified among 61 isolates, from different Portuguese health institutions (March 2010 to December 2011) were multidrug resistant, showing susceptibility only to colistin. The bla_{KPC-3} gene, conferring resistance to carbapenemes, was present alone or in combination with other *bla* genes: bla_{SHV-26}, $bla_{CTX-M-15}$, and the ESBL $bla_{SHV-164}$, here firstly described. The Tn4401-harbouring bla_{KPC-3} encountered in all isolates, showed a 68 bp deletion upstream of the *bla* gene. All but two KPC-3-containing plasmids revealed the following types: $IncF_{repB}$ plus IncFIIs (n=3) and $IncF_{repB}$ plus IncP (n=1). Dissemination of bla_{KPC} seems to be due to carriage of similar KPC-harbouring plasmids within genetically distinct *K. pneumoniae* (ST14, ST34, ST59, ST416 and the novel ST960, by MLST) and *E. cloacae* clinical strains.

Keywords Tn4401-KPC-3; $IncF_{repB}$

1. Introduction

The emergence and rapid dissemination of carbapenemase-producing *Enterobacteriaceae*, mostly identified among *Klebsiella pneumoniae*, has become an important therapeutic and infection control problem in Europe [1]. In Portugal, according to the European Antimicrobial Resistance Surveillance Network (EARS-Net) surveillance study, the occurrence of antibiotic resistance and multidrug resistance (MDR) in *K. pneumoniae* continues to raise [2]. Between 2009 and 2011, an increasing proportion of resistant *K. pneumoniae* isolates was noticed for all antibiotic categories, with MDR growing from 13.7% in 2009 to 20.7% in 2011. In 2011, from the 36.2% of third generation cephalosporins-resistant *K. pneumoniae* isolates, 93.3% were ESBL producers. Cases of carbapenem non-susceptible *K. pneumoniae* isolates were also detected in that period, with an increase from 0.72% in 2008 to 1.58% in 2010 (2). Hence, the understanding of the resistance mechanisms to carbapenems, a class of antibiotics considered as one of the last resort for treating infections caused by isolates presenting multidrug resistance, is imperative.

However, in Portugal, little is known on carbapenemase-producing *Enterobacteriaceae*. A recent study reported an outbreak of an ertapenem-resistant ST15 *K. pneumoniae* [3], where the carbapenem resistance was associated with deficiencies in major porins and, in some cases, with extended-spectrum β-lactamase (ESBL) or plasmid-mediated AmpC β-lactamases (PMAβ) production. With the exception of a VIM-2-producing *K. oxytoca* isolate [4], no carbapenemase-producing *Enterobacteriaceae* have been described in Portugal. KPC enzymes have not been described in clinical *Enterobacteriaceae* isolates, but a KPC-2-producing *E. coli* isolate was recently found in the aquatic environment [5].

Hence, the aim of this study was to investigate the resistance mechanisms in *Enterobacteriaceae* clinical isolate resistant to carbapenems identified in Portuguese hospitals, by the determination of the potential relatedness of the isolates and respective plasmids harbouring carbapenem resistance mechanisms.

2. Material and methods

2.1 Bacterial Strains

We report 6 KPC-3-producing *Enterobacteriaceae* isolates (5 *K. pneumoniae* and 1 *Enterobacter cloacae*), collected between March 2010 and December 2011 and identified among 61 isolates (26 *Klebsiella* spp, 15 *Escherichia coli*, 9 *Enterobacter* spp, 6 *Morganella morgannii*, 4 *Proteus mirabilis*, 1 *Serratia marcescens*), sent to the National Reference Laboratory of Antimicrobial Resistances at the National Institute of Health, as part of a laboratory surveillance program for carbapenem nonsusceptibility confirmation. The majority of KPC-3-producing isolates were collected from the urine (42.8%) of elderly (≥65 years old) male patients (85.7%), admitted at four geographic distant Portuguese hospitals.

2.2 Antimicrobial susceptibility tests

Antimicrobial susceptibility testing was performed by standard disk diffusion method, according to French Society of Microbiology (SFM) guidelines (www.sfm-microbiologie.org/), by using 32 commercial disks (Bio-Rad), after culture in a simple agar medium. Clinical isolates showing synergism between carbapenems (imipenem, meropenem and/or ertapenem) and boronic acid (and/or clavulanic acid) or with EDTA were considered presumptively carbapenemase-producers from class A or Class B, respectively. Minimal inhibitory concentrations (MICs) for different categories of antibiotics were determined by the microdilution broth method, for transformants and respective isolates, by using microdilution broth method, and the results were interpreted according to EUCAST breakpoints (http://www.eucast.org/clinical_breakpoints/). Isolates were considered multidrug-resistant when presented a reduced susceptibility to 3 or more structurally unrelated antibiotics.

2.3 Characterization of antibiotic resistance genes

PCR and sequencing were applied to detect and identify CARB-encoding genes from class A (bla_{KPC} and bla_{GES}), class B (bla_{IMP}, bla_{VIM} and bla_{NDM}) and class D (bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-48}, bla_{OXA-51} and bla_{OXA-58}) [6]. The presence of bla_{ESBL} (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{GES}) and $bla_{PMA\beta}$ (bla_{CMY}, bla_{MOX}, bla_{FOX}, bla_{LAT}, bla_{ACT}, bla_{MIR}, bla_{DHA}, bla_{MOR}, bla_{ACC}) genes, were also investigated, as previously described [7]. All PCR products were purified with ExoSAP IT, and sequenced directly on both strands using automatic sequencer ABI3100 (Applied Biosystems).

2.4 Genetic environment

The genetic context of the bla_{KPC-3} gene was examined by digestion with *Eco*RI and *Apa*I of plasmid DNA isolated from all clinical strains, using the QIAprep spin miniprep kit (Qiagen). The fragments were ligated into the kanamycin-resistant pBK-CMV (Stratagene), and the resulting plasmids were used to transform *E. coli* DH5α Δ*ampC,* using standards methods. That region was therefore amplified and sequenced by using primer walking. Sequence alignments and generation of resistance cassette contigs were performed using *Bionumerics* version 3.5 (Applied Maths); gene identity was confirmed at the NCBI website (http://www.ncbi.nlm.nih.gov/).

2.5 Plasmid analysis

Transfer experiments, either by conjugation (by liquid and solid mating assays) [7] or electroporation of plasmid DNA [8], was performed. PCR-based replicon typing (PBRT) was used to type the resistance plasmids of the KPC-3 producing isolates. The major incompatibility (Inc) groups, specifically FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA were detected as previously described [9].

2.6 Molecular epidemiology

Clonal relatedness of the 5 *K. pneumoniae* isolates was investigated by multilocus sequence typing (MLST), using the protocol developed by the Institute Pasteur (www.pasteur.fr/mlst/Kpneumoniae.html), through the identification of seven housekeeping genes (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB* and *tonB*). The resulting sequences were analysed using BioNumerics v.3.5 software (Applied Maths) and were assigned to sequence types (STs) using the tools on the *K. pneumoniae* MLST webpage.

2.7 Nucleotide sequence accession number

The sequence of the $bla_{SHV-164}$ β-lactamase gene was submitted to the EMBL Nucleotide Sequence Database under accession no. HE981194.

3. Results and discussion

The determination of antimicrobial susceptibility of the KPC-3 producers displayed a MDR phenotype with consistent susceptibility only to colistin (Table 1). All isolates demonstrated a positive combined disk test with meropenem and meropenem-boronic acid, indicative of serine carbapenemase production (data not shown).

Table 1 Characteristics of KPC-3-producing isolates and respective transformant/conjugants.

Isolates[a]	Hospital/ Year of isolation	Non-susceptibility profile[b]	MLST	PBRT	β-lactamases encountered
K. pneumoniae INSRA10302	C/2010	β-lact, Carb, Ag, S, T	ST14	Non-typable	KPC-3,TEM-1, SHV-1
E. cloacae INSRA11054	A/2010	β-lact, Carb, Qui, Ag, S, T	-	Non-typable	KPC-3
K. pneumoniae INSRA12031	A/2011	β-lact, Carb, Qui, Ag, F, S, T	ST34	IncFrepB, IncFIIs	KPC-3,TEM-1, SHV-26
K. pneumoniae INSRA12267	D/2011	β-lact, Carb, Qui, Ag, S, T	ST960	IncFrepB, IncFIIs	KPC-3,TEM-1, SHV-164[c]
E. coli DH5α-KPC-3 (p12267)	-	β-lact, Carb, Ag, S	-	IncFrepB	KPC-3,TEM-1
K. pneumoniae INSRA13165	B/2011	β-lact, Carb, Qui, Ag, S, T	ST416	IncFrepB, IncFIIs	KPC-3,TEM-1, SHV-14, OXA-30, CTX-M-15
K. pneumoniae INSRA14159	A/2011	β-lact, Carb, Qui, Ag, S	ST59	IncFrepB, IncP	KPC-3,TEM-1
E. coli K12 C600-KPC-3 (p14159)	-	β-lact, Carb, Ag, S	-	IncFrepB	KPC-3,TEM-1

[a] *E. coli* DH5α-KPC-3 (p12267) and *E. coli* K12 C600-KPC-3 (p14159) were transformant/conjugant of *K. pneumoniae* INSRA12267 (harbouring the plasmid p12267 containing the KPC-3 and TEM-1 enzymes) and *K. pneumoniae* INSRA14159 (harbouring the plasmid p14159 containing the KPC-3 and TEM-1 enzymes), respectively.
[b] β-lact (amoxicillin, amoxicillin/clavulanate, ticarcillin, piperacillin, piperacillin/tazobactam, cephalothin, cefuroxime, cefoxitine, cefixime, cefpodoxime, cefotaxime, ceftazidime, ceftriaxone, cefepime and/or aztreonam); Carb (imipenem, meropenem and/or ertapenem); Qui (nalidixic acid, norfloxacin, pefloxacin and/or ciprofloxacin); Ag (kanamicin, gentamicin and/or amikacin); F (fosfomycin), S (trimethoprim/sulfamethoxazole), T (tigecycline).
[c] β-lactamase here firstly described.

Following PCR and sequencing, the bla_{KPC-3} gene was confirmed in all isolates, alone or in combination with other *bla* genes, namely: (1) bla_{SHV-26}, an inhibitor-resistant SHV (IRS); (2) $bla_{CTX-M-15}$, the most frequent ESBL-type found worldwide; and (3) the ESBL $bla_{SHV-164}$ (HE981194), here firstly described (Table 1). No co-expression of KPC-3 with other carbapenemase or PMAβ was detected.
The genetic context of the bla_{KPC-3} genes, after digestion with *Eco*RI and *Apa*I of plasmid DNAs from all clinical strains, showed that the recombinant plasmids presented a 4,110 bp insert conferring resistance to carbapenems (Fig. 1).

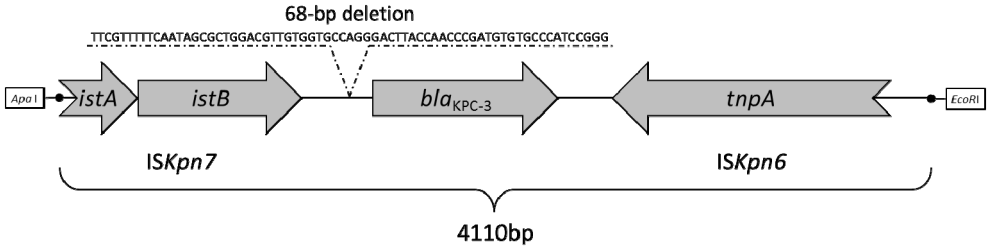

Fig. 1 Representation of the Tn4401-harbouring bla_{KPC-3} encountered in this study, with a 68 bp deletion upstream of the *bla* gene. Arrows are drawn to scale (GenBank accession no. HM769262).

Genetic mapping around the bla_{KPC-3} gene identified the Tn*3*-like Tn*4401* transposon, which has been suggested as an important factor in its worldwide dissemination [10]. Several isoforms of Tn4401 have been described: a, b, and c differing by a 100- to 200-bp sequence upstream of bla_{KPC} gene [10, 11]; isoform d, a truncated Tn*4401* structure with a 5.3-kb deletion (2); and isoforms with 68-bp [12, 13], 215-bp (GenBank accession no. DQ989640), and 255-bp deletions [14]. In this study, we detected, in all isolates, the 68-bp deletion isoform (Fig. 1), which ends upstream of the -35 region of the putative promoter identified by Yigit *et al*. [15]. This mobile structure was only described so far in KPC-3-producing *K. pneumoniae* strains with high-level carbapenem resistance, collected in the United States and Canada [12, 13].

In our study, the majority of the bla_{KPC-3}-harboring plasmids appear to be nonconjugative, since only one conjugant [*E. coli* K12 C600-KPC-3 (p14159)] and one transformant [*E. coli* DH5α-KPC-3 (p12267)] were obtained. In general, both transformants had antibiotic resistance profiles similar to those of their parental clinical isolates (Table 1).

The characterization of KPC-3-containing plasmids revealed that all (4 *K. pneumoniae*) but two KPC-3-producing isolates were positively typed: $IncF_{repB}$ plus IncFIIs (n=3) and $IncF_{repB}$ plus IncP (n=1). Both conjugant and transformant had only the $IncF_{repB}$, suggesting that this Inc group is associated with KPC-3-harbouring plasmids.

Clonal relatedness of the 5 *K. pneumoniae* isolates, obtained by MLST showed that the KPC-3-producing *K. pneumoniae* were from distinct STs, namely ST14, ST34, ST59, ST416 and the novel ST960 (Table 1). All clones were only sporadically detected (www.pasteur.fr/mlst/Kpneumoniae.html), although ST14 have been associated with NDM-producing *K. pneumoniae* [16, 17] and ST416 with an epidemic of DHA-1-producing *Klebsiella pneumoniae* [18]. This data suggests that the dissemination of bla_{KPC} is due to carriage of similar KPC-harbouring plasmids within genetically distinct *K. pneumoniae* and *E. cloacae* clinical strains.

4. Conclusions

In conclusion, this study provides new data regarding the molecular epidemiology of carbapenemase-producing *Enterobacteriaceae* in Portugal, which includes KPC-3-harbouring IncFrepB plasmids that are shared by polyclonal *K. pneumoniae* and *E. cloacae* clinical strains. These results show the importance of national laboratory surveillance programs to understand the emerging antibiotic resistance mechanisms and circulating clones; in addition, their link with European programs, such as EARS-Net, permit to correlate these data with non-susceptibility trends in a wide context. Overall, our results emphasize the need of a concerted action to manage carbapenem resistance.

Acknowledgements. We thank the laboratories participating in the Antibiotic Resistance Surveillance Program in Portugal (ARSIP) for sending isolates to the National Reference Laboratory of Antimicrobial Resistance at National Institute of Health. V. Manageiro was supported by grant SFRH/BPD/77486/2011 from Fundação para a Ciência e a Tecnologia (FCT), Lisbon, Portugal.

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Prevalence and management of fungicide resistance in *Fusarium* spp. infecting potatoes in Canada

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Strains of *Fusarium* spp., particularly *F. sambucinum*, with resistance to thiabendazole/thiophanate-methyl and fludioxonil fungicides have been recovered from potato tubers sampled from storages across Canada in recent years. This resistance has limited the effectiveness of seed-piece and post-harvest treatments resulting in increased seed-piece decay after planting and dry rot in storage. Surveys from 2007-2012 in Canada showed that *Fusarium sambucinum* was the most predominant seed-decay and dry rot pathogen, followed by *F. coeruleum, F. avenaceum* and *F. oxysporum*. Isolates of pathogenic *F. graminearum* were also recovered, although infrequently. Many weakly pathogenic or nonpathogenic *Fusarium* spp. were also frequently recovered, including *F. sporotrichioides, F. accuminatum, F. cerealis, F. equiseti*, *F. crookwellense, F. tricinctum*, and *F. torulosum*. Isolates of the various *Fusarium* spp. collected during the surveys were tested for their sensitivity to thiophanate-methyl (Senator ® PSPT), thiabendazole (Mertect ® SC) and fludioxonil (Maxim® PSP) in fungicide-amended agar assays. Most isolates of *F. sambucinum* recovered in recent years showed resistance to both thiabendazole/thiophanate-methyl and fludioxonil. Isolates of *F. coeruleum* with resistance to fludioxonil were also recovered. Isolates of *F. oxysporum* recovered in these surveys were always sensitive to thiabendazole and thiophanate-methyl, but resistant to fludioxonil. By contrast, most other *Fusarium* spp. were sensitive to all tested products. Field and storage studies were conducted to ascertain the impact of fungicide-resistant strains on crop loss and to define potential management strategies. In all cases, treatment of potato seed pieces with mancozeb, difenoconazole or prothioconazole prior to planting completely controlled seed-piece decay caused by a multi-class resistant isolate of *F. sambucinum*. Additionally, fungicide mixtures containing difenoconazole were consistently able to reduce post-harvest dry rot when applied as a liquid spray to tubers entering storage. At times, *Pseudomonas syringae* (Bio-Save®10LP) and oxysilver nitrate (Agress®) also provided post-harvest disease control efficacy. However, products with the active ingredients thiabendazole (Mertect®) or phosphite (Confine^{TM}) were not effective when applied as a post-harvest treatment. Fungicide resistance has limited the effectiveness of the most common fungicides used against *Fusarium* spp. on potatoes in Canada. A re-evaluation of management practices for control of *Fusarium*-induced potato disease is required. Knowing the predominant *Fusarium* spp. in a particular seedlot and their sensitivities to various chemical products would provide growers with important information to use to make disease management decisions.

Keywords: potato; *Fusarium*; fungicide resistance

Fusarium spp. are important pathogens of potato that cause yield losses at planting and in storage following harvest. *Fusarium* spp. can only infect potatoes through wounds. Thus, infection can occur when inoculum is spread from diseased to healthy seed during seed cutting and handling. As well, inoculum in soil attached to the surface of tubers can infect potatoes through wounds made during harvest and handling operations prior to storage.

Potatoes infected with *Fusarium* spp. develop a spreading external decay that usually becomes shrunken and wrinkled in appearance (Fig.1.). When diseased tubers are cut open, the brown decay can be seen spreading into the internal tissues of the tuber (Fig.2.). The internal decay is usually marked by open cavities which contain the white mycelium of the fungus.

Figure 1. External symptoms of Fusarium dry rot.

Figure 2. Internal symptoms of Fusarium dry rot showing cavities filled with white mycelium of the fungus within the tuber tissue.

Fusarium species causing dry rot and seed-piece decay

Potato seed surveys were coordinated by Agriculture and Agri-Food Canada, Crops and Livestock Research Centre in Charlottetown, Prince Edward Island, Canada from 2007-2012. Samples of infected potato seed tubers were sent by growers, provincial government personnel and potato industry representatives from different potato production regions across Canada. Each sample (representing a seed lot) contained 4 – 10 potato tubers with obvious signs of *Fusarium* infection. From these tubers, a 5mm x 10mm tissue sample was extracted from the border of a disease lesion. Tissue samples were surface-sterilized in 10% Javex (0.6% sodium hypochlorite) for 1 minute, followed by two rinses in sterile distilled water. Tissue pieces were then transferred to ½ strength potato dextrose agar (PDA) in Petri dishes. After 5 to 7 days, or when sufficient colony growth was observed, fungal colonies were hyphal-tipped and then transferred to fresh plates of ½ strength PDA to obtain pure cultures.

Isolates were identified to species level using micro-morphological features [1]. A sub-set of isolates representing the diversity of species in the collection was subjected to partial sequencing of the translation elongation factor 1-alpha for comparison with sequences of this gene in FUSARIUM-ID (http://fusarium.cbio.psu.edu; [2]) to confirm micro-morphological observations.

Surveys from 2007-2012 in Canada showed that *Fusarium sambucinum* was the most predominant seed-decay and dry rot pathogen, followed by *F. coeruleum, F. avenaceum* and *F. oxysporum*. Isolates of pathogenic *F. graminearum* were also recovered, although infrequently. Many weakly pathogenic or nonpathogenic *Fusarium* spp. were also frequently recovered, including *F. sporotrichioides, F. accuminatum, F. cerealis, F. equiseti*, *F. crookwellense, F. tricinctum*, and *F. torulosum*.

Resistance to control products

Isolates of the various *Fusarium* spp. collected during the surveys were tested for their sensitivity to thiophanate-methyl (Senator ® PSPT), thiabendazole (Mertect ® SC) and fludioxonil (Maxim® PSP) in

fungicide-amended agar assays. Senator ® PSPT and Maxim® PSP are applied to cut potato seed pieces at planting and Mertect ® SC is applied post-harvest to tubers entering storage. To determine the chemical sensitivity of isolates, agar plugs (5 mm diameter) were removed from the margins of 7-10 day-old cultures and transferred onto ½ strength PDA plates amended with 0 or 10 ppm of thiabendazole or fludioxonil. Chemicals were first dissolved in acetone and then added to the molten agar after autoclaving. Control plates (without fungicides) contained PDA with acetone only. Two replications were tested for each fungicide/isolate combination and the experiment was repeated.

After incubation at room temperature in the dark for 7 days, the diameter of hyphal growth in each plate (two observations per plate at right angles to each other) was recorded using digital calipers. The growth of isolates at 10 ppm of fungicide was compared with hyphal growth in the control plates (0 ppm). An isolate was considered to be resistant to the fungicide if growth in the fungicide-amended plate was >50% of the growth in the controls.

In 2012, samples were received from all provinces in Canada except Newfoundland. In total, 1,011 individual isolates of *Fusarium* spp. were obtained in 2012. The most common species identified were *F. sambucinum, F. oxysporum,* and *F. avenaceum* (Table 1). In addition, a high frequency of weakly pathogenic or nonpathogenic *Fusarium* spp. was also identified (Table 1). Most isolates of *F. sambucinum* showed resistance to both thiabendazole/thiophanate-methyl and fludioxonil. A significant number of isolates of *F. coeruleum* with resistance to fludioxonil were also recovered. Isolates of *F. oxysporum* recovered in these surveys were always sensitive to thiabendazole/thiophanate-methyl, but resistant to fludioxonil. By contrast, most other isolates of various *Fusarium* spp. were sensitive to all tested products (Table 1).

Table 1. Frequency and chemical sensitivity of isolates of *Fusarium* spp. recovered from infected seed potato tubers in Canada in 2012.

Species	Total number of isolates	Thiabendazole		Fludioxonil	
		Sensitive	Resistant	Sensitive	Resistant
F. sambucinum	189	45	144	71	118
F. coeruleum	21	18	3	12	9
F. avenaceum	59	57	2	55	4
F. oxysporum	165	164	1	1	164
F. graminearum	11	11	0	11	0
*F. spp.**	566	555	11	561	5
Total	1011	850	161	711	300

*Minor weakly pathogenic or nonpathogenic Fusarium spp. recovered include F. sporotrichioides, F. accuminatum, F. cerealis, F. equiseti, F. crookwellense, F. tricinctum, and F. torulosum.

Potato seed treatment trials

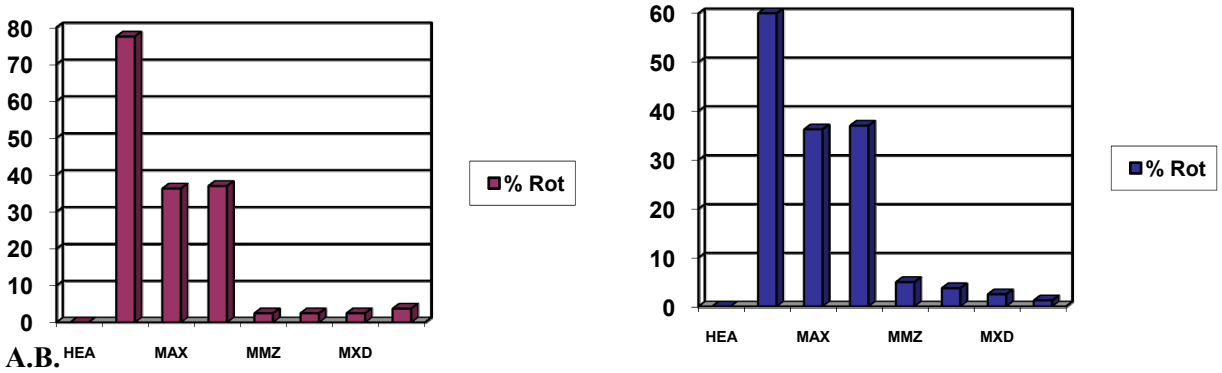
Field and storage studies were conducted in Prince Edward Island, Canada in 2011 to ascertain the impact of fungicide-resistant strains on crop loss and to define potential management strategies. Potato seed-pieces were inoculated with a fungicide resistant strain of *F. sambucinum* and then treated with various seed treatments including:

1. Healthy control = not inoculated [HEA]
2. Diseased control = inoculated but no seed treatment [DIS]
3. Maxim PSP (0.5% fludioxonil) [MAX]
4. Senator (10% thiophanate-methyl) [SEN]
5. Maxim MZ PSP (0.5% fludioxonil; 7% mancozeb) [MMZ]
6. Solan MZ (16% mancozeb) [SMZ]
7. MaximD (fludioxonil + difenoconazole) [MXD]
8. Emesto Silver (penflufen + prothioconazole) [SILV]

Seed pieces were then incubated at 10°C for 6 weeks after which they were rated for disease (Fig.3). In all cases, treatment of potato seed pieces with mancozeb, difenoconazole or prothioconazole prior to planting completely controlled seed-piece decay caused by a multi-class resistant isolate of *F. sambucinum* (Fig.4).

A. **B.**

Figure 3. Inoculation of potato seed-pieces with fungicide-resistant *F. sambucinum* followed by seed-piece treatment. **A.** good disease control **B.** poor disease control



A.B.

Figure 4. Percentage of rot in potato seed-pieces after inoculation with fungicide -resistant *F. sambucinum* followed by application of various seed treatments. **A**=Russet Burbank; **B**=Shepody

Post-harvest trials

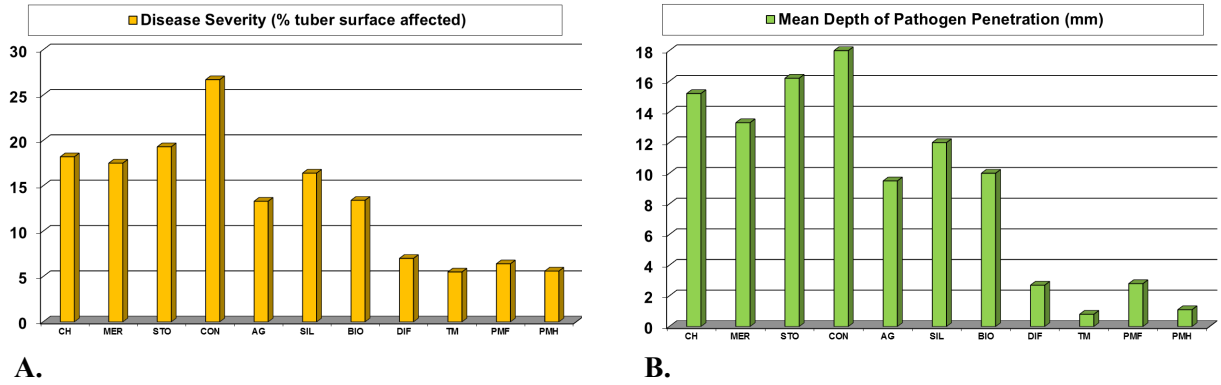
Post-harvest trials were conducted in 2012 at the Harrington Research Farm of Agriculture and Agri-Food Canada, Charlottetown, PE. The experimental design was a randomized complete block with four replications. Individual experimental units consisted of plastic, ventilated crates each containing 25 tubers of the cultivar Yukon Gold.

Tubers were inoculated (10,000 conidia/mL applied to tubers wounded with a scoring tool) with a local, fungicide-resistant (resistant to fludioxonil and thiabendazole/thiophanate-methyl) isolate of *Fusarium sambucinum* prior to fungicide application. Tubers were incubated overnight at room temperature after inoculation and then chemically treated using a spray volume of 210 mL/100 kg potato tubers. After treatment, tubers were stored for 4 months at 5°C and 95% RH.

After 4 months of storage, individual tubers were assessed for percent of tuber surface covered with lesions, and then cut longitudinally from the point of wounding to measure (in mm) pathogen penetration into internal tuber tissues causing visible necrosis.

Fungicide treatments were:

1. Untreated Check (inoculated with fungicide-resistant *F. sambucinum*) [CH]
2. Mertect SC (thiabendazole 500g/L)[MER]
3. Storox (27% hydrogen dioxide)[STO]
4. Confine (45.8% mono- and di-potassium salts of phosphorous acid)[CON]
5. Agress (experimental product)[AG]
6. Silver Periodate (experimental product)[SIL]
7. Bio-Save 10LP (*Pseudomonas syringae*)[BIO]
8. Difenoconazole[DIF]
9. Tank mix (difenoconazole + fludioxonil + azoxystrobin)[TM]
10. Premix (difenoconazole + fludioxonil + azoxystrobin - full rate)[PMF]
11. Premix (difenoconazole + fludioxonil + azoxystrobin - half rate)[PMH]



A. **B.**

Figure 5. A. Disease severity (%) and **B.** depth of pathogen penetration (mm) into tuber tissues following inoculation with fungicide-resistant *Fusarium sambucinum* and subsequent fungicide treatment prior to storage.

Fungicide mixtures containing difenoconazole were consistently able to reduce post-harvest dry rot when applied as a liquid spray to tubers entering storage (Fig.5.). At times, *Pseudomonas syringae* (Bio-Save®10LP) and oxysilver nitrate (Agress®) also provided post-harvest disease control efficacy. However, products with the active ingredients thiabendazole (Mertect®), hydrogen dioxide (Storox®), or phosphite (Confine^{TM}) were not effective when applied as a post-harvest treatment (Fig.5.).

Fungicide resistance has limited the effectiveness of the most common fungicides used against *Fusarium* spp. on potatoes in Canada. A re-evaluation of management practices for control of *Fusarium*-induced potato disease is required. Knowing the predominant *Fusarium* spp. in a particular seedlot and their sensitivities to various chemical products would provide growers with important information to use to make disease management decisions.

Acknowledgements: The authors would like to acknowledge funding provided by Agriculture and Agri-Food Canada and Syngenta Canada Ltd. They would also like to thank participating potato growers, provincial government colleagues and industry representatives that contributed to sample submission.

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Proteomic evaluation of Multiple Antibiotic-Resistant *Escherichia coli* recovered from slaughtered pigs

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In the present study, a proteomic survey of 66 *E. coli* isolates with different antibiotic resistance profiles, recovered from faecal samples of pigs slaughtered for human consumption was carried out. A total of 857 different spots were picked from the four different strains-analysed. Preliminary results based on the full proteome analyses of these strains revealed proteins, among others, which expression can be related with antibiotic resistance gene mechanism. Additionally, proteins involved in transport/ATP-binding, glycolysis, transcription/RNA processing, translation, proteolysis, fatty acid biosynthesis, and proteins related in oxidative and acid stresses responses were identified. These results reinforce the utility of antibiotic resistance proteomes from *E. coli* isolates in the identification of resistance mechanisms, even as potential diagnostic and therapeutic targets.

Keywords Proteomics; Multiple Antimicrobial Resistant; *Escherichia coli*; Pigs

1. Introduction

Escherichia coli is one of the most frequent cause of nosocomial and community-acquired, enteric infections, and systemic infections in humans [1]. The human population may be colonized and/or infected via contact, occupational exposure or through the food chain. In addition to the transmission of resistant bacteria, resistance genes from the resident bacterial flora in animals can be transferred to pathogens which may infect humans [1]. There is ongoing concern about the risks posed to human health by antimicrobial-resistant bacteria isolated from farm animals especially multidrug-resistant *E. coli* strains. There are three types of enzymes that inactivate antibiotics: beta-lactamases, responsible for hydrolyzing nearly all beta-lactams which have ester and amine bonds (e.g. penicillins and cephalosporins) thus inactivating the antibiotic action; transferases, a group of enzymes who inactivate aminoglycosides, chloramphenicol, streptogramin or rifampicin by binding adenylyl, phosphoryl or acetyl groups to the periphery of the antibiotic agent through the transport across the cytoplasmic membrane; oxidation and reduction processes are used by pathogenic bacteria as a resistance mechanism against antibiotics [2].

In addition to the difficulties in controlling infectious diseases, the phenotype of resistance can generate metabolic changes which, in turn, can interfere with host pathogen interactions [3]. The ability to determine the protein complement expressed by an organism at specific times and under specific conditions provides insights into the proteins the organism needs to selectively express to survive and thrive. Currently, proteomics is a challenging field that has been growing rapidly in the post genomic era, the evaluation of protein expression in response to various stress mechanisms, such as sensitivity to antibiotics or modifications related to antibiotic resistance, could represent a valid and integrating approach for the development of new therapeutic strategies [4].

Understanding mechanisms at the molecular level is extremely important to control multi-resistant strains. Accordingly, a proteomic survey of 66 *E. coli* isolates with different antibiotic resistance phenotypes, recovered from faecal samples of pigs slaughtered for human consumption was performed by two-dimensional electrophoresis (2-DE) and subsequent protein identification by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS).

2. Material and Methods

The 66 *E. coli* strains included in this study were recovered from faecal samples of pigs slaughtered for human consumption [5]. Overnight cultures were grown in Brain Heart Infusion (BHI) solid medium, were adjusted to 0.06 OD (550nm) with fresh BHI broth to yield a starting inoculum of approximately 1x 10^6 CFU/ml. Cultures were then incubated at 37°C for five hours, and cell pellets obtained when cell growth reached the exponential phase. The crude protein extract was obtained according to the methodology presented by Pinto *et al* [6].
SDS-PAGE was performed on vertical gel with constant gel concentration with a T=12.5% and C=0.97% according to a procedure described previously by Laemmli [7]. Electrophoresis was carried out with constant amperage of 30mA per gel until the dye-front reached the bottom of the gels that were stained with Coomassie Brilliant Blue R250.The mono-dimensional profile of these 66 strains was compared with each other and subsequently, four of these strains, selected randomly on base of their SDS-PAGE patterns and phenotypic/genotypic characteristics, were submitted to two-dimensional electrophoresis, namely Isoelectric Focusing followed by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (IEF x SDS-PAGE).

2-DE was performed with IPG (ImmobilineTM pH Gradient) technology [7]. Protein samples were previously prepared with 2-D Clean-Up Kit (GE Healthcare) and the protein pellet was resuspended in rehydration buffer (8M urea, 1% CHAPS, 0.4% DTT, 0.5% carrier ampholyte buffer pH 3-10). Samples were loaded in precast IPG strips with linear gradient of pH 3-10 (pH 3-10, 13 cm, Amersham Biosciences, UK), using an IPGphor fixed-length Strip Holder. For IEF, a first step of active rehydration, at 50 V for 12 h, was performed; followed by focused sequentially at 500 V for 1 h, gradient at 1000 V for 1 h, gradient at 8000 V for 2:30 h, and finally 8000 V during 30 minutes on an EttanTM IPGPhor IITM (Amersham Biosciences, Uppsala, Sweden). Seven IEF replicate runs were performed and the GE Healthcare protocol for IPG strips pH 3-10, in order to obtain the optimized running conditions, resulting in a final 17 hour run. Focused IPG strips were then stored at -80ºC in plastic bags. Before running the second dimension, strips were equilibrated twice for 15 minutes in equilibration buffer (6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS in 0.05 M Tris-HCl buffer (pH 8.8)). In the first equilibration it was added 1% DTT to the original equilibration buffer and to the second 4% iodoacetamide, and also bromophenol blue was added to both solutions. The equilibrated IPG strips were gently rinsed with SDS electrophoresis buffer, blotted to remove excessive buffer, and then applied onto a 12.52% polyacrylamide gels in a Hoefer^{TM} SE 600 Ruby$^{®}$ (Amersham Biosciences) unit. Some modifications were introduced in the SDS-PAGE technique previously reported by Laemmli [7], that allowed to increase its resolution, with proper insertion of the IPG strips in the stacking gel [6,7]. The 2-DE gels were stained overnight in Coomassie Brilliant Blue G-250 [6]. Coomassie-stained gels were scanned on a flatbed scanner (Umax PowerLook 1100; Fremont, CA, USA), and the resulting digitized images were analyzed using Image Master 5.0 software (Amersham Biosciences; GE Healthcare).

Spots of expression in all gel replicates were manually excised. After tryptic digestion, peptide fragments were analyzed using a MALDI-TOF/TOF UltraFlex model from Bruker Daltonics. For protein identification, peptide mass fingerprints were processed with the Flexanalysis software and searched using the MASCOT server (Matrix Science, London, UK) on the SwissProt database. Carbamidomethyl cysteine was set as fixed modification and oxidized methionine was searched as a variable modification, one missed cleavage was allowed, and a peptide mass tolerance up to 100 ppm was used. A match was considered successful when the protein identification score was located out of the random region with a significance threshold of $p < 0.05$.

3. Results and Discussion

E. coli strains included in this study were previously characterized for antibiotic resistance and virulence factors [5]. The 66 bacterial strains were clustered in four different protein profiles, with major differences in the positions higher than 60 kDa of molecular weight. Further, full proteomic studies were performed in the same IEF and SDS-PAGE conditions, for four strains which were selected randomly, on base of their SDS-PAGE patterns and phenotypic/genotypic characteristics (Table 1). Three of the selected strains (SU23, SU62 and SU60) present a phenotype of multidrug-resistance that included antimicrobial agents belonging to at least three different antimicrobial categories (such as tetracyclines, fluoroquinolones, penicillins, folate pathway inhibitors or phenicols), while and strain SU76 shows resistance only to tetracycline. The use of pH 3-10 IPG strips resulted in a well spread display of protein spots which allowed their safe and accurate excision and image identification. A total of 857 different spots were picked from the strains analysed, respectively: 320 (SU76), 199 (SU23), 150 (SU62) and 188 (SU60).

Table 1 Phenotypic and genotypic characterization of the four stains selected for IEF X SDS-PAGE.

Isolate	Phenotype of resistance[a]	Resistance genes detected	Phylogenetic group	Virulence factors
SU76	TET	*tet*A	A	*fim*A
SU23	AMP-TET-NA-STR- SXT	bla_{TEM}-*sul*3-*tet*A-*tet*B-*aad*A	A	
SU62	AMC-AMP- TET-STR-SXT-CHL	bla_{TEM}-*sul*1-*aad*A-*tet*B-*cml*A	B1	*fim*A-*aer*
SU60	AMC-AMP-CIP-TET-STR-NA-SXT-CHL	bla_{TEM}-*sul*3-*aad*A-*tet*A-*cml*A	B1	*fim*A-*aer*

aAMP, ampicillin; AMC, amoxicillin-clavulanic acid; TET, tetracycline; STR, streptomycin; SXT, sulfamethoxazole-trimethoprim; NA, nalidixic acid; CIP, ciprofloxacin; CHL, chloramphenicol.

To date, from the spots collected we could identify a total of 379 proteins, 116 (SU76), 94 (SU23), 75 (SU62) and 94 (SU60) respectively, corresponding to 36%, 47%, 50% and 52% of the identified spots. These preliminary results based on the full proteome analyses of these strains revealed proteins related to biosynthesis and regulation, glycolysis, transport, stress response, cellular metabolic processes and antibiotic resistance (Figure 1) and mostly are localized either at the membrane or cytoplasmic level.

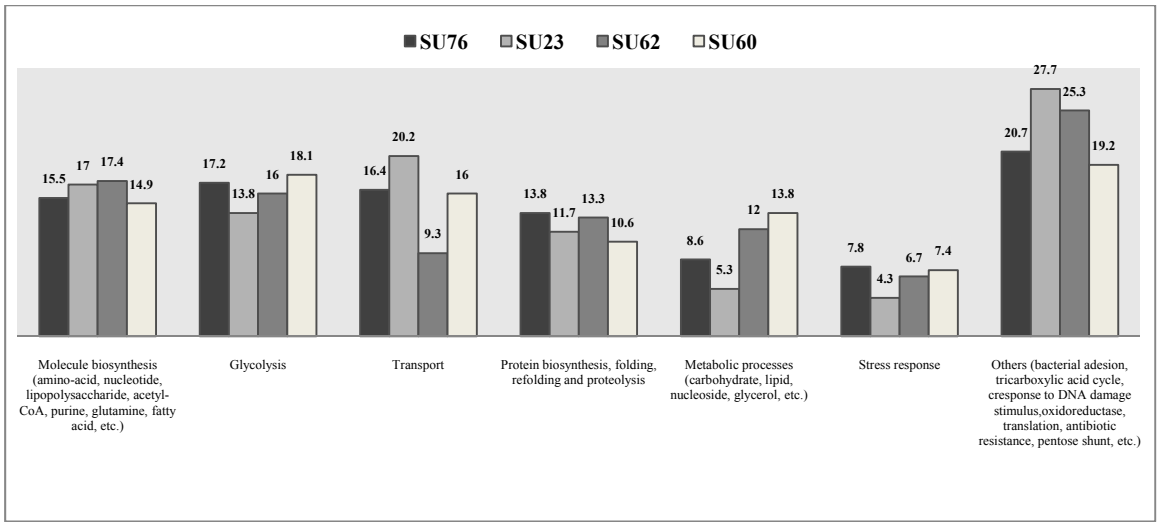

Fig. 1 Distribution of the biological processes related to the protein spots identified found in the 2-DE gels of the *E. coli*. strains, respectively SU76, SU23, SU62 and SU60.

Resistance can result from intrinsic features of the organisms, such as the outer membrane proteins (OMPs) in gram-negative bacteria and the expression of efflux pumps [4]. OMPs were identified in all strains studied by 2-DE, OmpA (like spots, 408 of SU76, 800 of SU62, protein molecular weight (MW) =37292 and pI=5.99, Figure 2) is required for the action of colicins K and L and for the stabilization of mating aggregates in conjugation, serve as a receptor for a number of T-even like phages and also acts as a porin with low permeability that allows slow penetration of small solutes. OmpA is also important for bacterial virulence and was also found overexpressed in others *E. coli* resistant strains [3]. The outer membrane protein assembly factor BamA, which is involved in assembly and insertion of beta-barrel proteins into the outer membrane, was also identified in all strains (like spots, 1 of SU23 and 523 of SU60, PW=90611 and pI=4.93, Figure 2). In addition, outer membrane proteins of the gram-negative porin family were identified in same of the strains, like OmpD (spots 395of SU76 and 788 of SU62, MW=40654 and pI=4.57, Figure 2) and OmpC (spots 79 of SU23 and 622 of SU60, MW=40343 and pI=4.58); they form pores that allow passive diffusion of small molecules across the outer membrane. In *E. coli* it has been well described that the OMPs are involved in streptomycin, nalidixic acid, chlortetracycline and tetracycline resistance [8] and, strains carried out present resistance to tetracycline, streptomycin and nalidixic acid.

Moreover, the heat resistant agglutinin 1 (HRA1), identified in strains SU76 and SU23 (spots 497 and 147, respectively) is a monomeric outer membrane agglutinin responsible for bacterial adhesion. The presence of HRA1 in these commensal strains, described as an accessory colonization factor in enteroaggregative *E. coli* strains, may enhance their pathogenicity of selecting pathogenic lineages [9].

Bacterial surface proteins are important for the host pathogen interaction, as they are frequently involved in disease pathogenesis and contribute to bacterial processes, many of which could be vital for survival within the host and for bacterial growth in stress situations [6]. Several chaperones, related to stress response were detected

in all studied strains. The chaperone protein ClpB (spots 716 of SU62 and 543 of SU60, MW=95697 and pI=5.37) is part of a stress-induced multi-chaperone system that is involved in the recovery of the cell from heat-induced damage, in cooperation with DnaK (spots 286 of SU76 and 12 of SU23, MW=69130 and pI=4.83), DnaJ (spots 281 of SU76 and 714 of SU62, MW=41589 and pI=7.97) and GrpE (spot 675 of SU60, MW=21727 and pI=4.68). The curved DNA-binding protein (spots 444 of SU76 and 111of SU23, MW=34404 and pI=6.33) functions under different conditions, probably acting as a molecular chaperone in an adaptive response to environmental stresses other than heat shock, this protein was found to be up regulated under high resistance conditions by Piras *et al* [8]. In addition, the trigger factor, involved in protein export, also acts as a chaperone by maintaining the newly synthesized protein in an open conformation, was expressed in all studied strains (like spots, 15 of SU23 and 747 of SU62 MW=47836 and pI=4.83). This protein was found to be decreased in abundance in piperacillin-tazobactam-resistant *E. coli* strain by others [3].

In Gram negative bacteria, quorum sensing involves the production, the release and the detection of auto-inducers, which are used for cell to cell communication and for regulation of gene expression of cell community. Quorum sensing has been already described in pathogenesis, virulence and in the biofilm formation [8]. S-ribosylhomocysteinelyase, also called Autoinducer-2 production protein LuxS, is part of the quorum sensing mechanisms since it is involved in the synthesis of autoinducer 2 (AI-2) which is secreted by bacteria and is used to communicate both the cell density and the metabolic potential of the environment. This protein was expressed in three of studied strains SU76, SU23 and SU62 (spots 514, 183 and 852, respectively, MW=19575 and pI=5.18).

A great number of proteins which were related to several functions within the cell metabolism were also found among these strains. Fructose-bisphosphate aldolase class 2, or FBPA, and Glyceraldehyde-3-phosphate dehydrogenase A, or GAPDH-A, are both glycolytic enzymes that were found among these strains (spot 610 of SU60 represent fba protein, MW=39351 and pI=5.52; while spot 806 of SU62 represent gapA protein, MW=35681 and pI=6.61). FBPA and GAPDH-A were found to be up regulated in high resistant *E. coli* bacteria [8]. On the other hand, Santos *et al*, found that the glyceraldehyde-3-phosphate dehydrogenase A protein was decreased in abundance in piperacillin-tazobactam-resistant *E. coli* strains, what could be a consequence of the biological cost that resistance imposes to fitness of bacteria [3].

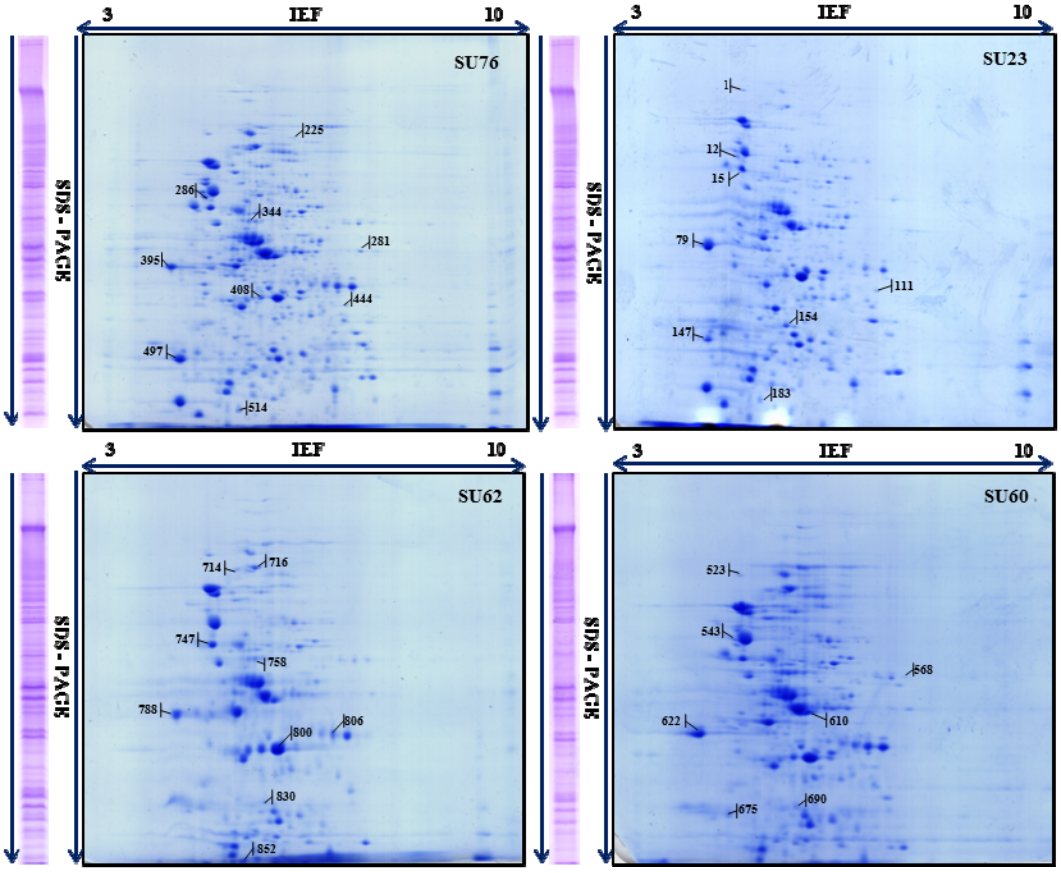

Fig. 2 First monodimensional profile (left) (Coomassie R-250 staining) and respective 2-DE gel image (Coomassie G-250 staining) obtained in strains.

Moreover, Glutamate decarboxylase alpha (or GAD-alpha) converts glutamate to gamma-aminobutyrate (GABA), consuming one intracellular proton in the reaction. The gad system helps to maintain a near-neutral intracellular pH when cells are exposed to extremely acidic conditions. This protein was found in stains SU76, SU62 and SU60 (spots 344, 758 and 568 respectively, MW=53221 and pI=5.22) and is essential for successful colonization of the mammalian host by commensal and pathogenic bacteria. For pathogenic bacteria to successfully colonize the intestine, they have to overcome the gastrointestinal tract barriers, including the primary bactericidal barrier, the gastric acidity [10]. The detection of this feature, in two commensal multi-resistant strains should be considered as a worrying factor.

In our study, three of the studied strains showed a phenotype of resistance to β-lactam antibiotics and presented the corresponding resistance gene bla_{TEM} (Table 1). In these strains three protein spots were identified as beta-lactamase TEM (spots 154, 830 and 690 respectively, MW=31666 and pI=5.69). This protein hydrolyzes the beta-lactam bond in susceptible beta-lactam antibiotics, thus conferring resistance to penicillins and cephalosporins, confirming that our strains possesses resistance to β-lactam antibiotics. Moreover, the protein DNA gyrase subunit B, identified in spot 225 (MW=90179 and pI=5.72) of strain SU76 is also associated with antibiotic resistance. DNA gyrase negatively supercoils closed circular double-stranded DNA in an ATP-dependent manner and in gram negative microorganisms it is identified as a quinolone target. Amino acid substitutions involved in the development of quinolone resistance have been described in subunits A and B of DNA gyrase [2].The emerging resistance to fluoroquinolones and the production of extended-spectrum b-lactamases (ESBL) by multi-drug resistant *E. coli* strains has caused increasing concern over the last decade due to the limited therapeutic options if infections with these strains occur [8].

Comparative proteomic approaches of *E. coli* strains with different genetic profile showed the same changes in the full proteome, especially in the outer membrane proteins, stress response proteins or proteins involved in metabolic processes that were expressed by the different strains studied.

Proteomic methodologies contribute towards determining antimicrobial resistance mechanism(s) through the capacity to analyse global changes of bacteria. In our study we could detect and confirm the expression of beta-lactamase proteins, in strains that showed to be resistant to β-lactam antibiotics. The identification and characterization of *E. coli* proteomes can be a valuable source of information and with possible applications in biochemical and biotechnological research areas for understanding the metabolic pathways leading to antibiotic resistance as well as protein expression in stress response.

Acknowledgements The support by national funds of Fundação para a Ciência e Tecnologia (FCT), cofinanced by POPH QREN Type 4.1–Advanced Training, and subsidized by the European Social Fund and National Funds of Ministry of Science and Technology for High Education (MCTES), to Sónia Ramos and Júlio Miranda (SFRH/BD/47706/2008 and SFRH/ BD/80001/2011, respectively) is gratefully acknowledged.

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Purification and characterization of antifungal lipopeptide from a soil isolated strain of *Bacillus cereus*

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An antifungal lipopeptides –producing strain was isolated from soil and identified as *Bacillus cereus* according to 16S r DNA analysis. The corresponding antifungal lipopeptides were purified to homogeneity by calcium chloride precipitation, Sephadex G-25 and RP-HPLC. The structures were elucidated by ESI-MS/MS and constituted by three main homologues: MW 1008.6, 1022.7 and 1036.7 Da respectively. The amino acid sequence showed that they differed from the surfactins, iturins and fengycins produced by the other *Bacillus* species. It demonstrated broad–spectrum, fungicidal activity and was active against clinically relevant yeasts and molds. The cytotoxic activity of lipopeptides against human erythrocytes was measured and did not show haemolytic activity even at the highest concentration tested. Also, we found that the lipopeptides showed significant cytotoxicity against the mouse fibroblast cancer cell line, L929.

Keywords: Antifungal; lipopeptides; *Bacillus cereus;* ESI-MS/MS

1. Introduction

The expanding size of the immunocompromised patients and emergence of drug resistant fungal pathogens underline the importance of new antifungal agents. In this connection much interest has been focused on novel therapeutic approaches using peptide antibiotics. Peptide antibiotics are quite diverse, amphipathic, either non-ribosomally synthesized (e.g., bacitracins, polymyxins, gramicidins) or ribosomally synthesized (b-defensins, magainins, thionins, etc.)[1]. Microorganisms including bacteria and fungi use non-ribosomal peptide synthetases to produce broad structural and biologically active peptides. This often contains unnatural aminoacids (D-aminoacids) and other molecules not found in ribosomally produced peptides [2].

The potential of *Bacillus* spp. to synthesize a wide variety of peptide antibiotics representing different basic chemical structures, they has been intensively exploited in medicine and industry [3]. In this context, we have been investigating the properties of antifungal lipopeptides purified from a soil isolate of *Bacillus cereus*. The present study describes the identification of an antifungal lipopeptide producing strain *Bacillus cereus* isolated from soil, and the purification, structural elucidation, and antifungal spectrum of the purified compound.

2. Materials and Methods

2.1Determination of 16S rDNA sequences of the isolate

Primers used for 16S rDNA sequence analysis were 16SF (AGAGTTTGATCCTGGCTCAG) and 16SR (ACGGCTACCTTGTTACGACTT). PCR was performed under the following condition: 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 59°C for 1 min and 72°C for 2 min, with a final 7 min extension at 72°C. Amplification of the PCR products of expected size was confirmed by electrophoresis. The PCR product was analyzed with an ABI Prism DNA sequence (ABI). The sequence of the PCR product was compared with the acquired sequence of GenBank using the BLAST programme.

2.2 Purification of the antifungal lipopeptide

Bacillus cereus strain was cultivated in a medium containing 3% peptone, 0.5% yeast extract, and 0.5% NaCl (pH 7.0) for 72 hours at 30°C at 150 rpm [4]. The culture was centrifuged at 15000 rpm for 15 min and supernatant was sterilized by membrane filtration. The sterile culture supernatant fluid was subjected to precipitation by addition of $CaCl_2$. The precipitated proteins were pelleted by centrifugation at 10,000xg for 20 min. The pellet was dissolved in 100mM EDTA–0.05 M Tris–hydrochloride buffer (pH 8.0), and dialyzed extensively against 0.05 M sodium phosphate buffer, pH 7.0, and dialysed. Ethanol (final concentration, 80%) was added to the dialysate, and the mixture was allowed to stand for 6h at 4°C. Precipitates were pelleted by centrifugation at 15000xg for 20 min. Then the supernatant was dried in vacuum, dissolved in minimal volume of water, and acidified with HCl to pH 3.0. Precipitates were collected by centrifugation (15000xg, 4°C),

resuspended in 0.05M ammonium bicarbonate, and dialyzed against deionized water. The dialysate as obtained above was submitted to filtration column of Sephadex G–25 (120 x 1.5cm, Biorad) using 0.05 M ammonium bicarbonate buffer with flow of 30 ml/h and fractions of 2.0 ml/tube were taken and monitored the absorbance at 220 nm. The collected fractions were tested for antifungal activity and freeze dried. Finally, concentrated active fractions from the sephadex G–25 gel filtration were applied to a C18 reverse–phase high–performance liquid chromatograph (RP–HPLC) column (Shimadsu, Japan) with a mobile phase, A (water/TFA (99.95:0.05, v/v) and B (acetonitrile/water/TFA (80:19.95:0.05)) and performed gradient elution starting with 100% A, 0% B changing to 0% A, 100% B over 80 min at a flow rate of 1.0 ml /min

2.3 Mass spectrometry

An Electrospray ionization mass spectrum (ESI–MS) was acquired on an Agilent ion trap mass spectrometer (6340 Series) coupled to an Agilent 1200 series HPLC. The samples were infused to the mass spectrometer through a reversed–phase column (Zorbax SB– C18, 2.1 x 35 cm) with solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The flow rate was maintained at 0.2 mL/min and the UV absorbance was monitored at 210 nm. MS data were acquired over an m/z range, 100– 1500. MS/MS data were collected using collision induced dissociation (CID) with the mass spectrometer operated in a data–dependent mode. All data were acquired in positive ionization mode and were processed with Bruker Data Analysis software, version 4.0.

2.4 Antifungal assay

The *in vitro* antifungal assay of the purified lipopeptide against various pathogenic yeasts was assessed by CLSI M27–A2 [5] and molds by M38–A2 [6]. RPMI 1640 Sigma– Aldrich, St. Louis, MO, USA) was used as the assay medium for *Candida* species, *Cryptococcus neoformans, Aspergillus fumigatus* and *Fusarium oxysporum.* The MIC was defined as the lowest concentration exhibiting no visible growth compared with the control cell. In order to determine the minimal fungicidal concentration (MFC), 100 µl of cell suspension was taken from each well, centrifuged, and washed three times with fresh Sabouraud broth. Then, each cell suspension was vortexed vigorously for 10 seconds, plated on a Sabouraud dextrose agar plate, and incubated at 30°C for 48 h. After incubation, the MFCs were assessed visually as the lowest concentration of the peptide at which there was no growth. All experiments were done in triplicates.

2.5 Cytotoxicity assays

The *in vitro* inhibitory activity on tumor cell line was performed as described previously [7] with L929 cells (mouse fibroblast cancer cell line). The percentage cell death and viability was calculated using the following equation: % cell death = control average – sample average / control average x 100; % cell viability = 100 – % cell death. Haemolytic activity of the lipopeptide was evaluated by determining the haemoglobin release from fresh human erythrocytes. The A blank was evaluated in the absence of additives and 100% haemolysis (A_{tot}) in the presence of 0.2% Triton X–100 in physiological solution. The haemolysis percentage was calculated using the following equation: % haemolysis = $(A_{pep} - A_{blank}) / (A_{tot} - A_{blank})$ x100.

3. Results and Discussion

The analysis of the 16S r DNA sequences indicates that the isolate is *Bacillus cereus*. The 1468 bp sequences were submitted to Gen Bank (Accession number JX512716).The antifungal lipopeptide was purified from supernatant fluid by calcium chloride precipitation, sequential Sephadex G–25, and C18 RP–HPLC. Total ion chromatogram and UV chromatogram revealed three major peaks, numbered from 1 to 3 as shown in Figure 1A and B. The mass spectra corresponding to peak 1, shows protonated masses at m/z 1008.6, (Figure 2). The mass difference of 14 Da was observed between the successive molecules which indicate that they are homologous in nature. The cyclic nature of the peptides was confirmed through saponification reaction, where an increase in mass of 18 Da indicates addition of a water molecule followed by a ring opening [8]. Figure 3 shows LC-ESI MS/MS of the saponified molecules corresponding to peak 1 and the sequences derived through *de novo* sequencing approach following Biemann's nomenclature [9]. By similarity, it can be envisaged that the lipopeptide belong to surfactin class of molecules that contain a β-hydroxy fatty acid with the back-bone cyclization occurred through an ester bond between the hydroxyl group of the fatty acid and carboxylic group of a c-terminal amino acid [10]. The lipopeptide composed of amino acids, Leu–Asp–Val–Leu–Leu–Leu–Leu and is unique in not having Glu or Gln among the repertoire, which is usually found in variants of surfactin family. The complete chemical structure remains to be determined. The *in vitro* activity of lipopeptide was assessed

against various yeasts and mold strains. Also, the antifungal activity was compared with conventional antimycotics like amphotericin B and fluconazole using microplate assay. As revealed in table 1, it inhibited the growth of *Candida* and Cryptococcal strains at concentrations ranging from 1–4µg/ml, while minimal fungicidal concentration (MFC) ranged from 2–4 µg/ml. Both *Aspergillus fumigatus* isolate and *Fusarium oxysporum* were susceptible to the lipopeptide at 4 µg/ml. Cytotoxic activity of a test material is often considered as the first step towards elucidating its anti cancer activity. Long term *in vitro* MTT assay showed that the lipopeptide was cytotoxic to L929 cell line with IC–50 value within the range of 150–200 µg/ml. The absence or less haemolytic activity is important for any antimicrobial agents for its applicability as therapeutic use in humans and animals. The haemolysis percentage was measured against human erythrocyte cells at various lipopeptide concentrations (Figure 4), and it showed very less haemolytic activity, while amphotericin B and fluconazole exhibited more haemolytic activity.

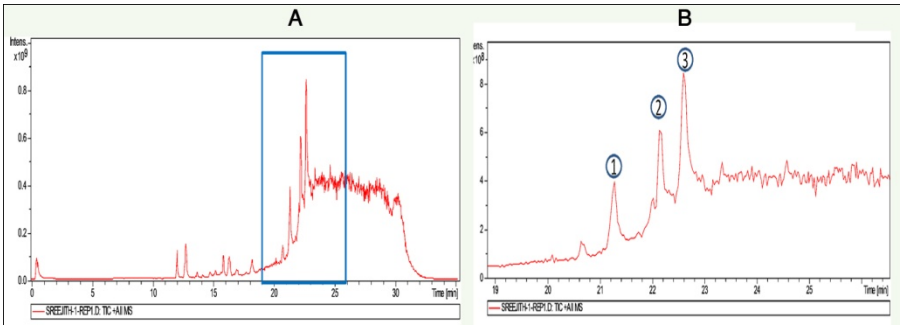

Figure 1: A) Total ion chromatogram showing the bacterial peptides and B) Highlighted region from the TIC which shows three major peaks (marked).

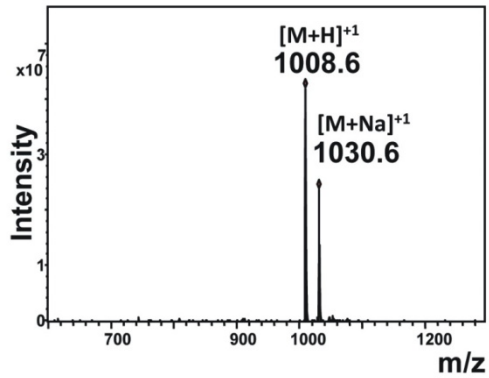

Figure 2: Mass spectra of the molecules corresponding to the peak 1.

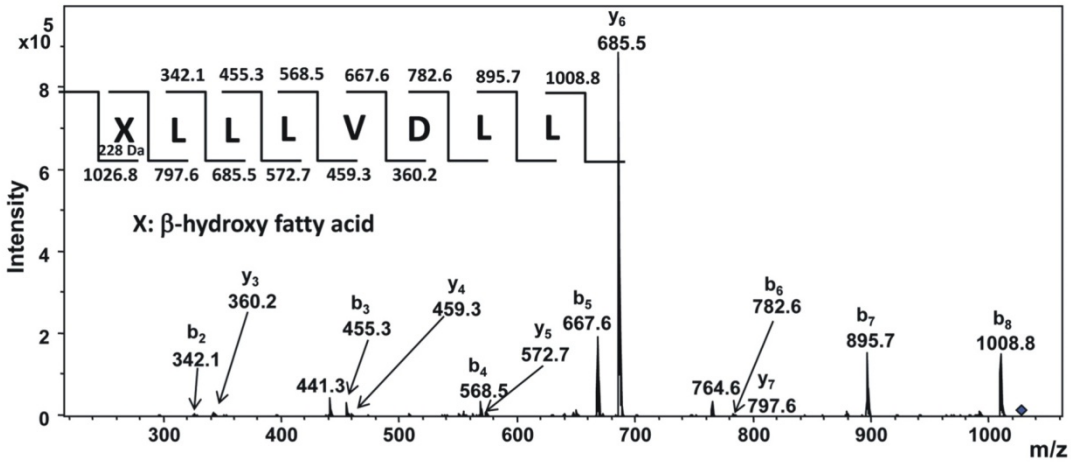

Figure 3: LC-ESI MS/MS spectra of the saponified molecules corresponding to the peak 1. Insets show the sequences derived through *de novo* approach. The b and y-ions are assigned according to Biemann's nomenclature [9].

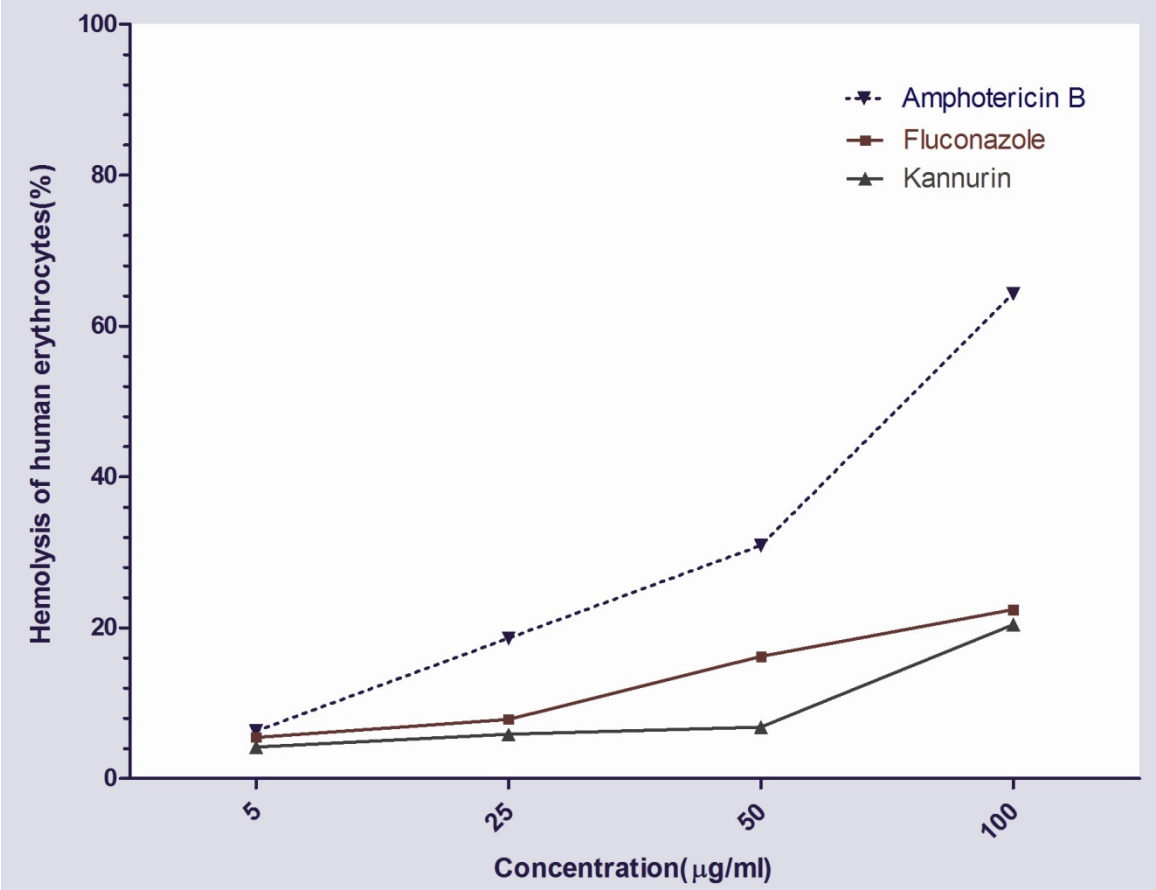

Figure 4: *In vitro* cytotoxicity of kannurin against mouse fibroblast cancer cell line. L929 cells were incubated at 37°C for 24 h in a CO_2 incubator with various concentrations of kannurin.

Table 1: Comparative *in vitro* activities of kannurin, amphotericin B, and fluconazole against yeasts and molds.

Species	MIC and MFC (μg/ml)				
	Kannurin		Amphotericin B		Fluconazole
	MIC	MFC	MIC	MFC	MIC_{80}
Yeast Pathogens					
Candida albicans ATCC 10231	1	2	1	1	2
Candida tropicalis MTCC 1406	2	4	1	1	4
Candida parapsilosis MTCC 1965	1	2	0.5	1	4
Cryptococcus neoformans MTCC 4406	2	4	0.25	1	4
Opportunistic Moniliaceous Molds					
Aspergillus fumigatus(Clinical isolate)	4	8	0.5	2	32
Fusarium oxysporum(Clinical isolate)	4	16	2	4	32

Though the lists of potent antifungal peptides are numerous, only a few of them made their entry as useful drugs. The development of novel antifungal agents with a broad spectrum antifungal activity, and less toxicity against mammalian cells has been anticipated. In conclusion, *Bacillus cereus* strain isolated from soil was shown to produce an antimicrobial peptide with a molecular mass of 1008.6 Da. Based on its unique physical, chemical and biological characteristics, this novel inhibitory substance has potential use for control of pathogenic fungi.

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Researchers, physicians, patients – don‘t be Lost in Translation

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Recognized problem on drugs and diseases terminology is translation from one language to another as well as usage among patients and healthcare practitioners. The aim of this work is to present HRANAFINA project as a part of STRUNA database of Croatian Special Field Terminology.

Keywords antimicrobials; pharmacology; nomenclature; linguistics; terminology

1. Introduction

Over the last decades, scientific research on the pharmacokinetics and pharmacodynamics of antimicrobial agents has increased. Unfortunately, definitions and expressions used by various authors differ in their meaning and various authors use different expressions to indicate the same meaning, so the comparison of the results of various experiments becomes more difficult. Efforts and progress were done on proper use and expression of commonly used expressions in pharmacokinetic and pharmacodynamic research [1]. Another recognized problem on drugs and diseases terminology is translation from one language to another and usage among patients and healthcare practitioners [2,3].

The translation process should ensure that the words have the same meaning as the original, in terms of semantics, either by using a direct equivalent. Grammatical problems associated with idiomatic expressions can hinder the literal translation of certain items. In the context of a linguistic validation, the translation must be applicable to use in the events and experiences of everyday life. Therefore, a literal translation may not be the most relevant kind. Often words do not have quite the same conceptual meaning in different languages. The process of translating and adapting scientific literature, textbooks for students, drug informations, leaflets for physicians or patients, or instruments for evaluating patients – as questionnaires, can be difficult in some circumstances due to the concepts evaluated, the wording of the phrases in the original version or phrasing in the target languages. There are few steps to be followed when translating in new languages [4]. Cultural adaptation is the first step and the second step consists in analyzing the properties of each new language version [5-7]. If the process of adaptation ensures a consistency in content and face validity, reliability and validity have to be confirmed for each new translation and compared to the properties of the previous languages.

The aim of this work is to present HRANAFINA project as a part of STRUNA database of Croatian Special Field Terminology.

2. Methods

The HRANAFINA project as a part of STRUNA database of Croatian Special Field Terminology was officially inaugurated on the web in February 2012 as open-acces database with the aim to gradually make available to the public the standardized Croatian terminology for all professional domains. HRANAFINA project is working on the Croatian terminology in fields of human anatomy and physiology including terms used in pharmacology as well, and grouped in sections like drugs, cell, therapy, receptor, protein, membrane, channel, enzyme. All terms with their definitions include their equivalents in English and are developed following the recommendations of the The Institute of Croatian Language and Linguistics as National Coordinator for Development of Croatian Special Field Terminology. The project supports two basic areas of the National Strategy for Science Development – development of information technology and sociocultural transition.

The Croatian Anatomy and Physiology Terminology Project (HRANAFINA Project) receives a grant from by The National Foundation for Science of the Republic of Croatia. Its lead institution is the University of Zagreb School of Dental Medicine and the main cooperating institution is the Institute of Croatian Language and Linguistics. The project fits into two basic areas of National strategy for Science Development: development of information technology and sociocultural transition from an industrial society to a knowledge-based society. The project has a two-pronged aims: building up of a Croatian terminology in fields of human anatomy and physiology including terms used in pharmacology, and Croatian terminology usage popularization among students of biomedical sciences, dentists, physicians, scientists, patients and all other interested parties.

3. Results

The project's two aims were well met: the building up of a Croatian terminology in fields of human anatomy and physiology including terms used in pharmacology and its usage popularization among students of biomedical sciences, dentists, physicians, scientists, patients and all other interested parties. The project has received good press: more than 20 doctors of medicine and dental medicine from four medical schools on three Croatian universities-the School of Dental Medicine University of Zagreb, the School of Medicine University of Zagreb, the School of Medicine University of Rijeka and the School of Medicine University of Split were joining the project and actively participating in the development of the Croatian terminology in fields of human anatomy and physiology including terms used in pharmacology. Collaborating with the Institute of Croatian Language and Linguistics, in project were included more than 2500 terms coming from foreign languages (mostly English and German). Terms were analyzed, edited and adopted according to Croatian grammar. In order to realize the aims, a project website with an online database of human anatomy and physiology terms, including terms used in pharmacology as well, was established, terminology manuals were prepared and terminology workshops were organized. As a permanent achievement of this project, and in cooperation with the Institute of Croatian Language and Linguistics and foreign language experts, an online multilanguage dictionary (Croatian, English, German, Italian and Latin) with advice on Croatian grammar will be developed and available. Since Spanish is the second worlds language, it is considered for inclusion in future project plans.

4. Discussion and Conclusion

The project will gradually improve the circulation of knowledge and information in the Croatian language as well as in the broader multilingual environment, facilitate the involvement of Croatian scientists, health care providers and medical students in international projects and become helpful official multi-language tool for international students coming to Croatian universities. In addition, offering medical terminology in the Croatian language, this open-acces database will facilitate physician-patient communication and provide user-friendly manual for informing people with no medical training. From these results, normative data in translating from and to Croatian language should be determined through using this new instrument in relevant populations in cross-national studies.

Community level approaches are less costly than patient-centered or physician-centered approaches, and have the potential to reach a broader population, raise general awareness of the issue, and shape social norms [8]. Access to care, language, literacy, and cultural factors compromise the receipt and acceptance of messages. Sociocultural and economic factors underlie cross-national differences in expectations, access to medications, and prescribing patterns [9-14]. Little is known about the effect of antibiotics related health campaigns on populations that are challenged by less access to care, lower education, low income, low English proficiency, or nonmainstream cultural backgrounds. Recent reports suggest a need to improve the general public's antibiotic-related knowledge, attitudes, and awareness [15, 16]. Limited English proficiency may contribute further to cultural distance from mainstream professionals. Since physicians frequently misinterpret their patients' expectations about antibiotics [17, 18], it is reasonable to assume that this effect may be exacerbated in encounters with patients who are socially and linguistically discordant with them. Providers, researchers, and policy makers, as well as patients, will benefit from a better understanding of the translation problems presented in this study, as well as from translation tool system proposed in Croatia.

Patients need a user-friendly format using straightforward, nontechnical language for what was systemic functional linguistics proposed [3, 19]. It is a theory of language which is concerned with the interaction between text and context [19]. Three dimensions of a situation are identified as having an impact on language: the mode of communication, the relationship between the people involved, and the topic or focus of the activity. This study has provided example that ananalytic linguistic framework for medical information enables a systematic understanding of translation. It can therefore be of use in guiding the production of text translations. Apart from provision of tool for translation, an equally important function of the project HRANAFINA was found to be provision of instructions for translators in organised terminology workshops. For both information and instructions to be clearly understood, the grammar needs to be consistent with the purpose of the term, phrase or text, achieved in supervision of the Institute of Croatian Language and Linguistics.

Although work on the online project database and the editing of terms in fields of human anatomy and physiology including terms used in pharmacology was time-consuming, project team members found enough free time to give their own contributions. They did so for free without any financial compensation. Some of the participants said that they were honoured to be a member of a team working on the development of the language of their profession, and that this was a chance to become "a part of the history". The vast majority of participants of the project considered it important and very important for the development and preservation of Croatian language and national identity. In conclusion, we express the wish that HRANAFINA project, which was

prepared by the long-term strenuous and meritorious work of an enthusiastic team of anatomists, physiologists, pharmacologists and linguists may gain general acceptance and may serve reliably for a long time for communication among experts and non-experts in Croatia, Europe and all over the world. We hope that this project contributes to overcome some terminology shortcomings that, in our opinion, have been avoided by intensive cooperation with linguists. Read more on http://hranafina.sfzg.hr/.

Acknowledgements The support of the Croatian Science Foundation is gratefully acknowledged.

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Screening of methanol Portuguese ethno botanical plant extracts for the antimicrobial activity

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In this work, we describe the generation of a plant natural product library based on the ethno botanical use of Portuguese plants. The systematic screening of antimicrobial plant extracts represents a continuous effort to find new compounds with the potential to act against multi-resistant bacteria and protozoa. The purpose of this experimental study was to observe the antimicrobial effect of methanol extracts of 55 plant samples selected from ethno botanical use of Portuguese plants. Two strains of Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and two strains of Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) were subject to screening on constructed Library using a micro-well assay and Agar plate assay. The extracts were also tested for mammalian cell cytotoxicity (using Vero cells). The results suggest that 2 to 4% of the extracts exhibit a 90 to 100% of bacterial inhibition in Gram-positive bacteria.

Keywords: Plant extracts, anti-bacterial, Ethnobotany, Portugal

Introduction

One of the major research focuses of the pharmaceutical industries is the quick and efficient detection of active compounds that can be used in Medicine. There is an increasing number of disease-related targets and consequentially the need for large and diverse compound libraries from which the active compounds could be selected [1]. In this article, we describe for the first time the generation of a library based on the ethnobotanic used of Portuguese plants.

Material and Methods

The selection of the Plants species used was based on [2,3]. Initially a total of 55 different species were collected from different regions of Portugal. These dried samples were bought from the company Antiga Ervanária - Alfredo A. Tavares, Sucessores., Lda, and the material was maintained at 4ºC until further use and a voucher specimen for each plant was deposited at IHMT-UNL (Table 1).The preparation of plant extracts libraries were performed by solvent extraction by percolation in 10 ml of methanol on 500 mg of dried plant samples, for 48 hours in the dark at room temperature. The extracts were evaporated to dryness and samples were re-suspended in 400 µl of dimethylsulfoxide (DMSO), giving a final concentration of 250 µge/µl [4]. Libraries extracts were stored in 1.8 ml freezing vials at -80 °C (fig 1). The extracts were tested for cytotoxicity by MTT assay [5] using Vero cells. Two strains of Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and two strains of Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*), present in the lab were used in the bioassays. The bacteria were subject to screening on constructed Library using a micro-well assay [6] followed by p-iodonitrotetrazolium chloride (INT) [7]. The initial screening for anti-bacterial activity concentration was 312 µge/mL; assuming that an active compound will have an average molecular weight of 500 and a yield of 0.1% of the extract, this correlates to the presence of low micro-molar compounds [4]. The activity of the selected extracts was subsequently confirmed by a screening in dose range response between 78 and 390 µge/mL.The Agar well diffusion assay, was performed according to [8]. The thin layer chromatography(TLC) that was developed using $CHCl_3$/MeOH (3:1) as solvent and the plate (MERCK NM300) detected with UV (254-350 nm). The Bio autography, using INT method as indicator of bacterial growth, were performed according to [9].

Table 1 Plant species selected and parts used in this study

Sample number	*Specie* (family)	Plant part used
1	*Achillea ageratum L.* Asteraceae (Compositae)	flower
2	*Adiantum capillus-veneris* (Pteridaceae)	leaves
3	*Agave americana* L. (Agavaceae)	Leaves, frut
4	*Agrimonia eupatoria* L. (Rosaceae)	Shoots, leaves
5	*Lippia citriodora L.* (Verbenaceae)	Leaves, floweres
6	*Borago officinalis* L. (Boraginaceae)	flower
7	*Chamaemelum nobile* var. *discoideum* (Boiss.), (Asteraceae)	flower
8	*Calamintha baetica* Boiss & Reuter (Lamiaceae)	leaves
9	*Centaurium erythraea* Rafn (Gentianaceae)	Shoots, leaves
10	*Chelidonium majus* L (Papaveraceae)	shoots
11	C*ymbopogon citratus* Poaceae (Gramineae)	leaves
12	*Cistus ladanifer* (Cistaceae)	flower, leaves
13	*Cytisus multiflowerus* (L'Hér.) Sweet (Fabaceae)	flower
14	*Datura stramonium* (Solanaceae)	leaves
15	*Datura stramonium* (Solanaceae)	shoots
16	*Equisetum arvense* L (Equisetacea)	shoots, leaves
17	*Eriobotrya japonica* (Rosaceae)	leaves
18	*Eucalyptus globulus Labill.* (Myrtaceae)	leaves
19	*Foeniculum vulgare* Miller subsp. *Piperitum* (Apiaceae)	shoots, leaves
20	*Fragaria vesca* L. (Rosaceae)	leaves
21	*Fraxinus angustifolia* Vahl (Oleaceae)	leaves
22	*Geranium purpureum* Vill. (Geraniaceae)	shoots, leaves
23	*Gomphrena globosa* L. (Amaranthaceae)	flowers
24	*Hypericum androsaemum* L Clusiaceae (Guttiferae)	leaves
25	*Hypericum androsaemum* L Clusiaceae (Guttiferae)	shoots
26	*Hypericum elodes* L. (Clusiaceae)	shoots, leaves
27	*Hypericum perforatum* L. (Clusiaceae)	Floweres, shoots
28	*Lithodora prostrata* (Loisel.) Griseb. (Boraginaceae	shoots, leaves
29	*Malva sylvestris* L., *Malva* L. spp. (Malvaceae)	leaves
30	*Malva sylvestris* L., *Malva* L. spp. (Malvaceae)	flower
31	*Marrubium vulgare* L. (Lamiaceae)	Shoots, leaves
32	*Melissa officinalis* L. (Lamiaceae)	Shoots, leaves
33	*Mentha* x *piperita* L. (Lamiaceae)	Shoots, leaves
34	*Mentha pulegium* L. (Lamiaceae)	Shoots, leaves
35	*Origanum virens* Hoffmanns (Labiatae)	shoots, leaves
36	*Parietaria judaica* L. (Urticaceae)	Shoots, leaves
37	*Persea gratissima Gaertn.* (Lauraceae)	Leaves
38	*Petroselinum crispum* (Apiaceae)	roots
39	*Phlomys lychnitis* L. (Lamiaceae)	Floweres, shoots
40	*Pistacia lentiscus* (Anacardiaceae)	Leaves
41	*Plantago coronopus* (Plantaginaceae)	Shoots, leaves
42	*Plantago major* L. (Plantaginaceae)	Leaves
43	*Potentilla erecta* (L.) Räuschel (Rosaceae)	Roots
44	*Prunus avium* L. subsp. *juliana* (DC.) Rchb. (Rosaceae)	pedicel
45	*Pterospartum tridentatum* (L.) Fabaceae (Leguminosae)	flower
46	*Pulicaria odora* (L.) Reichenb. (Asteraceae)	Leaves, flower
47	R*osmarinus officinalis* L. (Lamiaceae)	leaves floweres.
48	*Sambucus nigra* L. (Caprifoliaceae)	Flower
49	*Sanguisorba minor* Scop. (Rosaceae)	leaves ,flower
50	*Tilia cordata (*Tiliaceae)	leaves ,flower
51	*Urtica membranaceae* (Urticaceae)	Shoots, leaves
52	*Urtica membranaceae* (Urticaceae)	Roots
53	*Vinca difformis* Pourret (Apocynaceae)	leaves ,flower
54	*Zea mays* L. (Poaceae)	stamens
55	*Althaea officinalis* L. (Malvaceae)	Roots

Results and discussion

Plant samples were collected from Portugal representing biological diversity encompassing 34 plant families. Extracts chosen were prepared from plant parts based on their ethnobotanical used. From the plant list described in table 1 a natural plant library was made after methanol extraction and the dry pellets were dissolved in DMSO and maintained at -80ºC, ready to be used. (fig.1).

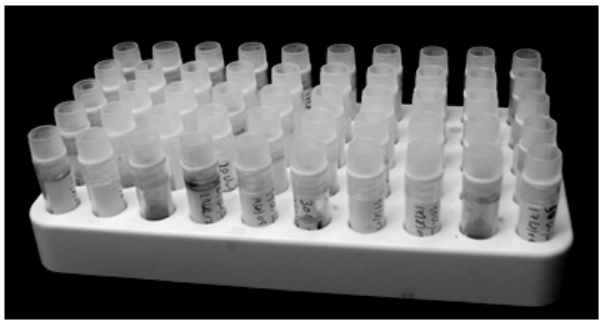

Fig.1 For all methanol fractions material archive was maintained and stored at −80 °C in sterile polypropylene freezing vials.

At the concentration used (300 µge/ml), the bacterial micro-well bioassay inhibition assay showed that *E. globulus Labill* , extracts at the defined concentration has effect on gram$^+$ bacteria (*S. aureus* and *B. cereus)*. This extract was select for the MTT assay using Vero cells. This concentration has no cytotoxicity effects on the Vero cells (data not show). Agar well diffusion assay using the Gram$^+$ *B. cereus* showed that *E. globus* methanolic (fig.2) (1cm) and with *H. androsaemum* L Clusiaceae (Guttiferae) leaves or shoots (data not showed) extracts has as well inhibitory activity on agar well assay (< 0.5 cm).

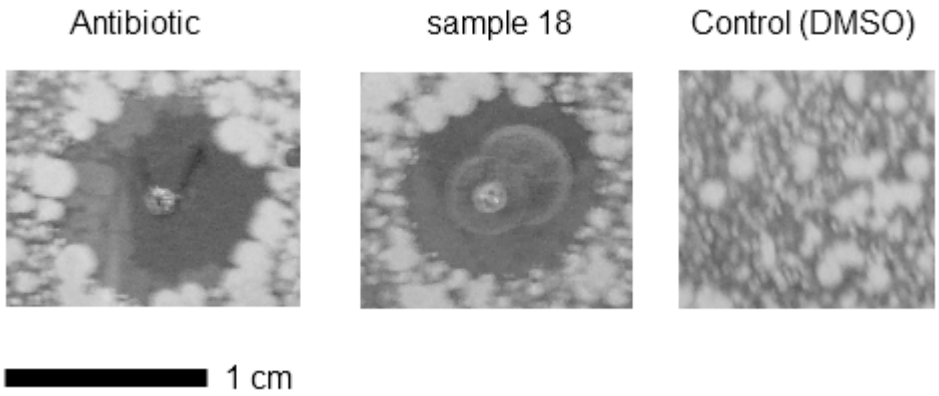

Fig. 2. Comparative activity by the well-diffusion agar method between Antibiotic (50mg/ml ampicillin), methanolic extract 18 *Eucalyptus globulus Labill,* leaves ((250 µge/µl) and DMSO; volume sample (3 µl), Bacteria tested: *B. cereus.* Bar=1cm

Using bacterial micro-well bioassay the inhibitory activity the *Eucalyptus globulus Labill* , methanolic extracts have an IC_{50} = 200 µge/ml. This methanolic extract was also tested by bio autography using TLC plates, in order to test the hydrophobicity versus hydrophilicity of the inhibitory compounds present on the extracts. The results (Fig. 3) suggest that the compounds from extract with inhibitory effect are of hydrophobic nature.

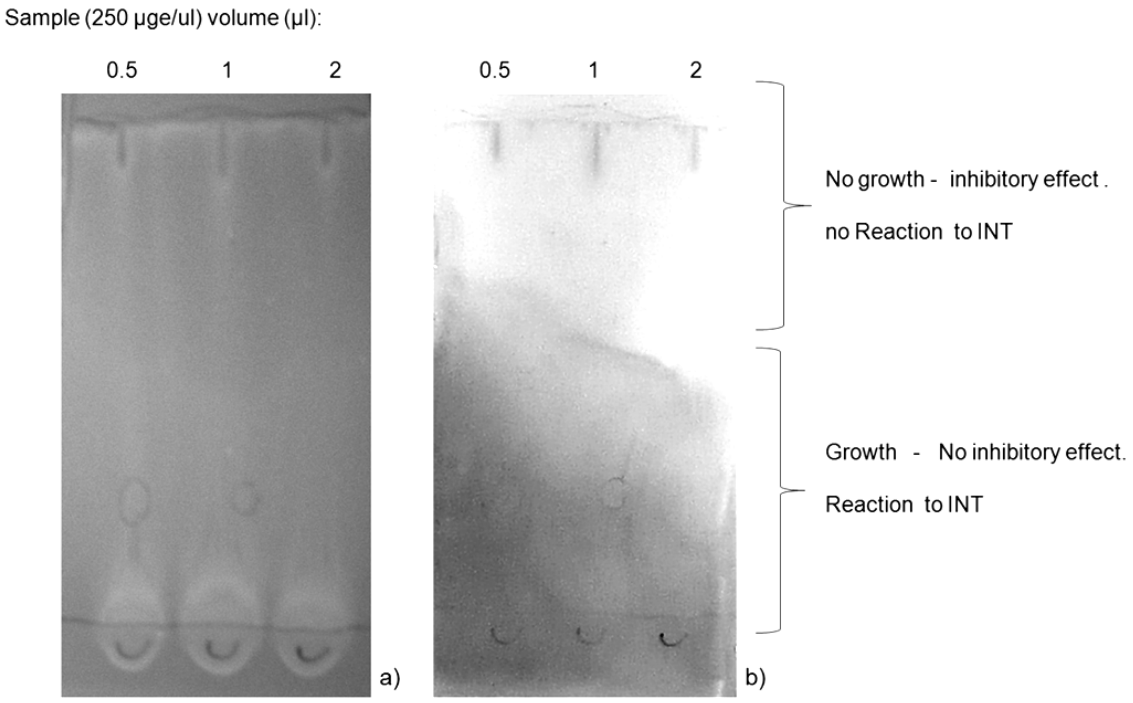

Fig 3. a) Plant extracts were applied and the chromatogram developed using CHCl3 / MeOH (3:1) as solvent and the Thin layer chromatography plate detected with UV (254-350 nm), b) The antimicrobial bioautography of total extract of *E. globus* against *Bacillus cereus* using the INT method.

The *E. globus* methanolic extract exhibit inhibitory effect on the gram^{+} bacteria in accordance with description made by [10]. Studies (GC-MS) are being performed to identify which compounds are responsible by that inhibitory effect. The future application of this library will be for the screening of inhibitors for cellular systems as well as in key enzymes involved in biological processes in parasite's infection [11] especially in *Toxoplasma gondii* infection.

Acknowledgements To Dr T. Conceição (IGC, Portugal) for the Methicillin susceptible *S. aureus* MSSA NCTC8325 and Dr. M Gaspar (FFUL, Portugal) for the *Pseudomonas aeruginosa* and *Bacillus cereus* samples is gratefully acknowledged.

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Specific functionalization of wool with ε-poly-L-lysine (ε-PL) for antimicrobial properties

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The prevention of microbial attack has become increasingly important in many fields of application, among which the bio(medical) and hygiene scope represent only a few areas for which there is a demand for improved antimicrobial activity. Currently, there is a growing concern in bio-functionalization of textile fabrics (including wool) with natural bioactive agents for antimicrobial protection, enable the production of safe, non-toxic, skin and eco-friendly textile products.
ε-poly-L-lysine (ε-PL) is an example of homopolymer with strong antimicrobial activity against most gram-positive and gram-negative bacteria, fungi and also some kinds of viruses. Moreover, it is harmless to humans, biodegradable and has antitumor effect. Thus, it has been already widely used in food manufacturing as a safe preservative. In this paper we present the investigation ofthe efficiency of wool surface modification with ε-PL through site-specific chemical coupling using nontoxic and zero-range reagents 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS).

Keywords: wool fabric, ε-poly-L-Lysine, grafting characterization, antimicrobial activity.

1. Introduction

As textile material surface is usually populated in the symbiotic relationship with both, bacteria and fungi, a lot of attention is recently paid to the development of various antimicrobial treatments of textiles in order to be the most effective protected against a variety of microorganisms [1]. However, the desired level of protection is to develop materials that would ensure not only effective and long-lasting antimicrobial protection to a wide range of pathogenic bacteria and fungi, but also to maintain a constant and well-defined biological environment. Materials should therefore reflect both antimicrobial activity and tissue (e.g. skin) biocompatibility, which means they should be toxic to prokaryotic and at the same time compatible with eukaryotic organisms. Among several antimicrobial agents that have been tested and are used in textiles, i.e. quaternary ammonium compounds, antiseptics and disinfectants, metallic compounds, as well as different colloidal nanoparticles for nano-functionalised surfaces [2], most of them being active not specifically and do not provide necessary security for human and environment. The use of antibacterial compounds like silver, copper or triclosan is a matter of debate despite they may interfere with non-specific defense mechanisms like antimicrobial peptides of skin [3]. Therefore, the profession has focused on the development of biocompatible equivalents. One of such perspective antimicrobial agents is ε-PL [4], which is due to its low minimum inhibitory concentration (MIC) and non-toxic nature, Generally Recognized as Safe (GRAS) and thus being applied in the fields of medicine, food, environment, agriculture, and lastly in the textile industry [5], as in the case, when microbial cellulose was modified with ε-PL, having the ability against infection [6]. Based on [7] poly-D-lysine can be immobilized onto chitosan/glycerophosphate salt hydrogels via azidoaniline photocoupling reaction resulting to biocompatible scaffold being useful for neural tissue engineering [7]. The amino groups of ε-PL were covalently coupled with the carboxyl groups of modified PET fabrics by carbodiimide (EDC/NHS) chemistry [8] resulting to PET fabric with excellent antibacterial activity. Antibacterially-active wool was also prepared by the molecular self-assembly method using ε-PL [9], forming an ultrathin layer on the wool surface with soaking wool into ε-PL solution, however showing low durability. Thus, the main goal of our study was to define the strategy for chemical coupling of ε-PL to wool by EDC/NHS mediated coupling approach bringing satisfy antimicrobial properties .Considering that wool is a proteinaceus fibre with amphoteric and consequently pH dependent functional properties (with the ip between pH 4,8-5,5, depending on its nature and pre-treatment), it offers many reactive sites for its functionalization. Many chemical reagents [1,10,11] or enzymes [5,12] can be applied to activate specific reactive groups in an aqueous environment producing low or high-reactive intermediates being able to react with an functional-active compounds [13]. In that respect Meade and co-workers [14] demonstrated successful covalent modification of wool with fluorescent compounds and micro and nanoparticles using EDC and NHS after controlled wool surface lipid removal. On Fig. 1 schematic presentation of different coupling chemistry of ε-PL on wool using ″grafting onto″ approach is presented where terminal carboxylate groups (pK=2,2) in ε-PL may be derivatized by different reagents (carbodiimides as EDC or acylimidazoles as DCI) through the formation of amide bond forming conjugates or reactive carbonyl intermediates, respectively. In our case EDC was used to convert carboxyl groups of ε-PL into unstable activated intermediates (o-acylisourea) that can react with the available non-protonated amino ($R\text{-}NH_2$ of Lys or

Arg), hydroxyl (R-OH of Ser, Thr or Tyr) or sulfhidril (R-SH of Cys) groups of wool amino-acid residues, depending on the pH of treatment solution and pK values of side reactive groups (pK R-NH_2 ≈ 10,8; pK R-OH ≈ 13; pK R-SH≈8,3) [15]. Water-soluble NHS can be added to increase stability and coupling efficiency through forming an active ester intermediate (N-hydroxy compound) which replace the o-acylisourea intermediate as presented on Fig. 2 [3].

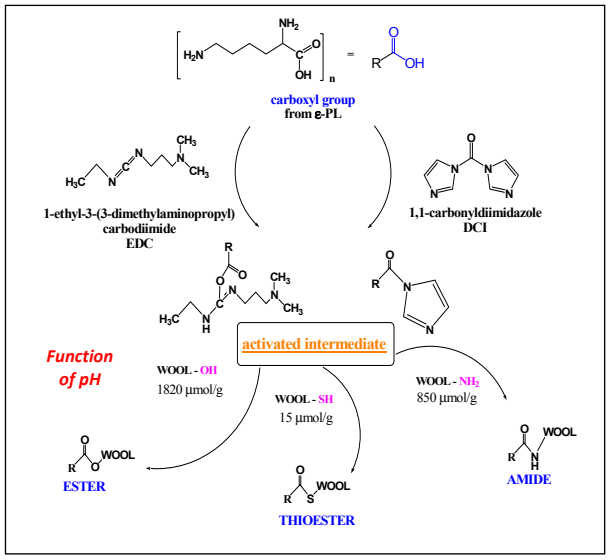

Figure 1: Possible mechanisms for ε-PL grafting onto wool amino acid residues [15,16].

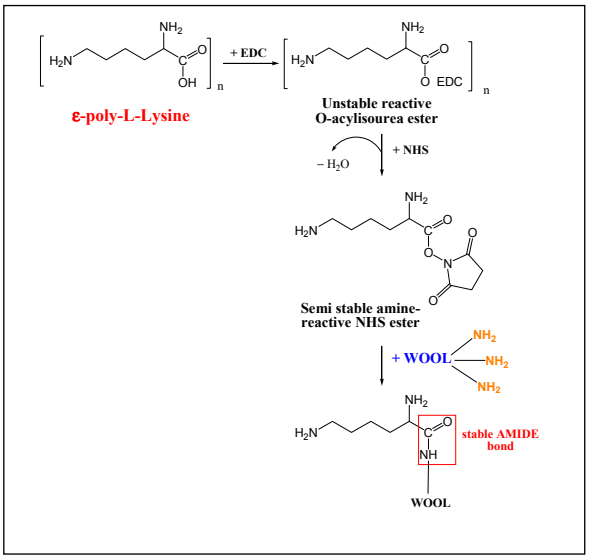

Figure 2: Mechanism that was used for the covalent grafting of ε-PL onto wool.

2. Materials and Methods

2.1 Materials used

The knitted wool fabric used was supplied by Lokateks d.o.o. (Škofja Loka, Slovenia) and was a fine rib1:1 knit with a mean fibre diameter of 23,5 µm and mass/unit area of 230 g/m^2. ε-PL with MW of ≈ 6067 g/mol was purchased from Zhengzhou Bainafo Bioengineering, China. *Escherichia coli* (ATCC 25922) was provided by the American collections of bacterial cultures American Type Culture Collection (ATCC). All other chemicals used in this work were of chemical grade without exception explanation.

2.2 Wool preparation and functionalization with ε-PL

In order to improve the functional chemical groups available for covalent attachment, the wool was pretreated to remove dirt, contaminants and oils as well as lipid layer. For that purpose washing with non-ionic detergent Sandoclean (washing twice at 40 °C for 30 min) and Soxhlet extraction (sequential extraction procedure with dichloromethane and rinsing with water) were used. The functionalization was carried out in two steps: first ε-PL was activated with EDC/NHS at 2,5/2,5/1 (EDC/NHS/COOH reactive groups in ε-PL) molar ratio in 0,15 M phosphate buffer (pH 4,5) for 1 hour. The wool was then reacted with ε-PL at 1/1 mass ratio in 0,15 M phosphate buffer (pH 7,5) for 24 hours at 23 °C by constant shaking. After-treatment procedure was performed with NaOH or EtOH, respectively, in order to remove all not-covalently or via ionic interactions bounded ε-PL being followed by measuring the conductance of washing solutions.

2.3 Methods used for determination of modification efficacy

The numbers of available amino groups, being responsible for the antimicrobial nature of the surface, were determined by a modified colorimetric method using Acid orange 7 dye (indirect analysis). The ε-PL could stochiometrically react with Acid orange 7 (AO7), which results in a decrease in absorbance of AO7 solution due to the formation of ε-PL-AO7 precipitation. Therefore, the concentrations of ε-PL can be obtained according to the decrease in absorbance of AO7 solution at its λ_{max} (484 nm). The standard curve of absorbance at 484 nm as a function of ε-PL concentration was made. Wool pieces with mass of 0,25 g were immersed in

mixture of 4 mL of 1% (mass of dye per mass of fabric) AO 7 dye solution ($c = 2 \cdot 10^{-3}$ mol/L) and 196 mL of acetate buffer (pH = 3˙6) and treated for 6 h at 40 °C in water bath shaker. The concentrations of amino groups were determined by measuring the absorbency at 484 nm and then calculated according to the adsorbed AO 7. The absorbance of the test solution against the blank (buffer) was measured using Infinite 200 series UV/Vis spectrophotometer (Tecan Group Ltd., Männedorf, Switzerland). As the concentration of amines is equal to the concentration of dye, the surface concentration can be calculated by multiplying the dye concentration by the desorbing volume [6].

Solubilized ε-PL was quantitated using colorimetric method of Bradford, using bovine serum albumin (BSA) as standard solution. Optical density of the samples and standards were measured at λ_{max} 595 nm against the reference (ref).

The staining test for the determination of ε-PL on wool fabric was a modification of the Coomassie protein assay (Bradford method described above). Wool fabrics with and without immobilized ε-PL were stained with ready-to-use dye in 1/50 surface to volume ratio at room temperature and constant shaking for 5 min followed by washing with distilled water for two cycles. Wool samples were colorimetrically evaluated by CIELAB colour space using a two-ray SF 600+ spectrophotometer (Datacolor). The source of light was a halogen lamp with xenon lightning that gives standardized daylight D65. Relative colour strengths (K/S values) were calculated from the scanned reflectance curves (R) using the Kubelka-Munk equation: $K/S = (1-R)^2/2R$ (1)

Surface charge of wool after treatment with ε-PL was evaluated as a function of pH medium in the presence of 10^{-3} M KCl by determination of ζ-potential in the contact surface between the wool sample and the fluid. The streaming potential experiments were carried out with the Electro kinetic analyzer (Anton Paar GmbH, Austria) using the cylindrical cell.

One of the most important aspects of our research was the study of interactions between the surface of modified material and pathogenic bacteria. The Gram-negative *Escherichia coli* ATCC 25922 was used to evaluate the antibacterial properties of the treated samples by ASTM E2149-01 Shake flask method.

The antibacterial effect was assessed in terms of the ratio of bacteriostasis, which was calculated using the following equation: reduction [%] = (A-B)/A x 100, (2)

where A and B are the numbers of bacteria colonies on the samples before and after shake flask test, respectively. For satisfactory antimicrobial activity means the reduction value exceed 60 %.

3. RESULTS AND DISCUSSION

3.1 Functionalization of wool with ε-PL

In order to quantify the coupling efficacy of ε-PL to wool fabric a variety of direct and indirect analytical methods, described under Chp.2.3 were used being collected in Table 1.

Table 1 Determination of coupling efficacy of ε-PL with direct and indirect analytical methods using different pre-treatment (washing vs extraction) and after-treatment (NaOH vs EtOH) processes for wool fabric. Legend: n.d.=not defined, W=wool

Pre-treatment: a) washing with nonionic detergent

After-treatment: NaOH			
Samples:	Indirect/solutions		Direct/ substrate
	Bradford g ε-PL/kg	AO7 mmol NH_2/kg	Bradford K/S [at λ=610 nm]
W + EDC/NHS + εPL	3,21	20,30 ± 0,58	17,13
W + εPL	0,56	17,54 ± 0,04	15,38
Ref W	n.d.	18,06 ± 0,64	0,61

b) Extraction

After-treatment: EtOH			
Samples:	Indirect/solutions		Direct/ substrate
	Bradford g ε-PL/kg	AO7 mmol NH_2/kg	Bradford K/S [at λ=610 nm]
W + EDC/NHS + εPL	46,34	36,46 ± 0,86	n.d.
W + εPL	15,83	37,76 ± 0,06	n.d.
Ref W	n.d.	35,45 ± 0,78	n.d.

The results of AO7 evaluation method indicated that the absorption of ε-PL onto wool increased for about 14 % compared to wool treated only with ε-PL and the reference sample, being more pronounced at washed and NaOH-after-treated wool. Measurements, obtained by Bradford analysis, also confirmed that wool treated with ε-PL and EDC/NHS poses more amino groups compared to the samples treated without the EDC/NHS.

The K/S values of the stained wool are showing that the reference wool is not stained by Coomassie dye (K/S = 0,61), probably due to the coulombic repulsions between negatively-charged wool and anionic dye in the acidic environment caused by their surface electric properties at low pH. The treatment of wool with ε-PL resulted in the existence of remarkably visual blue colour, being the deepest (K/S of 17,13) at wool sample treated with ε-PL and EDC/NHS.

Figure 3 shows changes in the electrokinetic potential of wool surface brought about by the treatment of wool (pretreated with extraction and aftertreated with EtOH) with ε-PL.

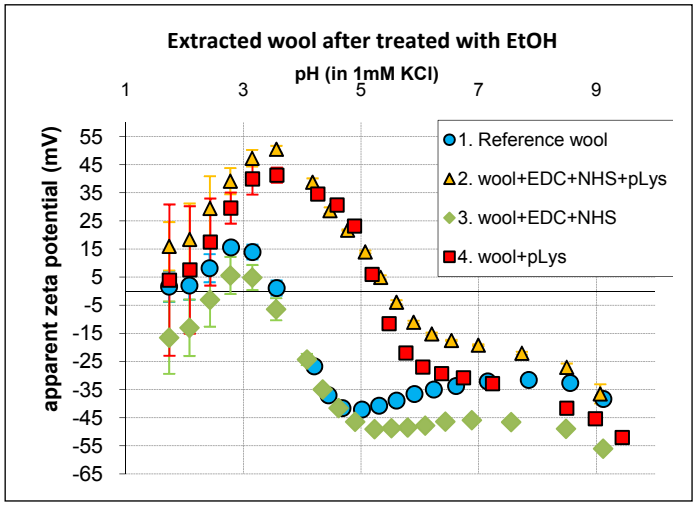

Figure 3: Zeta potentials (ζ) of wool samples. Reference wool and wool treated with EDC/NHS, but without ε-PL, showed the ip around pH 3,5 and the net negative ζ-potential which indicated that the surface is hydrophilic. The addition of ε-PL resulted to an increase in the ζ-potential at almost the entire pH area and the ip is shifted to around pH 5,5. The analysis also shown the difference in the negative ζ-potential at high pHs (pH 9) between samples treated only with EDC/NHS (-56,04±2,12) and ε-PL+EDC/NHS (-36,54±3,41) which increases by the pH decreasing, indicating on a tighter structure of wool.

3.2 Antibacterial measurement

Antibacterial activity evaluation of treated samples against reference wool is shown in Table 3.

Table 3 Antibacterial effect of wool treated with ε-PL at pH 7,5 and using EDC/NHS in molar ratio EDC/NHS/NH2=2,5/2,5/1 against *E. coli*

Sample:	Bacteria reduction [%]
Ref W	9,09
W + ε-PL	58,18
W + EDC/NHS + ε-PL	73,64

Results of Shake flask test presents evidence that wool functionalized with ε-PL using EDC/NHS reagents have higher antibacterial activity against *E. coli* than those treated only with ε-PL or the reference.

Based on the obtained results we can conclude that the treatment conditions significantly affected the performance properties of treated wool fabrics. The best performance properties were obtained when the wool fabric was pretreated with the extraction process. The wool fabric and the ε-PL molecules carried negative and positive charges, respectively, under the treatment conditions (pH = 7,5) because their isoelectric points are around pH 4,9 and pH 9,0, respectively. Thus, the electrostatic adsorption of ε-PL on wool occurred presumably simultaneously with the grafting reaction, resulting to comparable values of the amino groups on the wool surface treated without EDC/NHS, to the wool treated with EDC/NHS and ε-PL. However, the results clearly shown that by using EDC/NHS the deposition of ε-PL increased, meaning that treatment via EDC/NHS approach provided more effective functionalization of wool with ε-PL, resulting to an increased antibacterial activity.

Acknowledgements This research was supported by the Ministry of Higher Education, Science and Technology and 7FP [Era-Net Matera Plus ANTIMICROB PEPTIDES grant no. 3211-10-000369].

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Studies of the antibacterial and antifungal activity profiles of *Olea europaea* L. cv. *Arbequina*

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Arbequina is a cultivar of the olive tree. It was introduced in Tunisia for its high productivity in addition to its pest resistance. This present research revealed a supplementary potential of this cultivar which exhibited a wide range of antimicrobial activity, especially of its aerial part volatiles. This activity was expressed by the important zone of inhibition of the growth of several bacteria and fungi. No antifungal activity was noted against *Botrytis cinerea* which seemed to be insensitive to these volatiles at the concentration used. Chemical studies are needed to identify the active compounds involved in the antibacterial and antifungal activities.

Keywords Antimicrobial activity; olive tree; volatiles; inhibition zone; mycelial growth

Introduction

Olea europaea, the Mediterranean tree, is an important economic and social tree. Tunisia occupies the 4th world position as producer and the third rank as exporter [1]. Improvement, development and protection efforts are essential for a renovated, profitable and competitive olive sector. To meet these challenges, the strategic objective recommends in any way the introduction of new varieties of olive, known for a good production in quantities and qualities.

Arbequina is a cultivar of olives, introduced in Tunisia for its high productivity as well as its pest resistance [2]. This cultivar is adaptable to different conditions of climate and soil. Its relatively small cup, allows it to be cultivated under more intensified, high-density conditions than other olive plantings. However, intensive production in agriculture increases the opportunities for diseases to develop as compared with undisturbed natural ecosystems [3]. The aim of this research is to assess the antimicrobial potential of this introduced cultivar against several bacteria and fungi and qualify its eventual resistance. In the best of our knowledge, the essential oil of the aerial part of *Arboquina* (leaves, stems, flowers and mixed shoots) are studied for the first time in the current study.

Materials and methods

1. Plant material

Arbequina is a cultivar of olives (Figure 1). The fruit is highly aromatic, small, symmetrical and dark brown. This cultivar is very productive and enters early into production. *Arbequina* olives have one of the highest concentrations of oil (20-22%) and are, therefore, mostly used for olive oil production. *Arbequina* was conducted in intensive drip irrigation and spaced at 7 × 7 m, gathered at the flowering stage in orchards of Menzel el Mhiri, Kairouan, Centre of Tunisia (35° 40′ 12″ North and 10° 05′ 24″ East).

Fig. 1 Arbequina cultivar.

2. Essential oil extraction

Fresh leaves, stems, flowers and mixed shoots of the tree aerial parts were cut in small pieces and submitted to steam distillation for 5–6 h. The oils were collected by chloroformic extraction. After drying the extract over anhydrous Na_2SO_4, the solvent was removed and the oil samples were stored prior to analysis. Yield based on fresh weight of the sample was calculated.

3. Antibacterial activity

3.1. Bacterial agents

Three bacterial agents, isolated from Tunisian soils, were selected as test microorganisms of the antibacterial activity of the olive volatile fractions: *Pseudomonas aureofaciens* 499*, Burkholderia glathei* 35 and *Bacillus pumilus* 420. They were provided by the Laboratory of Phytopathology of the Regional Center of Research in Horticulture and Organic Agriculture (CRRHAB) of Chott-Mariem, Tunisia. They were cultured at 25°C on Nutrient Agar (NA) medium for 48 h before use.

3.2. Antibacterial activity test

The qualitative and quantitative antibacterial assay of the volatile fractions of *Arbequina* was carried out by the disc diffusion method [4]. Five hundred microliters of the inoculums were spread over plates containing Potato Dextrose Agar medium and the paper filter discs (5 mm) impregnated with the volatile fractions at a concentration of 1 mg/disc were placed on the surface of the media. The plates were incubated at 37°C for 18 h. The inhibition zone was measured.

Two controls were also included in the test. The first was a control involving the presence of microorganisms but the absence of the test material and the second is a standard antibiotic (Ampicillin) which was used in order to control the sensitivity of the tested microorganisms, and the developing inhibition zones were compared with those of reference discs.

4. Antifungal activity

4.1. Phytopathogenic fungal species

Five phytopathogenic fungal species were used for the assessment of the antifungal activity of olive volatiles namely: *Fusarium solani*, *Fusarium oxysporum* f. sp. *lycopersici*, *Penicillium italicum*, *Botrytis cinerea* and *Verticillium dahliae*. These fungi were obtained from the Laboratory of Phytopathology of CRRHAB of Chott-Mariem, Tunisia. They were cultured at 25°C on potato dextrose agar (PDA) medium one week before use.

4.2. Determination of antifungal activity of volatile fractions

The disc diffusion method was used for antifungal screening [5]. Fungal broth culture aliquots adjusted to 10^4–10^5 CFU/ml were added to Potato Dextrose Agar medium and distributed uniformly in 9 cm Petri plates. Oil

dilutions were made with chloroform. Under aseptic conditions, paper discs (5 mm) were impregnated with volatile oils at a concentration of 1 mg/disc and placed on the culture plates after removing the chloroform by evaporation.

The antifungal chemical reference product, carbendazim, was used as a positive control and chloroform as a negative control. The diameter of the inhibition zone (mm) was measured after incubation at 28°C for 4 days and compared with control.

Results and discussion

1. Volatile yields

Olea europaea c.v. *Arbequina* did not yield the same amount of oil from the different parts. The total yield of oils obtained from flowers, leaves, stems and mixed shoots was 1.99, 0.71, 0.47 and 0.63 10^{-2}%, respectively. Flowers seemed to be the richest in volatiles, comparatively to the other parts.

2. Antibacterial activity

The antibacterial activities of the volatile fractions were tested *in vitro* by using disc method with three microorganisms as shown in Table 1. The results of the bioassays revealed that all oils exhibited moderate to appreciable antibacterial activities against all cocci Gram-positive and rods Gram-negative bacteria. Inhibition zone diameters varied between 6 and 13 mm.

The flower essential oils were more biologically active against *Bacillus pumilus* than the other oils. A large inhibition zone diameter reaching 11 mm was recorded, which was approximately similar (12 mm) to that of the positive control i.e. ampicillin (Table 2).

Leaf volatiles exhibited interesting inhibition zones against the tested Gram-negative microorganisms i.e. *Pseudomonas aureofaciens* and *Burkholderia glathei.* The inhibition zone diameters were, respectively, 13 and 10.5 mm. *Arbequina* leaf volatiles showed an antibacterial activity against *P. aureofaciens* which was more important than that induced by mixed shoots. This result may be explained by a possible antagonist effect of the components existing in the mixed shoots or the low proportion of the active principles in this part. In fact, the inherent activity of a given essential oil can be expected to relate to the chemical configuration of the components, the proportions in which they are present and to their interactions [6].

3. Antifungal activity

Both *Fusarium solani* and *F. oxysporum* seemed to be sensitive to all tested volatiles of *Arbequina*. An inhibition zone diameter reaching 10 mm was noted. Similarly, powdered, dried olive, extracted with hexane, methanol and butanol, exhibited the strongest activity against *F. oxysporum*, comparatively to the other tested fungi [7]. An important antifungal activity of olive extracts was also reported against *Verticillium* sp. [7]. In our research, mixed shoot and stem volatiles of *Arbequina* were found to be biologically active against *V. dahliae* (Table 1). On the other way, *V. dahliae* appeared pathogenic on several other olive cultivars, such *Koroneiki* [8] and *Chemlali* [9]. Wilting disease of olive caused by *V. dahliae* is widespread wherever this crop is grown. The disease is most damaging on young olive plants, while on older trees, including those over 100-year-old, the disease does not normally kill the tree, but reduces vegetation and causes partial defoliation of one or more branches [10]. Consequentially, *Arbequina* seemed to be more resistant than many other cultivars.

All tested fractions failed to show antifungal activity against *Botrytis cinerea* which seemed to be insensitive to these volatiles at the concentrations used. Similarly, the *in vitro* growth of *Penicillium italicum* was not affected by the majority of *Arbequina* volatiles tested. Contrarily to reports from other researches, olive oil mill wastewater inhibited the *in vitro* mycelial growth of *B. cinerea* and *P. italicum* and significantly decreased fungus mold formation on the tested fruits [11, 12]. These recorded activities were probably due to the phenolic compounds contained on olive oil mill wastewater [11] and which were absent in olive tree volatiles. Tests of other *Arbequina* extracts against these both fungi are needed for eventual antifungal activities.

Table 1 Antimicrobial activity of *Arbequina* volatile oils used at the concentration of 1 mg/disc.

		Flowers	Leaves	Stems	Mixed shoots
Bacterial species					
Gram (+)					
Bacillus pumilus	+/-	(+)	(+)	(+)	(+)
	Φ (mm)	11	9	6	6
Gram (-)					
Pseudomonas aureofaciens	+/-	(+)	(+)	(+)	(+)
	Φ (mm)	9	13	6	7
Burkholderia glathei	+/-	(+)	(+)	(+)	(+)
	Φ (mm)	8	10.5	6	10
Fungal species					
Penicillium italicum	+/-	(-)	(+)	(-)	(-)
	Φ (mm)	0	9	0	0
Verticillium dahliae	+/-	(-)	(-)	(+)	(+)
	Φ (mm)	0	0	12	10.5
Fusarium solani	+/-	(+)	(+)	(+)	(+)
	Φ (mm)	6	8	10	9
Fusarium oxysporum f. sp. *lycopersici*	+/-	(+)	(+)	(+)	(+)
	Φ (mm)	8	9	8	9
Botrytis cinerea	+/-	(-)	(-)	(-)	(-)
	Φ (mm)	0	0	0	0

Table 2 Antimicrobial reference standards.

	B. pumilus	*P. aureofaciens*	*B. glathei*	*P. italicum*	*V. dahliae*	*F. solani*	*F. oxysporum*	*B. cinerea*
Ampicillin	12	25	30	-	-	-	-	-
Carbendazim	-	-	-	0	40	40	46	35

-: Not tested

In addition to its pest resistance, *Arbequina* presented a supplementary antimicrobial activity which increases its importance in olive sector. Chemical studies are needed to identify active compounds responsible for the recorded antibacterial and antifungal activities.

Acknowledgements The support of the professor Mounir MEKKI, Laboratory of Malherbology and Phytopharmacy of The Higher Agronomic Insitute of Chott-Mariem, is gratefully acknowledged.

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Synergistic effect of antibacterial peptide with virus vaccine on the immunity of pig against PCV2

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For sake of exploring a novel way to enhance the immunity of pig against virus infection, the experiment was carried out to immunize the thirty 6-week piglets with inactivated PCV2 and attenuated Hog cholera vaccine intramuscularly, and then they were orally feed with recombinant antibacterial peptides (ABP) expressed by Saccharomyces cerevisiae, which was mixed with feed at the ratio of 300 ppm and taken by piglets for 5 weeks after the vaccination. The blood was collected from the experimental pigs to detect the dynamic changes of specific antibody by ELISA, and to evaluate the expression level of IL-2, IFN-γ, IL-10, CD4 and CD8 gene in lymphocytes by Quantitative RT-PCR; the amount of PCV in blood was also assayed by real time PCR on 20, 22 and 24 weeks after the vaccination. The results were found that, compared with those of vaccinated control pigs, the expression levels of IL-2 gene did not show obvious difference with those of the control group, the expression level of CD4, CD8 and IFN-γ significantly elevated in the immune cell from the blood of treated pigs with ABP ($P<0.05$). Meanwhile, the IL-10 level markedly decreased in the immune cells of the treated pigs ($P<0.05$) in comparison with that of the control pigs. Furthermore, the amount of PCV2 was also significantly lowered in the blood of treated pigs with ABP from 20 to 24 weeks after inoculation ($P<0.01$), implying the enhancement of virus cleanup in the treated pig by ABP. These suggest that oral administration of ABP via feed is probably an easy inspiring method to promote the immunity of pig against virus infection and proliferation *in vivo*.

Key word: Antibacterial Peptide, Pig, PCV2, vaccination, immunity,

1. Introduction

In recent years, porcine circovirus-2 (PCV2) causes significant immune suppression of animal and causes huge economic damage to pig industry around the world. The high PCV2 infection rate not only affects pig's growth and weight gain, but also increase the potential risks of infection by other dangerous pathogens. Presently people are more deeply concerned about animal disease outbreak associated with the abuse of antibiotics in feeding and treatment of animal disease, which could bring serious food safety and health problems for human beings.

It was reported that PCV2 infected animals showed high expression of IL-10 mRNA and low CD4+T cell in blood. These suggested that infected animals suffered from immune suppression. Analyses by qPCR showed that pigs infected with PCV2 displayed a significantly increased mRNA expression for IL-10 ($P<0.05$) (M. Anderssona et al., 2011; W. Sipos, teal. 2004). A loss of CD3+CD4+CD8+ memory/activated Th lymphocytes was particularly notable. All T lymphocyte sub-populations CD3+CD4+CD8+ memory Th, CD3+CD4+CD8- naive Th, CD3+CD4-CD8+ Tc and CD3+CD4-CD8-γδTCR+ lymphocytes were susceptible to PCV2 infection-induced lymphopenia. (J.Nielsen et al., 2003).

Fusion peptide PGBD-2 is expressed in S. *cerevisiae* through genetic engineering techniques which sequences are from different gene of Protegrin-1 (PG-1) and pig defense-2（PBD-2). Long ABP genes were constructed through recombinant genetic engineering method and expressed in the yeast. In preliminary animal feeding experiments, fusion peptide PGBD-2 demonstrated good biological effects, such as increase of sow farrowing rate, drop of stillbirth rate and obvious improvement of weight gain of nursery pigs. So this inspire us to carry out animal experiments to observe the immunological changes in pig fed with fusion PGBD-2, and explore the molecular mechanism for enhancement of animal immunity for sake of development of novel method to restore the immune function of infected animal.

2. Materials and methods

2.1. Materials

Fusion peptide PGBD-2 was produced by solid state fermentation of recombinant Saccharomyces cerevisiae in Chengdu Gold Spring Biological Co., Ltd for animal experiment.

Bacteria: E. coli ATCC 25922, P. aeruginosa ATCC10211, S. aureus ATCC26112, S. pneumonia ATCC49619.

Supernatant of antimicrobial peptides of combined-fermentation yeasts: Weighing 2g recombinant antimicrobial peptide yeast preparation, add 4ml PBS buffer (snail nut enzyme 30mg/ml, sorbitol 1M). After refining and ultrasound, digest for 5h at 37 ℃. Then use 65 ℃ for 30min for inactivation of digestive enzymes and 10000×rpm centrifugal filter to collect the supernatant of antimicrobial peptides.

Pigs: 30 healthy piglets, 6 weeks old, weighing 9.5±0.5 kg, were provided by TQLS Livestock Technology Co., Ltd. Mianyang, Sichuan province. They were immunized with inactivated PCV and attenuated Hog cholera vaccine intramuscularly, and then orally feed with recombinant PGBD-2 peptides expressed by Saccharomyces cerevisiae, which was mixed with feed at the ratio of 300 ppm for 5 weeks after the vaccination.

2.2. Minimum inhibition concentration of PGBD-2 product

Broth dilution methods to determine the minimal inhibitory concentration (MIC) of solid state combined-fermentation products: antimicrobial peptides PGBD-2. The standardization of the bacterial cell (E. coli, P. aeruginosa, S. aureus, S. pneumonia) number used for susceptibility testing is of critical importance for obtaining accurate and reproducible results. The recommended final inoculums size for broth dilution is 5×10^5 colony-forming units (CFU) ml-1 (Robert E W Hancock et al., 2008) .

2.3. Experimental design and animals

30 healthy pigs were divided randomly into treatment and control group, five pigs in each group. The treatment group dietary source contained crude ferment products (at the ratio of 300 ppm PGBD-2) in addition to the same basic diet as the control group, and they were both reared under the same condition and management. The blood samples were collected from the experimental pigs before the addition of ABP and 14, 28 days after the feeding with ABP (20, 22 and 24 weeks respectively). The gene expression level of IFN-γ, IL-2, IL-10, CD4 and CD8 were detected in the immune cells by real time quantitative RT-PCR.

2.4. Nucleic acid extraction

Obtained material from the concentrated plasma, both whole blood cells and serum, stored in liquid nitrogen, were used for RNA and DNA extraction. The frozen samples ,25 mg each sample, were cut into small pieces and homogenized using a ground stick and approximate with 200 µl of TRIzol® (Invitrogen, Life Technologies, Carlsbad, CA, USA) added. When the final amount (800 µl) of TRIzol® was added the mixture was homogenized by passing it 10-15 times through a 1.2 mm needle fitted on a syringe. RNA was extracted using a combination of TRIzol® and E.Z.N.A. ® Total RNA kit I (Omega Bio-Tech, Norcross, GA, USA). The purified RNA was suspended in diethylpyrocarbonate (DEPC)-treated water. After analysis of purity, the RNA was reverse-transcript into cDNA and preserved at -20℃.

The DNA-containing phase, from the TRIzol® separation, was mixed with buffer (4 M guanidine thiocyanate, 50 mM sodium citrate and 1 M Tris). The aqueous phase was removed after centrifugation (12000 × g, 30 min) and the remaining phase was mixed with isopropanol as same volume and centrifugated (12000 × g, 15 min). The obtained DNA pellet was washed in 70% EtOH and resuspended in 1× TE-buffer. The DNA was used for analysis of the presence of PCV2 DNA in the intestine (M.Anderssona et al., 2011).

2.5. Quantitative PCR for detection of PCV-2 DNA in serum

Quantitative PCR (qPCR) was performed on the DNA extracted from the serum to determine the presence of PCV2 DNA using an in-house protocol. PCV2 DNA was detected with the following primer pairs: S-PCV2—Forward:ATA ACC CAG CCC TTC TCC TACC; Reverse: CTT CGG ATA TAC TGT CAA GGC T; PCV2-1010—Forward: GTC AAG GCT ACC ACA GTC AC; Reverse: GGC CTA CGT GGT CTA CAT TTCC. The real-time PCR mixture containing 1× SYBR Green I master-mix (TianGen™ QPCR mix, TianGen Gene, Peking, china), 400 nM primers and 1µL template was run on a Bio-Rad IQ5 Real-time PCR cycler (Bio-Rad, Hercules, CA, USA) using a program initiated by 95 °C for 5 min, followed by 40 cycles of 15 s at 95 °C, 2 s at 58 °C and 30 s at 72 °C. All reactions, including standard points and negative control (no template added) were

performed in triplicate. The fluorescence was measured at the last step of each cycle. After 40 cycles, a melting curve analysis was performed by raising the temperature 0.5 °C every 10 s, from 55 °C to 95 °C, and measuring the fluorescence at each cycle. The quantification was made from a standard curve generated by titration of a positive cDNA containing a full-mRNA genomic or a PCR product. In brief, PCV2-DNA was amplified from serum obtained from PCV2 infected pigs using the same primers. The restriction pattern of the PCR product was verified by gel electrophoresis before purified. The copy number was determined to 5.4×10^8 copies per ng DNA and the lower detection limit for both assays was set to 50 copies/100 ng DNA.

2.6. Quantitative real-time PCR to analyze mRNA expression of IFN-γ, IL-2, IL-10, CD4 and CD8

Pig peripheral blood mononuclear immune cells were separated by fico 400. Complementary cDNA of IFN-γ, IL-2, IL-10, CD8 and CD4 were synthesized in a Gradient thermo cycler using Superscript II Reverse Transcriptase (Invitrogen, Life Technologies, Carlsbad. CA. USA). Quantitative real-time PCR was performed to analyze the gene expression of IFN-γ, IL-2, IL-10, CD8 and CD4; and one reference genes: β -acting. In brief, 1 μL of cDNA, primers, 5′-6-carboxyfluorescein (FAM) were added to a total of 25 μL in 1× TianGen™ QPCR Mix (TianGen Peking, China). The samples were amplified in triplicate in an IQ5 Real Time PCR cycler (Bio-Rad) for 40 cycles. The FAM fluorescence signal was measured at the 60°C step for each cycle. A negative control, with no template added, was included in each run. The relative expression of cytokine mRNA was calculated relative to the mean expression of the cytokine mRNA in the control pigs using the geometric means method and the formula: relative level = $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). The ΔΔCt was calculated by the formula: $\Delta Ct_{\text{infected pig}} - \Delta Ct_{\text{control pi}}g = (Ct_{\text{cytokine}} - Ct_{\text{reference gene}})_{\text{infected pig}} - (Ct_{\text{cytokine}} - Ct_{\text{reference}}\ \text{gene})_{\text{control pig}}$, where Ct_{cytokine} is the mean Ct value of triplicates and each $Ct_{\text{reference gene}}$ is the mean Ct value of triplicates from the reference gene. Significant differences between the experimental groups were calculated for each cytokine using a Mann–Whitney rank sum test on the relative cytokine levels (M.Anderssona et al., 2011). Primers designed as following :

Table1: Primers of β-Acting, IFN-γ, IL-2, IL-10, CD4 and CD8.

Genes	Sequences of Primers
β-Actin F	5'-CTCCTCCCTGGAGAAGAGCTA -3'
β-Actin R	5'-CCTTCTGCATCCTGTCGGCAA -3'
$CD4^+$-F	5’ ACACAGCCTCAGTTACCGAGTTG 3’
$CD4^+$-R	5’ CCTCTTGTCTTCCACTTCGCAGAT 3’
$CD8^+$-F	5’ GCGAGGAGGACCAAGGCTACTAT 3’
$CD8^+$-R	5’ TGATGACCAGTGACAGGAGAAGGA 3’
IL-2-F	5’ CTGGAGCCATTGCTGCTGGAT 3’
IL-2-R	5’ CTGTAGCCTGCTTGGGCATGTA 3’
IFN-γ-F	5’ TGGTAGCTCTGGGAAACTGAATGA 3’
IFN-γ-R	5’ CTCTGGCCTTGGAACATAGTCTGA 3’
IL-10-F	5’ GCAGCCAGCATTAAGTCTGAGAAC 3’
IL-10-R	5’ GTCAGCAACAAGTCGCCCATCT 3’

2.7. Statistical analysis:

3. Results

3.1. MIC of antimicrobial peptides PGBD-2

As illustrated in Fig.1, with the increase of PGBD-2 in the medium, both growth of gram positive and negative bacteria were significantly inhibited *in vitro* ($P<0.01$). 5 ug/ml PGBD-2 started to markedly suppress the growth or proliferation of bacteria ($P<0.05$); and 15 ug/ml PGBD-2 resulted in extreme strong inhibitory effect on the growth or proliferation of bacteria in comparison with the blank control ($P<0.01$).

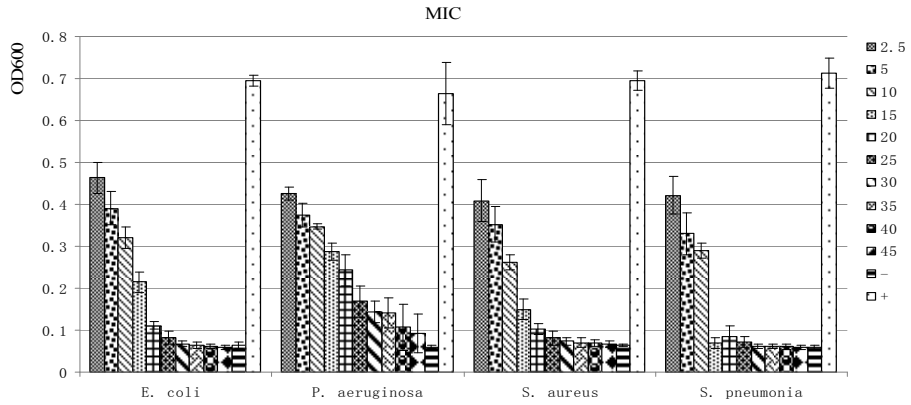


Figure1: Effect of different concentration of PGBD-2 peptide on bacteria growth *in vitro*.

3.2. Presence of PCV2 *in vivo*.

In PGBD-2 treatment group, the positive rate of PCV-2 was not only significantly decreased *in vivo* from 4/5 to 1/5, the content also dropped significantly ($P<0.05$). In contrast, PCV-2 positive rate increased significantly the control group (from 1/5 to 5/5), and content was also significantly higher than the experimental pigs ($P<0.01$). These proofs suggested that fusion peptide PGBD-2 significantly enhanced the immunity and resistance of sows to pathogen, which is probably facilitate the clearance of PCV-2 virus from pig.

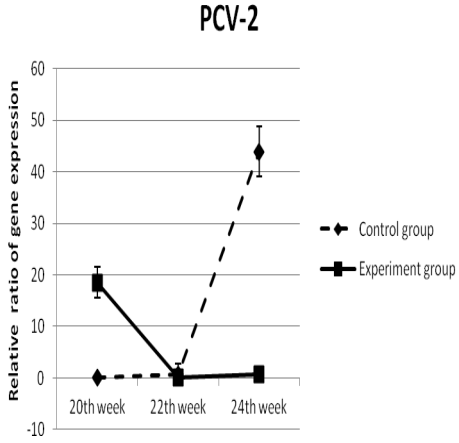
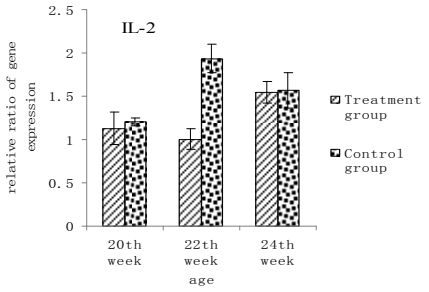


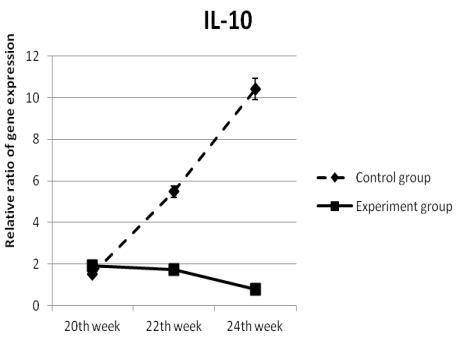
Figure2. The dynamic changes of PCV-2 level in the experimental pigs during the experimental period.

3.3. IL-10, IL-2 and IFN-γ gene expression change

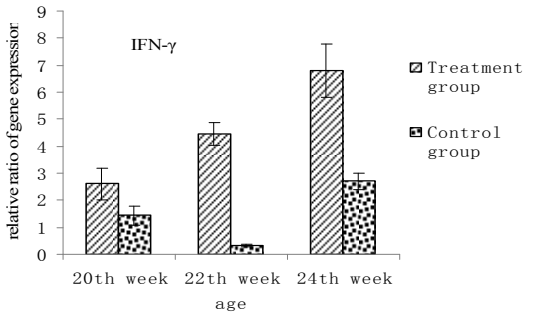
It was shown that compared with the control group, IL-10 gene expression of blood immune cells in pigs fed with fusion PGBD-2 peptide was significantly reduced ($P<0.01$); moreover, the expression level of IFN-γ gene also increased markedly in the treated pigs in comparison with that of the control ($P<0.01$), despite that IL-2 gene expression level did not manifest obvious difference between the treated and control group ($P>0.05$)(Fig.4).



a)



b)

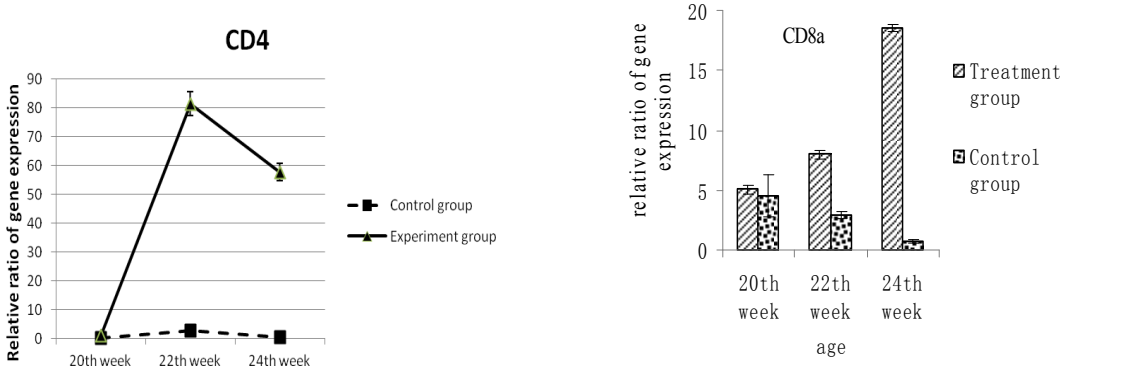


c)

Figure 3. Change of gene expression level of IL-2, IL-10 and IFN-γ from the immune cells of experimental pigs.

3.4. T-cell surface glycoprotein CD4 mRNA increased as ABP feeding.

In fusion peptide PGBD-2 feeding group, CD4 gene expression increased significantly ($P < 0.01$) in blood immune cells, comparison with control groups. Indicating that the body's CD4 immune regulation significantly enhanced, that will help to improve the level of immune defense, strengthen the viruses and bacteria and other pathogenic infections clearance capacity.



a) b)

Figure 4. CD4 and CD8 gene expression level of the immune cells *in vivo* from the blood of experimental pigs.

4. Discussion

IL-10 gene expression was maintained at a low level in the treated pigs during the entire experimental period. 28 days post treatment. While IL-10 levels of the control group were almost 7 times higher than that of the treated pigs at the end of experiment, indicating that the fusion PGBD-2 peptide has a good effect on the immunity of pig to enhance the resistance to PCV-2 replication *in vivo*. Similarly, the expression level of IFN-γ gene was also significantly raised in the immune cells of treated pigs; meanwhile, the IL-2 gene expression was not obviously changed by the treatment. Therefore, the IL-10 level of control group could significantly suppress the immune function of pig. It was reported that PMWS infected pigs manifested high level of IL-10 expression [J.Nielsen et al., 2003], which implying that over-expression of IL-10 is one of reason for immune failure for vaccination and protection of pigs due to various virus infection. The down-regulation of IL-10 gene expression and up-regulation of IFN-γ gene could benefit the immunity of pig with PGBD-2 peptide.

CD4 and CD8 positive cells are very important in animals immune cells, CD4 and CD8 are mainly expressed in T_h and Tc cells in the blood, which are critical for immunoregulation and virus clearance. Fusion PGBD-2 peptide induced the increase of CD4 and CD8 in competent T lymphocyte, which could facilitate the recognition of TCR for antigen, differentiation and proliferation of T cells, and therefore promote immune response to microbial infection, accelerate antigen-presentation process and specific cellular immunity, implying viruses and other pathogen clearance could be conducted more easily. The decrease of PCV2 DNA shows the antiviral immunity was raised in the treated group, and confirms that the resistance of pig against virus is potentiated *in vivo* by PGBD-2 feeding. Consequently, PCV-2 infection rate (4/5) and content were relatively high in the treated pigs before treatment. At last, the infection rate and content of PCV-2 markedly lowered to 1/5 compared to the increase of PCV-2 (from 1/5 positive to 5 / 5 positive) in the control pigs during the experimental period.

In order to avoid digestion of PGBD-2 and improve the effect of PGBD-2 peptide in the digestive tract, the fusion PGBD-2 peptides were coated by special molecules to be protected from digestion in stomach and slowly released in the small intestine. In this way, more PGBD-2 molecules could be absorbed and execute more powerful effect on the immune system.

In a word, our result confirms that fusion antimicrobial peptide is very useful for the development of effective bioproduct to control PCV2 and other viruses infection of animals.

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The chemical composition and the antimicrobial activity of the essential oil of cultivated and wild *Rosmarinus* and *Lavandula*

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Rosmarinus officinalis (R.O.) and *Lavandula stoechas (L.S.)* are two native medicinal plants in Lebanon, belonging to Lamiaceae family. They are largely used in the traditional medecine. The main focus of this study was to evaluate and compare the chemical composition and the antimicrobial activity of the essential oils (E.O) from wild plant and compare them with cultivated *R.O.* and *Lavandula angustifolia (L.A)* a cultivated plant. Another objective was the comparison between two methods of extraction: solvent free microwave extraction (SFME) and hydrodistillation (Clevenger). The E.Os were analyzed by GC-MS. This study showed different composition and antimicrobial activity of the E.Os within the same species, and a powerful antimicrobial activity of the whole (E.O) in comparison with the activity of each component alone.

Keywords *Rosmarinus officinalis; Lavandula stoechas; Lavandula angustifolia;* Essential oil; GC-MS analysis; chemical composition; antimicrobial activity.

1. Introduction

Diseases of bacterial origin are an important focus of research because of spontaneous or acquired bacterial resistance, particularly those following the prescription of antibiotics. Considering the failure to acquire new molecules with antimicrobial properties, the identification of antimicrobial agents from other natural sources is of great importance.

Lebanon, by its geographical location and climate, offers a rich and diverse flora. In fact, 143 families, 1449 genera and 7253 species have been mentioned in "The New Flora of Lebanon and Syria" [1]. Many among them are considered medicinal and aromatic plants and the majority is not yet studied.

The antimicrobial properties of some essential oils have been recognized for centuries and their extractions have found applications in several therapeutic areas. However, these preparations show various compositions depending on their country of origin and bioactive molecules and therefore their ability to overcome acquired microbial resistance can also change.

In recent years, demand for rosemary oil (*Rosmarinus officinalis*) has increased, because of its widespread use as a natural food additive for food preservation because of its antimicrobial, antiviral, antifungal [2] and antioxidant properties, and especially for its low cost and its ease of access [3]. Similarly, lavender oil has long been used for cosmetic and therapeutic purposes [4, 5, 6].

In a preliminary attempt aiming at gathering scientific evidence for the traditional indications we assessed the antimicrobial activity of two plant species belonging to the Lamiaceae family *Rosmarinus officinalis* (wild and cultivated) and *Lavandula (stoechas* and *angustifolia)* used in the traditional medicine as antimicrobial agents.
In this study, we have identified biologically active components in the E.Os of the above mentioned plants then we evaluated the antimicrobial effect of these oils and their major components using the broth dilution method against two certified ATCC bacteria: *Escherichia coli* and *Pseudomonas aeruginosa*.

On another hand, it seemed relevant to compare the yield and chemical composition of essential oils obtained with two methods of extraction: solvent free microwave extraction (SFME) and hydrodistillation (Clevenger) from both wild and cultivated plants.

2. Materials and Methods

2.1 Essential oil extraction

Wild and cultivated *R. officinalis* were harvested from Chouweifat, Lebanon and *L. stoechas* and *L. angustifolia* were harvested from Ain-Aar, Lebanon. The flowering tops of these plants were used to extract the essential oil. Wild *R. officinalis* was extracted by two methods: SFME and hydrodistillation for 3 hours according to the standard procedures described in the European Pharmacopoeia using a Clevenger type apparatus. The others plants were extracted only by SFME, using a "Milestone Dry Dist Sorisole" machine, the extraction duration was 30 min for each specimen and using a power of 500W.

2.2 Chemical composition analysis

E.O. composition was analyzed by GC using a Varian gas chromatograph CP3800 coupled with Mass Detector 1200 MS/MS. The split/splitless injector model 1177 was at 280 °C in split 1:100. The CP 8400 Autosampler was injecting each time 1 µl of each sample. The carrier gas was helium, at a working constant flow rate of 1 ml/min. GC/MS analysis were done using a fused silica capillary column Factor Four VF-5 ms, measuring 30 m x 0.25 mm internal diameter, film thickener of 0.25 µm. The temperature was programmed at 40^{o}C (5min) with an increase of 5^{o}C per minute until 310^{o}C (1 min). Mass spectra were recorded at 70 eV, Manifold 40°C, Ion Source temperature 280°C, Transfer line 320°C, Acquisition: Full Scan 40 – 800 amu.

Kovat's indices (KI) of all constituents were determined by Kovat's method by co-injection of the samples with a solution containing the homologous series of n-alkanes (C8–C20) (Fluka, Buchs/sg, Switzerland) and mass spectra with those from co-injected mixtures of standard individual compounds (purchased from Aldrich and Alfa Aesar) available in our laboratories and those reported in literature32. Standards of some EOs of known composition (such as EO of *Rosmarinus officinalis* L. - Phytosun' Aroms, Plelo, France) have been injected in similar conditions to check the retention times and the mass spectra. The percentage of each compound was determined using GC-FID.

2.3 Determination of antibacterial activity

The different essential oils and the main components were tested on certified strains *Escherichia coli* (ATCC 8739) and *Pseudomonas aeruginosa* (ATCC 9027) obtained from Epower™.

Antimicrobial activities of the Essential oil and its major compounds were evaluated by broth dilution and disc diffusion methods using MacConkey and Pseudomonas agars. Standard reference antibiotics were used in order to control the sensitivity of the tested bacteria. The incubation conditions used were 24h at 37°C. All the experiments were carried out in duplicate and averages were calculated for the inhibition zone diameters.

2.4 Culture and enumeration of bacteria

The certified strains presented in lyophilized form were reconstituted by suspension in suitable broth (Muller Hinton). The method used is the standard method "colony forming units" consisting of a serial dilution of the colonies. To test the effect of E.O. or compound, different concentrations of E.O./compound were randomly selected and put in three tubes each, corresponding to three different concentrations of bacteria. After a good homogenization, 0.1 ml of each tube is spread over the Petri dishes and the same operation is repeated twice.

To properly monitor the antimicrobial activity of extracts, the same culture was repeated after two hours, 24 hours and 48 hours of contact with the essential oil samples. Quantitative evaluation of the antimicrobial activity of extracts was used to determine the minimum bactericidal concentration MBC (minimal bactericide concentration).

The results were statistically analyzed using SPSS16. The adopted tests were ANOVA and T-test.

3. Results and discussion

3.1 Yield and chemical composition of essential oil of studied plants

Yields of E.O extracted by Clevenger (wild *R. officinalis*) and by microwave (wild and cultivated *R. officinalis*, *Lavandula angustifolia* and *Lavandula stoechas*) and the extraction conditions are represented in the table 1.

Table 1 Duration and yield of the Clevenger and microwave extractions.

	Wild *R. officinalis*		*Cultivated R. officinalis*	*Lavandula stoechas*	*Lavandula angustifolia*
Method	Microwave	Clevenger	Microwave	Microwave	Microwave
Extraction duration	20 min	3h	20 min	20 min	20 min
Yield	0.0059	0.0017	0.003	0.0005	0.0004

Tables 2 and 3 represent the experimental data, harvest date, yield, retention time, the names and percentages of the main components identified.

Table 2 Yield and some components from the chemical composition of essential oils of flowering tops of *Lavandula angustifolia* and *Lavandula stoechas*, extracted by microwave.

Essential oil		*L. angustifolia*	*L. stoechas*
Harvest Date		15-Mar-2011	21-Mar-11
Yield		0.04%	0.05%
RT	Components		
12.48	Eucalyptol	7.15	10.6
17.5	Linalool	48.55	26.57
23.18	Camphor	10.5	11.47
25.94	Borneol	5.68	0.73
27.66	Terpinen-4-ol	9.41	0.71
29.5	Myrtenol	-	6.39
34.7	(-)-Myrtenyl acetate	-	8.27
37.97	Cubenol	-	4.11
39.54	Ledol	-	5.01

Thirty-five compounds of the chemical profile of *L. angustifolia* were identified representing 99.49% of the whole oil.

Table 3 Yield and some components from the chemical composition of essential oils from flowering tops of *R. officinalis* wild and cultivated, extracted by microwave or Clevenger.

Essential oil		**Wild *R. officinalis* (milestone)**	**Wild *R. officinalis* (clevenger)**	**Cultivated *R. officinalis* (milestone)**
Harvest date		7-May-2011	07-May-11	15/7/2011
Yield		0.59%	0.17%	0.003
RT	Components			
8.4	α-pinene	9.6	12.31	4.36
8.98	Camphene	3.07	5.04	0.93
10.04	β-Pinene	3.79	4.19	1.72
12.48	Eucalyptol	40.13	34.31	49.06
23.18	Camphor	3.22	1.4	10.33
25.94	Borneol	16.57	24.25	8.35
30.39	Verbenone	-	-	4.61
33.4	Bornyl acetate	5.79	8.79	1.04
36.62	caryophyllene	8.79	1.31	6.58

Twenty compounds of wild R. O. extracted by milestone representing 99.29% of the whole oil and twenty compounds from wild *R. officinalis* extracted by Clevenger were identified. The difference in composition shows that the method of extraction of essential oil affects the chemical profile of the oil.
Regarding cultivated R.O., twenty compounds of its chemical profile have been identified representing 94.61% of the whole oil. Among them verbenone was a major component of cultivated R.O. was absent in the oil of wild R. O., this finding shows that the mode of cultivation of the plant affects largely its chemical composition.

3.2 Antibacterial effect of extracts by broth dilution method and statistical evaluation.

Table 4 represents a summary of the results showing the MBC of the studied products on *E. coli* and *P. aeruginosa*.

Table 4 The MBC of the studied products ($\mu l.ml^{-1}$).

	L. angustifolia	Wild *R. officinalis* (Milestone)	Wild *R. officinalis* (clevenger)	Cultivated *R. officinalis*	Linalool	Caryophyllene	Camphor	Eucalyptol	Borneol
E. coli	5	5	5	5	5	-	-	5	-
P. aeruginosa	25	50	-	-	-	-	-	-	-

-: no effect

We note that *Lavandula stoechas* didn't show any MBC but a MIC (minimal inhibiting concentration) equal to 50µl/ml for both *E. coli* and *P. aeruginosa*.
None of the main components showed an effect on *P. aeruginosa* while a bactericide effect was detected with *L. angustifolia* and wild *R. officinalis* (milestone). The effect of linalool and eucalyptol decreased when high amounts of camphor were present (E.O of *L. stoechas*).

L. angustifolia, wild and cultivated, *R. officinalis*, linalool and eucalyptol have shown the same MBC= 5µl/ml.
Only *L. angustifolia* and wild *R. officinalis* (milestone) have shown an effect on *P. aeruginosa* (MBC= 25 µl/ml and 50µl/ml respectively) with a significant difference confirmed by ANOVA test. Statistical tests have shown significant difference between the 2 extraction methods. ANOVA test has also shown a powerful antibacterial effect on *E. coli* for linalool and cultivated R. O.

4. Conclusion

In conclusion, this study has evaluated 2 species of Lamiaceae growing wild and cultivated in Lebanon and showed that their essential oils have an antibacterial activity with a synergetic effect between the components of the E.O especially shown on *P. aeruginosa.*

This study showed that among the oils and tested compounds, linalool, cultivated *R. officinalis*, wild *R. officinalis* extracted by milestone and cultivated *Lavandula* (*angustifolia*) were the most potent against *E. coli*. The most powerful antibacterial effect on *P. aeruginosa*, was detected with *L. angustifolia* and wild *R. officinalis* extracted by milestone. We note that our study demonstrated a significant difference between the two extraction methods: hydrodistillation (3h) and microwave (30 min). The latter leads to better results rapidly which save time and energy. Nevertheless further studies 'in vitro' and 'in vivo' are needed to demonstrate the antimicrobial effectiveness of these essential oils, the best combinations of components to achieve the desired antimicrobial activity and to extrapolate our results into the industrial and pharmaceutical domain.

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The influence of filling material “Radent” on the viability of microorganisms isolated from patients with periodontitis

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We studied the influence of different ways of filling material for root canals dressing under all types of calcium-containing materials “Radent” on the viability of the causative agents of periodontosis. In the course of the studies was found that the filling material “Radent” has antibacterial properties that can be statistically significantly increased in the filling material compounding with the use of the chlorhexidine fluid.

Keywords chronic periodontitis, calcium-containing filling materials, chlorhexidin.

1. Introduction

It is known that the root canal infection is polymicrobic, even after extensive biomechanical preparation it may contain pathogens. One of the causes of treatment failure of complicated forms of dental caries is resistance of some microbial species. Thus, some of them are highly resistant to antimicrobial agents due to their ability to penetrate the dentinal tubules and to adapt to changing environmental conditions, so the root filling material should possess strong antibacterial effect [4]. Accordingly, the search for a better alternative to endodontic agents resulted in various combinations of calcium hydroxide, hydroxyl radicals of which cause lipid peroxidation of cell wall membrane, and chlorhexidine; the latter has a sufficiently wide range of antibacterial effect against aerobic and anaerobic microorganisms, as well as different types of *Candida* [7].

One solution to the problem of sterility of root canals in the treatment of complicated caries is the use of a complex of medicamental and physical factors. Nowadays the antibacterial effect of low-intensity laser radiation is quite well studied and assessed as high [2]. It is known that laser radiation stimulates lipid peroxidation in cell membranes, which leads to the formation of free radicals [1, 6].

The purpose is to study the effectiveness of the root filling material "Radent" on the viability of *Escherichia coli* and *Staphylococcus aureus* under the influence of infrared laser radiation and magnetic field.

2. Materials and methods

We used in experiments the root filling material "Radent" ("Raduga R", Russia), which consists of zinc oxide (70%) and calcium hydroxide (30%). Powdered agent "Radent" or zinc oxide was dissolved in distilled water, and in another case - in 1% solution of chlorhexidine. To determine the antimicrobial effect of "Radent" and zinc oxide we used Lai et al. method [5] in our own modifications.

Sensitivity of microorganisms was determined by direct application of "Radent" and zinc oxide on inoculation of the test strain, which corresponds to a density of 0.5 McFarland standard and contains approximately 1.5×10^8 CFU/mL (colony-forming units per milliliter). Noted materials were deposited in the volume of 40 ml; the study results were assessed in 18-24 hours of cultures incubation at 35° C and expressed in millimeters of diameter of growth retardation area of test strain. The latter was obtained in microbiological research of contents of root canals of patients with chronic periodontitis.

In this work we used the infrared laser radiation (ILR) and the magnetic field of apparatus with a semiconductor transmitter at gallium-arsenide "Optodan" (SIE Vend, Russia). Laser wavelength was 0.85-0.98 microns; magnetic induction was 50 mT. *E. coli* or *S. aureus* inoculum was divided into two portions. First one was irradiated in a sterile Petri dish by ILR for 2 minutes at a distance of 1 cm, and the second one was held for 2 minutes free of physical influence. Then both portions of inoculum were seeded on Petri dishes; determination of test strains sensitivity to filling materials was made with the same procedure as before and after irradiation. Statistical processing of the data was performed using Student *t*-test.

3. Results and discussion

In the course of the studies it was found that "Radent" has antibacterial properties: clearly visible growth retardation area is formed around it in all strains. In the case of "Radent" with chlorhexidine the growth retardation area significantly increases for all strains of microorganisms (Table 1).

Table 1 Influence of "Radent" on viability of microorganisms.

Tested strains	Growth retardation area – "Radent", mm		*p* between groups
	distilled water	chlorhexidine	
Escherichia coli	10.1±1.0	15.3±2.6	<0.05
Staphylococcus aureus	7.4±0.5	14.3±0.8	<0.05

The agent of zinc oxide also has a strong antibacterial effect against Gram-negative as well as gram-positive bacteria (Table 2).

Table 2 Influence of zinc oxide on viability of microorganisms.

Tested strains	Growth retardation area - zinc oxide, mm		*p* between groups
	distilled water	chlorhexidine	
Escherichia coli	9.6±0.3	18.3±1.9	<0.05
Staphylococcus aureus	8.6±0.5	15.1±1.1	<0.05

After the combined effect of magnetic-laser radiation and "Radent" agent on *E. coli* the sensibility of strains of the latter significantly increased. This fact can be explained by the ability of these factors to reduce the number of viable bacterial cells. Our previous studies show that laser radiation and magnetic field significantly reduce the number of viable *E. coli* [3]. However, differences in the sensitivity of *S. aureus* to "Radent" before and after magnetic-laser irradiation are not identified (Table 3).

Table 3 Combined effects of medication and physical factors on the viability of microorganisms.

Tested strains	Growth retardation area – "Radent", mm		*p* between groups
	distilled water	chlorhexidine	
Escherichia coli	8.7±0.6	21.7±1.5*	<0.05
Staphylococcus aureus	7.7±0.5	14.3±0.7	<0.05

Note. * - $p<0.05$ when comparing with the samples without physical restraint (tabl. 1).

Similar changes in the sensitivity of *E. coli* and *S. aureus* before and after magnetic-laser radiation was detected in the study of zinc oxide (Table 4).

Table 4 Combined effects of medication and physical factors on the viability of microorganisms.

Tested strains	Growth retardation area - zinc oxide, mm		*p* between groups
	distilled water	chlorhexidine	
Escherichia coli	9.3±0.9	23.0±2.0*	<0.05
Staphylococcus aureus	7.9±0.9	15.6±1.1	<0.05

Note. * - $p<0.05$ when comparing with the samples without physical restraint (tabl. 2).

4. Conclusion

Thus, this study showed that the type of unit, where the hydroxide-contained agent "Radent" and zinc oxide were prepared as root filling material, influences their antimicrobial activity; it can be improved by using chlorhexidine solution through the formation of additive and synergistic effect. The combined effects of the filling material and magnetic-laser radiation in the clinical setting will provide strong antibacterial effect. Above-mentioned information gives us reason to recommend this method for the treatment of chronic forms of apical periodontitis in clinical practice.

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