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PhD Thesis

Antibiotic and Probiotic activity of Lactic Acid Bacteria isolated from Honeybee gut and Beebread

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Summary

Research papers published within the PhD thesis

Iorizzo, M., Testa, B., Lombardi, S. J., Ganassi, S., **Ianiro, M**., Letizia, F., ... & De Cristofaro, A. (2020). Antimicrobial activity against *Paenibacillus larva*e and functional properties of *Lactiplantibacillus plantarum* strains: Potential benefits for honeybee health. Antibiotics, 9(8), 442.

Iorizzo, M., Testa, B., Ganassi, S., Lombardi, S. J., **Ianiro, M**., Letizia, F., ... & Coppola, R. (2021). Probiotic Properties and Potentiality of *Lactiplantibacillus plantarum* Strains for the Biological Control of Chalkbrood Disease. Journal of Fungi, 7(5), 379.

Abstract

Lactic acid bacteria constitute a broad heterogeneous group of microorganisms historically used for their important properties. It suffices to think of the different sectors in which they are used: food and drink industry for the production of traditional and innovative fermented and non-fermented products, production of industrially relevant metabolites, and finally as probiotic organisms to improve health and strengthen of the host immune system.

In the last years, this last aspect has been evaluated also in the beekeeping sector, integrating these microorganisms in the diet of bees by means of sugar syrups, in order to evaluate a possible increase in the resistance to pathogenic organisms.

In this PhD thesis, lactic acid bacteria belonging to the species *Lactobacillus plantarum*, recently reclassified as *Lactiplantibacillus plantarum*, previously isolated from bee bread and from the digestive tract of *Apis mellifera ligustica*, were used

A preliminary screening was based on the of ability of 61 *L. plantarum* strains to inhibit some of the main pathogens for bees, such as *Peanibacillus larvae* and *Ascosphaera apis*, responsible for the American Foulbrood disease and the Chalkbrood Disease, respectively.

Based on the results obtained by this test, five strains were subsequently selected and used to evaluate their possible applicability in the beekeeping sector as probiotics. Data registered in this study highlighted the ability of these strains, with different degrees, to inhibit the two pathogens, to produce EPS and to form biofilm in different conditions and sugar concentrations. The biochemical characterization of the strains showed the presence of enzymatic patterns and carbohydrates assimilation that can improve the digestion and assimilation on nutrients by bees. Moreover, two out of five tested strains showed high auto-aggregation and adhesion to hydrocarbons, two important prerequisites for colonization and protection of the host digestive tract. Finally, almost all tested strains were able to survive the stress conditions given by high sugar concentrations.

Based on this knowledge, new biotechnological approaches could be developed to improve the bee health and the quality of hive products.

Riassunto

I batteri lattici costituiscono un ampio ed eterogeneo gruppo di microrganismi storicamente utilizzati per le loro importanti proprietà. Basti pensare ai diversi settori in cui vengono utilizzati: industria alimentare e delle bevande per la produzione di prodotti tradizionali e innovativi fermentati e non, produzione di metaboliti rilevanti a livello industriale e, infine, come organismi probiotici per migliorare la salute e rafforzare il sistema immunitario dell'ospite.

Negli ultimi anni quest'ultimo aspetto è stato valutato anche nel settore dell'apicoltura, integrando questi microrganismi nella dieta delle api mediante sciroppi di zucchero, al fine di valutare un possibile aumento della resistenza agli organismi patogeni.

In questa tesi di dottorato sono stati utilizzati batteri lattici appartenenti alla specie *Lactobacillus plantarum*, recentemente riclassificata come *Lactiplantibacillus plantarum*, isolati dal pane d'api e dal tratto digestivo di *Apis mellifera ligustica*.

Uno screening preliminare si è basato sulla capacità di 61 ceppi di *L. plantarum* di inibire alcuni dei principali patogeni per le api, come *Peanibacillus larvae* e *Ascosphaera apis*, responsabili rispettivamente della peste americana e della malattia di Chalkbrood.

Sulla base dei risultati ottenuti da questo test, sono stati successivamente selezionati cinque ceppi e utilizzati per valutare la loro possibile applicazione nel settore dell'apicoltura come probiotici. I dati registrati in questo studio hanno evidenziato la capacità di questi ceppi, con gradi diversi, di inibire i due patogeni, di produrre EPS e di formare biofilm in condizioni e concentrazioni di zucchero differenti. La caratterizzazione biochimica dei ceppi ha mostrato la presenza di pattern enzimatici e di assimilazione dei carboidrati in grado di migliorare la digestione e l'assimilazione dei nutrienti da parte delle api. Inoltre, due dei cinque ceppi testati hanno mostrato un'elevata auto-aggregazione e adesione agli idrocarburi, due importanti prerequisiti per la colonizzazione e la protezione del tratto digestivo dell'ospite. Infine, quasi tutti i ceppi testati sono riusciti a sopravvivere alle condizioni di stress date da elevate concentrazioni di zucchero.

Sulla base di queste conoscenze, potrebbero essere sviluppati nuovi approcci biotecnologici per migliorare la salute delle api e la qualità dei prodotti dell'alveare.

Chapter 1: *Apis mellifera ligustica*

1.1 Introduction

Bees are the most important insects worldwide and they contribute to the pollination of a large number of crops and wild plants (EFSA, 2017). In addition, they are also considered important bioindicators of environmental pollution (Celli & Maccagnani, 2003). The Food and Agriculture Organization of the United Nations (FAO) estimates that of the 100 crop species that provide 90% of food worldwide, 71 are pollinated by bees, and the majority of crops grown in the European Union depend on insect pollination (EFSA, 2017). The estimated annual monetary value of pollination is billions of dollars (Hedtke *et al.*, 2015).

Beekeeping is an ancient tradition, and honeybees are kept in Europe for several millennia (EFSA, 2017). In recent years, a growing interest has been reported for the urban beekeeping practice as a fascinating rewarding pastime, which allows people to increase biodiversity, produce local foods, and reconnect with nature (Moore & Kosut, 2013).

Given the importance of honeybees in the ecosystem, and in the food chain, and given the multiple services they provide to humans, their protection is pivotal.

Managed honeybees are highly social, frequent a multitude of environmental niches, and continually share food; conditions that promote the transmission of parasites and pathogens (Gaggìa *et al.*, 2018).

Although managed honeybee colonies are continuously increasing over the last 55 years, colony populations have significantly decreased in many European and North American countries (Aizen & Harder, 2009), as a result of several incoming stressors (agrochemicals, pathogens, climate change) and socioeconomic reasons (vanEngelsdorp & Meixner, 2010).

In the last decade, special attention has arisen toward “colony collapse disorder” (CCD) in the USA, with the alarming claims of the media describing the dramatic demise of honeybee colonies. Colony losses have exceeded 90% in some locations, and loss of pollination services has had major impacts on some fruit and vegetable production (Gaggìa *et al.*, 2018).

As reported by vanEngelsdorp and Meixner (2010), the honeybee can die in many ways, and CCD is just one of them. Finally, since an excellent genetic variability exists both in honeybee host and pathogens, the symptoms and could vary in several regions (Neumann & Carreck, 2010; Gaggìa *et al.*, 2018).

Concerning pesticides, in 2013 the EU imposed a temporary ban on the use of the most important neonicotinoids on some crops. However, the new proposals are for an entire ban

on their use in fields, with the sole exception being for plants entirely grown in greenhouses (EFSA, 2013; Gaggìa *et al.*, 2018). Although it is impossible to pinpoint the individual factors that may determine premature colony mortality, in different regions of the world certain bee diseases and parasites play a significant role in increased bee colony mortality and colony losses.

1.2 *Apis mellifera*: colony organization and global diffusion

The honeybee is certainly one of the most important insects of the terrestrial habitats. The honeybee belongs to the order Hymenoptera, family Apidae, and it is member of the genus *Apis*. The center of origin is presumably Southeast Asia, where most of the species are found. Phylogenetic analyses based on nuclear and mitochondrial DNA markers strongly support a cluster into three distinct groups: cavity-nesting bees (*A. mellifera*, *A. cerana*, *A. koschevnikovi*, *A. nulensis*), giant bees (*A. dorsata*, *A. laboriosa*, *A. binghami*, *A. nigrocincta*), and dwarf bees (*A. florea*, *A. andreniformis*) (Arias & Sheppard, 2005; Raffiudin & Crozier, 2007).

Originally, the insect geographic distribution included West Asia, Europe, and Africa, and only later in the Americas, Australia, and the Pacific Islands. *Apis mellifera* has also been introduced through much of the range occupied by *A. cerana*, including Japan and China (Breed *et al.*, 2010; Gaggìa *et al.*, 2018).

The honeybee is the most studied domesticated species of matriarchal type, in which it is possible to distinguish three individuals belonging to three distinct polymorphic castes, all winged. Normally, in a beehive lives a queen, the only fertile female who is entrusted with the task of laying eggs. Morphologically, the queen turns out to be larger than the other bees, besides it can survive even for 5 years and it can lay thousands of eggs in a lifetime. The workers, sterile females whose job is the maintenance of the colony and the defense of the same, are smaller in size than the queen, and on average their life is 30-45 days longer if born in the winter. The drones, finally, are male bees whose only function is reproduction. They are bigger than the workers but smaller than the queen; they are not able to collect pollen as they have smaller proboscis than workers and have no sting.

Bees live on wax honeycombs built within natural cavities, such as trunks, or containers supplied by man (hives). The honeycombs consist of a double series of hexagonal cells placed horizontally, made by the bees themselves, modeling the wax they produce, inside which the eggs laid by the queen and the food supplies are received. Once built, the honeycombs are used for several years, and their cells are cleaned and sanded for reuse.

1.3 Insect morphology and digestive system

From a morphological point of view, it is possible to distinguish between three well-defined parts of the body: head, thorax, and abdomen.

The **head** looks like a globular capsule on which are inserted several sense organs, including the compound eyes, of considerable size, formed by 4000-5000 ommatidia, which are indispensable for distance vision, three defined simple ocelli eyes, arranged in a triangle on the head necessary for close vision in the penumbra of the hive and to perceive the solar radiation. The antennae consist of thousands of sensili necessary for the perception of tactile, gustatory, olfactory, thermal, and hydropower. The mouthparts positioned at the bottom of the head are of the chewing-lapping type.

The **chest** is covered with hairs that mask its segmentation. It consists of three segments: the prothorax, the mesothorax and metathorax. Moreover, a dorsal, ventral, and two lateral laminae can be highlighted. The prothorax door hanging off the first pair of legs, in mesothorax there are the first pair of wings and the second pair of legs. Finally, in the metathorax are present the second pair of wings and the third pair of legs. The legs are used both for walking and for collecting pollen and cleansing the body. The wings are membranous and horizontally held in the rest position, the secondary wings, which are smaller, and are coupled to the preceding by a hook allowing the flight synchronization.

The **abdomen** is made up of 10 segments, of which the first is incorporated into metathorax. The last abdominal segment bears the sting, except in the drones that have none. The sting is one notched stylet connected to the venomous apparatus. Because of teeth that are facing backward, during the act of puncture, the organ penetrates the tissues and it gets stuck. In the effort exerted by the bee to collect it, the abdomen is stripped away and the bee within a few hours dies. The queen bee's sting appears to be smooth and not toothed, so that the act of the queen puncture outruns the risk of being killed. The digestive system of the honeybee extends from the mouth to the anus where the waste material is excreted (Figure 1).

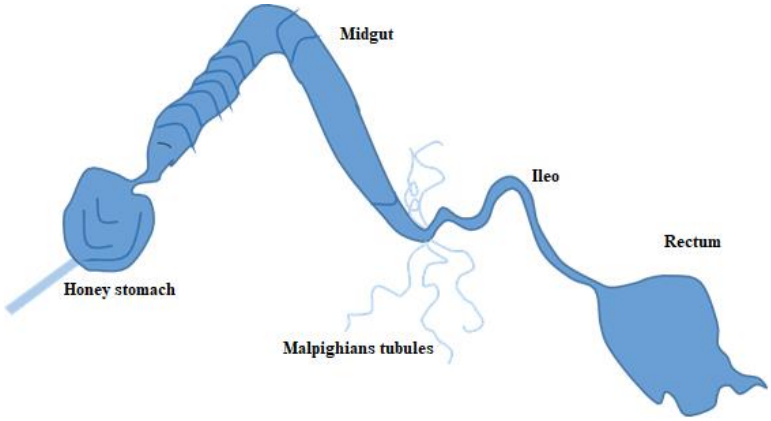

Figure 1. Representation of honeybee digestive system (Gaggìa *et al.*, 2018).

This can be divided into the front intestine (stomodeum), medium (mesentery), and rear (proctodeum). They are part of the intestine anterior pharynx, esophagus, the honey stomach, and proventriculus (Gullan & Cranston, 2014). The middle intestine is where the proper digestion takes place, and the rear intestine consists of the small intestine and rectum, which terminate with the anus. Nectar and substances of liquid nature, sucked in through the mouthparts, enter the honey stomach, and are then regurgitated into the hive. The pH is highly acidic, but this acidity is also influenced by the type of feeding of the insect (Gaggìa *et al.,* 2018).

Most of the nutrients from the digested feed are absorbed through the walls of the ventriculus (midgut), which is the functional stomach of bees, where most of the digestion and absorption takes place. Digestive enzymes work across a range of pH, but the pH optimum is 8. Thus, the proventriculus the drastic change in pH between the crop and the midgut define two major microbial niches, one coevolved with liquid transfer and food storage, and the other coevolved to reside within the enzymatically active and comparatively nutrient-rich midgut (Di Gioia and Biavati, 2018; Gaggìa *et al.,* 2018;).

From the midgut, the nourishment passes to the small intestine at the pyloric valve (the point where the transition from mesentery to proctodeum is marked); here are also about a hundred Malpighian tubules that, similarly to human kidneys, remove uric acid and other metabolites from the hemolymph. The pyloric valve regulates the passage of waste substances in the small intestine first and then in the rectal ampulla (a sort of variable volume sac), where these substances can remain for many days before being eliminated during the moments of flight, a particular mechanism useful in winter when many days pass before the bee can get rid of the feces during the so-called purification flights (in fact the bees never get rid of the feces inside the hive). The proctodeum, being the terminal part of the digestive system of insects, has the purpose of reabsorbing water and eliminating catabolites and undigested parts of the insect's diet.

1.4 Honeybee product of interest

Honey is a natural sweetener with a posh composition. Honey features vary counting on the botanical source and geographical origin, also as climatic, processing, and storage conditions (Machado De-Melo *et al*., 2018). Honey is mainly composed of carbohydrates (60-85%) and water (12-23%), its composition may influence its shelf life and some of its properties, including color, flavor, density, viscosity, hygroscopicity, and crystallization (Machado De-Melo *et al.,* 2018). Honey also contains small amounts of other components, like nitrogen

compounds, organic acids, minerals, vitamins, Maillard reaction products, volatile compounds, and several bioactive substances that affect sensory and physical characteristics, also biological potential (Machado De-Melo *et al*., 2018). In general, the vast majority of health benefits attributed to honey have been related to both antioxidant and antimicrobial activities (Osés *et al.,* 2016). Furthermore, honey has shown other potential functional properties that are worth commenting on, the most important of which are antihypertensive capacity, anti-inflammatory activity, as well as prebiotic and probiotic effects (Bogdanov, 2012).

Pollen comes in the form of granules with a flowery, sweet scent and flavor, similar to raw honey. It is one of the essential components of the diet of bees, the workers traveling from flower to flower collect the pollen in special baskets placed on their legs and involuntarily allow it to be moved on plants of the same species, thus allowing the plants to reproduce. Being a complete food, it is widely used as a tonic in phytotherapy, because it is a product rich in proteins (6-30%), amino acids (15-22%), lipids (1-10%), carbohydrates (up to 50%), simple sugars (4-10%), water (12-20%), vitamins, enzymes, unsaturated fatty acids, mineral salts and flavonoids (Almeida-Muradian *et al.,* 2005). Considering the chemical composition of the product, the use of dry pollen in human food generates a feeling of well-being by contributing to a functional balance of the human organism.

Bee bread is a unique product that is very important not only for humans but also for bees. The bee bread mainly includes pollen, honey, and secretions of bees' salivary glands (Vásquez & Olofsson, 2009; Barajas *et al.,* 2012). Bees pack the components in the cells of the honeycomb, then secure the mixture with wax and honey (Barene, 2015). Such gathered and preserved pollen is subject to lactic fermentation in the environment of the bee nest. Fermented bee pollen is called the bee bread (DeGrandi-Hoffman *et al.,* 2013; Fuenmayor B *et al.,* 2014). Bee bread is characterized by a higher nutritional value than pollen, better digestibility, and richer chemical composition (Habryka *et al.,* 2016). Bee bread contains considerably larger amounts of peptides and free amino acids. Due to the proportions of particular components, the bee bread is a superb foodstuff that would supplement the deficiency of vitamins and nutrients within the human organism. Because of the presence of all the essential amino acids, bee bread is characterized by better composition than many valuable products obtained supported animal proteins. Bee bread also has good properties that help eliminate various toxins from the organism (Nagai *et al.,* 2005; Habryka *et al.,* 2016). Bee bread contains about 30% protein on average (DeGrandi-Hoffman *et al.,* 2016), is different from fresh pollen, and it contains more sugars and much less starch. According

to Roulston and Cane (2000), the content of starch in pollen is in the range of 0–22%. Most sorts of pollen contain but 5% of starch, and pollen derived from sunflower contains only 0.4% starch (Roulston *et al*., 2000). Bee bread is rich in vitamins of group B, as well as vitamin K, which is not present in the fresh pollen (Kieliszek *et al.,* 2018). The content of lactic acid, which may be a preservative agent, in bee bread is above 3%. Carbohydrates constitute between 24 and 34% (Barene, 2015). Bee bread is more biologically active and easily digestible due to the high content of easily digestible sugars, fat, mineral components, and a better proportion of free amino acids in comparison to pollen (Nagai *et al.,* 2004).

Royal jelly is considered the most prized product of the hive. It has a white, milky, and viscous colour, with an aromatic, sour taste (pH 3.5-4.5), produced by the salivary glands of nurse bees; called royal as it is intended for feeding the queen and young larvae in the first 3 days of life. It is a protein product (15%), of which most are amino acids (there are 8 amino acids essential for human health), a moderate sugar content (about 16%) glucose and fructose, smaller traces of other sugars, lipids, and minerals, and among the vitamins the most abundant are those of group B. It has no contraindications and is widely used to fortify the body at certain times of the year (seasonal changes, periods of high stress, etc.), and for a very long time now, it has been used by pregnant women and elderly people because of its properties. beneficial and immunostimulating (Xue *et al.*, 2017).

1.5 The Honeybee microbiota

Gut commensal bacteria are microorganisms that colonize the digestive tracts of different animal species including insects. Given the importance of this insect to our economy, studying these insects and protecting them is the goal of much research, increasingly focused on their bacterial component. The microbiota composition of insect, vary with food sources, season and other environmental factors (Yun *et al.,* 2014; Hroncova *et al.,* 2015). Some are opportunistic, while others (the most important) are stable and widespread colonists that regulate many aspects of host physiology including nutrition, detoxification, development, and resistance to pathogenic infections (Flint *et al.,* 2012). Gut bacteria is transmitted and shared by the colony members through the fecal-oral route, trophallactic interaction, consumption of bee bread, encounters with old bees within the hive, and contact with hive materials during the early adult stage (Martinson *et al.,* 2012; Powell *et al.,* 2014; Kwong & Moran, 2016).

A deeper understanding of the microbiota of Western honeybees, as well as closely related pollinator species, such as *Apis cerana* and *Bombus terrestris*, is a research field with great

potential benefits, including the development of integrative techniques for their conservation (Romero *et al.,* 2019).

Recent studies have revealed that the gut microbiota of *Apis mellifera* is a unique and completely distinct environment from other insects. The microbiota of *Apis mellifera*, as well as for other social animals, is transmitted by direct contact already from the first social interactions between the members of the colony (Powell *et al.,* 2014). the intestinal environment of this social insect remains conserved from the point of view of the main microbial species, even if an environmental change occurs (Moran *et al.,* 2012); furthermore, the biochemical interactions between host and microorganisms have been seen as highly specialized (Martinson *et al.,* 2011). While species presence is fairly consistent, relative abundances vary slightly but significantly between the castes, hives, developmental stage, and anatomical regions (Jeyaprakash *et al.,* 2003; Martinson *et al.,* 2011, 2012; Moran *et al.,* 2012; Corby-Harris *et al.,* 2014; Powell *et al.,* 2014; Ludvigsen *et al.,* 2015; Kapheim *et al.,* 2015; Jones *et al.,* 2018; Yun *et al.,* 2018).

Over the years, increasingly in-depth studies have revealed differences in the relative abundance of some of the enteric microbial species, most significantly between a queen bee and kelp (Yun *et al.,* 2014; Kapheim *et al.,* 2015; Tarpy *et al.,* 2015) , while these differences were almost nil among worker bees (Martinson *et al.,* 2012; Powell *et al.,* 2014).

Understanding how the microbiota interacts at a community level can be useful in understanding how this community changes according to different external stimuli, and consequently how the host reacts to this change (Romero *et al.,* 2019).

Some commensals of *Apis mellifera* are not found in any other animal species, not even in other bee species (Kwong & Moran, 2016). These intimate symbiotic relationships lead to more rewarding collaborations than less specialized microenvironments, but they also result in significantly greater codependency (Powell *et al.,* 2016; Onchuru *et al.,* 2018; Raymann & Moran, 2018). The maintenance of a constant microbiota in *Apis mellifera* is also given by the eusocial nature of the insect the exchanges between the members of the colony (trophallaxis) allows a transfer of microorganisms, thus contributing to the maintenance of the microbiota of the constant colony (Engel *et al.,* 2012; Martinson *et al.,* 2012; Powell *et al.,* 2014).

Over time, some microbes may have evolved with *Apis mellifera*, bringing various benefits to the host, contributing to the wintering of the colony thus guaranteeing its survival (Engel & Moran, 2013a). The microbial community of *Apis mellifera* is closely linked to immune stimulation (Li *et al.,* 2017), metabolic functioning (Lee *et al.,* 2015; Wang *et al.,* 2015;

Zheng *et al.*, 2016, 2017), and resistance to pathogens (Evans & Armstrong, 2006; Koch & Schmid-Hempel, 2011; Schwarz *et al.*, 2016; Kwong *et al.*, 2017; Li *et al.*, 2017; Raymann & Moran, 2018). Other studies supported culture-dependent methods evidenced a microbiota composed of several bacterial species within the genera *Lactobacillus* and *Bifidobacterium* (Vásquez *et al.*, 2012; Di Gioia and Biavati, 2018) with new identified lactobacilli species (Olofsson *et al.*, 2014). The probiotic properties of those bacteria are notably recognized in vertebrates where *Lactobacillus* and *Bifidobacterium* strains exert beneficial activities within the gut microbiota (Gaggìa *et al.*, 2010).

Acetobacteraceae and *Apilactobacillus kunkeei* thrive in sugar-rich, acidic environments such as the crop, beebread, and honey and are considered core hive bacteria, as they are associated with nurse workers and developing larvae (Anderson *et al.*, 2013).

Olofsson and Vásquez (2008) have discovered a symbiotic lactic acid bacteria (LAB) microbiota in the honey stomach of the Western honeybee, *Apis mellifera* (Olofsson & Vásquez, 2008). This previously unknown microbiota is composed of several phylotypes within the genera *Lactobacillus* and *Bifidobacterium* that play a pivotal role in the production of honey (Olofsson & Vásquez, 2008; Vásquez *et al.*, 2012) and bee bread (Vásquez & Olofsson, 2009), a product that is stored for a long-term period and consumed by both adult honeybees and larvae. The research conducted by Gilliam (1997) has shown that these bacteria are endemic in the digestive tract of adult bees, regardless of the season, but variable depending on the sources of nectar and then based on flowers which bees feed on. The colonization of the intestinal tract is also influenced by the age of the insect; it is known now that the tasks performed by the bee workers during their existence change with age; then carrying out a multiplicity of tasks both outside and inside the hive, even the intestinal microflora change. The larvae of bees, initially sterile, acquire such microflora through the power supply, and social relations inside the hive, (the trophallaxis), before completing their development cycle.

These microorganisms are hosted in the various compartments of the insect gastrointestinal tract, and each specific compartment favors the development of certain microbial species (Figure 2). The bacterial flora "sour", represented by *Lactobacillus* and *Bifidobacterium*, plays a very important role in the life of bees. In fact, such microorganisms are involved in the processes that lead to the conversion of the nectar in honey and the production of bee bread, the latter starting from the pollen, through fermentation processes, also possible thanks to the optimal temperature (35°C) for the development of bacteria and their activities, which is found inside the cells where the bees regurgitate the freshly collected nectar. The

presence of lactic bacteria is also responsible for the well-known antimicrobial properties of honey (Silva *et al.*, 2017).

The research conducted by (Vásquez *et al.*, 2012) has also highlighted the possibility that these bacteria can play a key role in maintaining the health of the insect, protecting it from pathogens, with similar mechanisms highlighted in the human body.

One of the representatives of the gastrointestinal tract of bees is *A. kunkeei*, a newly identified fructolytic bacterial species (Endo *et al.*, 2012). It has been demonstrated (Vàsquez *et al.*, 2012) that *A. kunkeei* is one of the dominant bacterial species in several bees, both farmed and wild. Studies conducted in Malaysia on *Apis dorsata* (Indian giant bee), a highly aggressive species but reared in these areas by the local honey production (Tajabadi *et al.*, 2011) confirmed the presence of these lactic bacteria in association with other different microbes, also confirming the variability of the symbiont microflora based on the floral essences collected by the insect during the year, which has already been demonstrated by Olofsson *et al.* (2014) in *Apis mellifera*. Furthermore, these microorganisms have been found not only in bees but also in the gastrointestinal tract of tropical fruit fly and giant ants (He *et al.*, 2011), whose diets are rich in fructose.

Above other insects, bees are of particular importance, not only for the production of honey and beehive products that man consumes, but also for the task they perform at an ecological level in the pollination of the various botanical essences cultivated by man or spontaneously evolved.

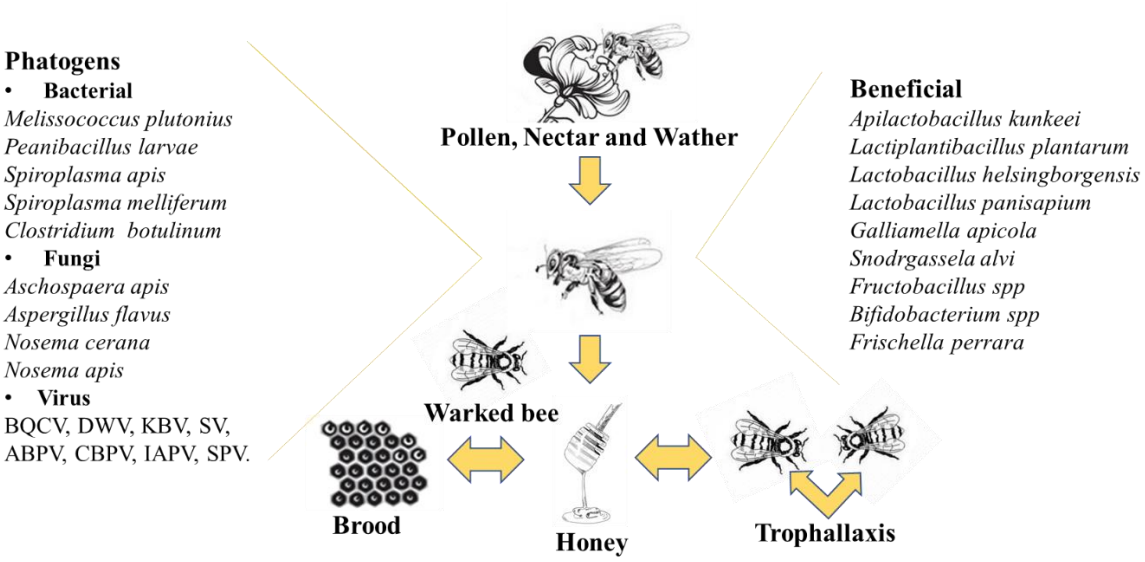

Figure 2. Nectar, pollen and water, are transported inside the hive; then processed and stored as food stocks; thanks to trophallaxis between the worker bees and brood, the transition of the microflora between the different members of the family is carried out. The viruses in the file list are *Black Queen Cell Virus* (BQCV), *Deformed Wing Virus* (DWV), *Kashmir Bee Virus* (KBV), *Sacrobrood Virus* (SV), Acute Bee Paralysis Virus (ABPV), Chronic Bee Paralysis Virus (CBPV), Israel Cute Paralysis Virus (ICPV) and Slow Paralysis Virus (SPV).

1.6 Importance of gut microbiota for the bee's health

Honey bees, like many other insects, lack an acquired immune response but have evolved effective immune responses to cope with infection by pathogens (Evans & Armstrong, 2006; Aggarwal & Silverman, 2008). In recent times, some researches have led to important discoveries into the roles of the gut microbiota in physiology, immunity, behavior, growth, development, and survival of the honey bee host (Kwong *et al.,* 2017; Zheng *et al.,* 2017). Raymann *et al.* (2017) reported that changes in the gut bacteria composition by antibiotics could increase the susceptibility to opportunistic bacterial pathogens and decreased the survivorship of honey bees. Other studies showed that the gut microbiota could affect honey bee growth, sugar metabolism, and pollen digestion (Engel *et al.*, 2012; Zheng *et al.*, 2016, 2017), enhancing the various metabolic functions of gut bacteria that are likely to contribute to bee growth and health (Li *et al*., 2017).

Balanced gut microbiota is necessarily associated with bee health since it provides countless enzymatic activities to make complex sugars available in the honeybees diet. Some studies evidenced that the lactobacilli and bifidobacteria community (LAB) in the crop vary numerically across seasons with the flowers visited by bees and with the health status of bees (Olofsson & Vásquez, 2008). Cox-Foster *et al.*, (2007) demonstrated a high relative abundance of the η-proteobacterial taxa in the bees from CCD-affected hives than in the healthy ones, while the presence of *Firmicutes* and *Alphaproteobacteria*, mainly represented by taxa related to the genus *Lactobacillus* was dramatically reduced in diseased bees. In three species of wild bumblebees, a low presence of *Snodgrassella alvi* and *Gilliamella apicola* strains was associated with a higher incidence of the pathogen *Crithidia* spp. (Cariveau *et al.,* 2014). *Snodgrassella* and *Gilliamella* form biofilm-like layers on the epithelium of the longitudinal invaginations of the ileum; *Snodgrassella* is in direct association with the host tissue followed by a thick layer of *Gilliamella*. Studies on gene functions showed significant enrichment in the categories of several activities associated with the formation of the biofilm on the gut epithelial surface and with the host interaction (Engel *et al.,* 2012). The microbial community of Bombus, which is dominated by *Gilliamella* and *Snodgrassella*, seems to protect the insect against a trypanosome (Koch & Schmid-Hempel, 2011), suggesting a possible role of the biofilm as a protective layer against parasite invasion.

1.7 Antibiotics and probiotics

LAB has been widely studied in animals and humans because of their probiotic properties,

which have led to their well-built commercial exploitation in the food, feed, and pharmaceutical market (Gaggìa *et al.*, 2018).

The bee's digestive system represents an optimal niche for LAB, which is obtained from the bee's diet suitable substrates for their growth. The *in vitro* antagonistic activity toward bee pathogens thank to organic acids and antimicrobial peptides (*M. plutonius, P. larvae,* and *N. ceranae*) is well documented (Yoshiyama & Kimura, 2009; Audisio *et al.,* 2011; Maggi *et al.,* 2013; Baffoni *et al.,* 2016; Iorizzo *et al.,* 2020a; Iorizzo *et al.,* 2020b).

Lactic acid bacteria show interesting properties just like the capability to grow and tolerate acidic pH, to supply organic acids, and to metabolize different sugars (Mozzi, 2016). These features explain the effectiveness of LAB in colonizing the sugar-rich digestive system of bees and suggest a potential for inhibiting the growth of acid-sensitive pathogenic bacteria. Taking under consideration that treatments with formic, lactic, and acetic acids are widely employed by beekeepers to prevent pathogen infections, and, within the light of the ultimate products of their metabolism, LAB may represent natural protecting bee symbionts of considerable importance (Olofsson & Vásquez, 2008).

The transfer of the probiotic concept from vertebrates to invertebrates still requires further considerations, and a number of other questions still got to be investigated and debated. Variation of the honeybee gut microbiota by supplementation of selected strains has originated special attention since it represents a strategy to increase the health status of colonies, both in terms of the presence of beneficial microorganisms within the bee gut, productivity and boosting the colony.

Antibiotics and probiotics can have functional effects on the host by altering the microbiota. There is also evidence that antibiotics can selectively deplete some microbial species more than others, leading to changes in composition (Gilliam, 1997; Raymann *et al.,* 2017).

In some areas, honeybees are continuously exposed to antibiotics treatment used for preventing outbreaks of American or European Foulbrood caused by *Paenibacillus larvae* or *Melissococcus plutonius*, respectively. Among them, oxytetracycline has been used for decades in beekeeping in the USA, and strains of bee gut bacterial species have acquired several tetracycline resistance loci, with high frequencies in colonies more exposed to it (Tian *et al.,* 2012). Tetracycline exposure results in severe gut dysbiosis, with pivotal effects on microbiome size and composition (Raymann *et al.,* 2017). The treatment also increases mortality in the hive, potentially due to greater susceptibility to opportunistic pathogens, as observed in the LAB (Raymann *et al.,* 2017). Some pesticides also impact the honey bee microbiome (Kakumanu *et al.,* 2016). In addition to downregulation of the immune system,

Li *et al.* (2017) also proven thtat the adverse effect of antibiotics on bee health was greater than the damage of *Nosema* infection. Other studies have found direct antagonism between members of the *Apis mellifera* microbial community and pathogens that threaten the host's health. Evans and Armstrong (2006) reported the inhibition of the growth of *Paenibacillus larvae* (the highly virulent bacteria that cause American foulbrood disease) by bacteria isolated from *Apis mellifera* (Kwong & Moran, 2016).

Forsgren *et al.* (2010) observed that bacteria belonging to the genus *Lactobacillus* and *Bifidobacterium*, isolated from *Apis mellifera*, were able to inhibit, *in vitro*, *Paenibacillus larvae*, as well as effectively reduce the number of infected larvae if used as probiotics.

In animals, generally, gut microbiome composition is affected by many factors, including diet, stress, immune responses, aging, and exposure to antibiotics.

The potential use of LAB as probiotics was also supported by Yoshiyama *et al.* (2013), which in their study used LAB isolated from fermented foods as probiotics. Probiotics, which in most cases are represented by bacteria of the genus *Lactobacillus*, have shown several positive effects, including an increase in honey production (Sabate *et al.*, 2012; Audisio, 2017; Fanciotti *et al.*, 2018), greater stimulation of the immune system (Evans & Lopez, 2004; Yoshiyama *et al.*, 2013), and greater stimulation in the brood of eggs from part of the queen bees, and a greater number of individuals in the colony (Audisio & Benítez-Ahrendts, 2011; Sabate *et al.*, 2012; Audisio *et al.*, 2015). There have also been recent findings on the contribution to the digestion and metabolism of the *Apis mellifera* diet (Lee *et al.*, 2015; Zheng *et al.*, 2016), including pectin (Engel *et al.*, 2012) and lignin (Rokop *et al.*, 2015) degradation.

These experiments are based on combining *in vitro* analyses of intestinal bacteria with *in vivo* experiments (gnotobiology) to characterize the functionality of individual strains and the microbiological community as a whole. The collective metabolic functions of the microbiota produce a significant positive effect on *Apis mellifera*, such as body weight gain, sensitivity to sucrose, and production of prostaglandins and vitellogenin (Kešnerová *et al.*, 2017; Zheng *et al.*, 2017).

Taxonomy and microbial abundance of a honey stomach and middle intestine have been shown to change with the season, and therefore with diet (Corby-Harris *et al.*, 2014; Ludvigsen *et al.*, 2015; Martinson *et al.*, 2012). Although the gut microbiota consistency is minimally affected by environmental factors (Martinson *et al.*, 2011; Kwong & Moran, 2016), this hypothesis has not been rigorously tested. This is partly due to the considerable difficulty involved in limiting the *Apis mellifera* diet to the plants of interest. *Apis mellifera*

is highly stressed when kept in captivity, and mitigating feeding behavior with laboratory-administered flower nectars and pollen further reduces the applicability of laboratory studies (Huang *et al.*, 2014).

The identification and characterization of the function of the bees' gut microbiome are not yet complete. Given that representative genomes of the honey bee microbiota have been sequenced, there is considerable variation between strains, indicating the existence of large pangenome within each microbial species that encode a diversity of genes and functionalities (Zheng *et al.*, 2016).

The main purpose of artificial feeding of beneficial bacteria to bees is to combat the most important pathogens affecting bees, both at larval and adult stages. *In vitro* tests evidenced interesting host protection properties by directly stimulating the bee's immune system and inhibiting pathogens through competitive exclusion and antimicrobial compound production (organic acids and secondary metabolites). Applications addressed to infected larvae showed a significant reduction of larvae mortality after supplementation of different beneficial bacteria. Forsgren (2010) successfully applied, in honeybee larvae previously infected with two different spore concentrations of *P. larvae*, a mixture of beneficial bacteria isolated from the social stomach, that is, *A. kunkeei*, *L. mellis*, *L. kimbladii*, *L. kullabergensis*, *L. helsingborgensis*, *L. melliventris*, *L. apis*, *L. mellifer*, *B. asteroides*, and *B. coryneforme*, with a final concentration of 10^7 bacteria/mL.

As already mentioned, the use of beneficial bacteria commercially exploited in humans and animals has also been tested. An improved wax gland cell development was observed by Pătruică *et al.* (2012) after the supplementation of organic acids and a probiotic product containing *Lactobacillus* and *Bifidobacterium* spp. Both individually and in combination, they positively influenced the number, morphology, and diameter of the wax cells (Gaggìa *et al.*, 2018). Surprisingly, Andrearczyk *et al.* (2014) found an increase of *Nosema* spp. infection, following administration in both winter and summer bees of a probiotic product, recommended for animals.

The production of antimicrobial compounds by gut symbionts for host protection is another interesting topic. A recent genomic analysis of 13 LAB strains, isolated from the honey crop, put in evidence that most of them produced extracellular proteins of known/unknown function related to antimicrobial action, host interaction, or biofilm formation. In particular, a putative novel bacteriocin with 51% homology with helveticin J was detected in *L. helsingborgensis* Bma5N (Butler *et al.*, 2013). At the same time, it has to be said that some strains did not evidence antimicrobial functions, thus confirming the high variability among

the gut microorganisms inhabiting the same niches. Vásquez *et al.* (2012) analyzed the interaction of some LAB symbionts with the honey crop by SEM and fluorescence microscopy. The resulting images evidenced biofilm formation and structures resembling extracellular polymeric substances (EPS), which are known to be involved in host protection/colonization and cellular recognition (Flemming & Wingender, 2010).

The use of antimicrobials to combat different pathogens is emerging as it is less invasive than normal antibiotic treatments, but it must be said that research on this front is still far from completion, although preliminary results are very promising. Nowadays, beekeepers too often rely on subspecies hybrids, with the false hope to increase disease resistance, but the resistance mechanisms against bee pathogens/parasites are usually a result of coevolution in local ecosystems (Ruottinen *et al.*, 2014).

Data available in the literature provide insight into the positive effect of these microorganisms on bee health. However, the main issue is how the modulation of the honeybee gut microbiota could influence the composition of the gut microbiota itself and also host immunity and physiology.

Chapter 2: *Lactobacillus* and probiotic cultures

2.1 Probiotics

The current definition of probiotic, formulated in 2002 by FAO (Food and Agriculture Organization of the United Nations) and WHO (World Health Organization) working group experts, states that they are "live strains of strictly selected microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). The definition was maintained by the International Scientific Association for Probiotics and Prebiotics (ISAPP) in 2014 (Hill *et al.,* 2014).

The criteria for the selection and assessment of probiotic microorganisms are the result of the collaboration between research institutions and universities with food industries. Markowiak *et al.* (2017), according to WHO, FAO, and EFSA suggestions, have identified the safety and functionality criteria, including the technological usefulness, of potential probiotic strains. Probiotic characteristics are not associated with the genus of a microorganism, but with few and specific strains belonging to a particular species (Hill *et al.,* 2014).

The beneficial effects of probiotics must be documented and must be consistent with the characteristics of the strain present in the marketed good. The strains with beneficial properties most frequently used belong to the genera *Bifidobacterium* and *Lactobacillus*. However, it must be considered that the probiotic potential of different bacterial strains, even within the same species, differs. Different strains of the same species are always unique and may have different areas of adherence (site-specific), specific immunological effects, and their action on healthy versus an inflamed mucosal milieu may be different (Soccol *et al.,* 2010).

Probiotics have numerous advantageous functions in animal organisms. Their main advantage is the effect on the development of the microbiota inhabiting the organism ensuring a proper balance between pathogens and the bacteria that are necessary for the normal functions of the organism (Oelschlaeger, 2010).

Probiotic microorganisms compete with pathogens for nutrient sources, adhesion sites, and space in colonized environments. They prevent the adherence of the pathogenic bacteria to the host cells by strengthening the barrier effect of the intestinal mucosa (Mangell *et al.,* 2002) and release gut-protective metabolites (arginine, glutamine, short-chain fatty acids, and conjugated linoleic acids) (Syngai *et al.,* 2015). Probiotic acts as an antimicrobial by

secreting the products called bacteriocins and substances such as organic acids (lactic, acetic, and butyric acid) and H_2O_2 (De Keersmaecker *et al.,* 2006). They also lower the intestinal pH, agglutinates pathogenic microorganisms, binds and metabolize toxic metabolites (Haskard *et al.,* 2001) or regulate intestinal motility (Marteau *et al.,* 2002) and mucus production (Vrese & Marteau, 2007). However, the transfer of the probiotic concept from vertebrates to invertebrates still requires further considerations, and several questions still need to be investigated and debated (Alberoni *et al.,* 2016).

2.2 Probiotic properties

2.2.1 Antimicrobial activity

Host protection is another important aspect that is frequently associated with balanced gut microbiota. It is a fact that different stress factors, such as parasites/pathogens, deficient nutrition, and pesticides, can cause immunosuppression (Antúnez *et al.,* 2009; Alaux *et al.,* 2010; Anbutsu & Fukatsu, 2010; Di Prisco *et al.,* 2013).

The honey bee has a simpler immune system compared to other model insects (Barribeau *et al.,* 2015), in favor of more convenient and less expensive social defence strategies (Cremer *et al.,* 2007). However, a big contribution to host protection is provided by the antagonistic activity of the gut microbiota and its interaction with the humoral and systemic immunity (Dillon *et al.,* 2005; Jaenike *et al.,* 2010).

Given that individual and social defence mechanisms are diverse and complex, one of the main effectors of the innate immunity in the honey bee is represented by antimicrobial peptides (AMPs), whose synthesis is under the control of the Toll and Imd signaling pathways (Lemaitre & Hoffmann, 2007). Antimicrobial activity is mainly achieved through alteration of the microbial membrane properties (Imler & Bulet, 2005) and intracellular metabolic processes (Brogden, 2005).

Evans and Pettis (2005) showed a higher abaecin (an antimicrobial peptide, AMP) expression in colonies with a lower incidence of *Paenibacillus larvae* (AFB). Jefferson *et al.* (2013) also found a strong positive correlation between the amount of total honey bee gut bacteria and transcript levels of two AMPs, defensin-1, and apidaecin. The hypothesis that the resident gut microorganisms may determine a basal immune response to regulate its proliferation and consequently harmful microorganisms through AMP synthesis has not yet be investigated in honey bees; however, studies on *Drosophila melanogaster* and *Anopheles mosquitoes* enter that direction (Jefferson *et al*., 2013).

A recent genomic analysis of 13 LAB strains, isolated from the honey crop, performed by Butler *et al.* (2013), put in evidence that most of these strains produced extracellular proteins of known/unknown function related to antimicrobial action, host interaction, or biofilm formation. At the same time, some strains did not evidence any "antimicrobial function", thus confirming the high variability among the gut microorganisms inhabiting the same niches (Alberoni *et al.,* 2016).

In addition, the action of several bacterial strains belonging to the Bacillaceae family, isolated from the stomach of bees, were also observed to show a strong in vitro antibacterial activity against bee pathogens. In this case, it is known from decades that inhibition activity is mainly due to the production of antibiotic molecules (lipopeptides and iturin-like lipopeptides) (Alippi & Reynaldi, 2006; Lee *et al.,* 2009; Yoshiyama & Kimura, 2009; Sabaté, Carrillo, & Audisio, 2009).

2.2.2 Cell surface properties: biofilm formation

Bacterial cells exhibit two types of growth modalities, namely planktonic cell and sessile aggregate which is known as a biofilm. Biofilm is an association of microorganisms in which cells attach on a living or non-living surface enclosed within a self-produced matrix of extracellular polymeric substance (Hall-Stoodley *et al.,* 2004). Biofilm formation is a very complex process, in which the cells of microorganisms are transformed from a planktonic to a sessile growth mode (Okada *et al.,* 2005). It is a multi-step process that begins with attachment to a surface, thus the formation of micro-colonies which leads to the formation of the three-dimensional structure and finally ends with the maturation followed by detachment. During biofilm formation, many species of bacteria can communicate with each other through a specific mechanism called quorum sensing that is a system of stimuli for coordinating the different gene expressions. Biofilm formation appears to be a survival mechanism that provides microorganisms with critical advantages, including greater access to nutritional resources, enhanced organism interactions, and greater environmental stability. Interactions of microorganisms in the biofilm matrix facilitate metabolic cooperation and genetic exchanges. Furthermore, microbial biofilms can thrive in extreme or hostile environments where individual microorganisms would have difficulty not only growing but also surviving (Dang & Lovell, 2016). In biofilms, bacterial cells exhibit 10 to 1.000 times less susceptibility to specific antimicrobial agents compared with their planktonic counterparts (Gebreyohannes *et al*., 2019).

This reduced susceptibility is caused by a combination of different factors, namely:

- poor antibiotic penetration into the polysaccharide matrix;
- the arbitrary presence of cells showing a resistant phenotype (known as “persisters”);
- the presence of either non-growing cells or cells that triggered stress responses under unfavorable chemical conditions within the biofilm matrix (Balcázar *et al.*, 2015).

2.3 Lactic Acid Bacteria and genus *Lactobacillus*

Lactic Acid Bacteria (LAB) constitute a group of gram-positive bacteria united by a hight number of morphological, metabolic, and physiological characteristics. The general description of the bacteria included in the group is gram-positive, non-sporing, non-respiring cocci or rods, which produce lactic acid as the major end product during the fermentation of carbohydrates. The LAB term is intimately associated with bacteria involved in food and feed fermentation, including related bacteria normally associated with the (healthy) mucosal surfaces of humans and animals.

At March 2020, the genus *Lactobacillus* comprised 261 species that are extremely diverse at phenotypic, ecological and genotypic levels. Zheng *et al.* (2020) evaluated the taxonomy of *Lactobacillaceae* and *Leuconostocaceae* on the basis of whole genome sequences. Parameters that were evaluated included core genome phylogeny, (conserved) pairwise average amino acid identity, clade-specific signature genes, physiological criteria and the ecology of the organisms (Zheng *et al.*, 2020). Based on this polyphasic approach, a reclassification of the genus *Lactobacillus* into 25 genera was proposed, including the emended genus *Lactobacillus*, which includes host-adapted organisms that have been referred to as the *Lactobacillus delbrueckii* group, *Paralactobacillus* and 23 novel genera or which the names *Holzapfelia, Amylolactobacillus, Bombilactobacillus, Companilactobacillus, Lapidilactobacillus, Agrilactobacillus, Schleiferilactobacillus, Loigolactobacilus, Lacticaseibacillus, Latilactobacillus, Dellaglioa, Liquorilactobacillus, Ligilactobacillus, Lactiplantibacillus, Furfurilactobacillus, Paucilactobacillus, Limosilactobacillus, Fructilactobacillus, Acetilactobacillus, Apilactobacillus, Levilactobacillus, Secundilactobacillus* and *Lentilactobacillus* were proposed (Zheng *et al.*, 2020). The amendment to the description of the family *Lactobacillaceae* to include all genera that were previously included in families *Lactobacillaceae* and *Leuconostocaceae* was also proposed, but the generic term “lactobacilli” was considered useful to designate all organisms that were classified as *Lactobacillaceae* until 2020 (Zheng *et al.*, 2020).

The reclassification proposed by Zheng *et al.*(2020) reflects the phylogenetic position of the microorganisms, and groups lactobacilli into robust clades with shared ecological and

metabolic properties, as exemplified for the emended genus *Lactobacillus* encompassing species adapted to vertebrates (such as *Lactobacillus delbrueckii, Lactobacillus iners, Lactobacillus crispatus, Lactobacillus jensensii, Lactobacillus johnsonii* and *Lactobacillus acidophilus*) or invertebrates (such as *Lactobacillus apis* and *Lactobacillus bombicola*).

2.4 Main sources of LAB isolation

LAB are an industrially important group of bacteria and are used as starter cultures for fermented milk products (yogurt and some cheeses) in the dairy industry, fermented meat (sausage, cured meat), and fermented vegetables.

Natural habitats where it is possible to find LAB, including the indigenous flora of raw milk and dairy products such as cheeses, yogurts, and fermented milk, can be a good source of novel LAB strains with the potential desirable properties for use in the production of novel fermented dairy products (Delavenne *et al.*, 2012; Perin & Nero, 2014; Tulini *et al.*, 2016; Wassie & Wassie, 2016; Aspri *et al.*, 2017; Perin *et al.*, 2019). The most frequently isolated LAB genera from raw milk and dairy products that were made from raw milk were *Enterococcus, Lactococcus, Lactobacillus, Leuconostoc*, and *Streptococcus* (Franciosi *et al.*, 2009). In another study, lactococcal strains were isolated from raw milk in the Camembert cheese area and identified by using both phenotypic criteria (physiological and biochemical tests) and genotypic (RAPD) criteria.

Without yet understanding the scientific basis, hundreds of years ago humans used lactic acid bacteria to produce fermented foods, noting that in such a way improved the shelf life of the food product, and in addition were exalted flavors and aromas that in normal conditions were not expressed. Similarly, today, these important microbial allies are widely used to produce a wide variety of fermented milk products including beverages such as kefir and semi-solid products such as yogurt. The preparation of these foods involves the development of these microbes during which the milk sugar, lactose, is converted into lactic acid. Following the accumulation of the acid, the structure of the milk proteins changes (curdling), and consequently the consistency of the product also changes.

Another LAB isolation tank is represented by meat and derivates (Drosinos *et al.*, 2005; Aro *et al.*, 2010; Mejri & Hassouna, 2016). As in the dairy industry, the production of cured meats in the meat industry is entrusted to microorganisms, naturally present or inoculated in the medium through starter cultures.

In modern times, the control of the processing conditions and quality of fermented meat products has greatly improved, especially because of the use of specific microorganisms

such as the LAB (Bis-Souza *et al.*, 2019; Bis-Souza *et al.*, 2020). The use of starter cultures can bring several benefits in comparison to spontaneous fermentation: better control of the fermentation, reduce the ripening time, reduce the possibility of pathogenic microorganism growth, and also improve the preservation of quality between batches (Laranjo *et al.*, 2017; dos Santos Cruxen *et al.*, 2019). The strategic selection of starter cultures consists of evaluating indicators: the fast and protracted colonization of meat mass, the production of organic acids (especially lactic acid), the inhibition of competitive microbiota (both spoilage and pathogenic microorganisms), prevailing at a reduced water activity (a_w), and also preserving or enhancing the sensory attributes of fermented meat product (Vinicius De Melo Pereira *et al.*, 2020).

The LAB are defined as ubiquitous and given the present importance that the buyer plays on so-called functional foods, it pushes researchers in isolating new microorganisms potentially useful even by non-common matrixes, for example in *Apis mellifera* (Gilliam, 1997; Olofsson & Vásquez, 2008) and beehive products (bee bread), which can be used not only as starter cultures in the food industry but also as potential probiotic microorganisms.

2.5 *Lactiplantibacillus plantarum*

Originally, *Lactiplantibacillus plantarum* was designated as *Streptobacterium plantarum* by Orla-Jensen (1919) and Holland (1920) it had been further described by Pederson (1957) as a typical LAB capable of using vegetables as a substrate. Based only on biochemical identification and before application of the genetic approach within the 1980s, *Lactobacillus pentosus*, *Lactobacillus arabinose*, *Lactobacillus rudensis*, and *L. plantarum* var. *mobilis* had been considered because the same species, thank to similarities in their phenotypic characteristics. *L. plantarum* is a microorganism with rod morphology, approximately 0.9–1.2×1.0–8.0 μm in size which occurs singly or grouped in briefly chains. *L. plantarum* is a heterofermentative, facultative anaerobe microorganism classified in Group B (Kandler and Waiss 1986). Fermentation of hexoses via the EMP metabolic pathway results in the formation of D- and L-lactic acids. On the other hand, pentoses are fermented to form lactic and acetic acid in the presence of inductive phosphoacetolase (Kandler *et al.*, 1986). In *L. plantarum*, both L- and D-stereospecific NAD-linked dehydrogenases operate with equal activity in metabolizing cells. Trace amounts of acetone, diacetyl, and 2,3- butanediol are produced. Pentoses are utilized by the induction of phosphor-ketolase, producing lactic and acetic acids. *L. plantarum* converts pyruvate to acetyl phosphate and carbon dioxide with the formation of hydrogen peroxide in the presence of flavin-containing pyruvate oxidase.

Acetyl phosphate is used in the production of ATP. The growth of *L. plantarum* can be stimulated by the inclusion of pyruvate as a component of complex and synthetic media. When growing in complex media, *L. plantarum* produces diacetyl, acetone, and carbon dioxide. Production of diacetyl is stimulated by high concentrations of glucose in the growth medium. The presence of carbohydrates can also contribute to other reactions, as sucrose also can be used as a substrate for the formation of polysaccharides (Todorov & Franco, 2010). *L. plantarum* can transform malic acid to carbon dioxide and thus find application within the wine industry for facilitating malo-lactic fermentation (Lonvaud-Funel, 1999).
L. plantarum is the most versatile species/strain with useful properties and is usually found in numerous fermented food products (Guidone *et al.*, 2014). Is a heterofermentative microaerophilic Gram-positive, catalase-negative, with rod morphology, occurring singly or grouped in short chains. *L. plantarum* is a mesophilic strain with the ability to grow at temperatures from 15°C up to 45°C (Kandler *et al.*, 1986). Good growth was recorded in the presence of 4 to 6% NaCl and at pH values between 4 and 9 (Kandler and Waiss 1986). Due to these characteristics, *L. plantarum* has been successfully isolated from various ecological niches such as different plant materials, milk, meat products, fish, and the stomach and intestinal tract of humans and animals (Kandler *et al.*, 1986; Balcázar *et al.*, 2008; Jiang *et al.*, 2016). This species has well-accepted GRAS (*Generally Recognised As Safe*) status and various strains of *L. plantarum* are isolated from different ecological niches including meat, fish, fruits, vegetables, milk, and cereal products (Todorov & Franco, 2010).

2.6 *Apilactobacillus kunkeei*

The name *Apilactobacillus kunkeei* (*A. kunkeei*) has been attributed to a replacement microbial species in honor of Dr. Ralph E. Kunkee, professor at the University of California at Davis (UCD), for his great contribution to the microbiology of wines. This new bacterial species was first identified by Edwards *et al.* (1998) by isolating the HY-15^{T} strain (type strain) from Cabernet Sauvignon wine which had undergone slow alcoholic fermentation. Cells are Gram-positive, rods approximately 0.5×1-1.5 mm. Colonies on MRS agar appear opaque, concave, and approximately 1-2 mm in diameter after 3 days of growth at 25°C. Facultatively anaerobic. They are obligate troctolitic lactic bacteria, as they prefer D-fructose to D-glucose as a carbon source, a feature not immediately highlighted, but only later emerged in the study performed by Endo *et al.* (2012); the presence of pyruvate, oxygen, and fructose, used as external electron acceptors, strengthen the growth of these microbes into glucose the growth of glucose in broth (liquid medium) is generally slow especially in

conditions of anaerobiosis, it is more rapid in the presence of fructose as reported in the research conducted by Neveling *et al.*, (2012). The strains produce nearly equimolar amounts of lactic acid and acetic acid, plus some traces of ethanol. D and L-lactic acids are produced in a 2:8 ratio from glucose, while the production of mannitol takes place by fructose. They do not produce ammonia from arginine and are unable to reduce nitrate. The carbohydrates fermented by this bacterial species are D-Fructose, D-Glucose, D-Raffinose (weakly), and sucrose, as far as mannitol is concerned it was fermented in the API 50 CHL Medium test but not using the modified medium by Jensen and Edward (1991); Esculin is not hydrolyzed, and also dextran is not produced from sucrose. These bacteria are unable to ferment N-acetylglucosamine, arabinose, arabitol (or Xylitol), plus some pentoses and hexoses, including galactose (Endo *et al.*, 2012). They are facultative anaerobic bacteria, whose growth capacity in MRS has been seen at pH 3.7 - 4.5 and 8.0 (at 25°C), the range of development temperatures is between 15 and 37°C, they are unable to grow at temperatures of 5°C and 45°C; furthermore, weak growth was found in an environment containing 5% NaCl. Bacterial cells have peptidoglycan type A4α (L-Lys-D-Asp) the amino acid L-lysine is present within the structure, while D-aspartic acid is a component of the characteristic structure of peptide bridges, this complex of elements thus described has been found in many *Lactobacillus* and *Pedicoccus*; also, the analysis on bacterial DNA showed a high number of G+C which varies from 36-37 mol%. The importance of this bacterial species was highlighted in the research conducted by Billiet *et al.* (2017), through artificial administration of *A kunkeei* and *L. crispatus* in Bumblebees (*Bombus Terrestris*) raised as pollinators; the positive correlation has been demonstrated in improving the vital conditions of bumblebees, favoring the digestive processes of raw pollen, the microorganisms supply certain enzymes capable of dissolving the thick outer wall of the pollen, thus making nutritional elements available (proteins and amino acids above all) in larger quantities (Roulston & Cane, 2000); furthermore, the research conducted by Rangberg *et al.* (2015) has shown that *A. kunkeei*, inoculated in *Apis mellifera*, can exert an antagonistic action against various pathogenic organisms of bees.

This bacterial species prefers environments rich in fructose it is possible to seek out in wine (Edwards *et al.*, 1999; Edwards *et al.*, 2000), on flowers, in honey, and on fruit. From the results obtained within the research carried out by Neveling *et al.* (2012), *A. kunkeei* is that the dominant species in nature among obligate fructolytic bacteria.

Chapter 3: Antimicrobical activity of lactic acid bacteria against bee diseases

3.1 Introduction

The eusocial nature of *Apis mellifera ligustica* has always facilitated the upkeep of relatively constant gut microbiota. this is often thanks to interactions among individuals within the hive environment, and mainly to trophallaxis. This term refers to the direct transfer of food or fluids from one individual to another; it's especially common among social insects like honeybees. along side nutrients, trophallaxis also allows the horizontal transmission of gut bacteria (Powell *et al*., 2014; Kwong & Moran, 2016). The stomachs of honeybees are filled with nutrients and are therefore a positive environment for symbiotic microorganisms. These participate in various processes, including food digestion, detoxification of harmful molecules, supply of essential nutrients, participation within the host defence system, and protection from pathogens and parasites. The gut microbiota is often influenced by various factors which will cause dysbiosis, including temperature, nutritional deficiencies, pesticides, parasites, or pathogens (Cox-Foster *et al*., 2007; Raymann *et al*., 2017). Gut microflora alteration may have a robust negative impact on bee immune defense, metabolism, and cognitive mechanisms (Hamdi *et al*., 2011). The honeybee intestine, which functions in digestion and food processing, is additionally the location of infections caused by pathogens like *Ascophaera apis* and *Paenibacillus larvae* (Hamdi *et al*., 2011; Raymann *et al*., 2017).

3.2 Main diseases in honeybees

Honeybees are attacked by numerous pathogens such as viruses, bacteria, fungi, and parasites who pose a significant threat to bees health. Due to the vital role honeybees play in the pollination of crops, fruit, and wildflowers, factors affecting bee health also affect sustainable and profitable agriculture as well as many non-agricultural ecosystems. Before the arrival of the parasitic mite, *Varroa destructor*, the economically most important diseases of honeybees worldwide were the bacterial brood diseases *American Foulbrood* (AFB), C*halkbrood* disease, and *European Foulbrood* (EFB).

- ***Paenibacilluls larvae:*** etiological agent of American Foulbrood

AFB is still among the most deleterious bee diseases (Hansen & Brødsgaard, 1999; Genersch, 2010). It not only kills larvae but is also potentially lethal to infected colonies.

Under normal beekeeping conditions, AFB is highly contagious since the spread of the disease is facilitated by exchanging hive and bee material between colonies (Hansen & Brødsgaard, 1999; Genersch, 2010; Ebeling *et al*., 2016).

The causative agent of AFB is that the Gram-positive, spore-forming bacterium *Paenibacillus larvae* (Genersch *et al*., 2006). The extremely tenacious endospores are the only infectious form of this organism. The spores are infectious only for larvae; adult bees do not become infected upon ingestion (Hitchcock *et al*., 1979). Larvae are most susceptible to infection during the first larval stages, i.e. 12-36 h after egg hatching. During this time window, the oral uptake of a dose of about ten spores or fewer via contaminated larval food is sufficient to successfully initiate a fatal infection (Genersch *et al*., 2006). Ingested spores pass through the foregut and germinate in the larval midgut around 12 h after ingestion (Yue *et al*., 2008; Stephan *et al*., 2020). Following ingestion, through spore-contaminated food, the spores germinate within the larval midgut lumen, where the vegetative bacteria massively proliferate before eventually breaching the midgut epithelium and invading the hemocoel, causing the death of the larvae, which during their decay releases an outsized number of spores (Hrabak, 2007; Yue *et al*., 2008). *P. larvae* contain active enzymes of the Embden-Meyerhof-Parnas, pentose phosphate, and Entner-Doudoroff pathways involved in carbohydrate metabolism and that it can metabolize different sugars including glucose and fructose to support vegetative growth (Neuendorf *et al*., 2004).

The spores remain infectious for more than 35 years and withstand heat, cold, drought, and humidity (Haseman, 1961). It is the tenaciousness of the spores and the production of extremely high numbers of spores in diseased colonies that make the effective control of AFB so difficult.

Within the further course of the disease within the colony, more and more larvae become infected and die in order that within the end, the shortage of brood and, hence, the shortage of progeny results in the collapse of the whole colony (Hansen & Brødsgaard, 1999; Ebeling *et al.*, 2016;). *P. larvae* often remain dormant in its spore-form and don't induce manifestation of AFB. It's been suggested that *P. larvae* may exist as a pathobiont within the native microbiota of adult worker bees and is then transmitted throughout the hive to fresh brood cells (Genersch, 2010).

Currently, since an efficient therapy against AFB isn't available, the authorities consider the burning of infected colonies because the only efficient control measure (Williams, 2000; von der Ohe, 2003; Pernal *et al.*, 2008). Over the previous couple of years, several different measures like the utilization of chemical fungicides, antibiotics, heterocyclic organic

compounds (indoles) and bacteriophages are tried against AFB disease (Kochansky *et al.*, 2001; Spivak & Reuter, 2001; Elzen *et al*., 2002; Beims *et al*., 2015; Alvarado *et al.*, 2017). Unfortunately, these approaches might be useful as therapy but are often ineffective for prophylactic purposes, and hives remain susceptible to diseases. Moreover, the prophylactic use of antibiotics has inevitably led to the onset of antibiotic resistance in *P. larvae* (Miyagi *et al*., 2000; Evans, 2003). Additionally, the utilization of chemical compounds should be limited, both because they're dangerous to honeybee health (Raymann *et al*., 2017) and since any residues present in honey also pose a significant risk to human health (Bargańska *et al.*, 2011). The utilization of natural compounds for disease control could represent a more suitable alternative (Grady *et al*., 2016; Alonso-Salces *et al*., 2017). Essential oils and other vegetable extracts from plants, herbs and spices exhibit antimicrobial activity against *P. larvae* (Flesar *et al*., 2010; Chaimanee *et al*., 2017) and this activity is especially thanks to the presence of phenolic and terpenoid compounds, which have well-known antimicrobial activity (Daglia, 2012; Solórzano-Santos & Miranda-Novales, 2012; Testa *et al*., 2019). However, the consequences of those substances on honeybee health and its symbiotic microflora aren't entirely known (Selma *et al*., 2009). Currently, there's an increased interest in investigating new, effective and safe control methods.

- ***Ascosphaera apis:*** **etiological agent of Chalkbrood disease**

Another disease most important is the chalkbrood, caused by the fungus *Ascosphaera apis* (Wynns *et al.*, 2013).

The fungus *Ascosphaera apis*, belonging to the heterothallic *Ascomycota* phylum, is a major and widespread pathogen of honeybee (*Apis mellifera*) broods, causing chalk-brood 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). The severity of the disease depends on various factors like environmental conditions, the genetic background and general health status of the honeybees, and therefore the virulence level of the fungal strains (Aronstein & Murray, 2010; Vojvodic *et al*., 2012; Engel & Moran, 2013b). 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).

Infections of *Ascosphaera* occur through the gut rather than externally through the cuticle (Wynns *et al.*, 2012). Honeybee larvae are initially infected by ingesting food contaminated by sexual spores of *A. apis*. The ascospores germinate within the anaerobic environment of

the alimentary tract, and therefore the hyphae of the mycelium subsequently penetrate the intestinal walls of the larvae and deprive them of nutrients (Bamford & Heath, 1982; Aronstein & Murray, 2010; Evison, 2015). After a couple of days, the fungus becomes visible as a fluffy white growth covering the larvae. *Ascosphaera* produces unique fruiting bodies comprised of spore balls held within a double-walled spore cyst, called cleistothecia. These develop on the cuticle and turn the larvae greenish-brown, grey, or black (Maxfield-Taylor *et al.,* 2015).

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; Iorizzo *et al.,* 2021). Honeybees have several defense mechanisms to resist chalkbrood disease, including hygienic behavior (Spivak & Reuter, 1998). However, if the potentially sporulating chalkbrood mummies are removed, hygienic behavior can increase rather than decrease transmission by exposing more individuals to the spores (Invernizzi *et al.,* 2011). Besides, social insect species, such as *A. mellifera*, exhibit behaviors 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, like 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 (Iorizzo *et al*., 2021). Outbreaks may be increased by the disruption of the beneficial microbial community within a colony (Engel & Moran, 2013a). Chalkbrood can cause a discount in honey production and a high percentage of larvae deaths, with significant economic consequences for beekeepers (Zaghloul *et al*., 2005; Aronstein & Murray, 2010; Vojvodic *et al.,* 2012).

- ***Melissococcus plutonius:*** **etiological agent of European foulbrood**

European foulbrood (EFB) is an economically important disease of honey bee (*Apis mellifera ligustica*) larvae caused by the anaerobic Gram-positive lanceolate bacterium *Melissococcus plutonius* (Bailey & Collins, 1982).

EFB is well distributed across every continent that honey bees inhabit (Matheson, 1993). Generally, *M. plutonius* is ingested with the food contaminated by the larvae under the age of 48 hours and multiplies in the middle intestine. Diseased larvae are sometimes removed from the colony by nurse bees. Some larvae die after being capped and are unable to transition to the pupal stage but eliminate the feces containing *M. plutonius*. Others, on the other hand, can pupate and develop to the adult stage, leaving the infected material in the

cell. EFB affects mainly unsealed larvae and kills them at the age of 4-5 days and in severe cases entire colonies are often lost (Ansari *et al*., 2017). The dead larvae turn yellowish, then brown, decompose, and become watery (Forsgren, 2010). The larval remains often give off a foul or sour smell due to secondary invaders, such as *Enterococcus faecalis* and *Paenibacillus sp*. (Arai *et al.,* 2012; Ansari *et al.,* 2017). Although the bacterium is the main cause of this disease, it is not the only factor that comes into play. A weak family under various stresses will tend to be more prone to the onset of the disease than strong families who have a good tendency to cleanliness. Moreover, the beekeeper also plays its role, especially when it creates imbalances between brood and adult bees. Other concomitant causes are food shortages and climatic-environmental factors. This disease can occur all year round, although it is more frequent in the spring season. Although *M. plutonius* is a non-spore-forming bacterium, it can survive in unfavorable conditions and remain infectious for several years in the wax (Cai & Collins, 1994). However, it is much less resistant to disinfection than spore-forming bacteria such as *Paenibacillus larvae*, the causative agent of American foulbrood. EFB did not create serious problems in many European countries since many infected and diseased colonies spontaneously recovered from the disease (Bailey, 1968). Nevertheless, a dramatic increase in the incidence of EFB has been recently observed, in particular in the United Kingdom, Switzerland (Wilkins *et al.,* 2007; Roetschi *et al.,* 2008), and Norway (Dahle *et al.,* 2011).

The protection against pathogens and/or parasites is yet another important trait frequently associated with a balanced intestinal flora. More specifically, microorganism's symbionts may play a role in the protection of their host by either stimulating the immune system of bees or inhibiting pathogens and parasites through the production of antimicrobial compounds. The presence of lactic acid bacteria (LAB) in the honeybee digestive system has been consistently reported in the literature, they are some of the most important intestinal symbionts of the honeybee. The use of intestinal microbial symbiont, such as dietary supplementation, can improve the health status of bees and increase their productivity, stimulating the immune defences and carrying out an antimicrobial action towards the unwanted and pathogenic microflora (Alberoni *et al.,* 2018; Iorizzo *et al.,* 2020b; Iorizzo *et al.,* 2020c; Romero *et al.,* 2019). In particular, Tejerina *et al.* (2021) recently demonstrated that the application of LAB (*L. melliventris, L. helsingborgensis* and *A. 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 diseases.

3.3 Antimicrobial properties of probiotic *Lactobacillus*

There is increasing knowledge on both the composition and therefore the functions of the honeybee gut microbiota, which has led to the invention of evidence of a link between balanced gut microbiota and honeybee health (Cox-Foster *et al*., 2007; Hamdi *et al*., 2011; Alberoni *et al*., 2016; Raymann & Moran, 2018). Especially, there's some evidence that *A. mellifera* gut microbiota may exhibit antifungal activity against *A. apis* e *P. larvae* (Sabaté, Carrillo, & Audisio, 2009; Li *et al*., 2012; Omar *et al*., 2014). In a stimulating review, Gaggìa *et al*. (2018) provided an summary of beneficial microorganism applications for the treatment of the most honeybee pathogens and their benefits in beekeeping production systems. For both infections, the utilization of probably probiotic bacteria within the prevention and biocontrol of bee pathogenic microorganisms offers interesting perspectives (Alberoni *et al*., 2016). The utilization of probably probiotic bacteria, unlike synthetic or natural chemical compounds, doesn't adversely affect the balance of gut microbiota and honeybee health (Crotti *et al*., 2012; Raymann & Moran, 2018). Moreover, the protection against pathogens and/or parasites is one among the aspects frequently related to a balanced microorganism (Cox-Foster *et al*., 2007; Wu *et al*., 2013; Fowler *et al*., 2020). The presence of lactic acid bacteria (LAB) within the honeybee alimentary canal has been consistently reported within the literature (Vásquez *et al*., 2012; Endo & Salminen, 2013; Kwong & Moran, 2016). Beneficial bacteria, belonging to LAB, are shown to market honeybee health through activating the honeybee's immune defenses and producing antimicrobial compounds inhibiting pathogenic microorganisms (Evans & Lopez, 2004; Evans *et al*., 2006; Rokop *et al*., 2015; Janashia *et al*., 2016; Olofsson *et al*., 2016; Kwong, Mancenido, *et al*., 2017; Filannino *et al*., 2019; Royan, 2019; Iorizzo *et al*., 2020a; Ramos *et al*., 2020). Some newer research has confirmed that the utilization of lactic acid bacteria (LAB) as probiotics could prevent certain diseases and improve honeybee health (Al-Ghamdi, *et al*., 2018; Daisley *et al*., 2020; Ramos *et al*., 2020; Tejerina *et al*., 2020). Especially, Tejerina *et al*., (2021) recently demonstrated that the appliance of LAB (*L. melliventris, L. helsingborgensis* and *A. kunkeei*) in syrup over 5 months reduced larval mummification in chalkbrood disease by over 80%. *A. kunkeei* may be a bacterium frequently present within the intestinal microbiota of honeybees. It colonizes fructose-rich niches and is really classified as a fructophilic lactic acid bacterium (FLAB) (Endo *et al*., 2012; Filannino *et al*., 2019). *A. kunkeei* seems to guard its niche against bacterial competitors, although the mechanism of its antimicrobial activity remains in many respects unknown (Olofsson *et al*., 2016). Some authors have assumed that the antimicrobial mechanisms of symbiotic bacteria

evolved synergistically with bees, to defend themselves and their hosts (Powell *et al.*, 2014; Schwarz *et al*., 2016). Inhibition might be supported a mixture of active compounds like proteins, peptides, fatty acids, organic acids, and peroxide (Berríos *et al*., 2018). Furthermore, the power of *A. kunkeei* to colonize the intestine and form a biofilm creates a barrier against unwanted microorganisms (Butler *et al*., 2013; Berríos et al., 2018). While there are many scientific data on the antimicrobial activity of *A. kunkeei* towards other microorganisms, and thereon of other bacterial species against *A. apis* (Sabaté *et al.*, 2009: Arredondo *et al*., 2018), reports of antifungal activity of *A. kunkeei* against *A. apis* are still few (Tejerina *et al*., 2018). The use of symbiotic FLAB within the prevention and biocontrol of honeybee pathogenic microorganisms, including chalkbrood disease, offers interesting possibilities (Audisio, 2017). The utilization of symbiotic bacteria, unlike synthetic or natural chemical compounds, doesn't adversely affect the balance of gut microbiota or impact honeybee health (Hamdi *et al.,* 2011; Vásquez *et al*., 2012; Omar *et al*., 2014; Raymann *et al*., 2017). Another potentially probiotic strain found within the bee gut is *Lactiplantibacillus* (Tajabadi *et al*., 2013; Javorský *et al.*, 2017; Parichehreh *et al.*, 2018). *L. plantarum* (formerly *Lactobacillus plantarum*) (Zheng *et al*., 2020) may be a versatile bacterium characterized by high adaptability to several different conditions, being isolated from various ecological niches including dairy, fruits, cereal crops, vegetables, fish and fresh meat (Mayo & Flórez, 2020). Several authors have proved that *L. plantarum* features a broad capacity to inhibit the expansion of various pathogens, and different strains exert inhibitory activity towards bacteria and fungi. Additionally, chemically different compounds with antibacterial and antifungal activity are characterized in culture filtrates (Niku-Paavola *et al*., 1999; Lavermicocca *et al*., 2000; Barbosa *et al*., 2016). *L. plantarum* also exhibits antagonist activity against *P. larvae*, the causative agent of the quarantine disease American foulbrood, which affects honeybee larvae and pupae (Niku-Paavola *et al*., 1999; Lavermicocca *et al*., 2000; Corby-Harris *et al*., 2007; Ren *et al.,* 2007; Ryu *et al*., 2008; Van Hoorde *et al*., 2008; Sharon *et al*., 2010; Storelli *et al*., 2011; Wouters *et al*., 2013; Tajabadi *et al*., 2013; Gotteland *et al*., 2014; Barbosa *et al*., 2016;; Javorský *et al*., 2017; Parichehreh *et al*., 2018; Daisley *et al*., 2020; Iorizzo *et al*., 2020a; Iorizzo *et al*., 2020b; Ramos *et al*., 2020; Zheng *et al*., 2020). Over the years, several studies have obtained relevant data supporting the probiotic properties of *L. plantarum* (Seddik *et al*., 2017; Behera *et al*., 2018). In conclusion, the mixing of *A. kunkeei* and *L. plantarum* in bee nutrition may represent a stimulating strategy to enhance bee and hive health.

Aim of the research

Paenibacillus larvae and *Ascosphaera apis* are both microorganisms able to cause disease in honeybees. The previous is the causative agent of the American foulbrood (AFB), a severe bacterial disease that affects larvae of honeybees, whereas the latter is an entomopathogenic fungus that can cause chalkbrood disease. In recent years, increasing attention was paid towards the use of lactic acid bacteria in honeybee diet to improve their health, productivity and ability to resist infections by pathogenic microorganisms.

The activities of this PhD thesis have moved in this context, specifically in the *in vitro* assessment of the antimicrobial activity exerted by different *Lactiplantibacillus plantarum* strains, previously isolated from various compartments of honeybee gastrointestinal tract or from hive products, against *Paenibacillus larvae* and *Ascosphaera apis*. Moreover, selected strains of *Lactiplantibacillus plantarum* showing antimicrobial activity against the two pathogens were further assayed for some physical and biochemical properties to assess their suitability as probiotics in the honeybee diet.

This study ultimately opens up interesting perspectives for new biocontrol strategies of honeybee diseases, such as the American foulbrood and the chalkbrood disease, through the use of probiotic lactic acid bacteria integrated in the bee diet.

Chapter 4: Materials and Methods

4.1 Microbial Cultures

In this study, sixty-one *Lactiplantibacillus plantarum* strains, isolated from bee bread and honeybee gut of *Apis mellifera ligustica*, were used. All *L. plantarum* strains belonged to the DiAAA (Department of Agricultural, Environmental and Food Sciences) collection of the University of Molise.

4.2 Antibacterial activity against *Paenibacillus larvae*

Strains of *L. plantarum* were tested for their antimicrobial activity against *Paenibacillus larvae* ATCC 9545 by using the agar spot test. The experiments were conducted by spotting 10 μL of 16 h LAB cultures (10^8 CFU/mL) onto the surface of MRS (Oxoid Ltd., Hampshire, UK) agar plates, which were then anaerobically incubated at 37°C for 24 h. *P. larvae* was cultured in 10 mL of Brain Heart Infusion (BHI-Oxoid Ltd., Hampshire, UK) at 37°C for 16 h. Subsequently, 100 μL of overnight cultures (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 on which each strain of *L. plantarum* was grown. Plates were incubated aerobically at 37°C. Tests were conducted in triplicate, and the inhibition was evaluated after 48 h by measuring the width (mm) of the clear zone (ZOI) around the colonies of *L. plantarum* strains.

4.3 Antibacterial activity of cultural broth, cell free supernatant, cell pellet and cell lysate against *Paenibacillus larvae*

L. plantarum strains producing a ZOI greater than 4 mm in the agar spot test were further tested by using the agar well diffusion assay for their antimicrobial activity against *P. larvae* ATCC 9545. In this case, the inhibitory activity was determined according to Iorizzo et al. (2020a) using broth cultures (BC), cell-free supernatants (CFS), cell pellets (CP) and cell lysates (CL) of each selected *L. plantarum* strain. For this purpose, *L. plantarum* strains were cultivated in MRS broth and incubated at 37°C for 16 h, that is, until a cell concentration of 10^8 CFU/mL (BC). The BC of each single strain was centrifugated (8000 rpm for 20 min at 4 C) and the supernatant (CFS) was sterilized by filtration (cellulose acetate membrane, pore size 0.22 μm, Sigma-Aldrich; St. Louis, Missouri, USA). The remaining pellet was washed and resuspended in 5 mL of physiological solution (CP). To obtain the CL, 5 mL of each

bacterial culture (BC) were 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 60 s intervals to promote cellular lysis (Ricci et al., 2018). The antimicrobial activity of BC, CFS, CP and CL was evaluated according to Tremonte et al. (2017). Briefly, 20 mL of BHI soft agar (0.7% agar) inoculated with an overnight culture of *P. larvae* ATCC 9545 (final concentration of about 10^7 CFU/mL) were poured into Petri plates. Wells of 5.0 mm in diameter were bored into a single plate and 50 μL of BC, CFS, CP or CL of each producer strain were placed into different wells. As control, 50 μL of MRS, adjusted to pH 3.5 with hydrochloric acid 1N (Sigma-Aldrich), were used. After incubation at 37°C for 48 h, plates were observed and the antibacterial activity was reported as width (mm) of clear inhibition zone (ZOI) around the inoculated wells (Sorrentino et al., 2018). For comparative purposes, an antimicrobial susceptibility test was performed by the disk diffusion method. Using oxytetracycline (OTC) (30 μg/disk). For this purpose, *P. larvae* ATCC 9545 was cultured as described by Krongdang et al. (2017). Briefly, after incubation for 48 h at 37˚C on the agar MYPGP, cells were suspended in sterile distilled water and adjusted to approximately 2.87×10^8 cells/ml (A620 nm = 0.388, equivalent to a McFarland standard no. 1. The diameter of the inhibition zone was measured and it was used to express the inhibition as percentage. The tests were conducted in triplicate.

4.4 Antifungal activity against *Ascosphaera apis*

The antifungal activity of sixty-one *Lactiplantibacillus plantarum* strains was assessed using the overlay method described by Magnusson et al. (2003) with some modifications. Strains were cultured on MRS (Oxoid Ltd., Hampshire, UK) broth at 37°C for 12 h. Then, they were inoculated with a central single streak of 2 cm on MRS agar plates, 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 *A. apis* 3117, both acquired from the German Collection of Microorganisms and Cell Cultures (DSMZ, GmbH) 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. One mL of the fungal suspension was then inoculated in a tube containing 10 mL of MEA soft agar (0.7% agar), which was overlaid on the MRS agar plates previously inoculated with each *L. plantarum* strain as described above. As a control, a plate containing MEA with the fungal suspension but without bacteria was

used. The inhibitory activity of *L. plantarum* strains was measured after 72 h of incubation at 37°C as the diameter (mm) of the clear zone around the bacterial streaks. The tests were performed in triplicate.

4.5 Assessment of spore viability and germination test

All chemicals were supplied by Sigma-Aldrich (St. Louis, MO, USA). Fungal cultures of *A. apis* DSM 3116 and *A. apis* DSM 3117 were cultured in aerobiosis in MEA medium at 28°C for 15 days. Spore suspensions were obtained by washing with 5-10 mL of 0.01% sterile Tween-80 the ascospores formed on the surfaces of plates. Suspensions were collected in a sterile 100 mL Erlenmeyer flask and dissolved by shaking with sterile glass beads for 2 h. The germination test was conducted according to Jensen et al. (2013) with some modifications. In short, sterile Teflon-coated slides (TEKDON, Myakka City, FL, USA) were placed in a sterile Petri dish lined with wet filter paper. One hundred μL of each spore suspension (about 10^7 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). Ten 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, Petri dishes were exposed to 9-13% CO_2 for 10 min (Heath and Gaze, 1987) using an AnaeroGen system (Oxoid; Basingstoke, UK). After 32 h at 34°C in aerobiosis, spores were counted directly on the Teflon slide. About 100 spores were counted in three different fields of view by using a phase contrast microscope at 400 X 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. The tests were conducted in triplicate.

4.6 Inhibition of Radial Mycelial Growth

The inhibitory activity against *A. apis* DSM 3116 and *A. apis* DSM 3117 was determined according to Iorizzo et al. (2020a) using broth cultures (BC), cell-free supernatants (CFS), cell pellets (CP) and cell lysates (CL) of each selected *L. plantarum* strain prepared as described above. Five mL of BC, CP, CFS or CL were added to 15 mL of MEA medium and 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 Petri dishes, which were then incubated at 37°C in aerobic conditions. The antifungal activity was evaluated by

measuring the hyphal radial growth (diameter) after 8 days of incubation and it was 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.

4.7 Exopolysaccharide (EPS) production and antimicrobial evaluation of EPS

Two hundred mL of MRS medium was inoculated with 1% (v/v) overnight precultures of each *L. plantarum* strain grown in the same medium. After incubation at 37°C for 48 h, cultures (10^8 CFU/mL) were centrifuged at 8000 rpm for 20 min at 4 C. The pellets were washed twice with sterile water and then centrifuged again (8000 rpm for 20 min at 4 C) and subjected to released exopolysaccharides (EPS-r) and bound exopolysaccharides (EPS-b) extractions as described by Tallon et al. (2003). Then, fractions were dried to constant weight. As a control, MRS broth without bacterial inoculum was used.

The fractions EPS-b and EPS-r were rehydrated with 5 mL of physiological solution and added to 15 mL of MRS or MEA to test their antimicrobial activity against *Paenibacillus larvae* ATCC 9545 or *A. apis* DSM 3116 and *A. apis* DSM 3117 using the same techniques described above. Non-inoculated MRS medium, treated as for EPS-r and EPS-b obtainment, were used as controls. The tests were conducted in triplicate.

4.8 Biofilm Production

L. plantarum strains were tested for biofilm production following the method described by Cozzolino et al. (2020) with some modifications. Briefly, strains were grown overnight at 37°C in MRS medium. Bacterial cells were harvested by centrifugation (8000 rpm for 10 min at 4°C), washed twice with phosphate-buffered saline (PBS) solution (Sigma–Aldrich, St. Louis, MO, USA), resuspended at 10^6 CFU/mL concentration in MRS broth without sugar and in MRS broth supplemented with 5%, 10% and 20% glucose, fructose or sucrose under aerobic and anaerobic conditions (GasPack anaerobic system, Sigma–Aldrich, St. Louis, MO, USA). Three aliquots of 200 μL for 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 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 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). Absorbance was measured at 580 nm using an automated Multilabel Plate Counter (Perkin Elmer 1420), and values represented the biofilm formation capacity. The experiments were performed in triplicate.

4.9 Antioxidant Activity

4.9.1 Cell Protein Assay

Overnight cultures (10^6 CFU/mL) of *L. plantarum* strains in MRS medium were centrifuged at 13000 rpm for 5 min at 4°C, and the obtained supernatants (CFS) 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 20 mM at pH 7.5, containing ethylenediaminetetraacetic acid (EDTA) 5 mM and MgCl2 5 mM, and then subjected to three cycles of sonication at 12Wfor 30 s, with 60 s intervals to promote cellular lysis, using a Labsonic M. The suspension was used for protein measurement according to Di Martino et al. (2020) using a BioSpectrometer (Eppendorf, Hamburg, Germany). 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). Cell pellet suspensions were sonicated as described above and, after 12 h of storage at -20°C, centrifuged

at 13000 rpm for 15 min at 4 C. The supernatants (CES) were used for the evaluation of the antioxidant activity. All the experiments were performed in triplicate.

4.9.2 Antioxidant Activity Assay

The total antioxidant activity (TAA) of the CFS 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 CFS 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 CFS 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 experiments were performed in triplicate.

4.10 Biochemical characterization of selected *L. plantarum* strains

L. plantarum strains were assessed for carbohydrate fermentation pattern using an API 50CHL system kit, and for enzymatic patterns, using an API ZYM system kit according to the manufacturer's instructions (bioMérieux SA, Marcy l'Etoile, France).

4.11 Auto-Aggregation

The auto-aggregation assay was performed according to Cozzolino et al. (2020). Fresh cultures (logarithmic growth phase) of selected lactobacilli were centrifuged (8000 rpm for 10 min at 4°C) and cells were subsequently washed three times with phosphate saline buffer (PBS, Sigma-Aldrich). Then, cells were washed twice and re-suspended in PBS to an optical density (OD) of approx. 0.5 (A580), in order to standardize the bacterial concentration at 10^8 CFU/mL. The tests were conducted in triplicate and the cell auto-aggregation was measured at 1, 2, 5 and 24 h of incubation at 37°C by measuring the OD at 580 nm of the upper suspension using a spectrophotometer (Multilabel Counter-PerkinElmer 1420, San Jose, USA). The percentage of auto-aggregation was calculated using the following formula:

Auto-aggregation%(A) = [(1-ODt/OD0) X 100]

where OD0 is the absorbance at time 0, and ODt is the absorbance detected after 1, 2, 5 or 24 h. The tests were conducted in triplicate.

4.12 Cell Surface Hydrophobicity

The determination of cell surface hydrophobicity, based on the bacterial ability to adhere to hydrocarbons (BATH), was evaluated on *L. plantarum* strains using xylene and toluene (Sigma-Aldrich) (Cozzolino et al., 2020). Hydrophobicity was calculated using the following formula:

% Hydrophobicity = [(OD0 - ODt/OD0) X 100]

where ODt represents the absorbance value after extraction with hydrocarbons (15, 30 and 60 min), and OD0 represents the absorbance value before extraction with hydrocarbons. The tests were conducted in triplicate.

4.13 Bacterial Survival in Sugar Syrup

To assess the osmotic tolerance of selected *L. plantarum* strains, a test was performed according to Iorizzo et al. (2020a), with some modifications. In detail, strains were grown in MRS broth at 37°C and after 24 h cells were harvested by centrifugation at 8000 rpm for 10 min at 4°C. The fresh pellets were washed twice with PBS buffer and were inoculated in sugar syrup in order to obtain an initial concentration of 10^7 CFU/mL. The experimental conditions were the following:

Test A: sugar syrup constituted by 40% glucose + 20% fructose (w/v) in distilled water at pH 4.2;

Test B: sugar syrup constituted by 50% (w/v) sucrose in distilled water at pH 4.2.

The sugar syrup was acidified using HCl 1N and sterilized using filtration (cellulose acetate membrane, pore size 0.22 μm, Sigma-Aldrich). The experiments were conducted in triplicate at 20°C and the bacterial viability was determined after 0, 24 and 48 h by plating in MRS agar (37°C for 72 h in anaerobic condition).

4.14 Statistical Analysis

All data, obtained by three independent experiments, were expressed as mean ± standard deviation (SD). Statistical analysis was performed through the analysis of variance (ANOVA) followed by the Tuckey's multiple comparison. Statistical significance was attributed to p-values < 0.05. The software SPSS (IBM SPSS Statistics 21) was used to analyse data.

Chapter 5: Results

5.1 Antimicrobial activity against *Paenibacillus larvae* and *Ascosphaera apis*

Table 1 reports the results of the antagonistic activity of sixty-one *L. plantarum* strains tested against *Paenibacillus larvae* ATCC 9545, *Ascosphaera apis* DSM 3116 and *A. apis* DSM 3117, two important pathogens causing disease in honeybees.

With regard to *P. larvae*, the results of this preliminary screening showed that 35 strains did not cause inhibition and twenty-one strains caused an inhibition zone (ZOI) < 4 mm. Only 5 *L. plantarum* strains, that is, P8, P25, P86, P95 and P100, demonstrated the greatest antagonism against the pathogen, showing inhibition zones > 4 mm.

As for results regarding *Ascosphaera apis*, inhibition zones were generally greater than those just described for *P. larvae*. In detail, all *L. plantarum* strains showed antifungal activity, but different intensities were registered (Table 1). In fact, only 12 strains showed ZOI ≤ 4 mm against both *A. apis* strains used as indicators. Interestingly, five *L. plantarum* strains, the same ones that showed the greatest antagonism against *P. larvae*, displayed a strong inhibitory activity (ZOI > 20 mm in diameter) against *Ascosphaera apis* DSM 3116 and *A. apis* DSM 3117. For this reason, *L. plantarum* P8, P25, P86, P95 and P100 were selected for subsequent analyses to explore the nature of the antimicrobial activity.

Table 1. Antimicrobial activity expressed by 61 *L. plantarum strains* against *P. larvae* ATCC 9545, *A. apis* DSM 3116 and *A. apis* DSM 3117 (n=3). N.D., not detected.

***L. plantarum* strains**	**Isolation Source**	**Antimicrobial activity *vs* *P.larvae* ATCC 9545 (ZOI mm)**	**Antimicrobial activity *vs A. apis* DSM 3116 (ZOI mm)**	**Antimicrobial activity *vs A. apis* DSM 3117 (ZOI mm)**
P3	beebread	< 4 mm	4.00±0.90	5.00±0.50
P4	beebread	N.D.	6.00±0.60	6.00±0.70
P5	beebread	< 4 mm	8.00±0.60	7.00±0.70
P7	beebread	< 4 mm	6.00±0.70	6.00±0.40
P8	**beebread**	**> 4 mm**	**26.0±0.7**	**22.0±0.90**
P9	beebread	< 4 mm	7.00±0.40	8.00±0.50
P21	beebread	< 4 mm	5.00±0.70	5.00±0.60
P36	beebread	< 4 mm	6.00±0.60	6.00±0.30
P37	beebread	N.D.	2.00±0.90	4.00±0.40
P39	beebread	N.D.	7.00±0.60	6.00±0.60
P57	beebread	< 4 mm	4.00±0.60	4.00±0.90
P61	beebread	N.D.	9.00±0.60	7.00±0.70
P81	beebread	N.D.	5.00±0.20	5.00±0.30
P82	beebread	< 4 mm	5.00±0.50	5.00±0.20
P86	**beebread**	**> 4 mm**	**22.0±0.90**	**21.0±0.50**
P87	beebread	< 4 mm	6.00±0.30	8.00±0.50
P88	beebread	< 4 mm	8.00±0.40	9.00±0.70
P92	beebread	< 4 mm	6.00±0.30	5.00±0.20
P94	beebread	< 4 mm	4.00±0.50	7.00±0.30
P95	beebread	< 4 mm	7.00±0.40	6.00±0.70
P97	beebread	< 4 mm	6.00±0.60	5.00±0.70
P101	beebread	N.D.	4.00±0.30	6.00±0.40
P103	beebread	N.D.	5.00±0.70	3.00±0.20
P106	beebread	N.D.	5.00±0.30	4.00±0.30
P108	beebread	N.D.	5.00±0.50	3.00±0.40
P1	honey stomach	N.D.	4.00±0.40	4.00±0.30
P3	honey stomach	N.D.	4.00±0.40	5.00±0.50
P10	honey stomach	< 4 mm	7.00±0.50	6.00±0.40
P15	honey stomach	N.D.	7.00±0.30	4.00±0.60
P21	honey stomach	N.D.	8.00±0.70	7.00±0.80
P24	honey stomach	N.D.	6.00±0.60	8.00±0.40
P26	honey stomach	N.D.	6.00±0.70	5.00±0.40
P27	honey stomach	N.D.	3.00±0.60	5.00±0.50
P35	honey stomach	N.D.	5.00±0.40	4.00±0.60
P38	honey stomach	N.D.	4.00±0.50	4.00±0.20
P40	honey stomach	N.D.	7.00±0.60	5.00±030
P59	honey stomach	N.D.	6.00±0.50	7.00±0.80
P60	honey stomach	N.D.	7.00±0.40	6.00±0.40
P69	honey stomach	N.D.	4.00±0.20	4.00±0.30
P70	honey stomach	N.D.	4.00±0.80	5.00±0.50
P73	honey stomach	N.D.	4.00±0.90	3.00±0.50
P74	honey stomach	N.D.	4.00±0.40	6.00±0.20
P80	honey stomach	< 4 mm	5.00±0.50	4.00±0.60
P81	honey stomach	N.D.	3.00±0.70	3.00±0.50
P82	honey stomach	< 4 mm	4.00±0.40	3.00±0.50
P83	honey stomach	N.D.	6.00±0.70	4.00±0.40
P92	honey stomach	N.D.	3.00±0.50	4.00±0.70
P94	honey stomach	N.D.	4.00±0.30	5.00±0.20
P95	**honey stomach**	**> 4 mm**	**21.0±0.70**	**22.0±0.20**
P97	honey stomach	N.D.	2.00±0.30	3.00±0.30
P100	**honey stomach**	**> 4 mm**	**23.0±0.80**	**25.0±0.90**
P103	honey stomach	N.D.	3.00±0.50	4.00±0.50
P104	honey stomach	N.D.	4.00±0.30	5.00±0.50
P106	honey stomach	N.D.	5.00±0.50	5.00±0.60
P108	honey stomach	N.D.	8.00±0.20	7.00±0.60
P111	honey stomach	N.D.	2.00±0.70	3.00±0.30
P10	mid-gut	< 4 mm	6.00±0.50	4.00±0.50
P25	**mid-gut**	**> 4 mm**	**22.0±0.10**	**24.0±0.30**
P48	mid-gut	< 4 mm	6.00±0.30	5.00±0.50
P54	mid-gut	< 4 mm	5.00±0.50	5.00±0.40
P55	mid-gut	< 4 mm	6.00±0.40	6.00±0.30

First of all, an anti-germinative test was conducted to ascertain the ability of *L. plantarum* to inhibit the germination of fungal spores of *Ascosphaera apis*. However, no significant differences were observed between the control (SG) and the samples containing the five cultures of *L. plantarum* strains (SGL) singly tested (Table 2).

Table 2. Spore germination (%) in SG (SPORE/GLENN) and in SGL (SPORE/GLENN/LAB) mix. Results are shown as mean ± standard deviation (n=3).

Sample tested	**Spore germination %**	
	***A. apis* DSM 3116**	***A. apis* DSM 3117**
SG (Control)	84±3	75±5
SGL-P8	83±1	70±3
SGL-P25	80±3	76±2
SGL-P86	82±4	75±6
SGL-P95	78±5	75±2
SGL-P100	84±2	78±6

A further screening was performed to detect the inhibitory activity exerted by broth cultures (BC), cell free supernatants (CFS), cell pellets (CP) and cell lysates (CL) of the five selected strains. This test was carried out against both pathogens *A. apis* and *P. larvae*, and the results are reported in Table 3.

Table 3. Inhibition (%) of culture broth (CB), cell pellet (CP), cell-free supernatant (CFS) and cell lysate (CL) from *L. plantarum* strains against *P. larvae* ATCC 9545, *A. apis* DSM 3116 and *A. apis* DSM 3117 (radial growth). Results are shown as mean ± standard deviation (n=3). For each tested indicator strains, different uppercase letters, in each column, and different lowercase letters, in each row, indicate significant differences ($p < 0.05$).

		L. plantarum strains				
		P8	**P25**	**P86**	**P95**	**P100**
Paenibacillus larvae ATCC 9545	**BC**	40.00 ± 2.1^{Cd}	33.33 ± 2.3^{Dc}	33.89 ± 4.0^{Cc}	23.33 ± 3.2^{Ba}	28.33 ± 2.0^{Cb}
	CFS	32.22 ± 3.0^{Ac}	20.56 ± 1.8^{Aa}	22.78 ± 3.4^{Ab}	18.89 ± 1.4^{Aa}	20.55 ± 3.3^{Aa}
	CP	37.22 ± 2.3^{Bd}	29.94 ± 2.6^{Cc}	28.33 ± 3.6^{Bc}	21.11 ± 2.4^{Ba}	25.83 ± 1.8^{Bb}
	CL	35.42 ± 3.1^{Bc}	26.21 ± 3.3^{Bb}	25.25 ± 2.4^{Bb}	19.83 ± 4.1^{Aa}	25.88 ± 2.6^{Bb}
Ascosphaera apis DSM 3116	**BC**	90.6 ± 0.7^{Dc}	92.4 ± 0.5^{Dc}	61.5 ± 2^{Ca}	60 ± 1.9^{Da}	75.3 ± 1.5^{Db}
	CFS	$< 1^{Aa}$	$< 1^{Aa}$	$< 1^{Aa}$	$< 1^{Aa}$	$< 1^{Aa}$
	CP	43.7 ± 2.8^{Bb}	62.1 ± 1.8^{Bc}	36.3 ± 1.7^{Ba}	37.8 ± 1.5^{Ba}	47.6 ± 2.6^{Cb}
	CL	77.2 ± 1.6^{Cc}	84.7 ± 0.6^{Cd}	81.8 ± 1.4^{Dd}	44.9 ± 3.3^{Cb}	38.8 ± 2.8^{Ba}
Ascosphaera apis DSM 3117	**BC**	92.5 ± 0.7^{Dc}	100 ± 0.0^{Dd}	62.9 ± 1.3^{Da}	88.1 ± 0.8^{Db}	88.2 ± 0.6^{Db}
	CFS	1.7 ± 0.8^{Aa}	2.7 ± 2.1^{Aa}	10.2 ± 4^{Ab}	1.7 ± 1.2^{Aa}	5.1 ± 2.4^{Aa}
	CP	50.0 ± 2.2^{Ba}	69.8 ± 1.7^{Bc}	57.4 ± 1.6^{Bb}	51.0 ± 2.2^{Ba}	57.3 ± 1.7^{Cb}
	CL	70.5 ± 1.8^{Cb}	79.2 ± 1.4^{Cc}	76.5 ± 1.3^{Cc}	70.4 ± 1.8^{Cb}	50.9 ± 2.2^{Ba}

As regards *Paenibacillus larvae* ATCC 9545, the antibiotic oxytetracycline (OTC, 30 μg/disk) was used as reference considering the inhibition halo produced against the pathogen

as 100% effective. On this basis, the percentages of inhibition given by *L. plantarum* strain products (BC, broth culture; CFS, cell free supernatant; CP, cell pellet; CL, cell lysate) were calculated.

The highest inhibitory activity against *P. larvae* ATCC 9545 was produced by the BC of *L. plantarum* P8. Moreover, it was noted that the BC of the five tested *L. plantarum* strains inhibited *P. larvae* ATCC 9545 more than CFS, CP and CL. The lowest inhibition was observed with the use of CFS, while the antagonistic activity exerted by CP and CL was similar and characterized by intermediate values between those reported for BC and CFS for all tested *L. plantarum* strains. The MRS broth at pH 3.5, used as control, did not produce inhibition.

The last antimicrobial screening regarded the activity of exopolysaccharides (EPS) against *Paenibacillus larvae* and *Ascosphaera apis*. Microbial EPS 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 this reason, the antimicrobial test was performed by using both EPS forms (Table 4).

The inhibition of *L. plantarum* products against *Ascosphaera apis* was evaluated after 8 days. As already reported in the screening against *P. larvae* ATCC9545, also in this case the BC of *L. plantarum* caused a greater inhibition than that showed by CFS, CP and CL. Moreover, inhibition values where higher against fungi than against *P. larvae*. In fact, BC produced an inhibition between 60.0% (P95) and 92.4% (P25) against *A. apis* DSM 3116, and between 62.9% (P86) and 100% (P25) against *A. apis* DSM 3117. Cell lysates (CL) inhibited *A apis* more than the CP and CFS matrices. In particular, they caused inhibition rates between 38.8% (P100) and 84.8% (P25) for *A. apis* DSM 3116, and between 50.9% (P100) and 79.2% (P25) for *A. apis* DSM 3117. The cell pellets (CP) showed inhibitory activity ranging from 36.3% (P86) to 62.1% (P25) against *A. apis* DSM 3116, and from 50.0% (P8) and 69.8% (P25) against *A. apis* 3117. Overall, the cell-free supernatants (CFS), showed the lowest inhibitory activity. In particular, all CFS were unable to inhibit *A. apis* DSM 3116 and inhibition ranged between 1.7% (P8 and P95) and 10.2% (P86) for *A. apis* DSM 3117.

Data show statistically significant differences ($p < 0.05$). The EPS-r values were between 0.82 (P95) and 1.56 mg/mL (P25), while the EPS-b values ranged between 1.96 (P95) and 8.82 mg/mL (P8). EPS in both forms did not show the ability to inhibit *Paenibacillus larvae* and *Ascosphaera apis.*

Table 4. EPS-r and EPS-b produced by *L. plantarum* P8, P25, P86, P95 and P100 and their antimicrobial activity against *Paenibacillus larvae* ATCC 9545 and *Ascosphaera apis* DSM 3116 and DSM 3117. All values are expressed as mean ± standard deviation (n = 3). Different lowercase letters(a–c) in each row indicate significant differences ($p < 0.05$).

	P8	**P25**	**P86**	**P95**	**P100**
EPS-r production	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}
Antimicrobial activity of EPS-r against:					
Paenibacillus larvae ATCC 9545	N.D.	N.D.	N.D.	N.D.	N.D.
Ascosphaera apis DSM 3116	N.D.	N.D.	N.D.	N.D.	N.D.
Ascosphaera apis DSM 3117	N.D.	N.D.	N.D.	N.D.	N.D.
EPS-b production	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}
Antimicrobial activity of EPS-b against:					
Paenibacillus larvae ATCC 9545	N.D.	N.D.	N.D.	N.D.	N.D.
Ascosphaera apis DSM 3116	N.D.	N.D.	N.D.	N.D.	N.D.
Ascosphaera apis DSM 3117	N.D.	N.D.	N.D.	N.D.	N.D.

EPS-r values are expressed as mg/mL; EPS-b values are expressed as the ratio of EPS-b (μg)/cell protein (μg BSA equivalents). N.D., Not Detected.

5.2 Biofilm production and antioxidant activity in *L. plantarum* strains

The ability of *L. plantarum* strains to form biofilms in different media and environmental conditions is illustrated in Figure 1 (Iorizzo et al., 2021). Generally, all tested *L. plantarum* strains were able to produce biofilms in all the conditions, but concentration and type of added sugar influenced this feature. *L. plantarum* P8 produced greater amounts of biofilm than the other strains under all the conditions, and in all the tests the anaerobic condition generally favoured the production of biofilms. In particular, the anaerobic condition promoted biofilm production in strain P25 when glucose and sucrose were used, in strain P86 in presence of sucrose and in strains P95 and P100 in presence of fructose. Moreover, all tested strains seemed to produce increasing amounts of biofilm with increasing sugar concentration, and this behaviour was particularly remarkable for the strain P8 except in the test conducted under aerobiosis with the addition of sucrose (Iorizzo et al., 2021).

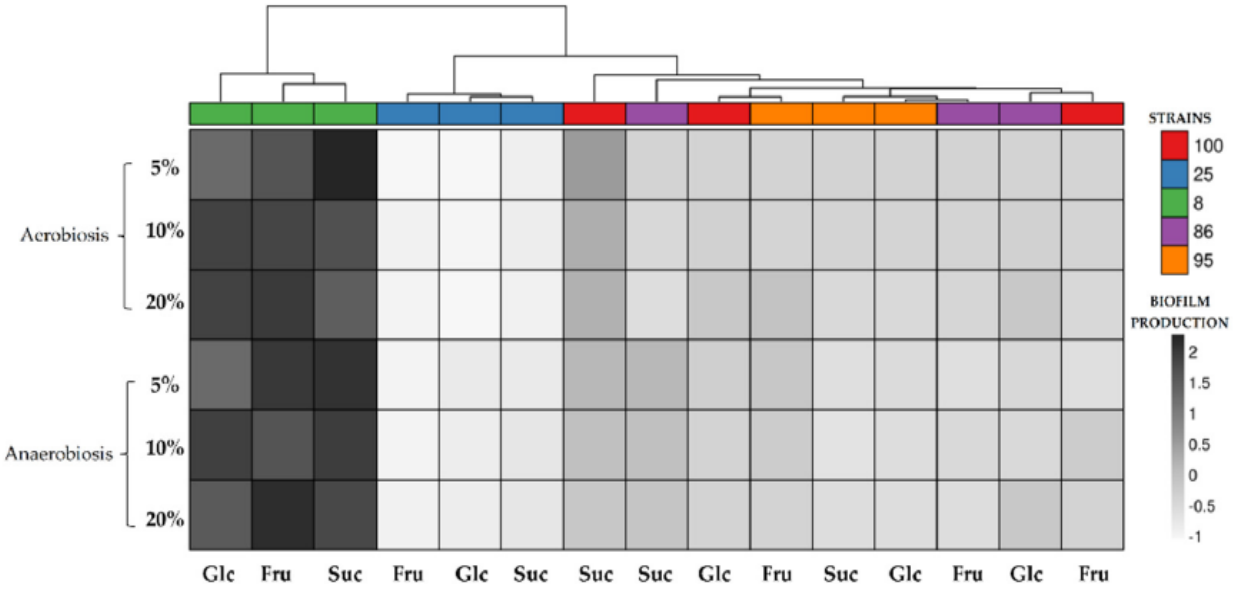

Figure 1. Biofilm production of *L. plantarum* P8, P25, P86, P95 and P100 in aerobic and anaerobic conditions and with different sugar concentrations (5%, 10% and 20%; Glc: glucose; Fru: fructose; Suc: sucrose). This figure was generated using ClustVis web tool https://biit.cs.ut.ee/clustvis/ (accessed on 4 December 2020).

The antioxidant activity of the CFS and CES obtained by *L. plantarum* strains are reported in Table 5. The antioxidant activity of the CFS, expressed as ng of ascorbic acid/mL, ranged between 20.01 (P100) and 37.45 (P8). CES values, expressed as the ratio between ascorbic acid (μg) and cell protein (μg BSA equivalents), were between 0.11 (P100) and 0.17 (P8).

Table 5. Antioxidant activity of *L. plantarum* P8, P25, P86, P95 and P100. All values are expressed as mean ± standard deviation (n = 3). Different lowercase letters(a–c) in each row indicate significant differences ($p < 0.05$).

	L. plantarum				
	P8	P25	P86	P95	P100
CFS 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}

CFS antioxidant activity expressed as ascorbic acid (ng/mL); ** CES antioxidant activity expressed as ratio of ascorbic acid (μg)/cell protein (μg BSA equivalents).

5.3 Biochemical characterization of *L. plantarum* strains

The results of the enzymatic profile of screened *L. plantarum* strains are reported in Table 6 (Iorizzo et al., 2020b). Strains P25 and P95 did not show esterase-lipase, leucine- and valine-arylamidase and α-fucosidase activity. In addition, strain P8 did not exhibit α-galactosidase and esterase activity, which were detected in the other strains. On the other hand, P8 resulted

the sole strain with Naphthol-AS-BI-phosphohydrolase activity. All the strains exhibited alkaline phosphatase, β-galactosidase, α- and β-glucosidase and N-acetyl--glucosaminidase activity.

Table 6. Enzymatic profile of five *L. plantarum strains* (API-ZYM system, BioMèrieux). + positive; - negative.

Enzyme assayed	***Lp. plantarum* strains**				
	P8	**P25**	**P86**	**P95**	**P100**
Alkaline phosphatase	+	+	+	+	+
Esterase (C4)	-	+	+	+	+
Esterase lipase (C8)	+	-	+	-	+
Lipase (C14)	-	-	-	-	-
Leucine arylamidase	+	-	+	-	+
Valine arylamidase	+	-	+	-	+
Cystine arylamidase	-	-	-	-	-
Trypsin	-	-	-	-	-
α-chymotrypsin	-	-	-	-	-
Acid phosphatase	-	-	-	-	-
Naphthol-AS-BI-phosphohydrolase	+	-	-	-	-
α-galactosidase	-	+	+	+	+
β-galactosidase	+	+	+	+	+
β-glucuronidase	-	-	-	-	-
α-glucosidase	+	+	+	+	+
β-glucosidase	+	+	+	+	+
N-acetil-β-glucosaminidase	+	+	+	+	+
α-mannosidase	-	-	-	-	-
α-fucosidase	-	-	+	-	+

The carbohydrate assimilation patterns of *L. plantarum* strains are shown in Table 7. All strains showed very similar profiles. Only P8 did not ferment the methyl-a-D-mannopyranoside. Strains P25 and P86 were able to ferment l-sorbose, and P100 was the sole strain able to ferment d-xylose.

Table 7. Carbohydrate assimilation patterns of five *L. plantarum* strains, (API 50 CHL system kit). + positive; - negative.

Carbohydrates	*Lp. plantarum* strains					Carbohydrates	*Lp. plantarum* strains				
	P8	**P25**	**P86**	**P95**	**P100**		**P8**	**P25**	**P86**	**P95**	**P100**
Glycerol	-	-	-	-	-	Esculine citrate de fer	+	+	+	+	+
Erythritol	-	-	-	-	-	Salicine	+	+	+	+	+
D-arabinose	-	-	-	-	-	D-Cellobiose	+	+	+	+	+
L-arabinose	+	+	+	+	+	D-Maltose	+	+	+	+	+
D-Ribose	+	+	+	+	+	D-Lactose	+	+	+	+	+
D-Xylose	-	-	-	-	+	D-Melibiose	+	+	+	+	+
L-Xylose	-	-	-	-	-	D-Saccharose	+	+	+	+	+
D-adonitol	-	-	-	-	-	D-Trehalose	+	+	+	+	+
Methyl-bD-Xylopyranoside	-	-	-	-	-	Inuline	-	-	-	-	-
D-Galactose	+	+	+	+	+	D-Melezitose	+	+	+	+	+
D-Glucose	+	+	+	+	+	D-Raffinose	+	+	+	+	+
D-Fructose	+	+	+	+	+	Amidon	-	-	-	-	-
D-Mannose	+	+	+	+	+	Glycogene	-	-	-	-	-
L-Sorbose	-	+	+	-	-	Xylitol	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	Gentiobiose	+	+	+	+	+
Dulcitol	-	-	-	-	-	D-Turanose	+	+	+	+	+
Inositol	-	-	-	-	-	D-Lyxose	-	-	-	-	-
D-Mannitol	+	+	+	+	+	D-Tagatose	-	-	-	-	-
D-Sorbitol	+	+	+	+	+	D-Fucose	-	-	-	-	-
Methyl-a-D-Mannopyranoside	-	+	+	+	+	L-Fucose	-	-	-	-	-
Methyl-a-D-Glucopyranoside	-	-	-	-	-	D-Arabitol	-	-	-	-	-
N-Acetyl-Glucopyranoside	+	+	+	+	+	L-Arbitol	-	-	-	-	-
						Potassium Gluconate	-	-	-	-	-
Amygdaline	+	+	+	+	+	potassium 2-Ketogluconate	-	-	-	-	-
Arbutine	+	+	+	+	+	potassium 5-Ketogluconate	-	-	-	-	-

5.4 Cell Surface Properties of *L. plantarum* strains: auto-aggregation and hydrophobicity

The auto-aggregation was assessed by measuring the optical density decrease of the five L. plantarum cultures suspended in phosphate saline buffer (PBS). As evidenced in Figure 2, the ability to aggregate and sediment increased progressively over time reaching, after 24 h, value ranges between 78.78% (strain P86) and 99.60% (strain P25) with significant differences among all strains.

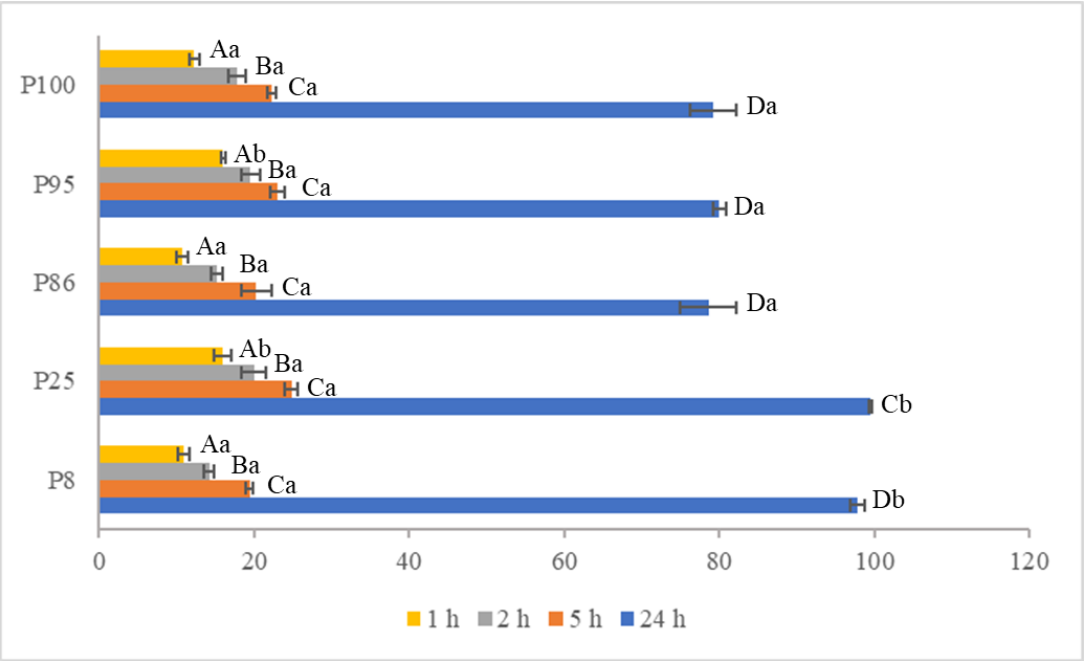

Figure 2. Auto-aggregation percentages of *L. plantarum* strains.

The hydrophobicity was assessed using the ability of the bacteria to adhere to hydrocarbons toluene and xylene. The results of the hydrophobicity (%) of the five *L. plantarum* selected strains (Figure 3) showed similar adhesion values to the two tested hydrocarbons and a gradual increase during over time. In detail, strains P8 and P25 showed a high adherence to toluene and xylene right from the start, as evidenced by hydrophobicity percentages greater than 90% already after 15 min. For these two strains, the hydrophobicity percentage was around 99% after 60 min. The other tested strains showed lower hydrophobicity percentages during the entire test. Specifically, the adherence rates for strain P86 and P95were less than 60% after 60 min to both xylene and toluene. The strain P100 showed the lowest hydrophobicity, as displayed by adherence values less than 40% to both hydrocarbons after 60 min.

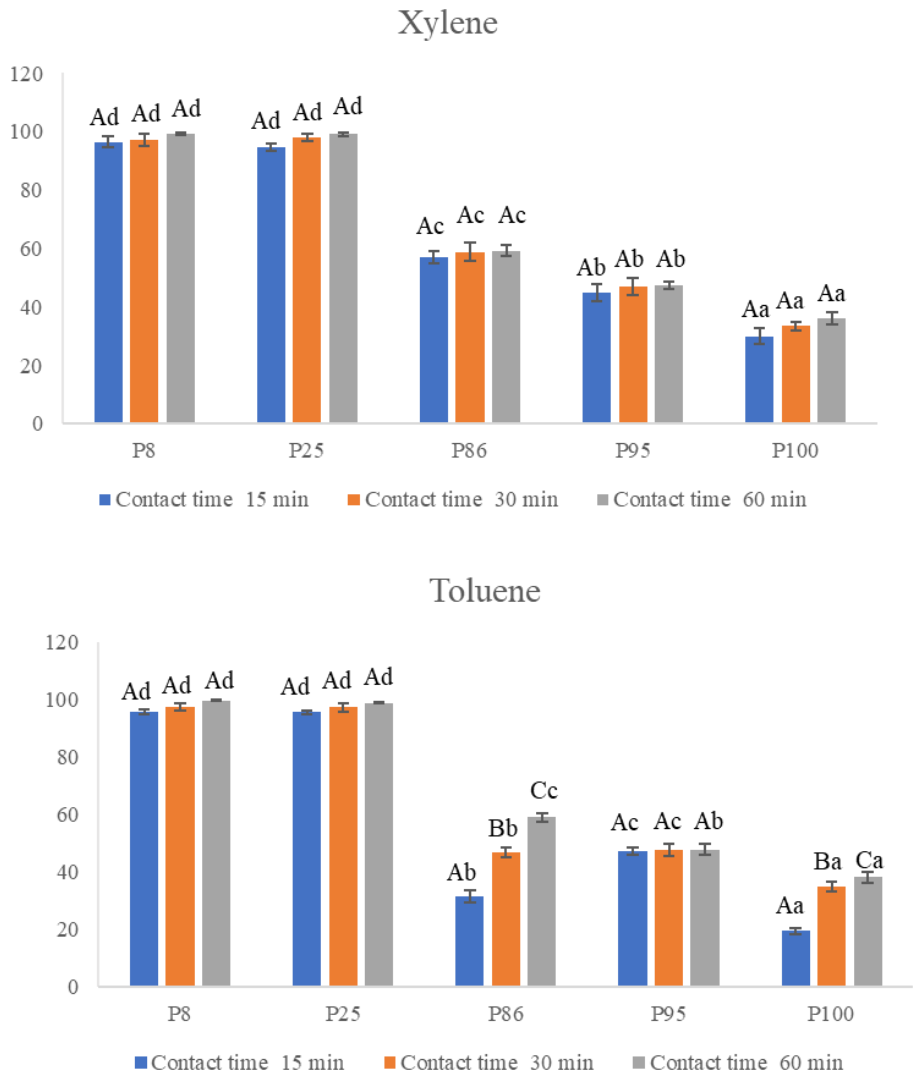

Figure 3. Adhesion (%) to hydrocarbons of *L. plantarum* strains as measured using the MATH assay.

5.5 Bacterial survival in sugar syrup

The results regarding the osmotic tolerance of L. plantarum strains are reported in Table 8. The screening based on the survival ability in two different sugar syrups, that is, 40% glucose + 20% fructose, pH 4.2, (Test A), and sugar syrup with 50% of sucrose at pH 4.2 (Test B). In both cases, the survival was evaluated for each strain at time zero and after 24 and 48 h of storage at 20°C. The highest reduction in viability was observed in the strain P25, which, after 48 h of storage, exhibited a reduction in cell viable of about 4 log units in Test A and about 2 log units in Test B.

As for the other strains, the survival was generally higher in Test B, as evidenced by count of about 6 - 6.5 Log CFU/mL after 48 h. In Test A, counts were lower than those just

described, and after 48 h of storage counts amounted to 4.28 and 5.84 Log CFU/mL (excluding the strain P25 whose behaviour was already annotated).

Table 8. Survival of *L. plantarum* strains in sugar syrups stored for 24 and 48 h at 20°C. Test A: 40% glucose + 20% fructose, pH 4.2; test B: 50% sucrose, pH 4.2. Results are shown as mean ± standard deviation (n = 3). For each sugar syrup, different uppercase letters (A–C in each column, and different lowercase letters (a–d) in each row, indicate significant differences ($p < 0.05$).

Time (hours)	Sugar syrup composition	Survival (log CFU/mL) of *L. plantarum* strains				
		P8	P25	P86	P95	P100
T_0	40% glucose 20% fructose	7.30 ± 0.06^{Ba}	7.29 ± 0.03^{Ac}	7.32 ± 0.04^{Ca}	7.34 ± 0.03^{Ca}	7.30 ± 0.04^{Ca}
T_{24}		7.23 ± 0.02^{Bb}	5.01 ± 0.04^{Ab}	7.11 ± 0.02^{Bb}	7.19 ± 0.04^{Bb}	7.20 ± 0.05^{Bb}
T_{48}		4.28 ± 0.04^{Ab}	3.22 ± 0.02^{Aa}	5.73 ± 0.05^{Ad}	5.84 ± 0.03^{Ae}	5.14 ± 0.02^{Ac}
T_0	50% sucrose	7.23 ± 0.06^{Ba}	7.15 ± 0.04^{Ca}	7.22 ± 0.04^{Ba}	7.20 ± 0.02^{Ba}	7.29 ± 0.02^{Ca}
T_{24}		7.21 ± 0.05^{Bc}	6.06 ± 0.05^{Ba}	7.17 ± 0.03^{Bc}	7.16 ± 0.02^{Bc}	7.06 ± 0.06^{Bb}
T_{48}		6.14 ± 0.02^{Ab}	5.15 ± 0.02^{Aa}	6.50 ± 0.05^{Ad}	6.35 ± 0.04^{Ac}	6.54 ± 0.04^{Ad}

Chapter 6: Discussion

6.1 Antimicrobial activity of tested *L. plantarum* strains

In the first part of this study, the ability of *L. plantarum* to inhibit two different honeybee pathogens was investigated. With regard to *Ascosphaera apis* DSM 3116 and DSM 3117, the results evidenced that the sensitivity to tested bacteria may be species- and not strain-dependent. In fact, the antimicrobial activity performed as preliminary test by using all the 61 *L. plantarum* strains showed very similar inhibition halos for both *A. apis* tested strains. In this context, it could be of interest to test the inhibitory activity exerted by *L. plantarum* strains against other *A. apis* strains to confirm this datum.

Five *L. plantarum* strains having high antimicrobial activity were further investigated. Data recorded in this study showed that they were unable to affect the germination capacity of fungal spores, while these five LAB exhibited the ability to inhibit *in vitro* the vegetative form of *A. apis*. This fact is of particular interest, considering that 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 favour the access to the interstitial space between the muscle fibers of infected larvae (Albo *et al.,* 2017; Maxfield-Taylor *et al.,* 2015). 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 (Bäumler & Sperandio, 2016; Crowley *et al.*, 2013; Deng *et al.*, 2020; Landini *et al.,* 2010).

Other researchers reported that the antimicrobial activity of LAB could be primarily attributed to their CFS, containing several antimicrobial compounds, including organic acids (lactic, acetic, formic, propionic, butyric, hydroxyl-phenylactic 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 concerning *A. apis* inhibition demonstrated that all five *L. plantarum* strains had strong antifungal activity. High inhibition occurred with the use of broth cultures (BC). Moreover, 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 ones, that substantially increases the overall antifungal activity. This fact was also hypothesized by other researchers (Cortés-Zavaleta *et*

al., 2014; Deng *et al.*, 2020; Iorizzo *et al.*, 2020; Landini *et al.*, 2010; Re *et al.*, 1999; Testa *et al.*, 2019). The tests performed with the use of EPS-b and EPS-r fractions showed that these fractions were unable to inhibit *A. apis* DSM 3116 and DSM 3117. This fact 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).

Considering the preliminary antimicrobial test carried out against *Paenibacillus larvae*, only five strains, that is, the same with the highest antimicrobial activity against *A. apis*, showed inhibition halos > 4 mm. For this reason, they were selected for further investigations, even if their antimicrobial activity can be considered as moderate if compared with that given by oxytetracycline (OTC), one of the most used antibiotics against *P. larvae* (Krongdang et al. 2017), for which an inhibition halo of 18 mm was registered.

Once again, the antimicrobial activity exerted by BC of the *L. plantarum* five strains showed greater antimicrobial activity against *P. larvae* ATCC 9545, compared to that shown by CFS, while CP and CL had intermediate inhibition values between BC and CFS, as already evidenced in the assay conducted against *A. apis*. Moreover, the absence of inhibition in the control test carried out with MRS acidified at pH 3.5 excludes that the inhibitory action of producer strains may be due to a low pH. In addition, the inability of many *L. plantarum* strains to inhibit *P. larvae* ATCC 9545 suggests that the antimicrobial activity is not due to nutritional competition. In this regard, previous research demonstrated that some LABs produce extracellular substances, secreted or tied to the cell wall, which can perform an inhibitory action against competing microorganisms (Neu & Lawrence, 2010; Olofsson *et al.*, 2016; Pannella *et al.*, 2020).

Some extracellular polymeric substances (polysaccharides, proteins, nucleic acids and lipids) are responsible for the cohesion of microorganisms and are involved in biofilm formation (Lembè *et al.*, 2012). The EPS production and the biofilm formation by LAB could be an effective strategy against biofilms and colonization of pathogenic bacteria since they compete with them for nutrients and space with different mechanisms of action (Berríos *et al.*, 2018; Mahdhi *et al.*, 2017). Fünfhaus *et al.* (2018) showed that *P. larvae* were able to

form biofilms at the beginning of the saprophytic phase, and this could promote optimal colonization of the honeybee larvae cadaver and access to all nutrients.

Several studies reported that some *L. plantarum* strains produce exopolysaccharides that, as well as contributing to biofilm formation, can exert an antimicrobial action (Li *et al.,* 2014; Liu *et al.,* 2017; Silva *et al.,* 2019). Based on these considerations, the ability of selected *L. plantarum* strains to produce extracellular polysaccharides (EPS) was evaluated. The tests also confirmed that EPS production is strain-dependent, as documented by other researchers (Lee *et al.,* 2016). In fact, *L. plantarum* P8 produced the largest amount of EPS-b. However, the results showed that, even if all the *L. plantarum* tested were able to produce EPS in bound and released form, none of the two forms was able to inhibit *A. apis* nor *P. larvae*.

6.2 Biofilm production and antioxidant activity in *L. plantarum* strains

Bacterial cells exhibit two types of growth modalities, known as planktonic cells and sessile aggregates, the latter also recognized as biofilm. The biofilm formation process takes place through a series of events that lead to adaptation under different nutritional and environmental conditions (Hentzer *et al*., 2005; Rivera *et al*., 2007). Biofilm is composed by association of microorganisms in which cells stick together on a living or non-living surface, and they are enclosed within a self-produced matrix of extracellular polymeric substance (Hall *et al*., 2004). Biofilm formation is a multi-step process that begins with the cell attachment to a surface, is followed by the formation of micro-colonies in a three-dimensional structure, and ends with its maturation followed by detachment. During biofilm formation, many species of bacteria are able to communicate each other through a specific mechanism called *quorum sensing*, a system of stimuli for coordinating the gene expression (Okada *et al*., 2005; Sauer *et al*., 2004). Biofilm formation represents an ancient, universal, and fundamental survival mechanism that provides microorganisms with advantages, including greater access to nutritional resources, enhanced organism interactions, and greater environmental stability. Interactions of microorganisms within the biofilm matrix facilitate metabolic cooperation and genetic exchanges. Furthermore, microbial biofilms can thrive in extreme or hostile environments where individual microorganisms would have difficulty not only growing but also surviving (Dang & Lovell, 2016). In fact, biofilms show an increased survival and resistance to environmental and chemical stressors mainly, but not only, by the protection conferred by the extracellular polysaccharide matrix.

The results reported in this study proved that *L. plantarum* ability to form biofilm is a strain-dependent character. Moreover, the anaerobic condition significantly favoured the biofilm production, and this fact suggests that the microaerophilic/anaerobic conditions of the intestinal tract can also favour the production of biofilms and the resulting intestinal colonization by these bacteria.

With regard to the antioxidant activity, it is important to underline that oxidative stress can cause severe negative effects in eukaryotic organisms. In particular, 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 (Collins *et al.,* 2004; Weirich *et al.,* 2002). Detoxifying enzymes can play a crucial role in honeybees exposed to biotic and abiotic stressors (Balieira *et al.,* 2018; Banerjee *et al.,* 2001). Moreover, oxidative stress has been reported to play an important 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 (Paris *et al.,* 2017; Seehuus, 2006). For instance, Li *et al.* (2020) recently reported that *A. apis* infection-induces oxidative stress in honeybee larvae, and decreases levels of metabolites involved in combating oxidative stress could compromise the antioxidant defences of the infected larvae. The specific activities of antioxidant enzymes 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. In this field, in the last decade increased attention was paid to the use of LAB as natural antioxidants. In fact, some LAB strains have enzymatic and nonenzymatic antioxidant activity and they are able to 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).

The assessment of the 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), showed that all five *L. plantarum* tested strains had antioxidant activity in CFS and CES. This datum suggests that their antioxidant activity may be due to different substances which deserve further investigations. Thus, these bacteria, if used as probiotics in the diet of honeybees, could limit oxidative stress due to pathogenic microorganisms or other biotic and abiotic factors.

6.3 Biochemical characterization of *L. plantarum* strains

The enzymatic activities involved in the honeybee gut are primarily responsible for the break down of the complex sugars assumed with the honeybees diet. Moreover, a balanced gut microbiota can improve the enzymatic pattern of the insect and it is associated with bee health (Engel *et al.,* 2012; Gaggìa *et al.,* 2018; Hamdi *et al.,* 2011). The enzyme profile of the five studied *L. plantarum* strains held α- and β-glycosidase activities. The β-glycosidase is capable to hydrolyse the glycosylated aromatic precursors, with formation of odorous compounds, including monoterpenes, and increased bioavailability of antioxidative plant metabolites in honey, beebread and royal jelly (Choi *et al.,* 2002; De Leonardis *et al.,* 2016, 2016; Kaškonienė *et al.,* 2020). β-glycosidase is also important for its contribution to the hydrolysis of cellulose in combination with other enzymes, including cellulase and hemicellulase (Zheng *et al.,* 2019). Instead, α-glycosidase converts maltose to glucose and is also directly involved, together with α-amylase, in the degradation of starch granules (Stanley *et al.,* 2011).

The impact of carbohydrates on bee survival is a well-studied field and it is established by now that bees live longer on syrup containing sucrose, glucose, or fructose (Haydak, 1970). Honeybees collect carbohydrate-rich food to support their colonies. Certain carbohydrates, such as the monosaccharides mannose, galactose, xylose, arabinose, rhamnose and the oligosaccharides lactose, melibiose, raffinose and melezitose present in their diet (Barker & Lehner, 1974), were described as toxic since these insects lack the appropriate enzymes for their digestion (Johnson, 2015). These carbohydrates are contained in natural nectar or derived from pectin hydrolysis or synthesized as melezitose. This sugar, composed of glucose and turanose and produced by aphids, is the primary trisaccharide in honeydew, where it can constitute up to 70% of the sugar fraction (Price *et al.,* 2007; Seeburger *et al.,* 2020). The results of carbohydrate assimilation test showed that all tested *L. plantarum* strains (P8, P25, P86, P95 and P100) are able to metabolize arabinose, galactose, lactose, mannose, melibiose, melezitose and raffinose, considered potentially toxic to honeybees. Given their ability to simultaneously participate in the breakdown of complex polysaccharides and metabolize toxic sugars, the role of these bacteria in improving dietary tolerance as well as maintaining the health of their hosts appears notable (Lee *et al.,* 2015; Ricigliano *et al.,* 2017; Zheng *et al.,* 2016).

6.4 Cell Surface Properties of *L. plantarum* strains: auto-aggregation and hydrophobicity

Selected *L. plantarum* strains were assessed for their adhesion ability to hydrocarbons (BATH), a method that determines hydrophobicity or hydrophilic nature of the cell surface (Schillinger *et al.,* 2005; Vinderola *et al.,* 2004). Based on the adherence to hydrocarbons, LAB hydrophobicity is classified as low (0 to 35%), moderate (36 to 70%), and high (71 to 100%) (Bouchard *et al.,* 2015; Ekmekci *et al.,* 2009). Under these ranges, *L. plantarum* P86, P95 and P100 showed a moderate hydrophobicity, while P8 and P25 showed a high hydrophobicity. These data suggest that the hydrophobicity appears to be strain-dependent and not species-dependent. In the environment, microorganisms live as planktonic cells, growing as aggregates. This self-binding structure is termed auto-aggregation or auto-agglutination. The ability to auto-aggregate (form floccules) of probiotic bacteria is correlate with adhesion, it is a prerequisite for colonization and protection of the host gastrointestinal tract, and appears to be the first step in the formation of biofilms (Kaushik *et al.,* 2009; Kragh *et al.,* 2016). Results reported in this study highlighted that the five tested *L. plantarum* strains had a high auto-aggregation ability, with percentage of aggregated cells increasing over time, in accordance with results obtained by other researchers on LAB strains belonging to the same species (García-Cayuela *et al.,* 2014). Moreover, significant differences were observed among the strains except for P8 and P25 strains, which showed a similar aggregation capacity. This datum confirms that also the auto-aggregation ability is a strain-dependent character (Trunk *et al.,* 2018; Tuo *et al.,* 2013).

6.5 Bacterial survival in sugar syrup

In recent years, beekeeping has become a fundamental need to intervene with an additional carbohydrate supplement for bees to integrate insufficient stocks, for spring and autumn stimulation of colonies, or to completely replace stocks (Iorizzo et al., 2020b) For this purpose sugar syrups are used, and the most widely used syrups contain sucrose, glucose and fructose (Smart *et al.,* 2019; Wang *et al.,* 2020). For this reason, the experimental design of this study included, as final step, the evaluation of the five *Lp. plantarum* selected strains to tolerate a high concentration of sucrose (50%) and of glucose and fructose mixture (40%+20%) at pH 4.2. In fact, honeybees are attracted by high concentrations of sugar syrup, and this behaviour becomes important to find a compromise between maximum attractiveness for honeybees and the survival of LAB. The results showed a good osmotic

tolerance of all strains in all combinations. This property would ensure a high vitality if bacteria were added in sugar syrups, used as additional food in hives.

Final considerations

The objective of this thesis is to contribute to the identification of new potential probiotic strains to improve the health of honeybees. The results obtained showed that selected strains of *Lactiplantibacillus plantarum* have not only an excellent inhibiting action against *Ascosphaera apis*, but also against *P. larvae*. Based on the preliminary results obtained, and subsequently deepened, excellent data emerged that allow to conceive of the possibility to integrate these microorganisms into the diet of honeybees. Moreover, given the excellent response to various tests of adhesion, production of EPS, biofilm formation and antioxidant activity, it seems that these strains can increase the state of health and consequently the "strength" of the colony and productivity. Moreover, the survival of tested strains in sugar syrups offers a possible way of administration of probiotic cultures to honeybees through the diet.

The future applicability of these microorganisms in the prevention of bee diseases, and therefore in maintaining the health status of colonies at optimal levels, will focus on *in vivo* tests able to reproduce under real conditions the data obtained up to now in the laboratory.

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Article

Antimicrobial Activity against *Paenibacillus larvae* and Functional Properties of *Lactiplantibacillus plantarum* Strains: Potential Benefits for Honeybee Health

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Abstract: *Paenibacillus larvae* is the causative agent of American foulbrood (AFB), a severe bacterial disease that affects larvae of honeybees. The present study evaluated, in vitro, antimicrobial activity of sixty-one *Lactiplantibacillus 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 *L. 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.

Keywords: *Lactiplantibacillus plantarum*; probiotics; *Paenibacillus larvae*; honeybee

1. Introduction

Paenibacillus larvae, the causative agent of the quarantine disease American foulbrood (AFB), is the most widespread fatal brood disease of honeybee (*Apis mellifera* L.) larvae and pupae [1]. This gram-positive, flagellated, spore-forming bacterium is highly adapted to honeybee larvae [1,2]. The honeybee gut is the site of *P. larvae* infection, as well as of pathogens such as *Ascosphaera apis*, *Nosema ceranae*, and probably many of the honeybee viruses [3]. Following ingestion, through spore-contaminated food, the spores germinate in the larval midgut lumen, where the vegetative bacteria massively proliferate before eventually breaching the midgut epithelium and invading the hemocoel, causing the death of the larvae, which during their decay releases a large number of spores. In the further course of the disease within the colony, more and more larvae become infected and die so that in the end, the lack of brood and, hence, the lack of progeny leads to collapse of the entire colony [1,2]. *P. larvae* often remains dormant in its spore-form and does not induce manifestations

of AFB. It has been suggested that *P. larvae* may exist as a pathobiont in the native microbiota of adult worker bees and is then transmitted throughout the hive to fresh brood cells [4]. The extreme contagiousness of AFB and the lethality for larvae and for entire colonies are the reasons why it is a notifiable disease in most countries. Currently, since an effective therapy against AFB is not available, the authorities consider the burning of infected colonies as the only efficient control measure [5]. Over the last few years, a number of different measures such as the use of chemical fungicides, antibiotics, heterocyclic organic compounds (indoles) and bacteriophages have been tried against AFB disease [6–10]. Unfortunately, these approaches could be useful as therapy, but are often ineffective for prophylactic purposes, and hives remain vulnerable to diseases. Moreover, the prophylactic use of antibiotics has inevitably led to the onset of antibiotic resistance in *P. larvae* [11,12]. In addition, the use of chemical compounds should be limited, both because they are dangerous to honeybee health [13] and because any residues present in honey also pose a serious risk to human health [14]. The use of natural compounds for disease control could represent a more suitable alternative [15,16]. Essential oils and other vegetable extracts from plants, herbs and spices exhibit antimicrobial activity against *P. larvae* [17,18] and this activity is mainly due to the presence of phenolic and terpenoid compounds, which have well-known antimicrobial activity [19–21]. However, the effects of these substances on honeybee health and on its symbiotic microflora are not entirely known [22]. Currently, there is an increased interest in investigating new, effective and safe control methods. In this context, the use of probiotic bacteria in the prevention and biocontrol of honeybee pathogenic microorganisms offers interesting perspectives [23]. The use of probiotic bacteria, unlike synthetic or natural chemical compounds, does not adversely affect the balance of gut microbiota and honeybee health [24,25]. Moreover, the protection against pathogens and/or parasites is one of the aspects frequently associated with a balanced intestinal flora [26–28]. It is well known that the initial phase of pathogen infection can be facilitated by any nutritional or environmental stress causing microbial dysbiosis [29–31]. The presence of lactic acid bacteria (LAB) in the honeybee gastrointestinal tract has been consistently reported in literature [32–34]. 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 [35–44]. The antagonistic effects of symbiotic LAB against *P. larvae* can be exploited to develop a new approach to AFB disease control [45–47]. Among LAB, *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) [48] is a versatile bacterium characterized by a high adaptability to many different conditions, being isolated from various ecological niches including dairy, fruits, cereal crops, vegetables, fish and fresh meat [49]. In addition, its presence in honeybee gut has been documented by several researchers [50–52], and its role, along with other bacteria and yeasts, in the transformation of fresh pollen into bee bread is well known [53,54]. *L. plantarum* pro-technological properties are exploited in different agri-food sectors [55–63]. Moreover, some strains of *L. plantarum* are known for their ability to produce several natural antimicrobial substances, thus inhibiting competitors that share the same niche [64–69]. The natural genomic architecture is the basis of its versatility and of its success in industrial applications, not only as starter culture but also as a bio-protective agent [70]. However, while numerous data on the functional and probiotic properties of *L. plantarum* in the diet of fish and mammals, including humans, were obtained [71–75], to our knowledge, its use as a probiotic in the honeybee diet and its antagonistic action against *P. larvae* has been little studied [76–78]. In this research we investigated the inhibitory properties of *L. plantarum* strains, isolated, in previous studies [79], from the honeybee (*A. mellifera L.*) gut and bee bread, against *P. larvae* ATCC 9545. In addition, some of their functional characteristics have been evaluated for a possible probiotication of the sugar syrups, to be used in the supplemental feeding of honeybees.

2. Results

2.1. Antimicrobial Activity

The antagonistic activity, of sixty-one *L. plantarum* strains, against *P. larvae* ATCC 9545 was investigated. The results of a preliminary screening, using the agar spot test, showed that thirty-five strains did not cause any inhibition and twenty-one strains caused an inhibition zone (ZOI) < 4 mm. P8, P25, P86, P95 and P100 *L. plantarum* strains, demonstrating the greatest antagonism against the pathogen (ZOI > 4 mm), were selected for the subsequent investigations (Table S1, Supplementary Materials). In this further analysis, the antimicrobial activity was carried out using agar well diffusion assay and the inhibitive capacity was assessed using cultural broth (BC) and cell free supernatants (CFS) of the five selected strains. The results of the antimicrobial activity are reported in Table 1.

Table 1. Antimicrobial activity (inhibition zone mm) of *L. plantarum* strains using cultural broths (BC) and cell free supernatants (CFS). Exopolysaccharides (EPS) amounts (μg/mL) in MRS broth after 48 h at 37 °C in aerobiosis. Results are shown as mean ± standard deviation (n = 3). Different uppercase letters (A–D), in each column, and different lowercase letters (a,b), in each row, indicate significant differences ($p < 0.05$).

L. plantarum Strains	Inhibition Zone (mm)		EPS Production (μg/mL)
	BC	CFS	EPS
P8	7.2 ± 0.2 ^{Db}	5.8 ± 0.3 ^{Ca}	174.0 ± 6.0 ^{C}
P25	6.0 ± 0.2 ^{Cb}	3.7 ± 0.1 ^{Aa}	140.0 ± 6.0 ^{B}
P86	6.1 ± 0.4 ^{Cb}	4.1 ± 0.3 ^{Aa}	167.0 ± 4.0 ^{C}
P95	4.2 ± 0.3 ^{Ab}	3.4 ± 0.1 ^{Aa}	76.0 ± 3.0 ^{A}
P100	5.1 ± 0.2 ^{Bb}	4.6 ± 0.3 ^{Ba}	135.0 ± 4.0 ^{B}

The BC, of five *L. plantarum,* inhibited *P. larvae* more than CFS. In particular, BC caused a ZOI between 4.2 and 7.2 mm wide, whereas the CFS caused a ZOI between 3.4 and 5.8 mm wide. The highest inhibitive activity was produced by *L. plantarum* P8 strain. The MRS broth at pH 3.5, used as control, did not produce any inhibition.

2.2. Exopolysaccharides Production

The exopolysaccharides (EPS) amounts, produced by the five *L. plantarum* selected strains, in MRS broth after 48 h at 37 °C in aerobiosis were generally significantly different among them (Table 1). In particular, P95 strain was the lowest EPS producer (76 mg/L); the other strains produced quantities greater than 130 mg/L with a maximum of 174 mg/L produced by the *L. plantarum* P100 strain.

2.3. Cell Surface Properties: Hydrophobicity and Auto-Aggregation

The hydrophobicity was assessed using the ability of the bacteria to adhere to toluene and xylene hydrocarbons. The results of the hydrophobicity (%) of the five *L. plantarum* selected strains are reported graphically in Figure 1 and numerically in Table S2 (Supplementary Materials). For each strain, the bacteria adhesion to the two hydrocarbons was similar and increased gradually during the test period (60 min). P8 and P25 strains, already after 15 min, showed a high adherence to toluene and xylene with a hydrophobicity percentage greater than 90%, and after 60 min, the percentage was around 99%. P86 and P95 strain adherence rates, after 60 min, were less than 60% both to xylene and toluene. P100 strain showed the lowest hydrophobicity with adherence values less than 40%, to both hydrocarbons, after 60 min.

The auto-aggregation (AA%) was assessed by measuring the optical density decrease of the five *Lactiplantibacilli* cultures suspended in phosphate saline buffer (PBS). The analyses showed that the ability to aggregate and sediment increased progressively over time, until reaching, after 24 h, value ranges between 78.78% (P86 strain) and 99.60% (P25 strain) with significant differences among all strains. The AA results are reported graphically in Figure 2 and numerically in Table S3 (Supplementary Materials).

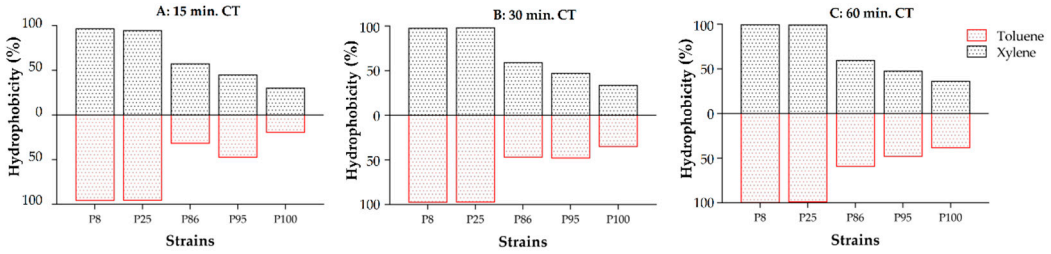

Figure 1. Adhesion of the *L. plantarum* five selected strains to toluene and xylene (expressed as hydrophobicity %) measured using bacterial ability to adhere to hydrocarbons (BATH) test after different contact times (CTs). (**A**) 15 min; (**B**): 30 min; (**C**) 60 min.

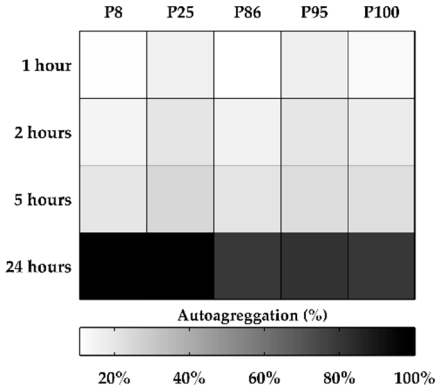

Figure 2. Auto-aggregation (AA%) of the *L. plantarum* strains expressed as optical density (OD) value at 580 nm.

2.4. Biochemical Characterization

The results of the enzymatic profile, obtained using an API ZYM kit, are shown in Table 2. P25 and P95 strains did not show esterase-lipase, leucine- and valine-arylamidase and α-fucosidase activity. In addition, P8 strain did not exhibit α-galactosidase and esterase activity, which were detected in the other strains. All the strains exhibited alkaline phosphatase, β-galactosidase, alpha- and β-glucosidase and N-acetyl-β-glucosaminidase activity.

The carbohydrate assimilation patterns, detected using API 50 CHL medium, are shown in Table 3. All the *L. plantarum* five strains showed very similar profiles. Only P8 strain did not ferment the methyl-a-D-mannopyranoside. P25 and P86 strains were able to ferment l-sorbose, and P100 was able to ferment the d-xylose, unlike the other strains.

Table 2. Enzymatic profile of the five *L. plantarum* strains performed using API-ZYM system (BioMèrieux). + positive; – negative.

Enzyme Assayed	*L. plantarum* Strains				
	P8	**P25**	**P86**	**P95**	**P100**
Alkaline phosphatase	+	+	+	+	+
Esterase (C4)	–	+	+	+	+
Esterase lipase (C8)	+	–	+	–	+
Lipase (C14)	–	–	–	–	–
Leucine arylamidase	+	–	+	–	+
Valine arylamidase	+	–	+	–	+
Cystine arylamidase	–	–	–	–	–

Table 2. *Cont.*

Enzyme Assayed	*L. plantarum* Strains				
	P8	**P25**	**P86**	**P95**	**P100**
Trypsin	−	−	−	−	−
α-chymotryspin	−	−	−	−	−
Acid phosphatase	−	−	−	−	−
Naphthol-AS-BI-phosphohydrolase	+	−	−	−	−
α-galactosidase	−	+	+	+	+
β-galactosidase	+	+	+	+	+
β-glucuronidase	−	−	−	−	−
α-glucosidase	+	+	+	+	+
β-glucosidase	+	+	+	+	+
N-acetil-β-glucosaminidase	+	+	+	+	+
α-mannosidase	−	−	−	−	−
α-fucosidase	−	−	+	−	+

Table 3. Carbohydrate assimilation patterns of the five *L. plantarum* strains, performed using API 50 CHL system kit. + positive; − negative.

Carbohydrates	*L. Plantarum* Strains				
	P8	**P25**	**P86**	**P95**	**P100**
Glycerol	−	−	−	−	−
Erythritol	−	−	−	−	−
D-arabinose	−	−	−	−	−
L-arabinose	+	+	+	+	+
D-Ribose	+	+	+	+	+
D-Xylose	−	−	−	−	+
L-Xylose	−	−	−	−	−
D-adonitol	−	−	−	−	−
Methyl-b-D-Xylopyranoside	−	−	−	−	−
D-Galactose	+	+	+	+	+
D-Glucose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Mannose	+	+	+	+	+
L-Sorbose	−	+	+	−	−
L-Rhamnose	−	−	−	−	−
Dulcitol	−	−	−	−	−
Inositol	−	−	−	−	−
D-Mannitol	+	+	+	+	+
D-Sorbitol	+	+	+	+	+
Methyl-a-D-Mannopyranoside	−	+	+	+	+
Methyl-a-D-Glucopyranoside	−	−	−	−	−
N-Acetyl-Glucopyranoside	+	+	+	+	+
Amygdaline	+	+	+	+	+
Arbutine	+	+	+	+	+
Esculine citrate de fer	+	+	+	+	+
Salicine	+	+	+	+	+
D-Cellobiose	+	+	+	+	+
D-Maltose	+	+	+	+	+
D-Lactose	+	+	+	+	+
D-Melibiose	+	+	+	+	+
D-Saccharose	+	+	+	+	+
D-Trehalose	+	+	+	+	+
Inuline	−	−	−	−	−

Table 3. *Cont.*

Carbohydrates	*L. Plantarum* Strains				
	P8	P25	P86	P95	P100
D-Melezitose	+	+	+	+	+
D-Raffinose	+	+	+	+	+
Amidon	−	−	−	−	−
Glycogene	−	−	−	−	−
Xylitol	−	−	−	−	−
Gentiobiose	+	+	+	+	+
D-Turanose	+	+	+	+	+
D-Lyxose	−	−	−	−	−
D-Tagatose	−	−	−	−	−
D-Fucose	−	−	−	−	−
L-Fucose	−	−	−	−	−
D-Arabitol	−	−	−	−	−
L-Arbitol	−	−	−	−	−
Potassium Gluconate	−	−	−	−	−
potassium 2-Cetogluconate	−	−	−	−	−
potassium 5-Cetogluconate	−	−	−	−	−

2.5. Bacterial Survival in Sugar Syrup

The bacterial osmotic tolerance was assessed based on survival ability in two different sugar syrups and the results are showed in Table 4. In test A (40% glucose + 20% fructose, pH 4.2), after 24h of storage, the P25 strain exhibited a reduction in cell viable density of about 2 log units, the other strains maintained a high cell density around 7.0 log CFU/mL; after 48 h of storage, the five strains maintained a cell density ranging between 3.22 (P25 strain) and 5.84 (P95 strain) log CFU/mL. In test B, using sugar syrup with 50% of sucrose at pH 4.2, after 24 h of storage, the viable cell density of the P8, P86, P95 and P100 strains remained similar to the initial one and decreased, by about 1 log unit, after 48 h. For the P25 strain, 1 log reduction, after 24 h, and 2 log reduction, after 48 h, of initial viability were detected.

Table 4. Survival of the *L. plantarum* strains in sugar syrups stored for 24-48 h at 20 °C. Test A: 40% glucose + 20% fructose, pH 4.2; test B: 50% sucrose, pH 4.2. Results are shown as mean ± standard deviation ($n = 3$). For every sugar syrup, different uppercase letters (A–C), in each column, and different lowercase letters (a–d), in each row, indicate significant differences ($p < 0.05$).

Storage Time (h)	Sugar Syrup Composition	Survival (log CFU/mL) of *L. Plantarum* Strains				
		P8	P25	P86	P95	P100
T_0	A	7.30 ± 0.06^{Ba}	7.29 ± 0.03^{Aa}	7.32 ± 0.04^{Ca}	7.34 ± 0.03^{Ca}	7.30 ± 0.04^{Ca}
T_{24}	40% glucose	7.23 ± 0.02^{Bb}	5.01 ± 0.04^{Ab}	7.11 ± 0.02^{Bb}	7.19 ± 0.04^{Bb}	7.20 ± 0.05^{Bb}
T_{48}	20% fructose	4.28 ± 0.04^{Ab}	3.22 ± 0.02^{Aa}	5.73 ± 0.05^{Ad}	5.84 ± 0.03^{Ae}	5.14 ± 0.02^{Ac}
T_0	B	7.23 ± 0.06^{Ba}	7.15 ± 0.04^{Ca}	7.22 ± 0.04^{Ba}	7.20 ± 0.02^{Ba}	7.29 ± 0.02^{Ca}
T_{24}	50% sucrose	7.21 ± 0.05^{Bc}	6.06 ± 0.05^{Ba}	7.17 ± 0.03^{Bc}	7.16 ± 0.02^{Bc}	7.06 ± 0.06^{Bb}
T_{48}		6.14 ± 0.02^{Ab}	5.15 ± 0.02^{Aa}	6.50 ± 0.05^{Ad}	6.35 ± 0.04^{Ac}	6.54 ± 0.04^{Ad}

3. Discussion

The role that probiotic bacteria can play as antagonists of honeybee pathogens, using the honeybee digestive tract as the site of infection, is very important [23,33,42,43]. The LAB antimicrobial action is often due to different factors: nutritional competition and compounds production as organic acids, fatty acids, proteinaceous compounds, phenolic acids and hydrogen peroxide [80,81]. In the inhibition test, carried out using the agar well diffusion method, the BC of the *L. plantarum* five strains showed a greater antimicrobial activity against *P. larvae* ATCC 9545, compared to that showed by CFS. Our results suggest that *Lactiplantibacilli* antagonistic action is due to different compounds

present in BC that could increase overall antimicrobial activity. The absence of inhibition in the control test, carried out with MRS acidified, excludes that the inhibitive action of bacterial cultures is due to a low pH. In addition, the inability of many *L. plantarum* strains to inhibit *P. larvae* ATCC 9545 (Table S1) suggests that the antimicrobial activity is not due to nutritional competition. In this regard, previous research demonstrated that some LABs produce extracellular substances, secreted or tied to the cell wall, which can perform an inhibitory action against competing microorganisms [43,82,83]. Some extracellular polymeric substances (polysaccharides, proteins, nucleic acids and lipids) are responsible for the cohesion of microorganisms and involved in biofilm formation [84]. The EPS production and the biofilm formation by LAB could be an effective strategy against biofilms and colonization of pathogenic bacteria, since they compete with them for nutrients and space with different mechanisms of action [85–92]. Fünfhaus et al. [93] showed that *P. larvae* were able to form biofilms at the beginning of the saprophytic phase, and this could promote optimal colonization of the honeybee larvae cadaver and the access to all nutrients. Several studies reported that some *L. plantarum* strains produce exopolysaccharides that, as well as contributing to biofilm formation, can exert an antimicrobial action [89,94–96]. Based on these considerations, we evaluated the ability of selected *L. plantarum* strains to produce extracellular polysaccharides (EPS). Our results showed that in MRS broth at 37 °C, and aerobiosis conditions, all the *L. plantarum* tested produced EPS and, in accordance with other researchers, this ability can be highly variable among *L. plantarum* strains [97]. Furthermore, the strains producing greater amounts of EPS also caused the strongest inhibitory action against *P. larvae* (Table 1). This suggests that there may be a correlation between these two properties. Further studies are needed to investigate EPS composition and to assess its capacity to inhibit spore germination, biofilm formation and vegetative growth of *P. larvae*. In addition to EPS production, also surface hydrophobicity and auto-aggregation are phenotypic traits that favor the biofilm formation and stability of microbial strains in the gastrointestinal tract [98,99]. The adhesion to intestinal epithelial cells is an important prerequisite for colonization of probiotic bacteria, preventing their immediate elimination by peristalsis and providing a competitive advantage in this ecosystem. In our work, we tested the selected strains potentiality to adhere to the intestinal tract, using bacterial adhesion to hydrocarbons (BATH), a method that determines the hydrophobicity or hydrophilic nature of the cell surface [100,101]. The hydrophobicity was assessed by carefully mixing a bacterial culture and hydrocarbon suspension (xylene and toluene) and then the decrease in optical density of the culture phase was evaluated. Based on the adherence % to hydrocarbons, the LAB could be classified into three groups: those with low (0 to 35%), moderate (36 to 70%) and high hydrophobicity (71 to 100%) [102,103]. Under these ranges, in the BATH test, P86, P95 and P100 *L. plantarum* strains showed a moderate hydrophobicity, on the contrary, P8 and P25 showed a high one. The variable values indicate that hydrophobicity appears to be strain-dependent and not species-dependent. In the future, it would be necessary to perform the assay with cell lines to confirm the ability of the selected strains to adhere to epithelial cells. In the environment, microorganisms live as planktonic cells and prefer growing as aggregates. This self-binding is termed auto-aggregation or auto-agglutination. The ability to auto-aggregate (form floccules) of probiotic bacteria is a correlate with adhesion, is a prerequisite for colonization and protection of the gastrointestinal tract and appears to be the first step in the formation of biofilms [104–106]. In general, our results highlighted that the five *L. plantarum* strains tested showed a high auto-aggregation ability and that the percentage of aggregated cells increased over time, in accordance with previous results obtained by other researchers, who have conducted similar studies on strains belonging to the same species [107]. Except for the P8 and P25 strains, which showed a similar aggregation capacity, significant differences ($p < 0.05$) were observed among the strains. This confirms that also the AA is a strain-dependent, not species-dependent, phenotypic character [99,108]. In recent years, beekeeping has become a fundamental need to intervene with an additional carbohydrate supplement for bees to integrate insufficient stocks, for spring and autumn stimulation of colonies or to completely replace stocks. The most widely used syrups contain sucrose, glucose and fructose [109,110]. The viability of probiotic organisms is a very important aspect; before

resisting the gastrointestinal tract, they must be able to survive during manufacturing and storage of probiotic products in order to express health benefits for the host. In our experimental studies we evaluated the capacity of five *L. plantarum* selected strains to tolerate a high concentration of sucrose (50%) and of glucose and fructose mixture (40 + 20%) at pH 4.2. Honeybees are attracted by high concentrations of sugar syrup, and this behavior becomes important to find a compromise between maximum attractiveness for honeybees and the survival of LAB. The results showed a good osmotic tolerance of all strains in all combinations. This property would ensure a high vitality if bacteria were added in sugar syrups, used as additional food in hives. Metabolic activities of the microbiota are key for symbiotic interactions in the honeybee gut and they have an impact on the health and disease of the host in different ways [111]. Gut microbiota participate in various processes, including defense systems and protection from pathogens, detoxification from harmful molecules, supply of essential nutrients and food digestion [112–115]. A balanced gut microbiota is necessarily associated with bee health since it provides countless enzymatic activities to break down the complex sugars of the honeybee's diet [29,113,116]. The enzyme profile, which we studied, showed that the five *L. plantarum* selected strains possess alpha- and beta-glycosidase activities. The beta-glycosidase is capable of hydrolyzing the glycosylated aromatic precursors, releasing odorous compounds including monoterpenes and increasing the bioavailability of antioxidative plant metabolites in honey, beebread and royal jelly [59,61,117–125]. In addition, beta-glycosidase is important, because in combination with other enzymes, including cellulase and hemicellulase, it contributes to the hydrolysis of cellulose [126]. Alpha-glycosidase enzyme converts maltose to glucose and is also directly involved, together with alpha-amylase, in the degradation of starch granules [127]. The impact of carbohydrates on bee survival has been studied, and it is well established that bees live longest on syrup containing sucrose, glucose or fructose [128]. Honeybees collect carbohydrate-rich food to support their colonies, and yet, certain carbohydrates present in their diet have been described as toxic because these insects lack the appropriate enzymes for their digestion [129]. These carbohydrates include the monosaccharides mannose, galactose, xylose, arabinose and rhamnose and the oligosaccharides lactose, melibiose, raffinose and melezitose [130–134]. They are contained in natural nectar or derived from pectin hydrolysis or synthesized as melezitose. This sugar, composed of glucose and turanose and produced by aphids, is primary trisaccharide in honeydew, where it can constitute up to 70% of the sugar fraction [135,136]. The results of carbohydrate assimilation tests showed that the P8, P25, P86, P95 and P100 *L. plantarum* strains are able to metabolize arabinose, galactose, lactose, mannose, melibiose, melezitose and raffinose, considered potentially toxic to honeybees. Given their ability to simultaneously participate in the breakdown of complex polysaccharides and metabolize toxic sugars, the role of these *L. plantarum* strains in improving dietary tolerance as well as maintaining the health of their hosts might be notable [131,137,138]. The selection and availability of new selected bacteria with good functional characters and with antagonistic activity against *P. larvae* always opens up interesting perspectives for new biocontrol strategies of diseases such as the AFB. Some researchers have highlighted the effectiveness of LAB in controlling this disease [45–47,76–78,139,140], and other researches have shown that effectiveness is not always certain in the hive [141,142] or that supplementation of honeybee diet, with improper probiotics, can be harmful to honeybees [143,144]. The functional properties, shown in vitro using *L. plantarum* strains, do not result axiomatically in health benefits for honeybee colonies. It is therefore necessary to assess in the future, in vivo/in situ, the role that these bacteria can have in maintaining the well-being of bees, and in particular, it is necessary to assess the contribution they can make in a prophylactic strategy against AFB disease.

4. Materials and Methods

4.1. Microbial Cultures

For this study sixty-one *L. plantarum* strains, isolated from bee bread and honeybee gut of *Apis mellifera* L., were used (Table S1, Supplementary Materials) [79]. These bacteria belong to the Di.A.A.A

(Department of Agricultural, Environmental and Food Sciences) collection of the University of Molise. In antimicrobial tests, *P. larvae* ATCC 9545 strain was used as indicator.

4.2. Screening of Antibacterial Activity

Sixty-one *L. plantarum* strain antimicrobial activity against *P. larvae* ATCC 9545 was investigated, using agar spot tests. The experiments were conducted by spotting 10 µL of 16 h LAB cultures (10^8 UFC/mL) onto the surface of MRS (Oxoid Ltd., Hampshire, UK) agar plates, which were then incubated anaerobically at 37 °C for 24 h, to allow colonies to develop. *P. larvae* was cultured in 10 mL of brain heart infusion (BHI-Oxoid Ltd., Hampshire, UK) at 37 °C for 16 h. Subsequently, 100 µL of overnight culture (10^7 UFC/mL) were inoculated into 7 mL of BHI soft agar (0.7% agar) maintained at 45 °C and poured over the MRS plates on which the selected *L. plantarum* were grown. The plates were incubated aerobically at 37 °C. The tests were conducted in triplicate, and after 48 h, the inhibition was evaluated by measuring the width (mm) of the clear zone (ZOI) around the colonies of the *L. plantarum* strains.

4.3. Determination of Antibacterial Activity

The *L. plantarum* strains producing a ZOI greater than 4 mm, in the agar spot test, were selected, and their antimicrobial activity against *P. larvae* ATCC 9545 was tested using agar well diffusion assay. The *L. plantarum* strains were grown in MRS broth and, after 16 h at 37 °C, the cultural broth (BC) of every single strain was centrifugated (8000 rpm for 20 min at 4 °C) and the supernatant (CFS) was sterilized by filtration (cellulose acetate membrane, pore size 0.22 µm, Sigma-Aldrich; St. Louis, Missouri, USA). The antimicrobial activity of the selected strains was evaluated according to Tremonte et al. protocol [65]. Briefly, 20 mL of BHI soft agar (0.7% agar) inoculated with an overnight culture of *P. larvae* (final concentration of about 10^7 CFU/mL) were poured into Petri plates. Wells of 5.0 mm in diameter were bored into a single plate and 50 µL of BC and of CFS, of each producer strain, were placed into different wells. As control, 50 µL of MRS, adjusted to pH 3.5 with hydrochloric acid 1N (Sigma-Aldrich), were used. After incubation at 37 °C for 48 h, the plates were observed and antibacterial activity was reported as width (mm) of clear zone of inhibition (ZOI) around the inoculated wells [19,81]. The tests were conducted in triplicate.

4.4. Biochemical Characterization

L. plantarum strains have been assessed for their carbohydrate fermentation pattern, using an API 50CHL system kit, and for enzymatic patterns, using an API ZYM system kit, according to the manufacturer's instructions (bioMérieux SA, Marcy l'Etoile, France).

4.5. Auto-Aggregation

The auto-aggregation assay was performed according to Cozzolino et al. [145]. Briefly, the *Lactobacilli* cultures were collected using centrifugation (8000 rpm for 10 min at 4 °C) during the logarithmic growth phase. Subsequently, the cells were washed three times with phosphate saline buffer (PBS, Sigma-Aldrich). Further, they were washed twice and re-suspended in PBS to an optical density (OD) of approx. 0.5 (A_{580}), in order to standardize the bacterial concentration at 10^8 CFU/mL. The tests were conducted in triplicate and the cell auto-aggregation was measured at 1, 2, 5 and 24 h of incubation at 37 °C, after which the OD at 580 nm of the upper suspension was measured using a spectrophotometer (Multilabel Counter-PerkinElmer 1420, San Jose, USA). The percentage of auto-aggregation was calculated using the following formula: Auto-aggregation % (A) = $(1 - OD_t/OD_0) \times 100$, where OD_0 is the absorbance at time 0, and OD_t is the absorbance detected after 1, 2, 5, and 24 h.

4.6. Cell Surface Hydrophobicity

The determination of cell surface hydrophobicity, based on the bacterial ability to adhere to hydrocarbons (BATH), was evaluated on *L. plantarum* strains, using xylene and toluene

(Sigma-Aldrich) [145]. Hydrophobicity was calculated as the percentage decrease in OD of the initial bacterial suspension and was expressed using the following formula: % Hydrophobicity = (OD_0 − OD_t/OD_0) × 100, where OD_t represents the absorbance value after extraction with hydrocarbons (15, 30 and 60 min), and OD_0 represents the absorbance value before extraction with hydrocarbons. The tests were conducted in triplicate.

4.7. Exopolysaccharides Production (EPS)

For exopolysaccharide production, 100 mL of MRS was inoculated with a 1% (*v*/*v*) of overnight pre-culture. After incubation, at 37 °C for 48 h, cells were separated using centrifugation at 7000 rpm for 30 min at 4 °C. Trichloroacetic acid was added to the supernatant, to a final concentration of 7% (*w*/*v*), which was then incubated at 4 °C for 12 h. The precipitated proteins were removed using centrifugation at 12,000 rpm for 30 min at 4 °C. The supernatant was then mixed with three volumes of pre-chilled ethanol (95%), vigorously stirred and kept at 4 °C overnight. EPS sediments were collected using centrifugation at 17,000 rpm for 30 min at 4 °C. As control, 100 mL of MRS broth without bacterial inoculum were used. The final polysaccharide fractions were lyophilized and their amount was determined by measuring the weight. The net quantity was obtained by subtracting the amount of EPS obtained from non-inoculated MRS broth. The tests were conducted in triplicate. All the chemical compounds used were supplied by Sigma-Aldrich.

4.8. Bacterial Survival in Sugar Syrup

To assess osmotic tolerance, a test was performed according to Iorizzo et al. [44], with some modifications. *L. plantarum* strains were grown in MRS broth at 37 °C and after 24 h the cells were harvested using centrifugation at 8000 rpm for 10 min at 4 °C. The fresh pellets were washed twice with PBS buffer and were inoculated in sugar syrup in order to obtain an initial concentration of 10^7 CFU/mL. The experimental conditions were the following; test A: sugar syrup constituted by 40% glucose + 20% fructose (*w*/*v*) in distilled water at pH 4.2; test B: sugar syrup constituted by 50% (*w*/*v*) sucrose in distilled water at pH 4.2. The sugar syrup was acidified using HCl 1N and sterilized using filtration (cellulose acetate membrane, pore size 0.22 µm, Sigma-Aldrich). The experiments, conducted in triplicate, were performed at 20 °C and the bacterial viability was determined after 0, 24 and 48 h by plating in MRS agar (37 °C for 72 h in anaerobic condition).

4.9. Statistical Analysis

All data, obtained by three independent experiments, were expressed as mean ± standard deviation (SD). Statistical analysis was performed through the analysis of variance (ANOVA) followed by the Tuckey's multiple comparison. Statistical significance was attributed to p-values < 0.05. The software SPSS (IBM SPSS Statistics 21) was used for the analysis.

5. Conclusions

The results of our research have shown that P8, P25, P86, P95 and P100 *L. 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 *L. plantarum* strains in vivo/in situ.

Supplementary Materials: The following are available online at http://www.mdpi.com/2079-6382/9/8/442/s1. Table S1: *L. plantarum* strains collection; Table S2: Hydrophobicity (%); Table S3: Auto-aggregation (%).

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B.T., S.P. and P.T.; methodology, S.J.L., S.G., B.T., F.V. and A.C.; project administration, S.P. and M.M.; resources, R.C. and A.D.C.; software, F.L. and M.M.; supervision, R.C. and A.D.C.; validation, M.S.; writing—original draft, M.I. (Massimo Iorizzo) and B.T.; writing—review and editing, M.I. (Massimo Iorizzo), S.G. and F.L. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest.

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Probiotic Properties and Potentiality of *Lactiplantibacillus plantarum* Strains for the Biological Control of Chalkbrood Disease

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Abstract: *Ascosphaera apis* is an entomopathogenic fungus that affects honeybees. In stressful conditions, this fungus (due not only to its presence, but also to the combination of other biotic and abiotic stressors) can cause chalkbrood disease. In recent years, there has been increasing attention paid towards the use of lactic acid bacteria (LAB) in the honeybees' diets to improve their health, productivity and ability to resist infections by pathogenic microorganisms. The screening of 22 strains of *Lactiplantibacillus plantarum*, isolated from the gastrointestinal tracts of honeybees and beebread, led to the selection of five strains possessing high antagonistic activity against *A. apis*. This study focused on the antifungal activity of these five strains against *A. apis* DSM 3116 and DSM 3117 using different matrices: cell lysate, broth culture, cell-free supernatant and cell pellet. In addition, some functional properties and the antioxidant activity of the five *L. plantarum* strains were evaluated. All five strains exhibited high antagonistic activity against *A. apis*, good surface cellular properties (extracellular polysaccharide (EPS) production and biofilm formation) and antioxidant activity. Although preliminary, these results are encouraging, and in future investigations, the effectiveness of these bacteria as probiotics in honeybee nutrition will be tested in vivo in the context of an eco-friendly strategy for the biological control of chalkbrood disease.

Keywords: *Ascosphaera apis*; chalkbrood disease; *Lactiplantibacillus plantarum*; biocontrol; honeybee

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1. Introduction

The fungus *Ascosphaera apis*, belonging to the heterothallic Ascomycota phylum, is a major and widespread pathogen of honeybee (*Apis mellifera*) broods, causing chalkbrood disease and larval death [1]. This disease is economically important since it results in significant losses of both honeybees (under certain circumstances, it can kill colonies) and colony productivity [2], and indications suggest that its incidence may be increasing [3]. 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 [4].

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 [5]. 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 [6]. Honeybees have several defense mechanisms to resist chalkbrood disease, including hygienic behavior [7]. However, if the potentially sporulating chalkbrood mummies are removed, hygienic

behavior can increase rather than decrease transmission by exposing more individuals to the spores [8]. In addition, social insect species, such as *A. mellifera*, exhibit behaviors such as flower sharing to collect pollen and nectar, which might increase the transmission of persistent chalkbrood spores between colonies [9]. Drifting workers and drones may also contribute to the spread of infection [10].

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 [11]. 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 [12–15]. In particular, there is some evidence that *A. mellifera* gut microbiota may exhibit antifungal activity against *A. apis* [16–18]. A broad range of chemotherapeutic compounds have been tested to control chalkbrood disease over the years [19–22], 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 [23]. Therefore, there is great interest in developing alternative chalkbrood-controlling strategies.

In an interesting review, Gaggìa et al. [24] 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 [25–28]. In particular, Tejerina et al. [29] 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. *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum* [30]) 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 [31–33]. *L. plantarum* strains have also been isolated from different honeybee species [34–37]. Several authors have demonstrated that *L. plantarum* colonizes the adult *Drosophila melanogaster* gut and that it influences different aspects of the insect's development and life, exerting a growth-promoting effect on larvae under nutrient scarcity [38–42]. Several authors have proved that *L. 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 [43–46], *L. plantarum* also exhibits antagonist activity against *Paenibacillus larvae*, the causative agent of the quarantine disease American foulbrood, which affects *A. mellifera* larvae and pupae [27,28,30–47]. Over the years, several studies have obtained relevant data supporting the probiotic properties of *L. plantarum* [48,49].

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 [50]. 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 [51–54]. The ability of probiotic bacteria to adhere to intestinal epithelial cells involves extracellular polysaccharide (EPS) production and biofilm formation [51–54], and several *L. plantarum* strains are able to do both [48,49].

In this research, the antagonistic activity of five *L. 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 *L. plantarum* strains as probiotics in the honeybee diet, and their potential use for the biocontrol of chalkbrood disease.

2. Materials and Methods

2.1. Microbial Cultures

In this study, 22 *L. plantarum* strains isolated from beebread, the midgut and the honey stomach of *A. mellifera* L. honeybees were used (Table S1). These bacteria belong to the Di.A.A.A. (Department of Agricultural, Environmental and Food Sciences) collection of the University of Molise [37]. As reference, *A. apis* DSM 3116 and *A. apis* DSM 3117 cultures (DSMZ: German Collection of Microorganisms and Cell Cultures GmbH) were used.

2.2. Screening for Antifungal Activity

The antifungal activity of the *L. plantarum* strains was assessed using the overlay method described by Magnusson et al. [55] 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 *A. apis* 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 *L. plantarum* strains was measured as the diameter (mm) of the clear zone around the bacterial streaks [56]. The tests were performed in triplicate.

2.3. Antifungal Activity Determination

2.3.1. Spore Viability and Germination Test

Fungal cultures of *A. apis* DSM 3116 and *A. apis* 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. [57] 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 10^7 spores/mL) was mixed with 400 µL of GLEN medium [57] 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 [58] 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.

2.3.2. 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. [59] 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 *L. plantarum* strain was cultivated in MRS broth and incubated at 37 °C for 12 h, reaching a cell concentration of 10^8 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 [60].

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: $\% I = [1 - (Ds/Dc)] \times 100$, 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.

2.4. Biofilm Production

Biofilm production was evaluated as described by Cozzolino et al. [61] with some modifications. The *L. 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 10^6 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.

2.5. Exopolysaccharide (EPS) Assay

2.5.1. 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

(10^8 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. [62]. 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).

2.5.2. 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.

2.6. Antioxidant Activity

2.6.1. Bacterial Culture Matrices and Cell Protein Assay

Overnight cultures (10^6 CFU/mL) of the *L. plantarum* strains in LM medium (Table S2) were centrifuged at 13,000 rpm for 5 min at 4 °C, and the obtained supernatants (CFS_{LM}) 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. [63] 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 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.

2.6.2. Antioxidant Activity Assay

The total antioxidant activity (TAA) of the CFS_{LM} 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. [64], 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 CFS_{LM} 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 CFS_{LM} 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.

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 [65].

3. Results

3.1. Antifungal Activity

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

The *L. 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 *L. 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 *L. plantarum* cultures are summarized in Figure 1.

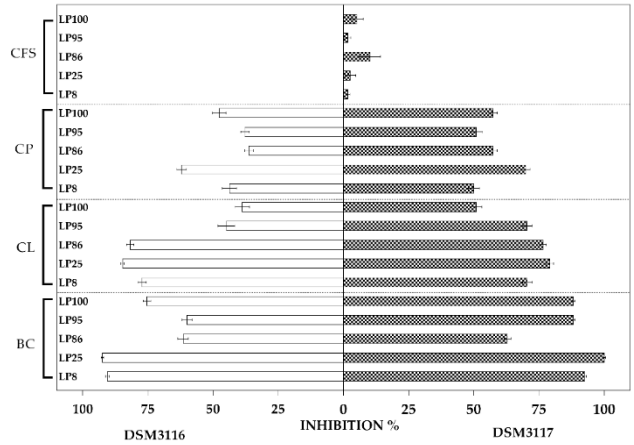

Figure 1. Inhibition (%) of *A. apis* DSM 3116 and *A. apis* DSM 3117 (radial growth) on Malt Extract Agar (MEA) plates after 8 days using culture broth (CB), cell pellet (CP), cell-free supernatant (CFS) and cell lysate (CL) from the *L. plantarum* strains.

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 *L. 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 *L. 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.

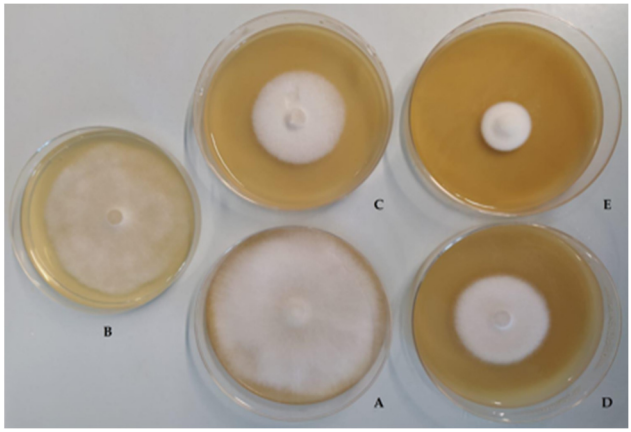

Figure 2. Inhibitory activity of *L. plantarum* LP100 against *A. apis* DSM 3116 after 8 days on MEA agar plates. A: *A. apis* (control); B: *A. apis* + CFS (cell-free supernatant); C: *A. apis* + CL (cell lysate); D: *A. apis* + CP (cell pellet); E: *A. apis* + BC (broth culture).

3.2. EPS and Biofilm Production

The amounts of EPS produced by the five *L. plantarum* strains are reported in Table 1. The EPS-r values, expressed as mg/mL, were obtained for each bacterial culture (10^8 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 (10^8 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 *L. plantarum* LP8, LP25, LP86, LP95 and LP100 strains. All values are expressed as mean ± standard deviation (n = 3). Different lowercase letters (a–d) in each row indicate significant differences ($p < 0.05$).

	***L. 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 *L. 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 *L. 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. *L. plantarum* LP8 produced, under all the conditions, greater amounts of biofilm than the other strains.

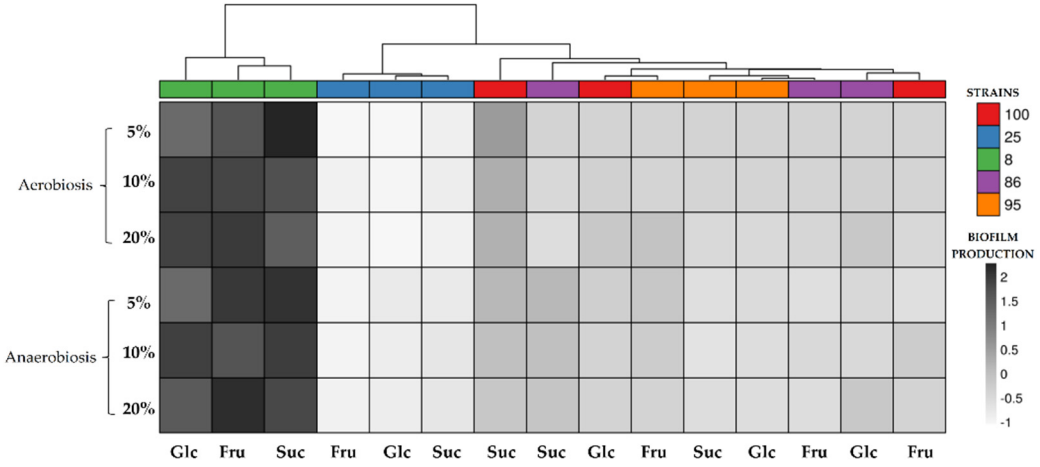

Figure 3. Biofilm production of *L. plantarum* LP8, LP25, LP86, LP95 and LP100 strains in aerobiotic and anaerobiotic conditions and with different sugar concentrations (5%, 10% and 20% (Glc: glucose; Fru: fructose; Suc: sucrose)). This figure was generated using ClustVis web tool [65] https://biit.cs.ut.ee/clustvis/ (accessed on 4 December 2020).

In all the tests, the anaerobiotic condition almost always favored the production of biofilms. In particular, the *L. 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.

3.3. Antioxidant Activity

The antioxidant activities of the CFS_{LM} and CES matrices are shown in Table 1. The *L. 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).

4. Discussion

4.1. Antifungal Activity

Our purpose was to investigate the ability of *L. 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 *L. plantarum* against wild *A. apis* strains.

Our study demonstrated that the *L. 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 [66,67]. 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 [51,52,68,69].

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) [70–82].

Our tests of *A. apis* inhibition demonstrated that all five *L. 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 [51–56,59–64,68–78]. 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 [83].

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 *L. 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 *L. plantarum* strains, added to the diets of honeybee colonies in vivo/in situ.

4.2. EPS and Biofilm Production

Our results show that these five *L. 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 [15,84–91]. 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 [3]. 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.

Our tests also confirmed that EPS and biofilm production are strain-dependent, as documented by other researchers [92]. *L. 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 [93–95].

The formation of EPSs and the development of a biofilm are also affected by other factors, including surface properties and environmental parameters [96–98]. 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 *L. plantarum* strains to the epithelial cells.

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 [99–101].

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) [102–110]. 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 [111–113]. Li et al. [4] 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 defenses 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 [114–118].

We assessed antioxidant activity using the ABTS assay, which is considered one of the most sensitive techniques [119] and a valid method for determining the antioxidant activity of both hydrophilic and lipophilic extracts [120]. All five *L. 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.

5. Conclusions

The *L. plantarum* strains used in our experiments have been shown to possess substances biologically active against *A. apis* fungi. These results confirm the potentially antagonistic role of *L. plantarum* against pathogenic microorganisms that use the digestive channels of honeybees as the sites of infection [47]. Moreover, our findings indicate the ability of the *L. 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 *L. plantarum* strains on honeybee health and productivity, and their efficacy in chalkbrood disease biocontrol in vivo/in situ.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jof7050379/s1. Table S1: List of 22 *L. plantarum* strains, Table S2: Letizia medium (LM) composition, Table S3: Spore germination, Table S4: Fungal inhibition, Table S5: Biofilm production.

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R.C.; investigation, B.T., S.P. and P.T.; methodology, S.J.L., S.G., B.T., F.V. and A.C.; project administration, S.P.; resources, R.C. and A.D.C.; software, F.L.; supervision, R.C. and A.D.C.; validation, M.S.; writing—original draft, S.G. and M.I. (Massimo Iorizzo); writing—review and editing, M.I. (Massimo Iorizzo), S.G. and F.L. Authorship must be limited to those who have contributed substantially to the work reported. All authors have read and agreed to the published version of the manuscript.

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