



## Original article

Characterization of gut bacterial flora of *Apis mellifera* from north-west Pakistan

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## ABSTRACT

Gut microbiota has been recognized to play a beneficial role in honey bees (*Apis mellifera*). Present study was designed to characterize the gut bacterial flora of honey bees in north-west Pakistan. Total 150 aerobic and facultative anaerobic bacteria from guts of 45 worker bees were characterized using biochemical assays and 16S rDNA sequencing followed by bioinformatics analysis. The gut isolates were classified into three bacterial phyla of Firmicutes (60%), Proteobacteria (26%) and Actinobacteria (14%). Most of the isolates belonged to genera and families of *Staphylococcus*, *Bacillus*, *Enterococcus*, *Ochrobactrum*, *Sphingomonas*, *Ralstonia*, *Enterobacteriaceae*, *Corynebacterium* and *Micrococcineae*. Many of these bacteria were tolerant to acidic environments and fermented sugars, hence considered beneficial gut inhabitants and involved the maintenance of a healthy microbiota. However, several opportunistic commensals that proliferate in the hive environment including members *Staphylococcus haemolyticus* group and *Sphingomonas paucimobilis* were also identified. This is the first report on bee gut microbiota from north-west Pakistan geographically situated at the crossroads of Indian subcontinent and central Asia.

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## 1. Introduction

As a pollinator, honey bee has a prominent role in sustainable agriculture in addition to production of honey and other natural products (Klein et al., 2007; Potts et al., 2010). Compared to other bee species, honey bees have been reported to increase the yield in animal pollinated crops which account for 35% of the global food production (Genersch, 2010; Klein et al., 2007). Hence, research related to physiology and pathology of honey bees in particular *Apis mellifera* has attracted a lot of attention (Muli et al., 2014).

Numerous causes of severe honey bee colony losses have been proposed, including pesticides toxicity (Desneux et al., 2007), poor

nutrition (Brodtschneider and Crailsheim, 2010) and genetic diversity (Mattila and Seeley, 2007). A high load of parasites and microbial pathogens, especially bacteria strongly connected with the disappearing of bee population (Core et al., 2012; Di Prisco et al., 2013; Olofsson and Vásquez, 2008). Therefore, characterization of bee gut microbiome can provide valuable insight about of parasites and bacterial pathogens.

Bacteriological analysis along with molecular techniques based on 16S rRNA sequences precisely characterize insects gut bacterial flora (Prabhakar et al., 2013; Ahn et al., 2012). The composition of bacterial assemblage in the digestive tract of honey bee *A. mellifera* is relatively simple compared with other gut-associated communities (Babendreier et al., 2007; Cox-Foster et al., 2007; Engel and Moran, 2013). A distinctive set of bacteria including Firmicutes, Actinobacteria,  $\alpha$ - and  $\gamma$ -proteobacteria found in the honey bee alimentary canal has been assessed by using Sanger as well as next generation sequencing techniques (Engel et al., 2012; Evans and Schwarz, 2011; Jeyaprakash et al., 2003; Li et al., 2012; Martinson et al., 2011).

Pakistan is located at the north-western frontier of the distribution range of the honey bees *A. cerana*, *A. dorsata* and *A. florae*.

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However, due to the low honey yields of eastern honey bees, commercial beekeepers in Pakistan use western honey bee *A. mellifera* since late 1980s (Waghchoure-Camphor and Martin, 2008). In this study, we characterized the aerobic and facultative anaerobic bacteria isolated from alimentary canal of *A. mellifera* from honey producing areas in north-west Pakistan.

## 2. Material and methods

### 2.1. Sample collection and dissection of the bees

In order to study the cultivable honey bee gut bacteria, 45 worker honey bees (*A. mellifera*) were collected from the bee farms located in cruciferous vegetation in districts of Kohat, Karak and Bannu in north west Pakistan. After collection, live bees were transported to the laboratory in the small cages containing sugar powder followed by storage at  $-20^{\circ}\text{C}$  until processing. Before dissection, whole bees were washed in 95% ethanol and complete alimentary canals of bees were aseptically dissected by clipping the stinger with sterile forceps. The dissected guts were macerated with sterile dissection scissors in 0.8% NaCl solution and immediately stored at  $-20^{\circ}\text{C}$ .

### 2.2. Culturing of bacteria

From the preserved bee gut samples, different dilutions (i.e. 1/10, 1/100 and 1/1000) were made and 100  $\mu\text{l}$  aliquots of the diluted sample were inoculated in LB agar plates and incubated for 24–48 h at  $37^{\circ}\text{C}$ . The separated colonies in master plates were sub-cultured in LB agar plates and incubated at  $37^{\circ}\text{C}$  and the morphology of isolated colonies in subculture plates was noted.

### 2.3. Biochemical tests

Various biochemical tests were performed including Coagulase test, Oxidase test, Urease test, Lactose fermentation test, and hydrogen sulfite production test etc. for the identification of bacterial isolates with the help of Bergey's Manual and API 20 NE identification system for non-fastidious, non-enteric Gram negative rods (Biomérieux, France).

### 2.4. Colony PCR and DNA sequencing

Isolated bacterial colony was subjected to amplification of the 16S rDNA gene according to Khan et al. (2014). The forward primer 5'-GGCTCAGAACGAACGCTGGCGGC-3' and the reverse primer 5'-CCACTGCTGCCTCCCGTAGGAGT-3' were used. These primers are highly specific for conserved regions of bacterial 16S ribosomal DNA. PCR product (10  $\mu\text{l}$ ) was subjected to electrophoresis and 40  $\mu\text{l}$  was purified with PCR clean up kit (Invitrogen Inc. USA). The DNA estimation was carried by using Qubit dsDNA Hs assay kit with Qubit 2.0 Fluorometer (Invitrogen, USA). The PCR products were sequenced using Big Dye Terminator kit and Genetic Analyzer ABI 377 (Applied Biosystems Inc., USA).

### 2.5. Sequence analysis

The resultant sequencing data was analyzed by Sequence Scanner v1.0 (Applied Biosystems Inc., USA). The 16S rDNA sequence of each bacterial isolate was compared using BLAST (Camacho et al., 2008) against '16S ribosomal RNA sequences (Bacteria and Archaea) database' (a subdivision of GenBank) and Ribosomal Database Project (Cole et al., 2014).

## 3. Results and discussion

Honey production is a profitable small enterprise in Khyber Pakhtoonkhwa province in north-west of Pakistan. Currently ~7000 beekeepers are involved in bee business in Pakistan with a total of 300,000 colonies of *A. mellifera* which produce ~7500 metric tons of honey each year (Waghchoure-Camphor and Martin, 2008). Due to importance of gut bacteria in the development, nutrition and immunity of honey bees, we carried out an analysis of gut bacteria in worker bees in different apiaries in north-west Pakistan.

We isolated 150 aerobic and facultative anaerobic bacteria from guts of 45 worker *A. mellifera* collected from different apiaries located in cruciferous vegetation in honey producing districts of Khyber Pakhtoonkhwa (i.e. Kohat, Karak and Bannu) in north-west Pakistan. Based on colony morphology and other bacteriological characteristics, 100 bacterial isolates were subjected to 16S ribosomal DNA (rDNA) amplification followed by sequencing. Consequently, 77 sequences of 16S rDNA were obtained and analyzed by BLAST (Camacho et al., 2008) and Ribosomal Database Project (Cole et al., 2014). In general agreement with previous studies (Martinson et al., 2011; Jeyaprakash et al., 2003; Li et al., 2012; Ahn et al., 2012; Prabhakar et al., 2013), these sequence analyses approaches classified isolated gut bacteria into three phyla i.e. Firmicutes (60%), Proteobacteria (26%) and Actinobacteria (14%) (Fig. 1). Among Firmicutes, most of the isolates belonged to genera *Staphylococcus*, *Bacillus* and *Enterococcus* (Fig. 2). Members of family Enterobacteriaceae and following genera belonging to  $\alpha$ -,  $\beta$ - and  $\gamma$ -Proteobacteria were also found i.e. *Ochrobactrum*, *Sphingomonas*, and *Ralstonia* (Fig. 2). Several actinobacterial isolates were classified in suborders of Corynebacterium and Micrococcineae. Hence culture based method adopted during this study revealed important members of "core" bacterial community present in alimentary canals of honey bees present in apiaries located in north-west of Pakistan (Fig. 3).

The phylogenetic tree of the partial 16S rDNA gene sequences of the bacterial isolates from the gut of honey bees showed relatedness among the bacteria when aligned with reference strains in GenBank (Fig. 4), which revealed that the bacterial population in the gut of honey bee foragers in North West Pakistan was diverse, including the phyla Firmicutes, Actinobacteria, and  $\alpha$ -,  $\beta$ -, and  $\gamma$ -proteobacteria. The richness of these bacterial assemblages suggests their ecological importance. For instance, the abundance of one representative of *Staphylococcus* was estimated at 29% of the total microbial gut samples analyzed. Additionally, most of the 16S rDNA gene sequences were found very comparable

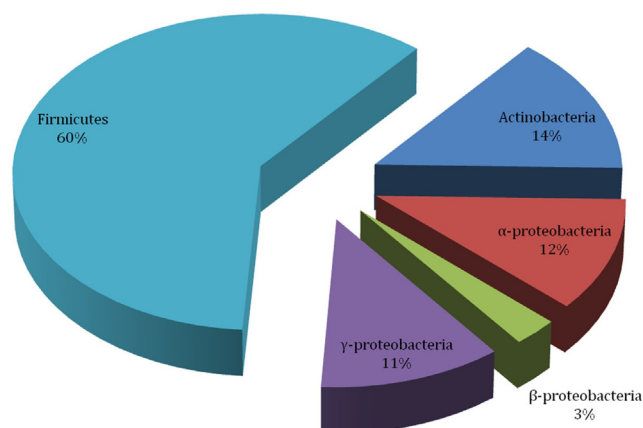


Fig. 1. Phyla-wise classification of honeybee gut bacteria obtained during the present study.

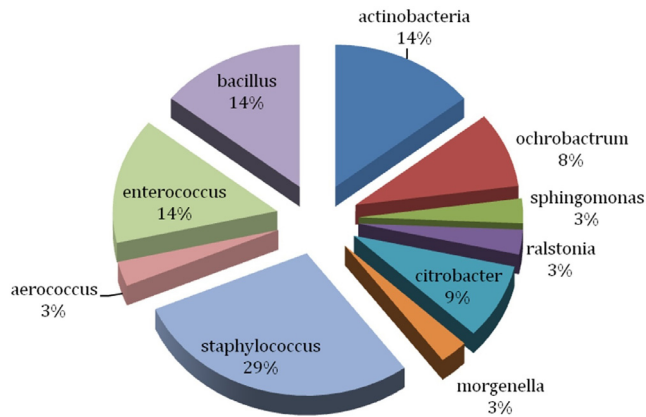


Fig. 2. Genera-wise classification of honeybee gut bacteria obtained during the present study.

to the sequences isolated from *Apis* sp that were already deposited in the NCBI database (Khan et al., 2017; Yoshiyama and Kimura, 2009; Martinson et al., 2012; Kacaniova et al., 2004)

Most of these bacteria were broadly classified as facultative anaerobes, tolerant of acidic environments and ferment sugars to produce lactic or acetic acid. 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 (Anderson et al., 2011). Some of the obligatory aerobic bacteria were adapted to highly acidic environments rich in sugar. As revealed by biochemical assays, many of these bacteria ferment sugars to produce lactic acid and various other end products, and several can reduce nitrate to nitrite suggesting a potential function in nitrogen metabolism within the gut. Beneficial honey bee bacterial strains are well represented in genomic databases, including 195 strains of *Bacillus*, 183 strains of *Lactobacillus* and 50 strains of *Bifidobacterium* (Anderson et al., 2011).

Bioinformatics analysis of 16S rDNA sequences in present dataset identified several opportunistic commensals that abound in the hive environment, but occur only sporadically within the honey bee gut. During this study, we frequently found Staphylococcal sequences. Careful sequence analysis showed occurrence of

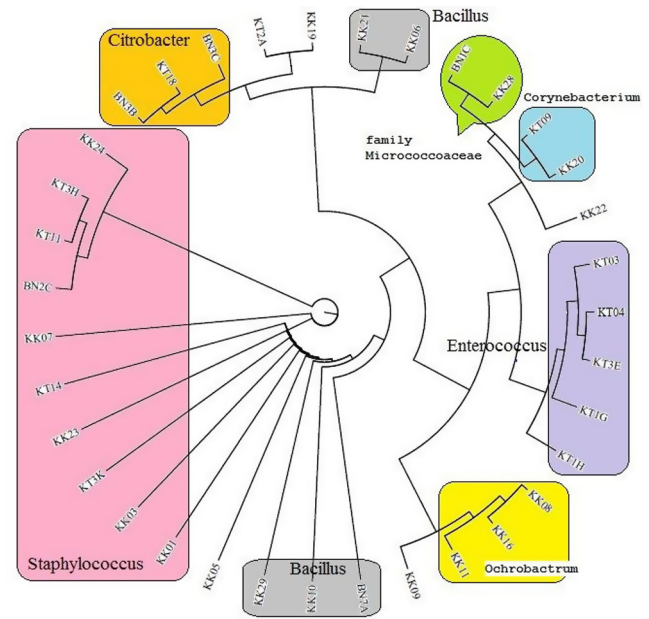


Fig. 4. Phylogenetic dendrogram of the 16S rDNA partial gene sequences of gut bacteria isolated from honey bees (*Apis mellifera*) in North West Pakistan. Groups (same genera) are shown in Color division. The tree was constructed using MEGA (V 6.), by the neighbor joining method. Bootstrap values (1000 replicates) expressed by most of all analysis.

members of *Staphylococcus haemolyticus* group (i.e. *S. devriesei*, *S. haemolyticus*, *S. hominis*). Staphylococcal strains are rarely and/or briefly mentioned in available literature on bee gut microbiota. However, we recurrently encountered *S. haemolyticus* group strains originated from apiaries located in study area. *S. haemolyticus* is the second-most clinically isolated coagulase-negative Staphylococcus bacterium which is considered an important nosocomial pathogen (Vignaroli et al., 2006). Like other coagulase-negative staphylococci, *S. hominis* has been considered a presumptive and opportunistic pathogen which may occasionally cause infection in patients whose immune systems are compromised (Jiang et al., 2012). Moreover, *Sphingomonas paucimobilis* which was confidently classified by 16S rDNA sequences, has been reported as a cause of nosocomial infections (Ryan and Adley, 2010).

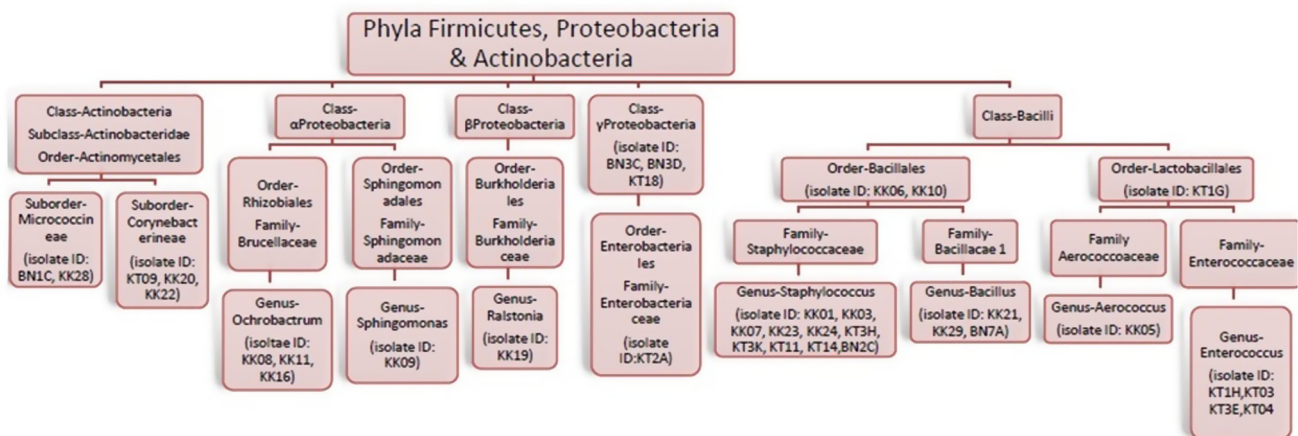


Fig. 3. Ribosomal Database Project based taxonomy of bacteria isolated from honeybee gut during the present study. Codes of representative isolates are given in parentheses.



We identified several actinobacterial species during this study. These species included (a) *Kocuria* sp., previously isolated from air in China (Zhou et al., 2008); (b) *Corynebacterium* sp., widely distributed in nature and are mostly harmless (Collins et al., 2004) while *Corynebacterium minutissimum* is a causative agent of erythrasma (superficial skin infection) and other infections (Granok et al., 2002; Dalal and Likhi, 2008); and *Micrococcus endophyticus*, previously isolated from plant roots (Chen et al., 2009). Many Actinobacteria have been reported in beebread indicative of their long live in stored food (Anderson et al., 2013; Corby-Harris et al., 2014). The slow growth of Actinobacteria produce spores and the transmission of these spores between bee life stages and hive surroundings may occur on the body of individual honey bees (Corby-Harris et al., 2014).

Like ants, bees also belong to hymenoptera which are eusocial insects that survive in colonies with queen and thousands of worker bees which can forage large distances for collecting nectars and pollens and return to the hive. Hence their contact with various environments acts as a vector of diverse bacterial flora. More than fifty bacterial species from 31 genera have been found associated with different ants including *Escherichia*, *Staphylococcus*, *Enterobacter*, *Pseudomonas*, *Bacillus*, *Streptococcus*, and *Klebsiella* (Pesquero et al., 2012).

A number opportunistic pathogenic bacteria have already been reported from different insects including phytophagous insect aster leafhopper (*Macrosteles quadrilineatus*) in association with plants (Soto-Arias et al., 2014). Nectar has considered as an environmental bacterial reservoir. An array of bacterial species belongs to Firmicutes and Enterobacteriaceae are reported from nectar. Different bacillus species were frequently reported from the nectars of Acacia and Mesquite (Anderson et al., 2013). Acacia plants (*Acacia Arabica*, *Acacia modesta* and *Acacia nilotica*) are major bee plants in local flora of the study area (Marwat et al., 2013). Therefore, interaction of worker bees in the study areas nectar from the blossom of Acacia flowers catalyzed the transmission of environmental bacteria in the bee guts (Anderson et al., 2013; Fridman et al., 2012).

The possible routes of bacterial contamination in honey bee and its by-products are human, hive tools, sugar feeders, wind and dust. Beekeepers skin infections, fecal contamination and sneezing can introduce pathogenic microbes into the hive environment. Hence bee guts can act as a carrier of opportunistic bacterial pathogens. Additionally, due to political and economic disruption in the study area caused by the war against terrorism, most of the beekeepers were not able to keep their bees properly. Like in Europe, a remarkable decline in the bee hives was observed during the early 1990s caused by the Soviet collapse, rather than from widespread ecological factors (vanEngelsdorp and Meixner, 2010). The socio-economic status of beekeepers drastically changed with the ending of the Soviet Union. Likewise, many beekeepers in north-west Pakistan were engaged in static jobs like shops, and left the migration of bees from one floral area for the next due to current war events. Therefore, proper management of existing bee farms will increase the productivity and quality of honey and their byproducts.

## Conflicts of interest

The authors declare no conflict of interest.

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