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Isolation, Characterization and Identification of Probiotic Lactic Acid Bacteria (LAB) from Honey Bees

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A B S T R A C T

Laboratory experiments were conducted to explore the diversity of naturally-occurring probiotic lactic acid bacteria (LAB) associated with honey bees and to understand whether they can be used as probiotics to manage honey bee stressors. Honey stomach, honey, bee bread, bee pollen and royal jelly from different species of honey bees (*Apis ceranaindica* Fabricius, *Apis melliefra* Linnaeus, *Apis florea* Fabricius, *Apis dorsata* Fabricius, *Tetragonula iridipennis* Smith) were examined for the presence of LAB. The results indicated a rich diversity of LAB in the samples analysed, with 42 isolates belonging to six genera, viz., *Enterococcus* (23.8%), *Micrococcus* (18.8%), *Streptococcus* (13.8 %), *Pediococcus* (13.8 %), *Lactobacillus* (10.0%), *Lactococcus* (10.0 %) and *Leuconostoc* (10.0%). Their scope in disease management is discussed.

Introduction

Lactic acid bacteria (LAB) are a group of Gram-positive, non-spore forming coccior rods which produce lactic acid as the major end product of the fermentation of carbohydrates (Axelsson, 1998). They are generally recognized as safe (GRAS) food grade microorganisms exploited as probiotics that confer health benefits on the host (FAO/WHO, 2001). Commensals within humans, insects and animals, these bacteria are common inhabitants of guts. Species of the genera such as *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* are associated with foods (Stiles and Holzapfel, 1997). They vary in cell types and physiological and biochemical characteristics (Savadogo *et al.*, 2006). Honey bees possess a diverse LAB microbiota in their honey crop, acquired by consuming pollen and nectar and through contact with older bees of the colony. All the extant honey bee species possess *Lactobacillus* and

Bifidobacterium within the crop in biofilms (Vasquez *et al.,* 2012), with potential to inhibit American Foul Brood-causing *Paenibacillus larvae* growth on agar plate (Forsgren *et al.,* 2009). Especially, *Lactobacullus kunkeei* is predominantly found in *A. laboriosa* honey in Nepal (10^8cfu per gram honey), followed by *A. mellifera* honey in Africa. This fructophilic, osmotolerant and acid-resistant bacterium originates from the flower nectar and/or pollen grains collected and ingested by the bees and are found in honey bees in stored pollen and honey (Endo *et al.,* 2012; Rangberg *et al.,* 2012; Corby-Harris *et al.,* 2014). This species was also predominant in the honey stomach of *Apisdorsata* (Tajabadi *et al.,* 2011). Now-a-days honey bees all over the world are reported to be in stress, leading to colony collapse disorder (CCD) (Ellis *et al.,* 2015). Among the stressors, viral diseases, especially Thai sacbrood (TSBV), European foulbrood (EFB) and the parasitic mite *Varroajacobsoni* (Oudemans) -associated diseases (varroosis), are important in Indian bees, *Apis cerana* (Rao *et al.,* 2016; Mathialagan *et al.,* 2017). In the absence of effective control measures and in view of antibiotics residues in honey, alternative strategies are needed to control them. Prophylactic feeding of probiotic LAB isolated from the honey bee environment may help the bees overcome the stress and sustain their health. Therefore this study was undertaken to isolate, characterize and identify the diversity of LAB species from honey stomach, honey, bee pollen, bee bread and royal jelly.

Materials and Methods

Collection of samples

Laboratory investigations were conducted at the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore during 2016-2017. Workers of the Indian bee (*Apis ceranaindica* Fab.), Italian bee (*Apis mellifera* Lin.), Little bee (*Apis florea* Fab.), Rock bee (*Apis dorsata* Fab.) and Dammer bee (*Tetragonula iridipennis* Smith) were collected alive from different places. They were kept in a refrigerator freezer for five minutes before they were dissected to detach the honey stomach (crop) from the abdomen (Plate 1). The detached crop was transferred to a sterilized Eppendorf tube containing 1.0 ml sterile water and crushed for LAB isolation. Unprocessed honey samples were collected from the feral colonies of *A. ceranaindica, A. mellifera A. florea, A. dorsata*and *T. iridipennis* (Plate 2). Workers of *A. ceranaindica* were collected to extract the royal jelly by carefully pressing the head of the nurse bees (Plate 3). Fresh royal jelly was scooped out from the queen cell cups of the Indian bees (Plate 4). Commercially available dry royal jelly sample was donated by a firm (Plate 5). Samples were collected using a micropipette and dissolved in 1.5 ml sterile water in a sterilized Eppendorf tube. Bee bread samples were collected from various bee species (*A. ceranaindica, A. mellifera, A. florea, A. dorsata* and *T. iridipennis*) with the help of a sterilized micropipette tip (Plate 6). Commercial bee pollen of *A. mellifera* was received as a donation (Plate 7). Foraging workers of different bee species were collected from the hives to collect pollen grains from the hind legs. They were transferred to a 1.0 ml Eppendorf tube before serial dilution and plating in the laboratory.

Isolation and enumeration of LAB

LAB populations were enumerated by serial dilution and plate count technique with 1.0 ml sample serially diluted to 10^{-5}dilutions before plating in Lactobacillus MRS Agar medium for isolation (Awan and Rahman, 2005). Calcium carbonate ($CaCO_3$) was added to MRS medium to induce more LAB growth (Aween *et al.,* 2012). Cycloheximide was

added to the above medium to avoid fungal contamination. The plates were incubated at 30 ± 2°C. The colonies that matured in 3 days were counted and expressed as Colony Forming Units (CFU / ml). Morphologically distinct and well isolated colonies were picked and transferred to new MRS agar plates by streaking to obtain pure colonies that were maintained on glycerol stocks. The cultures were stored at - 80˚ C for further studies. Gram staining was done as per the protocol described by Sharma (2007). The stained slides were examined under themicroscope.

Identification of the isolates

All the isolates were tested for Gram reaction and catalase test as described by Harrigan and McCance (1976). The isolates were then identified with reference to Bergey's *Manual of Determinative Bacteriology*. Identification was based mainly on gram staining, absence of catalase, cultural and morphological characteristics (such as its elevation, shape, colour and texture of the colonies). *Lactobacilli* species were taxonomically classified following the discriminatory schemes of Kandler and Weiss (1986), Hammes *et al.,* (1992) and Liu *et al.,* (2014).

Results and Discussion

A random survey of honey bee niches for the presence of LAB indicated that different species of LAB are commonly associated with *A. ceranaindica, A. mellifera, A. florea, A. dorsata* and *T. iridipennis*. As many as 42 LAB isolates were isolated from the honey stomach, honey, bee pollen, bee bread and royal jelly collected from different sources and their morphological details with population densities are presented in Tables 1, 2, 3, 4 and 5. Morphologically, most isolates (N=34) were cocci and the rest (N=8) rod-shaped, grouped into seven LAB genera, namely, *Enterococcus* (23.8 %), *Micrococcus* (18.8%), *Streptococcus* (13.8%), *Pediococcus* (13.8%), *Lactobacillus* (10.0%), *Lactococcus* (10.0%) and *Leuconostoc* (10.0%) (Figure 1). Their populations varied from 16.40 – 35.87 x 10^5 CFU/ml in honey stomach (Table 1), 10.43 - 31.30 x 10^5 CFU/ml in honey (Table 2), 5.14 - 14.35 x 10^5 CFU/ml in bee pollen (Table 3), 13.05 - 25.15 x 10^5 CFU/ml in bee bread (Table 4) and 10.23 – 20.15 x 10^5 CFU/ml in royal jelly (Table 5). Heterofermentative, these LAB grew typically in MRS agar medium, producing protective exopolysaccharides. They were all gram positive, catalase negative and non-spore forming rods and cocci in singles, pairs, chains or tetrads (Plate 8, 9, 10). Species from the LAB genera *Pediococcus, Aerococcus, Tetrageono coccus* are all capable of division in two planes and form tetrads (Holzapfel and Wood, 2014). *Enterococcus* in singles, pairs, small chains or small groups are cosmopolitan associated with insect gut, fermented foods and drinking water (Lebreton *et al.,* 2014). Rod-shaped LAB were rarely found in the honey stomach and honey of *A. ceranaindica, A. mellifera* and *A. dorsata* and the bee pollen of *A. ceranaindica* at Coimbatore. Generally, LAB species may differ from place to place. Hosny *et al.,* (2009) isolated several species of *Lactobacillus, Streptococcus, Micrococcus, Enterococcus* and *Micrococcus* from honey. Strains of *Lactobacillus acidophilus* were isolated from honey samples by Aween *et al.,* (2012). *Lactobacillus* is commonly associated with honey bees even in India (Pattabhiramaiah *et al.,* 2012). Elsewhere *L. kunkeei* has been reported as the most common fructophilic species (Vasquez *et al.,* 2012). Earlier different species of bacteria have been reported from the pollen (Corby-Harris *et al.,* 2014) and honey stomach (Tajabadi *et al.,* 2011). Royal jelly from queen cell cups, nurse bee heads and a bee product of commerce also harboured LAB in them (10.23 – 20.15 x 10^5 CFU/ml). Isolation of LAB from the fourth instar larval gut in this study

assumes significance as the larval gut is the target of highly pathogenic bacteria and fungi. Both royal jelly and worker jelly (bee bread + gland secretions + diluted honey) play an important role in the nature of bacterial composition as reported by Vojvodic *et al.,* (2013) who found that first and second larval instars contained almost exclusively Acetobacteraceae, while later instars were dominated by one or two very different *Lactobacillus* spp. Acetobacteraceae includes acetic acid bacteria (AAB), a large group of obligate aerobic gram-negative bacteria, well adapted to sugary and alcoholised fluid such as vinegar, fruit juice, sap water, alcoholic beverages and flowers. AAB of the genera *Gluconobacter, Acetobacter, Gluconacetobacter* and *Saccharibacter* have been reported as symbionts of bees that may also regulate the innate immune system homeostasis in insects (Crotti *et al.,* 2010). The sugar-loving and flower-associated *Gluconobacters* are among the predominant bacterial groups in bees (Hamdi *et al.,* 2011). While both AAB and LAB may play a role in nutrient processing in adult bees, they contribute to larval immunity during the early and fragile stage of honey bee development (Vojvodic *et al.,* 2013). Royal jelly is considered highly antimicrobial, possessing a pH between 3.6 and 4.2, with many peptides active against gram-positive and gram-negative bacteria, fungi and yeasts. However, LAB isolated from royal jelly in this investigation may be related to its water content which is around 67 per cent (Wongchai and Ratanavalachai, 2002).

Frequent occurrence of LAB associated with honey bees strengthens the need to exploit them in honey bee health management strategy. Symbionts play a major role in maintaining the health of the host animal, including insects, especially honey bees. LAB is a key symbiotic micro-organism in bees, providing not only adequate nutrition but also host immunity against pathogens (Vasquez *et al.,* 2012). At present, acaricides and antibiotics are extensively used in apiculture to manage the ectoparasitic *Varroa* mite syndrome. This leads to resistance in mites and pathogens (Thomson *et al.,* 2002; Berry *et al.,* 2013).

Fig.1 Population density and percentage of isolates identified in the survey from different genera of lactic acid bacteria (LAB). Vertical bars indicate the standard error. CFU, colony forming units

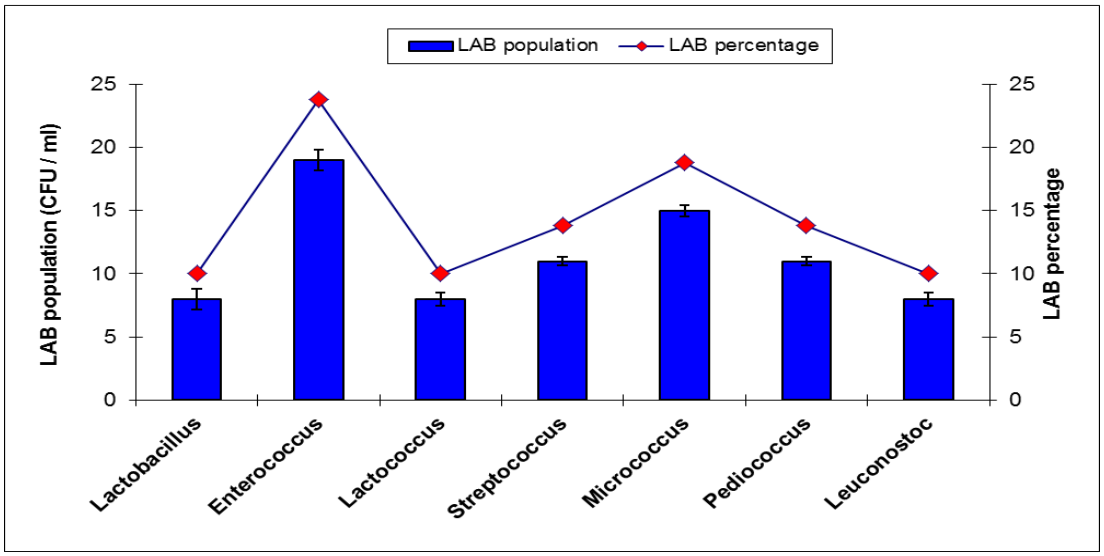

Table.1 Population and colony morphology of LAB in the honey stomach of honey bees

Bee species	Place of collection	Isolation source (caste)	Number of isolates	LAB (10^5 CFU/ml)	Colony morphology				Cell shape	Probable LAB genera
					Elevation	Colony texture	Edge	Colony colour		
A. ceranaindica	TNAU	Worker (healthy)	Honey stomach1	35.87 ± 10.76 (n = 3)	Raised	Coarse surface	Smooth	Whitish	Rods	*Lactobacillus*
			Honey stomach2	29.71 ± 8.54 (n = 3)	Flat	Shinny surface	Rough	Yellowish	Rods	*Lactobacillus*
			Honey stomach3	17.21 ± 5.90 (n = 3)	Flat	Coarse surface	Rough	Creamy	Cocci in singles	*Enterococcus Lactococcus Leuconostoc*
			Honey stomach4	15.41 ± 4.76 (n = 3)	Raised	Shinny mucoid surface	Smooth	Creamy	Cocci in chains	*Enterococcus Streptococcus Pediococcus*
			Honey stomach5	10.91 ± 2.90 (n = 3)	Flat	Shinny surface	Smooth	Whitish	Cocci in tetrads	*Micrococcus*
A. ceranaindica	TNAU	Worker (Diseased)	Honey stomach1	13.15 ± 2.25 (n = 3)	Flat	Coarse surface	Rough	Creamy	Cocci in singles	*Enterococcus Lactococcus Leuconostoc*
			Honey stomach2	9.50 ± 4.75 (n = 3)	Raised	Shinny mucoid surface	Smooth	Creamy	Cocci in chains	*Enterococcus Streptococcus Pediococcus*
A. florea	TNAU	Worker (healthy)	Honey stomach1	16.40 ± 5.65 (n = 3)	Flat	Shinny surface	Smooth	Whitish	Cocci in tetrads	*Micrococcus*
A. mellifera	TNAU	Worker (healthy)	Honey stomach1	24.67 ± 7.54 (n = 3)	Flat	Shinny surface	Smooth	Whitish	Cocci in tetrads	*Micrococcus*
			Honey stomach2	16.73 ± 5.64 (n = 3)	Raised	Coarse surface	Smooth	Whitish	Rods	*Lactobacillus*
A.dorsata	TNAU	Worker (healthy)	Honey stomach1	21.31 ± 3.80 (n = 3)	Flat	Shinny surface	Rough	Yellowish	Rods	*Lactobacillus*
T. iridipennis	TNAU	Worker (healthy)	Honey stomach1	14.78 ± 2.76 (n = 3)	Flat	Shinny surface	Smooth	Whitish	Cocci in tetrads	*Micrococcus*

(Mean ± SE; SE, standard error; n = number of replicates; CFU, colony forming units)

Table.2 Population and colony morphology of LAB in the unprocessed comb honey of honey bee species

Bee species	Place of collection	Number of isolates	LAB (10^5 CFU/ml)	Colony morphology				Cell shape	Probable LAB genera
				Elevation	Colony texture	Edge	Colony colour		
A. ceranaindica	TNAU	Honey 1	31.30 ± 6.61 (n = 3)	Flat	Coarse surface	Rough	Creamy	Cocci in singles	*Enterococcus Lactococcus Leuconostoc*
		Honey 2	25.28 ± 4.15 (n = 3)	Raised	Shinny mucoid surface	Smooth	Creamy	Cocci in chains	*Enterococcus Streptococcus Pediococcus*
		Honey 3	17.37 ± 3.90 (n = 3)	Raised	Coarse surface	Smooth	Whitish	Rods	*Lactobacillus*
A. mellifera	TNAU	Honey 1	18.20 ± 5.51 (n = 3)	Flat	Shinny surface	Smooth	whitish	Cocci in tetrads	*Micrococcus*
		Honey 2	15.10 ± 2.10 (n = 3)	Flat	Shinny surface	Rough	Yellowish	Rods	*Lactobacillus*
A. florea	TNAU	Honey 1	12.09 ± 2.41 (n = 3)	Flat	Coarse surface	Rough	Creamy	Cocci in singles	*Enterococcus Lactococcus Leuconostoc*
		Honey 2	8.23 ± 1.78 (n = 3)	Raised	Shinny mucoid surface	Smooth	Creamy	Cocci in chains	*Enterococcus Streptococcus Pediococcus*
A.dorsata	TNAU	Honey 1	15.37 ± 5.80 (n = 3)	Raised	Coarse surface	Smooth	Whitish	Rods	*Lactobacillus*
T. iridipennis	TNAU	Honey 1	10.43 ± 4.62 (n = 3)	Flat	Shinny surface	Smooth	whitish	Cocci in tetrads	*Micrococcus*

(Mean ± SE of 3 replicates; SE, standard error; CFU, colony forming units)

Table.3 Population and colony morphology of LAB in the bee pollen of honey bees

Bee species	Place of collection	Number of isolates	LAB (10^5 CFU/ml)	Colony morphology				Cell shape	Probable LAB genera
				Elevation	Colony texture	Edge	Colony colour		
A. ceranaindica	TNAU	Pollen 1	14.35 ± 2.31 (n = 3)	Raised	Shinny mucoid surface	Smooth	Creamy	Cocci in chains	*Enterococcus Streptococcus Pediococcus*
		Pollen 2	9.55 ± 4.45 (n = 3)	Flat	Shinny surface	Smooth	whitish	Cocci in tetrads	*Micrococcus*
		Pollen 3	6.14 ± 6.51 (n = 3)	Raised	Coarse surface	Smooth	Whitish	Rods	*Lactobacillus*
A. mellifera	TNAU	Pollen 1	11.21 ± 5.31 (n = 3)	Flat	Coarse surface	Rough	Creamy	Cocci in singles	*Enterococcus Lactococcus Leuconostoc*
	Commercial bee pollen (Dhalavaipuram)	Pollen 1	10.30 ± 2.86 (n = 3)	Flat	Shinny surface	Smooth	whitish	Cocci in tetrads	*Micrococcus*
A. florea	TNAU	Pollen 1	12.67 ± 6.43 (n = 3)	Flat	Shinny surface	Smooth	whitish	Cocci in tetrads	*Micrococcus*
		Pollen 2	8.70 ± 2.30 (n = 3)	Raised	Shinny mucoid surface	Smooth	Creamy	Cocci in chains	*Enterococcus Streptococcus Pediococcus*
A.dorsata	TNAU	Pollen 1	14.10 ± 7.51 (n = 3)	Flat	Shinny surface	Smooth	whitish	Cocci in tetrads	*Micrococcus*
T. iridipennis	TNAU	Pollen 1	5.14 ± 1.56 (n = 3)	Raised	Shinny mucoid surface	Smooth	Creamy	Cocci in chains	*Enterococcus Streptococcus Pediococcus*

(Mean ± SE of 3 replicates; SE, standard error; CFU, colony forming units)

Table.4 Population and colony morphology of LAB in the bee bread of honey bees

Bee species	Place of collection	Number of isolates	LAB (10^5 CFU/ml)	Colony morphology				Cell shape	Probable LAB genera
				Elevation	Colony texture	Edge	Colony colour		
A. ceranaindica	TNAU	Bee bread 1	25.15 ± 5.10 (n = 3)	Flat	Coarse surface	Rough	Creamy	Cocci in singles	*Enterococcus Lactococcus Leuconostoc*
		Bee bread 2	19.75 ± 7.23 (n = 3)	Raised	Shinny mucoid surface	Smooth	Creamy	Cocci in chains	*Enterococcus Streptococcus Pediococcus*
		Bee bread 3	10.32 ± 4.67 (n = 3)	Flat	Shinny surface	Smooth	whitish	Cocci in tetrads	*Micrococcus*
A. mellifera	TNAU	Bee bread 1	17.83 ± 5.43 (n = 3)	Flat	Coarse surface	Rough	Creamy	Cocci in singles	*Enterococcus Lactococcus Leuconostoc*
		Bee bread 2	10.56 ± 3.90 (n = 3)	Raised	Shinny mucoid surface	Smooth	Yellowish surround	Cocci in chains	*Enterococcus Streptococcus Pediococcus*
A. florea	TNAU	Bee bread 1	15.31 ± 5.71 (n = 3)	Flat	Coarse surface	Rough	Creamy	Cocci in singles	*Enterococcus Lactococcus Leuconostoc*
		Bee bread 2	9.11 ± 1.37 (n = 3)	Raised	Shinny mucoid surface	Smooth	Creamy	Cocci in chains	*Enterococcus Streptococcus Pediococcus*
A. dorsata	TNAU	Bee bread 1	21.32 ± 6.63 (n = 3)	Flat	Shinny surface	Smooth	whitish	Cocci in tetrads	*Micrococcus*
T. iridipennis	TNAU	Bee bread 1	13.05 ± 2.80 (n = 3)	Flat	Shinny surface	Smooth	whitish	Cocci in tetrads	*Micrococcus*

(Mean ± SE of 3 replicates; SE, standard error; CFU, colony forming units)

Table.5 Population and colony morphology of LAB in the royal jelly

Bee species	Place of collection	Isolation source	Number of isolates	LAB (10^5 CFU/ml)	Colony morphology				Cell shape	Probable LAB genera
					Elevation	Colony texture	Edge	Colony colour		
A. ceranaindica	TNAU	Queen cell cup	Royal jelly 1	10.23 ± 2.19 (n = 3)	Raised	Shinny mucoid surface	Smooth	Creamy	Cocci in chains	*Enterococcus Streptococcus Pediococcus*
A. ceranaindica	TNAU	Worker bee head	Royal jelly 1	14.30 ± 3.13 (n = 3)	Flat	Shinny surface	Smooth	whitish	Cocci in tetrads	*Micrococcus*
A. mellifera	Dhalavaipuram	Commercial bee product	Royal jelly 1	20.15 ± 2.50 (n = 3)	Flat	Shinny surface	Smooth	whitish	Cocci in tetrads	*Micrococcus*

(Mean ± SE; SE, standard error; n = number of replicates; CFU, colony forming units)

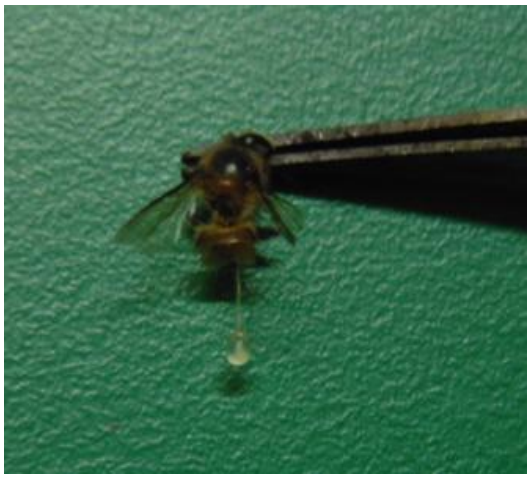

Plate.1 Detached honey stomach (crop) of *A. ceranaindica* worker bee.

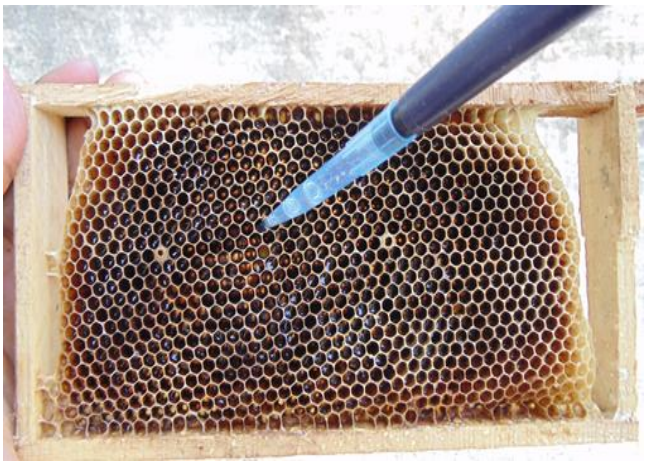

Plate.2 Collection of unprocessed honey in *A. ceranaindica*.

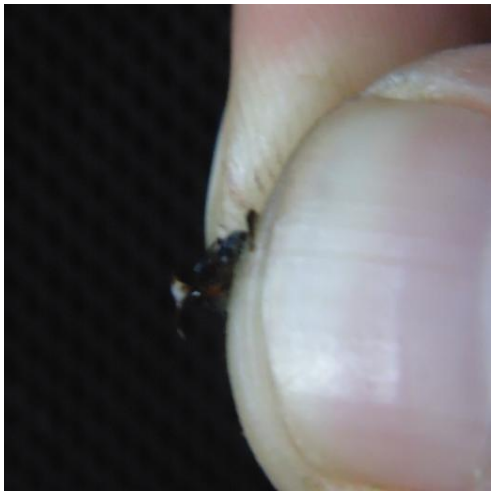

Plate.3 Extracting royal jelly by pressing the head of an *A. ceranaindica* nurse bee.

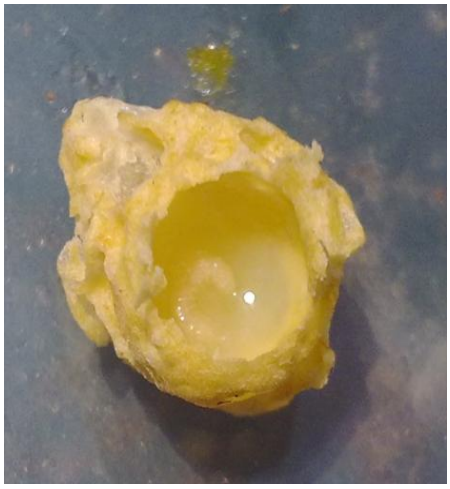

Plate.4 Royal jelly of *A. ceranaindica* in a queen cell cup before scooping it out.

Plate.5 Commercial sample of *A. melliefra* royal jelly (bee product)

Plate.6 Collecting the bee bread from an *A. ceranaindica* comb.

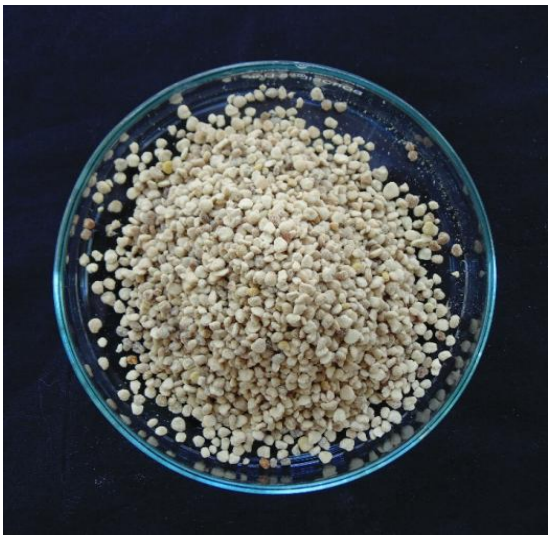

Plate.7 Commercial bee pollen of *A. mellifera*.

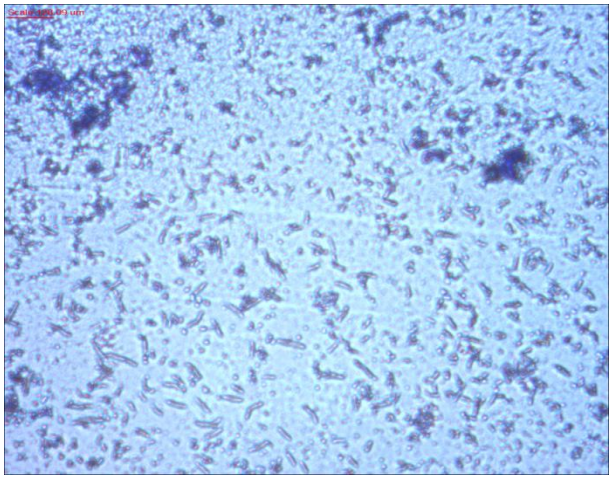

Plate.8 LAB in the honey stomach of *A. ceranaindica* worker bees. Cells were gram positive cocci and rods.

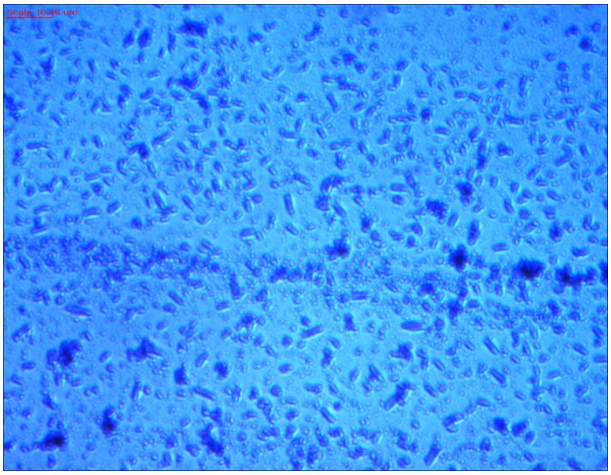

Plate.9 LAB in the honey bee stomach of *A. ceranaindica* worker bees. Cells were gram Positive cocci in singles and chains.

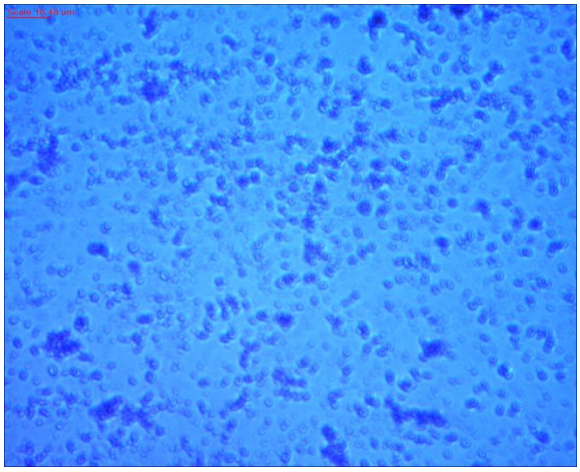

Plate.10 LAB in the honey stomach of *A. ceranaindica* worker bees. Cells were gram-positive cocci in tetrads

They also end up as residues in honey (Johnson and Jadon, 2010). The antibiotics may also kill the beneficial honey bee microflora that is part of the natural honey bee defence against pathogens, including probiotics (Yoshiama and Kimura, 2009), which produce antimicrobials (Hamdi *et al.*, 2011). Thus stress management strategies in honey bees need to focus on non-chemical and non-antibiotic measures such as probiotics (Moritz *et al.*, 2010). The results of this survey highlights the diversity of LAB associated with honey bees and their environment. As a non-chemical measure, dusting the bees with powdered table sugar has been reported to help them dislodge the mites by enhanced grooming (Fakhimzadeh, 2001; Conrad, 2013). Recently, it has been reported that powdered sugar acts a reservoir of probiotic LAB, probably suppressing diseases in bees through augmentation of LAB populations (Mathialagan, 2014; Mathialagan *et al.*, 2015). Prophylactic feeding of LAB isolated from the bee world will also help the bees resist the diseases from enhanced immunity.

Varroa infestation and both viral and bacterial diseases are key stressors in beekeeping that need urgent attention by the beekeepers in South India. In this background this investigation explored the diversity of LAB genera such as *Enterococcus*, *Lactobacillus*, *Micrococcus*, *Lactococcus*, *Streptococcus*, *Pediococcus*, and *Leconostoc* from the honey stomach, honey, bee pollen, bee bread and royal jelly collected from different bee species. However, these isolates need to be characterized and confirmed through molecular studies in future. Antibiotics, besides residue in honey, may cause elimination of symbiotic gut microbiota, or microbial dysbiosis. Key among them is the probiotic LAB that are significantly abundant in and around bees, overwhelmingly related to the well-being of honey bees. The symbiotic AAB are also likely to occur in association with LAB in honey bees. While the ecto parasitic *Varroa* mites and the diseases they transmit need to be controlled without pesticides and antibiotics, enriching the gut microbiota with prophylactic feeding of potential LAB strains as a bee feed will help the bees manage the stress from diseases.

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