

REVIEW ARTICLE

Gut microbiome dysbiosis and honeybee health

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Abstract

Since a few decades, apiculture is facing important economic losses worldwide with general major consequences in many areas of agriculture. A strong attention has been paid towards the phenomenon named Colony Collapse Disorder in which colonies suddenly disappear with no clear explanations. Honeybee colonies can be affected by abiotic factors, such as environmental pollution or insecticide applications for agricultural purposes. Also biotic stresses cause colony losses, including bacterial (e.g. *Paenibacillus larvae*) and fungal (e.g. *Ascosphaera apis*) pathogens, microsporidia (e.g. *Nosema apis*), parasites (i.e. *Varroa destructor*) and several viruses. In the light of recent research, intestinal dysbiosis, considered as the relative disproportion of the species within the native microbiota, has shown to affect human and animal health. In arthropods, alteration of the gut microbial climax community has been shown to be linked to health and fitness disequilibrium, like in the medfly *Ceratitis capitata* for which low mate competitiveness is determined by a gut microbial community imbalance. According to these observations, it is possible to hypothesize that dysbiosis may have a role in disease occurrence also in honeybees. Here we aim to discuss the current knowledge on dysbiosis in the honeybee and its relation with honeybee health by reviewing the investigations of the microbial diversity associated to honeybees and the recent experiments performed to control bee diseases by microbial symbionts. We conclude that, despite the importance of a good functionality of the associated microbiota in preserving insect health has been proved, the mechanisms involved in honeybee gut dysbiosis are still unknown. Accurate *in vitro*, *in vivo* and in field investigations are required under healthy, diseased and stressed conditions for the host.

Introduction

Among insects, honeybees (*Apis mellifera*) are of great relevance for humans, and the ecosystems, not only as honey producers but especially as pollinators of agricultural and horticultural crops and of wild

plants (Batra 1995; Dedej and Delaplane 2003). In a recent review, Klein et al. (2007) reported that most of the major crops for global food production are strictly dependent on animal pollination or are greatly favoured by pollinators, in terms of fruit size, quality and quantity. Even though the honeybee is

not the only insect pollinator, it still remains the most important one for most crops worldwide (vanEngelsdorp and Meixner 2010). In total, 22.6% of all agricultural production in the developing world, and 14.7% of that in the developed world is directly dependent on animal pollination (vanEngelsdorp and Meixner 2010). In total, 35% of the global food production comes from crops that depend on pollinators (Klein et al. 2007). Taking into account these numbers, the value of insect pollination has been estimated at 153 billion Euros per year worldwide (about 10% of the total value of agricultural production), approximately 14.2 billion and 14.4 billion Euros for EU25 and for USA and Canada, respectively (Gallai et al. 2009). Besides the great crop economic value, the contribute of bee pollination should be considered also within the overall biodiversity that has an inestimable economic value.

In recent years, a severe decline in bee populations and related economic damage has been reported. The international network of the European Cost Action FA0803 COLOSS (Colony LOSS), consisting of 161 members from more than 40 countries worldwide, has been created to coordinate efforts to study, explain and prevent honeybee colony collapse at a global scale. In a recent analysis the COLOSS team (Neumann and Carreck 2010) reported that, during the winter 2007–2008 honeybee losses reached 30% in USA, 1.8–53% in Europe, 25% in Japan and 10–85% in the Middle East.

A definitive cause of the losses has not yet been identified and actually the worldwide honeybee loss is supposed to be caused by the combination of different agents/factors: (i) environmental stress, such as unsuitable weather conditions, lack of forage (nectar and pollen) and the use of insecticides in agriculture (Naug 2009); (ii) biotic stresses, including infection by parasites (*Varroa destructor*, *Acarapis woodi*, *Tropilaelaps* spp.), microsporidia (*Nosema* spp.), pathogenic fungi (*Ascosphaera apis*) and bacteria (*Paenibacillus larvae*, *Melissococcus plutonius*) and more than 18 viruses (e.g. deformed wing virus – DWV) (Genersch et al. 2010); and (iii) the phenomenon known as colony collapse disorder (CCD), a syndrome characterized by the rapid disappearance from a colony of its adult bee population and severely contributing to the death of *A. mellifera* colonies in the United States, Europe and Japan. CCD was first reported in 2006 and despite many efforts, scientists are far from clarifying the causes of this phenomenon. However, in some works it was supposed that CCD could be caused by infectious agents (Cox-Foster et al. 2007; Higes et al. 2009; Bromenshenk et al. 2010).

The microbiome associated to the intestinal system of animals, in particular vertebrates and humans and its impact on host health, are receiving increasing attention. Due to its complexity and its large metabolic capacity, the intestinal microbial ecosystem has been recently defined as a separate organ within the host body, and more specifically an ‘exteriorized organ’ (Cani and Delzenne 2007; Possemiers et al. 2010).

As organs are constituted by cells organized in a specific structure (tissue) serving a common function, the microbes in the digestive tract exist mainly in complex associations adherent to the intestinal mucosa (Macfarlane and Dillon 2007). These structures, defined as mucosal biofilms, are usually multi-species consortia, in which bacteria communicate with each other, coordinate their activities and cooperate for identical functions. In humans, this complex microbial ecosystem is constituted by at least 1 800 different genera of bacteria, counting around 200 g of living cells, with a concentration of 10^{12} microorganisms per gramme content (Sartor 2008; Possemiers et al. 2010).

The structure and the composition of the microbiome are dynamic and several factors such as age (Biagi et al. 2010) and diet (Gibson and Roberfroid 1995) contribute to its shape and functionality. A study illustrating this point, has been recently conducted by Hehemann et al. (2010). They compared the ability of the gut microbiome of Japanese and North American individuals to produce porphyranase and agarases. These enzymes allow the digestion of substrates only present in marine red algae absent in terrestrial plants. According to the authors, genes coding for such enzymes were present only in the microbiome of Japanese. The consumption of seaweeds by Japanese allows the introduction of algae-associated bacteria, which transfer the genes involved in the degradation of the algal sulphated polysaccharides to competent gut resident bacteria with a process known as horizontal gene transfer. This is an example of co-evolutionary process in which the functionality of the microbiome can be influenced by the dietary habits of the host and the host can take advantage from its adapted and specialized microbiome.

In the light of these evidences and according to a recent review examining the complex evolutionary and ecological mechanisms governing human symbiotic communities (Dethlefsen et al. 2007), it is important to underline the following for a better understanding of the host-microbiome relationship, it is not enough to take into account the contribute

of individual microbial species, but more attention needs to be given to the community considered as a whole. Under this perspective, recent observations propose evidences that alterations in the relative proportions of the phyla constituting the microbial community are linked to specific diseases. These alterations have been defined as intestinal dysbiosis (Sartor 2008). Their consequences on host health have been studied mainly in animal models. For example, Turnbaugh et al. (2006) studied the gut microbiome of obese and lean mice revealing a correlation between obesity and abnormal dominance of *Firmicutes* compared to *Bacteroidetes*. The same authors observed an identical imbalance in obese humans. Moreover, they reported that after a therapy, in which the microbial community of obese people was enriched with *Bacteroidetes*, the patients lost between 2% and 6% of their body weight without changes in their diet. In conclusion the study proposed that dysbiosis of the two dominant gut bacterial divisions (*Bacteroidetes* and *Firmicutes*) is an additional contributing factor to the pathophysiology of obesity (Ley et al. 2006).

As well as for obesity, other diseases, such as inflammatory bowel disease (Tamboli et al. 2004), Crohn's disease (Sokol et al. 2008) and alcohol-induced endotoxemia (Mutlu et al. 2009), have been reconducted to intestinal dysbiosis in animals and/or humans.

Similarly to vertebrates, the gut microbiome of invertebrates is a complex association of microbial cells that collectively performs essential functions for the host physiology, biology, ecology and evolution. In recent years great efforts have been devoted to the investigation of the intimate relationships established between insects and their symbiotic microorganisms (Dale and Moran 2006).

Relatively recent studies reported how, in different arthropods (insects included), indigenous gut bacteria can prevent the growth of non-indigenous microbes by a process known as colonization resistance (CR) (Dillon and Charnley 2002) and how the addition of probiotic bacteria in shrimp diet can significantly improve disease resistance by enhancing immunity, as well as presumably modulating the microflora in the shrimp gut (Li et al. 2009). A phenomenon, increasingly observed in insect natural populations, is the 'symbiont-mediated protection', in which vertically transmitted symbionts act in the protection of their hosts against pathogens or predators (Brownlie and Johnson 2009), acting in some cases as an additional non-canonical exogenous immune system (Schneider and Chambers 2008).

Ryu et al. (2008) discovered a mutualistic relationship between the endogenous gut microbiome and the immune system of the *Drosophila* fruit fly, revealing that the fly's normal microbiome has a major role in controlling the growth of pathogenic bacteria.

The essential role of the native gut microbiome of insects is exemplified by the studies performed on the gut microbial symbionts of the Mediterranean fruit fly (medfly) *Ceratitis capitata* and their importance in the reproductive fitness of the host (Ben-Ami et al. 2010; Gavriel et al. 2011). The sterile insect technique is a well established technology used for the biological control of the medfly. However, the irradiated sterile medfly males are less competent in mating with females than wild males, leading to the necessity of a massive male release in the field to outcompete the natural male population. By using molecular microbial ecology methods, Ben-Ami et al. (2010) observed that the irradiation procedure, besides sterilizing the males, affects the gut bacterial community structure of the medfly resulting in a significantly decreased level of *Klebsiella* sp. and in an overgrowth of *Pseudomonas* sp. Based on the concept that a regenerated microbiome could enhance the performances of the sterile flies, following irradiation the authors fed the treated males with a diet enriched with *Klebsiella oxytoca* living cells, previously isolated from the medfly's gut. When compared to untreated males, those exposed to the diet supplemented with the 'probiotic' bacterium presented a stable colonization of *K. oxytoca*, a decreased presence of potentially pathogenic pseudomonads and a higher mating fitness (Ben-Ami et al. 2010). On the whole, these studies indicate that when the indigenous biota of arthropods is present in a suitable climax community, it acts as a barrier against pathogens and, as well as in the vertebrates, intestinal dysbiosis affects the host's health.

Considering the growing attention in honeybee disease causing microorganisms and the fragmentary information of the role of non-pathogenic bacteria within the gut microbiota, the aim of the present paper is to review the recent developments in the study of bacterial diversity in the honeybee's gut, in order to understand better the ecology and the dynamics of these bacterial communities, and the symbiont-mediated mechanisms potentially diminishing pathogen infections and parasites. Honeybee gut dysbiosis and its relationship with the animal health, as well as the possible role of those classes of bacteria emerging as symbionts that contribute to the maintenance of gut homeostasis are discussed.

Microbial Symbiosis in *Apis Mellifera*

The microbial community associated with bees have been investigated and analysed at first by using culture-based techniques (Gilliam and Valentine 1976; Gilliam and Morton 1978; Gilliam et al. 1990). Several microorganisms, including Gram-variable pleomorphic bacteria, *Bacillus* spp. and *Enterobacteriaceae*, together with moulds and yeasts were identified from pollen, nectar, bees, as well as from honey, wax and royal jelly (Snowdon and Cliver 1996; Gilliam 1997). Moulds, particularly the genera *Penicillium* and *Aspergillus*, were commonly found in the alimentary canal of worker honeybees (Gilliam and Prest 1972; Gilliam et al. 1974, 1977). Intestinal yeasts were most frequently encountered in worker bees from colonies that were diseased, fed with nutrient-deficient diets or with antibiotics, or exposed to pesticides; yeast presence appeared to be an indicator of stress conditions in honeybees (Gilliam 1997).

By the development of molecular tools, the possibilities of analysing the composition and the structure of the microbiota inhabiting the honeybee's gut have greatly increased. The application of molecular techniques evidenced at least six phylogenetic groups, i.e. α -, β - and γ -*Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria* as the major bacterial taxa of the honeybee microbial community (Jeyaprakash et al. 2003; Mohr and Tebbe 2006; Babendreier et al. 2007; Yoshiyama and Kimura 2009). The finding that bee specimens sampled in different geographic areas, such as South Africa (Jeyaprakash et al. 2003), Germany (Mohr and Tebbe 2006) and Switzerland (Babendreier et al. 2007) present a similar community composition suggests that *A. mellifera* has a core bacterial microbiota worldwide.

A recent metagenomic approach using high-throughput pyrosequencing technology has been applied to analyse the microbial diversity of honeybees in hives affected or not by CCD (Cox-Foster et al. 2007). Consistently with the previous cultivation-based analyses, the metagenomic approach showed that γ -*Proteobacteria*, *Firmicutes* and α -*Proteobacteria* are the dominant taxa inhabiting the honeybee's gut. In particular γ -*Proteobacteria* accounted for the most abundant group (Jeyaprakash et al. 2003; Babendreier et al. 2007; Cox-Foster et al. 2007).

Molecular studies have been mainly performed on adult bees (Jeyaprakash et al. 2003; Mohr and Tebbe 2006; Babendreier et al. 2007; Cox-Foster et al. 2007) and in few cases larvae close to pupation or eggs have been taken into account (Jeyaprakash

et al. 2003; Mohr and Tebbe 2006). Several severe bee diseases affect the larval stage. For instance the bacteria *P. larvae* and *M. plutonius*, the causative agents of the American Foul Brood disease (AFB) and the European Foul Brood disease (EFB), respectively, affect larvae in their earliest stage even if larval death occurs usually within 3–5 days in the case of EFB and within 6–14 days for AFB (Rauch et al. 2009; Budge et al. 2010). The larval stage is also affected by fungal pathogens like the ascomycete *A. apis*. The relationship between pathogen infections and gut microbial diversity are poorly explored and more detailed knowledge of the larval bee bacterial community is mandatory for the development of novel biological control strategies.

Among the endemic bacterial groups frequently found within the honeybee microbiome, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) are recently generating a great interest (Olofsson and Vásquez 2008; Crotti et al. 2010). Lactic acid bacteria are the most common microbes used as probiotics, proposed to enhance bee immunity, whereas in the case of AAB, their common prevalence in insects with a sugar-based diet, suggests a possible relevant role in the host's biology (Evans and Lopez 2004; Crotti et al. 2010).

Lactic acid bacteria are Gram-positive, low-GC, acid-tolerant, non-respiring bacteria that produce lactic acid as the major metabolic end-product of carbohydrate fermentation. They are normal inhabitants of the gastrointestinal tract of many insects, as well as of vertebrates and have a beneficial activity for the host due to their involvement in the immunomodulation and maintenance of a healthy intestinal microflora (Mitsuoka 1992). The presence of bacteria related to the genera *Lactobacillus* and *Bifidobacterium* in the honeybee digestive system has been consistently reported in the literature (Rada et al. 1997; Jeyaprakash et al. 2003; Mohr and Tebbe 2006; Olofsson and Vásquez 2008). The bee's digestive system represents an optimal niche for the LAB, which, as a counterpart, obtain from the bee's diet suitable substrates for their growth.

Acetic acid bacteria are a large group of obligate aerobic Gram-negative bacteria within the α -*Proteobacteria* clade, commonly found in association with various kinds of sugar matrices. AAB of the genera *Gluconobacter*, *Acetobacter*, *Gluconacetobacter* and *Saccharibacter* have been reported as symbionts of bees (Crotti et al. 2010). Among these, the sugar loving and flower-associated gluconobacters are among the predominant bacterial groups in bees. Mohr and Tebbe (2006) isolated from the honeybee's gut about

one hundred bacterial strains belonging to different bacterial divisions. All isolates of the α -*Proteobacteria* were AAB, closely related to *Gluconobacter oxydans* or *Saccharibacter floricola*, an osmophilic bacterium previously isolated from pollen (Jojima et al. 2004).

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

Microbial Dysbiosis in Honeybee

Among the aforementioned studies, Cox-Foster et al. (2007) compared the gut associated bacteria of bees sampled in CCD and non CCD-affected hives from different geographic areas, over a period of 3 years. The authors' purpose was to identify candidate pathogens significantly associated with CCD. According to their data, one organism, Israeli Acute Paralysis Virus, appeared to be strongly correlated with CCD, while in healthy colonies it was not detected. However, the causal relationship between the virus and CCD was not proved.

The same metagenomic survey demonstrated a high relative abundance of the γ -proteobacterial taxa in the bees from CCD-affected hives than in the healthy ones, while the presence of *Firmicutes* and α -*Proteobacteria*, mainly represented by taxa related to the genus *Lactobacillus* and AAB, respectively, was dramatically reduced in diseased bees. This observation led the authors to suppose that such a dysbiosis may reflect physiological changes accompanying CCD and affecting the commensal community. Under another perspective, it can be hypothesized that the unbalanced species distribution within the gut microflora, and in particular the low presence of LAB and AAB (thought to have beneficial effects on the honeybee's health), may negatively influence the physiology of the bees and directly or indirectly increase the host's susceptibility to diseases.

This hypothesis presents a possible new reading key not only for CCD but also for other diseases such as, the direct negative effect of the *Varroa* mite

and its indirect effect by virus transmission, the foul-brood diseases and fungal, microsporidial and viral infections. The insight that the bee's health may be modulated by symbiotic microorganisms colonizing the gut in balanced relative proportions, is a not yet explored starting point to understand colony losses and develop effective countermeasures. With this intent it will be essential to examine the nature of the relationship occurring between pathogenic and non-pathogenic microorganisms and the host, in order to identify those traits that in the host are able to provide increased tolerance to the pathogens.

Under this perspective, and following the example of Ben-Ami et al. (2010), it seems realistically possible to use molecular diagnostic techniques to identify an altered microbial composition in bee individuals and, once a dysbiosis is observed, apply treatments based on competent active bacteria in order to restore the primitive climax community.

Honeybee Disease Control by Symbiotic Microbes

Insects are commonly co-infected by multiple microbes, beneficial or pathogenic, which, having different and conflicting requirements, interact with each other competitively or synergistically. The recent awareness of the potential use of symbionts as biological control agents against insect pathogens or agricultural and human parasites, vectored by arthropods, has stimulated the research not only to explore the microbial diversity of insects, but also to study the interactions between beneficial symbionts and pathogen agents and to unveil the mechanisms by which native symbionts can compete with pathogens.

The use of beneficial bacteria as probiotics, against pathogens, is commonly implemented in human and animal health care. A clear definition of probiotic has been given by Roy Fuller according to which a probiotic is 'a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance' (Fuller 1989). In insects an excellent example of the use of probiotic symbionts for the enhancement of the competitiveness of the male sterile flies of *Ceratitis capitata* has been recently published (Ben-Ami et al. 2010). In the case of honeybees the probiotic approach has been only theorized but few works have been conducted in this field of study.

Recent studies are taking into account the possibility to use the bee microbial symbionts in order to fight against bee pathogens and parasites or to

enhance bee immunity in disease episodes. In several studies, among the microbial symbionts associated to bee, LAB have been proposed to have a probiotic effect on bees stimulating their immunity, helping the larvae to overcome attacks of pathogens (Evans and Lopez 2004). Lactic acid bacteria are well known producers of antimicrobial compounds, such as organic acids, hydrogen peroxide and antimicrobial peptides (de Vuyst and Vandamme 1994).

Other symbionts, well documented as part of the bee microbiota, are aerobic sporeformers related to the genus *Bacillus* spp. and related genera; these microorganisms have been documented to secrete peptide antibiotics, synthesized either through a ribosomal or non-ribosomal mechanism and antibiotic-like compounds, that in some cases have been shown to possess an antagonistic activity against *P. larvae*.

Also AAB present features that can make them suitable candidates for controlling pathogen infections. They are acid-tolerant bacteria able to acidify the pH during their growth; changes in the environmental pH of surroundings can likely affect the growth of pathogens that share with the AAB symbionts the same gut micro-niche. In different insect models like dipterans and hemipterans, several AAB symbionts have been shown to be capable of intimately colonizing the gut epithelia also by the way of a massive production of extracellular polysaccharides (Marzorati et al. 2006; Favia et al. 2007, 2008; Damiani et al. 2008, 2010; Crotti et al. 2009; Kounatidis et al. 2009; Chouaia et al. 2010).

In recent years a wide range of bacteria isolated from honey samples and other apiarian sources have been tested as biocontrol agents for the capability to inhibit *in vitro* the growth of *P. larvae* or other bee pathogens, such as *A. apis*. Most of the bacteria that showed an antagonistic effect belonged to *Bacillus* sp. (Alippi and Reynaldi 2006; Evans and Armstrong 2006; Cherif et al. 2008; Sabaté et al. 2009). Other effective bacteria belong to the genera *Brevibacillus*, *Paenibacillus*, *Stenotrophomonas*, *Acinetobacter*, *Serratia*, *Providencia* and *Sphingomonas* (Alippi and Reynaldi 2006; Evans and Armstrong 2006; Lee et al. 2009; Yoshiyama and Kimura 2009). A recent work showed the high antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria, including the bee pathogen *P. larvae*, of a strain isolated from US domestic honeybee and classified as *Paenibacillus polymyxa* (Lee et al. 2009).

Despite the description of several bacteria that can counteract honeybee pathogens, very few *in vivo* experimental works have been done. Lactic acid

symbionts of the genera *Lactobacillus* and *Bifidobacterium* have been investigated for their capability to inhibit *P. larvae in vitro* and a combination of selected strains resulted in a complete inhibition of the bee pathogen on agar plates (Olofsson and Vásquez 2008; Forsgren et al. 2010). The addition of bacterial strains to larval food significantly decreased the number of the larvae infected by *P. larvae*. This result showed that probiotic bacteria can modulate gut homeostasis in order to protect bee larvae from diseases. The mechanisms by which this protection is mediated are different, ranging from a direct antagonism with the pathogen by the production of antimicrobial compounds to the activation or stimulation of the innate immune response. Evans and Lopez showed how the administration of non-pathogenic LAB to the larval diet stimulates the transcription of genes usually involved in the immune response (Evans and Lopez 2004). Thus, if the symbiotic microflora activates the immune response, this produces an already immune alarmed situation and may lead to a more prompt immune response in the case of an infection episode.

In all of the aforementioned studies the mechanisms involved in the biocontrol activities of the proposed strains were: (i) the direct inhibition of the pathogen by production of antimicrobial compounds; (ii) the activation/stimulation of the bee's immune system. Moreover, only the LAB's beneficial activity has been evaluated *in vivo* in larval specimens.

In order to better understand the role of population dynamics and of microbial ecology of the native microflora in preserving the honeybee's health, several other aspects need to be investigated. For example the role of other bacterial genera, such as AAB and LAB, the mechanisms related to the competitive exclusion (the competition for nutrients or for the adhesion to the epithelia), the modulation of host intestinal pH, the cell-to-cell communication between microbes and between microbes and host cells and the microbial prebiotic effect on the insect's gut consortium (fig. 1).

Conclusions

Humans and vertebrates are far to live as self-supporting individuals and the symbiotic microbes within the intestinal gut system are essential for their survival (Macfarlane and Dillon 2007). As demonstrated in recent years, they have a co-operative behaviour that is oriented to enhance the fitness of the entire community and to overcome biotic and abiotic stresses. The importance of a good

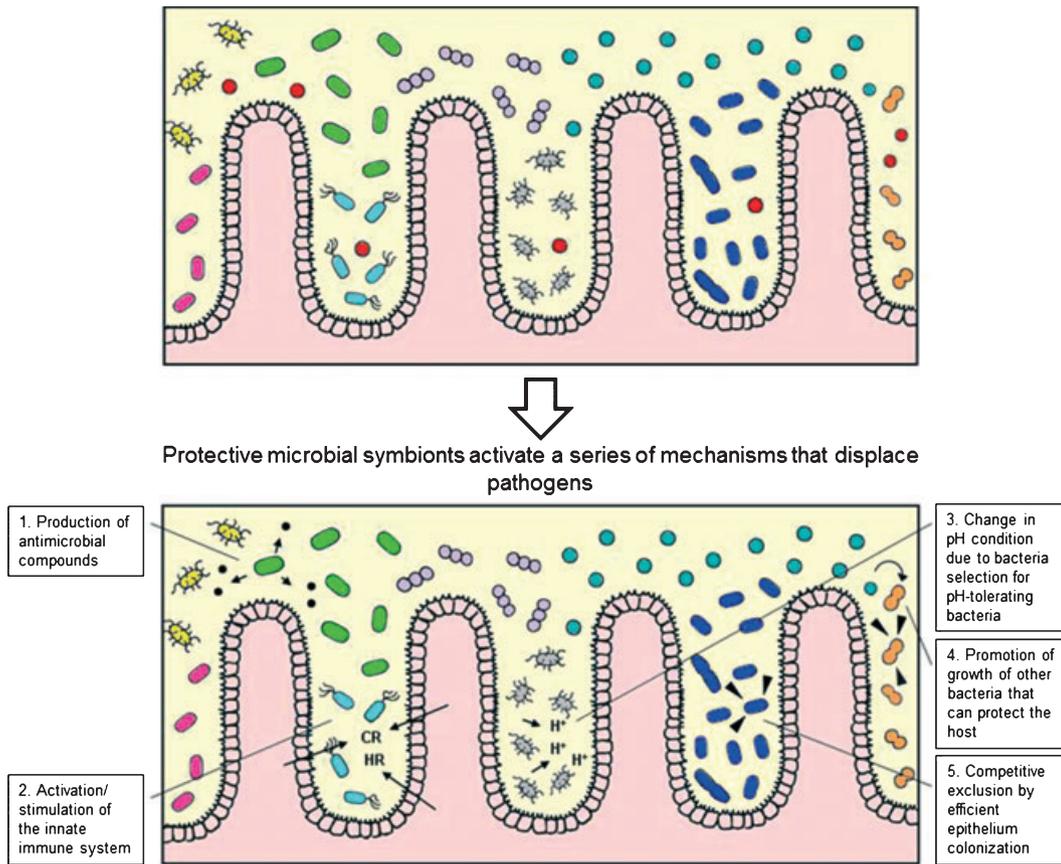


Fig. 1 Mechanisms of action of biological control agents. In the scheme five microbial mechanisms of action for mediating the protection of the gut epithelium are represented: (1) direct inhibition of pathogens by production of antimicrobial compounds; (2) activation/stimulation of the bee's immune system mediated by the microbial symbionts against the pathogens (HR, humoral response, which includes the production of antimicrobial peptides, the cascades that regulate coagulation and melanization of the hemolymph and the production of reactive intermediates of oxygen and nitrogen; CR, cellular response, which refers to hemocyte-mediated responses, like phagocytosis and encapsulation); (3) modulation of host intestinal pH by the microbial symbionts; (4) microbial prebiotic effect on the insect's gut consortium; and (5) competitive exclusion of microbial symbionts counteracting the pathogens. Pathogens are indicated in red. Microorganisms mediating mechanisms one to five are represented in the scheme with cells with different colour mechanisms: (1) green cells; (2) turquoise cells; (3) grey cells; (4) light blue cells; and (5) blue cells. Orange cells can displace the pathogenic red cells by using different mechanisms alternatively or in combination (mechanisms one to three and five) after a probiotic stimulation determined by the prebiotic cells marked in light blue. Other colours indicate neutral non-pathogenic, non-probiotic commensals.

functionality of the gut's microbiome, defined as an 'external organ' is mainly related to its influences on the global health of the host. This concept, that is now well established and validated for vertebrates and humans, is emerging as valid for invertebrates as well, insects included.

Among the insects, particular attention is now growing on honeybees because of their economic relevance in human society. Due to recent honeybee colony losses and the consequent economic damages, scientific research has focused on management strategies to contrast honeybee diseases. A promising, but still undeveloped strategy, is the management of the natural bee microbiome.

Initial clues are now emerging, indicating that under unstressed conditions a protective bee gut microbiome develops in a suitable climax community that could contribute to erect a barrier against diseases, while an unbalanced microbiome or, in other words, an intestinal dysbiosis occurs in presence of pathogen invasions (Cox-Foster et al. 2007). These observations suggest that the alteration in the microbiome is linked to the occurrence of diseases. However, there is still a paucity of *in vivo* experimental data on the potential of probiotics as a prophylactic treatment contributing to the prevention of honeybee bacterial infections (Forsgren et al. 2010). Moreover, the mechanisms implicated in this

process are far to be clarified. Many aspects such as the population dynamics and the ecological drivers that determine the balance or unbalance of the microbiome need to be deeply investigated *in vitro*, *in vivo* and in field experiments under healthy, diseased and stressed conditions.

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