Integrative and Comparative Biology

*Integrative and Comparative Biology*, volume 52, number 1, pp. 89–99
doi:10.1093/icb/ics089

*Society for Integrative and Comparative Biology*

SYMPOSIUM

# Paratransgenesis: An Approach to Improve Colony Health and Molecular Insight in Honey Bees (*Apis mellifera*)?

Anbjørg Rangberg,* Dzung B. Diep,* Knut Rudi* and Gro V. Amdam[1,*,†]

*Department of Chemistry, Biotechnology and Food Science, University of Life Sciences, P.O. Box 5003, N-1432 Aas, Norway; †School of Life Sciences, Arizona State University, P.O. Box 874501, 427 East Tyler Mall, Tempe, AZ 85287, USA

From the symposium "The Impacts of Developmental Plasticity on Evolutionary Innovation and Diversification" presented at the annual meeting of the Society for Integrative and Comparative Biology, January 3–7, 2012 at Charleston, South Carolina.

[1]E-mail: Gro.Amdam@asu.edu

**Synopsis** The honey bee (*Apis mellifera)* is highly valued as a commercial crop pollinator and a model animal in research. Over the past several years, governments, beekeepers, and the general public in the United States and Europe have become concerned by increased losses of honey bee colonies, calling for more research on how to keep colonies healthy while still employing them extensively in agriculture. The honey bee, like virtually all multicellular organisms, has a mutually beneficial relationship with specific microbes. The microbiota of the gut can contribute essential nutrients and vitamins and prevent colonization by non-indigenous and potentially harmful species. The gut microbiota is also of interest as a resource for paratransgenesis; a Trojan horse strategy based on genetically modified symbiotic microbes that express effector molecules antagonizing development or transmission of pathogens. Paratransgenesis was originally engineered to combat human diseases and agricultural pests that are vectored by insects. We suggest an alternative use, as a method to promote health of honey bees and to expand the molecular toolbox for research on this beneficial social insect. The honey bees' gut microbiota contains lactic acid bacteria including the genus *Lactobacillus* that has paratransgenic potential. We present a strategy for transforming one *Lactobacillus* species, *L. kunkeei*, for use as a vector to promote health of honey bees and functional genetic research.

## Introduction

Insects vector several disease-causing pathogens, creating a global challenge through their negative impact on human health and agriculture (Robinson et al. 2004; Coutinho-Abreu et al. 2010). To halt the spread of these pathogens, scientists have attempted to genetically transform insect-associated symbiotic microorganisms, a process called the paratransgenic method (Durvasula et al. 1997). Paratransgenesis is a Trojan-horse approach in which symbiotic bacteria, fungi, or viruses of the vector insect are genetically manipulated to deliver effector proteins that block development or transmission of the pathogen (Fig. 1). The ultimate goal of paratransgenesis is to combat the disease vectored by the insect, thereby reducing its ability to damage human health or economic interests (Robinson et al. 2004; Coutinho-Abreu et al. 2010).

Although general, in principle, the paratransgenic method has so far been exclusively applied to insect systems, starting with the kissing bug *Rhodnius prolixus* and its endosymbiotic bacterium *Rhodococcus rhodnii* (Durvasula et al. 1997). *R. rhodnii* was transformed to express cecropin A, a peptide lethal to the *R. prolixus* parasite *Trypanosoma cruzi*, which is responsible for Chagas disease. Malaria is another insect-vectored disease for which paratransgenic control has been tested. The genus *Plasmodium* contains four species of parasitic protozoa that cause malaria, and the bacterium *Pantoea agglomerans*, a symbiont of the insect vector *Anopheles* mosquitoes, was recently engineered to secrete anti-*Plasmodium* effector proteins (Bisi and Lampe 2011). Another example is the transformed fungus *Materhizum anisopliae* that carries effector genes against *Plasmodium* development (Fang et al. 2011). Symbiotic densonucleosis

Advanced Access publication June 1, 2012

viruses (or densoviruses) are also attractive agents for paratransgenic malaria control (Ren et al. 2008). In protection of economically important agricultural crops, transgenic *Enterobacter* strains have been developed into vehicles that target populations of the larvae of cane beetles (*Dermolepida albohirtum)*, corn borers (*Pyrausta nubialis*), and cotton bollworms (*Helicoverpa armiger*) (reviewed by Pittman et al. 2008).

Developing a paratransgenic strategy involves several steps: the isolation of suitable microorganisms; identification of effector proteins; transformation of a bacterium, fungus, or virus; effective delivery of the transformed microorganism (transformant) back into the insect host; and when desirable, the spread of the transformant within and between host populations (Fig. 1). Overall, and so far, paratransgenesis has not been a widely successful method against insect-vectored disease or against the decimation of crops (Coutinho-Abreu et al. 2010). A recurrent issue is implicit negative effects on host fitness that impair the spread of the paratransgenic control mechanism in the target insect population (Fang et al. 2011). Due to these negative effects on the host's health, the potential of this method to further human interests has thus far failed to meet expectations.

Here, we propose an alternative scope of paratransgenesis: the genetic manipulation of symbiotic microorganisms with the goal of enhancing a host organism's *positive* effects on human health and economic interests. We propose using the honey bee, *Apis mellifera*, and its symbiotic gut microbes. Several of these microorganisms are potential candidates for genetic transformation and can, in theory, be modified into delivery vehicles for effector proteins against honey bees' pathogens. Furthermore, the microbes may be engineered to secrete active metabolites, peptides, or enzymes that can influence the physiology or behavior of honeybees, with the purpose of promoting their health or advancing the understanding of their biology. The latter use would expand the molecular genetic toolbox of honey bees to include functional paratransgenetics.

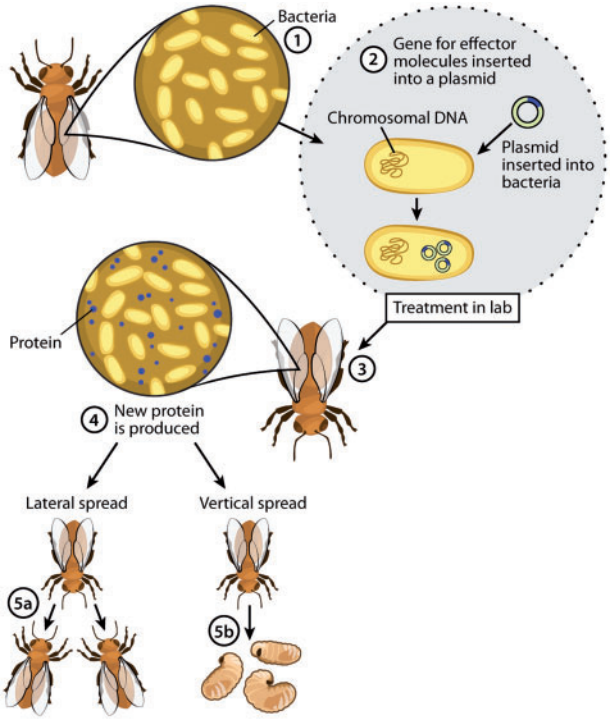


**Fig. 1** The paratransgenic method, using bacteria as delivery vehicles. (**1**) The first step is to isolate and identify commensal or mutualistic microorganisms living within the insect host. (**2**) Next, the isolated bacterium, fungus, or virus is transformed with modified plasmids containing a gene (blue inset) or genes encoding effector molecules, e.g., molecules that block development or transmission of the pathogen. (**3**) The transformed microorganisms are then transferred back to the host by feeding or injection of either larvae or adults. (**4**) Recolonization and expression of effector molecules (blue dots) within the insect host. (**5**) When desired, the transformed microorganism can be transferred laterally (**5A**), e.g., among social insects by social feeding, or vertically (**5B**) from caregiver to offspring, e.g., by feeding with feces (common in termites) or rearing of larvae (such as in honey bees).

## Honey bees

Honey bees are highly social insects (Fig. 2). They live in colonies of about 10,000–50,000 females and a few hundred males (Wilson 1971). Each colony has one reproductive queen (Fig. 2A), which lays eggs and produces several pheromones that are essential for the integrity and functioning of the colony. The remaining females are functionally sterile helpers, called workers. These workers perform many different tasks during their lifetimes, including rearing of larvae (Fig. 2B), colony defense (Fig. 2E), and foraging (Fig. 2F). Foraging workers utilize advanced navigation, communication, learning, and memory skills to ensure optimal influx of resources to the colony (Frisch 1967; Seeley 1995; Menzel et al. 2006). Through the domestication of honey bees, these skills are exploited by human societies for pollination of crops (Fig. 2G), as well as for basic research (Fig. 2H).

Honey bees are of considerable value to agriculture, with a yearly impact estimated to be worth more than $15 billion in the United States and at least €15 billion in Europe (van Engelsdorp and Meixner 2010; European Parliament 2011). Several

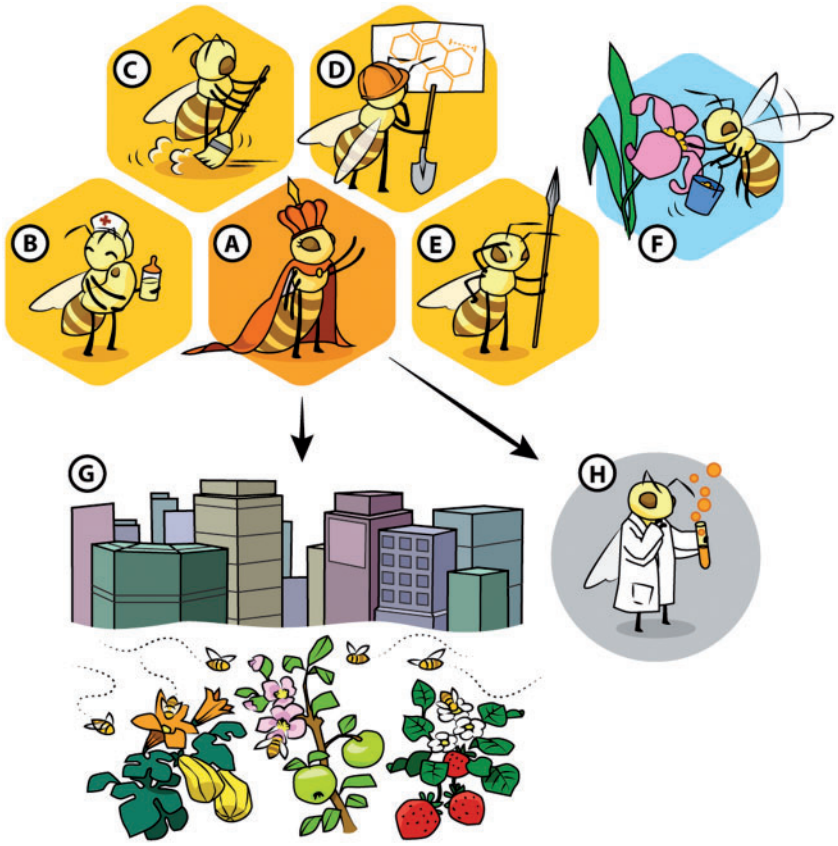


**Fig. 2** The honey bee society. A honey bee colony consists of one reproductive queen (**A**), 10,000–50,000 functionally sterile worker bees, and a few male drones. All the workers are females that perform different tasks during their lifetime. Young workers generally nurse larvae (**B**) and clean the nest (**C**), before they progress to the building and maintenance of wax combs (**D**), colony defense (**E**), and receiving and processing foods collected by foragers. Foraging is usually performed toward the end of the workers' lifespan (**F**). Honey bees are highly appreciated by human societies for their pollination services (**G**) and as a model in research (**H**).

varieties of crops such as almonds, apples, blueberries, oranges, strawberries, sunflower, and canola are dependent on pollination by honey bees to give economically valuable yields. In consequence, massive mortality of honey bees due to colony collapse disorder (CCD) can lead to dramatic crop failures (Cox-Foster et al. 2007). Although CCD is not fully understood, several explanatory models involve gut pathogens such as the microsporidium *Nosema cerana* (Tokarz et al. 2011). These models are debated and more knowledge is needed, for instance, on *N. cerana*'s cycle of infection in CCD (Traver and Fell 2011; Dainat et al. 2012). *Paenibacillus larvae* is another pathogen that has severe effects on honey bees' health. This bacterium invades the guts of larvae causing American Foulbrood disease, which can lead to the demise of the colony (Chan et al. 2011).

The use of a paratransgenic method to promote honey bee health requires knowledge of the bees' "microbiota." The microbiota is the complete assembly of microorganisms in a particular organ or region. Until recently, the microbiota had been considered a black box, especially regarding how microbial species in the gut affect their host, and vice versa. This lack of data was primarily due to limited availability of tools for assessing microbial diversity (Hamady and Knight 2009). Recent technological advancements have shed light on previously unknown microbiota, revealing an important interplay between microorganisms and their hosts (Shi et al. 2010).

## The microbiota

A microbiota is defined as a collection of microorganisms living in a limited region or habitat. For example, all microbes inhabiting the gastrointestinal (GI)-tract of an organism are referred to as a gut microbiota (Fig. 3). The composition of the gut microbiota in its eukaryotic host is not static but subject to dynamic changes during the course of

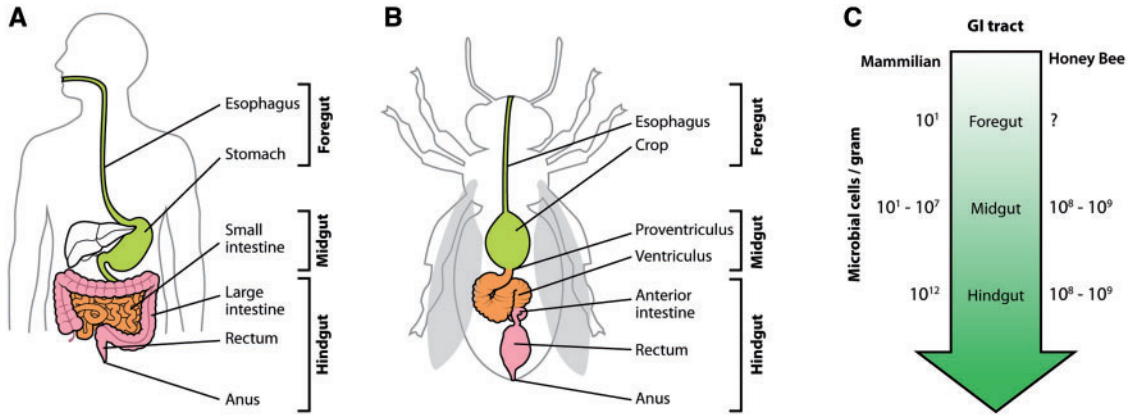


**Fig. 3** Composition of the gastrointestinal (GI)-tracts of humans and honey bees. The human GI tract (**A**) and the honey bee GI tract (**B**) can be generalized into foregut, mid-gut, and hindgut. The foregut functions as a food-storage organ in the form of stomach (A) and crop (B). The small intestine (A) and ventriculus (B) in the mid-gut is the primary site of digestion and of the absorption of nutrients, while the hindgut is the final site of absorption prior to defecation. (**C**) A substantial number of bacteria are found in all segments of the GI tract. The distribution is not homogenous, varying in mammals from $10^1$ bacteria/g in the stomach to $10^{12}$ bacteria/g in the large intestine (Sekirov et al. 2010). Two surveys in honey bees estimated that both the mid-gut and hindgut contained $10^8$–$10^9$ bacteria/g (Rada et al. 1997; Kacaniova et al. 2004).

the host's development or the host's physiological status or health. For instance, *Bifidobacterium* (often referred to as a probiotic) is a predominant bacterium in newborn infants that is found in low numbers in elderly people (Tannock 2010). Antibiotic-associated diarrhea is often caused by compromised gut microflora leading to altered carbohydrate metabolism and an osmotic imbalance in the gut. A disturbed gut microflora is also implicated in heart disease, obesity, and food allergy in humans. Research reveals that the human gut microbiota can interfere with the host's metabolism (Ley et al. 2006); healthy gut microbiota are absent or distorted in many disease-states (Arumugam et al. 2011), and some chronic inflammatory bowel diseases such as Crohn's diseases and ulcerative colitis are associated with distinct changes in the biostructure of the microbiota (Qin et al. 2010).

Animals, including humans (Fig. 3A), are largely dependent on gut microbes to extract nutrients from food and to produce some essential vitamins. Ruminants, for example, rely entirely on their microflora of bacteria, archaea, protozoa, and fungi to digest cellulose. Invertebrates like honey bees (Figs. 3B and 3C) also rely on their microflora. Insects with highly restricted diets containing limited nutrition (e.g., plant sap, nectar, wood, chitin-rich shell) use symbiotic microbiota to assimilate nitrogen and phosphate as well as to produce fatty acids, digestive enzymes, amino acids, and other necessary nutrients (Akman Gunduz and Douglas 2009). The microbiota of insects is commonly less complex than that of vertebrate animals (Dillon and Dillon 2004) and perhaps this is due to the lack of immunological memory (acquired immunity) in insects, leading to an inability to host a complex microbiota (McFall-Ngai 2007). However, such differences between vertebrates and invertebrates could also be partly caused by technical artifacts stemming from greater sampling efforts for vertebrates, especially mammals.

## The microbiota of honey bees

The sugar-loving bacterium *Gluconobacter* was identified as a significant component of the gut microbiota of honey bees as early as the beginning of the 20th century (White 1921). In the 1980s, the principal microbiota of this insect was described as Gram-negative, Gram-positive, and Gram-variable bacteria, and molds and yeast (Gilliam 1997). Up until the turn of the century, however, almost all identifications and quantifications of the honey bee gut microbial assemblage were determined by cultivation-dependent techniques, including selective and specially formulated culture media, different atmospheric conditions, and different taxonomic keys (for an example, see Rada et al. [1997]). Unfortunately, these methods are biased toward culturable microorganisms and thus fail to capture the microbial assemblage accurately.

Over the past decade, some new molecular methods (reviewed by Shi et al. [2010]) have increased our ability to correctly describe microbial assemblages, including that of the honey bee gut. By utilizing

**Table 1** Microorganisms of the honey bee gut

| Alphaproteobacteria | Betaproteobacteria | Gammaproteobacteria | Bacteroidetes | Actinobacteria | Frimicutes |
|---|---|---|---|---|---|
| *Acetobacter* | *Curvibacter* | *Salmonella* | *Pedobacter* | *Corynebacterium* | *Planococcus* |
| *Gluconobacter* | *Cormamonas* | *Serratia* | | *Bifidobacterium* | *Lactobacillus* |
| *Bartonella* | *Janthiobacterium* | *Erwinia* | | *Steptomyces* | *Bacillus* |
| *Gluconacetobacter* | *Ralstonia* | *Actinobacillus* | | *Nocardiopsis* | *Enterococcus* |
| *Saccharibacter* | *Defita* | *Klebsiella* | | | *Brevibacillus* |
| | *Simonsiella* | *Enterobacter* | | | *Stenotrophomonas* |
| | *Neisseria* | *Pantoea* | | | *Staphylococcus* |
| | | *Acinetobacter* | | | |

Divisions and genera of bacteria identified so far from *Apis mellifera* workers and larvae by molecular approaches based on 16s rDNA and metagenomic surveys. Data from Jeyaprakash et al. (2003), Evans and Armstrong (2006), Mohr and Tebbe (2006), Babendreier et al. (2007), Cox-Foster et al. (2007), Olofsson and Vasquez (2008), Crotti et al. (2010), Patil et al. (2010), and Audisio et al. (2011).

16S rDNA (a gene encoding the rRNA involved in the small subunit of the bacterial ribosome), researchers have identified species belonging to the genera *Bifidobacterium* and *Lactobacillus* in honey bee gut extracts (Jeyaprakash et al. 2003; Mohr and Tebbe 2006; Babendreier et al. 2007; Cox-Foster et al. 2007; Olofsson and Vasquez 2008). Despite environmental, geographic, and subspecies-specific differences among honey bees, 95% of the *A. mellifera* microbiota can be assigned to eight characteristic bacterial phylotypes, representing five bacterial classes; Gammaproteo-bacteria, Betaproteobacteria, Alphaproteobacteria, Actinobacteria, and Firmicutes (Martinson et al. 2011), see Table 1 for an overview.

The exact functions of the microbiota of the honey bee gut remain largely unknown, including whether some of the microbiota is indigenous and permanently established or transient and only found for a limited period of time. It is also unclear whether the dominant phylotypes are commensal, parasitic, or mutualistic. The consistent association with a limited number of phylotypes, however, may point toward mutualistic symbiosis (Martinson et al. 2011). Most likely, mutualistic microbes are protective against pathogens and provision nutrients and vitamins. Honey bees require relatively high levels of vitamins, including the vitamin B complex, and gut bacteria represent a likely source of B vitamins (Snyder et al. 2010).

There is now a growing interest in the potentially beneficial antagonistic effect that the gut microbiota have toward honey bees' pathogens (Evans and Armstrong 2006). Nonpathogenic bacteria can stimulate innate immune response in larvae (Evans and Lopez 2004), and bacteria isolated from Argentinean bees can inhibit the pathogen *Ascosphaera apis* (Sabaté et al. 2009). These effects might be driven by competitive exclusion, whereby the pathogens are outcompeted by the normal flora (Saxelin et al. 2005), or production of a range of antimicrobial compounds such as bacterocins, which may protect the gut from pathogenic invasion (Patil et al. 2010; Audisio et al. 2011). Considerable efforts have focused on finding an inhibitory bacterium against the bacterial pathogen *P. larvae*, the causal agent of debilitating and infectious American foulbrood disease (Evans and Armstrong 2006; Yoshiyama and Kimura 2009; Forsgren et al. 2010; Benitez et al. 2012). However, no efforts have so far been directed toward transforming microbes from the microbiota of honey bees to produce paratransgenic tools that can promote gut health and fight disease.

## Honey bee paratransgenesis—a feasible approach

A project that aims to succeed with honey bee paratransgenesis could unfold in three phases. First, one must identify and describe bacteria from the gut that have substantial potential to serve as a transgenic vehicle. This potential must include compatibility with the gut microbiota of honey bees in major ecogeographic regions such as Europe and the United States. During the next phase, the bacterium is transformed with an inert reporter protein, tested, and described for its performance. Measures of performance should include efficient return to the honey bee, appropriate retention time in the gut, lack of interference with the established microflora of the gut or with the health, and survival of the bees. In the final phase, the selected bacterium is transformed with effector genes against honey bee pathogens or used in a functional (paratrans-)genetic approach to produce active metabolites, peptides, or enzymes that promote healthy honey bee physiology or that dissect the phenotypic effects of specific

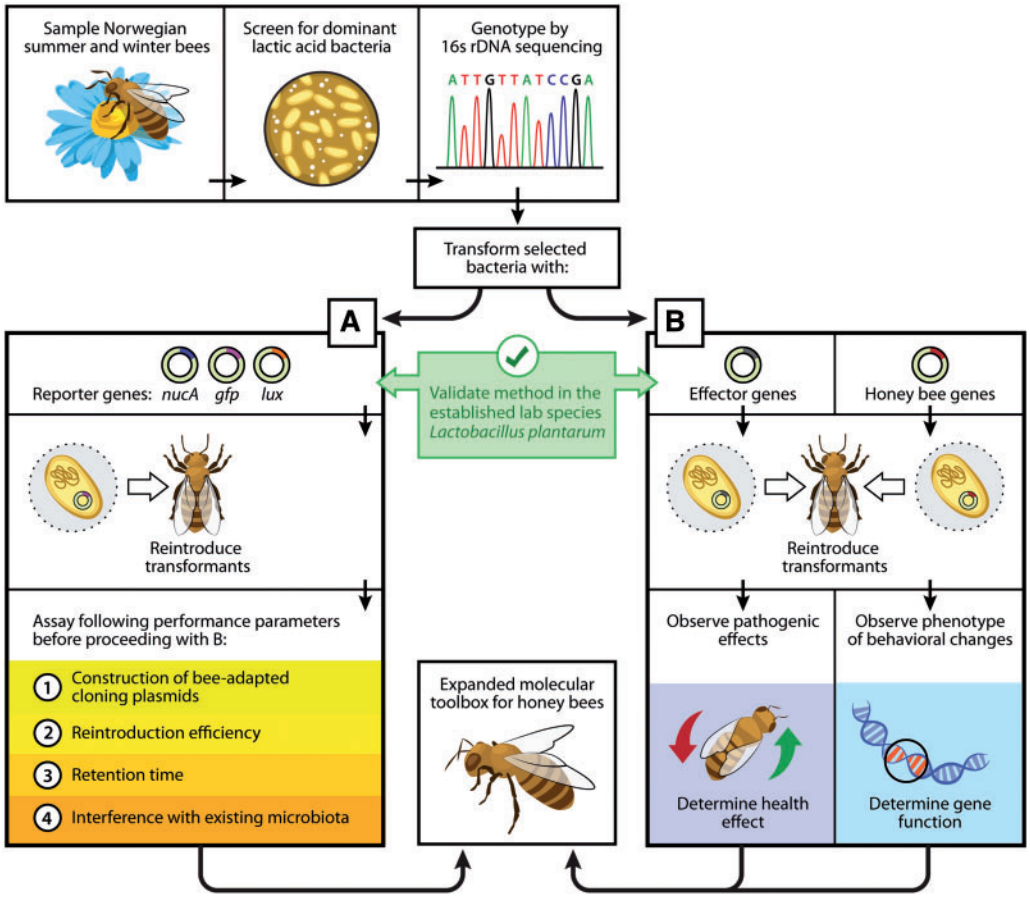


**Fig. 4** Flow-chart for developing a paratransgenic method for honey bees. Honey bee workers from diverse summer and winter environments are screened for dominant lactic acid gut bacteria by selective culture media and by bacterial genotyping. The paratransgenic potential of the selected bacterium is first tested with reporter genes (**A**), and several important performance parameters are assayed before proceeding with the next step (**B**), in which the bacterium is transformed into a paratransgenic delivery vehicle for effector genes against pathogens, or for the production of active metabolites, peptides, or enzymes that can reveal causal connections in the genes, physiology, and behavior of honey bees (B). In our example, the established laboratory bacterium *L. plantarum* is transformed in parallel, with both reporters and functional genes, as a reference species for the development of methods.

honey bee genes (Fig. 4). The full potential of these methods remains to be discovered, but recombinant gut bacteria should be generally well-suited to produce factors that can act in the mid-gut lumen or in the columnar cells that line the lumen of the gut.

Members of the lactic acid bacterial group are appropriate candidates for paratransgenesis. These bacteria are common inhabitants of guts where many of them can have probiotic properties. They are also found in diverse vegetables, meat products, and especially in fermented dairy products, such as yogurt and cheese, where they play a key role in the formation of texture and exert preservative effects, the latter being due to the production of organic acids (lowering pH) and of antimicrobial peptides such as bacteriocins. Lactic acid bacteria are, therefore, "generally regarded as safe" (GRAS) for human consumption (Salminen et al. 1998).

Due to their GRAS status and a sizable molecular tool box available for these organisms, many recent studies have attempted to use lactic acid bacteria as delivery vehicles for therapeutic proteins and a variety of vaccines (Seegers 2002; Bermudez-Humaran et al. 2011). Amongst lactic acid bacteria, the genus *Lactobacillus* has been a preferred delivery vehicle because it is mostly nonpathogenic and robust. The discovery of lactic acid bacteria, including *Lactobacillus,* in the honey bee gut (Olofsson and Vasquez 2008; Audisio et al. 2011), therefore, encourage us to assess them for use as delivery vehicles.

## Development of a *Lactobacillus* vehicle system

The mid-gut (Fig. 3) is the primary site of enzymatic digestion and uptake of nutrients into the hemolymph (blood) that circulates throughout the insect

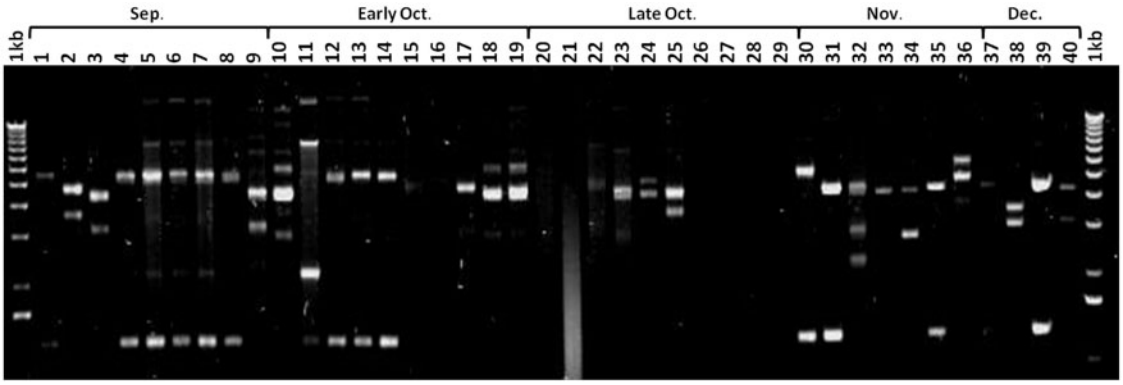


**Fig. 5** The plasmid profile of 40 *L. kunkeei* isolates. Isolates of *L. kunkeei* were purified from the mid-guts of honey bees. The bees were collected between September and December 2010. Each sampling included at least 2 hives from the apiary of the Norwegian University of Life Sciences. The isolates were assessed for plasmid content, which was visualized on 0.8% agarose gels. The 1 kb DNA ladder from Invitrogen was provided at both edges. Highly heterogeneous plasmid content was observed between the isolates, indicating high plasmid diversity.

body. We performed a screening of samples from honey bee mid-guts with growth conditions selective for *Lactobacillus*. Over a period of about 4 months (September to December 2010), more than 200 isolates were genotyped by 16s rDNA sequencing, and about 75% of them were identified as *Lactobacillus kunkeei*. To examine whether the *L. kunkeei* isolates were clonally related or diverse, 40 isolates were randomly chosen for plasmid profiling. We found that the number and size of these plasmids varied greatly. Some isolates contained none, while others carried 5–6 plasmids (Fig. 5). This pattern suggests that the *L. kunkeei* of the honey bee gut represents a relatively heterogeneous group.

*Lactobacillus kunkeei* is described as a fructophilic bacterium (i.e., fructose is its preferred carbon source, Endo et al. [2012]). This bacterium has been identified in honey bees before (Olofsson and Vasquez 2008) and is also found during wine fermentation and on flowers (Edwards et al. 1998; Endo et al. 2009; Endo et al. 2012). Our *L. kunkeei* isolates may therefore originate from nectar and/or pollen grains collected and ingested by the bees. Pollen is the bee's major source of proteins, lipids, vitamins, and minerals, whereas nectar is their main source of energy. Most nectars are dominated by a limited number of sugars: sucrose, glucose, and fructose (Nicolson 2011). Using the API50 CHL system from BioMèrieux that assesses the fermentation of 49 different sugars, we found that the 40 *L. kunkeei* isolates (Fig. 5) fermented only 4 to 7 carbohydrates (Table 2). The strongest activity was measured on fructose and disaccharide sucrose that is composed of one unit glucose and one unit fructose, corresponding to major constituents in nectar.

To our knowledge, genetic manipulation on *L. kunkeei* has not been attempted before. For transformation, several isolates of *L .kunkeei* were made electro-competent using a growth medium containing a higher concentration of glycine to weaken the bacterial cell wall, a protocol that has been applied successfully to other lactic acid bacteria (Aukrust et al. 1995). A set of isolates were transformed with a broad-spectrum plasmid containing a replicon derived from the lactococcal plasmid pSH71 (de Vos 1987; Sorvig et al. 2005). For heterologous gene expression, we used the secretion signal of a protein (annotated as lp_3050) from the genome-sequenced *L. plantarum* WCFSI strain to drive secretion of the reporter protein nuclease A (encoded by *nucA*) (Mathiesen et al. 2009). Our transformed *L. kunkeei* secreted this reporter protein in both low and high sugar containing growth media. Transformed bacteria were fed back to individual bees in the laboratory, and we could recover a substantial number of transformants from mid-gut samples 24 h after feeding. Presence of the transformed *nucA* plasmid was confirmed by PCR. These observations suggest that *L. kunkeei* provides a basis for the development of paratransgenic methods in honey bees (Fig. 4, detailed data to be reported elsewhere).

## Future work

The genetic tool box for *L. kunkeei* must be expanded. This work will include more effective systems for plasmid replication and the construction of new reporter systems, including visual markers such as green fluorescent protein or luciferase that can be

**Table 2** Metabolic profiling of 40 *L. kunkeei* isolates using the API50 CHL system from BioMèrieux

| Isolate | D-glucose | D-fructose | Mannitol | Sucrose | Trehalose | Rafinose | Potassium-gluconat |
|---|---|---|---|---|---|---|---|
| 1 | + | ++ | + | ++ | ++ | − | + |
| 2 | + | ++ | + | ++ | + | − | + |
| 3 | + | ++ | w | ++ | − | − | + |
| 4 | + | ++ | + | ++ | + | − | + |
| 5 | + | ++ | w | ++ | ++ | − | + |
| 6 | ++ | ++ | w | ++ | ++ | − | + |
| 7 | + | ++ | w | ++ | ++ | − | + |
| 8 | + | ++ | + | ++ | + | − | + |
| 9 | + | ++ | + | ++ | + | − | + |
| 10 | + | ++ | + | ++ | + | − | + |
| 11 | + | ++ | + | ++ | ++ | − | + |
| 12 | + | ++ | + | ++ | + | − | + |
| 13 | + | ++ | w | ++ | − | − | − |
| 14 | ++ | ++ | + | ++ | ++ | − | + |
| 15 | ++ | ++ | + | ++ | ++ | − | + |
| 16 | ++ | ++ | + | ++ | ++ | − | + |
| 17 | + | ++ | − | ++ | + | − | + |
| 18 | + | ++ | − | ++ | + | − | + |
| 19 | + | ++ | − | ++ | + | − | + |
| 20 | ++ | ++ | w | ++ | + | − | + |
| 21 | + | ++ | + | ++ | w | − | + |
| 22 | ++ | ++ | + | ++ | ++ | − | + |
| 23 | + | ++ | + | ++ | ++ | + | + |
| 24 | + | ++ | + | ++ | ++ | − | + |
| 25 | + | ++ | + | ++ | ++ | − | + |
| 26 | + | ++ | − | ++ | + | − | w |
| 27 | ++ | ++ | + | ++ | + | + | + |
| 28 | + | ++ | + | ++ | ++ | w | + |
| 29 | + | ++ | + | ++ | ++ | w | + |
| 30 | + | ++ | w | ++ | + | − | w |
| 31 | + | ++ | + | ++ | + | − | + |
| 32 | + | ++ | + | ++ | + | − | + |
| 33 | + | ++ | + | ++ | ++ | w | + |
| 34 | + | ++ | + | ++ | ++ | − | + |
| 35 | + | ++ | + | ++ | ++ | − | + |
| 36 | + | ++ | w | ++ | + | − | w |
| 37 | + | ++ | + | ++ | ++ | − | + |
| 38 | + | ++ | + | ++ | + | − | + |
| 39 | + | ++ | − | ++ | + | − | + |
| 40 | + | ++ | + | ++ | ++ | w | + |

This panel tests 49 different carbohydrates, but only seven of these produced positive fermentation reactions in our experiment (listed): "++" indicates strong positive reaction, "+" intermediate, "w" weak, and "−" no observable reaction.

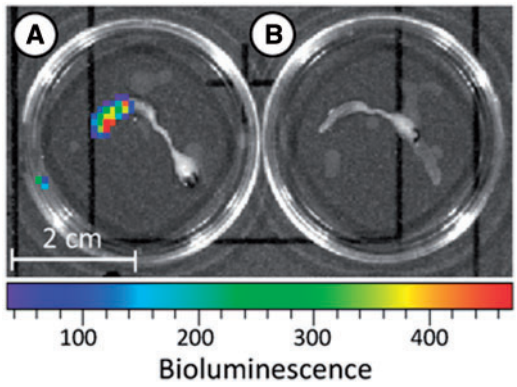


**Fig. 6** Transformed bacteria imaged through the wall of the honey bee gut. We used *Enterococcus* transformed with a plasmid containing the *luxABCDE* operon, which encodes luminescent luciferase, to determine the reporter potential of luminescence light. The bacterium was grown in GM17 medium until the optical density of ~0.5 was reached. Ten microliters of the bacterial suspension was subsequently fed to individual honey bees. Bees fed with 10 µl GM17 medium only, i.e., without luminescent bacteria, were included as a control. *Enterococcus* was not originally isolated from the honey bee gut, and its retention time is short in bees. Thus, the gut was removed after 1 h and assayed for bioluminescence using the IVIS® Lumina Imaging System from Caliper (Binding: M, exposure time: Auto, f/stop: 1). Emitted light was detected through the gut wall of bees fed with transformed *Enterococcus* (**A**) and was absent in the control (**B**). We conclude that luminescent light has reporter potential in the honey bee gut. It can be worthwhile to transfer this system to a more appropriate vector, such as *L. kunkeei*.

monitored through the gut wall (Fig. 6). These aids are requirements for future work with effector genes.

Moreover, although the honey bee is an increasingly important model for studies of behavior, neurobiology, and aging, few methods are available for studying its functional genetics. RNA interference technology is currently the only tool for gene silencing, and no method is available for gene over-expression (reviewed by Amdam [2011]). In principle, a transformed gut bacterium may not only provide a system for fighting disease but it can also deliver proteins and enzymes that test functional genetic hypotheses. This makes the development of honey bee paratransgenesis all the more important (Fig. 4).

Finally, a field that has not yet been widely explored is the transmission and persistence of the microbiota in populations. For humans, it has been suggested that the increase in immunological disorders such as asthma and allergy can partly be attributed to the loss of beneficial bacteria within populations, although specific mechanisms have not been derived (Blaser and Falkow 2009). Social insects such as honey bees represent a particularly attractive model for investigating the dispersal of the microbiota within populations in relation to health and disease. Like humans, honey bees have evolved hygienic behavior that limit and prevent the spread of pathogens (Evans and Spivak 2010), but unlike humans, honey bees may have evolved mechanisms that secure the selective transmission of beneficial bacteria (Evans and Armstrong 2006). Understanding these mechanisms in honey bees could facilitate the development of strategies for preventing the loss of beneficial bacteria in human populations.

## Acknowledgments

We thank Kirsten Traynor, Heli Havukainen, Marije Oostindjer, and Øyvind Halskau for helpful comments on the manuscript. Sabine Deviche made invaluable contributions to figures and graphic design.

## Funding

This work was supported by the Research Council of Norway (to G.V.A.) and the Norwegian University of Life Sciences (to A.R.). The SICB 2012 symposium was generously supported by a grant from the National Science Foundation Division of Integrated Organismal Systems (IOS# 1153657).

## References

Akman Gunduz E, Douglas AE. 2009. Symbiotic bacteria enable insect to use a nutritionally inadequate diet. Proc Biol Sci 276:987–91.

Amdam GV. 2011. Social context, stress, and plasticity of aging. Aging Cell 10:18–27.

Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto JM, et al. 2011. Enterotypes of the human gut microbiome. Nature 473:174–80.

Audisio MC, Torres MJ, Sabate DC, Ibarguren C, Apella MC. 2011. Properties of different lactic acid bacteria isolated from *Apis mellifera* L. bee-gut. Microbiol Res 166:1–13.

Aukrust TW, Brurberg MB, Nes IF. 1995. Transformation of *Lactobacillus* by electroporation. Methods Mol Biol 47:201–8.

Babendreier D, Joller D, Romeis J, Bigler F, Widmer F. 2007. Bacterial community structures in honeybee intestines and their response to two insecticidal proteins. FEMS Microbiol Ecol 59:600–10.

Benitez LB, Velho RV, de Souza da Motta A, Segalin J, Brandelli A. 2012. Antimicrobial factor from *Bacillus amyloliquefaciens* inhibits *Paenibacillus larvae*, the causative agent of American foulbrood. Arch Microbiol 194:177–85.

Bermudez-Humaran LG, Kharrat P, Chatel JM, Langella P. 2011. Lactococci and lactobacilli as mucosal delivery vectors for therapeutic proteins and DNA vaccines. Microb Cell Fact 10(Suppl 1):S4.

Bisi DC, Lampe DJ. 2011. Secretion of anti-*Plasmodium* effector proteins from a natural *Pantoea agglomerans* isolate by using PelB and HlyA secretion signals. Appl Environ Microbiol 77:4669–75.

Blaser MJ, Falkow S. 2009. What are the consequences of the disappearing human microbiota? Nat Rev Microbiol 7:887–94.

Chan QW, Cornman RS, Birol I, Liao NY, Chan SK, Docking TR, Jackman SD, Taylor GA, Jones SJ, de Graaf DC, et al. 2011. Updated genome assembly and annotation of *Paenibacillus larvae,* the agent of American foulbrood disease of honey bees. BMC Genomics 12:450.

Coutinho-Abreu IV, Zhu KY, Ramalho-Ortigao M. 2010. Transgenesis and paratransgenesis to control insect-borne diseases: current status and future challenges. Parasitol Int 59:1–8.

Cox-Foster DL, Conlan S, Holmes EC, Palacios G, Evans JD, Moran NA, Quan PL, Briese T, Hornig M, Geiser DM, et al. 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318:283–7.

Crotti E, Rizzi A, Chouaia B, Ricci I, Favia G, Alma A, Sacchi L, Bourtzis K, Mandrioli M, Cherif A, et al. 2010. Acetic acid bacteria, newly emerging symbionts of insects. Appl Environ Microbiol 76:6963–70.

Dainat B, Evans JD, Chen YP, Gauthier L, Neumann P. 2012. Predictive markers of honey bee colony collapse. PLoS One 7:e32151.

de Vos WM. 1987. Gene cloning and expression in lactic streptococci. FEMS Microbiol Rev 46:281–95.

Dillon RJ, Dillon VM. 2004. The gut bacteria of insects: nonpathogenic interactions. Annu Rev Entomol 49:71–92.

Durvasula RV, Gumbs A, Panackal A, Kruglov O, Aksoy S, Merrifield RB, Richards FF, Beard CB. 1997. Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. Proc Natl Acad Sci USA 94:3274–8.

Edwards CG, Haag KM, Collins MD, Hutson RA, Huang YC. 1998. *Lactobacillus kunkeei* sp. nov.: a spoilage organism associated with grape juice fermentations. J Appl Microbiol 84:698–702.

Endo A, Futagawa-Endo Y, Dicks LM. 2009. Isolation and characterization of fructophilic lactic acid bacteria from fructose-rich niches. Syst Appl Microbiol 32:593–600.

Endo A, Irisawa T, Futagawa-Endo Y, Takano K, du Toit M, Okada S, Dicks L. 2012. Characterization and emended description of *Lactobacillus kunkeei* as a fructophilic lactic acid bacterium. Int J Syst Evol Microbiol 62:500–4.

European Parliament. 2011. Report on honeybee health and the challenges of the beekeeping sector. Committee on Agriculture and Rural Development (http://www.europarl.europa.eu/sides/getDoc.do?type=REPORT&reference=A7-2011-0359&format=XML&language=EN).

Evans JD, Armstrong TN. 2006. Antagonistic interactions between honey bee bacterial symbionts and implications for disease. BMC Ecology 6:4.

Evans JD, Lopez DL. 2004. Bacterial probiotics induce an immune response in the honey bee (Hymenoptera: Apidae). J Econ Entomol 97:752–6.

Evans JD, Spivak M. 2010. Socialized medicine: individual and communal disease barriers in honey bees. J Invertebr Pathol 103(Suppl 1):S62–72.

Fang W, Vega-Rodriguez J, Ghosh AK, Jacobs-Lorena M, Kang A, St Leger RJ. 2011. Development of transgenic fungi that kill human malaria parasites in mosquitoes. Science 331:1074–7.

Forsgren E, Olofsson TC, Vasquez A, Fries I. 2010. Novel lactic acid bacteria inhibiting *Paenibacillus larvae* in honey bee larvae. Apidologie 41:99–108.

Frisch KV. 1967. The dance language and orientation of bees. Cambridge (MA): Harvard University Press.

Gilliam M. 1997. Identification and roles of non-pathogenic microflora associated with honey bees. FEMS Microbiol Lett 155:1–10.

Hamady M, Knight R. 2009. Microbial community profiling for human microbiome projects: tools, techniques, and challenges. Genome Res 19:1141–52.

Jeyaprakash A, Hoy MA, Allsopp MH. 2003. Bacterial diversity in worker adults of *Apis mellifera capensis* and *Apis mellifera scutellata* (Insecta: Hymenoptera) assessed using 16S rRNA sequences. J Invertebr Pathol 84:96–103.

Kacaniova M, Chlebo R, Kopernicky M, Trakovicka A. 2004. Microflora of the honeybee gastrointestinal tract. Folia Microbiol 49:169–71.

Ley RE, Turnbaugh PJ, Klein S, Gordon JI. 2006. Microbial ecology: human gut microbes associated with obesity. Nature 444:1022–3.

Martinson VG, Danforth BN, Minckley RL, Rueppell O, Tingek S, Moran NA. 2011. A simple and distinctive microbiota associated with honey bees and bumble bees. Mol Ecol 20:619–28.

Mathiesen G, Sveen A, Brurberg MB, Fredriksen L, Axelsson L, Eijsink VG. 2009. Genome-wide analysis of signal peptide functionality in Lactobacillus plantarum WCFS1. BMC Genomics 10:425.

McFall-Ngai M. 2007. Adaptive immunity: care for the community. Nature 445:153.

Menzel R, Leboulle G, Eisenhardt D. 2006. Small brains, bright minds. Cell 124:237–9.

Mohr KI, Tebbe CC. 2006. Diversity and phylotype consistency of bacteria in the guts of three bee species (*Apoidea*) at an oilseed rape field. Environ Microbiol 8:258–72.

Nicolson SW. 2011. Bee food: the chemistry and nutritional value of nectar, pollen and mixtures of the two. African Zool 46:197–204.

Olofsson TC, Vasquez A. 2008. Detection and identification of a novel lactic acid bacterial flora within the honey stomach of the honeybee *Apis mellifera*. Curr Microbiol 57:356–63.

Patil PB, Zeng Y, Coursey T, Houston P, Miller I, Chen S. 2010. Isolation and characterization of a *Nocardiopsis* sp. from honeybee guts. FEMS Microbiol Lett 312:110–8.

Pittman GW, Brumbley SM, Allsopp PG, O'Neill SL. 2008. Assessment of gut bacteria for a paratransgenic approach to control *Dermolepida albohirtum* larvae. Appl Environ Microbiol 74:4036–43.

Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T, et al. 2010. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464:59–65.

Rada V, Machova M, Huk J, Marounek M, Duskova D. 1997. Microflora in the Honeybee digestive tract: counts, characteristics and sensitivity to veterinary drugs. Apidologie 28:357–65.

Ren X, Hoiczyk E, Rasgon JL. 2008. Viral paratransgenesis in the malaria vector *Anopheles gambiae*. PLoS Pathogens 4:e1000135.

Robinson AS, Franz G, Atkinson PW. 2004. Insect transgenesis and its potential role in agriculture and human health. Insect Biochem Mol Biol 34:113–20.

Sabaté DC, Carrillo L, Audisio MC. 2009. Inhibition of *Paenibacillus larvae* and *Ascosphaera apis* by *Bacillus subtilis* isolated from honeybee gut and honey samples. Res Microbiol 160:193–99.

Salminen S, von Wright A, Morelli L, Marteau P, Brassart D, de Vos WM, Fonden R, Saxelin M, Collins K, Mogensen G, et al. 1998. Demonstration of safety of probiotics—a review. Int J Food Microbiol 44:93–106.

Saxelin M, Tynkkynen S, Mattila-Sandholm T, de Vos WM. 2005. Probiotic and other functional microbes: from markets to mechanisms. Curr Opin Biotechnol 16:204–11.

Seegers JF. 2002. Lactobacilli as live vaccine delivery vectors: progress and prospects. Trends Biotechnol 20:508–15.

Seeley TD. 1995. The Wisdom of the Hive. Cambridge (MA): Harvard University Press.

Sekirov I, Russell SL, Antunes LC, Finlay BB. 2010. Gut microbiota in health and disease. Physiol Rev 90:859–904.

Shi WB, Syrenne R, Sun JZ, Yuan JS. 2010. Molecular approaches to study the insect gut symbiotic microbiota at the 'omics' age. Insect Sci 17:199–219.

Snyder AK, Deberry JW, Runyen-Janecky L, Rio RV. 2010. Nutrient provisioning facilitates homeostasis between tsetse fly (Diptera: Glossinidae) symbionts. Proc Biol Sci 277:2389–97.

Sorvig E, Mathiesen G, Naterstad K, Eijsink VG, Axelsson L. 2005. High-level, inducible gene expression in *Lactobacillus sakei* and *Lactobacillus plantarum* using versatile expression vectors. Microbiology 151:2439–49.

Tannock GW. 2010. Analysis of bifidobacterial populations in bowel ecology studies. In: Mayo B, editor. Bifidobacteria. Genomics and molecular aspects. Norfolk: Caister Academic Press. p. 1–15.

Tokarz R, Firth C, Street C, Cox-Foster DL, Lipkin WI. 2011. Lack of evidence for an association between Iridovirus and colony collapse disorder. PLoS One 6:e21844.

Traver BE, Fell RD. 2011. Prevalence and infection intensity of *Nosema* in honey bee (*Apis mellifera* L.) colonies in Virginia. J Invertebr Pathol 107:43–9.

van Engelsdorp D, Meixner MD. 2010. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. J Invertebr Pathol 103:S80–95.

White PB. 1921. The normal bacterial flora of the bee. J Pathol Bacteriol 24:64–78.

Wilson EO. 1971. The insect societies. Cambridge (MA): Belknap Press of Harvard University Press.

Yoshiyama M, Kimura K. 2009. Bacteria in the gut of Japanese honeybee, *Apis cerana japonica*, and their antagonistic effect against *Paenibacillus larvae*, the causal agent of American foulbrood. J Invertebr Pathol 102:91–96.