Exploiting gut microbial diversity of honey bees of temperate climate of Kashmir valley

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Abstract: Invertebrates host numerous micro-organisms with interactions ranging from symbiosis to pathogenesis. The digestive tract of insect represents a large source of yet unexplored microbial diversity. These micro-organisms utilize a wide range of organic polymers and can be involved in methanogenesis and nitrogen fixation .The gut microflora also play significant part in pheromone production, degradation of pesticide, vitamin synthesis and pathogen prevention. Microbial communities in insect intestine have been studied by cultivation dependant techniques; however these methods do not reflect entire communities. This diversity is attributed to different feeding habits resulting in different gut structures and functions and promotes the establishment of different phylotypes. The focus of the study is to provide comprehensive data regarding micro-flora harboring honey bees of temperate climate.

[Bhat SA, Nazir R, Shah FA, Bhat GA. **Exploiting gut microbial diversity of honey bees of temperate climate of Kashmir valley.** *Nat Sci* 2014;12(6):105-110]. (ISSN: 1545-0740). http://www.sciencepub.net/nature. 15

Key Words: Pathogenesis, symbiosis, microflora, honeybees, phylotypes.

Introduction

Animals living in social communities typically harbor a characteristic gut microbiota important for nutrition and pathogen defense. Accordingly, in the gut of the honey bee, *Apis mellifera*, a distinctive microbial community, composed of a taxonomically restricted set of species specific to social bees, has been identified. Despite the ecological and economical importance of honey bees and the increasing concern about population declines, the role of their gut symbionts for colony health and nutrition is unknown. Agriculture is the lifeline of economy for the people of Jammu and Kashmir. Majority of the population in villages earn their livelihood from agriculture and other allied sectors like apiculture, sericulture, pisciculture etc. All these practices serve as the backbone of the GDP (gross domestic product) of the state. As a pollinator, the honey bee, Apis is a key species for agricultural production and contributes significantly to the human food supply.

As the health of honey bees and the quality of honey is affected by many microbes, thus there is the need of isolating and identifying these microbes so as to find out the means of controlling them. Insect gut micro biota plays essential role in the growth, development, pathogenesis and environmental adaptation of host insects. The molecular and systems level analysis of insect gut symbiotic microbial community will allow us to discover novel biocatalysts for biomass deconstruction and to develop innovative strategies for their management. In many animals, the gut microbial community, in particular, confers functions related to nutrition and susceptibility to disease and thus might also play an important role in the health and resilience of honey bees.

Viruses, fungi, protozoa and bacteria are all known to cause infections in bees, sometimes leading to collapse of colonies, and causing serious threats to the beekeeping industry (Glinski, and Buczek, 2003). Composition of the digestive microflora of honey bees is the result of feeding pollen and nectar, but also it is a consequence of interaction among the bees in the hive (Glinski and Jarosz, 1995). In adult bees it consists of Gram positive, Gram-negative and Gram-variable bacteria, fungi and in some circumstances, yeasts. Apicultural economic development strongly relies on the health status of honey bee colonies. Honey bees face many diseases and consequently rely on a diverse set of individual and group-level defenses to prevent disease. One route by which honeybees and other insects might combat disease is through the shielding effects of their microbial symbionts. The intestinal flora of most organisms plays a crucial role in nutrient assimilation and immune function. So far, most studies on honeybee microflora have focused on disease causing microorganisms (Alippi et al., 2002); while much less emphasis has been given to non-pathogenic microorganisms and their potential benefit for individual bees or whole colonies. However, there is growing awareness of the importance of the composition of the intestinal micro-flora for health and growth of honeybees (Gilliam, 1979; Gilliam et al., 1988a; Gilliam et al., 1997; Dillon & Dillon, 2004).

In recent years there has been renewed interest in the understanding of insect gut microorganisms for two reasons. First, this diverse microbiota is a

potential source of novel bioactive compounds such as antimalarial, antiviral and antitumour peptides (Chernysh *et al* 2002), enzymes (Zhang and Brune 2004) and novel metabolites (Wilkinson 2001). Second, manipulating these microbial symbionts is thought to be an effective strategy for controlling the spread of pathogens that use insects as hosts (Ferguson 1961; Lehane *et al* 1997; Beard *et al* 2002; Dillon *et al* 2005).

Systemic overview of Literature

Honey bees are greatly valued as honey producers for human consumption and even more importantly, as pollinators in natural and agricultural ecosystems. However, there is clear evidence of recent declines in both wild and domestic pollinators, including honey bees (Potts *et al.*, 2010). The suggested reason for this phenomenon include changes in land use, agrochemicals, pathogens, foreign species, climate change, and the interactions between them. Thus, research on honeybee health is increasing, especially with respect to both the beneficial and harmful symbionts harbored by honeybees, including bacteria, fungi, yeasts, protozoans, mites, and viruses (Gilliam, 1997; Cox-Foster *et al.*, 2007). The gut bacteria of insects are known to be essential for host nutrition and defense against pathogens (Dillon and Dillon**,** 2004), and therefore, it is thought that the health status of the host can be monitored by observing the microbial communities in the guts, as revealed by the human gut microbiome studies (Turnbaugh *et al.*, 2008). Recent studies have shown that several characteristic bacterial groups occupy most of the bacterial communities in the guts of honey bees and bumble bees (Mohr and Tebbe, 2006; Koch and Schmid-Hempel, 2011; Martinson *et al.*, 2011), and some of the groups may contribute to the host defense against known bee pathogens (Koch and Schmid-Hempel, 2011). The simple bacterial communities in the honey bee guts contrast with those in the human guts (Rajilić-Stojanović *et al.*, 2007), and this may be attributed to their simple food requirements of nectar and pollen (Winston, 1987). The gut microbiota represents all aspects of microbial relationships, from pathogenic to obligate mutualism. Earlier emphasis on studies of insect-bacteria symbiosis was replaced by a drive to study insect–microbial pathogen relationships and produce microbial insecticides. The recent upsurge in research into endosymbionts such as Wolbachia and Buchnera spp. has moved the focus away from the pathogenic relationship and re-emphasized the extent to which microbes have integrated with insects.

Life stages and associated micro-organisms

Bees have two distinct life forms, brood (egg, larva and pupal stages which develop within the hive) and adult. Most diseases are specific to just one of these life stages. While the list of diseases is quite long, only a few are of serious concern to apiculturists. Viruses, fungi, protozoa and bacteria are all known to cause infections in bees, sometimes leading to collapse of colonies, and causing serious threats to the beekeeping industry (Glinski and Buczek, 2003). These studies were carried out over three years and included different developmental stages. There were substantial qualitative as well quantitative differences in the microbial types depending on the species, developmental stage and the diet. *Apis mellifera* adults predominantly contained *Lactobacilus* where as larval SSCP patterns had a predominance of bands corresponding to *Salmonella enterica* var *typhi*, uncultured *Simonesiella* and uncultured *Serratia*. This is presumably because the food source for forager bees (honey and nectar) has a low pH of approximately 3.9 and lactobacilli can tolerate this pH. The pH of larval gut is around 7 and is less favourable for Lactobacilli. On the other hand, the gut from the larvae of solitary bee *O. bicornis* showed SSCP patterns quite different from the other two species, which could be due to different social habit and also difference in development. The gut of this species opens during the early development of the larvae whereas for the other two species it opens much later, just before pupation. This would result in differences in physicochemical conditions and thus differences in the microbiota.

Table 1: Micro-organisms commonly associated with bee comb, body and gut of honeybee

S. No	Bacteria	Viruses	Moulds
1	Staphylococcus sp.	Slow paralysis virus	Mucor sp.
2	Streptobaccillus sp.	Chronic paralysis associate virus	Penicillium sp.
3	Baccillus sp.	Egypt bee virus	Aspergillus sp.
4	*Paenibacillus alvei*	Deformed wing virus	Rhizopus sp.
5	*Melissococcus pluton*	Thai sacbrood virus	Geotrichums sp.
6	*Paenibacillus larvae larvae*	Kashmir bee virus	Botryotricum sp.
7	Pseudomonas aeruginosa		*Aspergillus* spp.
8	Achromobacter euridice		*Ascosphaera apis*
9	Enterococcus facecalis		*Nosema apis*
10			*Nosema ceranae*

Pattabhiramaiah et al., (2012) carried out detection of novel probiotic bacterium Lactobacillus spp. in the workers of Indian honeybee, Apis cerana indica collected from different parts of Karnataka, India which play a very significant role in the general health maintenance of the host. Total bacterial genomic DNA was extracted from the midguts of the worker honey bee sub species Apis cerana indica, collected from different parts of Karnataka and amplified using PCR, with 16S rRNA primers. The amplified PCR products were purified and sequenced directly. This partial, 16Rdna sequences from Apis cerana indica revealed the presence of novel bacterial flora composed of lactic acid bacteria (LAB), which originated in the honey stomach of the Indian honeybee. The findings of Mahesh et al., (2007) indicate that two microbial genera, Lactobacillus and Wolbachia, were predominantly present in significant numbers in the midgut of Apis mellifera carnica.

Nowadays genetic tools are available for each of the major honey bee pathogens and these tools offer new avenues for screening bees and colonies to predict ailments and causes of colony declines. In addition, the sequencing of the honey bee genome (Honey Bee Genome Sequencing Consortium, 2006) and associated efforts to define the genetic and protein makeup of bees have generated informative genetic tags for bee proteins involved with development, immunity, physiology, and behavior. These resources for bees and their disease agents can be exploited in order to improve bee breeding schemes, manage diseases or bee nutrition, and regulate the movement of viruses, bacteria, fungi, and other infectious agents.

Ajao A.M and Babatunde S. K (2013) carried out the isolation and identification of microorganisms in comb and body parts of wild and domesticated honey bees of two ecozones of Nigeria. Thirty honeybees were collected (10 each from the wild, modern and traditional beekeeping sites) using sweep nets and the bees were killed with chloroform. The body and the bee comb were swabbed using sterile normal saline moistened swab stick. The swabs were diluted and homogenized bees from each of the habitats and passed through microbial studies. The body of each bee was disinfected by swabbing with iodine followed by 70% ethanol to avoid contamination of the gut with external microbes the dissection of the bee gut was carried out with sterile scissors following the method previously described by Youdeowei (1974). Like all other invertebrate, insects are always dissected from the dorsal side, this is because the nerve cord lies ventrally and this exposes the required internal parts. For the dissection to expose the gut, the parts were dismembered to make observation and subsequent culturing easier. The contents of the fore, mid and hind guts were emptied using flame sterilized forceps. The contents were homogenized separately and culture on appropriate media. One ml of homogenized sample was diluted in 9 ml of sterile normal saline. From this, four sterile dilutions were further made to give. 1/201/401/80 and 1/160 1.0 ml each of dilutions which was inoculated into molten sabourad dextrose agar (SDA) containing tetracycline for bacteria growth, while nutrient agar and De Ma Ro (MRS) agar were inoculated for the bacteria growth. SDA plates were incubated at 30oC for 3 day while NA and MRA were incubated at 37oC for 3 days. The plates were prepared in duplicates. The plates were examined De Ma Rogossa Sharbroth (MRS) day for growth. These media were prepared following the manufacturer instructions. The total number of colonies forming units (CFU) per plate was counted using colony counter and was correlated to dilution factor. Each different colony after counting were sub-cultured to obtain pure culture and were identified using morphological and biochemical methods as previously described (Cowenand Steel,1998). Colony forming unit (CFU) growth on SDA was counted, sub-cultured on fresh new SDA and identified using staining morphology and bio chemical tests (Cheesbough, 1987; Mahon and Manuselis).

The microbial examination of the comb, body and gut of the bees showed the presence of six fungi and three bacteria species in the three habitats in both ecological zones. The species of the mould encountered were *Mucor hiemalis, Penincillium frequentans, Aspergilusrepens, Rhizopus stolonifer, Geotrichums sp.* and*Botryotricum sp*. while *Staphylococcus aureus, Streptobaccillus sp.* and *Baccillus pulyifacien* were thebacteria encountered.

Independent studies of bacterial community profiles based on 16S rRNA sequences show that workers of *A. mellifera* and some *Bombus* species consistently harbor a distinctive gut microbiota not shared with solitary bees (Cox-Foster, Koch H, Schmid-Hempel P, Martinson VG). This microbiota consists of eight distinct species or phylotypes (i.e., closely related strains with ≥97% sequence identity in 16S rRNA sequences, hereafter referred to as species): three Gram-positive species (two closely related Firmicutes within *Lactobacillus* and one within *Bifidobacterium*) and five Gram-negative species (one β-proteobacterium with the *Candidatus* name "*Snodgrassella alvi*," two closely related γ-proteobacteria, one with the *Candidatus* name "*Gilliamella apicola*," and two α-proteobacteria) (Martinson VG, Moy J, Moran NA).

The reports of Audisio & Benítez-Ahrendts (2011) revealed that Lactobacillus johnsonii isolated from Apis mellifera L. bee-gut exhibited a beneficial

effect on honeybee colonies. Tobias et al (2008) detected the novel probiotic Lactobacillus spp. in the stomach of the honeybee Apis mellifera and also from the fresh honey from Sweden. Jeyapraksh et al (2003) established the presence of Lactobacillus sp in the worker adults of Apis mellifera capensis and Apis mellifera scutellata assessed using 16SrRNA sequences. The findings of Mahesh et al (2007, 2011) indicate that two microbial genera, Lactobacillus and Wolbachia, were predominantly present in significant numbers in the midgut of Apis mellifera carnica. Lactobacilli are important for the maintenance of the intestinal microbial ecosystem (Sandine, 1979). Species of Lactobacillus form the most numerous genus in the heterogeneous group of Lactic Acid Bacteria (LAB). The members of the genus Lactobacillus are important residents of the gastrointestinal (GI) microbiota and have been subjects of increasing interest due to their possible role in the maintenance of GI health. Because of this positive health promoting properties, Lactobacillus species are widely used as probiotics (Ouwehand et al., 2002).

Mohr and Tebbe (2006) compared the gut communities of three species of bees using a culture independent approach. Here, the SSCP technique was used for community dynamics analysis and the relevant bands were sequenced to determine the phylogenetic affiliations of uncultured gut bacteria. The bee species used in the study can co-exist in the same habitat but had different social behavior and feeding habits. In Apis mellifera (the European honey-bee), workers have mouth-to-mouth contact with larvae and with each other. Adult forager bees use nectar as a food source; the larvae are first fed on secretions from the hypopharyngeal gland and later on the secretion is mixed with pollen and nectar. In Bombus terrestris (the bumble-bee), adult workers have only indirect contact with larvae and each other by feeding on the same food, which has been collected and mixed with nectar by forager bees. In the third species, Osmia bicornis (red mason bee) is a typical solitary bee. The female lays eggs on a stored mass of pollen, separates them with mud and dies before the eggs hatch. The emerged larvae feed on the stored pollen and thus there is no direct feeding contact between larvae and adults at all.

Puerta et al reported that the fungal species Ascosphaera apis causes chalkbrood or ascosphaeriosis (Apis mellifera) in Spain. Chalkbrood probably occurs in all the countries of the Mediterranean, although it has not been reported to be present in Portugal, Algeria, Albania and Morocco (Bradbear, 1988). Until 1968, it was considered to be a primarily European disease, but in 1971 it became recognized as economically important in the USA (Hitchcock and Christensen, 1972). It can be assumed that the disease is distributed worldwide.

White (1912) described that European foulbrood disease is caused by Bacillus pluton. The name was later modified to Melissococcus plutonius (Truper & dé Clari, 1998). Cai and Collins (1994) made a comparative sequence analysis revealing that M. plutonius is a close phylogenetic relative of the genus Enterococcus. Furthermore, isolates of M. plutonius are remarkably homogenous based on morphological, physiological and immunological (Allen & Ball, 1993; Bailey & Gibbs, 1962) as well as genetic studies (Djordjevic et al., 1999; Dancer & Barnes, 1995).

Conclusion and future prospects

Beekeeping is practised over a greater area of the earth's surface than perhaps any other single branch of agriculture and on it depends the success of many other branches of agriculture. Beehive microbes may shed light on honey bee diseases that are taking heavy to all of industry throughout world. They may serve as source of novel microbial species also. The microbiota of the gut can contribute essential nutrients and vitamins and prevent colonization by non-indigenous and potentially harmful species. The intestinal floras of most organisms play a crucial role in nutrient assimilation and immune function.. However, there is growing awareness of the importance of the composition of the intestinal micro-flora for health and growth of honeybees (Gilliam, 1979; Gilliam et al., 1988a; Gilliam et al., 1997; Dillon & Dillon, 2004).Current research focuses on understanding of insect gut microorganisms as a potential source of novel bioactive compounds such as antimalarial, antiviral and antitumour peptides enzymes and novel metabolite. Also manipulating these microbial symbionts is thought to be an effective strategy for controlling the spread of pathogens that use insects as hosts.

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5/23/2014