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Relationships between probiotic bacteria and honeybee diseases

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A mio padre Carlo,
A mio fratello Gianluigi,
a voi, insieme, per sempre nell'eternità

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INTRODUCTION

The gut microbial composition of honey bees (*Apis mellifera* L.) is simple, unique and highly specialized; the dominant bacterial phyla belong to the *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicutes* (Kwong *et al*., 2014; Kwong & Moran, 2016). In social insects, such as honey bees, intestinal bacteria are transmitted and shared by colony members through oral-faecal transmission, oral trophallactic interaction, consumption of stored pollen or beebread, interactions with older bees within the hive and contact with hive material during the adult phase (Engel & Moran, 2013; Powell *et al*., 2014). With its simple and characteristic composition, the honey bee gut microbiota provides an excellent model for studying the functional and structural aspects of the bacterial communities associated with the gut (Martinson *et al.*, 2012; Kwong *et al.*, 2017). These microorganisms participate in various processes such as food digestion, detoxification from harmful molecules, supply of essential nutrients, participation in the host defense system and protection from pathogens and parasites (Engel & Moran, 2013). An imbalance of this microbiota (dysbiosis) can lead to a greater susceptibility to disease and an alteration of the genetic-immune system of bees. Dysbiosis can lead to a weakening of bees that are unable to cope with external stressors and pathogens and opportunistic diseases, contributing to the phenomenon of Colony Collapse Disorder (CCD) (Evans & Lopez, 2004; Kwong *et al.*, 2017; Zheng *et al*., 2017). The presence of Lactic Acid Bacteria (LAB) in the honey bee digestive system has been consistently reported in the literature (Vásquez *et al*., 2012; Rokop *et al*., 2015).

The environment of the honey bee stomach, characterized by the microaerobic condition, the presence of sugars (from nectar, honeydew, artificial nutrition) and sufficiently optimal temperature (i.e. about 32-35°C, when the bees heat the brood), independent from the outside air temperature, is the optimal niche for LAB. Based on the received data, Olofsson *et al*. (2016) suggested that bees and LAB flora developed with a mutual dependence on each other: LAB received a niche in which nutrients were available, and the bees obtained a protection from harm (Andersen *et al*., 2011; Filannino *et al*., 2019). LAB originate from the flower nectar and/or pollen grains collected and ingested by the bees and are found in the gut, stored pollen and honey. The pollen collected from flowers changes both microbiologically and biochemically immediately after harvesting, thanks to the contribution of lactic acid bacteria that ferment sugars. The pollen is thus transformed into beebread to support the young growing bees that are the future of the colony (Endo *et al*., 2009, 2013; Vásquez *et al*., 2009, 2012). Besides, the LAB microbiota has been linked to the immune system and defense against pathogens (i.e. Iorizzo *et al*., 2020 a, c). A greater knowledge on the relationship between the composition and function of the intestinal microbiota with the health and productivity of bees is highly desirable (Evans & Lopez, 2004; Pattabhiramaiah *et al*., 2012;

Fanciotti *et al*., 2018). The ever-deeper knowledge of the LAB populations, which colonize the intestines of bees, will surely enrich our understanding on insect–microbe symbiosis and on the evolutionary dynamics of these microorganisms in the social life of the hive. *Apis mellifera ligustica* Spinola is a native and an endemic Italian subspecies easily bred by beekeepers because of its adaptability to a wide range of climatic conditions, its ability to store large quantities of honey without swarming and its docility if disturbed.

In recent years, a major concern has arisen in several countries for the conservation of local honey bee species and of their original genetic structure (Fontana *et al*., 2018). The knowledge about LAB species diversity, also at subspecies level, could be crucial for the development of biotechnological approaches able to improve the health of honey bees. On this basis, the aim of a first study, preparatory to the PhD thesis, was the phenotypic and genotypic characterization of the predominant LAB species from the social stomach and from midgut of *A. m. ligustica* honey bee foragers and from the beebread produced in its hives (Iorizzo *et al*., 2020 b). The research was conducted in Molise region (Italy), an area with a temperate climate in Southern Italy (annual average minimum temperature: 9.7°C; annual average maximum temperature: 16,6°C; annual average temperature: 13.15°C).

The LAB isolation was carried out by dissecting the social stomach and midgut of foragers and from the beebread. The DNA extraction (Querol *et al*., 1992) was obtained by two milliliters of overnight seven isolated cultures previously centrifuged. The DNA analysis was carried out by PCR-DGGE (Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis). After the isolate were amplifyied with primers P1V1 (Cocolin *et al*., 2001) and P4V3 (Klijn *et al*., 1991). Sequences were aligned with those in GeneBank utilising the Blast program (Altschul *et al*., 1997) to determine the closest known relatives, based on the partial 16S rRNA gene homology.

The strains belonging to *Apilactobacillus kunkeei* (Edwards *et al.*) Zheng *et al.* (n. 85), *Lactiplantibacillus plantarum* (Orla-Jensen) Zheng *et al.* (n. 61) and to *Fructobacillus fructosus* (Kodama) Endo & Okada (n. 16) were biotyped using Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) analysis. All the strains belonging to *Al. kunkei*, *Lp. plantarum* and to *F. fructosus* were assessed for their carbohydrate fermentation pattern using API 50CHL system kit and enzymatic patterns using API ZYM system kit, according to the manufacturer's instructions (BioMérieux SA, Marcy l'Etoile, France). The reported research (Iorizzo *et al*., 2020 b) strongly enriched the knowledge on *A. mellifera* microbiota. The levels of inter-species variability depend on the isolation sources: beebread, honey stomach and midgut. So far, several authors have described the inter-species diversity in *A. mellifera* subspecies (Olofsson *et al*., 2011; Di Cagno *et al*., 2019; Filannino *et al*., 2019; Cilia *et al*., 2020).

First results showed the beebread highlighted the presence of all the detected species. Specifically, *Al. kunkeei*, *Lp. plantarum* and *F. fructosus* were detected as predominant species and *Levilactobacillus brevis* (Orla-Jensen) Zheng *et al*. and *Lactococcus lactis* (Lister) Schleifer *et al.* as a minority. The presence of isolates belonging to four species was also detected in honey stomach, where *Al. kunkeei* and *Lp. plantarum* represented the predominant species, while isolates identified as *F. fructosus* as well as *Lb. lactis* were the minority. Finally, in midgut the presence of only two species was detected and *Al. kunkeei* represented the predominant species. In fact, 56 isolates were identified as *Al. kunkeei* and only 4 isolates as *Lp. plantarum.*

In conclusion, first results showed a low biodiversity in species confirming the predominance of *Al. kunkeei* and *Lp. plantarum* in the social stomach and in midgut. *F. fructosus,* along with *Al. kunkeei* and *Lp. plantarum,* were mainly associated with beebread. For all the three species a reasonable intra-species biodiversity was found and a relationship between biotypes and compartments has been detected. An exhaustive study on genomic diversity of honey bee gut microbiota has been reported by Ellegaard and Engel (2019) evidencing that the bacterial population from honey bee guts are not only organized into discrete genetic clusters, so-called "sequence-discrete populations" (SDPs), but each SDP of the honey bee gut microbiota harbor very high levels of strain diversity. However, as also evidenced by other authors (Zhu *et al*., 2015; Ellegaard *et al.*, 2016), to identify the causes and consequences of intra-species diversity, experimental and phenotypic data must be linked to each LAB biotype profile. Moreover, with our knowledge, only few studies (Ellegaard *et al*., 2020; Sinotte *et al*., 2020), if any, link the biotype's diversity to specific ecological niches in honey bee foragers. On this basis, the main important scientific result obtained in this study was the finding that the specific biotypes, through their metabolic profile, contribute to the correct physiology of the associated honey bee organs or to the definition of beebread features. In fact, regardless of species, the biotypes associated with the social stomach and midgut were able to metabolize several sugars considered toxic to bees. In addition, the biotypes detected in beebread could contribute to the release of odorous compounds or to the increase in antioxidative metabolites.

The inter- and intra-species diversity of LAB-specific as well as functional diversity could present a new horizon to consider the honey bee microbiome. Therefore, the results obtained in these preliminary studies could be considered as important knowledge for the development of biotechnological approaches able to improve the health of honey bees or to enhance the quality features of their products.

With the isolates available at the Department of Agricultural, Environmental and Food Sciences of the University of Molise, the activity against *Paenibacillus larvae* White using *Lp.*

plantarum strains whose functional properties were also determined (Iorizzo *et al*., 2020 c) and the antagonistic activity against *Ascosphaera apis* (Maasen ex Claussen) L.S. Olive & Spiltoir of *Al. kunkeei* strains whose functional properties were also determined (Iorizzo *et al*., 2020 a) were preliminarily evaluated.

In the first case *P. larvae*, the causative agent of the quarantine disease American foulbrood (AFB), is the most widespread fatal brood disease of Western honeybee (*A. mellifera*) larvae and pupae (Hansen & Brødsgaard, 1999). It is well known that the initial phase of pathogen infection can be facilitated by any nutritional or environmental stress causing microbial dysbiosis (Genersch, 2010; Hamdi *et al*., 2011). The presence of lactic acid bacteria (LAB) in the honeybee gastrointestinal tract has been consistently reported in literature (Endo *et al*., 2013; Kwong & Moran, 2016). Beneficial bacteria, belonging to LAB, have been shown to promote honeybee health through activating the honeybee's immune defenses and producing antimicrobial compounds inhibiting pathogenic microorganisms (Filannino *et al*., 2019; Iorizzo *et al*., 2020 a). The reported study evaluated, *in vitro*, antimicrobial activity of sixty-one *Lp. plantarum* strains, against *P. larvae* ATCC 9545. Five strains (P8, P25, P86, P95 and P100) that showed the greatest antagonism against *P. larvae* ATCC 9545 were selected for further physiological and biochemical characterizations. In particular, the hydrophobicity, auto-aggregation, exopolysaccharides production, osmotic tolerance, enzymatic activity and carbohydrate assimilation patterns were evaluated. The five *Lp. plantarum* selected strains showed suitable physical and biochemical properties for their use as probiotics in the honeybee diet. The selection and availability of new selected bacteria with good functional characteristics and with antagonistic activity against *P. larvae* opens up interesting perspectives for new biocontrol strategies of diseases such as AFB. The results of this research have shown that P8, P25, P86, P95 and P100 *Lp. plantarum* strains are able to inhibit *P. larvae* ATCC 9545 and they own some physiological and functional properties that make these strains candidate as a probiotic for the honeybee. However, we may consider the preliminary and preparatory results for future studies that can consolidate the acquired knowledge and assess the benefits these bacteria may have on honeybee health in the hive. In addition, it will be important to investigate the factors that determine antimicrobial action and to evaluate antagonist activity of *Lp. plantarum* strains *in vivo/in situ*.

The second case dealt with *A. apis*, that is an important pathogenic fungus of honeybee larvae; it can colonize the intestine, especially in conditions of nutritional or environmental stress that cause microbial dysbiosis. In this work, some functional properties of nine selected *Al. kunkeei* strains were evaluated. The study focused on the antifungal activity of these strains against *A. apis* DSM 3116, using different matrices: cell lysate, broth culture, cell-free supernatant, and cell pellet.

The cell lysate showed the highest antifungal activity. Moreover, the strains were shown to possess good cell-surface properties (hydrophobicity, auto-aggregation, and biofilm production) and a good resistance to high sugar concentrations. These *Al. kunkeei* strains were demonstrated to be functional for use in "probiotic syrup", useful to restore the symbiotic communities of the intestine in case of dysbiosis and to exert a prophylactic action against *A. apis*. The results showed the capacity of selected *Al. kunkeei* strains to inhibit *A. apis* and highlighted their important properties, such as ability to form biofilms, high auto-aggregation, and hydrophobicity, all prerequisites for candidacy as probiotic microorganisms in a honeybee diet. In addition, the selected *Al. kunkeei* strains showed high osmotic tolerance, a functional requirement for the probiotication of sugar syrups to restore or strengthen the symbiotic communities in honeybee guts in case of microbial dysbiosis. On the basis of this knowledge, the experimental plan of the doctoral work was designed.

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1. CHAPTER 1

Probiotic properties and potentiality of *Lactiplantibacillus plantarum* strains for the biological control of chalkbrood disease (*J. Fungi*, 2021, *7*, 379).

1.1 INTRODUCTION

The fungus *Ascosphaera apis* (Maasen ex Claussen), belonging to the heterothallic Ascomycota phylum, is a major and widespread pathogen of honeybee (*Apis mellifera* L.) broods, causing chalkbrood disease and larval death (Evison & Jensen, 2018). This disease is economically important since it results in significant losses of both honeybees (under certain circumstances, it can kill colonies) and colony productivity (Evison, 2015) and indications suggest that its incidence may be increasing (Aronstein & Murray, 2010). Recent research demonstrated that *A. apis* infection, together with other biotic and abiotic factors, induces oxidative stress and impairs the antioxidant defensive capacity of honeybee larvae (Li *et al.*, 2020).

Pathogenesis occurs when larvae ingest sexual spores of *A. apis* with their food. Inside the gut, the spores find the necessary anaerobic environment for their germination and extend into hyphal growth (Bamford & Heath, 1989). The infected larvae rapidly reduce their food consumption and then stop eating. The persistence of ascospores, which remain viable for many years on all surfaces inside the hive, provides a continuous source of infection (Bailey & Ball, 1991). Honeybees have several defence mechanisms to resist chalkbrood disease, including hygienic behaviour (Spivak & Reuter, 1998). However, if the potentially sporulating chalkbrood mummies are removed, hygienic behaviour can increase rather than decrease transmission by exposing more individuals to the spores (Invernizzi *et al*., 2011). In addition, social insect species, such as *A. mellifera*, exhibit behaviours such as flower sharing to collect pollen and nectar, which might increase the transmission of persistent chalkbrood spores between colonies (Manley *et al*., 2015). Drifting workers and drones may also contribute to the spread of infection (Castagnino *et al*., 2020).

Chalkbrood disease depends on several interacting aspects, such as the environment, the biological characteristics of both the host and the fungus (which may influence fungal pathogenesis and the transmission of the disease) and possible co-infections. Outbreaks may be increased by the disruption of the beneficial microbial community within a colony (Engel & Moran, 2013). There is increasing knowledge on both the composition and the functions of the honeybee gut microbiota, which has led to the discovery of evidence of a link between balanced gut microbiota and honeybee health (Cox-Foster *et al*., 2007; Raymann & Moran, 2018). In particular, there is some evidence that *A. mellifera* gut microbiota may exhibit

antifungal activity against *A. apis* (Sabaté *et al.*, 2009; Omar *et al.*, 2014). A broad range of chemotherapeutic compounds, not permitted in Italy, have been tested to control chalkbrood disease over the years (Heath, 1982; Davis, 2003), but none have been able to control it properly. Furthermore, pesticides and antifungal chemicals have had serious impacts on the environment, honey quality and honeybee colonies themselves (Frazier *et al.*, 2008). Therefore, there is great interest in developing alternative chalkbrood controlling strategies.

In an interesting review, Gaggìa *et al*. (2018) provided an overview of beneficial microorganism applications for the treatment of the main honeybee pathogens and their benefits in beekeeping production systems. Some more recent research has confirmed that the use of lactic acid bacteria (LAB) as probiotics could prevent certain diseases and improve honeybee health (Al-Ghamdi *et al*., 2018; Ramos *et al*., 2020). In particular, Tejerina *et al*. (2021) recently demonstrated that the application of LAB (*Lactobacillus melliventris*, *Lactobacillus helsingborgensis* and *Lactobacillus kunkeei*) in sugar syrup over 5 months reduced larval mummification in chalkbrood disease by over 80%.

These data highlight that the administration of probiotic lactic bacteria in the honeybee diet can be a valid strategy for the biological control of chalkbrood disease. *Lp. plantarum*, formerly *L. plantarum* (Zheng *et al.*, 2020), is an important and ubiquitous LAB species characterized by extreme adaptability and genome plasticity. It has been isolated in many different environmental niches, such as fruit, vegetables, all types of fermented foods, meat and fish (Van Hoorde *et al*., 2008; Gotteland *et al*., 2014). *Lp. plantarum* strains have also been isolated from different honeybee species (Tajabadi *et al*., 2014; Iorizzo *et al*., 2020b). Several authors have demonstrated that *Lp. plantarum* colonizes the adult *Drosophila melanogaster*Meigen gut and that it influences different aspects of the insect's development and life, exerting a growth-promoting effect on larvae under nutrient scarcity (Corby-Harris *et al*., 2007; Storelli *et al*., 2011). Several authors have proved that *Lp. plantarum* has a broad capacity to inhibit the growth of different pathogens, and different strains exert inhibitory activity towards bacteria and fungi. In addition, chemically different compounds with antibacterial and antifungal activity have been characterized in culture filtrates (Niku-Paavola *et al*., 1999; Barbosa *et al*., 2016). *Lp. plantarum* also exhibits antagonist activity against *Paenibacillus larvae*, the causative agent of the quarantine disease American foulbrood, which affects *A. mellifera* larvae and pupae (Daisley *et al*., 2020; Iorizzo *et al*., 2020c; Ramos *et al*., 2020; Zheng *et al*., 2020). Over the years, several studies have obtained relevant data supporting the probiotic properties of *Lp. plantarum* (Seddik *et al*., 2017; Behera *et al*., 2018).

Suggested mechanisms by which probiotics may benefit the gut environment and the health of the host include improving intestinal barrier function through effects on the epithelium and mucus lining, producing antimicrobial substances, competing with pathogenic bacteria and antioxidative activity (Royan *et al*., 2019). The ability of microorganisms to colonize is often considered one of the main selection criteria for potential probiotics, as their colonization is important for their activity. In addition, both their longer permanence in the mucosa of the host and their action as a biological barrier reduce or prevent pathogen colonization (Vu *et al*., 2009; Deng *et al*., 2020). The ability of probiotic bacteria to adhere to intestinal epithelial cells involves extracellular polysaccharide (EPS) production and biofilm formation (Vu *et al*., 2009; Deng *et al*., 2020), and several *Lp. plantarum* strains are able to do both (Seddik *et al*., 2017; Behera *et al*., 2018).

In this research, the antagonistic activity of five *Lp. plantarum* strains, isolated from the honeybee gut and beebread toward *A. apis* was assessed. The abilities of these lactic bacteria to produce EPSs and biofilms, as well as their antioxidant activity, were also evaluated. The final goal of this study was to evaluate the use of these *Lp. plantarum* strains as probiotics in the honeybee diet, and their potential use for the biocontrol of chalkbrood disease.

1.2 Materials and Methods

1.2.1 Microbial cultures

In this study, 22 *Lp. plantarum* strains isolated from beebread, the midgut and the honey stomach of *A. mellifera* workers were used (Table S1). These bacteria belong to the Dept. AAA (Department of Agricultural, Environmental and Food Sciences) collection of the University of Molise (Iorizzo *et al*., 2020b). As reference, *A. apis* DSM 3116 and *A. apis* DSM 3117 cultures (DSMZ: German Collection of Microorganisms and Cell Cultures GmbH) were used.

1.2.2 Screening for antifungal activity

The antifungal activity of the *Lp. plantarum* strains was assessed using the overlay method described by Magnusson *et al.* (2003) with some modifications. The LAB strains were cultured in De Man, Rogosa and Sharpe (MRS) broth (Oxoid Ltd., Hampshire, UK) at 37°C for 12 h. Then, they were inoculated with a central single streak of 2 cm on MRS agar plates, which were then incubated at 37°C for 24 h under anaerobic conditions (GasPack anaerobic system, Sigma-Aldrich; St. Louis, MO, USA).

Fungal cultures from *A. apis* DSM 3116 and 3117 were cultured in Malt Extract Agar (MEA) medium (Oxoid Ltd., Hampshire, UK) under aerobic conditions at 28°C for 5 days. Then, a 6 mm-diameter mycelial disc was removed, dissolved in physiological solution (0.9% NaCl) and

vortexed for 5 min; 1 mL of the fungal suspension was then inoculated in a tube containing 10 mL of MEA soft agar (0.7% agar), which had been overlaid on the MRS agar plates previously inoculated with the LAB strains as described above. As a control, a plate containing MEA with the fungal suspension but without bacteria was used. After 72 h of incubation at 37°C, the inhibitory activity of the *Lp. plantarum* strains was measured as the diameter (mm) of the clear zone around the bacterial streaks (Testa *et al*., 2019). The tests were performed in triplicate.

1.2.3 Antifungal activity determination

Spore viability and germination test

Fungal cultures of *A. apis* DSM 3116 and 3117 were cultured in MEA medium at 28°C for 15 days in aerobiosis. A spore suspension was obtained by washing the ascospores that formed on the surfaces of plates with 5-10 mL of 0.01% sterile Tween-80. The suspension was collected in a sterile 100 mL Erlenmeyer flask and loosened by shaking with sterile glass beads for 2 h. The germination test was conducted according to the procedure described by Jensen *et al.* (2013) with some modifications. Briefly, sterile Teflon-coated slides (Tekdon, Myakka City, FL, USA) were placed in a sterile Petri dish lined with wet filter paper. Then, 100 µL of spore suspension (about 107 spores/mL) was mixed with 400 µL of GLEN medium (Jensen *et al.*, 2013) and 100 µL of LAB culture (grown in MRS broth at 37°C for 24 h), and 10 µL of this spore/GLEN/LAB (SGL) mixture was placed onto the Teflon-coated slides. A spore/GLEN (SG) mixture without LAB cultures was used as a control. To stimulate germination, the Petri dish was exposed for 10 min to 9–13% CO_2 (Heath & Gaze, 1987) using an AnaeroGen sachet in a 3.5 L jar (Oxoid; Basingstoke, UK), and after 32 h at 34°C in aerobiosis, we counted the spores directly on the Teflon slide. About 100 spores were counted in three different fields of view on the slide using a phase contrast microscope at 400 magnification (Axioplan, Zeiss; Göttingen, Germany). Spores were considered germinated when the length of a hypha was longer than the length of the diameter of the spore. All the chemical compounds were supplied by Sigma-Aldrich (St. Louis, MO, USA). The tests were conducted in triplicate.

Inhibition of radial mycelial growth

The inhibitory activity against the *A. apis* 3116 and *A. apis* 3117 strains was determined according to Iorizzo *et al.* (2020a) using the following matrices of LAB cultures: broth culture (BC), cell-free supernatant (CFS), cell pellet (CP) and cell lysate (CL).

To obtain the matrices, each *Lp. plantarum* strain was cultivated in MRS broth and incubated at 37°C for 12 h, reaching a cell concentration of 108 CFU/mL. This culture, without any treatment, was the BC matrix. Then, 5 mL of this bacterial culture was centrifuged at 8000 rpm

for 15 min at 4°C; the resulting supernatant was sterilized by filtration (0.22 µm-pore-size cellulose acetate filter) to obtain the CFS matrix. For the CP matrix, the remaining pellet was washed and resuspended in 5 mL of physiological solution. To obtain the CL matrix, 5 mL of bacterial culture (BC) was centrifuged, and the pellet was washed, resuspended in 5 mL of physiological solution and then subjected to three cycles of sonication (Labsonic M; Sartorius, Germany) at 12 W for 30 s, with a 60 s pause between the cycles to promote cellular lysis (Ricci *et al*., 2018).

For each matrix (BC, CP, CFS and CL), 5 mL was added to 15 mL of MEA; this preparation was then poured into 90 mm Petri dishes. After the solidification of the medium, a mycelial disc (6 mm in diameter) of each *A. apis* strain was placed in the middle of the Petri dish, which was then incubated at 37°C under aerobiotic conditions. The antifungal activity was evaluated by measuring the hyphal radial growth (diameter) after 8 days of incubation and expressed as the percentage of inhibition using the following formula: I % = [1 - (Ds/Dc)] x 100, where Ds is the hyphal diameter of the sample and Dc is the hyphal diameter of the control (MEA with fungus only). The experiments were performed in triplicate.

1.2.4 Biofilm production

Biofilm production was evaluated as described by Cozzolino *et al.* (2020) with some modifications. The *Lp. plantarum* strains were grown overnight at 37°C in MRS medium. The bacterial cells were harvested by centrifugation at 8000 rpm for 10 min at 4°C, washed twice with phosphate-buffered saline (PBS) solution (Sigma–Aldrich, St. Louis, MO, USA), resuspended at 106 CFU/mL in MRS broth without sugar and in MRS broth supplemented with 5%, 10% and 20% glucose, fructose or sucrose under aerobiotic and anaerobiotic conditions (GasPack anaerobic system, Sigma–Aldrich, St. Louis, MO, USA). Three 200 µL aliquots of each bacterial suspension were transferred to a 96-well polystyrene microtiter plate. Wells filled with uninoculated culture media were used as negative controls. The microtiter plates were incubated for 24 h at 37°C. The medium was then removed from each well, and the plates were washed three times with a sterile physiological solution to remove unattached cells. The remaining attached cells were fixed with 200 µL of 99% methanol (Sigma–Aldrich, St. Louis, MO, USA) per well. After 15 min, the methanol was removed, and the cells were left to dry. Then, 200 µL of 2% Crystal violet (Liofilchem; Roseto degli Abruzzi, Italy) was placed in the wells for 5 min. The excess stain was then removed by washing three times with sterile saline solution. After the plates were air-dried, the adherent cells were resuspended in 160 µL of 33% (*v/v*) glacial acetic acid (Sigma–Aldrich, St. Louis, MO, USA). The values of absorbance at 580 nm, measured using an automated

Multilabel counter (PerkinElmer 1420), represented the biofilm formation capacity. The experiments were performed in triplicate.

1.2.5 Exopolysaccharide (EPS) assay

Production and isolation of EPSs

Microbial EPSs are not permanently attached to the microbial cell surface and exist in two forms depending on their location: cell-bound EPSs, which closely adhere to the bacterial surface (bound exopolysaccharides; EPS-b), and EPSs that are released into the surrounding medium (released exopolysaccharides; EPS-r).

For each bacterium, 200 mL of MRS medium was inoculated with 1% (*v/v*) overnight precultures grown in the same medium. After incubation at 37°C for 48 h, the cultures (108 CFU/mL) were centrifuged at 15,000 g for 15 min at 4°C. The pellets were washed twice with sterile water and then centrifuged again at 15,000 g for 15 min at 4°C and subjected to EPS-r and EPS-b extractions. The screening and extractions of EPS-r and EPS-b were carried out as described by Tallon *et al.* (2003). The final fractions were dried to constant weights. As a control, MRS broth without bacterial inoculum was used. The tests were conducted in triplicate. All the chemical compounds were supplied by Sigma–Aldrich (St. Louis, MO, USA).

Antifungal activity of EPSs

The fractions of EPS-b and EPS-r, obtained from 20 mL of MRS medium, were rehydrated with 5 mL of physiological solution and added to 15 mL of MEA for antifungal activity tests against *A. apis* 3116 and *A. apis* 3117 using the same technique described in Section 2.3. The corresponding fractions of non-inoculated MRS medium were used as controls. The tests were conducted in triplicate.

1.2.6 Antioxidant activity

Bacterial culture matrices and Cell protein assay

Overnight cultures (106 CFU/mL) of the *Lp. plantarum* strains in LM medium (Table S2) were centrifuged at 13,000 rpm for 5 min at 4°C, and the obtained supernatants (CFSLM) were used directly for the antioxidant activity assay.

Cell pellets (CPs) were divided into two aliquots to determine their protein content and antioxidant activity. For total cell protein extraction, the CP was resuspended in 1 mL of Tris-buffered saline (TRIS) solution at pH 7.5; 20 mM containing ethylenediaminetetraacetic acid (EDTA) 5 mM and $MgCl_2$ 5 mM, and then subjected to three cycles of sonication at 12 W for 30 s, with a 60 s pause between the cycles, using a Labsonic M. The suspension was used for protein measurement according to Di Martino *et al.* (2020) using a BioSpectrometer (Eppendorf,

Hamburg, Germany). The total protein concentrations, expressed as µg/mL, were calculated by means of a calibration curve where bovine serum albumin (BSA) was used as a standard.

For antioxidant activity, the CP was washed twice with sterile water and resuspended in 200 µL of ethanol/water (40/60). The cell pellet suspensions were sonicated (12 W for 30 s, with a 60 s pause between the three cycles) and, after 12 h of storage at 20°C, centrifuged at 13,000 rpm for 15 min at 4°C. The supernatants (CES) were used for the evaluation of antioxidant activity. All the reagents used in this experiment were from Sigma–Aldrich (St. Louis, MO, USA). All the experiments were performed in triplicate.

Antioxidant activity assay

The total antioxidant activity (TAA) of the CFSLM and CES, obtained as described above, was evaluated using the 2,2 azino-*bis* 3-ethylbenzothiazoline-6-sulfonic acid (ABTS +) radical cation method according to Re *et al.* (1999), with some modifications. Briefly, ABTS was dissolved in water to a concentration of 7 mM. ABTS radical cations (ABTS +) were produced by reacting the ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 24 h before use. The ABTS + solution was diluted with citrate buffer (pH 4.0) to an optical density (OD) of 0.700 at 734 nm. Then, 100 µL of CFSLM and CES were mixed with 900 µL of the ABTS + solution. The OD was measured at 734 nm after 4 min in the dark at room temperature using a BioSpectrometer (Eppendorf, Hamburg, Germany). Ascorbic acid was used as the standard for the calibration curve. The antioxidant activity of CFSLM was expressed as µg ascorbic acid/mL; the antioxidant activity of CES was expressed as the ratio (*w/w*) between ascorbic acid (ng) and protein (µg; BSA equivalents). All the reagents used for this experiment were from Sigma–Aldrich (St. Louis, MO, USA). All the experiments were performed in triplicate.

1.2.7 Statistical analysis

All the data obtained from three independent experiments are expressed as mean standard deviation (SD). Statistical analysis was performed using an analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Statistical significance was attributed to p-values < 0.05. SPSS software (IBM SPSS Statistics 21) was used for the analysis. The heatmap of biofilm production was generated using ClustVis web tool (Metsalu & Vilo, 2015).

1.3 RESULTS

1.3.1 Antifungal activity

In a preliminary antifungal test, all 22 *Lp. plantarum* strains showed antifungal activity but with different intensities (Table S1).

The *Lp. plantarum* strains LP8, LP25, LP86, LP95 and LP100 caused inhibition zones more than 2 cm in diameter and were selected for subsequent analysis.

In the anti-germinative tests, no significant differences were observed between the control (SG) and the samples containing the cultures (SGL) of the *Lp. plantarum* strains (Table S3).

The results of the inhibition of the radial mycelial growth of the *A. apis* 3116 and *A. apis* 3117 strains by the various matrices of *Lp. plantarum* cultures are summarized in Figure *1*.

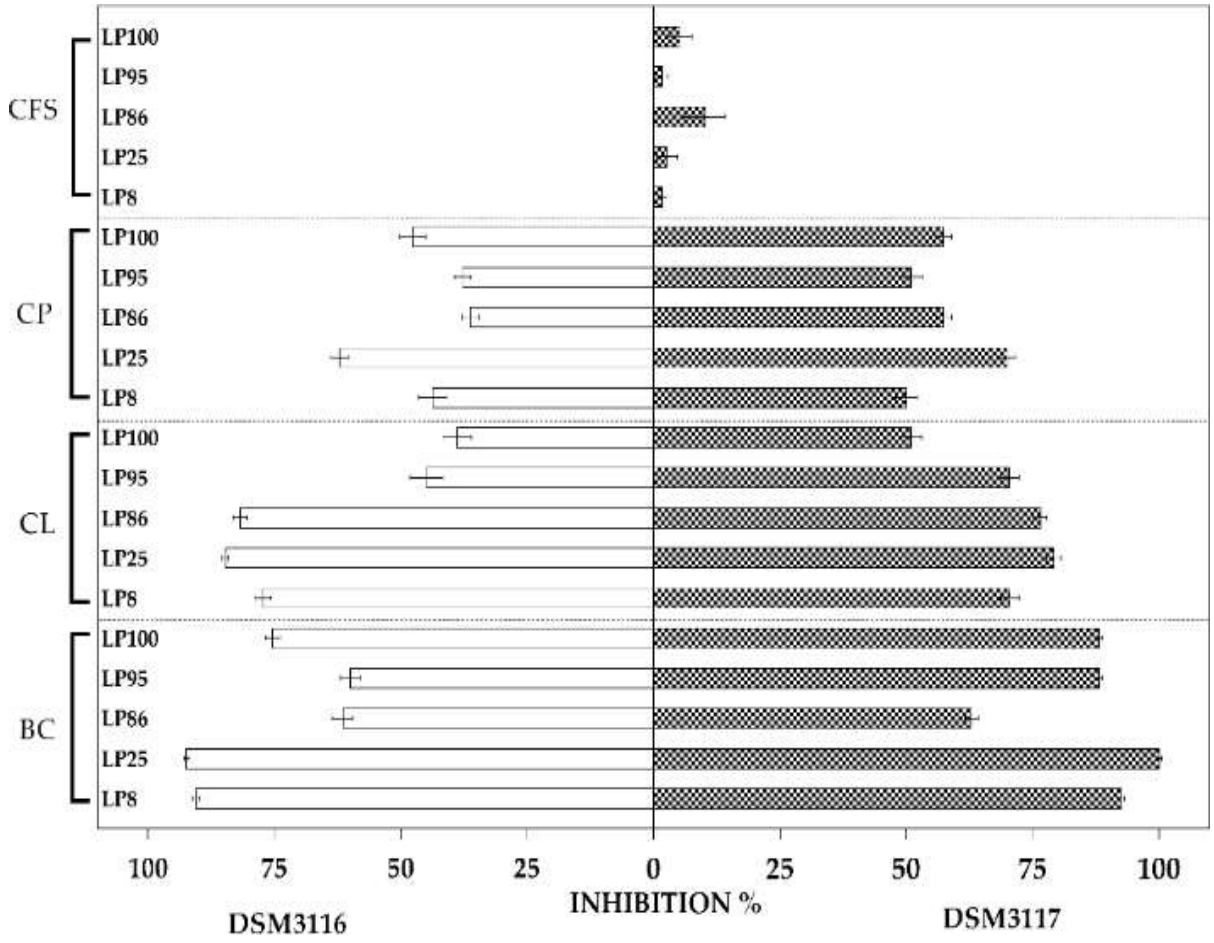


Figure 1. Inhibition (%) of *A. apis* DSM 3116 and *A. apis* DSM 3117 (radial growth) on Malt Extract Agar (MEA) plates after 8 days using culture broth (CB), cell pellet (CP), cell-free supernatant (CFS) and cell lysate (CL) from the *Lp. plantarum* strains.

The results of the various tests show that there were significant differences between the radial growth percentage values obtained using different matrices. The numerical data are reported in Table S4.

After 8 days, the *Lp. plantarum* broth cultures (BCs) caused greater inhibition of the two fungi than the other matrices did, with values between 60.0% (LP95) and 92.4% (LP25) against *A. apis* 3116, and 62.9% (LP86) and 100% (LP25) against *A. apis* 3117.

The cell lysates (CLs) inhibited the fungi more than the CP and CFS matrices. In particular, they caused inhibition rates between 38.8% (LP100) and 84.8% (LP25) for *A. apis* 3116, and between 50.9% (LP100) and 79.2% (LP25) for *A. apis* 3117. The cell pellets (CPs) showed inhibitory activity ranging from 36.3% (LP86) to 62.1% (LP25) against *A. apis* 3116, and from 50.0% (LP8) and 69.8% (LP25) against *A. apis* 3117. The cell-free supernatants (CFSs), overall, showed less inhibitory activity, which was found to be between 1.7% (LP8 and LP95) and 10.2% (LP86) for *A. apis* 3117, and they did not inhibit *A. apis* 3116. Figure *2* shows the inhibitory activity of *Lp. plantarum* LP25 against *A. apis* DSM 3117 after 8 days on MEA agar plates. EPS-b and EPS-r did not inhibit *A. apis* 3116 or *A. apis* 3117.

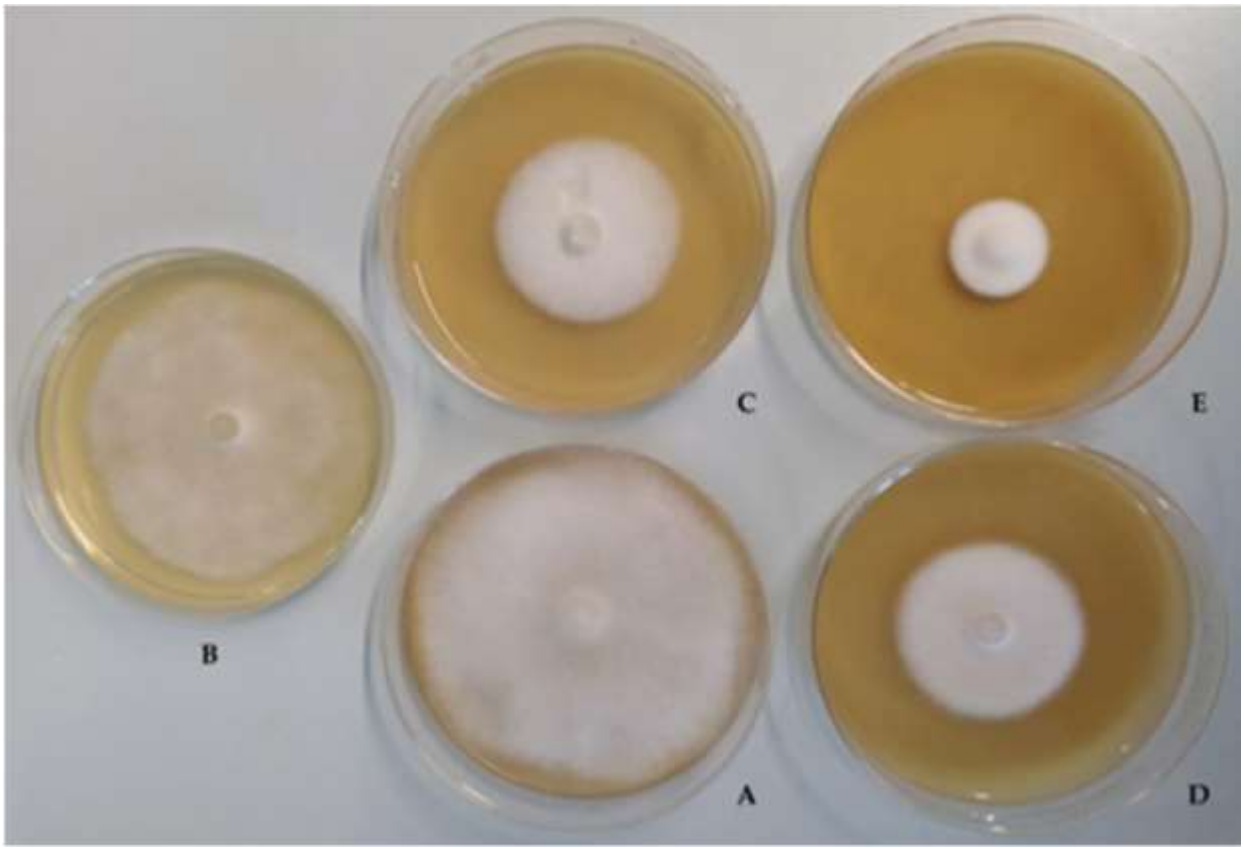


Figure 2. Inhibitory activity of *Lp. plantarum* LP100 against *A. apis* DSM 3116 after 8 days on MEA agar plates. A: *A. apis* (control); B: *A. apis* + CFS (cell-free supernatant); C: *A. apis* + CL (cell lysate); D: *A. apis* + CP (cell pellet); E: *A. apis* + BC (broth culture).

1.3.2 EPS and Biofilm production

The amounts of EPS produced by the five *Lp. plantarum* strains are reported in Table 1. The EPS-r values, expressed as mg/mL, were obtained for each bacterial culture (108 CFU/mL) in MRS broth. The EPS-b values are expressed as the ratio between EPS-b (µg) and cell protein (µg, BSA equivalents) obtained for each bacterial culture (108 CFU/mL) in MRS broth. The data show

statistically significant differences ($p < 0.05$). The EPS-r values were between 0.82 (LP95) and 1.56 mg/mL (LP25), while the EPS-b values were between 1.96 (LP95) and 8.82 mg/mL (LP8).

Table 1. EPS production in MRS medium after 48 h and antioxidant activity in LM medium of *Lp. plantarum* LP8, LP25, LP86, LP95 and LP100 strains. All values are expressed as mean ± standard deviation (n = 3). Different lowercase letters (a–c) in each row indicate significant differences ($p < 0.05$).

	***Lp. plantarum* strains**				
	LP8	**LP25**	**LP86**	**LP95**	**LP100**
CFS_{LM} antioxidant activity *	37.45 ± 0.40 ^{c}	36.88 ± 0.40 ^{c}	25.73 ± 0.81 ^{b}	22.30 ± 0.05 ^{a}	20.01 ± 0.81 ^{a}
CES * antioxidant activity **	0.17 ± 0.02 ^{b}	0.16 ± 0.01 ^{b}	0.12 ± 0.01 ^{a}	0.14 ± 0.01 ^{a}	0.11 ± 0.00 ^{a}
EPS-r	1.40 ± 0.03 ^{b}	1.56 ± 0.50 ^{c}	1.28 ± 0.10 ^{b}	0.82 ± 0.07 ^{a}	1.49 ± 0.06 ^{b}
EPS-b	8.82 ± 0.11 ^{d}	2.23 ± 0.09 ^{a}	5.05 ± 0.12 ^{b}	1.96 ± 0.08 ^{a}	5.54 ± 0.10 ^{c}

* CFS_{LM} antioxidant activity expressed as ascorbic acid (ng)/mL; ** CES antioxidant activity expressed as ratio of ascorbic acid (µg)/cell protein (µg BSA equivalents); EPS-r values expressed as mg/mL; EPS-b values expressed as the ratio of EPS-b (µg)/cell protein (µg BSA equivalents).

Figure *3* shows a heatmap in which the *Lp. plantarum* strains are clustered based on their different capacities to form biofilms in different media and environmental conditions. The biofilms were assessed by measuring the optical density (OD), and the numerical data are shown in Table S5. All the tested *Lp. plantarum* strains were able to produce biofilms in all the conditions, but to different degrees, depending on the concentration and type of the added sugar. *Lp. plantarum* LP8 produced, under all the conditions, greater amounts of biofilm than the other strains.

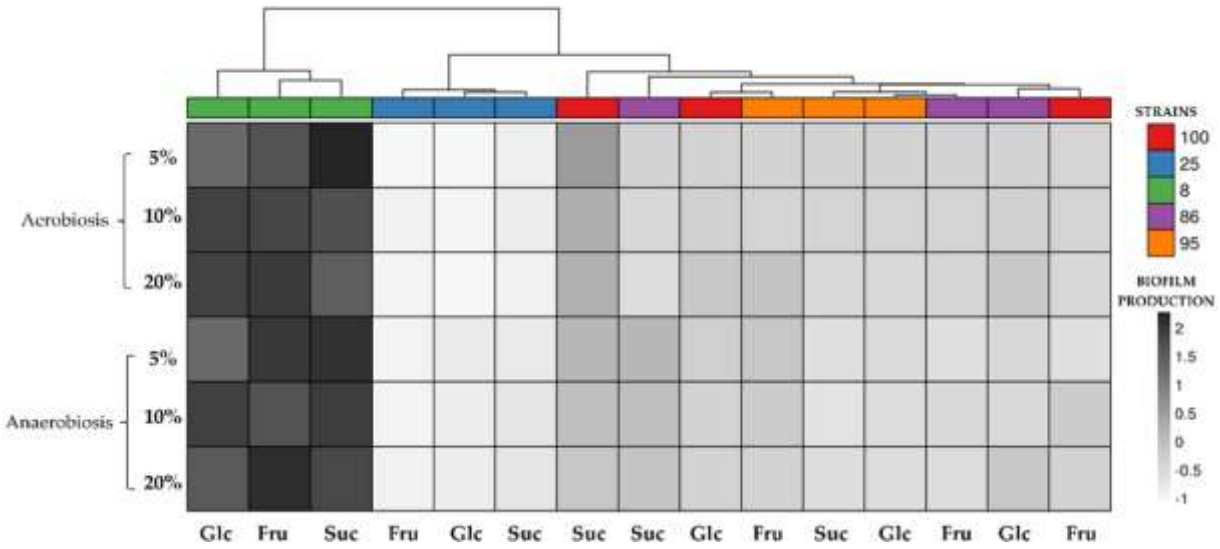


Figure 3. Biofilm production of *Lp. plantarum* LP8, LP25, LP86, LP95 and LP100 strains in aerobiotic and anaerobiotic conditions and with different sugar concentrations [5%, 10% and 20% (Glc: glucose; Fru: fructose; Suc: sucrose)]. This figure was generated using ClustVis web tool (Metsalu & Vilo, 2015) https://biit.cs.ut.ee/clustvis/ (accessed on 4 December 2020).

In all the tests, the anaerobiotic condition almost always favoured the production of biofilms. In particular, the *Lp. plantarum* LP8 strain always produced more biofilm than the other strains under this condition. The anaerobiotic condition favored biofilm production in

the tests with both glucose and sucrose for the LP25 strain, in the sucrose tests for the LP86 strain and in the fructose tests for the LP95 and LP100 strains.

All the bacterial strains tended to produce increasing amounts of biofilm as the sugar concentration increased, although there were often no significant differences ($p > 0.05$). Once again, the LP8 strain stood out because it produced increasing amounts of biofilm under the conditions of greater osmolarity of the sugar syrup; the differences for this strain were almost always significant ($p < 0.05$), except in the test conducted under aerobiosis with the addition of sucrose.

1.3.3 Antioxidant activity

The antioxidant activities of the CFS_{LM} and CES matrices are shown in Table 1. The *Lp. plantarum* strains produced different results for each of the two matrices. The antioxidant activity values of the CFS_{LM}, expressed as ng of ascorbic acid/mL, were between 20.01 (LP100) and 37.45 (LP8). Those of the CES, expressed as the ratio between ascorbic acid (µg) and cell protein (µg BSA equivalents), were between 0.11 (LP100) and 0.17 (LP8).

1.4 DISCUSSION

1.4.1 Antifungal activity

The purpose of this study was to investigate the ability of *Lp. plantarum* to inhibit two different *A. apis* strains, DSM 3116 and DSM 3117. The results suggest that sensitivity to the bacterial cultures may be species- and not strain-dependent. Future *in vivo* tests will be performed to verify the antifungal activity of *Lp. plantarum* against wild *A. apis* strains.

Results demonstrated that the *Lp. plantarum* strains did not affect the germination capacity of fungal spores, while these LAB exhibited the ability to inhibit the vegetative form of *A. apis* in vitro. The mycelial hyphae of this fungus, which are responsible for its virulent action, penetrate the peritrophic membrane and gut wall barrier to enter the honeybee hemocoel. The pressure caused by the septate hyphae and the enzymatic activity favor access to the interstitial space between the muscle fibers of infected larvae (Maxfield *et al*., 2015; Albo *et al*., 2017). The epithelial cells of the larval gastrointestinal tract are protected from pathogen colonization by several mechanisms exerted by commensal microbiota, including competition for adhesion sites or nutrient sources and producing antimicrobial substances (Landini *et al*., 2010, Crowley *et al*., 2013; Bäumler *et al*., 2016; Deng *et al*., 2020).

Many other researchers have shown that the antimicrobial activity of LAB is primarily attributed to the CFS, in which several antimicrobial compounds are found, including organic

acids (lactic, acetic, formic, propionic, butyric, hydroxylphenylactic and phenylactic acids) and other inhibitory substances (e.g., carbon dioxide, hydroperoxide, fatty acids and bacteriocins) (Bulgasem *et al*., 2016; Janashia *et al*., 2018).

The tests of *A. apis* inhibition demonstrated that all five *Lp. plantarum* strains had strong antifungal activity. High inhibition occurred with the use of the broth cultures (BC), which was most likely due to an interaction between several factors. In addition, the inhibitory effects obtained using the cell pellet (CP) and cell lysate (CL) were stronger than those obtained with the cell-free supernatant (CFS). Our results suggest that there may be synergy between various compounds, extra- and intracellular, that substantially increases the overall antifungal activity. This has also been hypothesized by other researchers (Re *et al*., 1999; Landini *et al*., 2010; Cortés-Zavaleta *et al*., 2014; Testa *et al*., 2019; Deng *et al*., 2020; Iorizzo *et al*., 2020a). Our tests showed that the EPS-b and EPS-r fractions did not inhibit *A. apis* 3116 and *A. apis* 3117. This suggests that the higher inhibitory effect of the CL compared to the CFS was probably due to the release of antifungal compounds from the bacterial cytoplasm after cell lysis.

The mechanisms behind the inhibition may involve some individual compounds that can cause membrane destabilization (such as fatty acids or peptides), proton gradient interference (such as organic acids or peptides) or enzyme inhibition (such as hydroxy acids). In addition, there may be some synergistic and/or additive effects involving various compounds (Siedler *et al*., 2019).

The antifungal compounds contained in the BC and CL matrices need to be investigated in future research, and after their identification and purification, we plan to use them in anti-germination tests on *A. apis* spores.

The antifungal properties of the *Lp. plantarum* strains shown *in vitro* do not axiomatically result in health benefits for honeybee colonies. It is therefore necessary to assess the role that these bacteria play in maintaining honeybee wellbeing and the contribution they can provide for the biological control of chalkbrood disease. In particular, we are testing the effects of sugar syrups enriched with lysates or live and active cultures of these *Lp. plantarum* strains, added to the diets of honeybee colonies *in vivo/in situ*.

1.4.2 EPS and Biofilm production

Results show that these five *Lp. plantarum* strains are able to produce EPSs and biofilms. As a result, these bacteria can persist in the intestine, where there is an abundant flow of sugars, enzymes and water and the constant invasion of foreign microbes following the

ingestion of flower nectar during foraging (Vásquez *et al*., 2012; Lee *et al*., 2016; Raymann & Moran, 2018). The germination of *A. apis* spores occurs in the midgut lumens of infected honeybee larvae. The hyphae penetrate the peritrophic membrane and gut epithelium, and then invade larval tissues (Aronstein & Murray, 2010). The inhibition of *A. apis* mycelial growth is an important key step for preventing the colonization of the intestinal cavity. Adhesion to the intestinal wall and the formation of biofilms by probiotic bacterial antagonists of pathogenic fungi could constitute an obstacle to the development and consequent invasive action of fungal mycelia.

The tests also confirmed that EPS and biofilm production are strain-dependent, as documented by other researchers (Lee *et al*., 2016). *Lp. plantarum* LP8 produced the largest quantities of EPS-b and biofilm, demonstrating that exopolysaccharides linked to the bacterial wall are important in the composition and architecture of biofilms (Flemming *et al*., 2007; Neu *et al*., 2010).

The formation of EPSs and the development of a biofilm are also affected by other factors, including surface properties and environmental parameters (Butler *et al*., 2013; Caggianiello *et al*., 2016). Our results show that anaerobiotic conditions and increased osmolarity often significantly favor biofilm production (Table S5). This suggests that the microaerophilic/anaerobic conditions of the intestinal tract can favor the production of biofilms and the resulting intestinal colonization by these bacteria.

In the future, it will be necessary to perform this test with cell lines to confirm the adhesion of the five selected *Lp. plantarum* strains to the epithelial cells.

1.4.3 Antioxidant activity

Oxidative stress is important in eukaryotic organisms and can cause severe negative effects. Reactive oxygen species (ROS) are the causative agents of oxidative stress, and they are produced during normal metabolic processes. Insects have a range of antioxidant enzymes, mainly composed of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD). Glutathione peroxidase (GPX) and glutathione reductase (GSR) can also remove ROS (Weirich *et al*., 2002; Collins *et al*., 2004).

Detoxification enzymes play a critical, crucial role in honeybees exposed to biotic and abiotic stressors through ecological interactions with their environments (nutritional and thermal stress, parasites, heavy metals and/or pesticides) (Balieira *et al*., 2018; Li *et al*., 2019). Oxidative stress has been reported to play an important pathological role in honeybee diseases. Even during the excessive proliferation of pathogens, the intestinal epithelium produces and

releases high levels of ROS, causing significant oxidative stress (Seehuus *et al*., 2006; Paris *et al*., 2017). Li *et al.* (2020) recently reported that *A. apis* infection induced oxidative stress in honeybee larvae, and decreased levels of the metabolites involved in combating oxidative stress could compromise the antioxidant defences of the infected larvae. The specific activities of antioxidant enzymes (CAT, GST and SOD) and the levels of metabolites (taurine, docosahexaenoic acid and L-carnitine) involved in combating oxidative stress were significantly decreased in the guts of infected honeybee larvae.

Increased attention has been paid over the last decade to the use of LAB as natural antioxidants. Some LAB strains have enzymatic and nonenzymatic antioxidant activity and promote the production of antioxidant enzymes, decreasing the risk of ROS accumulation during the ingestion of food, thereby reducing oxidative damage (Feng & Wang, 2020; Kullisaar *et al.*, 2002).

It was assessed antioxidant activity using the ABTS assay, which is considered one of the most sensitive techniques (Chanput *et al.*, 2016) and a valid method for determining the antioxidant activity of both hydrophilic and lipophilic extracts (Arnao *et al.*, 2001). All five *Lp. plantarum* strains showed antioxidant activity in the CFS_{LM} and CES matrices, and this suggests that their antioxidant activities may be due to different substances (e.g., intracellular antioxidant enzymes, nonenzymatic antioxidant components such as glutathione, cell surface proteins or polysaccharides, etc.), which need to be investigated in greater detail.

These bacteria, if used as probiotics in the diets of honeybees, could limit oxidative stress due to pathogenic *A. apis* fungi and other biotic and abiotic factors.

1.5 CONCLUSIONS

The *Lp. plantarum* strains used in our experiments have been shown to possess substances biologically active against *A. apis*. These results confirm the potentially antagonistic role of *Lp. plantarum* against pathogenic microorganisms that use the digestive channels of honeybees as the sites of infection (Iorizzo *et al.*, 2020c). Moreover, our findings indicate the ability of the *Lp. plantarum* LP8, LP25, LP86, LP95 and LP100 strains to produce EPSs and form biofilms, which are prerequisites for potential candidates to be used as probiotics in the honeybee diet. In addition, the antioxidant properties of the tested bacterial strains can help to increase the tolerance of these insects to endogenous and exogenous oxidative stress. The obtained results encourage the design of strategies to improve honeybee health through nutritional approaches or the modulation of the gut microbiota using beneficial microbes and open up a new horizon for fighting honeybee pathogens.

Future research activities will involve the investigation of the nature of the antifungal compounds and evaluate the effects of these *Lp. plantarum* strains on honeybee health and productivity, and their efficacy in chalkbrood disease biocontrol *in vivo/in situ*.

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2. CHAPTER 2

Antimicrobial activity from putative probiotic lactic acid bacteria for the biological control of American and European foulbrood diseases (*Vet. Sci.*, 2022, *9*, 236).

2.1 INTRODUCTION

Apis mellifera L. is an insect of great ecological importance on a worldwide level both for its role as a pollinator of crops, fruit, and wildflowers and for maintaining the biodiversity of numerous ecosystems. Moreover, as a pollinating insect, it holds a high economic value for hive products (Klein *et al*., 2007; EFSA Panel, 2012). Whilst global stocks of managed honeybee colonies have apparently been increasing, considerable losses of wild and domestic bees have been reported in many parts of the world (Moritz *et al*., 2010; Gray *et al*., 2016).

Biotic and abiotic factors that adversely impact bees' welfare and survival include changes in land use and management intensity, climate change, beekeeper's management practices, lack of forage (nectar and pollen), the use of pesticides in agriculture, parasites and pathogen infections (Morimoto *et al*., 2011; Steinhauer *et al*., 2018). The gut microbiome is crucial for the honey bee's growth and development, immune function and defence against pathogens. Furthermore, a well-balanced microbiota is important for sustaining the health and vigour of honey bees (Raymann & Moran, 2018). The gut microbiota can be influenced by the aforementioned factors and its imbalance (dysbiosis) can lead to a weakening of honey bees and contribute to Colony Collapse Disorder (CCD) (Anderson *et al*., 2017; Raymann & Moran, 2018).

The digestive tract of honey bees is the site of infections triggered by pathogenic agents of brood disease, such as *Paenibacillus larvae* and *Melissococcus plutonius*, which can cause considerable losses in beekeeping and agriculture as they adversely influence the survival of both managed and wild honey bees (Evison & Jensen, 2018). *M. plutonius* (Order: Lactobacillales; Family: Enterococcaceae), a Gram-positive bacterium (Bailey *et al*., 1982; Trüper *et al*., 1998) resulting in the outbreak of European foulbrood (EFB), a globally damaging brood disease occurring in *A. mellifera* (Ellis *et al*., 2005; Forsgren *et al*., 2010).

P. larvae (Order: Bacillales; Family: Paenibacillaceae), a flagellated, spore-forming Gram-positive bacterium, is the causative agent of quarantine disease American foulbrood (AFB), the most severe and globally distributed brood disease affecting *A. mellifera* in the larval and pupal stages (Genersch *et al*., 2006; Ebeling *et al*., 2016). Heavy brood losses, colony collapse and extreme contagiousness render EFB and in particular AFB economically important and notifiable diseases in many world regions (Genersch, 2010).

To date, to prevent the further spread of the two pathogens, the destruction of symptomatic colonies and the surveillance of nearby apiaries are the only means to address them (Genersch *et al*., 2010; Alippi *et al*., 2014). However, the beekeeping practice-based management of EFB is considered since this is a conditioned disease, e.g., feeding, adding healthy honey bees, and artificial replacement of the queen (Amiri *et al*., 2017). Since it is only effective against the vegetative form of the above-mentioned pathogens, the use of antibiotics for controlling these diseases is an unsustainable strategy. Moreover, long-lasting treatments and their common use in prophylaxis, as well as failing to eliminate bacterial spores, may cause or accelerate mechanisms of antibiotic resistance (Alippi *et al*., 2014; Miyagi *et al*., 2000). Additionally, antibiotics may leave residues in hive products (Lopez *et al*., 2008; Bargańska *et al*., 2011) and also cause honey bee gut community dysbiosis (Mutinelli *et al*., 2003; Jia *et al*., 2022). On this matter, a growing number of research studies have revealed the relationship between disease states and variation in the native microbiome of honey bees, suggesting that the integrity of the microbiome correlates with disease susceptibility and, on a more general level, with the health status of honey bees (Lopez *et al*., 2008; Hamdi *et al*., 2011; Raymann & Moran, 2018; Sopko *et al*., 2020). For all these reasons, the use of antibiotics is now tightly regulated and not recommended in most European countries (Lopez *et al*., 2008; Bargańska *et al*., 2011; Floyd *et al*., 2020). Further measures to control AFB disease include enzymes (e.g., glucose oxidase) (Sagona *et al*., 2015); heterocyclic organic compounds, such as indoles, to inhibit the germination of *P. larvae* spores (Alvarado *et al*., 2017); bacteriophages (Tsourkas, 2020); and selective breeding for hygienic behaviour (Spivak & Reuter, 2001). However, these methods are often inefficient or not applicable for prophylactic purposes, leaving honey bee colonies susceptible to AFB (Grady *et al*., 2016; Alonso-Salces *et al*., 2017). The adoption of biological alternatives for disease control could represent a more viable management strategy. Herbal and propolis extracts, as well as spices and essential oils, showed antimicrobial action towards *P. larvae* and *M. plutonius* (Alonso-Salces *et al*., 2017; Kim *et al*., 2018). Nevertheless, their efficacy is often limited by the resilient nature of *P. larvae* spores that can remain infectious for more than 35 years (Genersch, 2010a;). Recently, research has focused on the intestinal microbial composition of bees as a possible natural alternative for the control of different diseases (Evans *et al*., 2006; Anderson *et al*., 2011). The structure of the gut bacterial community can be used as an indicator of honey bee health, and maintaining its balance is the key to supporting the health and well-being of honey bees (Hamdi *et al*., 2011; Vásquez *et al*., 2012; Forfert *et al*., 2015, Raymann & Moran, 2018). Several studies have shown that the dysbiosis of the gut microbial community can

promote the incidence of EFB and AFB diseases (Erban *et al*., 2017; Floyd *et al*., 2020). Other investigations have reported that the use of probiotics as a supplement in the honey bee diet could improve the health status of bees (Alberoni *et al*., 2016, 2018; Gaggìa *et al*., 2018), counteract bee pathogens and parasites, or enhance bee immunity (Evans *et al*., 2006; Anderson *et al*., 2011; Zheng *et al*., 2018).

Lactic acid bacteria (LAB) are common inhabitants of the gastrointestinal tract (GIT) of numerous insects and their presence in the honey bee intestinal tract has been consistently reported in the literature (Vásquez *et al*., 2012; Anderson *et al*., 2013; Iorizzo *et al*., 2020b). The importance of LABs is also underscored by their ecological distribution, which is not limited to the gut of the adult bee. They have been isolated from the gut of larvae (Gaggìa *et al*., 2015) and the honey stomach of adult bees (Olofsson *et al*., 2008), which is a further relevant microbial niche associated with food storage and liquid transfer (water, nectar, and royal jelly), adjacent to the midgut. Moreover, LABs are also dominant in the hive environment (Cilia *et al*., 2020). Given this background, there is an urgent demand for new approaches to developing an innovative and safe management strategy for the biocontrol of such serious diseases. The use of a beneficial bacteria-based procedure for the prevention and biocontrol of pathogenic microorganisms in honey bees offers interesting prospects (Alberoni *et al*., 2016; Iorizzo *et al*., 2020b). It is well established that LAB exerts an antagonistic effect against various bee pathogens through the production of antibacterial metabolites, e.g., organic acids and bacteriocins (Gaggìa *et al*., 2018; Iorizzo *et al*., 2020a, b). Recently, this activity has been assessed against *P. larvae* and *M. plutonius* (Vásquez *et al*., 2012; Killer *et al*., 2014; Daisley *et al*., 2020), the causal agents of the AFB and EFB diseases, respectively. Among LABs associated with bee gut microbiota, *Lactiplantibacillus plantarum* (previously *Lactobacillus plantarum*) and *Apilactobacillus kunkeei* (formerly classified as *Lactobacillus kunkeei*) are two of the most abundant species (Iorizzo *et al*., 2020b). *Al. kunkeei* is a bacterium that colonizes niches rich in fructose and is therefore classified as fructophilic. Fructophilic lactic acid bacteria (FLAB) are a very recently defined group of LABs (Endo *et al*., 2018). Being fructose, their optimal substrate, they can be found in fermented foods, flowers, and fruits. They are also present in the intestine of insects whose diets are rich in fructose (Endo *et al*., 2013). *Lp. plantarum* is an important and ubiquitous LAB species characterized by extreme versatility (Siezen *et al*., 2010). This bacterium is normally detected in a wide variety of niches, including fermented foods, plants, and the gastrointestinal tract (GIT) of mammals, fish, and insects, including honey bees (Iorizzo *et al*., 2016; Garcia-Gonzalez *et al*., 2021). Various authors have proven that *Lp. plantarum* and *Al. kunkeei* have antagonistic activity against several pathogens, including *P.*

larvae and *M. plutonius* (Vasquez *et al*., 2012; Lamei *et al*., 2019; Iorizzo *et al*., 2020c; Bielik *et al*., 2021).

Several studies have highlighted that the use of generic probiotics may not be beneficial to bees, but rather cause alteration of the symbiotic microflora and depress the immune system of honey bees (Andrearczyk *et al*., 2014; Ptaszyńska *et al*., 2016). Therefore, the isolation of bacteria that normally colonize the gut of honey bees is highly recommended for the selection of suitable strains that are functional to enhance the welfare of honey bees and increase their resistance to pathogens.

Based on these considerations, *in vitro* inhibitory activities of four *Lp. plantarum* and four *Al. kunkeei* strains isolated from the gut of the honey bee (*A. mellifera*) against *P. larvae* and *M. plutonius* were evaluated. Furthermore, several functional and biochemical characteristics of these LABs were investigated for feasible application as probiotics in bee diets.

2.2 Materials and Methods

2.2.1 Bee Sample Processing

Worker bees (*A. mellifera* subsp. *ligustica*) were sampled in spring 2020 at an apiary located in the Molise region, in south-eastern Italy, housed in queen bee cages and immediately transported to the laboratory. The apiaries were owned by beekeepers belonging to a beekeeping association (CONAPROA, National Consortium of Beekeeping Producers, Campobasso, Italy).

To obtain the social stomach, the midgut, and the ileum from the honey bee samples, these were killed by removing the head; the removal of the head is necessary not only to facilitate the operator in the dissection, but also to avoid contamination of the samples by allochthonous bacteria (of environmental origin).

The insects were then put in a glass Petri dish containing sterile saline (NaCl 0.9%) and dissected at room temperature using a pair of stainless-steel microdissection scissors and tweezers with fine tips (both washed in alcohol and flame-sterilized). To obtain the social stomach, the distal part of the oesophagus and near the proventriculus were cut; the midgut was obtained by cutting at the level of the pyloric valve; and the ileum was removed by performing incisions in the initial part of the rectum (Iorizzo *et al*., 2020b). Anatomical specimens from five worker bees were placed in tubes containing sterile physiological solutions in order to isolate bacteria.

2.2.2 Bacterial isolation

Bacterial colonies were isolated from MRS (Oxoid, Milan, Italy) and modified MRS (2% fructose) agar plates incubated at 35°C under anaerobic conditions. After 48-72 h, for each of the two-culture media, 10% of the total microbial colonies were picked at random and purified by successive streaking onto MRS agar plates. Gram-positive and catalase-negative strains were selected as presumptive LABs and subsequently characterized.

2.2.3 Genotypic analysis

Genomic DNA extraction from pure bacterial cultures and PCR amplification was accomplished as described by Iorizzo *et al*. (2021). Molecular identification of the bacteria was carried out based on 16S rRNA nucleotide sequencing. The 16S rRNA gene region of the genomic DNA was amplified using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′). Sequencing of purified amplified products was analysed using the Basic Local Alignment Search Tool (Zhang *et al*., 2000) in the NCBI (National Center for Biotechnology Information) database (NCBI, 2018). The phylogenetic analysis of the 65 16S rRNA sequences was performed with MEGA X software (Kumar *et al*., 2018), using *Al. kunkeei* ATCC 700308 and *Lp. plantarum* ATCC 14917 as reference strains.

2.2.4 Screening of antimicrobial activity

A preliminary screening of the antimicrobial activity of 65 LABs against *P. larvae* ATCC 9545 and *M. plutonius* ATCC 35311 was carried out by spotting 10 µL overnight LAB cultures (10^8 CFU/mL) onto the surface of MRS (Oxoid Ltd., Hampshire, UK) agar plates, which were then anaerobically incubated at 35°C for 24 h to allow colonies development. The pathogens were cultured in 10 mL of *Brain Heart Infusion* (BHI-Oxoid Ltd., Hampshire, UK) at 35°C for 16 h. Subsequently, 100 µL of overnight culture (10^7 CFU/mL) were inoculated into 7 mL of BHI soft agar (0.7% agar), maintained at 45°C and poured over the MRS plates. After incubation at 35°C for 48 h, the diameter of the clear zone around the LAB colonies, resulting from a lack of pathogen growth, was measured with a calibre in millimetres and expressed as the zone of inhibition (ZOI). The experiment was conducted in triplicate and activity was reported as the diameter of the ZOI ± SD. The LAB strains that showed the greatest antagonistic activity against the two pathogens (ZOI > 4 mm) were used for subsequent tests.

2.2.5 Antimicrobial activity test

LAB strains (*Lactiplantibacillus plantarum:* LP 31, LP 42, LP 148 and LP 179; *Apilactobacillus kunkeei:* ALK 181, ALK 222, ALK 268 and ALK 385) were grown in MRS broth for 16 h at 35°C, reaching a cell density of 10^8 CFU/mL. The bacterial cultures were centrifugated (8,000 rpm for 15 min at 4°C) to separate the cell pellet from the cell-free supernatant (CFS). The supernatant (CFS) was sterilized by filtration (cellulose acetate membrane, pore size 0.22 µm, Sigma-Aldrich; St. Louis, MO, USA) and used for subsequent tests. The antimicrobial activity was tested following Iorizzo *et al.* (2002a) protocol and its intensity has been expressed as ZOI (Testa *et al*., 2019). The tests were conducted in triplicate.

2.2.6 Biochemical characterization

LABs possessing antimicrobial activity against *P. larvae* and *M. plutonius* were screened for their carbohydrate fermentation and enzymatic patterns, using the API 50CHL and the API ZYM system kit (bioMérieux SA, Marcy l'Etoile, France), respectively.

Prior to API ZYM use, the LABs were grown in MRS broth at 35°C and after 18 h, the cultures were centrifuged, washed and resuspended in sterile saline. The bacterial suspension (BS) was adjusted to a 6 on the McFarland turbidity scale and used for the subsequent phases according to the manufacturer's instructions. Positive evidence of 19 enzymatic activities was found as a result of suspension chromatic change.

For the application of the API 50CHL, the BS was adjusted to 2 on the McFarland scale and used for the subsequent analysis. The ability of 49 different carbohydrates to be assimilated caused the pH to decrease and generated a colorimetric change in the medium, the composition of which was determined.

2.2.7 Cell surface properties

Bacterial cultures

The LAB strains were grown in MRS broth at 35°C. After 12 h, the cultures were centrifugated (8,000 rpm for 15 min at 4°C). Subsequently, the bacterial cells were washed three times with physiological solution (NaCl 0.9%) and resuspended in the same solution to an optical density of 0.5 on the MacFarland scale (OD_{580}), to standardize the bacterial density at 10^8 CFU/mL. The OD_{580} of the BS was measured using a spectrophotometer (Multilabel Counter—PerkinElmer 1420, San Jose, CA, USA). The subsequent tests were conducted in triplicate and the measurements were carried out in duplicate.

Auto-aggregation

The evaluation of the auto-aggregation (AA) capacity was performed according to Cozzolino *et al*. (2020). Briefly, the BSs were incubated at 35°C and their OD_{580} was measured using a spectrophotometer (PerkinElmer 1420 Multilabel Counter) after 1, 2, 5 and 24 h. The percentage of AA (AA%) was calculated using the following formula: $AA\% = [1 - (OD_t/OD_0)] \times 100$, where OD_0 is the absorbance at time 0 and OD_t is the absorbance detected after 1, 2, 5 and 24 h.

Hydrophobicity

The determination of cell surface hydrophobicity was evaluated on LAB strains based on the bacterial ability to adhere to hydrocarbons (BATH), as described by Iorizzo *et al.* (2020a), using xylene and toluene. Every single organic solvent was added (1:1 *v/v*) to the BSs and mixed (vortex-type mixer) for 5 min. After 15, 30, and 60 min of incubation at room temperature, the aqueous phase was carefully removed and the values of absorbance were detected at 580 nm using a spectrophotometer (PerkinElmer 1420 Multilabel Counter). Hydrophobicity was expressed as the percentage decrease in the optical density using the following formula: $H\% = [1 - (OD_0/OD_t)] \times 100$, where OD_t represents the absorbance value after the addition of xylene or toluene (15, 30 and 60 min), while OD_0 represents the absorbance value before the addition of the hydrocarbons.

Statistical analysis

All the data obtained from the three independent experiments are expressed as mean ± standard deviation (SD). Statistical analysis was performed using an analysis of variance (ANOVA). The obtained data, normally distributed, were analysed using Tukey post hoc tests with ANOVA. Statistical significance was attributed to p-values < 0.05. The software SPSS (IBM SPSS Statistics 21) was used for the analysis.

2.3 RESULTS

2.3.1 Lactic Acid Bacteria species diversity

In total, 65 Gram-positive and catalase-negative bacterial strains were assumed to be LABs. The partial 16S rRNA gene sequences of these bacteria were determined and compared with related bacteria in GenBank (http://www.ncbi.nlm.nih.gov/BLAST/, accessed on 2 January 2022). Sequence matches that showed the highest identity scores (99% and above) were considered acceptable for taxonomic placement at the species level. According to the 16S rRNA gene sequences, all isolated strains were used to construct a phylogenetic tree (Figure 1) using the MEGA X program (Kumar *et al.* 2018) via the maximum likelihood method and Kimura 2-parameter model (Kimura *et al.* 1980).

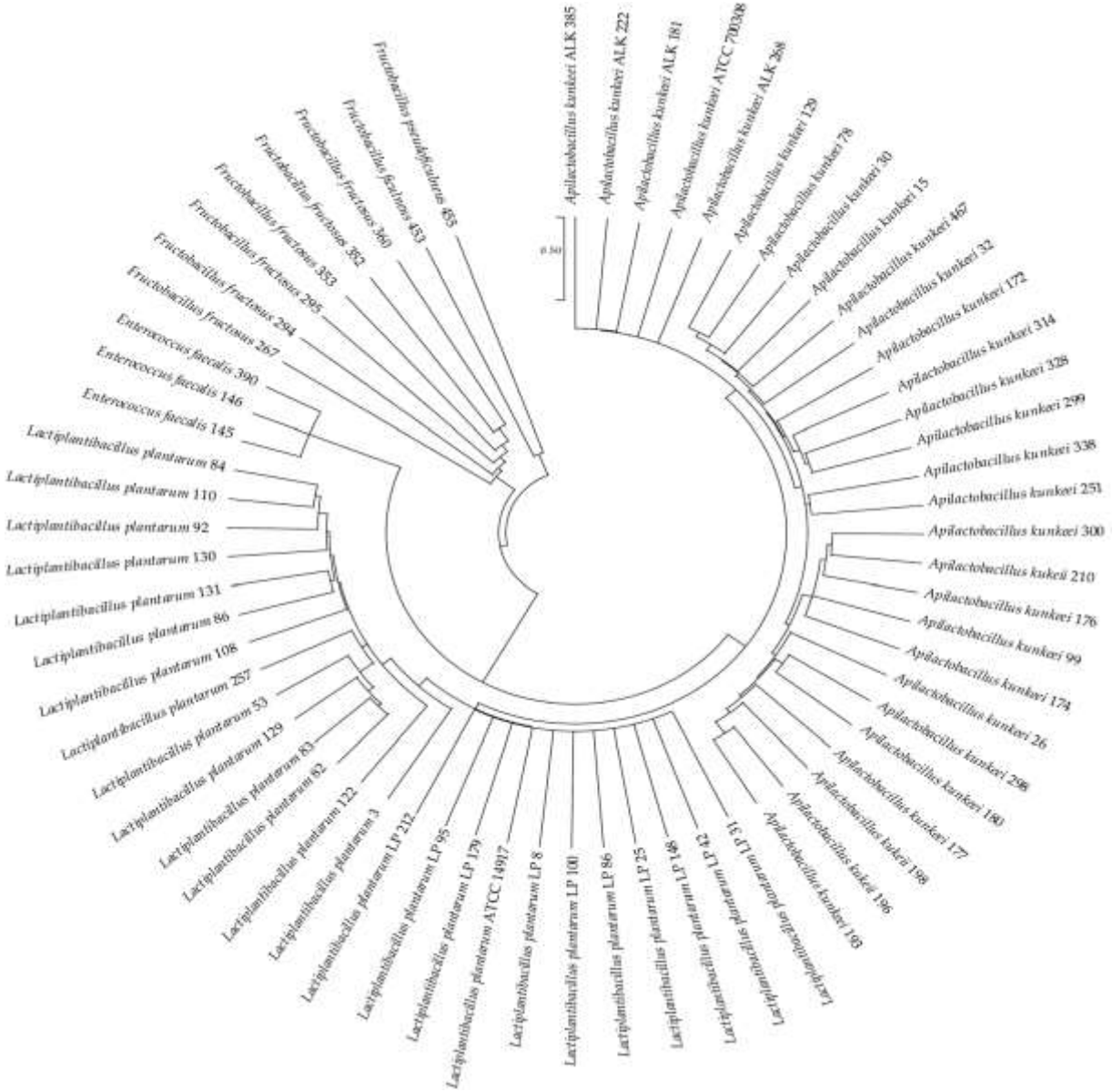


Figure 1. Phylogenetic analysis of the 16S rRNA gene sequences of the sixty-five LAB isolates. The type strains *Lactiplantibacillus plantarum* ATCC 14917 and *Apilactobacillus kunkeei* ATCC 700308 have been used as references. The analysis was conducted with the MEGA X (Kumar *et al.*, 2018) program using the maximum likelihood method and the Kimura 2-parameter model (Kimura *et al.*, 1980). The scale bar represents a 0.5% nucleotide sequence difference.

The phylogenetic analysis showed that the bacterial strains considered in this study belonged to 4 genera: *Apilactobacillus*, *Lactiplantibacillus*, *Fructobacillus* and *Enterococcus*. The predominant species were *Al. kunkeei* (28 strains) and *Lp. plantarum* (24 strains), followed by *Fructobacillus fructosus* (7 strains), *Enterococcus faecalis* (3 strains), *Fructobacillus ficulneus* (1 strain) and *Fructobacillus pseudoficulneus* (1 strain).

2.3.2 Antimicrobial activity

The antagonistic activity of 65 LABs against *P. larvae* ATCC 9545 and *M. plutonius* ATCC 35311 was investigated. Four *Lp. plantarum* (LP 31, LP 42, LP 148 and LP 179) and four *Al. kunkeei* (ALK 181, ALK 222, ALK 268 and ALK 385) strains demonstrated the greatest antimicrobial activity against the two pathogens (ZOI > 4 mm) and were selected for a subsequent agar well diffusion test using the CFS of the LAB cultures.

CFS matrices, obtained from the *Lp. plantarum* and *Al. kunkeei* strains, caused the growth inhibition of both pathogens. The numeric data of the antimicrobial activity (mm ZOI) are reported in Table 1 and highlight significant strain-specific differences, not species-related differences.

Table 1. Antimicrobial activity by cell-free supernatant (CFS) of the tested *Lactiplantibacillus plantarum* (LP) and *Apilactobacillus kunkeei* (ALK) strains against *Paenibacillus larvae* and *Melissococcus plutonius*. The data (mean ± SD; $n = 3$) were expressed as zone of inhibition-ZOI (mm). Different lowercase letters (a–d) in each row indicate significant differences ($p < 0.05$).

Matrices (CSF)	LP31	LP42	LP148	LP179	ALK181	ALK222	ALK268	ALK385
P. larvae	14.9 ± 0.2 ^{b}	5.1 ± 0.5 ^{b}	15.8 ± 0.3 ^{a}	13.9 ± 0.4 ^{c}	15.5 ± 0.5 ^{a}	16.3 ± 0.2 ^{a}	15.0 ± 0.4 ^{b}	13.7 ± 0.2 ^{c}
M. plutonius	15.1 ± 0.3 ^{a}	5.1 ± 0.3 ^{a}	16.0 ± 0.1 ^{a}	12.0 ± 0.2 ^{d}	15.9 ± 0.4 ^{a}	14.0 ± 0.3 ^{b}	14.2 ± 0.3 ^{b}	13.0 ± 0.5 ^{c}

The ZOI values of the CFSs against *P. larvae* ranged from 13.7 mm to 16.3 mm. The lowest inhibition values were recorded for strains LP 179 and ALK 385 while the highest value was recorded for the CFS of the strain ALK 222. In the tests against *M. plutonius*, ZOI ranged from a minimum of 12.0 mm (LP 179) to a maximum of 16.0 mm (LP 148).

2.3.3 Hydrophobicity and auto-aggregation

The results obtained in the BATH assay are graphically reported in Figure 2 and numerically in Table 2. Adhesion to the two hydrocarbons gradually increased during the duration of the test. After 60 min, *Lp. plantarum* LP 42, LP 179 and *Al. kunkeei* ALK 181 showed an adhesion to xylene and toluene greater than 90%. *Al. kunkeei* ALK 268 exhibited particular behaviour, as it already showed the highest adhesion to xylene (> 96.62%) and the lowest adhesion to toluene (47.34%) after 15 min. The overall data showed similar behaviour among the two tested bacterial species.

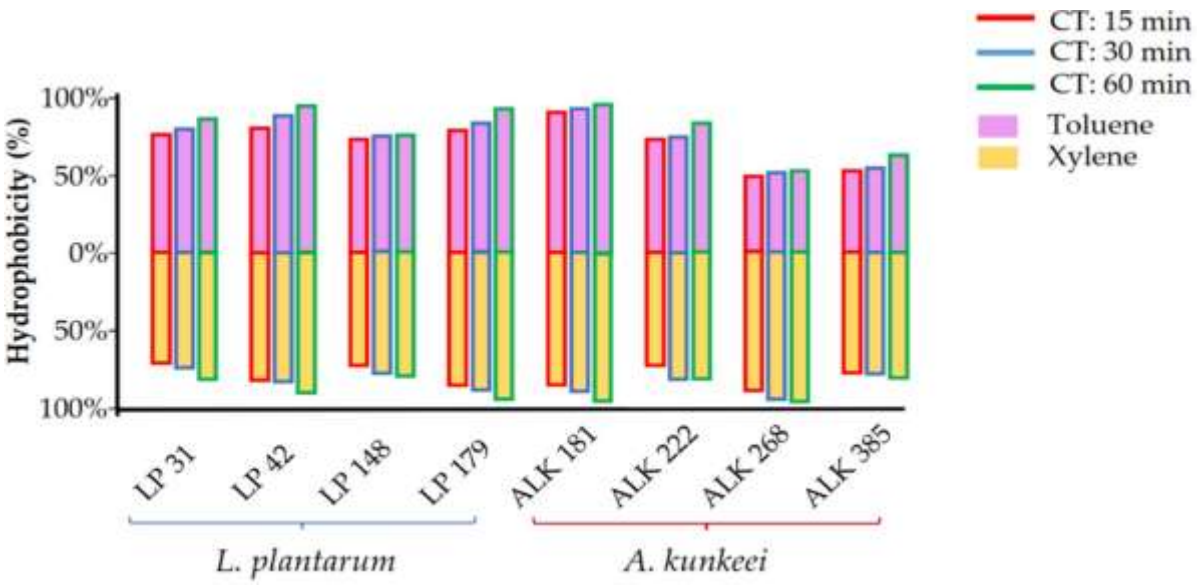


Figure 2. Adhesion of the *Lactiplantibacillus plantarum* and *Apilactobacillus kunkeei* strains to toluene and xylene expressed as hydrophobicity (%) after different contact times (CT; 15, 30, and 60 min).

In Figure 3, the heat map depicts the results of the auto-aggregation tests. The numeric data are reported in Table 3. The eight LAB strains showed progressive aggregation over time with often significant differences among them. The auto-aggregation values after 2 h had a similar trend for the two LAB species. After 5 and 24 h, significant differences between strains were observed. After 1, 2, and 5 h, ALK 181 strain had the highest percentage of auto-aggregation (19.0%, 24.40% and 36.1%, respectively). After 24 h, all strains showed aggregation values above 50%. The best performing strain was LP 31 (68.8%), while the lowest value was recorded for strain ALK 222 (51.1%).

Table 2. Adhesion to toluene and xylene of the *Lactiplantibacillus plantarum* and *Apilactobacillus kunkeei* tested strains (LP 31, LP 42, LP 148, LP 179, ALK 181, ALK 222, ALK 268 and ALK 385) after different contact times. The data (mean ± SD; $n = 3$) were expressed as percentage of hydrophobicity, different lowercase letters in each row and uppercase letters in each column indicate significant differences ($p < 0.05$).

					Hydrophobicity (%) Xylene			
Time	**LP31**	**LP42**	**LP148**	**LP179**	**ALK181**	**ALK222**	**ALK268**	**ALK385**
15	70.8 ± 1.3 ^{Cc}	80.5 ± 2.6 ^{Bb}	72.2 ± 1.8 ^{Bc}	85.3 ± 1.1 ^{Bb}	84.0 ± 1.7 ^{Bb}	72.3 ± 2.7 ^{Bc}	95.4 ± 1.5 ^{Aa}	76.1 ± 2.6 ^{Ac}
30	76.0 ± 2.2 ^{Bc}	81.4 ± 1.9 ^{Bc}	77.9 ± 1.7 ^{Ac}	87.8 ± 2.9 ^{Ab}	88.6 ± 1.8 ^{Ab}	79.6 ± 2.6 ^{Ac}	97.3 ± 1.5 ^{Aa}	76.2 ± 1.0 ^{Ac}
60	82.8 ± 3.9 ^{Ac}	90.9 ± 2.3 ^{Ab}	78.7 ± 1.2 ^{Ac}	91.3 ± 1.4 ^{Ab}	91.9 ± 1.9 ^{Ab}	81.9 ± 3.3 ^{Ac}	98.0 ± 0.5 ^{Aa}	80.1 ± 2.3 ^{Ac}
					Hydrophobicity (%) Toluene			
Time	**LP31**	**LP42**	**LP148**	**LP179**	**ALK181**	**ALK222**	**ALK268**	**ALK385**
15	.7 ± 2.0 ^{Cc}	77.9 ± 0.5 ^{Cb}	72.2 ± 1.7 ^{Ac}	79.1 ± 0.8 ^{Cb}	92.1 ± 1.6 ^{Aa}	72.8 ± 0.4 ^{Cc}	47.7 ± 1.0 ^{Ae}	53.1 ± 0.4 ^{Bd}
30	.4 ± 2.5 ^{Bd}	88.4 ± 0.4 ^{Bb}	73.6 ± 1.2 ^{Ae}	84.0 ± 0.5 ^{Bc}	92.8 ± 0.3 ^{Aa}	75.5 ± 1.2 ^{Bd}	47.8 ± 1.9 ^{Ag}	54.9 ± 2.3 ^{Bf}
60	.5 ± 2.4 ^{Ab}	94.1 ± 0.9 ^{Aa}	74.4 ± 0.4 ^{Ac}	93.5 ± 0.7 ^{Aa}	93.2 ± 0.3 ^{Aa}	83.1 ± 1.7 ^{Ab}	50.0 ± 0.3 ^{Ae}	60.2 ± 1.2 ^{Ad}

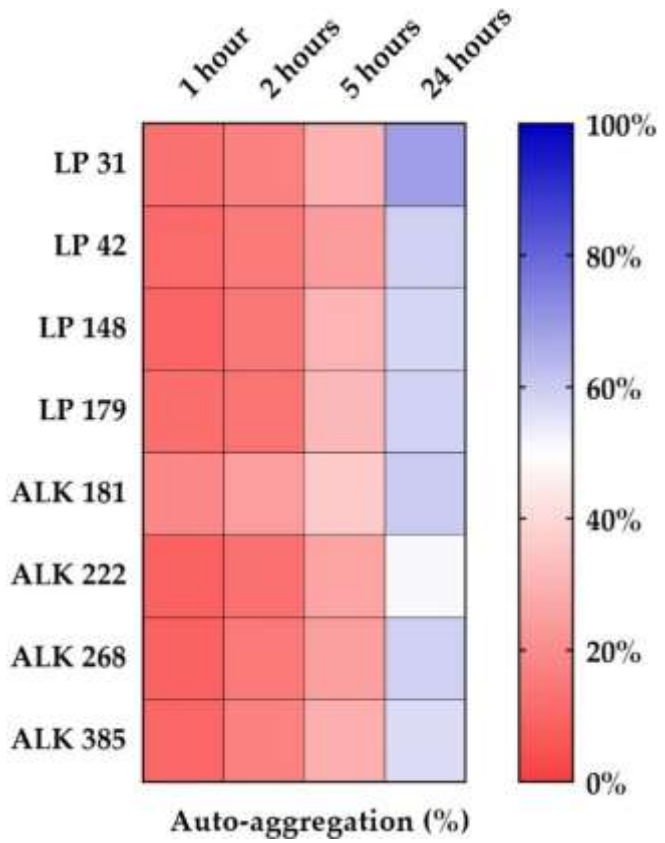


Figure 3. Percentage of auto-aggregation (AA%) of the *Lactiplantibacillus plantarum* and *Apilactobacillus kunkeei* strains.

Table 3. Auto-aggregation (AA) at 35 °C of the tested lactic acid bacteria (*Lactiplantibacillus plantarum*: LP 31, LP 42, LP 148 and LP 179; *Apilactobacillus kunkeei*: ALK 181, ALK 222, ALK 268 and ALK 385). The data (mean ± SD; $n = 3$) were expressed as percentage of AA, different lowercase letters in each row and uppercase letters in each column indicate significant differences ($p < 0.05$).

Time (h)	LP31	LP42	LP148	LP179	ALK181	ALK222	ALK268	ALK385
1	12.4 ± 0.3 ^{Db}	11.2 ± 0.5 ^{Cb}	9.5 ± 0.5 ^{Db}	12.3 ± 0.9 ^{Cb}	19.0 ± 0.9 ^{Da}	8.93 ± 0.2 ^{Cb}	9.1 ± 0.1 ^{Db}	10.2 ± 0.4 ^{Db}
2	17.0 ± 1.3 ^{Cb}	15.1 ± 1.8 ^{Cb}	14.5 ± 1.6 ^{Cb}	13.8 ± 1.2 ^{Cb}	24.4 ± 1.9 ^{Ca}	12.4 ± 0.7 ^{Cb}	14.7 ± 0.8 ^{Cb}	17.0 ± 1.6 ^{Cb}
5	29.4 ± 3.0 ^{Bb}	24.0 ± 2.6 ^{Bc}	30.2 ± 2.5 ^{Bb}	31.5 ± 1.8 ^{Ba}	36.1 ± 2.4 ^{Ba}	26.3 ± 1.7 ^{Bb}	24.8 ± 1.0 ^{Bc}	28.8 ± 2.1 ^{Bb}
24	68.8 ± 3.6 ^{Aa}	59.2 ± 2.5 ^{Ab}	57.9 ± 2.0 ^{Ab}	58.7 ± 2.9 ^{Ab}	60.0 ± 2.3 ^{Ab}	51.1 ± 2.2 ^{Ac}	59.2 ± 1.7 ^{Ab}	56.8 ± 2.4 ^{Ab}

2.3.4 Biochemical characterization

The carbohydrate assimilation patterns, detected using the API 50 CHL kit, were determined. Unlike the *Al. kunkeei* strains, the four *Lp. plantarum* strains were able to assimilate raffinose, while only the *Al. kunkeei* strains were capable of assimilating 5-keto-gluconate. *Lp. plantarum* LP 179 and *Al. kunkeei* ALK 268 were the only tested bacteria capable of assimilating the rhamnose. *Lp. plantarum* LP 31 was the only tested bacterium capable of assimilating the pentose sugar L-xylose. The disaccharides gentiobiose and turanose were

assimilated by the four *Al. kunkeei* strains. Gentiobiose was also assimilated by *Lp. plantarum* LP 31 and LP 42, while *Lp. plantarum* LP 42 and LP 148 were capable of assimilating turanose. Gluconate was only assimilated by *Lp. plantarum* LP 31 and LP 179 strains. All LP strains assimilated sorbose, methyl-α-D-mannopyranoside and methyl-α-D-glucopyranoside. Moreover, the strain LP 31 was able to assimilate most of the tested carbohydrates.

Enzymatic profiles were detected with the API ZYM kit. The four *Al. kunkeei* exhibited very similar enzyme profiles. The differences among the ALK isolates were recorded regarding α-galactosidase, β-glucosidase and β-glucuronidase activities; however, *Al. kunkeei* ALK 181 and ALK 268, showed no β-glucuronidase activity. The four *Al. kunkeei* strains did not produce lipase, trypsin, α- chymotrypsin and α-mannosidase.

With regards to the *Lp. plantarum* enzymatic profile, the four tested strains showed N-acetyl-β-glucosaminidase, α-glucosidase, β-galactosidase and β-glucosidase activities. Only the LP 31 strain did not possess the alkaline phosphatase, α-galactosidase and esterase enzymes. Moreover, L42 and L179 strains were negative for esterase-lipase, leucine and valine arylamidase activities. The four *Lp. plantarum* strains did not produce the following enzymes: acid phosphatase, cystine arylamidase, lipase, trypsin, naphthol-AS-BI-phosphohydrolase, α-chymotrypsin, α-fucosidase, α-mannosidase and β-glucuronidase.

2.4 DISCUSSION

Our investigation of the LAB community in the honey bee gut showed that *Al. kunkeei* and *Lp. plantarum* were the most numerically representative species. *Al. kunkeei* is a highly versatile bacterium and can be found in fructose-rich niches, including honey, beebread, flowers and the gastrointestinal tract of honey bees (Olofsson *et al.*,2008; Endo, 2012; Vásquez *et al.*,2012; Corby-Harris *et al.,* 2014). *Lp. plantarum* is also a LAB usually isolated from the honey bee gut (Tajabadi *et al.*,2014; Chandran, 2018) and, as highlighted in one of our previous studies, is sometimes numerically very representative (Iorizzo *et al*., 2020b). This confirms the extreme adaptability of this species to different environmental niches (Siezen *et al*., 2010; Martino *et al*., 2016), including those rich in fructose (Gustaw *et al.*, 2018).

LABs' antimicrobial activity is due to several factors: nutritional competitiveness and the production of compounds such as organic acids, fatty acids, protein compounds, phenolic acids and hydrogen peroxide (Reis *et al.*, 2012). In this investigation, the inhibition test was performed by utilizing the CFS of LAB cultures by the agar well diffusion method. The eight LAB strains showed inhibitory activity against the vegetative growth of *P. larvae* ATCC 9545 and *M. plutonius* ATCC 35311. Our results show the absence of inhibition in

the control test with MRS pH 3.8, suggesting that the antagonistic action is not related to organic acids but to the presence in CFS of other metabolites capable of inhibiting the two pathogens. Accordingly, further studies are required to determine the exact nature of these antimicrobial compounds and to assess their mechanism of action against *P. larvae* and *M. plutonius.* It is also important to test the antigerminative efficacy of these compounds against *P. larvae* spores.

A balanced gut microbiota, in addition to playing an antagonistic role against pathogens, can also be involved with its metabolic activities in the digestive process of bees (Raymann & Moran, 2018; Powell *et al.,* 2014). In our analysis of enzyme activity profiles, determined using the API-ZYM test, the eight strains of *Lp. plantarum* and *Al. kunkeei* were found to possess glycosidase activity. Beta-glycosidase is important because, in combination with other enzymes, including cellulase and hemicellulose produced by honey bee gut symbionts such as *Gilliamella*, it contributes to the hydrolysis of cellulose (Zheng *et al.,* 2016). Alpha-glycosidase is an enzyme that can hydrolyze maltose to glucose and is also directly involved in the degradation of starch granules (Zheng *et al.,* 2019; Stanley *et al.,* 2011). Several carbohydrates present in the honey bee diet are toxic because these insects do not possess functional enzymes to metabolize them (Lee *et al.,* 2015). The results obtained in the carbohydrate assimilation test showed that the four *Lp. plantarum* strains are capable of metabolizing the monosaccharides L-arabinose, galactose, mannose, and the oligosaccharides melibiose, lactose, melezitose and raffinose. The four *Al. kunkeei* assimilated L-arabinose, galactose, mannose, melibiose, lactose and melezitose.

Lp. plantarum LP 179 and *Al. kunkeei* ALK 268 were also able to metabolize rhamnose and *Lp. plantarum* LP 31 was the only bacterium able to assimilate L-xylose.

All of the above-mentioned carbohydrates are considered to be potentially toxic to the honey bee and may be contained in traces in the natural nectar derived from the hydrolysis of pectin, or synthesized as melezitose (Lee *et al.,* 2015). This sugar is a trisaccharide, composed of monosaccharide glucose and disaccharide turanose. It can be produced by aphids and is the main carbohydrate contained in honeydew (Zheng *et al.,* 2016; Seeburger *et al.,* 2020; Johnson *et al.,* 2015). The use and the role of the selected LAB strains as probiotics, thanks to their specific enzymatic activities, can contribute to the breakdown of complex polysaccharides and metabolize toxic sugars and consequently improve the dietary tolerance of honey bees (Zheng *et al.,* 2016).

The ability of probiotics to adhere to the intestinal *epithelium* is an important pre-requisite for biofilm formation in the host intestinal tract. This ability, along with their antimicrobial activity are important features that can hinder the colonization of undesirable

microorganisms (Iorizzo *et al.,* 2022a, b, c;). The gut adherence ability of probiotic bacteria involves different types of surface properties, including hydrophobicity and auto-aggregation (Collado *et al.,* 2008, Trunk *et al.,* 2018). Auto-aggregation mechanisms generally involve various molecules including cell sur- face proteins, exopolysaccharides, carbohydrates, glycoproteins, teichoic and lipoteichoic acids (Salas-Jara *et al.,* 2016). The eight LAB strains were observed to have a high level of autoaggregation, which is a recommended characteristic for a good probiotic strain (Kos *et al*., 2003). The adherence capacity is a strain-specific property due to several interactions between hydrophobic and hydrophilic components of the cell bacterial surface (Collado *et al.,* 2008). In our tests, the hydrophobicity was evaluated by the BATH method using xylene and toluene as hydrocarbons. The results showed that the LAB strains had high values of hydrophobicity and were in line with previous investigations (Iorizzo *et al.,* 2022a). Thanks to their cell surface properties, these LABs used as probiotic supplements in honey bees' diets can persist in the intestinal tract where, especially during foraging, there is an intense flow of water and nectar.

The virulent action of *P. larvae* and *M. plutonius* is based on a few key steps: growth dynamics; attachment to host cells by the production of biologically active compounds, such as adhesins; and the production of enzymes degrading the peritrophic matrix, enabling the pathogens to directly attack the epithelial cells (Garcia-Gonzalez *et al*., 2013; Nakamura *et al*., 2021).

Probiotic bacteria, by binding to receptors in the intestinal mucosa, can inhibit the adherence of pathogenic microorganisms that are subsequently eliminated from the intestine.

2.5 CONCLUSIONS

In this scientific investigation, the evaluation of the functional properties of the eight strains of *Al. kunkeei* and *Lp. plantarum* was carried out *in vitro* and therefore the good probiotic potential highlighted does not axiomatically result in health benefits for the honey bee colonies. Therefore, further investigations are needed to evaluate, *in vivo* or *in situ*, the role that these LABs, used as dietary supplements, can play in safeguarding and improving honey bee health. In particular, it is necessary to evaluate the contribution that these bacteria can make to the biocontrol strategy against EFB and AFB diseases.

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3. CHAPTER 3

Diversity of plant pollen sources, microbial communities and phenolic compounds present in bee pollen and bee bread (*Environ. Sci. Pollut. Res.*, 2024, https://doi.org/10.1007/s11356-024-34517-x).

Abstract

The pollination of several crops, as well as wild plants, depends on honeybees. To get the nutrients required for growth and survival, honeybee colonies are dependent on pollen supply. Bee pollen (BP) is partially packed in honeycomb cells, and processed into beebread (BB) by microbial metabolism. The composition of pollen is highly variable and is mainly dependent on ecological habitat, geographical origin, honey plants, climatic conditions, and seasonal variations. Although there are important differences between the BP and the BB, little comparative chemical and microbiological data on this topic exists in the literature, particularly for samples with the same origin. In this study, BP and BB pollen samples were collected from two apiaries located in Campania and Molise regions of southern Italy. Phenolic profiles were detected via HPLC, while antioxidant activity was determined by ABTS assay. The Next Generation Sequencing (NGS) based on RNA analysis of 16S (rRNA), and Internal Transcribed Spacer (ITS2) regions was used to investigate the microbial community (bacteria and fungi) and botanical origin of the BP and BB. Chemical analysis showed a higher content of flavonols in BP (rutin, myricetin, quercetin, and kaempferol), while in BB there was a higher content of phenolic acids. The NGS analysis revealed that the microbial communities and pollen sources are dependent on the geographical location of apiaries. In addition, a diversity was highlighted between the microbial communities present in the BP and BB samples collected from each apiary.

3.1 INTRODUCTION

Pollination is a key component in agricultural food production and ecosystem maintenance (Goulson *et al.,* 2015; Vaudo *et al.,* 2015; Stevenson *et al.,* 2017). In this context, honeybees hold an essential role in pollinating wild plants and various agricultural crops (Goulson *et al*. 2015). The virtuous relationships between flowering plants and their pollinators have an extensive co-evolutionary history. In this long-standing plant-pollinator mutualism, honeybee colonies rely on the availability of pollen as a floral resource, from which they obtain the nutrients they need for their development and survival (Dötterl & Vereecken 2010). Bee pollen (BP) is flower pollen gathered as a load by the bee for the purpose of nourishing its larvae in the early stages of development. In addition, bee pollen (BP) is partially packed into honeycomb cells, where it undergoes a chemical change mediated by microbial metabolism to form a product called bee bread (BB), which is mainly used by nurse bees to produce royal jelly, which is high in protein and essential for the nutrition of larvae and adult queens (Vaudo *et al*. 2015). The main phytometabolites of pollen include carbohydrates, proteins and amino acids, lipids, polyphenols, enzymes

and coenzymes, vitamins and minerals (De-Melo & De Almeida- Muradian 2017; Bakour *et al*. 2019; El Ghouizi *et al*. 2023). This composition is highly variable and is dependent on ecological habitat, geographical origin, honey plants present, climatic conditions, and seasonal variations (Araújo *et al*. 2017; Donkersley *et al*. 2017; Alimoglu *et al*. 2021; Bayram *et al*. 2021). The beneficial effects and functional properties of the pollen are well-known and have been mainly attributed to its high content of polyphenols (Leja *et al*. 2007; Rzepecka-Stojko *et al*. 2015). Phenolic compounds, including flavonoids and phenolic acids, are recognized as important natural antioxidants; in vivo and in vitro studies support the role of these compounds in preventing DNA damage induced by reactive oxygen species (ROS) (Yonekura *et al*. 2016; Albishi *et al*. 2019). Therefore, other than providing immense benefits to the diet of honeybees (Stevenson *et al*. 2017), in recent years pollen has also become an important component of animal and human nutrition (Abdelnour *et al*. 2019; Kostić *et al*. 2020). The biological process of BP's transformation into BB is not yet fully elucidated. However, it is known that enzymes from bees' glands (e.g., amylases that are responsible for starch hydrolysis), as well as bacteria (mostly lactic acid bacteria - LAB) and yeasts sourced from bees' saliva and surfaces of pollen loads, play a crucial role in BP fermentation and BB production (Vásquez & Olofsson 2009; lorizzo *et al*. 2020; Ispirli & Dertli 2021; Ghosh *et al*. 2022). Important gaps in our knowledge remain regarding the microbial ecosystem of bee products, including both bacteria and fungi. Still very little is known about species composition and the role of these microorganisms in the biotransformation of BP to BB (Vásquez and Olofsson 2009; Friedle et al. 2021). Moreover, although there are important chemical differences between the BP and BB, little comparative data on this topic exists in the literature, particularly for samples with the same origin (Bayram et al. 2021). Therefore, in the present study, by means of HPLC-PDA, the phenolic profiles of BP and BB samples collected from Apis mellifera ligustica hives located in Campania and Molise, southern Italy, were characterized. Moreover, we have applied the Next Generation Sequencing (NGS) technology, based on the 16S ribosomal RNA (rRNA) and ITS2 (Internal Transcribed Spacer) analysis to identify and compare the microbial communities and plant pollen sources present in BP and BB.

3.2 Materials and Methods

Samples collection

The sampling areas are located in two regions of southern Italy at different latitudes and altitudes, Castelpoto, Campania region (41°08′42″ N; 14°42′14″ E; 280 m a.s.l.) and Montenero, Molise region (41°59′40″ N; 14°47'09" E; 109 m a.s.l.). Bee pollen (BP) and bee bread (BB) samples were collected from two apiaries of Apis mellifera ligustica belonging to the beekeeping association CONAPROA (Consorzio Nazionale Produttori Apistici; Italy) in spring 2020. From three different hives of each individual apiary, at intervals of fifteen days, six samples of BP and BB were collected. To minimize

contamination, samples were extracted from the honeycomb cell with a separate, sterile sampling instrument and placed in sterile 50 mL tubes for transport to the laboratory within 2 hours after collection. The BP and BB samples were mixed separately prior to analysis to have one representative sample from each hive.

Next-Generation Sequencing (NGS)

DNA extraction

DNA extraction from BP and BB was carried out using the Stool DNA Isolation Kit (Norgen, Biotek Corp., Thorold, ON, Canada) according to the manufacturer's instructions. The concentration and purity of the extracted nucleic acids were visualized and quantified, after agarose gel electrophoresis, by Nanodrop (NanoDrop™ 2000/2000c; Thermo Fisher Scientific, Italy). DNA quantity was standardized to a concentration of 10 ng/µl.

Illumina MiSeq amplification and sequencing

For each sample, the V3-V4 region of the 16S rRNA gene was amplified using primers 341F and 805R (Takahashi et al. 2014), while the ITS2 (Internal Transcribed Spacer) region of the rRNA was amplified using primers 2024F and 2409R (Zhang et al. 2018). Amplicons for each library were purified and mixed in equal proportions. Then, the amplicons were sequenced using the Illumina MiSeq PE300 platform (Illumina, San Diego, CA, USA) at Biodiversa s.r.l. (Rovereto, Italy).

Statistical Analysis

Total antioxidant activities and total phenolic compounds were measured in triplicate and results were expressed as mean ± standard deviation (SD). The statistical analyses were performed with the SPSS Statistics 21 (IBM Corp., Armonk, NY, USA) software. Student t-test was used to assess statistical comparison among the experimental groups. Differences were considered significant at $p<0.05$.

Bioinformatic analysis

Raw paired-end reads were analyzed with the QIIME 2 (ver. 2019.4) pipeline (Caporaso et al. 2010) using the DADA2 plugin. In detail, read quality filtering and trimming, error rate estimation, dereplication, read merging and chimera detection were conducted. Features were filtered at a threshold value of 0.005% for bacteria and 0.05% for eukaryotes. Bacteria taxonomy was assigned using trained OTUs at 99% from the GreenGenes database (version 13-8), whilst eukaryotes were classified using the UNITE database (version 8.2) at 99% of identity. The resulting feature tables were processed with the phyloseq package (Version 1,40.0) using the R software (version 4.2.2). The graphical representation of the data was conducted with the ggplot2 package (Wickham 2016).

Phenolic profile and antioxidant activity of pollen

Pollen extracts

The extraction of polyphenols from bee pollen samples was accomplished according to the procedure reported by Fanali et al. (2013). Briefly, 10 g of pollen were dissolved in 25 ml. of ethyl acetate. Subsequently, 12.5 mL of 40% ammonium sulfate and 2.5 mL of 20% H_3PO_4 were added. The mixture was stirred for 20 min at room temperature and finally filtered through a Buchner funnel. The aqueous nhase was mived with 20 ml of ethyl acetate and centrifuged (4000mmx10 min) All the organic phases were collected and then dried by vacuum rotary evaporation (Büchi Rotavapor R-114, Büchi Labortechnik AG, Flawil, Switzerland) for 2 hours at 30°C. The viscous residual was dissolved in 4 ml of MeOH and after filtration through a 0.45 µm cellulose acetate membrane, the pollen extract (PE) was stored at -20°C until it was used for further analysis. All the chemical compounds used were supplied by Sigma-Aldrich, Merck (KGaA Darmstadt, GE), and the tests were conducted in triplicate.

Antioxidant Activity (ABTS Assay)

PE antioxidant activity determination was assayed using the ABTS method according to the procedure reported by Letizia et al. (2023). In the ABTS test, the antioxidant capacity of the BP and BB samples (20 mg) was assessed using the reaction between the radical cation ABTS and the antioxidant compounds. Briefly, ABTS was dissolved in pure methanol to a concentration of 7 mM. ABTS radical cations (ABTS+) were produced by reacting the ABTS methanol solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 24 h before use. The ABTS+ solution was diluted with cold pure methanol to an optical density (OD) of 0.700 at 745 nm. Then, 100 µL of PE were mixed with 900 µL of the ABTS+ solution. The OD was measured at 745 nm after 6 min in the dark at room temperature using a BioSpectrometer (Eppendorf, Hamburg, GE). Trolox was used as the standard for the calibration curve. Antioxidant activity was expressed as Trolox Eq./g.

Determination of Total Phenolic Content (TPC)

Polyphenols were determined by the Folin-Ciocalteu colorimetric method using the Polyphenol Assay Kit (Steroglass; San Martino in Campo-Perugia, Italy). The principle of the method is based on the responsiveness of the Folin-Ciocalteu reagent to oxidize the hydroxyl groups of polyphenols in the sample, in a highly alkaline medium. The results were expressed as mg of gallic acid equivalents (GAE)/g.

Determination of phenolic compounds by HPLC-PDA

Chromatographic conditions

The HPLC chromatographic method for the separation and analysis of phenolic compounds was operated using a Phenomenex Synergy C18 (150 mm x 46 mm) with a binary gradient elution system

consisting of A) 0.1% formic acid in water and B) 0.1% formic acid in ACN, starting with 90% A to 60% A in 40 min at a flow rate of 1 mL/min. The HPLC system was coupled to a Photodiode Array Detector (PAD), which allowed working at 268 and 330 nm, obtaining UV spectra of the phenolic compounds. Confirmation of the compounds was obtained by combining spectra matching with purity test results. PDA was used to confirm the identity of the peaks and to check their purity. Peak identity was confirmed by superimposing the spectrum of each peak with the corresponding standard. The peaks were also identified by comparing their retention times (R.) with the retention times of the pure standards.

Evaluation of analytical parameters

Limits of detection (LOD) and quantification (LOQ) were determined by analyzing subsequent diluted solutions of the standard compounds, up to reach the analyte concentrations which give a signal-to-noise ratio of 3 and 10, respectively. Calibration curves were constructed by linear regression by plotting the relative areas as a function of analyte concentration. All the chemical compounds used were supplied by Sigma-Aldrich, Merck (KGaA Darmstadt, GE). Dynamic range was evaluated considering the concentrations between LOQ values and 100mu*g / m * L The retention time of each reference standard, relative standard deviation in retention time (RSD %), R^ 2. LOD, and LOQ were reported in Table 1.

Table 1 - Retention time, relative standard deviation in retention time (RSD %), R^2 LOD, and LOQ of the phenolic standards.

Phenolic compounds	Retention time (R_t min)	RSD % (R_t min)	R^2	LOD (µg/mL)	LOQ (µg/mL)
Gallic acid	5.18	0.38	0.9998	0.05	0.15
Protocatechuic acid	8.15	0.45	0.9995	0.05	0.15
p-OH benzoic acid	11.56	0.68	0.9998	0.05	0.15
Chlorogenic acid	12.76	0.74	0.9998	0.15	0.35
Vanillic acid	13.64	0.78	0.9996	0.10	0.25
Caffeic acid	14.49	0.84	0.9996	0.10	0.25
p-coumaric acid	20.57	0.91	0.9998	0.10	0.25
Ferulic acid	23.14	0.96	0.9995	0.10	0.15
o-coumaric acid	26.28	1.02	0.9993	0.10	0.15
Rutin	27.36	1.08	0.9997	0.18	0.25
Myricetin	32.05	1.14	0.9983	0.20	0.30
Quercetin	35.56	1.19	0.9976	0.18	0.25
Cinnamic acid	39.06	1.23	0.9989	0.10	0.15
Kaempferol	41.40	1.28	0.9985	0.20	0.30

3.4 RESULTS

NGS results for plant, bacteria and fungi families and genera and in pollen samples are graphically shown in Figs. 1, 2, 3. In collected samples from hives of Montenero (BP_M; BB_M), three plant families were identified in BP_M (Asteraceae 14.20%, Brassicaceae 46.07, Convolvulaceae 37.98), while the BB_M samples had predominant pollen from Fabaceae (34.82%), Asteraceae (33.37%), Myrtaceae (14.15%) and Rosaceae (8.91%). At the genus level, in BP_M, mainly *Helianthus* (14.08%), *Convolvolus* (37.90%) and *Diplotaxis* (46.0%) were identified, while in BB_M, *Trifolium* (34.6%), *Rubus* (8.9%), *Eucalyptus* (14, 15%) and *Dittrichia* (13.5%) and *Helianthus* (12.68%) were identified. In the BP collected from Castelpoto hives (BP_C), mainly Fabaceae (63.5%) and Rosaceae (22.7%) families were identified. On the genus level, *Trifolium* (63.4%) and *Rubus* (22.66%) were identified as the main plant sources in BP_C and *Borago* (62.22%) with *Trifolium* (32.6%) in BB_C.

As for fungi, the Cladosporiaceae and Erysiphaceae families were the most relevant in the pollen collected from Castelpoto hives. The most representative taxonomic groups at the genus level were *Cladosporium* (37.8%), *Botrytis* (13.7%), *Alternaria* (11.0%) in BP and *Cladosporium* (38.8%), *Sporobolomyces* (7.8%), *Alternaria* (6.5%) in BB_C. In the pollen collected from the Montenero hives, the families with a relative greater abundance were Malasseziaceae (38.0%) and Pleosporaceae (27.1%) in BP_M while Pleurotaceae (24.9%) and Malasseziaceae (7.5%) in BB_M, with the predominance of the following genera: *Alternaria* (27.1%) and *Malassezia* (38.0%) in BP_M and *Pleurotus* (25.0%) and *Malassezia* (7.5%) in BB_M.

The main bacterial phylum in pollen was Proteobacteria with relative abundances of 90.44% in BP_C, 66.3% in BP_M, 88.0% in BB_M and 62.2% in BB_C. Other representative phyla were Firmicutes in pollen samples of Montenero (BP_M 31.6%; BB_M 9.84%) and Tenericutes in BP_C (7.8%) and BB_C (33.4%) collected from Castelpoto hives. The family of Enterobacteriaceae is the main representative of Proteobacteria phylum in BP_C (78.4%) belonged to the following families: Entomoplasmataceae (7.8%), Sphingomonadaceae (5.4%) Moraxellaceae, (5.0%), Burkholderiaceae (2.0%) and Pseudomonadaceae (1.7%). The bacteria most present in BP_M belonged to the following families: Pseudomonadaceae (17.05%), Rhizobiaceae (11.45%) Moraxellaceae (10.22%), Enterobacteriaceae (6.11%), with a substantial presence of unclassified Lactobacillales (26.0%). While the families of bacteria predominant in BB_M were: Pseudomonadaceae (23.05%), Sphingomonadaceae (10.1%), Moraxellaceae (5.66%), Alcaligenaceae (3.0%) and Lactobacillaceae (2.82%). At the genus level *Candidatus Phlomobacter* and *Mesoplasma* were highly represented in BP_C and in BB_C. In the pollen collected from the Montenero hives, a more diverse but numerically more homogeneous presence of bacterial groups belonging to the genera *Agrobacterium*, *Acinetobacter*, *Anoxybacillus*, *Burkholderia*, *Delftia*, *Pseudomonas*, *Sphingomonas* and unclassified *Lactobacillales* was found.

Total phenols and antioxidant activity

Total phenolic content and antioxidant activity were higher in the BB than in the BP (Table 2). In particular, ABTS values increased from 0.42 (BP_M) to 3.58 (BB_M) mg Trolox Eq./g and the total phenols from 3.73 mg (BP_M) to 5.19 mg (BB_M) GAE/ g. In the pollen collected from Castepoto hives, the values of antioxidant activity increased from 6.78 mg (BP_C) to 9.86 mg (BB_M) Trolox Eq./g, while total phenol amounts increased from 7.55 mg (BP_M) to 14.08 mg (BB_M) GAE/g.

Table 2 - Antioxidant capacity and total phenols content in the bee pollen and bee bread samples collected from Castelpoto and Montenero apiaries.

	Castelpoto		Montenero	
	ABTS	Total phenols	ABTS	Total phenols
	(mg Trolox Eq./g)	(mg GAE/g)	(mg Trolox Eq./g)	(mg GAE/g)
Bee pollen	6.78 ± 1.80^{a}	7.55 ± 2.49^{b}	0.42 ± 0.24^{b}	3.73 ± 0.15^{a}
Bee bread	9.86 ± 1.40^{a}	13.72 ± 2.86^{a}	3.58 ± 1.04^{a}	5.19 ± 2.16^{a}

*Data were expressed as mean ± SD (n = 3) Different lowercase letters (a-b) in each column indicate significant differences D < 0.05

Phenolic profiles

Fourteen phenolic compounds (phenolic acids and flavonoids) were quantified in the pollen samples (Table 1). Overall, we observed that BB contains mainly phenolic acids, while BP predominantly contains flavonoids. In particular, as shown in Table 3, in BP_M samples the highest concentration was determined for rutin (33.23 µg/g) followed by ferulic acid (27.10 µg/g). Regarding the phenolic acids detected in BB_M samples *p*-OH benzoic acid was the most abundant compound (65.81 µg/g), followed by lower quantities of 3,4-dihydroxy-benzoic (28.6 µg/g) 2,5-dihydroxy-benzoic (27.71 µg/g) caffeic (13.31 µg/g) and vanillic (3.75 µg/g) acids. As for flavonoids, in BB_M samples, kaempferol (10.26 µg/g) and myricetin (1,66 µg/g) were detected. In the BP_C samples, taken at the apiary of Castelpoto, flavonoids such as rutin (35.90 µg/g) and quercetin (13.40 µg/g) were mainly detected. They were followed by lower quantities of myricetin (4.50 µg/g) and kaempferol (2.56 µg/g), while in BB_C samples only the kaempferol (20,10 µg/g) was detected. As phenolic acids, in BB_C ferulic acid (41.80 µg/g) was the most abundant compound, followed by lower quantities of 3,4-dihydroxy-benzoic (34.60 µg/g), 2,5-dihydroxy-benzoic (30.30 µg/g), o-coumaric (25.20 µg/g), cinnamic (23.80 µg/g) caffeic (13,80 µg/g), p-OH benzoic (11.20 µg/g) ferulic (3.75 µg/g), gallic (3.10 µg/g) and chlorogenic (2.45 µg/g) acids.

Table 3 - Phenolic profile of bee pollen (BP) and bee bread (BB) samples collected from Castelpoto and Montenero apiaries.

	Castelpoto		Montenero	
Phenolic compounds (µg/g)	BP	BB	BP	BB
Gallic acid	0.26 ± 0.46	3.10 ± 0.57	n.d.	n.d.
3,4-dihydroxy-benzoic acid	0.52 ± 0.90	34.60 ± 3.72	n.d.	28.60 ± 12.54
2,5-dihydroxy-benzoic acid	n.d.	30.30 ± 5.50	n.d.	27.71 ± 11.13
p-OH benzoic acid	n.d.	11.20 ± 3.22	n.d.	65.81 ± 18.02
Chlorogenic acid	n.d.	2.45 ± 0.53	n.d.	n.d.
Vanillic acid	n.d.	n.d	n.d.	3.75 ± 2.07
Caffeic acid	n.d.	13.80 ± 5.44	n.d.	13.31 ± 7.86
p-coumaric acid	n.d.	n.d	n.d.	n.d.
Ferulic acid	n.d.	41.80 ± 2.99	27.10 ± 1.47	n.d.
o-coumaric acid	n.d.	25.20 ± 1.76	n.d.	n.d.
Rutin	35.90 ± 2.97	n.d	33.23 ± 0.81	n.d.
Myricetin	4.50 ± 0.93	n.d	2.89 ± 0.45	1.66 ± 0.54
Quercetin	13.40 ± 2.20	n.d	9.87 ± 0.32	n.d.
Cinnamic acid	n.d.	23.80 ± 5.32	n.d.	n.d.
Kaempferol	2.56 ± 0.84	20.10 ± 1.58	2.90 ± 0.37	10.26 ± 2.28

*Data were expressed as mean ± SD (n = 3).

3.5 DISCUSSION

The identification of pollen origin is a central issue in pollination ecology studies and agroecological research. Currently, conventional pollen identification uses light microscopy and discriminates species based on morphological characteristics. This requires specialized knowledge in palynology, is time-consuming and lacks discriminatory power at lower taxonomic levels (Sickel *et al.* 2015). In this work, pollen identification through molecular sequencing and DNA barcoding has been proposed as an alternative approach. Our results confirm that for qualitative analysis, NGS presents itself as a useful and efficient workflow for pollen identification at the genus level, as shown in previous research (Galimberti *et al*. 2014; Keller *et al*. 2015; Richardson *et al.* 2015; Bell *et al.* 2016). In particular, we have highlighted considerable differences in pollen samples collected from Castelpoto and Montenero hives, evidently due to the different plant species present in these sites. In addition, significant differences were shown in the

BP and BB samples from each individual site, probably due to the summation effect created in the BB obtained from BP collected by bees at different time periods. Regarding the phenolic compounds, our results demonstrate that in BP and BB were primarily found phenolic acids and flavonoids, as evidenced in other studies (Campos *et al*. 1997; Bayram *et al*. 2021). In particular, our study showed that the range of phenolic components during the biotransformation of BP to BB is significantly modified. From the data reported (Table 3), the BP samples mainly contain the flavonoids rutin, myricetin, quercetin and kaempferol, no significative presence of phenolic acids was detected. Similar to those found in our investigation other studies show that rutin and myricetin and kaempferol are the phenolic compounds mainly found in BP (Negri *et al*. 2018; De-Melo *et al*. 2018; Carpes *et al*. 2013). Regarding the BB, in our study, we can observe that the pollen extract contains mainly phenolic acids: gallic acid, 3,4-dihydroxy- benzoic acid; 2,5-dihydroxy-benzoic acid; p-OH benzoic acid; vanillic acid; caffeic acid; p-coumaric acid, ferulic acid and kaempferol as flavonoid, c, luteolin and quercetin (Silva *et al*. 2009); Bayram *et al*. (2021) reported that rutin was the phenolic compound with the highest rate of occurrence in both BP and BB samples. However, concentrations of protocatechuic acid, 2,5-dihydroxybenzoic acid and kaempferol compounds were significantly higher in BB samples than in BP samples from the same hive (Bayram *et al*. 2021). Different results have been reported in a recent study published by Urcan *et al.* (2018), which demonstrated that despite the biochemical transformation that occurs during the fermentation of bee bread, the phenolic compounds are unaffected and remain unchanged. Furthermore, Urcan *et al*. (2018) have shown that there is a high correlation between floral pollen, bee pollen and bee bread and that there is no correlation between soil/climate and phenolic composition. Nevertheless, Sobral et al. (2017) and Bayram *et al*. (2021) studied BP and BB from different origins and demonstrated that similarities and differences in bee pollen samples from different regions may vary depending on many factors, such as differences in regional flora, climatic conditions, altitude and storage conditions of pollen. Phenolic acids and flavonoids are synthesized in plants through the phenylpropanoid pathway (Mandal *et al*. 2010; Falcone Ferreyra *et al.* 2012). Plant-derived phenylpropanoids compose the largest group of secondary metabolites produced by higher plants, mainly, for the protection against biotic or abiotic stresses such as infections, wounding, UV irradiation, exposure to ozone and pollutants and also act as key chemical modulators of plant communication with insects and microbes. In particular, the presence of flavonoids, and their derivatives, plays an important role in plant protection through their role as attractants to pollinators through flower color and as repellents to pathogens, plant-feeding insects and natural plant predators (herbivores) (Harborne and Willians 2000; Noel *et al*. 2005; Mierziak *et al*. 2014). The species- specific flavonol, and phenolic acid profiles, may be more important than flavonol content to determine the particular antioxidant capacity of pollen of different botanical origins. This beehive product can be considered as an important source of natural antioxidants. The prevalence of phenolic acids

in the BB can be interpreted as an effect of the degradation of procyanidin, molecules of the flavonoid class. In this way, polyphenols that cannot be absorbed by the digestive system, during the fermentation phase of BB, are transformed by the enzymes of the microbiota (esterase, glucosidase, demethylation, dihydroxylation and decarboxylation) into a wide range of lower molecular weight phenolic compounds, such as small phenolic acids (Tarko *et al*. 2013). This data suggests that there is a link between the fermentation process and the formation/degradation mechanism of phenolic compounds in the formation phase of BB. This correlation includes the ability of some bacteria to break down phenolic compounds and the positive-negative effects of phenolic compounds on bacterial growth (Bayram *et al*. 2021; Peng *et al*. 2014; Zhang *et al*. 2014; Shabbir *et al*. 2021). Polyphenols, with their selective microbial action release ecological niches occupied by competing bacteria, thus allowing the flowering of beneficial intestinal bacteria (Didaras *et al*. 2020). Polyphenols' direct beneficial impact on the gut microbiota is based on two main modes of action: a direct bacterial stimulatory effect and a direct antimicrobial effect. The genome of several beneficial bacteria encodes a series of polyphenol-associated enzymes (PAZymes) specifically involved in polyphenol metabolization (Yang *et al*. 2023). In the presence of polyphenols, PAZymes- producing bacteria can utilize these compounds to improve their fitness and their persistence in intestinal niches. In addition, polyphenols can also selectively inhibit the development of potentially pathogenic species often associated with metabolic disorders (Rodríguez-Daza *et al*. 2021). Furthermore, our results showed that total phenols and antioxidant activity were significantly higher in BB than in BP. Among the antioxidants in BB, low molecular weight compounds were the most significant. Flavonoids and phenolic acids are characterized by high antioxidative potential, which is closely related to their chemical structure (Speisky *et al*. 2023). The high antioxidant potential of phenolic acids is due to the presence and position of hydroxyl groups, a carboxyl group in close proximity to the ortho-diphenolic substituents, and the ethylene group between the phenyl ring and the carboxyl group (Rzepecka-Stojko *et al*. 2015). In recent decades, and with the increased accessibility of DNA sequencing methods, fungal and bacterial communities in hive products have been characterized and their role in bee health and ecology has begun to be examined. Floral resources are the main food source for honeybees, therefore fungi and bacteria in nectar and pollen directly contribute to microbial communities in hives and in the honeybees' gastrointestinal tract. In this study, we analyzed the microbial community of BP and BB using NGS technology, based on 16S rRNA and ITS2 analysis. The different microbial communities in the BP and BB samples, as illustrated in this study, are presumably due to the different geographical locations of the hives and the processing of BB, which involves fermentation, and the different environmental conditions, such as maintained temperature, higher humidity, anaerobic conditions, the addition of secretions, and the high-sugar environment in the hive (Ghosh *et al*. 2022).

3.6 Conclusion

In this study, it is presented a comparative characterization of plant sources, microbial community, and phenolic compounds present in BP and BB samples from two different apiaries. The results revealed that the microbial communities of BP and BB samples differed significantly according to the geographical location of the apiaries and the plant sources available. On the other hand, regardless of the sampling site, a higher presence of flavonoids was detected in BP and phenolic acids in BB. These differences in phenolic profile are probably largely due to the metabolism of bacteria and yeasts present in the early stages of pollen fermentation. Sufficient information on the presence and correlation between microorganisms and polyphenols during pollen maturation is lacking. Therefore, we suggest future research aimed at expanding our understanding of how pollen composition and quality may affect bee health.

3.6 References

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4. Concluding Remarks

As already well known, a variety of adverse factors, among which pests and pathogens over all, but also pesticide exposure, diet quantity, quality, and diversity as well as unfavorable weather and fodder conditions, contribute to the decline in honey bee populations. In addition, the beekeeper′s management practices strongly influence the health of honey bee colonies. All these factors, acting alone or in combination, affect honey bee colonies, causing possible serious disturbances in the composition of the honey bee microbiota. Moreover, honey bees, like all living things, are constantly in contact with many microorganisms that can exert an impact on their health and well-being.

Several researchers have investigated the use of the gut microbial symbionts as a supplement in the honey bee diet and the results have shown that such microorganisms (multiple strains or single strain), or their metabolites, could improve the health status of the honey bees. The honey bee symbionts could be exploited to actively counteract bee pathogens and parasites, or to enhance immunity, and thus indirectly, increase the protection of the honey bees' health.

The modulation of the honey bee gut microbiota, by supplementation of selected LABs, has aroused special attention since it represents a strategy to improve the health status of colonies, in terms of productivity, as well as boosting the presence of beneficial microorganisms within the gut of new-generation bees. Moreover, appropriate probiotics could be exploited to actively counteract bee pathogens and parasites, or to enhance immunity, and thus indirectly, increase the protection of the honey bees' health. However, it is extremely clear that further studies are needed to gain a better understanding of how host–microbe–environment interactions influence honey bee mortality and colony loss.

Preliminary studies (conducted at the Department of Agricultural, Environmental and Food Sciences of the University of Molise) showed that *Al. kunkeei* strains could be functional for use in "probiotic syrup", useful to restore the symbiotic communities of the intestine in case of dysbiosis and to exert a prophylactic action against *A. apis*. Selected *Al. kunkeei* strains are able to inhibit *A. apis* and highlighted their important properties, such as ability to form biofilms, high auto-aggregation, and hydrophobicity, all prerequisites for candidacy as probiotic microorganisms in a honeybee diet. In addition, the selected *Al. kunkeei* strains showed high osmotic tolerance, a functional requirement for the probiotication of sugar syrups to restore or strengthen the symbiotic communities in honeybee guts in case of microbial dysbiosis.

The *Lp. plantarum* strains used in our first experiments (Chapter 2) have been shown to possess substances biologically active against *A. apis*. These results confirm the potentially antagonistic role of *Lp. plantarum* against pathogenic microorganisms that use the digestive channels of honeybees as the sites of infection. Five strains of *Lp. plantarum* are able to produce Extracellular Polymeric Substances (EPSs) and form biofilms, which are prerequisites for potential candidates to be used as probiotics in the honeybee diet. In addition, the antioxidant properties of the tested bacterial strains can help to increase

the tolerance of these insects to endogenous and exogenous oxidative stress. These first results suggest the possibility to set up new strategies to improve honeybee health through nutritional approaches or the modulation of the gut microbiota using beneficial microbes and open up a new horizon for controlling honeybee pathogens.

The study of the LAB community in the honey bee gut showed that *Al. kunkeei* and *Lp. plantarum* were the most numerically representative species. *Al. kunkeei* is a highly versatile bacterium and can be found in fructose-rich niches, including honey, beebread, flowers and the gastrointestinal tract of honey bees. *Lp. plantarum* is also a LAB usually isolated from the honey bee gut and it is sometimes numerically very representative. This confirms the extreme adaptability of this species to different environmental niches, including those rich in fructose.

LABs' antimicrobial activity is due to several factors: nutritional competitiveness and the production of compounds such as organic acids, fatty acids, protein compounds, phenolic acids and hydrogen peroxide. In further investigation (Chapter 3), focused on European Foulbrood (EFB) and American Foulbrood (AFB), the eight LAB strains tested showed inhibitory activity against the vegetative growth of *P. larvae* and *M. plutonius*. Results show the absence of inhibition in the control test, suggesting that the antagonistic action is not related to organic acids but to the presence of other metabolites capable of inhibiting the two pathogens. Accordingly, further studies are required to determine the exact nature of these antimicrobial compounds and to assess their mechanism of action against *P. larvae* and *M. plutonius.* It will be also important to test the antigerminative efficacy of these compounds against *P. larvae* spores.

A balanced gut microbiota, in addition to playing an antagonistic role against pathogens, can also be involved with its metabolic activities in the digestive process of bees. The analysis of enzyme activity profiles, determined using the API-ZYM test, showed that the eight strains of *Lp. plantarum* and *Al. kunkeei* possess glycosidase activity. Several carbohydrates present in the honey bee diet are toxic because these insects do not possess functional enzymes to metabolize them. The results obtained in the carbohydrate assimilation test showed that the four *Lp. plantarum* strains are capable of metabolizing the monosaccharides L-arabinose, galactose, mannose, and the oligosaccharides melibiose, lactose, melezitose and raffinose. The four *Al. kunkeei* assimilated L-arabinose, galactose, mannose, melibiose, lactose and melezitose. *Lp. plantarum* LP 179 and *Al. kunkeei* ALK 268 were also able to metabolize rhamnose and *Lp. plantarum* LP 31 was the only bacterium able to assimilate L-xylose.

All of the above-mentioned carbohydrates are considered to be potentially toxic to the honey bee and may be contained in traces in the natural nectar derived from the hydrolysis of pectin, or synthesized as melezitose. This sugar is a trisaccharide, composed of monosaccharide glucose and disaccharide turanose. It can be produced by aphids and is the main carbohydrate contained in honeydew. The use and the role of the selected LAB strains as probiotics, thanks to their specific enzymatic activities, can contribute to

the breakdown of complex polysaccharides and metabolize toxic sugars and consequently improve the dietary tolerance of honey bees.

The ability of probiotics to adhere to the intestinal *epithelium* is an important pre-requisite for biofilm formation in the host intestinal tract. This ability, along with their antimicrobial activity are important features that can hinder the colonization of undesirable microorganisms. The gut adherence ability of probiotic bacteria involves different types of surface properties, including hydrophobicity and auto-aggregation. Auto-aggregation mechanisms generally involve various molecules including cell surface proteins, exopolysaccharides, carbohydrates, glycoproteins, teichoic and lipoteichoic acids. The eight LAB strains were observed to have a high level of autoaggregation, which is a recommended characteristic for a good probiotic strain. The adherence capacity is a strain-specific property due to several interactions between hydrophobic and hydrophilic components of the cell bacterial surface. In our tests, the hydrophobicity was evaluated by the BATH method using xylene and toluene as hydrocarbons. The results showed that the LAB strains had high values of hydrophobicity and were in line with previous investigations. Thanks to their cell surface properties, these LABs used as probiotic supplements in honey bees' diets can persist in the intestinal tract where, especially during foraging, there is an intense flow of water and nectar. The virulent action of *P. larvae* and *M. plutonius* is based on a few key steps: growth dynamics; attachment to host cells by the production of biologically active compounds, such as adhesins; and the production of enzymes degrading the peritrophic matrix, enabling the pathogens to directly attack the epithelial cells. Probiotic bacteria, by binding to receptors in the intestinal mucosa, can inhibit the adherence of pathogenic microorganisms that are subsequently eliminated from the intestine.

However, the evaluation of the functional properties of eight strains of *Al. kunkeei* and *Lp. plantarum* was carried out *in vitro* and, as a consequence, the good probiotic potential highlighted does not axiomatically result in health benefits for the honey bee colonies. Therefore, again, further investigations are needed to evaluate, *in vivo* or *in situ*, the role that these LABs, used as dietary supplements, can play in safeguarding and improving honey bee health. In particular, it is necessary to evaluate the contribution that these bacteria can make to the biocontrol strategy against EFB and AFB diseases.

Finally, a comparative characterization of plant sources, microbial community, and phenolic compounds present in Bee Pollen and Bee Bread samples from two different apiaries (preparatory to the use of LABs in the Western honeybee diet), revealed that the microbial communities of BP and BB samples differed significantly according to the geographical location of the apiaries and the plant sources available. On the other hand, regardless of the sampling site, a higher presence of flavonoids was detected in BP and phenolic acids in BB. These differences in phenolic profile are probably largely due to the metabolism of bacteria and yeasts present in the early stages of pollen fermentation. Sufficient information on the presence and correlation between microorganisms and polyphenols during pollen maturation is

lacking. Therefore, obtained results suggest future research aimed at expanding our understanding of how pollen composition and quality may affect bee microbiota and health.

In the last year of the PhD course (see List of publications), also bees and honey as bioindicators of environmental contamination by polycyclic aromatic hydrocarbons, heavy metals and plasticizers residues were used (Di Fiore *et al*., 2023). In addition, also the chemical composition and markers in the mediterranean orange blossom honey by UHPLC-HRMS metabolomics study integrating melissopalynological analysis, GC-MS and HPLC-PDA-ESI/MS were studied (Kasiotis *et al*, 2023). In light of these studies, whether or not the bee gut microbial flora is influenced by different pollutants and whether or not the microbial flora influences the chemical composition of honey should be investigated.

Future research activities will involve also the investigation of the nature of the antifungal compounds and evaluate the effects of these *Lp. plantarum* strains on honeybee health and productivity, and their efficacy in bee diseases biocontrol *in vivo/in situ*. Furthermore, it could bee appropriate to chacaracterize the microbiota of local population, also of the different subspecies, to verify possible symbiosis between microbes and subspecies/populations. In addition, it would be interesting to study the microbiota of wild colonies that have demonstrated to survive for several years in the natural environment.

4. LIST OF PUBLICATIONS

Full papers with IF

1. Iorizzo M., Albanese G., Letizia F., Testa B., Di Criscio D., **Petrarca S**., Di Martino C., Ganassi S., Avino P., Pannella., Aturki Z., Tedino C., De Cristofaro A., **2024**. Diversity of plant pollen sources, microbial communities, and phenolic compounds present in bee pollen and bee bread. *Environ. Sci. Pollut. Res.* https://doi.org/10.1007/s11356-024-34517-x
2. Kasiotis K.M., Baira E., Iosifidou S., Manea-Karga E., Tsipi D., Gounari S., Theologidis I., Barmpouni T., Danieli P.P., Lazzari F., Dipasquale D., **Petrarca S**., Shairra S., Ghazala N.A., Abd El-Wahed A.A., El-Gamal S.M.A., Machera K., **2023**. Molecules fingerprinting Chemical Markers in the Mediterranean Orange Blossom Honey: UHPLC-HRMS Metabolomics Study Integrating Melissopalynological Analysis, GC-MS and HPLC-PDA-ESI/M, *Molecules*, 8, 3967.
3. Di Fiore C., De Cristofaro A., Nuzzo A., Notardonato I., Ganassi S., Iafigliola L., Sardella G., Ciccone M., Nugnes D., Passarella S., Torino V., **Petrarca S**., Di Criscio D., Ievoli R., Avino P., **2023**. Biomonitoring of polycyclic aromatic hydrocarbons, heavy metals, and plasticizers residues: role of bees and honey as bioindicators of environmental contamination. *Environ. Sci. Pollut. Res. Int.* 30(15): 44234-44250.
4. Ganassi S., De Cristofaro A., Di Criscio D., **Petrarca S**., Leopardi C., Guarnieri A., Pietrangelo L., Venditti N., Di Marco R., Petronio G., **2022**.The new invasive mosquito species *Aedes koreicus* as vector-borne diseases in the European area, a focus on Italian region: What we know from the scientific literature, *Frontiers in Microbiology* 13: 931994.
5. Iorizzo M., Ganassi S., Albanese G., Letizia F., Testa B., Tedino C., **Petrarca S**., Mutinelli F., Mazzeo A., De Cristofaro A., **2022**. Antimicrobial Activity from Putative Probiotic Lactic AcidBacteria for the Biological Control of American and EuropeanFoulbrood Diseases, Veterinary sciences, 9, 236
6. Iorizzo, M.; Letizia, F.; Ganassi, S.; Testa, B.; **Petrarca, S**.; Albanese, G.; Di Criscio, D.; De Cristofaro, A. **2022**. Functional Properties and Antimicrobial Activity from Lactic Acid Bacteria as Resources to Improve the Health and Welfare of Honey Bees. *Insects* 13: 308.
7. Iorizzo, M.; Letizia, F.; Ganassi, S.; Testa, B.; **Petrarca, S**.; Albanese, G.; Di Criscio, D.; De Cristofaro, A. **2022**. Recent Advances in the Biocontrol of Nosemosis in Honey Bees (*Apis Mellifera* L.). *J. Fungi* 8, 424.
8. Iorizzo M., Testa B., Ganassi S., Lombardi S.J., Ianiro M., Letizia F., Succi M., Tremonte P., Vergalito F., Cozzolino A., Sorrentino E., **Petrarca S**., De Cristofaro A., Coppola R., **2021**. Probiotic Properties and Potentiality of *Lactiplantibacillus plantarum* Strains for the Biological Control of Chalkbrood Disease Journal of Fungi, 7, 379.
9. Iorizzo M.; Lombardi S.J.; Ganassi S.; Testa B.; Ianiro M.; Letizia F.; Succi M.; Tremonte P.; Vergalito F.; Cozzolino A.; Sorrentino E.; Coppola R.; **Petrarca S**.; Mancini M., De Cristofaro A. **2020**. Antagonistic Activity against *Ascosphaera apis* and Functional Properties of *Lactobacillus kunkeei* Strains. *Antibiotics 9*, 262.
10. Iorizzo M.; Pannella G., Lombardi S.J.; Ganassi S.; Testa B.; Succi M.; Sorrentino E., **Petrarca S**., De Cristofaro A.; Coppola R., Tremonte P., **2020**. Inter- and Intra-Species Diversity of Lactic Acid Bacteria in *Apis mellifera ligustica* Colonies. *Microrganisms* 8, 1578.
11. Iorizzo M.; Testa B.; Lombardi S.J.; Ganassi S.; Ianiro M.; Letizia F.; Succi M.; Tremonte P.; Vergalito F.; Cozzolino A.; Sorrentino E.; Coppola R.; **Petrarca S**.; Mancini M., De Cristofaro A. **2020**. Antimicrobial Activity against *Paenibacillus larvae* and Functional Properties of *Lactiplantibacillus plantarum* Strains: Potential Benefits for Honeybee Health. *Antibiotics 9*, 442.

Abstracts

12. Di Criscio D., Ganassi S., Tedino C., Grazioso P., **Petrarca S.**, Hatjina F., De Cristofaro A., **2023**. EAG

test on *Apis mellifera* as a future prospect of application for *Varroa destructor* control. European Congress of Entomology, Crete (Greece), Poster presentation, Book of abstracts.

13. Di Criscio D., Ganassi S., Tedino C., **Petrarca S.**, Mancini M., Grazioso P., Hatjina F., De Cristofaro A., **2023**. Evaluation of the efficacy of essential oils against *Varroa destructor* and their bioactivity on *Apis mellifera*. XXVII Congresso Nazionale Italiano di Entomologia, Palermo (Italy), Oral presentation, Book of abstracts.

Informative articles

14. De Cristofaro A., **Petrarca S**., **2021**. Apicoltura sostenibile: un'opportunità da reddito, e non solo per il Molise. In: Un altro Molise è possibile. Ed. Il Bene Comune; Morcone (BN), 157-166.
15. De Cristofaro A., **Petrarca S**., **2021**. *Apis mellifera ligustica*: una esperienza concreta di selezione condivisa territorialmente. In: Api e biodiversità. Tutela delle sottospecie autoctone di *Apis mellifera* Linnaeus, 1758. Fontana P., Zanotelli L. (a cura di). Fondazione Edmund Mach Ed., San Michele all'Adige (TN). pp. 71-112.

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