

Detection of novel probiotic bacterium *Lactobacillus* spp. in the workers of Indian honeybee, *Apis cerana indica*Mahesh Pattabhiramaiah¹, Reddy.M.S², Dorothea Brueckner³

1 & 2 - Centre for Apiculture studies, Dept. of Zoology, Bangalore University, Jnana Bharathi, Bangalore-560056, India.

3 - Honeybee Research Unit, University of Bremen, FB 2, 28334 Bremen

reply2mahesh@gmail.com

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ABSTRACT

Many insects are known to have microorganisms in the gut which can play an important role in their nutrition. In the present study, we report the presence of probiotic bacterium *Lactobacillus* spp. in the gut of the honeybee sub species *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 honeybee 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, 16S rDNA 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 (Genbank accession number: EU392167) which has a putative health-conferring properties of probiotics.

Keywords: *Apis cerana indica*, LAB, PCR, Genbank, Karnataka

1. Introduction

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 symbiont.

The intestinal floras of most organisms play 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).

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 (Ouweland *et al.*, 2002).

Members of the genus *Lactobacillus* are of commercial importance, involved in a range of industrial products and applied as probiotics. They are a major part of the Lactic acid bacteria group, named as such because most of its members convert lactose and other simple sugars to lactic acid. The production of lactic acid makes its environment acidic which inhibits the growth of some harmful bacteria. Among these lactobacilli, the so-called *Lactobacillus acidophilus* complex is the predominant species and has received considerable industrial and medical interest because of its putative health-conferring properties as probiotics.

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 worker 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) indicates that two microbial genera, *Lactobacillus* and *Wolbachia*, were predominantly present in significant numbers in the midgut of *Apis mellifera carnica*.

This study was undertaken to assess the prevalence of the probiotic bacterium *Lactobacillus* in Indian honeybee *Apis cerana indica* collected from different parts of Karnataka.

2. Materials and Method

2.1 Colony sources

Honeybee species, *Apis cerana indica* were collected from different parts of Karnataka, India (Figure 1). Bee workers were stored in pure ethanol at -20°C . Whole guts were dissected out by separating the abdomen from the thorax, cutting open the abdomen with a micro scissor along both sides, removing the ventral cuticula and transferring the gut to a 1.5-ml Eppendorf tube. All instruments used in the dissection process were flame-sterilized between each individual.



Figure 1: Workers of the honeybee species, *Apis cerana indica* collected from different geographic regions of Karnataka, India

2.2. DNA extraction and PCR protocols

Total genomic DNA was extracted from the gut of an individual worker and using **Aquapure Genomic DNA kit** (catalog number 7326343) reagents following the procedure suggested by the manufacturer and the genomic DNA was resuspended in 50 µl of sterile water. For PCR amplification 1 µl of genomic DNA was used.

Eubacterial Universal primers 16S rRNA primers: eub2F (5'- gagagtttgatcctggctcag-3') and eub1407R (5'- ctacggctacctgttacga-3') were used to amplify from the genomic DNA of honeybees.

Standard PCR was performed by a hot start method in a 25 µl reaction volume containing 1 µl DNA sample, 1 µl forward and reverse primers, 5 µl 10X Buffer containing 15 mM of MgCl₂, 1 unit of *Taq* polymerase (Roche), and 1 µl dNTPs (10mM). Deionized MilliQ water was added to a final volume of 25 µl. The PCR reaction mix was prepared in one batch and then added to each sample. A sample containing deionized water in place of template DNA was included in all reactions as a negative control. PCR amplification was done on a Master Cycler Gradient (Eppendorf) under the following thermal profile: 95^o C for 2min @ 1 cycle, 95^o C for 30sec @ 30 cycles, 40^o C for 30sec @ 30 cycles, 73^o C for 3min @ 30cycles and an extension cycle of 73^o C for 1min @ 1cycle. The amplified products were detected by running a 1.5% agarose gel (TAE buffer) with a 1kb molecular weight marker. The gels were stained in ethidium bromide, observed under UV Transilluminator and then photographed (Figure -2).

Clean laboratory practices, sealed pipette tips, and fresh reagents were used to avoid contamination. Negative controls (consisting of all components except the DNA template) were conducted on each date to detect potential contamination, but positive controls were not carried out to reduce the likelihood of contamination.

2.3. DNA sequencing and Sequence deposition

To authenticate the PCR result, random sequencing (sample 4) of 16S rRNA products was done. The purified PCR samples from *Apis cerana indica*, positive for *Lactobacillus* were purified with a Prep-A-Gene PCR Clean-Up System kit (Bio-Rad) according to the manufacturer's instructions. A 500-ng amount of template (amplification product) was combined with 10 ng of primer, 2 µl of Sequence buffer, and water to 10 µl. Sequencing was carried out using Big Dye 2.0 (Applied Biosystems) end-terminal cycle sequencing, followed by separation and analysis on an Applied Biosystems 3130 DNA Analysis machine.

The nucleotide sequences of *Lactobacillus* from the worker of honeybee *Apis cerana indica* was deposited in GenBank (Genbank accession number: EU392167) using Bankit software (<http://www.ncbi.nlm.nih.gov/BankIt/index.html>). Sequences were also compared directly to all 16S rRNA sequences deposited in GenBank using BLASTN, the NCBI homepage (<http://www.ncbi.nlm.nih.gov/BLAST/>).

3. Results and Discussions

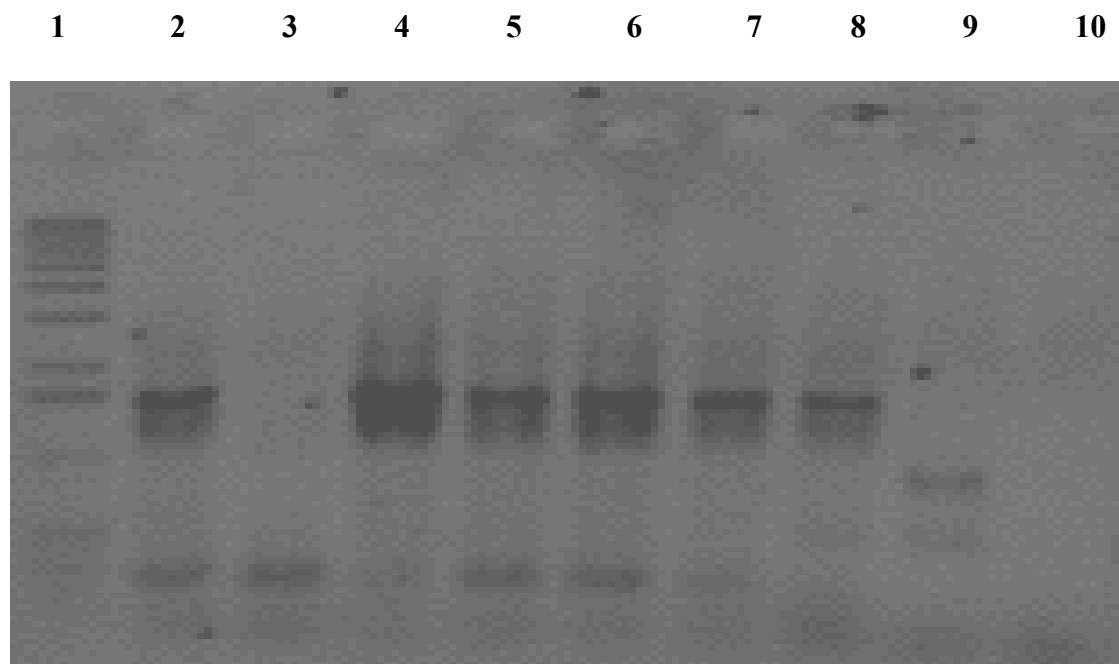


Figure 2: PCR amplification of *Lactobacillus* from the workers of honeybee sub species *Apis cerana indica* collected from different parts of Karnataka, India.

Lane - 1: Mol. wt marker,

Lane -2 :Ankola and Sirsi in Uttara Kannada District, 3. Purappamane in Shimoga District, 4. Sakaeshpur in Hassan District, 5. B.R.Hills in Chamarajanagar District, 6.Panakana Halli in Mandya District, 7. Kantanakunte in Bangalore Rural District, Doddaballapur Taluk, 8. Nelamangala, in Bangalore District, 9. Sringeri in Chikmagalore District, 10. Negative control respectively.

The PCR result using *Lactobacillus* specific primers revealed that the *Lactobacillus* genera were present in *Apis cerana indica* with its presence in 75% of the honeybee workers examined by the PCR (Figure -2). It provided a PCR product of the expected length of ~1100bp, while the failure to detect *Lactobacillus* in samples 3 and 9 by standard PCR(Figure 2) may be due to inhibition of the reaction or to a low titer of *Lactobacillus* or absence of *acidophilus* sp. in the workers of honeybees is unknown. Lane-10 showed negative for PCR, clearly indicating there was no contamination in the PCR carried out. Results of the study revealed the presence of *Lactobacillus* spp. in worker honeybee of the species *A. cerana indica*, which might act as a probiotic in the midgut of honeybee.

The Basic Local Alignment Search Tool (BLAST) results of the deposited query sequence confirmed the presence of the endosymbiont and also helped in estimating the identity percentage of the homologous sequence present in the nucleotide database of NCBI. The BLAST results revealed regions of local similarity between sequences of *Lactobacillus* Sp and confirmed the presence of *Lactobacillus* in honeybees, which falls in a predominant group of acidophilus, which is widely used as probiotics.

The BLAST result of sequence of *Apis cerana indica*, Accession number **EU392167**, showed 100% identity with *Lactobacillus Apis mellifera* (EF032161), 92% identity with *Lactobacillus alvei* strain (Accession number AY667698), identity of 90% with *Lactobacillus apis* strain (Accession number AY667701), 100% identity with *Lactobacillus acidophilus* strain (Accession number CP000033), 90% identity with *Lactobacillus crispatus* strain (Accession number AF257097, AF257096) , and 93% identity with *Lactobacillus* Sp in the stomach of honeybees(HM113262, HM113277, HM113308, HM113311, HM113343, HM534800, HM534801, HM534803) with total query coverage of 100% and E-Value of 0.0 .

The detailed nucleotide sequence analysis of sequence (Accession no: EU392167) revealed that the length of the nucleotide was 548 bp with a molecular weight of 178,256 kDa (Table-1).The G+C content were higher than the A+T content (Table-2) with 25 unknown (N) nucleotide. It revealed considerably higher G+C content of 50% as compared to A+T content of only 46%, revealing that the sequence was GC rich.

Table-1: Individual nucleotide sequence statistics of *Lactobacillus* Spp(Accession No EU392167.1) from worker honeybee *Apis cerana indica* (EU392167.1) analyzed by CLC Workbench (Version 5.1.1)

Sequence type	DNA
Length	548 bp
Name/Accession No	gi 118162031 gb EU392167.1
Description	<i>Lactobacillus</i> endosymbiont of <i>Apis cerana indica</i> , 16S ribosomal RNA gene, partial sequence.
Nucleotide count (A)	135
Nucleotide count (T)	116
Nucleotide count (G)	162
Nucleotide count (C)	110
A+T	251
G+C	272
Any nucleotide (N)	25
Weight	178,256 kDa

Our study aimed to assess the midgut of honeybee adults, PCR results and direct sequencing revealed the presence of lactic acid bacteria which may inhibit the growth of harmful pathogens. The PCR survey conducted revealed the presence of *Lactobacillus* spp. in 75% of the honeybee worker of the subspecies *Apis cerana indica* collected from different parts of Karnataka, India which might act as a probiotic in the midgut of honeybee. Nucleic acid sequencing suggests that the indigenous bacterial flora in the honey stomach is dominated by *Lactobacillus* phylotype.

The role of microbes in honeybee colonies is essential in maintaining their health and survival. Microbes are most commonly found in the digestive system of worker bees and vary with location and season. Researchers in Sweden recently found twelve different live lactic acid bacteria (LAB) in the honey stomachs of bees (Olofsson & Vásquez, 2008).The organisms most often isolated from pollen are the bacteria, *Pseudomonas* spp., *Lactobacillus* spp., *Bacillus* spp. and *Enterobacteriaceae*, and the yeasts *Saccharomyces* spp., *Torulopsis* spp., *Candida* spp. and *Cryptococcus* spp. (Gilliam, 1979; Glinski and Jarosz, 1988). These

organisms colonize the bee intestine and create a permanent microflora in the alimentary tract (Glinski and Jarosz, 1988).

The bacteria belonging to the genus *Lactobacillus*, are normal inhabitants of the gut of honeybees and are GRAS (Generally Regarded As Safe). Strains of this genus have been shown to have important metabolic and protective functions in the gastrointestinal tract, interfering with enteric pathogens and maintaining a healthy intestinal microflora. These bacteria are considered beneficial gut inhabitants of humans and other animals and are involved in immunomodulation, interference with enteric pathogens and the maintenance of a healthy microbiota (Mitsuoka, 1992).

It is also well known that *Lactobacillus* spp., produce antimicrobial substances (Kim *et al.*, 1997). These facultative anaerobes tolerate the acidic environments and ferment sugars to produce lactic or acetic acid. The production of lactic acid makes the environment acidic which inhibits the growth of some harmful bacteria. These bacteria could be cultured as microbial food supplement, which benefits the host by improving its intestinal microbial balance (Fuller, 1989). These beneficial bacteria have a role to play in disease control/prevention, enhancement of growth rates, and nutrition. These right microbes could be used to inoculate the hive and the bee gut to aid colony health and diet.

The midgut of honeybees is the only part of the alimentary tract, which is not lined by chitin, and thus is extremely exposed to toxic substances and improper food. The colonization of bee intestine by bacteria such as *Lactobacillus* spp. and yeasts is a very important process; the micro-organisms participate not only in food digestion but also in the production of vitamins and antibiotic substances which eliminate pathogenic micro-organisms (Tomasik & Tomasik, 2003). In order to create a proper microfloral environment in the bee intestine, pollen substitutes need to be supplemented with microorganisms, which colonize the alimentary tract and determine proper digestion.

Gilliam (1997) recorded the presence of a consistent honey bee gut microbiota, occurring independent of season and geography using culture based techniques. For honeybees this starts with specific microorganisms that are added to the beebread when they store it on the comb and continues in their digestive tract. These cultures of microorganisms could be a very beneficial addition to the pollen supplements. Máchová *et al.* (1997) put probiotics into sugar syrup used to feed bees and noticed that this improved bee survival.

The use of probiotic *Lactobacillus* in addition to pollen supplement may stimulate the appetite and improve nutrition by the production of vitamins, detoxification of compounds in the diet, and by the breakdown of indigestible components. They prevent potential pathogens from establishing infection by numerous mechanisms, which include: production of short-chain fatty acids and bacteriocin (an endogenous antibiotic), induction of a low oxidation-reduction potential, competition for nutrients, deconjugation of bile acids (which renders them bacteriostatic), blockade of adherence receptors and degradation of bacterial toxins (Savage, 1980). In addition, there is evidence that probiotics induce an immune response in honeybees (Evans and Lopez, 2004).

Bacteria inhabit the intestines and protect against some unhealthy organisms. Forsgren *et al.* (2010) demonstrated the use of novel lactic acid bacteria inhibits the growth of pathogen in honey bee larvae. Furthermore, they also demonstrated that the addition of LAB to young honey bee larvae exposed to *P. larvae* spores decreases the proportion of larvae that succumb

to American Foul Brood infection. The breakdown of food by *L. acidophilus* produces lactic acid, hydrogen peroxide, and other byproducts that make the environment hostile for undesired organisms. Thus *Lactobacillus* are also known to inhibit the growth of major honey bee pathogens (Promnuan *et al.*, 2009; Audisio *et al.*, 2010; Forsgren *et al.*, 2010).

Treatment by formic, lactic, and acetic acid is widely employed by beekeepers to guard against such honeybee pathogens as *Varroa destructor* and *Nosema apis*. Organic acids such as formic acid, which is produced by bifidobacteria (Van der Meulen *et al.*, 2006), and both lactic and acetic acid, which are produced by LAB discovered in the honey stomach are antimicrobial substances, meaning that these bacteria may be of considerable importance in protecting honeybees against pathogens.

Thus, information about the interactions that occur between different bacterial species inside the hive and the dynamics of the bacterial community could be important in order to develop new approaches for disease control, avoiding the use of commercial antimicrobial substances such as antibiotics.

4. Conclusion / Suggestions/ Findings

In conclusion, we have provided the first detailed survey of novel nonpathogenic bacteria in the honeybee gut of *Apis cerana indica* collected from various parts of Karnataka, India. Thus, the preliminary studies showed that *Lactobacillus* endosymbiont were invariably present in populations of *Apis cerana indica*. The frequent presence of native *Lactobacillus* strains associated with Indian honey bees confirmed in this research study could encourage research related to the development of probiotic treatments for bee colonies. The resident gut bacteria are apparently highly specialized to this group of organisms. The novel LAB flora discovered in our studies has evolved a mutual dependence with honeybees. There is a mutual symbiosis between the LAB and honeybees. The LAB draws available nutrients from the bees, and the honeybees are protected by the LAB from harmful pathogens.

5. References

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