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Beneficial bacteria as biocontrol agents for American foulbrood disease in honey bees (*Apis mellifera*)

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American foulbrood (AFB) is a cosmopolitan bacterial disease that affects honey bee (*Apis mellifera*) larvae and causes great economic losses in apiculture. Currently, no satisfactory methods are available for AFB treatment mainly due to the difficulties to eradicate the tenacious spores produced by the etiological agent of AFB, *Paenibacillus larvae* (Bacillales, Paenibacillaceae). This present review focused on the beneficial bacteria that displayed antagonistic activities against *P. larvae* and demonstrated potential in AFB control. Emphases were placed on commensal bacteria (genus *Bacillus* and lactic acid bacteria in particular) in the alimentary tract of honey bees. The probiotic roles lactic acid bacteria play in combating the pathogenic *P. larvae* and the limitations referring to the application of these beneficial bacteria were addressed.

Key words: *Paenibacillus larvae*, American foulbrood disease, honey bee, beneficial bacteria, biocontrol

Introduction

American foulbrood (AFB) disease is by far the most virulent and deleterious bacterial disease that causes fatal brood infection in honey bees (*Apis mellifera*). Its etiological agent is the pathogenic Gram-positive, spore-forming bacterium *Paenibacillus larvae* (Bacillales, Paenibacillaceae). Spores produced by *P. larvae* can infect honey bee larvae, especially those newly hatched, but not adult honey bees. Once being ingested by honey bee larvae, *P. larvae* spores will germinate in the midgut, develop into vegetative cells and proliferate, then, breach the epithelium and enter the hemocoel, finally leading to the death of larvae due to bacteremia and the production of additional infective spores (Genersch 2010, Ebeling et al. 2016).

P. larvae spores are highly resistant to various environmental adversities (Genersch 2008) and can be horizontally transmitted within and between colonies through both natural routes (i.e., through adult honey bees' activities such as cell cleaning, larvae nursing, honey robbing, and honey bee drifting) and artificial routes (i.e., through beekeeping activities such as combination of colonies, exchange, and reuse of contaminated beekeeping equipment) (Lindström et al. 2008). *P. larvae* spores also demonstrated a vertical transmission mode between colonies, i.e., from mother colonies to daughter swarms (Fries et al. 2006). AFB is a major problem in apiculture because tenacious *P. larvae* spores presented in various apiarian reservoirs are difficult to eliminate. If left untreated, AFB can annihilate the whole colony due to the lack of viable offspring and cause great economic losses.

Unfortunately, no cure exists for this notorious disease. The most commonly applied strategy, especially outside the European Union, is the prophylactic application and supplementary feeding of antibiotics to suppress clinical AFB symptoms. For colonies not yet displaying AFB clinical symptoms, the shook swarm procedure (shaking all of the worker bees together with the queen into new and empty hives to get rid of any potential contamination on the comb, honey, and pollen) is recommended as a way of sanitizing the colony (Ohe 2003). For clinically diseased colonies, hive (as well as the potentially contaminated equipment) incineration is commonly adopted to prevent the spread of AFB.

During the past two decades, there is growing awareness of the problems associated with long-term indiscriminate use of antibiotics for AFB control in beekeeping practice. These problems mainly include the emergence of resistant *P. larvae* strains (Miyagi et al. 2000, Alippi et al. 2007, 2014), disturbed honey bee microbiota and reduced lifespan of honey bees (Raymann et al. 2018, Powell et al. 2021), spread of antibiotic resistance genes and immune deficits in honey bees (Daisley et al. 2020a), and undesirable presence of residues in beehive products destined for human consumption (Reybroeck et al. 2012). These concerns, together with the need for sustainable development of apiculture, have urged intensified research for identifying safe alternatives for AFB control.

So far, various approaches have been sought to combat this viciously contagious disease. A thorough review regarding the potential use of plant extracts, essential oil, propolis, royal jelly, and

isolated compounds as natural strategies for the prevention and control of AFB has been documented (Alonso-Salces et al. 2017). Other strategies concerning selective breeding of honey bees for hygienic behavior (Spivak and Reuter 2001, Behrens and Moritz 2014), use of honey bee venom (Fernández et al. 2014), bacteriophage therapy (Tsourkas 2020), fatty acids and probiotics (Kuzyšinová et al. 2016) have also been proposed.

Probiotics are 'live microorganisms that, when administered in adequate amounts, confer beneficial effects on the host' (Hill et al. 2014). The alimentary tract of honey bees is a promising reservoir of probiotic bacteria. Commensal bacteria isolated from honey bees have demonstrated beneficial functions on the host via stabilizing microbiota equilibrium (i.e., reducing the number of potential pathogens and increasing the population of the beneficial microorganisms), facilitating the breakdown and utilization of pollen grains (Engel et al. 2012), promoting weight gain of individual honey bees (Zheng et al. 2017), producing essential nutrients (vitamins for example), neutralizing dietary toxins, and enhancing the host's innate immunity through up-regulated expression of antimicrobial peptides (AMPs) genes (Kwong et al. 2017, Royan 2019). Accumulated documents demonstrate that probiotics not only have trophological values to the host but also act as a therapeutically microbial-based solution to reduce disease burden in honey bees.

This review article provides an overview of beneficial bacteria that either have the potential to fight against *P. larvae* (in vitro) or have exerted beneficial impacts on honey bees and the whole colony (in vivo). Emphasis is placed on studies of using commensal bacteria in the alimentary tract of honey bees as probiotics to combat AFB in beekeeping. The probiotic roles lactic acid bacteria play in fighting against *P. larvae* are elaborated. The limitations with regard to the potential application of these beneficial bacteria are also addressed.

Beneficial Bacteria Exhibiting Probiotic Potential in AFB Control

Functions of the Alimentary Microbiota in Honey Bees

The honey bee microbiome plays a beneficial role in bee health, fitness, metabolism, and immunity (Nowak et al. 2021). A disrupted microbiome, for example, gut dysbiosis resulting from exposure to agrochemicals, was associated with compromised honey bee innate immunity and increased susceptibility to bacterial infection (Motta et al. 2022a), while supplementation of bee gut microbiome (BGM) or natural gut strains from honey bee microbiota helped enhance honey bees' resistance to pathogen challenge and replenish perturbed gut communities (Powell et al. 2021, Steele et al. 2021). The worker bee bacterial community is significantly influenced by AFB (Erban et al. 2017). AFB-infected honey bee larvae displayed a perturbed microbiome depleted of bacterial genera *Lactobacillus* (Lactobacillales, Lactobacillaceae) and *Stenotrophomonas* (Xanthomonadales, Xanthomonadaceae) which were abundant in healthy honey bee larvae (Ye et al. 2021). In this sense, supplementation of endogenous beneficial bacteria with anti-*P. larvae* activities will prime the host's innate immune system and strengthen their ability to combat AFB.

The alimentary canal of adult honey bees is divided into four regions (honey crop, midgut, ileum, and rectum) with each compartment containing distinct niche-adapted microbial communities (Martinson et al. 2012). A highly specialized set of bacteria, consisting of five dominant and recurring phylotypic clusters, has been found to colonize mainly in the midgut and hindgut (including ileum and rectum) of adult worker honey bees. Of which, one cluster

was from Firmicutes (Firm-4 and Firm-5, genus *Lactobacillus*) (Cox-Foster et al. 2007). The latter (Firm-5) is now classified as *Lactobacillus melliventris*. Levels of and strain identities of bacteria were highly variable between hives (Ellegaard et al. 2015). The consistent presence of these distinctive phylotypes in individual honey bees implicates their central functions on host health and a coevolved symbiotic relationship between bacteria and honey bees (Moran et al. 2012, Sabree et al. 2012). To date, the mutualistic relationship between gastrointestinal bacteria and the host has been well recognized (Crotti et al. 2013). The symbiotic roles nonpathogenic alimentary microbiota play in honey bees can be summarized as enhancement of host metabolic competency, contribution to growth and development, provision of protection from pathogens, and modulation of systemic immunity provision (Kwong and Moran 2016).

Potential Probiotic Bacteria for AFB Control

Certain bacterial species are potential biocontrol agents for AFB due to their antagonistic activities against the infectivity and pathogenicity of *P. larvae*, which include the genus *Bacillus* (Bacillales, Bacillaeeae), one of the major antibiotic-producing groups (Bérdy 2005), genera *Lactobacillus* and *Bifidobacterium* (Bifidobacteriales, Bifidobacteriaceae), the producer of lactic acid as well as other organic acids (Quinto et al. 2014). The application of these bacteria either as a prophylactic supplement or as a therapeutic treatment presents a potential approach for AFB control.

Bacteria from the genus Bacillus *and their anti-*P. larvae *activities.*

Gram-positive, spore-forming *Bacillus* spp. commonly occur in the alimentary tract of adult honey bees (Gilliam 1997). For example, at the genus level, *Bacillus* was reported to account for 14% of the adult honey bee gut bacteria (Anjum et al. 2018). *B. sonorensis*, *B. tequilensis*, and *B. aryabhattai*, as well as *Brevibacillus laterosporus* (previously classified as *B. laterosporus*) were isolated and identified from the digestive tract of healthy honey bees (Khaled et al. 2018).

Bacteria in genus *Bacillus* can generate a vast array of biologically active molecules, including antimicrobials (lipopeptides, bacteriocins) (Vezina et al. 2020) and enzymes (protease, catalase, lipase, levansucrase) predicted to participate in the breakdown of macromolecules in honey bees (Lee et al. 2015, Cochrane and Vederas 2016). These attributes are crucial for their antibiotic properties and are of trophic importance for their hosts. The most well-known *Bacillus* species used for insect manipulation is *B. thuringiensis* (*Bt*), which is widely used as bioinsecticides due to its capacity to produce a wide range of toxins (Malovichko et al. 2019). The commercially developed formulations of *Bt* products are mainly targeted for controlling leaf-feeding insects (Lepidoptera), beetle pests (Coleoptera), and mosquitoes (Diptera) (Bravo et al. 2011). The toxicity of *Bt* products on honey bees varied with *Bt* strains, concentrations of toxins applied, test duration, and exposure routes (Steinigeweg et al. 2021). The majority of the studies showed no meaningful negative impact of *Bt* on honey bees. Due to the low UV resistance of *Bt* spores, *Bt* products generally have a short half-life period under field conditions ranging from a few hours to two days. For example, under controlled laboratory conditions, dietary exposure of honey bee larvae and adults to *Bt* toxins (*Bt* Cry9Ee and *Bt* Cry78Ba1) did not affect survival or larval weight, pollen or syrup consumption, or the core midgut bacterial structure and composition in adult honey bees (Dai et al. 2019, Han et al. 2021). In field bioassays, commercial *Bt* products (Dipel and Xentari) were proven to be safe for foragers and newly merged honey bees (Libardoni et al. 2021). In fact, the high frequency of honey bee harboring *Bacillus*

species (mainly the *B. cereus* group) suggested a stable symbiosis established between honey bees and this bacterial taxon, which may partly explain honey bees' stronger capacity to tolerate *Bt* than that of other insects (Evans and Armstrong 2006).

Studies (Supplemental Table S1) have demonstrated that *Bacillus* spp., both exogenous (soil or bacterial collections for example) and endogenous (of apiarian sources), exhibited in vitro antagonistic potential against the growth of *P. larvae*. They could either inhibit the germination of *P. larvae* spores through the production of iturin-like peptide (Benitez et al. 2012) or displayed in vitro bactericidal and bacteriolytic effects toward *P. larvae* cells through the production of antibiotic-like compounds (Alippi and Reynaldi 2006), bacterioncin (for example entomocin 110) (Cherif et al. 2008), lipopeptide surfactin (Sabaté et al. 2009, 2012a), or other antimicrobial peptides (Bartel et al. 2019). In an *in vivo* experiment, when administered once a month (from May to December) at the concentration of 10^5 spores/ml in supplemental sugar syrup, a honey-originated strain *B. subtilis* subsp. *subtilis* Mori2 strain, which exhibited *in vitro* inhibitory activity against *P. larvae* growth, improved colony performance by increasing honey storage and the sanitary status of the hive which had the effect of reducing spore counts of *Vairimorpha* (*Nosema*) *sp.* and the percentage of infestation with *Varroa* sp. *foretica* (Sabaté et al. 2012b).

These abovementioned spore-formers from genus *Bacillus*, when applied as probiotic supplements, present advantages in formulae preparation due to their spores' abilities to withstand a relatively wide range of temperatures and remain viable in the acidic environment of the honey crop. The limitations of current research are that the majority of the currently available results were obtained through *in vitro* assays, the chemical nature of substances involved in the anti-*P. larvae* activities, in most cases, remained to be clarified, and more *in vivo* research is necessary to evaluate the inhibitory effects of *Bacillus* spp. against *P. larvae*. Furthermore, the GRAS (Generally Recognized As Safe) (Food and Drug Administration 2016) status of each *Bacillus* spp. still needs to be evaluated at the hive level through *in vivo* assays before they can be used as probiotic supplements in honey bees.

Beneficial effects of lactic acid bacteria (LAB) for honey bee health.

LAB are a biologically defined group of Gram-positive bacteria functionally related by their phenotypic characteristics of producing large amount of lactic acid as the major end-product of carbohydrate metabolism. The LAB genera (currently classified in phylum Firmicutes, class Bacilli, and order Latobacillales) include 14 members with *Lactobacillus* being the largest genus (Mokoena 2017). They are generally recognized as safe (GRAS) and widely used in food and feed industry (Rashid and Sultana 2016).

The beneficial effects LAB exert on honey bees have been well understood. Firstly, as important symbionts in honey bees, LAB help to maintain intestinal homeostasis and potentially diminish pathogen infections (Hamdi et al. 2011). Isolated from the honey crop, *L. kunkeei* (currently classified as *Apilactobacilus*) significantly decreased the mortality of honey bee larvae exposed to the causative agent of European foulbrood (EFB) disease, *Melissococcus plutonius* (Lactobacillales, Enterococcaceae) (Vásquez et al. 2012). *Lactobacillus* strains from the gut of honey bees helped reduce the spore load of *Vairimorpha* (*Nosema*) *ceranae* (Baffoni et al. 2015) and the incidence of both *Nosema* and *Varroa* (Audisio et al. 2015) in worker honey bees. These LAB strains can provide honey bees with protection against pathogenic bacterial and fungal infections, as well as parasites.

As is known, LAB can produce a range of antimicrobial metabolites including organic acids (such as lactic acid, acetic acid, and formic acid), volatiles, hydrogen peroxide, diacetyl, carbon dioxide, and

bacteriocins (Vieco-Saiz et al. 2019). These substances are produced in a species- and strain-dependent manner, which makes it possible for LAB to work synergistically (namely proto-cooperation) and provide honey bees with more antimicrobial capabilities to defend pathogenic threats (Butler et al. 2013, Olofsson et al. 2016).

LAB also play an important role in host-microbe interactions. They produce exopolysaccharides (the main component of extracellular polymeric substances that are involved in biofilm formation, cellular recognition, and host colonization), which offers LAB colonization advantage (Pătruică and Mot 2012) and limits the virulence and spread of pathogenic bacteria through niche competition under a quorum sensing mechanism (Kareb and Aïder 2020).

Secondly, LAB are of trophic importance to honey bees. LAB can synthesize amino acids and vitamins during metabolic processes. They are involved in the breakdown and further fermentation of polysaccharides and oligopeptides that exist in honey bee diet, thus facilitating the uptake of nutrients indispensable to honey bees. Enzymes synthesized by LAB can even help detoxify some carbohydrates (arabinose, xylose, galactose, mannose, lactose, melibiose, and raffinose) that may be toxic to honey bees (Lee et al. 2015).

Furthermore, LAB can prime honey bees' innate immune system and provide protection against attacks from potential pathogens. The exposure of honey bee larvae to LAB strains from the genus *Lactobacillus* spurred the immune response in larvae as evidenced by the up-regulated transcriptional expression of AMPs components, *abaecin* (Evans and Lopez 2004) and *Apidaecin*1 (Janashia and Alaux 2016). The administration of *Leuconostoc mesenteroides* TBE-8 (Lactobacillales, Lactobacillaceae) (isolated from the hindgut of bumble bee *B. eximius*) to honey bees significantly increased the transcriptional expression of nutrition-related genes (major royal jelly protein 1 in the head and vitellogenin in the abdomen) and AMPs genes (hymenoptaecin and apidaecin in the abdomen) (Huang et al. 2021). All the above mentioned modes of actions LAB exert on honey bees are summarized in Fig. 1.

In addition to these nonspecific effects, there are extra properties possessed by LAB that enable them to specifically inhibit the germination of *P. larvae* spores. Some LAB are capable of producing enzymes that can break down two co-germinants (uric acid and L-tyrosine) that are essential for the germination of *P. larvae* spores in the midgut of honey bee larvae (Alvarado et al. 2013). For example, some *Lactobacilli* can vigorously synthesize enzymes involved in the catabolism of uric acid. These enzymes, including uricase, allantoinase, and allantoicase, can degradate uric acid to urea (Guo et al. 2016). *Lactobacillus plantarum* Lp39 can produce tyrosine decarboxylase, which can break down tyrosine, the other key germinant of *P. larvae* spores (Daisley et al. 2020b). Taken together, these characteristics make LAB a promising tool for prophylactic and therapeutic treatment of AFB.

Beneficial effects of exogenous and autochthonous LAB strains in fighting against P. larvae.

LAB were commonly present in healthy larval instars at different developmental stages (Vojvodic et al. 2013), as well as in the alimentary tract of adult honey bees, bee products, beehive, and other apiarian sources (Ramos et al. 2019). The elaborated documents regarding the occurrence of LAB in honey crop, honey bee guts, and bee products are summarized in Supplemental Table S2.

To date, a few exogenous LAB strains have displayed *in vitro* inhibitory properties against *P. larvae* growth, including 34 strains from the genus *Enterococcus* (Lactobacillales, Enterococcaceae) isolated from nonfermented ecosystems (Jaouani et al. 2014) and various strains of *L. plantarum* from fermented food matrices (Lazzeri et
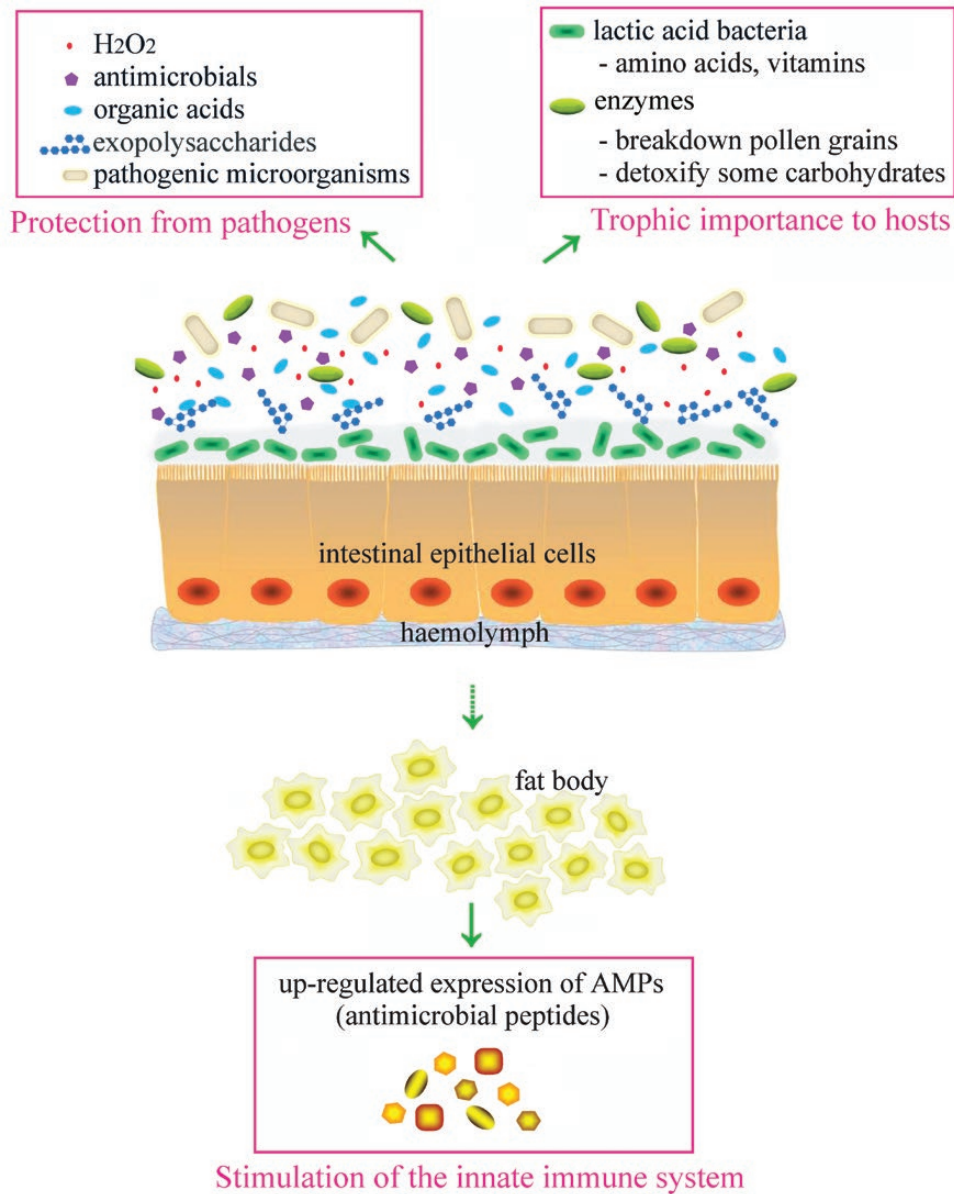
Fig. 1. Three modes of nonspecific actions that LAB exert beneficial effects on honey bees. First, the production of substances endowed with antimicrobial or biofilm-formation activities helps to protect honey bees against pathogens (Evans and Lopez 2004, Pătruică and Mot 2012, Janashia and Alaux 2016, Vieco-Saiz et al. 2019). Secondly, the production of essential nutrients and syntheses of enzymes facilitating the utilization of dispensable nutrients in honey bees diet play important roles in host's nutrition (Lee et al. 2015). Thirdly, the up-regulation of the expression of antimicrobial peptides helps to prime honey bees' innate immune system and promote immunomodulation (Kareb and Aïder 2020).

al. 2020). For the nine LAB strains (*Lactobacillus* spp., *Enterococcus* spp., and *Weissella* spp.) isolated from fermented feeds and food, the *in vivo* oral administration of them to honey bee larvae or adult honey bees (at a density of 10^7 cfu/ml) stimulated hosts' innate immune response by significantly increasing the transcriptional expression of AMP genes (including *abaecin*, *defensin*, and *hymenoptaecin*) (Yoshiyama et al. 2013). Additionally, *L. reuteri* strain ATCC 23272 (from a culture collection) demonstrated *in vitro* antagonistic activity against the growth and biofilm formation of *P. larvae* due to the acidic nature of its cell free supernatant (CFS) (Betesho et al. 2019).

In the meantime, quite a few autochthonous LAB strains isolated from honey bee related sources (*Lactobacilus* spp., the main representatives of LAB in particular) also demonstrated *in vitro* anti-*P. larvae* activities, which included a *Lactobacillus* strain (*L. apis* sp.

nov.), isolated from the honey crop (stomach) and detected mainly in the digestive tracts of 3-day-old honey bees, foraging workers and honey bee drones (Killer et al. 2014), the potent lactic acid producers of *L. plantarum* and *L. brevis* (Mudroňová et al. 2011), three *L. johnsonii* strains (including *L. johnsonii* CRL1647) (Audisio et al. 2011) and a set of LAB isolated from the gut (from esophagus to rectum) of worker honey bees (Kačániová et al. 2018, 2020, Al-Ghamdi et al. 2020, Iorizzo et al. 2020, Bielik et al. 2021, Zeid et al. 2022, Iorizzo et al. 2022), *Enterococcus faecium* EFD (Dimov et al. 2020) and *Enterococcus durans* EDD2 (Lactobacillales, Enterococcaceae) (Gyurova et al. 2021) isolated from freshly collected pollen granules. In addition, metabolites and peptides, produced by LAB in honey, endowed polyfloral honeys with anti-*P. larvae* activities (Erler et al. 2014).

In lab experiments or field tests, exposing honey bees or their larvae to beneficial LAB has proved to be able to decrease the infection rate and mortality of *P. larvae*-infected larvae, stimulate the innate immune responses, improve colony development, and confer health benefit to honey bees (Audisio 2017). Table 1 summarizes reports obtained from *in vivo* bioassays that demonstrated the beneficial effects of anti-*P. larvae* LAB on honey bee larvae, adult honey bees, and the colony. The inhibitory properties of these honey bee-specific LAB (hbs-LAB) were attributed to their secretome (extracellular fraction) (Lamei et al. 2019). Accumulated results (detailed in Supplemental Table S3) demonstrated that the supplementary feeding of LAB to honey bees can not only help prevent *P. larvae* infection but also increase the health level of the whole colony.

Other potential probiotic symbionts in honey bees against P. larvae.

There are other honey bee-borne microbes that demonstrated probiotic potential in AFB control. Honey bee larvae-originated *Stenotrophomonas maltophilia* (Xanthomonadales, Xanthomonadaceae), *Acinetobacter* sp. (Moraxellales, Moraxellaceae),

Brevibacillus formosus (Bacillales, Paenibacillaceae), and *B. fusiformis* (Evans and Armstrong 2006), *Paenibacillus polymyxa* TH13 of honey origin (Lee et al. 2009), *Brevibacillus laterosporus* that are consistently detected in the whole body of honey bees at immature (larvae and pupae) and mature (emerging workers and foragers) stages (Alippi and Reynaldi 2006, Marche et al. 2016), and *Streptomyces* sp. (Kitasatosporales, Streptomycetaceae) AmelAP-1 isolated from pollen (Grubbs et al. 2021) all exhibited a high level of in vitro inhibitory activity against *P. larvae*. *Brevibacillus laterosporus* could inhibit both the *in vitro* vegetative growth and spore germination of *P. larvae* due to the production of bacteriocin laterosporuli and other antimicrobial substances (Marche et al. 2019a). This bacterium was envisioned to contribute to the maintenance of a balanced gut microbiota in honey bees and relate to health improvement (Marche et al. 2019b).

Other symbionts of honey bees, such as the acetic acid bacteria (AAB) of genus *Gluconobacter* (Rhodospirillales, Acetobacteraceae), which also metabolize sugars and produce various organic acids, may have the potential to inhibit the growth of acid-sensitive *P. larvae* (Crotti et al. 2010). These abovementioned microbes (detailed information shown in Supplemental Table S4) deserve further

Table 1. Beneficial effects of anti-*P. larvae* lactic acid bacteria (LAB) on honey bees in *in vivo* assays

Strains (sources)	Methods (A, larval exposure assay; B, field bioassay)	Effects	References
Nine strains (fermented feeds/ food)	**A:** oral administration maintained for 24 h at a density of 10^7 cfu/m either in an artificial worker diet fed to honey bee larvae or in a 50% w/v sucrose solution in ddH$_2$O fed to adult honey bees	Stimulation of the innate immune response by up-regulating the expression of immune-related genes	Yoshiyama et al. 2013
Eleven strains (honey crop)	**A:** mixture of strains added into the larval food at a concentration of 10^7 cells/ml, co-administered with 5×10^3 or 5×10^4 *P. larvae* spores per ml to one-day instar larvae and maintained for 7 days	Decreased the number of larvae succumbing to AFB infection irrespective of the infective dose	Forsgren et al. 2010
Four strains (honey bees' guts)	**A:** one-day instar larvae were challenged with *P. larvae* spores at day 1; individual bacterial suspensions diluted to 1×10^6 cfu/ml in ddH$_2$O were administered to larvae from day1 to day 6	Decreased the mortality percentage of larvae challenged with *P. larvae* spores	Al-Ghamdi et al. 2018
Four *L. kunkeei* (*Apilactobacilus*) strains (honey bees' midgut)	**A:** one-day instar larvae challenged with 1×10^6 cells/ml *P. larvae* spores and co-administered with individual/mixed strains (1×10^7 cells/ml) for 48 h; switched to normal diet from day 3 to day 6 **Safety of bacteria:** newly emerged workers treated with individual and mixed bacteria (1×10^7 and 2×10^7 cells/ml for 7 and 10 days, respectively	Reduced mortality of *P. larvae*-infected larvae; no toxic effects on larvae and honey bees	Arredondo et al. 2018
L. johnsonii CRL1647 (whole gut of honey bees)	**B:** monoculture suspension (10^5 cfu/ml) in sugarcane syrup was consumed within 24-48 h and administered every 14–15 days for 3 consecutive months, or monthly for 13 consecutive months	Stimulation of egg-laying and honey storage, and enhancement of the colonization of beneficial bacteria belonging to *Lactobacillus*	Audisio and Benítezahrendts 2011; Audisio et al. 2015
	B: cell free supernatant (CFS), containing 128.1 mM lactic acid, 38 mM acetic acid, and 0.3 mM phenyl-lactic acid, supplemented in syrup at the dose of 20, 30, 40, and 60 ml CFS per honey bee, evaluated at 24, 48 and 72 h post treatment	Improved honey bees' health status implied by possessing more fat bodies per honey bee and increased population size of treated colonies	Maggi et al. 2013
LX3 (Lp39 and LGR-1 from culture collection and LkBR-1 from healthy hive)	**A:** one-day instar larvae were orally supplemented with LX3 (1×10^7 cfu/ml of each strain) for 24 h before infection; second instars were challenged with *P. larvae* spores (1×10^4/ml); third instars were switched to normal diet.	Reduced pathogen load, up-regulated expression of key immune genes, and improved larval survival during *P. larvae* infection.	Daisley et al. 2020b
	B: LX3 was delivered through Biopatty (250 g of base pollen patty ingredients infused with three strains each at a final concentration of 10 cfu/g); hive supplementation occurred twice on day 0 and day 7	Improved honey bees' survival, primed the host's innate immune system, and lowered opportunistic pathogenic *E. coli* loads	

investigations to evaluate their potential as biocontrol agents in AFB prevention.

Limits About the Current Application of Probiotics in AFB Control

The abovementioned beneficial microbes are promising in fighting against *P. larvae* infection in honey bees and negating the concerns arising from the long-term application of antibiotics. However, further research needs to be performed before specific suitable probiotic products can be applied for AFB control in apiculture.

Firstly, similar to the functions of antibiotics, these beneficial microbes mainly target the vegetative forms of *P. larvae*, but do not destroy the infectious *P. larvae* spores. They can mitigate and prevent the outbreak of AFB, but cannot eliminate the disease. Combined use of other methods that can synergistically function together with these beneficial microbes to intervene in the spore stage of *P. larvae* and impede its vegetative growth would be an effective strategy to deal with AFB.

Secondly, the majority of currently available results were obtained under well controlled laboratory conditions using newly emerged bees or individual larvae. Probiotic effects of hbs-LAB observed at the individual level may fail to be validated at the colony level (Stephan et al. 2019). Further research conducted in AFB-infected colonies in open fields will give more convincible results.

Thirdly, there is a lack of standardization as to the protocol of how to evaluate the efficacy of a probiotic product for honey bees. The discrepancies in the methods used by different researchers, which included the enterobacterial repetitive intergenic consensus (ERIC) type of *P. larvae*, the number of spores used to challenge larvae, the composition of the probiotic strains, the dose, the timing and the duration of application, make accurate comparisons of currently available results almost impossible. Taken the dosage of human probiotic products as an example, the concentrations of 10^6 cfu/ml in the small bowel and 10^8 cfu/g in the colon are quoted as necessary to achieve clinical effects (Minelli and Benini 2008). However, to our best knowledge, no consensus has reached as to the appropriate dosage of probiotics applied on honey bees. Therefore, the standardization of application procedures would be the key link allowing results obtained by different researchers to be compared with each other.

It is worth mentioning that some commercial probiotics for animals and humans have demonstrated beneficial effects on tested honey bees (Kaznowskia et al. 2005, Mishukovskaya et al. 2020), while others, even hbs-LAB, have been reported to increase pathogen susceptibility (Schmidt and Engel 2016) and bee mortality (Borges et al. 2021), and fail to reduce background loads of *P. larvae* spore in honey bee colonies (Lamei et al. 2020). The application of potential probiotic strains does not necessarily confer expected positive health effects on the host. Improperly selected probiotics products may even dysregulate honey bees' immune systems, increase their mortality, and promote pathogen infections. Healthy honey bees supplemented with a commercial probiotic (*Lactobacillus rhamnosus*) 9 days before Varimorpha (Nosema) *ceranae* infection had a 25-times higher load of microsporidian spores and a shorter lifespan than the control (Ptaszyńska et al. 2016). In an *in vitro* larval rearing assay, the commensal and probiotic strain, *Parasaccharibacter apium* strain C6 (Corby-Harris et al. 2016), failed to improve larval survival of honey bee larvae infected with virulent *Melissococcus plutonius* (Floyd et al. 2020). Under laboratory-controlled conditions, newly emerged honey bees fed with the sugar syrup supplemented with 10% EM for bees (a commercial probiotic product) had a significantly higher

mortality than honey bees fed with pure sugar syrup (Tlak Gajger et al. 2020). These results highlight the importance for proper selection and application of probiotics, as well as the discreetness of translating probiotic effectiveness from the individual level to the colony level.

More importantly, the probiotic properties of these beneficial microbes, in most cases, have not been assessed. Some researchers suggested the use of 'beneficial microorganisms' or 'apipromotor', instead of 'probiotics' to refer to these beneficial microbes before their probiotic status has been proven (Alberoni et al. 2016). Researchers have begun to evaluate the probiotic capacities of hbs-LAB regarding their abilities to survive and colonize in the intestinal environment of honey bees, their hemolytic activities, and detailed technological characteristics in the production of not only probiotics targeted for honey bee use (Iorizzo et al. 2020) but also probiotic food products for human consumption (Elzeini et al. 2021, Toutiaee et al. 2022). Their findings will greatly promote the utilization of specific probiotics targeted for AFB control and health improvement in honey bees. Therefore, future probiotics research in honey bees needs to focus on selecting honey bee-derived strains capable of re-establishing and persisting in honey bee hosts (Motta et al. 2022b), optimizing delivery system, timing and dosage of application, and validating in field trials (Chmiel et al. 2021) before the reproducibility of relevant research can be achieved and the efficacy of beneficial bacteria in beekeeping can be claimed.

Finally, it should be pointed out that the aim of disease reduction in honey bees is not always consistent with the improvement of colony productivity (honey yield in particular). The activation of immune system (even by endogenous bacterial LAB strains) is costly in honey bees, which may occur at the expense of bee development, reduced productivity, and longevity of honey bees (Evans and Pettis 2005, Janashia and Alaux 2016). There is a trade-off between colony health and productivity which needs to be taken into consideration.

Conclusions

Collectively, beneficial bacteria have great potential in serving as biological alternatives for AFB control. The determination of their GRAS status and detailed probiotic properties, their combined application with other disease-controlling methods to achieve synergistic functions, and the evaluation of their practical effects in more field tests will be necessary before incorporating them into the integrated strategy of AFB prevention and treatment.

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Conflict of Interest

None declared.

Author Contributions

Manhong Ye (Conceptualization-Lead, Writing – original draft-Lead, Writing – review & editing-Supporting), Xiaoyuan Li (Data curation-Equal, Writing – review & editing-Supporting), Fengping Yang (Data curation-Equal, Writing – review & editing-Supporting), Bin Zhou (Conceptualization-Equal, Data curation-Supporting, Writing – review & editing-Lead)

Supplementary Material

Supplementary material is available at *Journal of Insect Science* online.

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