



Microbial symbionts of honeybees: a promising tool to improve honeybee health

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Among pollinators, honeybees are the most important ones and exert the essential key ecosystem service of pollination for many crops, fruit and wild plants. Indeed, several crops are strictly dependent on honeybee pollination. Since few decades, honeybees are facing large-scale losses worldwide, the causes of which are found in the interaction of several biotic and abiotic factors, such as the use of pesticides, the habitat loss, the spread of pathogens and parasites and the occurrence of climate changes. Insect symbionts are emerging as a potential tool to protect beneficial insects, ameliorating the innate immune homeostasis and contributing to the general insect wellbeing. A review about the microbial symbionts associated to honeybees is here presented. The importance of the honeybee microbial commensals for the maintenance and improvement of honeybee health is discussed. Several stressors like infestations of *Varroa* mites and the use of pesticides can contribute to the occurrence of dysbiosis phenomena, resulting in a perturbation of the microbiocenosis established in the honeybee body.

Introduction

Non-conventional habitats, among which extreme environments (like hot or cold deserts, inland or coastal saline systems), polluted sites and animal gut, have been less explored in terms of biodiversity, richness and functionality as compared to other well-studied conventional habitats, such as soil- and water-associated matrices. Nonetheless, they represent a considerable source of compounds and microorganisms with interesting biological and biotechnological potential [1,2]. Growing attention has been recently directed to the study of these niches and, among these various non-conventional habitats, to the animal gut or, in general, body intended as niches in which microorganisms survive and flourish [3].

All metazoans hosting a gut microbiota, including arthropods, establish with their microbes complex and dynamic symbiotic interactions, which recently have been shown to go beyond a mere nutritional complementation of the host diet, embracing a wide set of aspects related to the host physiology, behavior, reproduction, evolution and immunity [3,4]. Insects are the most diverse animal group on earth and during their evolutionary history they adapted to feed on a variety of substrates and matrices, ranging from wood or phloem sap to blood. These nutritionally unbalanced diets are exploited and/or complemented through insect microbiota [see the review 5]. Microorganisms also played a major role in insect adaptation and evolution [6].

Among insects, honeybees are of great importance worldwide due to their pollination activity for crops, fruit and wild plants. They offer a key ecosystem service, essential for a sustainable

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productive agriculture and for the maintenance of the non-agricultural ecosystem. Pollination services are mandatory for the production of crops like fruits, nuts and fibers, whereas the results of many other agricultural crops are significantly improved by pollination. It has been estimated that without pollinators a decrease by more than 90% of the yields of some fruit, seed and nut crops could occur [7]. In the case that wild bees do not exert their pollination service in a specific agricultural crop, managed honeybees, which are versatile, cheap and convenient, represent the only solution to ensure pollination [8]. The dependence of worldwide crops on pollinators is extremely deep and during 2005 the global economic value of insect pollination was estimated to be €153 billion a year, which corresponds to 9.5% of the total economic value of agricultural crops for human consumption [9].

Since few years, concerns are rising over honeybee health and, consequently, over its impact on economy [10]. Large-scale losses have been reported worldwide and related to several causes, i.e., the habitat loss of pollinators, the increasing use of agrochemicals, the outbreak of diseases, the attacks of parasites, the alarm related to climate change, the introduction of alien species and the interaction among all of these factors [10]. Managed honeybees are facing increasing threats of diseases, pests and reluctance among younger generations to learn the skills of beekeeping. In the last past years, to define and to calculate the vulnerability of world agriculture pollinator decline have become a primary point of action [8,9,11,12]. Recently, Colony Collapse Disorder [CCD] has attracted the attention of academic and public opinion, but this poorly understood syndrome is just one cause of the colony losses. Recent studies suggest that several factors are involved in CCD, as parasites, pathogens, pesticides (and other environmental stressors) and, above all, the interactions among them [13,14].

Honeybee symbionts could be exploited to actively counteract bee pathogens and parasites or to enhance bee immunity, and thus indirectly to increase the protection of honeybees' health. Probiotic bacteria, such as lactic acid bacteria (LAB), have been administered in laboratory conditions to honeybees, resulting in the stimulation of the innate immune system and the prevention of attacks by pathogen [15]. Recent studies in the insect model *Drosophila* emphasize how complex, intimate and multifaceted is the relation subsisting between the host and the microbiota, which, if well balanced, leads to the optimal insect wellness [4].

In this review, we present the current understanding of the importance of honeybee symbionts for the maintenance and improvement of the insect health. In particular, the microbiota involvement in the stimulation of the insect immune system and body homeostasis – with a special focus on the gut dysbiosis – and how this may be related to the use of pesticides, the spread of viruses and the occurrence of parasites is discussed.

Microbial community associated to the honeybee *Apis mellifera*

Cultivation-dependent and -independent approaches have been long used to define the composition and the structure of the honeybee microbiota, analyzing different honeybee developmental stages, such as larvae, pupae, newly emerging adults and adults; different genders, such as females and drones; and different social individuals, such as queens, nurses or foragers [16]. Six phylogenetic groups, i.e. α -, β - and γ -Proteobacteria, Firmicutes, Bacter-

oidetes and Actinobacteria, have been found as the major bacterial taxa of the honeybee bacterial community, representing moreover the bacterial core maintained in honeybees worldwide [16].

The recent technological innovations in the genomics and metagenomics fields revolutionized the potential of applications and the throughput of the analyzed data, allowing DNA sequencing of high numbers of nucleotides with low costs and high accuracy. The microbial composition and structure of a specific community can be evaluated with high sensitivity, low cost and short times, thanks to new sequencing technologies and the multiplexing approach [17,18]. Also honeybee microbiota has been evaluated by the use of these techniques [19–23, Table 1]. Interestingly, eight bacterial phylotypes have been retrieved as major constituents of honeybee bacterial community, i.e. Alpha-1, Alpha-2, Beta, Gamma-1, Gamma-2, Firm-4, Firm-5 and Bifido, which correspond to the six phylogenetic groups mentioned above.

The metagenomic survey on honeybees from CCD-affected and not affected hives performed by Cox-Foster *et al.* [19] revealed that in non-affected honeybees Firmicutes and α -Proteobacteria are more abundant than in CCD colonies. Similarly, in the work by Cornman *et al.* [20], deep sequencing on honeybees showed a high proportion of Alpha-1, Alpha-2 and Bifido phylotypes in individuals from not affected hives compared to those from CCD-affected hives. Cloning libraries of 16S rRNA by Martinson *et al.* [21] revealed that the most abundant taxon in *A. mellifera* samples was represented by Firm-5 phylotype. *A. mellifera* showed a distinctive bacterial pattern, made up of the eight typical phylotypes, some of which are also present in closely related corbiculate bees of the genera *Apis* and *Bombus*. Lately, pyrotag analysis, quantitative PCR (qPCR) and fluorescent *in situ* hybridization (FISH) confirmed Beta, Firm-5 and Gamma-1 phylotypes (BFG phylotypes) as main members of *A. mellifera* microbiota, with a characteristic distribution along the gastrointestinal tract [22]. The crop resulted poor in microbial species, due to continuous filling and emptying for nectar supply, and also the midgut showed a low BFG load, due to the presence of the digestive enzymes and the peritrophic membrane that prevents microbial attachment. By contrast, the ileum and the rectum were rich in microbes. The ileum showed a defined microbial distribution with Gamma-1 phylotype gathered in a thick mat, between Beta phylotypes and the ileum wall, and with Firm-5 phylotype located in small pockets along the ileum wall. The rectum showed the majority of BFG phylotypes together with the majority of bacterial diversity [22].

A deep sampling of gut microbiota from 40 individuals has been performed by Moran *et al.* [23]. Four phylotypes were present in all samples, even if with different frequencies, i.e. one γ -Proteobacterium, classified as *Gilliamella apicola* [24], one β -Proteobacterium corresponding to *Snodgrassella alvi* [24] and two Firmicutes classified in *Lactobacillus* genus.

Yeasts, wide spread microorganisms in the honeybee environment, such as flowers, fruits and plant leaves [25,26], are also important components of the bee microbiota. Recently by the use of molecular tools, sequences related to the genera *Saccharomyces*/*Zygosaccharomyces* and to the family Saccharomycetaceae have been identified [20], confirming previous results obtained by cultivation-dependent methods that showed the association of yeasts with honeybee [27].

TABLE 1

Actual knowledge on the bacterial species associated to the honeybee *Apis mellifera* according to cultivation-independent and -dependent methods. Data from cultivation-independent studies and some data from cultivation-dependent studies are from Sabree *et al.* [50]. Other cultivation-dependent data are from studies that identified the isolates by partial or complete 16S rRNA gene sequencing

| Case study | Origin | Sample | Method | Total <i>n</i> sequences | % known bee species groups ^a | Alpha-1 ^a | Alpha-2 ^a | Beta ^a | Gamma-1 ^a | Gamma-2 ^a | Firm-4 ^a | Firm-5 ^a | Bifido ^a | Other bacteria |
|---|------------------------|---|--------------------|--------------------------|---|----------------------|----------------------|-------------------|----------------------|----------------------|---------------------|---------------------|---------------------|----------------|
| Cultivation-independent techniques | | | | | | | | | | | | | | |
| Jeyaprakash <i>et al.</i> [51] | South Asia | Dissected guts | Sanger | 8 | n/a | +(3) | +(1) | +(2) | +(2) | – | – | +(1) | +(1) | ^b |
| Mohr and Tebbe [52] | Germany | Dissected guts | Sanger | 13 | n/a | – | +(1) | +(1) | +(2) | – | – | – | – | ^b |
| Babendreier <i>et al.</i> [53] | Switzerland | Midgut and hindgut | Sanger | 27 | n/a | +(3) | +(2) | +(6) | +(8) | +(1) | +(2) | +(4) | – | ^b |
| Disayathanoowat <i>et al.</i> [54] | Thailand | Midgut | Sanger | 17 | n/a | – | – | + | +(1) | – | – | +(2) | +(1) | ^b |
| Cox-Foster <i>et al.</i> [19] | Australia, USA, Hawaii | Pooled whole bees | Pyrotags 454 | 428 | 97.4 | 1.9 | 3.2 | 16.9 | 60.9 | 9.6 | 0.6 | 2.8 | 1.7 | 2.6 |
| Martinson <i>et al.</i> [21] | Arizona | Single whole bees | Sanger | 271 | 98.5 | 0.0 | 1.1 | 11.1 | 11.8 | 0.0 | 10.0 | 63.8 | 0.7 | 1.5 |
| Martinson <i>et al.</i> [21] | Arizona | Bacterial cells isolated from pooled guts | Sanger | 267 | 98.5 | 0.7 | 0.0 | 3.7 | 9.7 | 0.0 | 10.5 | 60.7 | 13.1 | 1.5 |
| Martinson <i>et al.</i> [22] | Arizona | Dissected gut sections | Pyrotags 454 | 96,505 | 99.9 | 0.0 | 0.3 | 20.3 | 10.1 | 24.2 | 0.2 | 44.0 | 0.8 | 0.1 |
| Sabree <i>et al.</i> [50] | Massachusetts | Dissected guts | Pyrotags 454 | 106,344 | 94.8 | 0.0 | 0.0 | 6.74 | 49.10 | 1.12 | 11.05 | 21.36 | 5.41 | 5.2 |
| Moran <i>et al.</i> [23] | Arizona, Maryland | Dissected guts | Pyrotags 454 | 329,550 | 99.1 | 1.0 | 1.0 | 9.1 | 11.9 | 2.0 | 45.4 | 23.2 | 5.4 | 0.9 |
| Engel <i>et al.</i> [37] | Arizona | Hindguts of worker bees | Illumina sequences | 76.6 Mb ^d | 82.4 | 13.8 | 3.4 | 4.9 | | 23.9 | | 9.7 | 3.4 | 17.6 |
| Cultivation-dependent techniques^c | | | | | | | | | | | | | | |
| Evans and Armstrong [55] | USA | Individual larvae | Sanger | 11 | n/a | – | – | – | – | – | – | +(1) | – | ^b |
| Olofsson and Vasquez [56] | Sweden | Guts | Sanger | 17 | n/a | – | – | – | +(3) | +(1) | +(1) | +(4) | +(5) | ^b |
| Vásquez and Olofsson [57] | Arizona | Guts | Sanger | 11 | n/a | – | – | – | +(1) | – | +(1) | +(2) | +(4) | – |
| Sabaté <i>et al.</i> [58] | Argentina | Pooled intestines | Sanger | 1 | n/a | – | – | – | – | – | – | – | – | ^b |
| Loncaric <i>et al.</i> [59] | Austria | Honey sac | Sanger | 11 ^e | n/a | – | – | – | – | – | – | – | – | ^b |
| Carina Audisio <i>et al.</i> [60] | Argentina | Intestines | Sanger | 5 | n/a | – | – | – | – | – | – | – | – | ^b |
| Vásquez <i>et al.</i> [44] | Sweden and Kenya | Dissected honey crops | Sanger | 137 ^e | n/a | – | – | – | v | – | +(4) | +(7) | +(29) | ^b |

n/a=indicates not available.

^a For studies with deep sequencing methods, percent values of phylotype abundance are indicated. In those studies where methods do not allow percent representation, '+' indicates the presence of a phylotype. Figures in parentheses indicate the number of sequences associated to a bacterial group. '-' indicates no presence of a phylotype.

^b Sequences of other bacteria, besides the phylotypes presented in the table, have been retrieved but the frequencies cannot be calculated due to the methods employed in these case studies.

^c Cultivation-dependent methods do not allow to represent all bacteria in the gut.

^d These numbers are from a dataset of metagenomic data.

^e These numbers are from a dataset including also, but not only, sequences from *A. mellifera*.

Emerging stressors for honeybee health

Currently, a renewed attention has been directed to the relationship between honeybee health and the use of pesticides, the occurrence of parasitic mites and the outbreak of viral disease, emphasizing their interconnection in determining the insect health status [14,28].

Pesticides, especially neonicotinoids, which are widely used for their excellent systemic properties, are indicated by scientists to play a role in CCD phenomenon and, in general, in weakening the processes of the colony, interacting with other stressors, such as parasites [28]. Honeybees are exposed to neonicotinoids at sublethal doses, and this results in insect behavioral disturbances, orientation difficulties and impairment in social activities [28,29]. Experiments to prove these difficulties have been performed not only in laboratory conditions – by ingestion tests and indirect contact tests [29] – but also in field trials, where honeybees were exposed to a direct contamination with the pesticides during the foraging activity or to an indirect contamination with the pesticide-contaminated materials stored in the hive or exchanged with the sister bees [28]. Sublethal doses of pesticides resulted to be dangerous also for bumble bees, inducing a weight loss of the insect, a low number of pupae and a reduced number of queens, thus impacting lastly the bumble bee populations [30].

The worldwide-spread, obligate-ectoparasitic mite *Varroa destructor* represents a severe threat for apiculture. It can lead to a colony collapse within a two- to three-year period. Periodic treatments with chemicals increase on the one hand the costs for beekeeping, and on the other hand the risk of the presence of chemical residues in the environment and in the honey [31]. Moreover, *Varroa* mites act as disseminators of viruses between and within bee colonies [32]. Recent publications highlighted the multifactorial origin of the honeybee collapse. For instance, *Varroa* can destabilize Deformed Wing Virus (DWV) dynamics making the virus a rapidly replicating killer [14]. When DWV dynamics are destabilized, a host immunosuppressive status with the down-regulation of the transcriptional factor NF- κ B is recorded. The authors suggest that the DWV-mediated immunosuppressive effect shows a DWV-threshold dependency; below a certain threshold, DWV infection is maintained under control. If a stress factor, like *Varroa*, subtracts the transcriptional factor NF- κ B, the concentration of the latter becomes too low to keep under control DWV that can finally outbreak, bringing to the collapse the bee population [14].

Pesticides, mites and viruses have a serious impact on the health of honeybees, but in all these studies there is a missing actor, represented by the gut microbial community. We will show in the next paragraphs how deeply correlated is the insect health with the gut microbiota and the immune system. Microorganisms could be a key element in managing and preserving honeybee health status toward different biotic and abiotic stressors.

Roles of the microbial partners

Recent research has shown that the gut microbiota is strictly linked to host homeostasis and metabolic diseases, e. g. diabetes and obesity [33]. The gut microbial community is involved in several aspects of the host life, ranging from the nutritional contribution to the energy salvage through fermentation, from influencing mating preferences (e.g. this is the case of the gut bacteria in

Drosophila [34]) to immunity [5]. The animal immune system works synergistically to contain the pathogens and to preserve the symbiotic relationships between host and microbiota. A fine regulation of signaling networks, which control the presence of antimicrobial compounds in the gut, allows the host to tolerate commensals and to block the proliferation of food-borne pathogens [35].

As presented above, the honeybee microbiota shows a consistency which leads to hypothesize the possibility of a neutral or beneficial involvement of it, or at least with some members of the microbiota, in the honeybee's life. Several of the taxa identified in honeybees are known to produce short chain fatty acids, such as lactic or acetic acid (Lactobacilli, Bifidobacteria, Acetobacteraceae and *Simonsiella*). These products may act as supplements to honeybee diet. Moreover, gut bacteria could allow to degrade pollen, which is covered by exine layers recalcitrant to most of digestive enzymes, using then the intine as a nutrient source [36,37].

While nutritional symbioses between insects and bacteria are well documented [5], the correlation that exists between the proper function of insect innate immune system and its microbiota is less explored. Symbionts are recently receiving increasing attention because of their recognition as strong and effective immunomodulators of insects [38–40].

In their work Ryu *et al.* [38] found that there is a fine equilibrium between the acetic acid bacterial commensals and the *Drosophila* innate immune system. The normal flora suppresses the growth of pathogenic bacteria, unless the system is perturbed. If a perturbation of the gut bacterial community occurs, an increased number of pathogenic bacteria could lead to gut apoptosis. In a normal condition the fly's immune system allows the dominance of an Acetobacteraceae strain, which in turn keeps down, by competitive exclusion, the proliferation of the gut apoptosis inducer.

Another case study is represented by the tsetse fly and its obligate symbiont *Wigglesworthia*. The latter complements the deficient diet of the fly with the products of its metabolism. However, the symbiosis at the base of tsetse–*Wigglesworthia* interactions goes beyond the nutritional role: larvae deprived of *Wigglesworthia* are immunocompromised when they reach the adult stage. Weiss *et al.* [39] show that in aposymbiotic tsetse flies the cellular innate immune system is seriously compromised and consequently the insects are highly susceptible to infections. When hemocytes from wild type individuals are transplanted in aposymbiotic adults or *Wigglesworthia* cell extracts are administered to the aposymbiotic mothers, the innate immune system functionality is restored.

Another study that highlights the multidimensionality of symbionts–host interactions has been performed on the Hawaiian squid *Euprymna scolopes* and the luminous bacterium *Vibrio fischeri* [40]. *V. fischeri* is the exclusive partner of the squid light organ and the symbiosis follows a dynamic balance of symbionts expulsion and regrowth. The well-known mediators involved in animal–microbe interactions, called 'microbe associated molecular patterns' (MAMPs), specifically lipid A component of lipopolysaccharide (LPS) and peptidoglycan component, interplay synergistically with the luminescence of symbionts to sustain the host development. Researchers found that MAMPs and luminescence interactions are both crucial for the maintenance of the symbiosis.

All these findings contribute to state that a finely regulated dialog exists among the symbiotic partners to reach a symbiostasis.

This is done through the regulation of pathways implicated in the substrate availability and pathways that govern host/symbionts population dynamics. Recently, artificial microcosms have been employed to prove that the high functionality of a specific system could be maintained, even during stress events, if microorganisms are distributed in a suitable climax community [41]. In the case of the microbiota associated to the digestive system, the maintenance and improvement of the host health against pathogens infection depends on the functionality of the system, which lastly relies on the presence of a suitable climax community [16]. Cox-Foster *et al.* [19] showed that CCD non-affected honeybees are mainly colonized by Firmicutes and α -Proteobacteria, while in CCD-affected bees a high abundance of γ -Proteobacteria is measured. This could be related to a case of dysbiosis, i. e. an unbalance of the gut microbiota, with the consequent loss of the proper functionality, which in turn negatively impacts the health status. Further studies are needed to unveil the strict and dynamic interplay existing between host and symbionts.

Microbial involvement in the general insect health status

Recent publications highlighted that in different *Drosophila* strains two taxonomically different bacteria, i. e. *Acetobacter pomorum* and *Lactobacillus plantarum*, modulate the insulin signaling and TOR pathway, respectively, through different bacterial products [4,42,43]. In *A. pomorum*, the acetic acid produced by the activity of the pyrroloquinoline quinone-dependent alcohol dehydrogenase (PQQ-ADH) modulates the insulin signaling which in turn controls several host homeostatic programs, as the developmental rate, the body size, the energy metabolism and the intestinal stem cell activity [42]. By contrast, *L. plantarum* promotes protein assimilation from the diet, regulating diet-derived branched-chain amino acid (BCAA) levels in the hemolymph. BCCA activates TOR signaling: (i) in the fat bodies, which results downstream into the promotion of growth rate and (ii) in the prothoracic glands, which has an impact downstream on the length of growth phase [43]. In fat bodies TOR pathway normally acts stimulating the systemic production of insulin-like peptides and thus promoting the growth. It has been hypothesized that (1) the stimulation of the

insulin signaling in presence of commensals could be the result of the evolution conflict between the host and its microbiota; (2) bacterial metabolites are cues for the host to be informed on the environmental nutritional availability for the host development [4]. Thus according to this second hypothesis the host would exploit its microbiota to sense the environment. Bacteria are known to communicate through quorum sensing which allows the regulation of their activity and physiological processes. Quorum sensing outcomes in important advantages for bacteria, i. e. host colonization, formation of biofilms, defense against competitors, and adaptation to changing environments. The kind of interaction here hypothesized implies a higher level of interaction between symbionts and hosts.

The molecular mechanisms that regulate the host microbe crosstalk are still poorly understood. However, all these studies highlight the key role of microbial partners in influencing the systemic growth of the host and preserving its health. As in *Drosophila*, it is possible to hypothesize that commensals in honeybee could have a higher level of interaction with the host, acting on the growth regulation of the insect. Components of *Drosophila* microbiota, as Lactobacillales and Acetobacteraceae members, are widespread in *A. mellifera*. LAB have been shown to exert a probiotic effect on honeybee larvae, eliciting the innate immune system to overcome pathogen attacks [15], and have been indicated as major modulators of honeybee health [44]. Like LAB, well-known for their ability to produce antimicrobial factors, other symbionts such as sporeforming bacteria are indicated as producers of peptide antibiotics and antibiotic-like compounds, which in some case possess antagonistic activity [45,46]. Finally, acetic acid bacteria (AAB), widespread in nature [47], can compete with the pathogen along the host epithelia, physically occupying the available niches and nutritionally competing with the pathogens. Moreover, acid and exopolysaccharide production may contribute to AAB successful colonization of the insect gut [48,49].

Perspectives

There is increasing evidence that there is a strict interconnection between the intestinal microbiota balance and the health status of the host [4]. Commensal microbiota drives immune and health

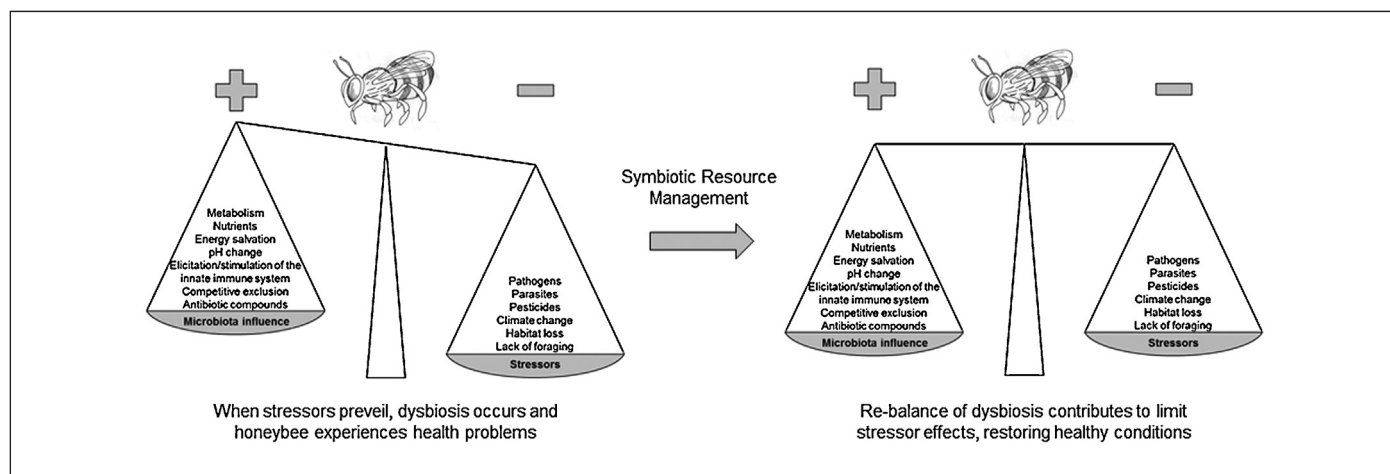


FIGURE 1

A graphical representation of the ecological concept of Symbiont Resource Management (SRM) which foresees the management of the insect gut microbiome to improve host health.

homeostasis by mechanisms that are yet poorly understood and a great effort has to be done in this direction. Insect symbionts are indeed emerging as a potential tool in biocontrol programs to protect beneficial insects, ameliorating the innate immune homeostasis and contributing to the general insect wellbeing [4]. The employment and exploitation of microorganisms in a defined environment or niche to solve practical problems have been termed as Microbial Resource Management (MRM) and MRM concepts are applicable to the maintenance and promotion of insect health [3]. A novel MRM application, the Symbiont Resource Management (SRM), can be defined as the application of microbial symbionts to manage insect-related problems [3; Fig. 1]. Symbiotic microorganisms can exert their beneficial contribution toward the host to sustain its health in different ways, i.e. by competitive exclusion, production of antibiotic compounds, activation/stimulation of the innate immune system and communication to the host of the environmental conditions. However, to come able to manage these complex microbial communities

within the body of the insects it is imperative to understand how they interact with the host. Therefore, further research has to be conducted to clarify the molecular mechanisms at the base of the symbiosis.

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