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Studies on the culturing and isolation of gut bacteria of *Apis mellifera* L.

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Abstract
The honey bee species *Apis mellifera,* commonly known as Italian honeybee, is a prominent pollinator among other species. These are found to be associated with the symbionts in their gut, which make them a powerful organism. The present investigation on the culturing of the *Apis mellifera* gut bacteria and their isolation for the identification of the phylum of the bacteria. It was observed that three media type namely, Nutrient Agar, Tryptone soy Agar and Actinomycetes isolation agar were most suitable one by using spread plate method. The pure bacterial culture plates were obtained streaking the individual colony. Form the individual colony 16S rRNA gene sequence analysis showed that *A. mellifera* gut is a home for Firmicutes, Proteobacteria and Actinobacteria.

Keywords: Bacterial phyla, *A. mellifera*, culturable method

Introduction
The honeybees are most beneficial insect having the extensive distribution in different geographical areas across the globe. The distribution pattern ranges from Plains to tropical forest to the highest peak of Himalayan cliff. The honeybees are the most dominant group of insect which are involved in pollination of wide variety of crop plants and fruit trees on which we humans are dependent. Across the globe they have gained the eminence as effectual pollinating agent in the natural ecosystem. Being the anthropocentric source as a servicer of pollination and benefactor of several bee products for human use, they have wangled apiculture to a gainful agro enterprise. Among the honey bee species, the Italian honeybee, *Apis mellifera,* is a important pollinator among other species, which play major role in pollinating crop plants (Aizen *et al*., 2008) [1].

The hymenopteran evolved from past million years ago to till now in this due bees during the course of evolution, have obtained social behavior and also started acquiring microorganisms and established symbiotic association with them. The social evolution of *A. mellifera* resulted them to be a eusocial insect with noble traits. In the *A. mellifera* colony, population is differentiated into the only reproductive female as queen, few hundreds of male reproductive as drones and thousands of altruistic, non-reproductive female taking care of colony as workers (Page and Peng 2001) [2]. *A. mellifera* has a gut microbial community entailed with its physiological functions related to nutrition and susceptibility to disease and thus take part in controlling the health and resilience (Genersch, 2010) [3]. So a better understanding of gut microbiota would help in healthy rearing of these bees and offer scope for developing innovative techniques for conserving the bee's population. Diversity of gut microbiota of Italian honey bees have elucidated and found that bacterial symbionts play a significant role in nutrition and development in workers (Raymann and Moran, 2018) [4].

As Insect microbiotas, particularly gut microbiotas, are complex and varied according to insect phylogeny and ecology. Many studies revealed that microorganisms from all domains, including bacteria, fungus, archaea, protozoa and viruses, found in the insect gut, with bacteria being the most prevalent one (Gurung *et al.*, 2019) [5]. Around 18 bacterial phyla were discovered in a single study of the microbiome of 21 insect groups. (Yun *et al.*, 2014) [6]. Some of the most common order with high abundance were Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Tenericutes. But, the knowledge on the gut microbiota associated with *A. mellifera* is meagre. As the research on Insect intestinal microbiota is gaining tremendous interest among the scientific faculty of agricultural stream (Mereghetti *et al*., 2017) [7]. Thus, a better understanding of gut microbiota of Italian honeybee would help in healthy rearing of these bees and offer scope for developing innovative techniques for

conserving the bee's population. This work was conducted to know major bacterial groups present in *A. mellifera* gut by culturable approach.

Materials and Methods

The experiment was conducted in Insect physiology laboratory, Division of Entomology and Division of Microbiology ICAR-Indian Agricultural Research Institute, Pusa, New Delhi, during the year 2020-2021. Honey bee samples were collected from hives maintained at division of entomology, IARI, New Delhi. Adult worker bee were used for studying the gut bacteria diversity.

Insect dissection and preparation of homogenates

The collected bees then will be washed in double distilled water for 60 seconds, surface sterilized with 70% (v/v) ethanol for 60s followed by thorough rinsing with double distilled water again to remove the disinfectant. The surface sterilized adult bee will be dissected using a sterilized micro scissor under lamina flow to extract the whole digestive tract (Fig 2). The extracted gut will be divided into foregut, midgut and hindgut after which the different sections will be homogenized separately in 0.85% NaCl with a sterile homogenizer and stored at -20 ºC until further analysis for gut bacterial diversity.

Media preparation

Isolation of gut bacteria was carried out by using three types of media like nutrition agar, Tryptone soya agar, Actinomycete isolation agar. All the media were prepared at 1 liters w/v and were sterilized at 121 ºC for twenty minutes. And poured on petri plates and allowed for solidification in the laminar flow.

Serial dilution and bacteria culturing

For culture dependent studies, serial dilutions of each gut section will be spread plated on at least three media types (Nutrient Agar, Tryptone soy Agar and Actinomycetes isolation agar) for colony forming units (CFU). The gut homogenates will be serially diluted (6-fold) by transferring homogenized sample into 0.85% NaCl, vortexing vigorously after which 50 µl of each dilution series (10^{-6}) will be spread in triplicate series.

The inoculated plates will be incubated at 37 ºC for 24-48 hours. Gut bacterial isolate enumeration will be done by calculating the Colony Forming Units (CFU). Mean colony counts will be used to calculate CFU. The total viable count will be expressed as the number of CFU in 1ml of sample. The bacterial isolates will be picked-up after specified hours of incubation for purification by repeated streaking on the corresponding agar plates.

Enumeration and isolation of culturable gut bacteria

DNA extraction

Based on 16S rRNA gene sequence analyses, distinct representative purified colonies of bacteria were chosen for identification. Individually purified bacterial isolates were grown for 24 hours at 37 degrees Celsius in nutrient broth. The pellet and supernatant were separated from the broth cultures after 24 hours of growth by centrifugation at 13,000 rpm. The supernatant was discarded and a modified cetyltrimethylammonium bromide (CTAB) method was used to extract DNA from the pellet. On an agarose gel, the extracted DNA quality was checked.

PCR amplification

The 16S rRNA of each isolate was amplified by PCR using Bio Line Master Mix and eubacterial primers 27F-(10µM), (5'→AGAGTTTGATCCTGGCTCAG→3) and 1492R-(10µM), (5→AAGGAGGTGATCCAGCCGCA→3'). Each reaction contained approximately 50ng DNA, 25 µl Master Mix (2X) and 0.5 mM of each primer. The following PCR protocol was used in a Bio-Rad C1000-thermal cycler (Bio-Rad Laboratories Inc, Berkeley, CA, USA): one cycle at 94 C for 5 min, 35 cycles at 94 C for 1 min, 52 C for 1 min and 72 C for 1 min 40 sec, followed by 72 C for 10 min and 4 C forever. Electrophoresis of PCR products in a 1.2 percent agarose gel was performed, and bands were visualised with ethidium bromide staining. The gels were run at 100 V for 1 h in TAE buffer (40 mM Tris-acetate, 1 mM ethylene-diamine-tetra-acetic acid (EDTA); pH 7.4). Gels were visualized under UV in the Gel Documentation system of Alpha ImagerTM gel imaging system (Alpha Innotech, San Leandro, CA, USA). The products were outsourced for sequencing.

Result and Discussion

Culture of gut bacteria

The gut bacteria (aerobic) associated with the Italian honey bee, *Apis mellifera* (Hymenoptera), were cultured by spread plated method on three media types, Nutrient Agar, Tryptone soy Agar and Actinomycetes isolation agar. Incubated at 37ºC and the growth of bacteria was obtained on culture plate after 24hrs after inoculation Figure: 1. on these plates multiple colonies of different bacteria was grown which were further subjected for purification.

Fig 1: Pic showing general colony obtained after 24 hrs of incubation

Purification of gut bacteria associated with *A. mellifera*

From the spread plate colonies the purification and isolation of single phyla of the gut bacteria associated with *A. mellifera* were isolated using commercially available bacteriological media from gut. The pure bacterial culture plates were obtained streaking the individual colony separately Figure: 2 and later each bacterial isolates were treated as different strains, these were utilized further for molecular level identification.

Fig 1: Pure colony obtained after streaking individual bacterial colony by streak plate method

Bacterial groups

The pure culture grown by streak plate method were utilized to develop 16S rRNA gene by DNA extraction and PCR amplification. Based on the sequences obtained, the comparative analysis of the sequences with their closest relatives from GenBank. Where, it was found that majority were from Firmicutes, followed by Proteobacteria and Actinobacteria. Among the different regions of gut it was observed that in Foregut, firmicutes were abundant phyla (61.7%), followed by proteobacteria (32.3%) and actinobacteria (5.8%). In Midgut firmicutes were abundant phyla (55.5%), followed by proteobacteria (40.7%), actinobacteria (3.7%). In Hindgut firmicutes (69.2%), proteobacteria (23.07%) and actinobacteria (7.69%). Thus showing that these phyla were localized in the gut of *Apis mellifera*.

Arthropods are the most numerous animal species on the planet, occupying nearly every ecological niche. They face a variety of challenges, including nutritionally deficient and recalcitrant food diets, toxins, environmental extremes, and threats from natural enemies. Herbivorous insects, in particular, have the flexibility to eat a variety of foods, which has been attributed in part to their gut microbial symbionts, which play important roles and some of which have been linked to lignocellulosic digestion. Among the different groups of microbes, bacterial microflora predominantly associates with insects, transiently or permanently (Liu *et al*., 2018) [8]. Insects form symbiotic microbial associations with their hosts through vertical transmission or diet, and these symbionts may be involved in food digestion, nitrogen fixation, cellulose digestion and host nutrition support, among other things. (Yalashett *et al*., 2017) [9]. The majority of microbes that enter the gut pass through quickly or are managed by the host immune system; however, a core community may be allowed to colonise the host and form the gut microflora, with each species performing a variety of functions that may influence host biology.

The results of this study have shown that the majority of culturable aerobic gut bacteria associated with *A. mellifera* comprises of three phyla: Firmicutes, Proteobacteria and Actinobacteria. The midgut of hymenopteran insects is reported to be slightly acidic to slightly alkaline (Heimpel 1995) [10] and in view of this, the gut compartments act as unique niches such that it allows specific gut microbiome adopted to those niches to thrive. The different bacteria identified from *A. mellifera* were enriched different based on their spatial colonization. It is presumed that all these different groups of microflora are enriched albeit different in the different compartments and may be playing different physiological roles according to the physiological need of the insect.

Conclusion

According to the study, *A. mellifera* gut has association with Firmicutes, Proteobacteria and Actinobacteria which were culturable by three media types, Nutrient Agar, Tryptone soy Agar and Actinomycetes isolation agar. Thus, it decipher that *A. mellifera* gut is associated with bacterial diversity, which may play an important role in nutrient assessment and growth of *A. mellifera* population.

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