Chapter 11

Microorganisms in Honey

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Additional information is available at the end of the chapter

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Abstract

Honey is a product with low water activity because of the great amount of sugars (fructose and glucose), and also it has antimicrobial compounds derived from flowers or because of its transformation process in the beehive. Despite all the honey microorganism barriers, some species of microorganisms are able to survive and may cause damage to honeybees or consumers. Techniques of pathogenic microorganism identification by DNA using PCR are recommended and required for sanitary and customs control. It is important to know the diversity of contaminating microorganisms in honey, especially due to disseminate pathogenic microorganisms in the international traded marketing. In contrast, beneficial microorganisms such as yeasts can remain latently in this product waiting for the moment in which the environment is suitable for their development. Among the beneficial bacteria found in honeybee products, we can mention some lactic acid bacteria that act as prebiotics when ingested. The microorganisms in the digestive tract of honeybees are important for their health. Thus, we present the knowledge of microbiota associated with honey from honeybees and stingless bees (*Hymenoptera*, *Apidae*) and the techniques available for the detection of microorganisms in honey.

Keywords: microbiota, prebiotics, pathogenic microorganisms, yeast, bacteria

1. Introduction

Honey is used as a therapeutic product since ancient times. Its properties are chemically evidenced by its composition. Among features that make this product effective against microorganisms, we can quote high osmotic pressure by low water activity (average 17.2%); low pH because of the presence of organic acids, mainly gluconic acid (average 3.9); the presence of hydrogen peroxide generated by action of enzyme glucose oxidase; low protein content;

low redox potential due to the presence of reducing sugars; and chemical agents present as lysozyme, phenolic acids, pinocembrin, terpenes, benzyl alcohol, and volatile substances [1, 2].

High osmotic pressure results from its composition: 85–95% of sugar, of which it has 28–31% of glucose, 22–38% of fructose, 1–4% of sucrose, and 1–9% maltose [3]. Isomaltose and some oligosaccharides are also present in honey and vary according to flowering, climate, and local production [4, 5]. As honey is a product developed from changes in nectar, the bees incorporate the glucose oxidase enzyme that converts glucose into hydrogen peroxide and gluconic acid; this compound is indeed important for the taste of products as well as their bioactivity [5, 6]. The presence of acids and other chemicals varies with the composition of the transformed nectar; for this reason, some honeys have higher antimicrobial activity with respect to other different blossoming [7].

About these conditions, few microorganisms have the capacity to develop or remain in honey. These microorganisms are derived from primary or secondary sources of contamination. The primary sources are related to digestive tract of honeybees, which have natural microorganisms and sources of material collection such as nectar, pollen and propolis, air, flowers, and the environment inside the beehive, while the secondary sources are incorporation of honey microorganisms postharvest, processing plants, and appliances [5].

2. Human pathogenic microorganisms found in honey

Due to characteristics cited above, only pathogenic bacteria capable of sporulation have the ability to keep in honey, but they have no reproductive capacity or vegetative cells. Fungi and yeasts are able to maintain their vegetative form [2].

Fungal growth is followed by the production of mycotoxins, which are secondary metabolites of filamentous fungi and toxic to humans and animals even in small concentrations. These are produced by fungi to reduce the incidence of competitors in environment [8]. The main producers of mycotoxins are fungi of the genus *Aspergillus*, *Alternaria*, *Fusarium*, and *Penicillium* [9]. Among which we should highlight *Aspergillus* spp. and *Penicillium* spp. because they are the most commonly found in honey. Articles about these microorganisms in honey record these genera in isolated colonies in the United Kingdom, Pakistan, Italy, and Brazil [10–13]. They are also associated with disease in honeybees.

In research performed with honey samples of different blossoming, fungi of different species were isolated, *Alternaria alternata*, *Aspergillus niger*, *Aspergillus proliferans*, *Aspergillus spelunceus*, *Chaetomium globosum*, *Cladosporium cladosporioides*, *Daldinia concentrica*, *Emericella discophora*, *Emericella qinqixianii*, *Penicillium corylophilum*, *Penicillium decumbens*, *Penicillium polonicum*, and *Penicillium echinulatum*, of which *P. corylophilum* and *A. niger* were the most frequent, but in low count, indicating that the honey is capable of containing multiplication of these fungi [13]. The presence of fungi does not imply the presence of mycotoxin; it has necessary ideal conditions such as high water activity, the presence of sugars, and the presence of organic acids capable of reducing pH. Necessary conditions for fungal growth are not

always the necessary conditions for production of mycotoxins [9]. As an example, we can cite the patulin produced by species of *Penicillium*, *Aspergillus*, and *Byssochlamys* whose optimum temperature for production is 23–25°C, with minimal water activity of 0.82–0.83. Aflatoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* have ideal temperature of 30–52°C and 0.80–0.95 water activity, and ochratoxin that is produced by species of *Aspergillus* and *Penicillium* needs temperature between 30–35°C and 0.93–0.99 of water activity [9].

Despite of inappropriate condition found in honey for mycotoxin production, it is important to say that the presence of fungus can also cause disease in different ways, as induction of allergic responses and infections. The fungi of genus *Aspergillus* are able to causing bronchopulmonary allergies among other forms of invasive aspergillosis. They are also related in acquired disease by immunocompromised patients in hospital. *Aspergillus fumigatus* is the most pathogenic followed by *A. flavus*, *Aspergillus terreus*, and *A. niger* [14]. The allergies and asthma may be caused by inhaled or ingested spores. For example, *Aspergillus clavatus* and *A. fumigatus* are responsible for allergies from malt workers who inhaled large amounts of spores during the malt handling for contaminated barley [15]. Foods with acidic pH, low humidity, and high concentration of sugars, such as honey, are sources for growth of the fungi *Aspergillus glaucus* [15].

Regarding the *Penicillium*, this fungus was first associated as producer of mycotoxins. They are saprophytic fungi able to grow at water activities less than 0.9; they can invade plants and animals but not as obligate parasite. They have vegetative reproduction by spores. However, the most important aspect concerns the production of toxins as aflatoxins, patulin, and ochratoxins [16]. In humans, only a minority of fungal species has pathogenicity, i.e., *Penicillium marneffei* (Southeast Asia), which is assigned lung infections in people with HIV virus in South Asia and China, and opportunistic infections—keratitis, ear infections, and endocarditis [17].

With respect to yeasts, only *Debaryomyces hansenii*, *Zygosaccharomyces rouxii*, *Zygosaccharomyces mellis*, *Aureobasidium pullulans*, and *Cryptococcus uzbekistanensis* species were isolated from honey [13]. Among them only *Cryptococcus* species was associated with human pathogenicity, i.e., the yeast *Cryptococcus neoformans* is characterized as opportunistic human pathogen able to infect the central nervous system [18].

Among bacteria, *Bacillus* sp. and *Clostridium* sp. were described in honey. *Clostridum perfringens* is known as an enterotoxin producer that happens in final stages of sporulation; thus, in adverse conditions for their development, the toxin will be released together with spore. Vegetative cells also produce enterotoxin but at low levels. Unlike *C. perfringens*, the toxin produced by *Clostridium botulinum* is stronger and produced during propagation. Thus, the best condition for propagation is the same for toxin production, which is 4.5 pH, water activity of 0.93, and temperature varying with strain [19].

There are about 200 species of *Clostridium*; a lot of them has pathogenicity and produce one or more toxins, assimilated by the gut and transported by blood [20]. Only *Clostridium botulinum* was found in honey [2], but was hardly detected with conventional methods; however, with molecular techniques as PCR, the detection was more accurate. In this way, samples that seem

negative showed positive with molecular test [21, 22]. This microorganism enters the beehive through the contaminated water or even by contact of product with ground. This organism does not cause damage to honeybees, but it is responsible for the development of botulism in humans, especially in children or people with weakened immune systems and can lead to death [23].

Genus *Bacillus* comprises rod-shaped Gram-positive bacteria with the ability to form spores. There are 60 species of huge genetic diversity, and most of them are nonpathogenic; the pathogenicity associated with others is in opportunistic form. These pathogens belong to group *Bacillus cereus*, a subgroup *Bacillus subtilis*; however, *Bacillus licheniformis*, *Bacillus pumilus*, and *Bacillus majavensis* can cause poisoning by food too [24]. *Bacillus cereus* is an important pathogen in honey; it is an enterotoxin producer in pH 6.0–8.0 and temperature ranging from 6°C to 21°C, but it is necessary to ingest 10^7 cells/mL to reach toxic effect [19].

Researchers isolated some bacteria in honey samples of different geographical and botanical origins. "They found *B. pumilus* (ML374), *B. licheniformis* (ML103A and ML104B), *B. amyloliquefaciens*, *B. subtilis*, *B. cereus*, *B. thuringiensis*, *B. licheniformis*, *B. megaterium*, and *B. pumilus* [13]." The bacteria of species *B. cereus* are enterotoxin producers; the others of *Bacillus* species are considered safe. Due to their ability of producing bacteriocins, they are promising in the study of new antimicrobial [25].

3. Beneficial microorganisms in honey for humans

Human metabolism is dependent of symbiotic microorganisms, known as the indigenous microflora capable of favoring the production and absorption of essential nutrients to our body such as K and B12 vitamins, pentatonic acid, pyridoxine, and biotin, and acts by modulating the immune system [26]. This microbiota lives in the gut, due to high acidity of the stomach (pH 1.5); the most microorganisms are unable to grow, while in the gut we can found a lot of microorganisms with 500–600 different species [26]. There is no oxygen in gut; for this reason, the gut bacteria are aero-tolerant and facultative anaerobic. We can find bacteria of genus *Actinomyces*, *Bacteroides*, *Clostridium*, *Enterobacter*, *Enterococcus*, *Escherichia*, *Klebsiella*, *Lactobacillus*, *Proteus*, *Pseudomonas*, *Staphylococcus*, and *Streptococcus*; many of them are opportunistic pathogens when move to other parts of the body [26].

For honey production, honeybees ingest nectar and turn this with help of enzymes. Beyond the enzymes, they incorporate some symbiont microorganisms associated with gastrointestinal tract that can bring benefit to human health [27]. The natural human microbiota is stable; so it is necessary for daily intake of the new symbiont to be able to populate the human body and maintain its benefits [28]. These microorganisms are known as probiotics and, when they grow in human gut, can make nourishment benefits, like fermentation, and broke nutrients facilitating absorption of short-chain fatty acids, ions, amino acids, and vitamins; protective effect, preventing invasion of pathogenic microorganisms; and trophic effect in the gut epithelium and in the system [28].

Bacterium *Gluconobacter oxydans* was isolated from honey harvested directly from beehive. Also, *Pseudomonas* spp. and *Bacillus* spp. were found [29, 30]. However, *G. oxydans* is highlighted because they showed 100% of survival in pH of 5.0 and 50% of survival after 3 h of contact in pH of 2.0 and showed resistance in 2% of bile salts. This is atypical behavior for bacteria, because normally they have low resistance in acidic environments. For this way, a bacterium resistant to condition of the stomach is promising to arrive in the gut, where it will grow and will make benefit [29]. These bacteria can assimilate cholesterol reducing absorption of this component by the body, and it can be used as probiotics in food [29]. In addition to this, as honey is rich in fructose, some bacteria that live in there possess the ability to degrade fructose more easily; these bacteria are known as fructophilic lactic acid that prefer to metabolize fructose and not glucose as normally is observed. In the gut, these bacteria produce bacteriocins that act as a barrier to other microorganisms and contribute to the immune system. *Lactobacillus kunkeei*, fructophilic lactic acid bacteria, were found in the stomach of honeybees, as well as in their hives [27].

Besides these microorganisms is necessary consumption of substances that promote their development, known as prebiotics. These prebiotics are components, like oligosaccharides, that are not digested by humans, but they serve as a substrate for the growth and performance of probiotics [28, 31]. Currently, there is a great interest in combining probiotics with oligosaccharides acting like prebiotic. There are studies with probiotic *Lactobacillus* sp., which show that when they are grown in the presence of oligonucleotides, they show an increase in growth and antibacterial activity with production of bacteriocins [32].

The most-studied prebiotics are fructo-oligosaccharides, inulin, and oligofructose especially [33, 34]. However, there are others recognized as prebiotic, like galacto-oligosaccharides, trans-galactosylated oligosaccharide, isomalto-oligosaccharides, lactulose, pyrodextrin, and soy-oligosaccharides [28]. In honey we can find malto-oligosaccharides [35], specifically in Brazilian honey samples that were found in isomaltose, cellobiose, panose, maltotriose, melezitose, raffinose, maltose, turanose, and maltotriose, which are characterized as prebiotics [36].

In addition to probiotics, there are microorganisms associated with honey that can produce bacteriocins, which are substances able to reduce or eliminate competing microorganisms. These are peptides produced by bacteria producers of lactic acid, to reduce competition for nutrients, making inappropriate environment for development of other bacteria; for this reason, they are studied as an option for replacing antibiotics, and as usual these can cause harsh effects to humans also. Bacteriocins have high potency *in vivo* and *in vitro* and have low toxicity, and they can be produced in situ through consumption of probiotics or purified through bioengineering [37]. In 2013, a study was conducted with a new bacterium strain isolated from honey, able to produce bacteriocins fungicides called *Bacillus* BH072. These bacteriocins were tested and showed inhibitory character against *A. niger* CGMCC3.03928, *Fusarium oxysporum* CGMCC3.2830, *Pythium*, and *Botrytis cinerea* CGMCC3.4584 [25]. In another search, 13 lactic acid bacteria were isolated from honey and honeybees, and they were tested against bovine mastitis; they observed that the synergism between lactic acid bacteria and honey was able to inhibit growth of bacteria that cause mastitis, even those that were resistant to other antibiotics, and this is a promising preventive treatment to be studied [38].

Studies suggested that the antimicrobial character of honey is attributed to activity of these bacteria in honey; these are also present in the stomach of honeybees. *Lactobacillus* spp. were isolated from the stomach of honeybees and honey, they were then tested against *Escherichia coli* and *Salmonella enterica*, and they showed inhibitory effect. It is important to say that *Lactobacillus helsingborgensis* and *L. kunkeei* are the most candidate promisors like probiotic producers of bacteriocins [39]. Direct application of honey was also effective against *Serratia marcescens* and *Candida albicans* [40]. Beyond health benefits, discovery and application of microorganisms able to develop biotechnological products must be studied because they can improve lifestyle and human survival, becoming in this way beneficial microorganisms.

Besides the microbiota associated with honey, it is worth mentioning that this product alone is highly beneficial by features from its composition. This makes the honey effective activity like antimicrobial, antioxidant, anti-inflammatory, anticancer, antihyperlipidemic, cardioprotective properties, for ocular treatment, gastrointestinal tract disorders, neurological disorders and wound healing [1]. Honey has a series of phenolic acids like caffeic, ellagic, ferulic, and p-coumaric acids; flavonoids, such as apigenin, chrysin, galangin, hesperetin, kaempferol, pinocembrin, and quercetin; and antioxidants, such as tocopherols, ascorbic acid, superoxide dismutase, catalase, and reduced glutathione [41]. These compounds are known for their ability to reduce free radicals; this composition may vary depending on floral source that honeybees have visited for honey production [42]. Its antimicrobial activity makes it an important substance for the treatment of wounds as a result of carbon, lipids, amino acids, proteins, vitamins, and minerals active in healing. Components such as hydrogen peroxide, high osmolarity, acidity, non-peroxide factors, nitric oxide, and phenols are active in their healing effect. It also promotes growth of tissue in the human body, and it has anti-inflammatory activity [43]. However, it is important to note that honey directed to the treatment of wounds and inflammation should undergo irradiation treatment, so that microbiota will not interfere negatively on treatment [44].

Finally, it is important to note that consumption of foods able to bring health benefits, beyond nutrition, is a current practice that should be encouraged; honey is characterized as such, and it should be ingested daily.

4. Microorganisms in honey for industrial use

The yeasts that were found in honey are able to withstand high concentrations of acids and sugar, and it can be a problem for the honey processing industry; however, they are promising for fermentative processes. Furthermore, the low concentrations of these nutrients in honey characterize yeasts as nutritionally less demanding. *Saccharomyces* is widely found in honey, as well as *Rhodotorula*, *Debaryomyces*, *Hansenula*, *Lipomyces*, *Oosporidium*, *Pichiu*, *Torulopsis*, *Trichosporon*, *Nematospora*, *Schizosaccharomyces*, *Schwanniomyces*, *Torulu*, and *Zygosaccharomyces*. The amount of these yeasts will be increased in relation to the humidity of honey; honey with higher humidity, we will have higher population of yeasts [2]. Species of *Zygosaccharomyces* are recognized as osmophilic; *Zygosaccharomyces gambellarensis* (a new species of yeast),

Zygosaccharomyces favi sp. nov., and *Zygosaccharomyces clade* were isolated from honey and bee-bread. They are obligatory osmophilic, and they do not have the ability to grow in high water activity [45]. In another study, during the isolation of 20 strains of yeasts from honey, all of them were characterized as *Zygosaccharomyces rouxii* [46]. Studies show that this yeast has high productivity of glycerol, a common characteristic in osmophilic yeast [47].

Besides yeast, filamentous fungi are also significant because they are known for their ability to produce extracellular substances such as enzymes and acids; they must be studied, as they are able to produce substances of industrial interest in osmotic stress condition. The genera *Aspergillus* and *Penicillium*, previously mentioned pathogens [10], are able to produce numerous extracellular compounds with biotechnological importance due to their characteristic of digest food externally before absorption of nutrients; for this reason, they produce organic acids and extracellular enzymes such as amylases and citric acid [15]. These fungi are capable of degrading starch, hemicellulose, cellulose, pectin, and sugars among other polymers. Some of them are able to degrade fats, oils, chitin, and keratin [16, 48].

5. The gut microbiota as an environmental factor for honeybee health

Honeybees have a beneficial anaerobic and micro-aerobic natural microbiota acquired and installed in their body. This includes Gram-negative groups like species *Gilliamella apicola*, *Snodgrassella alvi*, and *Frischella perrara* and Gram-positive groups like species of *Lactobacillus* and *Bifidobacterium* [49, 50]. That is, *Acetobacteraceae*, *Parasaccharibacter apium* confers resistant to *Nosema* [51] and *Bartonella apis*, a honey bee gut symbiont of the class *Alphaproteobacteria* [52, 53]. So it is natural for bees to acquire these microorganisms through feeding [49]. This honeybee normal microbiota comes from food, pollen, and honey consumption or through contact with other worker honeybees.

The microbiota associated to the honeybee *A. mellifera* is complex, and it has been described as being mainly composed of yeasts, Gram-positive bacteria (such as *Lactobacillus rigidus apis*, *S. constellatus*, *Bacillus* spp., *Streptococcus*, and *Clostridium*), and Gram-negative or Gram-variable bacteria (*Achromobacter*, *Citrobacter*, *Enterobacter*, *Erwinia*, *Escherichia coli*, *Flavobacterium*, *Klebsiella*, *Proteus*, and *Pseudomonas*) [54–58].

There are several bacterial species negatively affecting honeybee health—*Paenibacillus larvae*, *Melissococcus plutonius*, *Spiroplasma apis*, and *Spiroplasma melliferum* [59–61]. Besides bacteria, there are many fungi, viruses [62], and protozoa, i.e., *Apicystis bombi*, *Crithidia mellificae*, and *Lotmaria passim* (**Figure 1**) [63–65]. *P. larvae* is a sporulated Gram-positive *Bacillus* that causes the American foulbrood disease in larvae.

Gilliam reported that these bacteria could be endemic of the digestive tract of adult honeybees and independent of seasons and nutritional factors [11]. They are different depending on the sources of nectar and the presence of other bacterial genera in the stomach of the honeybee. It seems that bees and lactic acid bacteria developed mutualism. Lactic acid bacteria prepare the environment to make nutrients available for honeybees; on the other hand, intestinal tract

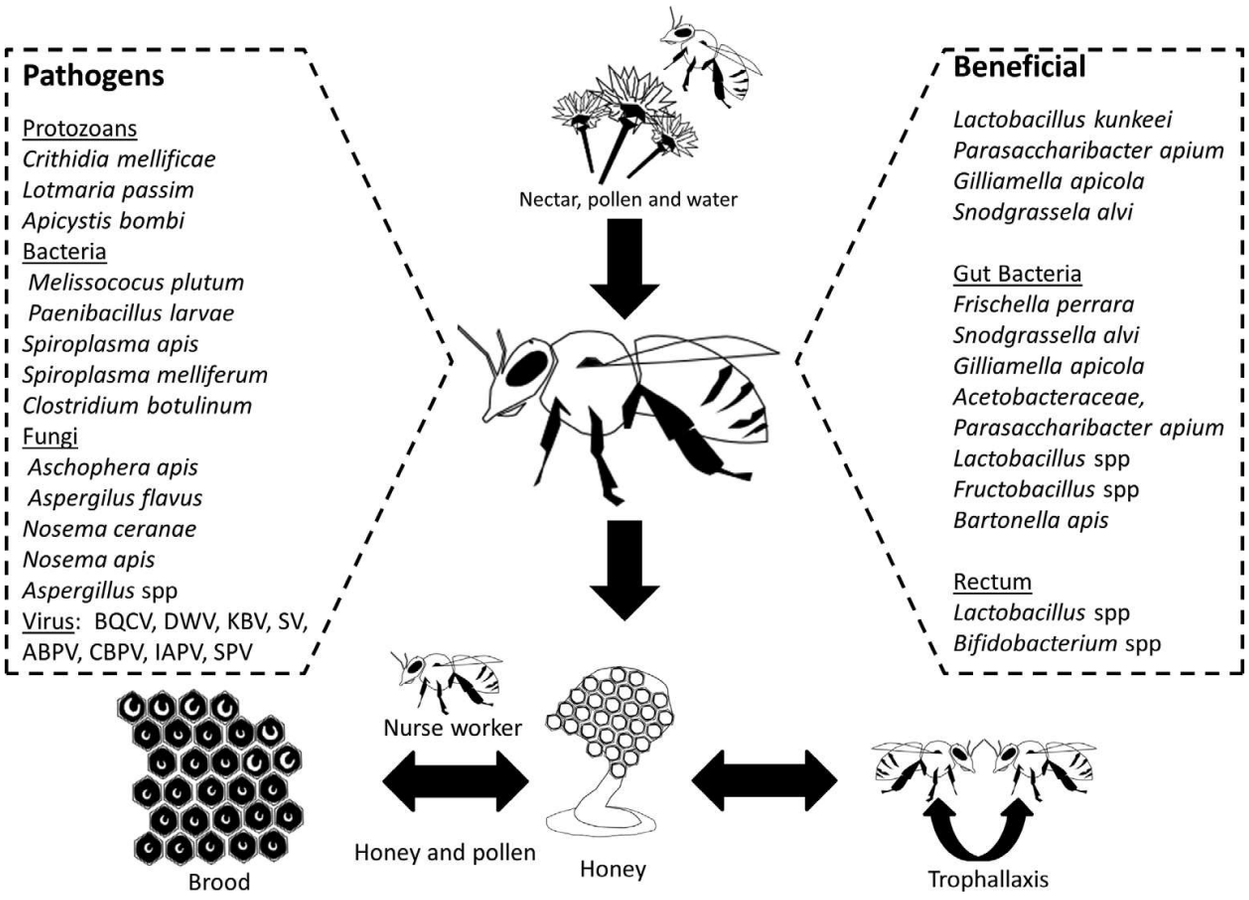

Figure 1. The pathogens and beneficial microorganisms in honeybee: one pathway of bee food contamination comes from environmental nectar, pollen (on flowers), and water collected by worker honeybees. The food is stored in beehive and can be transferred by trophallaxis among workers and brood. Another pathway is the consumption by honeybees of contaminated honey and/or pollen from other beehives. Common viruses: black queen cell virus (BQCV), deformed wing virus (DWV), Kashmir bee virus (KBV), Sacbrood virus (SV), acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), Israel acute paralysis virus (IAPV), and slow paralysis virus (SPV). Niche of beneficial microbiota on alimentary tract (gut). The arrows indicate the transfer of microorganisms by food among individuals (larva and adults) in the beehive. For detail, see in the text.

of honeybees is protected from harmful microorganisms. The honeybee regurgitates the nectar stocked in the crop in the hive honeycomb that has an optimum temperature of 35°C [66] for the development of lactic acid bacteria.

The honeybee larvae probably are sterile initially, but as feed on honey from nurse workers, honeybees gain over time this intestinal flora before completing their life cycle [67]. Honeybees harbor a number of commensal or beneficial bacteria distributed throughout the different compartments of their gastrointestinal tract. Each compartment of the honeybee gastrointestinal tract has a distinct environment favoring specific microorganisms [68]. Several findings have indicated that the honeybee gut is colonized by a distinctive set of bacterial species designated as the core gut microbiota [69]. Because the community composition changes through the life cycle of honeybee, the colonization of the gut is believed to be influenced by the age [68]. During the course of their life span, worker honeybee performs many different tasks that can contribute to these variations. Newly emerged worker honeybees nurse larvae within the hive, whereas

older worker honeybees build and maintain the wax combs, defend the colony, and receive and process food that is collected by foragers. In addition to the microbiota in the gut, a novel lactic acid bacterial flora composed of 13 taxonomically well-defined *Lactobacillus* and *Bifidobacterium* species were discovered in the crop of honeybees [70, 71]. The crop functions as an inflatable bag that can transport the nectar back to the hive for storage and honey production. It is hypothesized that lactic acid bacteria play a key role in the conversion of both nectar to honey and pollen to beebread (stored food rich in protein) due to their fermentation properties [70, 72]. The lactic acid bacterial microbiota is of great importance to the honeybee health, protecting them against bee pathogens [73, 74] and contributing to the antimicrobial properties of honey [71].

Lactic acid bacteria are found in two distinct phyla: *Firmicutes* and *Actinobacteria*. The most important genera of lactic acid bacteria within the *Firmicutes* are *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, and *Weissella*, which all have a low G+C content. Lactic acid bacteria in the *Actinobacteria* phylum only include species of the *Bifidobacterium* genus that in contrast to the *Firmicutes* members have a high G+C content [75, 76].

Lactic acid bacteria are important inhabitants of the intestinal tract of man and other mammalian and vertebrate animals. *Lactobacillus* and *Enterococcus* are members of this family and are also present in food and fermentation processes [77]. These microorganisms disclose interesting properties not only for the food industry but also for health. The antimicrobial potential of these bacteria includes, among others, the synthesis of compounds such as lactic acid, short-chain volatile fatty acids, and bacteriocin-like molecules [78, 79]. Antagonistic studies are generally directed toward food spoilage and/or pathogenic microorganisms related to the host or product from which the lactic acid bacteria were isolated. Fructophilic lactic acid bacteria are a special group of lactic acid bacteria, which prefer fructose over glucose as growth substrate [80]. They are found in fructose-rich niches, e.g., flowers and fruits. Moreover, the microorganisms can be found in fermented foods made from specific fruits, including wine, fermented cocoa beans, and fermented durian-based condiments [81–83]. *Fructobacillus* spp. and *L. kunkeei* are representatives of these microorganisms, and a few novel species have recently been classified as members of this interesting group [84, 85].

Quite recently fructophilic lactic acid bacteria were found in the gastrointestinal tract of several flower- or fructose-related insects, including honeybees, tropical fruit flies, and giant ants [86–88], whose diets are fructose rich. Of these insects, honeybees are economically and agriculturally important for honey production and especially for crop pollination, which links to human food production. However, despite the importance of these insects in nature and in our lives, populations of honeybees are reported to have decreased considerably during the last decade and to be still decreasing worldwide, mainly by colony collapse disorder [89]. To understand and to prevent the disorder, microbial interactions, both symbiotic and pathogenic, have recently been studied [90, 91], and findings have indicated that honeybees carry specific microbiota dissimilar to other animals, including humans. Fructophilic lactic acid bacteria, especially *L. kunkeei*, have been found to be one of the dominating bacterial species in several honeybees kept or captured in different regions [73, 90]. Lactic acid bacteria have been successfully applied as probiotics to contribute to health in humans and various companion and farm animals [92, 93]. As lactic acid bacteria are important components in their gastrointestinal tract,

with a reported impact on the intestinal barrier mechanism [94], it is not surprising that lactic acid bacteria, especially fructophilic lactic acid bacteria, may be involved with honeybee health.

Symbiosis is common in nature, in which symbionts as commensals or mutualists evolved to benefit each other. Culture-independent studies of the human microbiota identified recently a complex symbiotic environment with more than 1000 bacterial phylotypes representing more than 7000 strains [95]. The composition of this microbiota has been suggested to be a result of a highly coevolved symbiosis and commensalism influenced by nutrition, physiology, and immunological factors. It varied with the sources of nectar and the presence of other bacterial genera within the honeybee and ended up eventually in the honey (**Figure 1**).

6. Microorganisms in stingless bee honey

Products of stingless bees are consumed since before the discovery of the Americas to the present day. Honey of these bees has activities against microorganisms, having importance in the colony maintenance as a microbiologically stable environment [96]. Stingless bee honey has characteristics that confer antimicrobial character, i.e., activity against Gram-negative and Gram-positive bacteria such as *Enterococcus*, *Staphylococcus faecalis*, *Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa*, *Bacillus cereus*, and *Candida albicans* [1, 97, 98], which justifies its use in popular medicine [6, 41, 99–101].

However, *Meliponini* also feature mutualistic interaction with microorganisms, i.e., lactic acid bacteria are found in Australian species as *Tetragonula carbonaria*, *T. hockingsi*, and *Austroplebeia australis* [102]. Yeasts such as *Starmerella meliponinorum*, *Starmerella neotropicalis*, *Candida apicola*, and *Zygosaccharomyces* spp. are commonly found in the Neotropical species of stingless bees such as *Tetragonisca angustula*, *Frieseomelitta varia*, *Melipona quinquefasciata*, and *Melipona quadrifasciata* [103–105] and provide sensory and conservation to food characteristics [106–109].

About fungi, the interesting fact is that bees cultivate them as food [110] and protection against other pathogenic microorganisms [111], i.e., *Scaptotrigona aff. depilis* young larvae, needs to be fed from the mycelium of *Monascus* genus (*Ascomycotina*) to complete their development [112], which reinforces the intrinsic evolutionary relationship between microorganisms and these bees.

Little is known about pathogens in stingless bees; however, there are no pathogen transfer record from *A. mellifera* [113], which shows the lack of information about microorganisms in *Meliponini*.

7. Microorganism detection methodologies in honey and honeybee products

7.1. Microbial diversity

Much has been discussed about the succession of gut microbiota among queens, workers, and larvae and the role of the diversity on the quality of honey, safety, and health of the colony [11, 53, 114–117]. New methodologies have made it possible to access information about the

differences in the profile of this microbiota in different apiculture sources [118–121], species [53, 122] and genetic diversity [116] of honeybees, development stages [53, 68, 117, 122–126], nutrition [116, 127], location inside the gut [49, 53, 68] and digestive system [120], ontogenetic stage and geographic location [118, 122, 125], environmental conditions [128], health control [129], and individual [116, 125].

This access has been carried out mainly by sequencing the coding region of the 16S subunit of the bacterial ribosome [53, 121, 130], both from genomic DNA from microorganisms growing on selective media as Man-Rogosa-Sharpe agar, Sabouraud dextrose agar, and *Candida* agar [117, 120, 131, 132], such as process-independent culture as specific PCR [68], denaturing gradient gel electrophoresis [124, 125], mixed and deep 16S sequencing [49, 128], pyrosequencing [53, 116, 121], and clone library [115, 118, 120, 122]. While culture-dependent methods are ideal for quantification of microorganisms and phenotypic testing, culture-independent methods generally have greater coverage in relation to the amount of different species accessed and are ideal for fingerprinting studies, and the identification of these species may be performed by real-time PCR analysis [49, 68, 125, 128]. These methods, although they have different principles, were able to distinguish similarly the narrow niche of bacterial species and the diversity of strains present in these matrices [120]. In some works, the complete genome [132, 133] or metagenome [114, 115] of the narrow range of species of microorganisms is accessed, enabling the search for specific functions of these bacteria for beehives by gene annotation, PCR screening [114], and Post-Light TM ion semiconductor sequencing [127]. Fluorescent in situ hybridization microscopy has also been used to characterize distribution and abundance of specific phyla across the life cycle and among gut organs [68]. Changes in the diversity of microbial populations found by these authors would be able to explain the transformations that occur in honey and pollen, as well as strategies of these insects to combat pathogens and invaders [11, 114, 116, 121] and beebread preservation [11, 120].

Several microorganisms present in the honey and in the gut of honeybees have antagonistic effects on honeybees and human pathogens, especially of *Bacillus* genus [123, 134], lactic acid bacteria as *Lactobacillus* [71, 121, 124, 130–132, 135], *Enterococcus* [130], *Bifidobacteria* [116, 132, 136–138], and *Acetobacteraceae* [117, 121, 133]. These same microorganisms can be accessed for other purposes, such as its potential as fermenters [116, 130, 133] or probiotics [116]. In this case, direct detection strategies of these microorganisms are not the analysis priority since their isolation is of interest to researchers for the antagonism studies. "This isolation is mainly done using traditional selective media, especially Man-Rogosa and Sharpe agar to *Lactobacillus*; *Streptococcus* selective medium and MTPY or Wilkins-Chalgren medium for *Bifidobacterium*" [71, 130, 136, 138, 139], with or without prior enrichment [133], and the identification of the isolates is mainly performed by sequencing 16S rRNA amplicons. However, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry profiling was used for acetobacterium identification from bumble bee crop [133] and clustering of lactic acid bacteria of a bumble bee gut microbiota [139]. Several studies have shown the effectiveness of these microorganisms to inhibit human pathogens such as *Staphylococcus aureus*, *Escherichia coli* O157: H7, *Salmonella*, and *Listeria* [130, 140] or pathogens of honeybees as *Melissococcus plutonius* [124, 138], the causative agent of European foulbrood and *Paenibacillus*

larvae [123, 124, 130, 134], and the causative agent of American foulbrood, among others. This effectiveness is generally associated with the production of acid, bacteriocins [130], and other antimicrobial molecules [140].

7.2. Monitoring of the microbiological honey quality

Traditional methods are often still used for monitoring the microbiological quality of honey used for human consumption, even as the rates established by the laws use these methods. Potato dextrose agar and yeast extract glucose chloramphenicol agar are media normally used for aerobic count and the total fungi (yeasts and molds), while Violet Red Bile and MacConkey medium agars are normally used for counting coliforms, which can also be done by the most probable number technique [119, 141, 142]. These media have recently been used to monitor the efficiency of a new filter-based method based in reducing the microbial burden and to improve the microbiological quality of honey [143]. Potato glucose agar in Brazil was also used for monitoring the honey contamination by yeast and fungi [144]. Standard plate count agar is used for monitoring of mesophilic bacteria, such as that was done in honey samples of Portugal [141, 142] and Argentina [119, 145].

7.3. Detection of honeybee pathogens in honey

The honey is an important route of contamination of honeybees, spreading many microorganisms, particularly pathogens that infect the honeybees. Several molecular techniques have been developed for the detection of pathogens like *Paenibacillus larvae*, *Melissococcus plutonius*, *Nosema ceranae* and *Nosema apis* [129, 146, 147], *Ascophera apis* and *Ascophera ceranae*, and *A. flavus* [129, 148]. Among them can highlight the simple PCR [149–151], NESTED-PCR [152], RT-PCR [153, 154], immunology-based tests (ELISA), and probe-based hybridization analysis [155]. The main advantages of these techniques would be less needed for sample treatment which often can be applied directly to the honeybee products, fast technique, specificity, and sensitivity of detection.

The use of these techniques and the detection of this pathogen have allowed the control of mortality of honeybee populations around the world, restricting the dissemination of pathogens in bee products. For example, the diagnosis of American foulbrood and European foulbrood usually occurs through visual inspection of brood combs and detection of diseased larvae, subjective criteria that could be confused with other beehive conditions [155, 156]. The traditional methods of detection of these pathogens include the visualization by microscopy and detection in tissues [155]; culture on selective medium [151, 155, 156], including *P. larvae* agar [151]; bacteriophage sensitivity; immunotechniques; and microscopy of suspect bacterial strains have been considered adequate for routine identification purposes [151]; these methods are time-consuming and laborious but especially require that the infection is in progress so that the pathogen is detected and confirmed. The detection of pathogens before any clinical signs of disease to be visible in the colony would not only control these diseases but also the prevention of their consequences for the hive. That is, *M. plutonius* was detected in healthy colonies by RT-PCR in England and Wales, showing that the extent of the prevalence of this pathogen in hives goes beyond the clinical signs [157].

RT-PCR has been used to simultaneously detect multiple viruses such as in cases of honey-bee parasitic mite syndrome where five out of seven viruses were detected in sample mite in Thailand [158]. Also, different multiplex RT-PCR were developed for the simultaneous detection of i) black queen cell virus (BQCV), deformed wing virus (DWV), Kashmir bee virus (KBV) and Sacbrood virus (SV) [159], ii) acute bee paralysis virus (ABPV), BQCV and SV [160], iii) ABPV and SV [161] iv) ABPV, chronic bee paralysis virus (CBPV), BQCV, DWV, KBV, and SV [162]. The effectiveness of this method in the detection of these pathogens was demonstrated in the simultaneous detection of these viruses in colonies [159, 160] and queens [162], where up to 93% of the queens have multiple infections [162].

Even more efficiently nine viruses (ABPV, BQCV, CBPV, DWV, KBV, SV, Israel acute paralysis virus (IAPV), *Varroa destructor* virus 1 (VDV-1), and slow paralysis virus (SPV)) were detected simultaneously in a single test developed by Glover and coworkers. These authors used a microarray technique with oligonucleotides based on DNA sequences of each of these viruses, but the time and cost of the technique are still unfeasible with its use for routine diagnosis [163].

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