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Criblage des bactéries d'intérêts biologique du microbiote intestinal d'*Apis mellifera intermissa*

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Abbreviations list

CFU: colony-forming unit

DNA: Deoxyribonucleic acid

EMB: Eosin Methylene Blue

GST: Glutathione S-transferase

GYC: Glucose yeast extract calcium carbonate

IPM: Integrated pest management

LAB: Lactic Acid Bacteria

LB: Luria Bertani

MRS: Man–Rogosa–Sharpe

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Introduction

Introduction

Insects are the most diverse and abundant animals worldwide [1]. Bees are among the small insects that play a vital and important role in the Earth's ecosystems. Bees have been found for millions of years, a subset of the order Hymenoptera characterized by social insects able to adapt and to develop in different climates and habitats. They are also characterized by their wondrous ability to transmit information and complex details about food sources, for example, through simple movements or so-called complex communication dance [2].

Bees, including honey bees are divided into three categories including, queens, female workers, and drones. Bee's social organization and labor division between the queen, working bees, and drones create a complex hierarchy that keeps the colony functioning and stable. The queen bee's role is to sole egg layer and the role of drones is limited to mating. For worker bees, their roles include nectar and pollen collecting, creating and protecting cells, and tending to the young [2].

In the majority of ecosystems, the honeybees are considered the most important pollinators [3] where they play a role in agricultural production and the diversity of wild plants [4], in addition to pollinating plants and increasing the production of agricultural crops, honey bees are economically important for producing significant commercial products including, honey, royal jelly, propolis, wax, and pollen [5].

This most important insect is threatened by numerous pathogenic germs and harmful factors. In fact, to survive against all these threats, honey bees rely on their microbiome [6]. The microbiome is defined as the community of microorganisms that occupy a host organism [7].

These microorganisms play a role in improving nutrient-poor diets, also helping to digest food, protecting the organism from parasites, pathogens and even predators, and also contributing to integrand intraspecific communication[1]. Moreover, the diversity and relative evolution of insects is due to their relationship with a number of beneficial microorganisms [1].

In living beings, microbial communities are mainly present in the digestive tract because it is a nutrient-rich environment [1,8]. There are nine bacterial species dominating the gut microbiota in the eusocial western honey bee *(Apis mellifera*). This gut microbiota is acquired by adult honey bees through their nestmates or contact with the nest and its materials[9].Lactic acid bacteria (LAB) representing by *Lactobacillus and Bifidobacterium* forms an integral part of honey bee microbiota, where we find them in the respiratory tract, gut and genitals of various animals

[10]. Within the gut, they coexist with their host and provide several benefits, including improved metabolism and immune defense [8].

Studies have found that bee gut microbes are associated with physiology and bee health, as well as potentially having a role in protecting against a number of pathogens and also parasites [11].

The Studies conducted by each of Olofsson and Vasquez, showed the presence of 13 types of bacterial species of LAB, within honey bees [12], all these bacteria contribute to a variety of functions in the host, with the primary impact being on immune modulation, which inhibits reproduction of pathogens by competing with them for nutrients as well as their ability to produce proteinaceous molecules called bacteriocins that inhibit the growth of most bacteria [10]. Moreover, research reported that gut microbiome can produce enzymes including the amylase enzyme [13]. As the study by Sun et al showed the presence of *Bacillus sp* bacteria produces amylase enzyme [14].

All these finding motivate us to investigate on the screening of bacteria from *Apis mellifera intermissa* gut microbiome able to produce bioactive compounds including, enzymes and bacteriocins molecules.

The current document is divided into parts including theory part and practical part. The theory part is divided in four main chapters, the first chapter treated *Apis mellifera* description and their importance in ecosystems. We also highlighted the diversity of their gut microbiota and the significant role it plays. For the second chapter addresses to the biological interests of honey bees gut microbiota. Then showed some ways of phenotypic identification that we had adopted to identify some bacterial species. While in the practical part, we mainly focused on the methods carried to isolate and identify the isolates bacteria, then we described the screening of bacterial germs able to produce bioactive compounds including, enzymes and bacteriocins molecules.

Bibliography part

Chapter I

Apis mellifera intermissa description

I.1. the honey bees [*Apis mellifera intermissa*] :

Insects that belong to one of the seven bee families of the superfamily *Apoidea* are referred to as "bees" [15]. Is a major group of the order *Hymenoptera* -- the Section *Aculeata*--. *Hymenoptera* whose females have stings— modifications of the ovipositors of ancestral groups of *Hymenoptera* [16].

Bees are mostly pollinators and only a small portion of bees globally are identified as honey bees[15].

Apis mellifera intermissa also named Tellian bee, Punic or the black bee, it was described and classified by Buttel Reepen in 1906 [17].*A. mellifera intermissa* the indigenous honey bee species found in North of Africa(Algeria, Morocco, Tunisia)and along the Mediterranean coast[18]. This breed of bee can also be found from the Atlantic to Libya, in the islands off the coast of Malta, and in the Canary Islands [17].

This type of honey bee is noticeably darker, it is prone to swarming, characterized by his aggressive defensive behavior, and copious propolis consumption, also has a high ability to adjust to significant changes in climatic circumstances. *Apis mellifera intermissa* can produce up to one hundred queen cells and several broods [18].

The honey bees are an essential component of the global ecological balance, due to their vital function in pollinating a wide variety of plant species. It has additional pursuits like beeswax, honey, propolis, and royal jelly manufacturing [19].

I.2. *Apis mellifera intermissa* description *:*

Morphologically, the honey bee has a small to medium- sized body with black or dark brown color. A. mellifera intermissa is covered with dense, fine hairs. The A. mellifera intermissa mouth is located on the underside of the head. A. mellifera intermissa is distinguished by its smaller hind wings compared to its front wings, as well as robust hind legs that are adapted for gathering pollen, often seen carrying yellow pollen balls. [2,15].

A. mellifera intermissa is characterized by uniformly dark pigmentation, with some unclear markings on abdominal tergites and the scutellum. The average tongue length is 6.5 mm, and the hair is short [17]. The body of *A. mellifera intermissa* is characterized by an exoskeleton composed mainly of the polysaccharide chitin and various structural proteins [20]. The body

structure of *A. mellifera intermissa* consists of three main parts: the head, thorax, and abdomen (Figure n°1) [21].

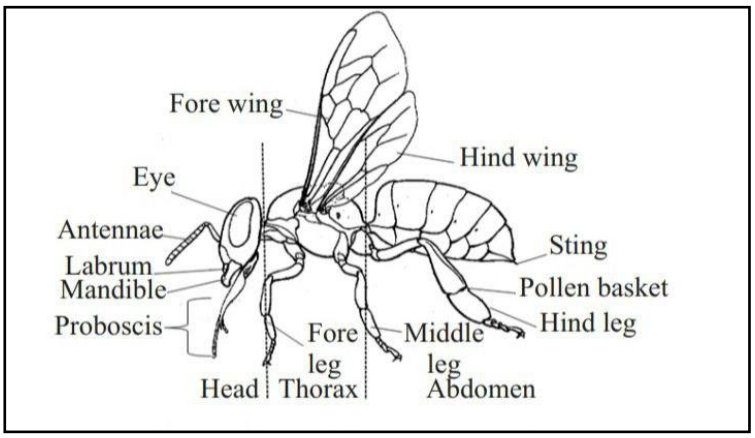


Figure n°1:Morphology of honey bees (Sharma et al ,2021) [6].

I.3. *Apis mellifera intermissa* classification :

There are currently ten or eleven families of bees, comprising roughly 700 genera and 20,000 living species [22]. Most bees with typical names belong to the Apidae family, which includes honey bees (Apini), bumble bees (Bombini), stingless bees (Meliponini), and orchid bees (Euglossini) [23].

The *Apis* mellifera intermissa belongs to the order *Hymenoptera* and the family *Apidae*. Below, we provide the details of its classification [24].

The classification of *Apis mellifera intermissa* is:

Kingdom: *Animalia*
Phylum: *Arthropoda*
Class: *Insecta*
Order: *Hymenoptera*
Sub-order: *Apocrita*
Sup- family: *Apoidea*
Family: *Apidae*
Genus: *Apis*
Species: *Apis mellifera*
Sub-species: *Apis mellifera intermissa*.

I.4. Reproduction and life cycle:

The queen bee is responsible for oviposition. Over its three to four years of life, it can lay up to 1,500 eggs every day [25]. Honey bees belong to holometabolous insects, which are characterized by four phases in their life cycle [23] Honey bees undergo the stage of transformation known as Holometabola (Complete Metamorphosis) [26]. Every bee goes through egg, larval, pupal, and adult stages, as do all insects that undergo complete metamorphosis [16].Honey bees begin their life cycle by laying eggs in honeycomb cells. For worker bees, the life cycle from egg to adult takes approximately 21 days, while for drones, it takes about 24 days [27].

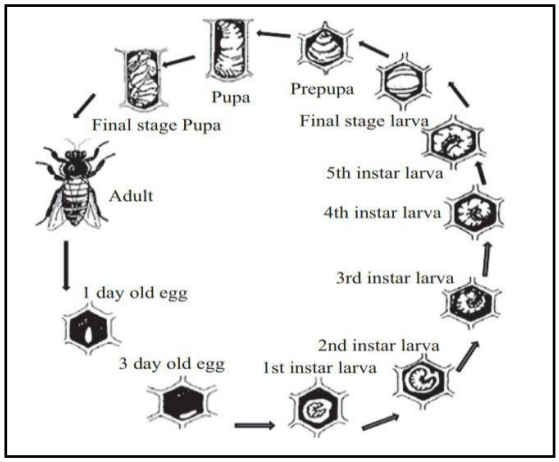


Figure n°2:The honeybees life cycle (Sharma et al ,2021) [6].

I.5. *Apis mellifera intermissa* society:

The Honey bees live in eusocial perpetual colonies with overlapping generations, a reproductive labour portion, and a brood care division. Every colony consists of three castes: the female reproductive queen bee, Female worker bees' range in numbers from 15,000 in winter to 50,000 in summer, while male drones are only present in the spring and usually number in the hundreds (Figure n°3) [28].

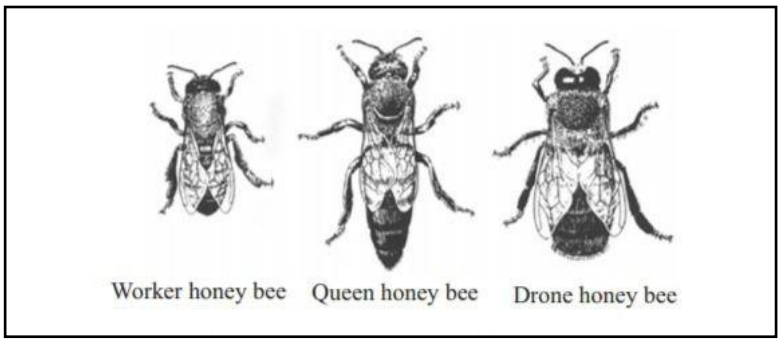


Figure n°3:The different castes of honeybees (Sharma et al ,2021) [6].

I.6. The importance of honey bees in the environment:

Honeybees are most known for their hive products, which include honey, wax, royal jelly, propolis, and pollen. Honey bees are important in agriculture as they are required for the development of seeds and fruits, and more broadly, for maintaining ecosystems and biodiversity. Honey bees actively assist in the pollination of both wild and cultivated plants due to their intense foraging activity. Each year, billions of bees depart from their hives to visit flowers in the surrounding areas. They find pollen and nectar, which are two essential elements of their nourishment [16].

Pollination is the process of transferring pollen from the male parts (anthers) to the female part (stigma) of a flower, either on the same flower or a different one [29]. Pollen is primarily transferred through passive self-pollination, as well as by wind and insects, with most species being pollinated by insects [30].

Bee pollination is crucial for both the quantity and quality of pollination. Bees are especially effective and the most important pollinators due to their morphology, which includes branching hairs on their bodies, their nutrition primarily consisting of nectar and pollen, and their vigorous foraging activities, during which they visit and pollinate numerous plant species [29,30,31].

Bees play a crucial role in preserving flora in natural environments and supporting the entire trophic cascade that depends on them. More than 16% of the world's flowering plant species and nearly 400 agricultural plants rely on bee pollination. Additionally, over 20,000 species of bees contribute to the conservation and evolution of 80% of plant species [29,30,31].

Good pollination enhances crop yields and contributes to the production of visually appealing fruits that are more resistant to falling and exhibit superior organoleptic characteristics such as taste and texture, as well as improved shelf life. The direct impact of bees on crop quality and quantity, as well as their role in preserving floral biodiversity, is well supported. Bees play a crucial part in ecosystem function [17].

Chapter II

Apis mellifera intermissa gut microbiome

II.1. Description of the gut microbiota of honey bees:

Bees are known to live in symbiosis with a variety of microorganisms that are responsible for specific activities such as nutrition degradation, defense against pathogenic agents, and bee behavior [32]. The analysis of the microbial gut community of bees using metagenomics and genomics revealed that the microbiome harbors genes that may play a role in defending against pathogens, detoxifying environmental pollutants, and digesting pollen cell walls [33].

Corbiculate bees, such as honey bees, bumble bees, and stingless bees, possess an essential microbiota composed of two Gram-negative species from the *Proteobacteria* phylum: *Snodgrassella alvi and Gilliamella apicola*. These species are commonly found in the core gut community. Additionally, *Frischella perrara*, belonging to the *Gammaproteobacteria* class *Orbales,* is also a member of this core community. In Gram-positive bacteria, there are also two species: *Lactobacillus* Firm-4 and *Lactobacillus* Firm-5 clades, which are widespread and clustered within the *Firmicutes* phylum. Although it is often less abundant, the species Bifidobacterium asteroides is present in the majority of adult worker bees [28,33,34,35].

The fundamental microbiota of the honey bee's intestine also includes five known bacterial species from the *Proteobacteria* phylum: *Frischella perrara*, *Bartonella apis*, *Parasaccharibacter apium*, and the *Gluconobacter*-related species group designated as *Alpha*2[34]. The three species of *Alphaproteobacteria* (“Alpha-1,” “Alpha- 2.1,” and “Alpha-2.2” are also present in the core microbiota of honeybees’ gut [33,36].

Although most bacterial phylotypes found in the honeybee gut are associated with those found in other insects, these three-unique bacterial phylotypes including; *Gilliamella apicola, Frischella perrara, and Snodgrassella alvi* are found exclusively in honey bees [28].

The microbiome composition in honey bees varies significantly depending on their age and roles within the colony [32].

The 16S rDNA community analysis revealed that nine bacterial species groups constitute 95% to 99.9% of the bacteria in the digestive tracts of most worker honey bees[34].*Lactobacillus mellis and Bifidobacteriaceae* were found to be more abundant in bees engaged in nest activities[32].

The midgut, which is the main site of digestion, contains a limited number of bacteria. This may be attributed to the presence of intestinal enzymes and the peritrophic membrane [37] .The ileum of honey bees consists of two dominant Gram-negative bacterial species: *Snodgrassella alvi and Gilliamella apicola* [36]. The distal rectum of honey bees is primarily inhabited by Gram-positive *Lactobacillus spp*. and the Gram-negative bacterium *Gilliamella apicola* [36].

II.2.The diversity of honey bee's microbiome:

II.2.1. The variability of honey bees gut microbiota on each individual:

Animals in social societies often possess a unique gut microbiota essential for feeding and pathogen defense. Honeybees (Apis mellifera) exhibit a distinct gut microbial community, consisting of a specific set of species unique to social bees [38].

The main factors that shape bee gut microbiome communities include exposure to pesticides and chemicals in the hive, as well as interactions between different microbial communities, such as bacteria and fungi. These factors significantly impact the composition and coexistence patterns of the bee gut microbiota [39].

Honeybee intestinal microbiomes vary drastically between queens, workers, and males. However, nurses and foragers have similar gut microbiomes despite differences in diet, activity, and environment. The queens had a distinct gut microbiome enriched with bacteria that could enhance the metabolic conversion of energy from food to egg production. Additionally, males had a significantly different gut microbiome compared to workers, but not queens. Differences in gut microbiomes are also reflected in the relative abundance of specific bacterial phylotypes, such as *Parasaccharibacter* [40].

Queens are dominated by *Acetobacteraceae and lactobacilli* lineages from the Firm-5 clade. Workers have an important gut microbiome composed of eight primary bacterial species transmitted through social contact. A comparison was made between the gut microbiota of queens isolated from their nurse bees and those with unrestricted access to attendants during hatching. The results showed that isolated queens had larger and more diverse gut microbial communities than non-isolated queens[41].A higher relative abundance of the *alpha-proteobacteria* including; *Parasaccharibacter apium* in the gut microbiome of queen honeybees was identified [40].

II.2.2.The distribution of honey bees gut microbiota:

The honey bee gut community is comprised of 8-10 bacterial phylotypes, including *Gilliamella, Snodgrassella, Bifidobacterium, Lactobacillus Firm-4 and Firm-5, and Bartonella*. These bacterial phylotypes make up more than 97% of the microbial population and have specific roles in regulating host metabolism and neurological functions. The organization of bacterial populations in the bee's intestine is heterogeneous and plays a crucial role in its health and overall activity[42].

The communities of bacteria are most prevalent in the posterior gut, comprising the ileum and rectum. The distribution of bacterial communities in the posterior gut is as follows [43] :

The ileum Region is dominated by specific bacterial species such as Snodgrassella alvi and Gilliamella apicola. These bacteria form a continuous layer along the longitudinal folds of the ileum, where they aid in the digestion of dietary carbohydrates and provide defense against pathogens [43], in the other hand *Frischella perrara* exhibits distinct colonization of the pyloric region near the junction of the middle and posterior intestines. This bacterium has been shown to stimulate immune pathways, including the melanization response, and influence cell replication in intestinal epithelial cells [43], also Gram-positive species such as *Lactobacillus and Bifidobacterium* are more abundant in the rectal area of the posterior gut. These bacteria assist in the digestion of carbohydrates from plants and produce short-chain fatty acids in both the gut and hemolymph [43].

The uneven distribution of bacterial communities within these regions reflects their specialized functions in metabolism, immune function, growth and development, and protection against pathogens. The unique distribution of bacterial species within each location contributes significantly to the overall health and resistance to illness of bee workers [43].

II.3. The main role of the honey bee's gut microbiota:

The distinctive distribution of bacterial species within each location contributes to the overall health and disease resistance of bee workers [42].

Gilliamella is renowned for its role in carbohydrate metabolism and has the ability to digest mono- and polysaccharides in the bee stomach. It aids in the liberation of nutrient-rich substances from pollen, thus benefiting the host's diet [42], as for *Bifidobacterium* which is a bacterium breaks down polysaccharides from food in honey bee guts, facilitating the puncturing of pollen and releasing nutrients [42], mentioning also that lactobacillus Firm4 and Firm5 are bacterial phylotypes are involved in amino acid metabolism pathways in the honey bee gut. They are

associated with the regulation of specific metabolites related to amino acid metabolism in the hemolymph [42].

These bacterial phylotypes are critical for controlling host metabolism, hormone signaling. and food consumption, they improve the nutritional status of honey bees and impact neurologic operations, notably neurotransmitter levels in the brain [42].

The major functions of the honeybee intestinal microbiota are:

1. Metabolism: The intestinal microbiota of honey bees plays a fundamental role in digesting nutritional carbohydrates. Bacterial species such as *Snodgrassella alvi, Gilliamella apicola, Lactobacillus, and Bifidobacterium* possess the ability to digest and metabolize a wide range of carbohydrates produced by plants. This metabolic function significantly contributes to the overall nutrition and energy balance of the bee [43]

2. Immune function: The gut microbiome of honey bees stimulates immune responses such as the production of antimicrobial peptides. For example, bacterial species like *Frischella perrara* contribute to enhancing the bee's defense against pathogens.[43].

3. Protection against pathogens: The intestinal microbiota plays a crucial role in protecting bees from pathogenic infections. Studies have demonstrated that bees with a healthy gut microbiome exhibit greater resistance to infections caused by pathogens such as the trypanosomatid intestinal parasite *Crithidia bombi*. Additionally, the gut microbiota has been implicated in defending bees against infections caused by bacterial and protozoan pathogens [43] .

4. Nutrient uptake and hormonal signaling: The gut microbiota plays a significant role in promoting nutrient uptake and influencing hormonal signaling in honeybees. Research has demonstrated its impact on factors such as gut size, weight gain, insulin signaling, and sucrose sensitivity in bees, thereby influencing their overall physiology and health [43]

5. Intestinal development and stability: The gut microbiota plays a crucial role in the growth and stability of the honeybee gut. It regulates the structure and composition of the gut microbiome, particularly during the transition from larval to adult worker stages and helps maintain the stability of adult workers' microbiomes throughout their lives. In honeybees, the gut microbiome also influences hormone signaling and facilitates nutrient assimilation. Research has shown its impact on factors such as gut size, weight gain, insulin signaling, and sucrose sensitivity in bees, thereby influencing their overall physiology and health. [43]

6. Organic acid production: The gut microbiota of honeybees produces organic acids, which play significant roles in honeybee physiology. [44]

7. Honey Freshness: The gut microbiota is involved in transforming nectar into honey and contributing to the preservation of its freshness [44]

In general, the gut microbiota of bees influences metabolism, immunological responses, defense against infections, and overall physiological balance, all of which are crucial for bee health and resilience against illnesses. [43]

Individuals adapt to their environment on several levels, including metabolism, immunology. behavior at the individual and collective reactions [45]. Unraveling the intricate relationships between honeybee colonies and their surroundings calls for a variety of instruments as well as ongoing advancements in nutrigenomics and epigenetics to pinpoint the stressors putting honeybees in jeopardy. After all, bee health and human health are closely related. [46,47,48]

Multiple strategies may be taken to manipulate the microbiome of bees to improve their productivity and overall health[49], among them the Gut Microbiota Protection by avoiding substances that upset the equilibrium of gut microbiota , such as pesticides, antibiotics, and unfavorable environmental circumstances [49] ,Using healthy bacteria, including Lactobacillus species, can help detoxify pesticides, defend against diseases, and improve bee health in general. Modified microorganisms such as Snodgrassella alvi have demonstrated potential in assisting bees in fending off mites and lowering virus burdens [49],Dietary Supplements, using the Probiotics ,Environmental Considerations and Collaborative Research [49] .

Within the honey bee's gut microbiota, there are bacteria capable of producing the enzyme amylase, among them *Bacillus sp*, *Bacillus subtilis* and *Bacillus amyloliquefaciens*, these two *Bacillus* species are those that secrete amylase [50].

Chapter III

Material and methods

III.1. Presentation of sampling sites:

The sampling area is a small university farm located in the city of Bouira, at latitude 36.378600° N and longitude 3.876077° E (Figure n°4) The site includes one of the three marked hives situated on the farm (figure n° 5).

Figure n°4: the 3 hives where we did the collect of samples (Link 1)

Figure n°5: the university of bouira Akli Mouhaned oulhadj bouira (Link 1)

III.2. *Apis mellifera intermissa* sampling :

On March 6th, 2024, during the winter, honey bee samples were collected from the Faculty of Nature, Life, and Earth Sciences at Bouira University, located in the central northern part of Algeria. The climate in the region was temperate, and the weather was sunny.

The beehive is situated in a designated area surrounded by various plants that provide nutrition for the bees, including flowering rosemary (*Rosmarinus officinalis*), *Carpobrotus chilensis*, *Oxalis pes-caprae*, and orange trees.

Eleven live honey bees were collected and placed in a securely closed box. The box was then stored in a refrigerator at 0°C to immobilize the bees.

III.3. Extraction of the gut from the honey bees:

To study the microbiome of the honey bees, the guts of eleven bees were extracted. All steps of the extraction process were conducted under aseptic conditions.

Using sterile tongs, the extracted gut was placed in an Eppendorf tube. Then, 500 µL of Luria Bertani (LB) broth was added to the tube. After the extraction, the gut was crushed using a sterile metal rod. Once well crushed, the volume was brought up to 1 mL by adding an additional 500 µL of Luria Bertani (LB) broth. The prepared suspensions were used for counting, isolating, purifying, and identifying the different bacteria present in the gut microbiome of our samples[53 modified].

III.4. The study of *Apis mellifera intermissa* gut microbiota diversity:

To identify the different bacterial species, present in the suspensions, it is necessary to obtain well-isolated colonies. The Eppendorf tubes containing the suspensions were incubated at 37°C for 48 hours to promote the enrichment of microorganisms, facilitating the isolation and purification of the bacteria. Then, a one-fold enrichment suspension (10^{-1}) was prepared.

As a second step, 100 µL of the (10^{-1}) dilution of each suspension were inoculated onto selected culture media: MacConkey agar, MRS agar (de Man, Rogosa, Sharpe agar), and GYC agar (Glucose, yeast extract, calcium carbonate agar). The plates were incubated in the oven at 37°C for 24 hours [51 modified].

After isolation, bacterial strains were purified to obtain pure cultures on each petri dish. This purification step was essential to identify the bacterial diversity present in the gut of honeybees.

After obtaining the pure culture dishes, the strains were conserved. One colony from each petri dish was picked and mixed with 500 µL of LB medium in an Eppendorf tube. The tubes were incubated in the oven at temperature 37°C for 24 hours to promote the growth of bacteria.

After incubation, 500 µL of glycerol was added to these tubes, thoroughly mixed, and then stored in the refrigerator until needed.

III.5. *Apis mellifera intermissa* gut microbiota identification :

To identify the pure cultures based on their characteristics, microbiological and biochemical tests were conducted. These included examining the fresh state to determine bacterial shape, motility, and grouping. Additionally, Gram staining and catalase tests were performed.

III.6. The screening of the microbiota of honey bees with biological interest:

III.6.1. Determination of the production of bacteriocin by lactic acid bacteria strains:

Strains isolated from MRS medium and identified as lactic acid bacteria according the phenotypic characters including A4, A5 and A6 were used to determine their ability to inhibit the growth of pathogenic strains including "*Klebsiella pneumonia*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* ". Thus, the agar overly method was performed [According to 52].

As first step, the bacterial suspension was prepared from the strains isolated in the MRS medium. Then, loop colony of each strain was inoculated in a tube containing 5 ml of the LB broth and incubated at 37°C for 24 hours. After the incubation, 1µL of bacterial suspension inoculated on LB agar plate and petri dishes were incubated for 24 hours at 37°C. Then, colonies of each strain obtained on LB medium were inoculated into MRS broth and incubated at 37°C for 24 hours. Following this, positive and negative strains were streaked onto MRS agar by forming a thick horizontal. The petri dishes were then incubated for an additional 24 hours at 37°C. After incubation, the MRS agar plates were covered with 1 mL of the bacterial suspension (10^8 CFU/mL) of the pathogenic strains. The petri dishes were then placed in a 37°C incubator for 24 to 48 hours. After incubation, the dishes were examined to find a clear inhibition zone around the lactic acid bacteria line [According to 52], All steps were performed under aseptic conditions to avoid contamination and the essay was triplicated.

III.7. Determination of amylase production from *Acetobacteriaceae* :

At first steps, a starch-based culture medium was prepared,[According to 53, modified], Then bacterial suspension was prepared from strains obtained on GYC medium. After that, 10 µL of each bacterial suspension was taken and placed on the starch-based agar following the spot method. The petri dishes were incubated at 37°C for 24 hours. After incubation, iodine was poured onto the surface of the petri dish and left for a few minutes. The dishes were examined by the appearance of light brown coloration, if the coloration becomes blue so the test is negative [According 54,modified]. All steps were performed under aseptic conditions to avoid contamination and the essay was triplicated.

Chapter IV

results and discussion

In order to carry out this study, the sampling was done in March 2024 in winter from the region of bouira precisely in the university of bouira akli mouhaned oulhadj and 11 bees were used to release the phenotypic characterization of the digestive tract microbiota of these bees following Gram staining and biochemical tests such as catalase [table II]. Then we conducted a screening method to evaluate the amylolytic activity of the isolates obtained on GYC medium [Table VI]. Additionally, antimicrobial assays were carried out on the isolates obtained from MRS medium to select isolates producing bacteriocin [table VIII].

IV.1. Presentation of suspension after crushing of digestive tubes:

After crushing the digestive tubes, it was observed that some suspensions exhibited a color change from light to black or nearly black. This color change persisted after each subsequent incubation. This observation, as indicated in [Table I], may suggest the presence of fungi in the isolates.

Table I : Sample table

Table of simples				
Ech	**Code ISO**	**Appearance**	**Color**	**Note**
A1	A1S1	Normal and full	Clear and after 24h of crushing and incubation becomes black	Incomplete digestive tract
A2	A2S1	Normal and full	Clear and after 24h of crushing and incubation becomes black	
A3	A3S1	Normal and full	Clear and after 48h of crushing and incubation becomes black	
A4	A4S1	Normal and full	Clear after 48h of incubation	
A5	A5S1	Normal and full	Clear and after 24h of crushing and incubation becomes black and after 24h of incubation becomes clear one more time	Incomplete digestive tract
A6	A6S1	Normal and full	Clear after 48h of incubation	
A7	A7S1	Normal and full	Clear after 48h of incubation	Incomplete digestive tract
A8	A8S1	Normal and full	Clear after 48h of incubation	
A9	A9S1	Normal and full	Clear and after 48h of crushing and incubation becomes black	
A10	A10S1	Normal and full	Clear and after 48h of crushing and incubation becomes black	
A11	A11S1	Normal and full	Clear and after 48h of crushing and incubation becomes black	

IV.2. The bacterial diversity form *Apis mellifera intermissa* gut microbiota:

From 11 honey bee samples, we obtained 49 isolates from the three types of agar media. After incubating at 37°C for 24 hours, there was significant growth with notable bacterial diversity (Figure n°6). Specifically, MacConkey medium yielded 32 isolates (65.31%), MRS medium yielded 5 isolates (10.20%), and GYC medium yielded 12 isolates (24.49%) (Figure n°7). The isolates displayed various appearances, colors, and sizes, indicating a diverse bacterial microbiota in the digestive tracts of the bees[Results are shown in Table II].

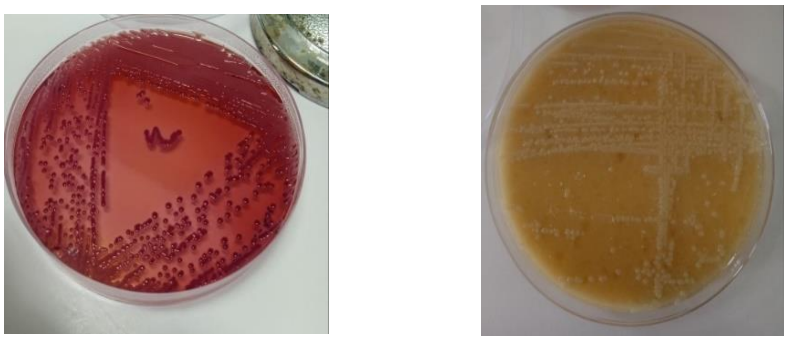


Figure n°6: isolates on MacConkey and GYC

Table II : Table of the development of isolates on different media

Table of the development of isolates on different media			
Isolates	Macconkey	MRS	GYC
A1	+	No growth	+
A2	+	No growth	+
A3	No growth	+	+
A4	+	+	+
A5	+	+	+
A6	+	+	+
A7	No growth	No growth	+
A8	+	No growth	+
A9	+	No growth	+
A10	+	+	+
A11	+	No growth	+

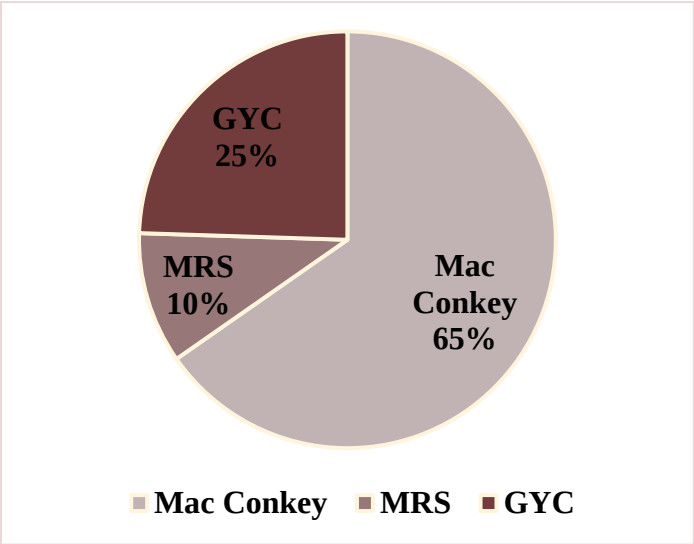


Figure n°7: the development of isolates on different media

From 49 isolates we got a dominance of non-mobile bacteria with 63.27 % while 36.73% were mobile and 1 isolate was ND. Regarding the catalase essay, we found a dominance of catalase positive with 95.92% while 4.08% were catalase negative (Figure n°8).

To be more specific, for the 32 isolates of MacConkey we got 56.25 % non-mobile and 46.88 % mobile bacteria and 100% catalase positive which indicate that the most of those bacteria has the catalase enzyme (Table IV).

In the other hand and on MRS medium we got 100% non-mobile bacteria and 40% catalase negative which indicate the absence of the catalase enzyme for this bacterium and 60% catalase positive which indicate the presence of the catalase enzyme (Table III).

As for the GYC medium we got 66.67% non-mobile bacteria while 25% were mobile and 1 isolate was ND, and 100% of the bacteria were catalase positive which indicate the presence of the catalase enzyme for all the bacteria that grow on GYC medium (Table III).

While form Gram staining, we found that from 49 isolates we got a dominance of Gram-negative bacteria with 53.06% while Gram positive bacteria were 44.90%.

Figure n°8: *catalase test*

While form Gram staining, we found that from 49 isolates we got a dominance of Gram-negative bacteria with 53.06% while Gram positive bacteria were 44.90% (Figure n°9).

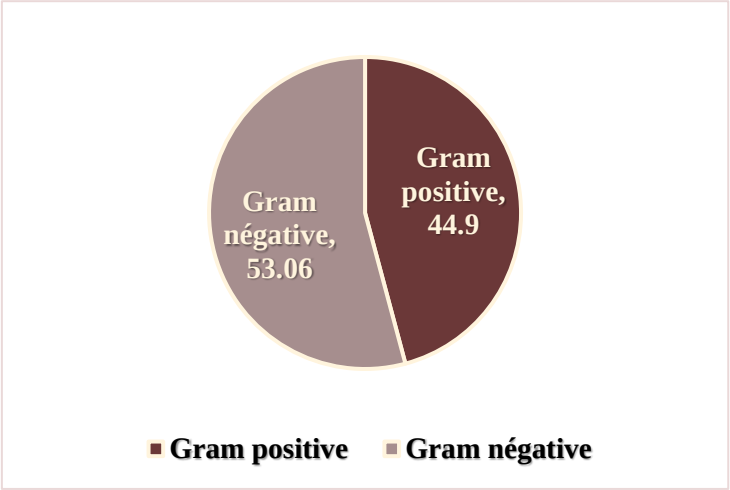


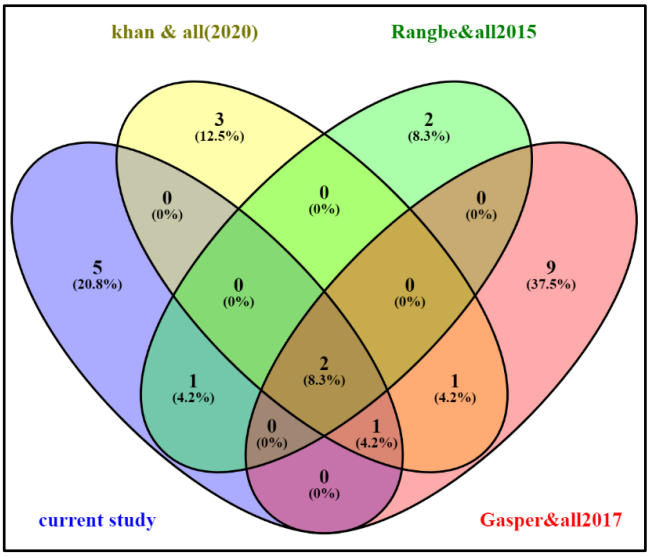
Figure n°9: Gram staining distribution of all the isolates

To be more specific on Macconkey medium we got 62.5% of Gram-negative bacteria while 37.5% of Gram-positive bacteria(Table IV). On another hand, 60% of gram-positive bacteria and 40% of Gram-negative bacteria were detected on the MRS medium (TableV)

As for the GYC medium, we have 58.33% Gram-positive bacteria and 33.33% Gram-negative bacteria (Table III).

To verify the initial identification results obtained from the MacConkey medium, the same bacterial strains were streaked onto CHROMagar medium and EMB (Eosin Methylene Blue) agar. This process helps to presumptively identify the bacterial isolates by observing their growth patterns and characteristics on these additional selective and differential media.

From 33 isolates we could identify 25 bacteria with the microbiological identification, we found a predominance of [*Klebsiella pneumonia*] with 12 bacteria or 48%, [*E. coli*] with 8 bacteria or 32%, [*Enterobacter*] with 2 bacteria or 8% and [*Staphylococcus aureus*] with 3 bacteria or 12%, this indicates the bacterial diversity of our honey bees' gut microbiome (Figure n°11).



. ***Figure n°10:*** *Venn diagram representing the common and the differences between other studies*

In comparison with others investigations, we found that our results or diversity from Apis mellifera gut microbiota from Algeria is different according to the results obtained by Rangberg and coworkers from the Norway, khan coworkers from Australia, Gasper coworkers from Estern Slovakia, we have only 2 common genera bacteria including "*Enterobacter and Klebsiella"* [55,56,57]

Also, two common bacteria "*Klebsiella* and Bacillus" according to Khan and coworkers, in the other hand we only found 1 common bacteria "*Lactobacillus*" between and the results of Rangberg and coworkers. The presence of this bacterial variety is caused by variations in location, climate,

nutrition, time and date of sampling, as well as the health of the bees treated in the various studies[58]

In our study we found some isolates of "*Staphylococcus"* which indicate a contamination from the small parts of exoskeletons linked to the digestive tubes of our honey bee samples (Figure n°10).

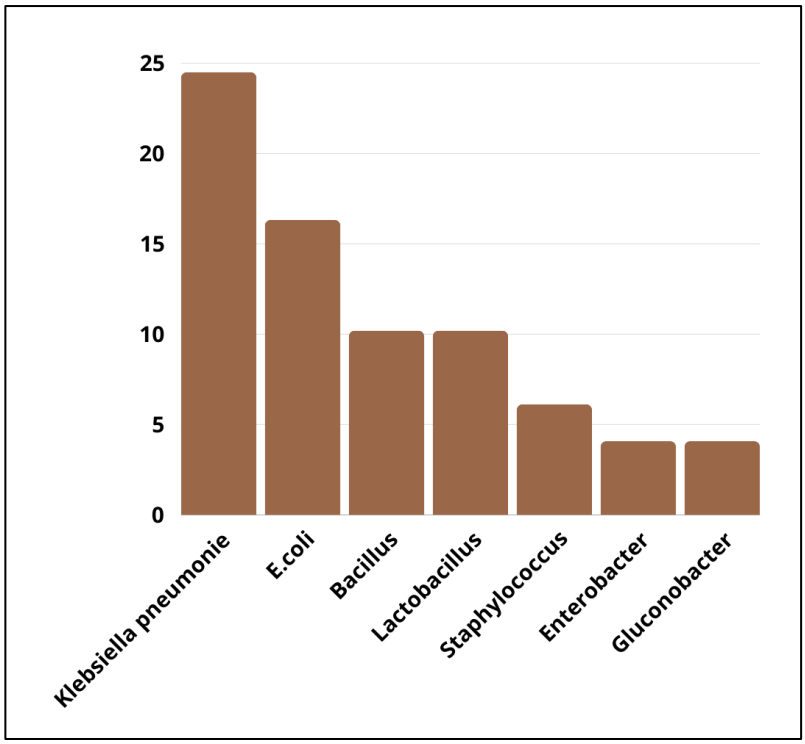


Figure n°11: Different bacteria identified distribution on the 3 different medium

VI.3. The microbiota of honey bees with biological interest:

VI.3.1. the antimicrobial potential of MRS medium isolates :

Among the 3 strains of lactic acid bacteria [LAB] tested we observed a zone of inhibition with A4 that inhibit *Pseudomonas aeruginosa and Klebsiella pneumonia*, while A5, A6 inhibit *Staphylococcus aureus* (Figure 12).

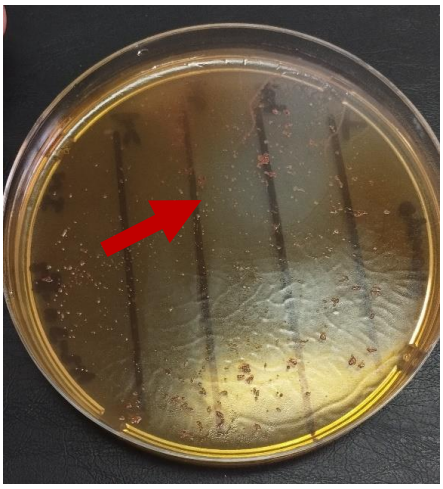


Figure n°12: antibacterial activity on LAB against pseudomonas

According to the study of Elzeini& all.2020 it showed that the isolates of LAB were all Gram positive and catalase negative and showed antagonism against "*Staphylococcus aureus, pseudomonas aeruginosa, klebsiella pneumonia"* [52].

Which corroborate with our obtained results. From the MRS; the isolates are Gram positive and catalase negative, except one isolate (A4) was Gram positive while catalase positive and also showed an antibacterial activity against "*Pseudomonas, Klebsiella and Staphylococcus*". The A4 could be attributed to others bacterial strains other than LAB group bacteria (Table IV) [52,59].

Figure n°13: Amylolytic activity result on GYC isolates

VI.3.2. the amylase production bacteria from GYC medium *:*

In order to determine whether those bacteria produce the amylase enzyme or not, we caried the test of the activity amylolitique on the 12 isolates of the GYC medium.

The obtained results revealed that the isolates [A6, A7, A8, A9, A10 and A11] may produce amylase enzyme. After we added the Lugol solution, we observed with the isolates [A6, A7, A8, A9, A10 and A11] clear zone of starch degradation with a clear brown color. While with the isolates [A1, A2, A3, A4, A5. S1, A5. S2] any zone of starch degradation with a dark brown color was observed, that indicate that the isolates weren't amylase producers (Table III).

According to Ganeshprasad and coworkers, amylolytic activity is observed with the *gluconobacter* isolates. While the study carried out by Shi and collaborator, revealed that *Acetobacter* is a member of the acetic acid bacteria (AAB) group and is commonly present in the guts of insects and a variety of fruits, flowers, and fermented foods [60,61].

That's indicate that the isolates [A6, A7, A8, A9, A10 and A11] may be affiliated to the *gluconobacter.*

Table V : microscopies result on GYC and activity amylolytic results.

Ech	Gram staining	Cell shape and organization	Mobility	Catalase	Enzymatic activity (alpha amylase)
A1	(-)	Bacilli	(+)	(+) weak	(-)
A2	(+)	Cocci	ND	(+)	(-)
A3	(+)	Cocci	(-)	(++)	(-)
A4	(+)	Cocci	(-)	(++)	(-)
A5	(+)	Bacilli	(-)	(+)	(-)
	(-)	Bacilli	(-)	(+)	(-)
A6	(+)	Cocci	(-)	(+)	(+)
A7	(+)	Short bacilli	(-)	(++)	(+)
A8	(+)	Short bacilli	(+)	(+)	(+)
A9	(-)	Bacilli	(-)	(+++)	(+)
A10	(-)	Cocci	(+)	(+)	(+)
A11	(-)	Cocci	(-)	(+)	(+)

Table VI : microscopies and microbiological results on mac Conkey.

Ech	Code ISO	Gram staining	Cell shape and organization	Mobility	Catalase	Suspected isolates
A1	A1 S2	(+)	Cocci	(-)	(++)	*Klebsiella pneumonia*
	A1 S1.1.1	(+)	Bacilli	(+)	(++)	ND
	A1 S1.1.2	(-)	Cocci	(-)	(++)	*Klebsiella pneumonia*
A2	A2 S1.1	(+)	Diplococci	(+)	(+)	*Escherichia coli*
	A2 S1.2	(+)	Cocci	(-)	(+)	*Klebsiella pneumonia*
	A2 S2.1.1	(-)	Cocci	(-)	(+)	*Klebsiella pneumonia*
	A2 S2.1.2	(-)	Bacilli	(-)	(+)	ND
A3	ND					
A4	A4 S1.1.1	(-)	Diplococci	(+)	(+)	*Klebsiella pneumonia*
	A4 S1.1.2	(-)	Bacilli	(+)	(++)	*Escherichia coli*
	A4 S1.2.2	(+)	Cocci	(-)	(+++)	*Klebsiella pneumonia*
	A4 S1.2.1	(-)	Diplococci	(+)	(+)	*Klebsiella pneumonia*
A5	A5 S1	(-)	Bacilli	(-)	(++)	ND
	A5 S2	(+)	Cocci	(-)	(+)	*Escherichia coli*
A6	A6 S1.1	(+)	Bacilli	(+)	(+)	*Staphylococcus aureus*
	A6 S1.2	(-)	Diplococci	(+)	(+)	*Staphylococcus aureus*
	No growth					
A7	No growth					
A8	A8 S1,1	(-)	Diplobacilli	(-)	(+)	ND
	A8 S1.2.1	(+)	Cocci	(+)	(+)	*Escherichia coli*
	A8 S1.2.2	(+)	Bacilli			*Escherichia coli*
	A8 S2.1.2	(-)	Bacilli	(+++)	(+++)	ND
	A8 S2.1.1	(-)	Cocci	(-)	(+)	ND
A9	A9 S1	(-)	Cocci clusters	(-)	(+)	*Klebsiella pneumonia*
	A9 S2.1.1.1	(-)	Cocci clusters	(-)	(++)	*Enterobacter*
	A9 S2.1.1.2	(-)	Cocci	(-)	(+++)	*Escherichia coli*
	A9 S2.1.2.2	(+)	Bacilli	(+++)	(+)	ND
	A9 S2.1.2.3	(-)	Cocci	(+)	(+++)	*Enterobacter*
	A9 S2.1.2.1	(-)	Cocci	(-)	(+++)	ND
	A9 S2.2.1.2	(+)	Cocci	(-)	(+)	*Klebsiella pneumonia*
	A9 S2.2.1.1	(+)	Bacilli	(+)	(+)	*Klebsiella pneumonia*
	A9 S2,3	(-)	Cocci	(+)	(++)	*Escherichia coli*
A10	A10 S1.1.1	(-)	Cocci clusters	(-)	(+) weak	ND
	A10 S1.1.2	(+)	Bacilli	(+)	(++)	*Escherichia coli*
	A10 S2,1	(-)	Cocci clusters	(+)	(+)	*Klebsiella pneumonia*
	A10 S2,3	(-)	Bacilli	(-)	(+)	*Staphylococcus aureus*
A11	A11 S1	(-)	Cocci	(-)	(+++)	*Klebsiella pneumonia*

Table VII : microscopies and the antimicrobial activity results on MRS.

Ech	Gram staining	Cell shape and organization	Mobility	Catalase	Antibacterial activity
A1	ND				
A2	ND				
A3	(-)	Cocci clusters	(-)	(+)	(-)
A4	(+)	Bacilli	(-)	(++)	(+)
A5	(+)	Cocci chain	(-)	(-)	(-)
A6	(+)	Diplobacilli	(-)	(-)	(-)
A7	ND				
A8	ND				
A9	ND				
A10	(-)	Bacilli	(-)	(+)	(-)
A11	ND				

Conclusion

Conclusion

In order to investigate on the screening of bacteria from *Apis mellifera intermissa* gut microbiome able to produce bioactive compounds including, enzymes and bacteriocins molecules. We examined 11 samples of bees of the species *Apis mellifera*, in particular *Apis Mellifera intermissa*, from the Bouira region.

Our study revealed a wide variety of the intestinal microbiota of honey bees, with the following dominant species: *Enterobacteriacae, Lactobacillaceae* and *Acetobacteraceae*. It also demonstrates the benefits of these bacteria and their importance in the maintenance of the intestinal system of honey bees and in ecosystems.

We have a special focus on the species that have a biological interest, which are an integral part of honey bee gut microbiota such as the Bacteria that have grown on MRS agar, which is suspected to be a lactic acid lactic acid bacterium that can produce pathogen-suppressing substances and thus maintain honey bees' health and reduce its recently increasing seasonal mortality rate. Also, the Bacteria that have grown on GYC agar, which is suspected to be *Acetobacter* and *Gluconobacter* bacteria, that have the ability to produce amylase enzyme which is of great importance currently.

Ce Project présente des perspectives prometteuses dans la découverte de nouvelles espèces bactériennes et leur caractérisation fonctionnelle. En explorant la diversité microbienne à l'aide de techniques avancées telles que le séquençage métagénomique, le projet vise à identifier des bactéries potentiellement bénéfiques pour la santé de l'abeille et leur potentiel application dans l'agriculture ou en médecine. Cependant, il fait face à des défis tels que la complexité de l'échantillonnage précis du microbiote intestinal, les exigences techniques et analytiques élevées, ainsi que la nécessité d'une interprétation approfondie des résultats pour comprendre pleinement les interactions bactérie-hôte et leur impact.

En résumé, le projet offre des perspectives intéressantes pour la découverte de nouvelles espèces bactériennes et leur potentiel application, mais il nécessite une approche méthodologique rigoureuse et une compréhension approfondie des défis techniques associés à l'étude du microbiote intestinal d'Apis mellifera intermissa.

Conclusion

We hope that this study will serve as a model for a better understanding of the bacterial diversity found in the honeybee gut microbiota and also highlight its effective role in the host's health and environment.

It also aims to help others to enhance knowledge regarding the microbiota of bee intestines and their biological interests, and to open prospects for other scientific endeavors to bridge the gap about the distinction and stability of this microbial community of honey bees, which would provide a new addition to the preservation of honey bees in Algeria.

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Abstract

Abstract :

The Honey bees host a variety of bacteria in its intestines, making it a valuable model of study and scientific research where these bacteria play an active role in preserving the host's health. Despite much research to understand this distinctive diversity, there is still ambiguity around this microscopic world. The aim of our study is to identify some of these stable bacterial species in honey bee Apis mellifera intermissa and also determine their biological interests through the phenotypic identification of 11 samples. Through the results of the phenotypic identification in comparison with previous studies, it can be said that the bacteria obtained mostly belong to species Enterobacteriaceae, with 32 isolates which is 65.31%,Acetobacteraceae with 12 isolates which is 24.49%, and Lactobacillaceae with 5 isolates which is 10.20% . Speaking of the biological interest of this bacterial diversity and through our results, we found that the bacteria we got at MRS medium which is suspected to be LAB it has the ability to produce bacteriocins as an inhibitor of pathogen growth. Also, through bacteria that grew on the GYC medium, we found productive strains for amylase enzyme, these strains suspected to be Acetobacter and Gluconobacter bacteria. This work provided insight into the bacterial diversity of the honey bee's Apis mellifera intermissa gut microbiota and its biological interests, which could open doors to future prospects and experiences in this field in Algeria.

Keywords: Honey Bee, *Apis mellifera intermissa*, bacterial diversity, gut microbiota LAB, *Acetobacter, Gluconobacter*, biological interests, bacteriocins, amylase enzyme.

Résumé :

Les abeilles domestiques hébergent une variété de bactéries dans leurs intestins, ce qui en fait un modèle précieux d'étude et de recherche scientifique où ces bactéries jouent un rôle actif dans la préservation de la santé de l'hôte. Malgré beaucoup de recherches pour comprendre cette diversité distinctive, il y a encore une ambiguïté autour de ce monde microscopique. L'objectif de notre étude est d'identifier certaines de ces espèces bactériennes stables chez l'abeille mellifère (Apis mellifera intermissa) et de déterminer leurs intérêts biologiques à travers l'identification phénotypique de 11 échantillons. Grâce aux résultats de l'identification phénotypique en comparaison avec les études précédentes, on peut dire que les bactéries obtenues appartiennent principalement aux espèces Enterobacteriacae avec 32 isolats qu'est 65,31%, Acetobacteraceae avec 12 isolats qu'est 24,49%, et Lactobacillaceae avec 5 isolats qu'est de 10,20%. Parlant de l'intérêt biologique de cette diversité bactérienne et à travers nos résultats, nous avons constaté que la bactérie que nous avons obtenue au milieu MRS qui est suspecté d'être LAB a la capacité de produire des bactériocines comme un inhibiteur de la croissance des pathogènes. Également à travers des bactéries qui se sont développées sur le milieu GYC, nous avons trouvé des souches productives pour l'enzyme amylase, ces souches suspectées d'être des bactéries Acetobacter et Gluconobacter. Ce travail a permis de comprendre la diversité bactérienne du microbiote intestinal de l'abeille mellifère Apis mellifera intermissa et ses intérêts biologiques, ce qui pourrait ouvrir des perspectives et des expériences futures dans ce domaine en Algérie.

Mots-clés : Abeille domestique, *Apis mellifera intermissa*, diversité bactérienne, microbiote intestinal, LAB, *Acetobacter, Gluconobacter*, intérêts biologiques, bactériocines, enzyme amylase.

الملخص:

يستضيف نحل العسل مجموعة متنوعة من البكتيريا في أمعائه، مما يجعله نموذجًا قيمًا للدراسة والبحث العلمي حيث تلعب هذه البكتيريا دورًا نشطًا في الحفاظ على صحة المضيف. على الرغم من الكثير من الأبحاث لفهم هذا التنوع المميز، لا يزال هناك غموض حول هذا العالم المجهري من خلال نتائج التعرف على النمط الظاهري مقارنة بالدراسات السابقة، يمكن القول إن البكتيريا التي تم الحصول عليها تنتمي في الغالب إلى الأنواعEnterobacteriacae ، مع 32 عزلة بنسبة 65.31٪، و Acetobacteraceaeمع 12 عزلة بنسبة 24.49٪، و .Lactobacillaceaeمع 5 عزلات بنسبة 10.20٪. الهدف من دراستنا هو تحديد بعض هذه الأنواع البكتيرية المستقرة في نحل العسل Apis mellifera intermissa وأيضًا تحديد اهتماماتها البيولوجية من خلال التعرف على النمط الظاهري لـ 11 عينة. بالحديث عن الاهتمام البيولوجي لهذا التنوع البكتيري ومن خلال نتائجنا، وجدنا أن البكتيريا التي حصلنا عليها في وسط MRS والتي يشتبه في أنها LAB لديها القدرة على إنتاج Bactériocines كمثبط لنمو العوامل الممرضة. أيضًا من خلال البكتيريا التي نمت على وسطGYC ، وجدنا سلالات منتجة لإنزيم الأميلاز، ويشتبه في أن هذه السلالات هي بكتيرياAcetobacter و Gluconobacter.

قدم هذا العمل نظرة ثاقبة على التنوع البكتيري لميكروبات نحل العسل Apis mellifera intermissa واهتماماتها البيولوجية، مما قد يفتح الأبواب أمام الآفاق والتجارب المستقبلية في هذا المجال في الجزائر.

الكلمات المفتاحية: نحل العسل، *Apis mellifera intermissa*، التنوع البكتيري، ميكروبيتا الأمعاءLAB، Acetobacter، *Gluconobacter*، الاهتمامات البيولوجية، Bactériocines، إنزيم الأميلاز.