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# Gram-Positive Bacteria with Probiotic Potential for the *Apis mellifera* L. Honey Bee: The Experience in the Northwest of Argentina

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**Abstract** *Apis mellifera* L. is one of the most important natural pollinators of significant crops and flowers around the world. It can be affected by different types of illnesses: american foulbrood, nosemosis, varroasis, viruses, among others. Such infections mainly cause a reduction in honey production and in extreme situations, the death of the colony. Argentina is the world's second largest honey exporter and the third largest honey producer, after China and Turkey. Given both the prominence of the honey bee in nature and the economic importance of apiculture in Argentina and the world, it is crucial to develop efficient and sustainable strategies to control honey bee diseases and to improve bee colony health. Gram-positive bacteria, such as lactic acid bacteria, mainly *Lactobacillus*, and *Bacillus* spp. are promising options. In the Northwest of Argentina, several *Lactobacillus* and *Bacillus* strains from the honey bee gut and honey were isolated by our research group and characterized by using in vitro tests. Two strains were selected because of their potential probiotic properties: *Lactobacillus johnsonii* CRL1647 and *Bacillus subtilis* subsp. *subtilis* Mori2. Under independent trials with both experimental and commercial hives, it was determined that each strain was able to elicit probiotic effects on bee colonies reared in the northwestern region of Argentina. One result was the increase in egg-laying by the queen which therefore produced an increase in bee number and, consequently, a higher honey yield. Moreover, the beneficial bacteria reduced the incidence of two important bee diseases: nosemosis and varroosis. These results are promising and extend the horizon of probiotic bacteria to the insect world, serving beekeepers worldwide as a natural tool that they can administer as is, or combine with other disease-controlling methods.

**Keywords** *Lactobacillus* spp. · *Bacillus* spp. · Apiculture · *Apis mellifera* · Honey yield · Beneficial effect

## Introduction

*Apis mellifera* L., commonly known as the honey bee, is one of the most important natural pollinators of significant crops and flowers. Close to 75 % of important crops over the world depend on pollinator insects. Many scientists hold that if the honey bee, its relative the bumblebee, or housefly disappears, fruit and seed varieties will be greatly affected. Moreover, their nutritional properties would also be reduced [1, 2] and would put not only honey bees, but also humans at risk as well.

In recent years, different factors have strongly affected the stability and presence of bee colonies all around the world. This social insect is a host for a large number of living organisms such as bacteria, molds, viruses and mites [3–11]. As in humans and other animals, the bee microbiota is complex and its health can be seriously affected by disturbances in the balance of the beneficial microbiota or because of ingestion of or contact with pathogenic microorganisms [3, 5, 12–14].

Globally, Argentina is the second largest honey exporter and the third largest honey producer, after China and Turkey [15]. To maintain such a high level of production, beekeepers often use (and unfortunately sometimes abuse)

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chemical compounds to fight against honey bee diseases; as a result, different bacterial bee pathogens, mainly *Paenibacillus larvae* [16, 17], have developed chemical resistance. Because of the economic importance of apiculture not only in Argentina, but around the world, it is crucial to develop efficient and sustainable strategies to control honey bee diseases and to improve the health of the bee colony. Therefore, the use of bacterial strains as biological alternatives, such as a bee probiotic or an "apipromotor" (i.e., that enhances honey bee colony integral status), is a promising option. This solution would serve beekeepers worldwide as a natural and innovative alternative that they can then administer or combine with the current methods.

## *Apis mellifera* L.

Not all bees that we observe in nature are similar; however, all of them are very important to maintain the world ecological equilibrium as we know it. The production of fruit crops such as apples, pears, melons, strawberries, as well as cotton or alfalfa, several clovers, onions, peas, asparagus, celery, among other crops, is dependent upon bee populations, either the domesticated honey bee or other native, wild, solitary or social species [18].

There are at least three different species of bees that belong to the genus *Apis*. In particular, the occidental honey bee, also called "European bee", *Apis mellifera* has existed for at least 30 million years. It was initially found in Europe, western Asia and Africa, but due to human migration over time, nowadays it can be found in almost all the countries of the world [19].

Honey bees are social, colony-forming insects that have a complex biological structure. Bee colonies can be considered "super organisms", composed of the individual, group and hive components. Even though the queen and worker are born from fertilized eggs and have the same genotype, the phenomenon of caste determination in honey bees present vast differences in behavior, life span, development, function, physiology, morphology and metabolism [19–21]. A typical small hive has about 20,000 bees, which are divided into three types: queen, drone and workers. The queen's main role is to keep the order of the whole colony, which it achieves by secreting a pheromone and laying eggs. The worker bees, on the other hand, have various responsibilities: they secrete wax from their abdominal glands; tend the queen, young drones and brood, and often work as foragers according to the season of the year. Foraging consists of flying out of the hive and visiting flowers and trees in search of nectar, pollen and propolis, which they then take to the hive and use to produce honey.

Keeping the bees healthy is of critical importance. In the last 10 years, the gut microbiota of the honey bee has been receiving increased attention due to the positive effect it can have on the health and performance of this social insect [12, 14, 21]. Consequently, the impact and the relevance of gut microbiome in the whole organism, whether complex as a human or "simpler" as an insect, is becoming a new area of study [14, 22–25]. Kwong and Moran reported that the honey bee microbiota has relevant roles in the digestion of food and in the protection of bees against parasites or other pathogens (virus and bacteria) [25].

It is believed that honey bees and other insects such as *Bumble* acquire their microbiota through consumption of pollen, food and through contact with older bees in the same colony or from other environments [3, 26–29]. However, other researchers have reported that honey bees (*Apis* spp.) possess a highly specialized gut microbial community, comprised of about eight bee-specific phylotypes, where bacteria is naturally occurring and not produced by food consumption [30–34]. Those microorganisms most likely come from pollen, the digestive tracts of honey bees, flowers, dirt, dust and the air [4, 35].

## Apiculture and Beekeeping Problems

As stated previously, Argentina is recognized worldwide not only as a significant honey producer but also as an exporter. Official data reveal that our country produces around 80–90 thousand tons of honey per year, and close to 95 % of that production is exported [15]. The Argentinean honey is known worldwide due to its organoleptic characteristics and its quality parameters (i.e., humidity, HMF, taste, flavor). However, Argentinians themselves are not large honey consumers (about 200 g per capita per year) compared to other countries where honey consumption is over 2 kg per capita per year.

In many countries, such as the USA, during a typical migratory beekeeping operation, the same population of honey bee colonies can be exposed to 5–10 different ecosystems over the course of 1 year. This means that foragers are in frequent contact with different ecological conditions: different climates, floral components, biocides, and water sources [9, 36]. All these factors can negatively affect the health and performance of each travelling colony.

Even though it may seem that the main problem in apiculture and for beekeepers is any disease that bees can contract (usually by a living agent), pesticides are actually proving to have a more devastating impact on bee colony stability [37, 38]. A recent scientific report, published by Kiljanek et al. [39], stated that in Europe, colony collapse disorder (CCD) might be closely related to specific chemical compounds, such as neonicotinoids. These compounds can reduce the developmental rate of queen honey bees, increase the occurrence of queen rejection, lower queen weight, affect honey bee cardiotoxicity and affect forager bee mobility and communicative ability [40].

1. Control of honey bee infectious diseases

Different pests can alter the natural and normal lifecycle of bees and, consequently, their health. Viruses, bacteria, molds, protozoa and mites are all diseases determined to be infectious. However, pesticides, pollution, deforestation and electromagnetic pollution from phone masts are considered other sources of bees "diseases" or reasons for CCD.

With regard to the aforementioned infectious diseases, some of them are host specific (i.e., they attack one specific insect, in this case honey bees). *Paenibacillus larvae*, a Gram-positive, sporulated bacterium is the etiological agent of American foulbrood, the worst bacterial disease for the honey bee brood [41].

*Nosema* spp. and *Varroa* spp. are among the greatest threats for apiculture [6, 42–44]. It is well known that their presence weakens a bee colony, causing loss in weight, malformation and weakening of the bees [45]. These bee parasites have also been associated with winter colony mortality, and they are dominant vectors of several honey bee viruses. Several researchers believe that *Nosema* spp. are also a possible cause of CCD. However, the specific causes of most losses are still undetermined [46–48]. In a recent scientific article, Maggi et al. reported that *Varroa destructor* is the main biological hazard for honey bees in temperate climates and countries like Argentina, Chile and Uruguay suffer from the devastating effects of this mite. They have also suggested that *Varroa* spp. are associated with colony losses [44].

In order to control and fight against these diseases, beekeepers frequently use antibiotics and pesticides that not only develop pathogen resistance, but also cause an imbalance of the normal bee microbiota [16, 49, 50]. The latter affects the bees' health and may alter their orientation, eventually reducing the number of hive members [51]. Moreover, the use of antibiotics or chemicals increases the risk of contamination of the products obtained from the hive because they may remain in the honey and thus affect its quality for human consumption and commercialization [52]. This is the case of chloramphenicol, an antibiotic that has already been detected in honey and other apiculture products in numerous countries [37, 53].

2. Natural and novel alternatives. GRAS Gram-positive bacteria

Different Gram-positive bacteria belonging to the genus *Lactobacillus*, *Enterococcus*, *Bacillus* and *Bifidobacterium* have been studied extensively, and many species have been given the "Generally Recognized As Safe" status (GRAS). This is significant due to their potential applications not only to food development but also as part of probiotic supplements or bioprotectors [54–56].

In particular, many species belonging to the genus *Lactobacillus* have been a part of common foods and beverages for centuries (such as yogurt, cheese, wine, fermented meat) or as a key ingredient of probiotic supplements or formulae for humans and different animals [54, 57–59].

On the other hand, *Bacillus* spp. have mainly been known for their metabolic potential and they have often been considered microbial factories for the production of a vast array of biologically active molecules such as enzymes, cyclic lipopeptides, antibiotics, bacteriocins. [60–64]. Moreover, their spore-forming ability also makes these bacteria some of the best candidates for developing efficient biotechnological products such as biopesticides [65]. Since 1996, the species *subtilis*, *licheniformis* and *clausii* have gained increased scientific interest as part of probiotic formulas [66–68], due to their beneficial effects not only for humans [67–69] but also for animals [70–72]. In addition, *Bacillus* cells possess an intrinsic ability to sporulate, providing an extra advantage which allows them to withstand the steps involved in creating stable biotechnological products [73, 74].

## A Potential Solution Toward a Bee Probiotic

Developing a bee probiotic requires isolating, studying and selecting viable and culturable bacteria from the aforementioned genders *Lactobacillus* or *Bacillus*, among others. Several studies have focused on the honey bee microbiome. However, the majority only used metagenomic analysis or genomic methods, without cultivating the microbiota [24, 31, 75]. In order to devise a bee probiotic formula, culturable and viable microorganisms must be selected. In this regard, Kwong and Moran [25], in a recent article, have reported that all of the bacterial species in the bee gut are able to be recovered and cultured in the laboratory.

Today, several species of *Lactobacillus* have been found in honey bees and have produced stunning results. Different researchers have found new *Lactobacillus* species in the honey bee gut and stomach, such as *apinorum, mellis, melliventris, kimbladii, kunkeei, helsingborgensis,* among others [23, 25, 30, 33, 34, 76]. In particular, *L. kunkeei* has been described as major member of honey bee microbiota. Endo and Salminen observed that fructophilic lactic acid bacteria (FLAB), a special group of lactic acid bacteria that prefer fructose, were found in flowers, varying fermented foods prepared from specific fruits, and in honey bee gut; they also determined that *L. kunkeei* predominates in bee products and in larvae [33]. Additionally, Audisio et al. [8] reported the presence of known viable and culturable *Lactobacillus* species, mainly *L. johnsonii*, and some *Enterococcus faecium* strains associated with the bee gut.

Another key point in formulating a honey bee probiotic supplement is related to the criteria selections. If we focus on the probiotic potential of both genera, *Lactobacillus* and *Bacillus*, the mechanisms of the positive effects they produce cannot always be exactly understood. However, several scientific articles have proposed some of the following features of the mechanisms: the ability of the bacteria to synthesize metabolites with antagonistic properties against surrounding microbiota, competition for nutrients, stimulation of the immune system, control of a defined illness and competitive exclusion mechanisms, among others [56, 57, 77–79]. Taking the proposed mechanisms into account, one of them may be chosen in order to select the isolated strains. Therefore, according to each research group selection criteria, several and different bee probiotic supplements can be devised. As an additional point, it is important to remember that even though the isolated, viable, culturable strains belong to the *Lactobacillus* or *Bacillus* genera, they cannot automatically be named "probiotic microorganisms"; that status must be proven.

Several studies have been conducted on using microorganisms as potential bee probiotics. Máchová et al. were pioneers in evaluating the potential of different microorganisms as bee probiotics; in their experiments, they worked on bees reared under laboratory conditions. The studied strains were anaerobic intestinal bacteria isolated from bees, as well as strains from several other sources [80] (see Table 1). They evaluated the viability of syrup-based probiotic formulas and determined the location and the impact on bee colon microbiota [80]. In turn, Evans and López studied the effect of known probiotic cultures on honey bee larvae: a commercial human probiotic culture was selected, and they observed the impact of those lactobacilli at inducing an immune response of the bee larvae after being exposed to *Paenibacillus larvae*. They proposed that nonpathogenic bacteria can be used as a probiotic to enhance honey bee immunity, specifically bee larvae [81]. Pătriucă and Mot [82] evaluated the effect of two commercial probiotics, designed for human consumption, on honey bee colonies, mainly observing their effect on the composition of the bee gut microbiota. In another scientific study, Pătriucă et al. [83] analyzed the effect of prebiotic and probiotic feed supplementation on the development of the wax glands of worker bees. Andrearczyk et al. studied the impact of a commercial probiotic, used by veterinarians, on honey bee survival after artificial *Nosema* spp. infestation. The assays were done under laboratory conditions, and they reported variable and sometimes negative effects [84] (see Table 1). Finally, Ptaszyńska et al. evaluated the effect of a commercial human probiotic strain, combined with a prebiotic (inulin), on the survival rates of honey bees infected with *Nosema ceranae* under laboratory conditions. They observed that, as a result of an improper selection of the probiotic strain and its combination with a prebiotic source, there was a negative impact on honey bee health, without preventing nosemosis development; moreover, that specific combination de-regulated bee immune systems, and significantly increased its mortality [85]. Such a situation had also been observed by Andrearczyk et al., but with another combination of probiotic strains which had not been isolated from the honey bee environment [84].

On the other hand, different researchers have proposed to consider the host-specific characteristic as a key factor in devising an effective probiotic and in order to ensure location specificity and colonization [86–88]. Therefore, to develop a probiotic for the honey bee, one option could be to look for promising bacterial strains from the honey bee or its environment. However, there are few scientific articles where honey bee-associated microorganisms were selected and tested due to their probiotic properties or as "apipromotor". *Lactobacillus johnsonii* CRL1647, AJ5 and IG9 were isolated from worker guts and selected due to their in vitro antagonistic activities against *Paenibacillus larvae* and *Ascosphaera apis*, among other microorganisms [8]. This in vitro step was followed by in vivo study, mainly with *L. johnsonii* CRL1647. Several assays were done, in experimental and commercial hives, during different years and with distinct doses (every 15 days or monthly). The most significant result was the one related to honey yield and until now has not been evaluated by another research group. The delivery of viable *L. johnsonii* CRL1647 to bee colonies, once a month, had a beneficial effect on the health of the bee colony, the stimulation of queen egg-laying, an increase of bees in the colony and higher honey production [89, 90].

The studies related to potential probiotic bacteria for bees that include another type of bacteria such as *Bacillus* spp. as potential bee probiotic have not been explored in depth yet. This type of bacteria, due to its physiology, is commonly found in and on honey bees and their environment [9]. Gilliam and Valentine [91] studied this genus in honey bees and honey. Sabaté et al. [10] in vitro described the significance of different *Bacillus subtilis* isolated from both honey and honey bee gut, as an antagonistic agent against *Paenibacillus larvae* and *Ascosphaera apis*. Their next study evaluated that the in vivo administration of *B. subtilis* subsp. *subtilis* Mori2 had three beneficial effects on bee colonies: an increase in open and operculated brood, greater accumulation of honey compared to the control hives, and a healthier hive, due to the reduction of *Varroa* and *Nosema* incident rates [92]. Even though the use of

**Table 1** Gram (+) bacteria as potential honey bee probiotics: state of the art of in vivo assays

| Strain[a] | Origin | Relevant reported results | *Apis mellifera* subspecies analyzed | References |
|---|---|---|---|---|
| *Lactobacillus* spp., *Eubacterium* spp., *Streptococcus* spp., *Bifidobacterium* spp., *Leuconostoc* spp.; *Lactococcus* spp., *Bacillus subtilis,* (*Enterobacter* spp., *Saccharomyces* spp.; *Escherichia coli*) | Anaerobic strains associated with honey bee gut//commercial probiotic for farm animals//collection strains//used in crops | Low viability of the microorganisms tested in 50 % sugar syrup<br>Presence of potential probiotic strains in bee colon<br>Mild increment in bee survival when fed with anaerobic strains associated to honey bee gut<br>Higher mortality of bees fed with *B. subtilis* | *carnica* | Máchová et al. [80] |
| *Bifidobacterium bifidum, Bif. longus*, *L. acidophilus*, *rhamnosus* and *reuteri* | Commercial human probiotic product | Probiotic strains able to elicit an immune response on bee larvae<br>Mild increment in defensin and abaecin levels of bee larvae | *ligustica* | Evans and López [81] |
| *L. johnsonii* CRL1647 | Worker bee gut | Queen egg-laying stimulation<br>Higher number of bees<br>Decrease in the *Nosema* spp. index<br>Swarming effect<br>Significant increase in honey yield<br>Healthier bee colony | *mellifera* | Audisio and Benítez [89]; Audisio et al. [90] |
| *L. acidophilus* LA-14 and *Bif. lactis* BI-04<br>*L. casei* | Commercial human probiotic products (Enterobiotics)<br>(Enterolactis Plus) | Significant reduction in the total number of bacteria in the digestive tracts of treated bees<br>Intestinal colonization by the beneficial bacteria contained in the probiotic products<br>Improvement in the bee health status | *carpatica* | Pătruică and Mot [82][a] |
| *L. acidophilus* LA-14, *Bif. lactis* BI-04<br>*L. casei* | Commercial human probiotic products (Enterobiotics)<br>(Enterolactis Plus) | Increase in size of wax cells | *carpatica* | Pătruică et al. [83] |
| *Bacillus subtilis* subsp. *subtilis* Mori2 | Honey sample (Morillos, Argentina) | Queen egg-laying stimulation<br>Higher number of bees<br>Lowering in the *Nosema* spp. index<br>Significant increase in honey yield<br>Healthier bee colony<br>Reduction of *Varroa* incident rate | *mellifera* | Sabaté et al. [92] |

**Table 1** continued

| Strain[a] | Origin | Relevant reported results | *Apis mellifera* subspecies analyzed | References |
|---|---|---|---|---|
| *L. casei, L. plantarum,* (*Saccharomyces cerevisiae, Rhodopseudomonas palustris*) | Commercial probiotic for veterinary use | Higher mortality in bees treated with the probiotic formula<br>Increase in *Nosema* spp. infection on bees previously treated with the probiotics | Not reported | Andrearczyk et al. [84] |
| *L. rhamnosus* | Commercial human probiotic product | Lower survival of bee feed with the probiotic<br>Rapid development of nosemosis in bees fed with the probiotic<br>Higher mortality of bees infected with *Nosema* and protected with the probiotic | *carnica* | Ptaszyńska et al. [85] |

[a] Strains enclosed by parentheses are either non-Gram-positive bacteria or other microorganisms also included in the probiotic formulations

lactic acid bacteria on bees has been reported before, few papers have evaluated the effect of the different probiotic supplements on honey production [80, 89, 90]. In turn, only one scientific article has studied the effect of *Bacillus* spp. strains, isolated from the honey bee environment, in honey bee colonies [92]. Table 1 presents the most significant results of studies where in vivo assays were performed, regarding the use of Gram-positive bacteria in the honey bee probiotic field.

Another natural option for beekeepers lies in the study of biological properties of metabolites produced by Gram-positive bacteria and the effects on honey bees. It is well known that the majority of this type of bacteria synthesizes a large variety of molecules with potential inhibitory effects on biological activities [57, 59, 60, 78, 79, 93–96]. The study of the chemical nature of those compounds revealed that they are mainly toxins, organic acids, hydrogen peroxide, bacteriolytic enzymes, bacteriocin, cyclic lipopeptides, etc. Endo and Salminen reported the anti-*Melissococcus plutonius* activity of a culture supernatant from *L. kunkeei* FF30-6, a honey bee-associated strain. They proposed that this biological effect was due to a combination of antibacterial peptides and a low pH [33]. Vásquez et al. [34] also reported that a different *L. kunkeei* strain, which they had isolated from the bee gut, had growth inhibition properties not only against *M. plutonius* but also several bacterial and yeast strains in vitro; the principal mechanism for the inhibition was not achieved. Yoshiyama et al. [97] evaluated the potential application of lactic acid bacteria (LAB) isolated from fermented feeds and foods for use as probiotics against *P. larvae*, and they suggested that the organic acids produced from LAB might be one cause of that inhibition. Porrini et al. [98] studied the use of bacteriocins and cyclic lipopeptides, mainly for the surfactin family, on *A. mellifera* individuals, under laboratory conditions, in order to determine both the effects of those metabolites on *Nosema* spp spores and on honey bees. Interestingly, those molecules were not toxic to worker bees even after 30 days of consumption. The surfactin sample from *B. subtilis* subsp. *subtilis* C4 was the only compound administered that decreased the parasitism intensity after 40 h of metabolite-*Nosema* spore contact [98].

In another study, Maggi et al. evaluated the organic acids synthesized by *L. johnsonii* CRL1647 (which can produce around 138 mM lactic acid), but this time on *A. mellifera mellifera* colonies. They found that organic acids enhanced colony fitness in those colonies fed with the metabolites produced by this strain. Moreover, fat bodies were also significantly increased after two applications of bacterial metabolites when compared to control groups [99]. Fat bodies play a major role in the life of insects, as they are involved in multiple metabolic functions: (a) storing and releasing energy in response to energy demands of the insect and (b) producing several antimicrobial peptides, acting similar to a vertebrate liver [100].

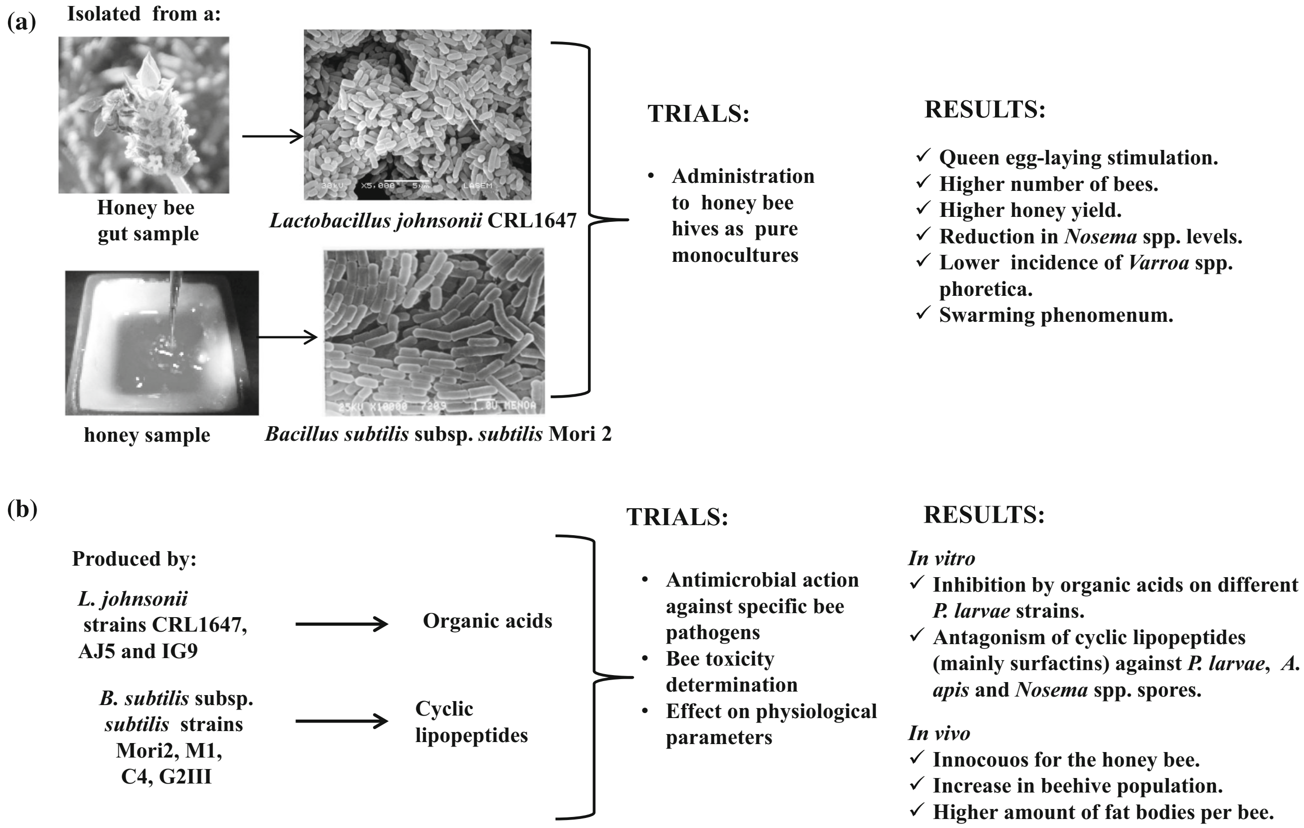


**Fig. 1** Description of the trials done and the different effects registered on the honey bee *Apis mellifera* with: **a** viable and culturable cells of *L. johnsonii* CRL1647, and *B. subtilis* subsp. *subtilis* Mori2 spores [89, 90, 92]; and **b** the administration of bacterial metabolites [8, 10, 98, 99]

Finally, the researchers also evaluated the impact of the bacterial metabolite on *Nosema* levels and observed that application of the bacterial metabolite strongly reduced the spore loads per bee [99]. Figure 1 shows a summary of the results obtained when viable cells of *L. johnsonii* CRL1647 and *B. subtilis* subsp. *subtilis* Mori2 spores were administered to hives (Fig. 1a) and when honey bee and hives received different bacterial metabolites.

## Final Remarks

*Lactobacillus* and *Bacillus*, both honey bee-associated strains and/or from the honey bee environment, as well as some of their metabolites, are becoming significant, safe and eco-friendly tools to help honey bee life. More information is being collected daily about the beneficial and protective effects of Gram-positive bacteria as part of probiotics for honey bee colonies. These bacteria are promising, innovative treatments which can not only increase honey yield per hive but also re-balance the microbial ecology of the bee gut, protect against pathogen colonization, and strengthen bee immunology.

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**Compliance with Ethical Standards**

**Conflict of interest** Marcela Carina Audisio declares that he has no conflicts of interest.

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