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Research Article

Gut-associated *Enterococcus faecalis* from *Apis cerana indica*: Antibiotic resistance patterns and biocontrol potential against *Paenibacillus larvae*

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ABSTRACT: The strain-level functional variability within certain key bacterial taxa remains underexplored, despite the fact that the microbiota of the honeybee is critical to the host's overall health and ability to resist disease. This study examined some of the biochemical properties, the patterns of antibiotic resistance, and the possible antagonism of *Enterococcus faecalis* isolates from the gut of *Apis cerana indica*, drawn from two different ecological zones of the Western Ghats of India, Shivamogga (Shac) and Kodagu (Kac). The isolates were determined through the 16S rRNA sequence, supplemented with a biochemical profile. The patterns of multidrug resistance were distinct, with *Enterococcus faecalis* Shac displaying resistance to beta-lactams, macrolides, and glycopeptides, and *Enterococcus faecalis* Kac showing resistance to rifampicin and reduced sensitivity to vancomycin and ampicillin, but *E. faecalis* Kac had comparatively broader susceptibility. *E. faecalis* Kac also had a higher inhibition of *Paenibacillus larvae*, the bacteria responsible for American Foulbrood than *E. faecalis* Shac. The pronounced antagonistic activity of *E. faecalis* Kac against *P. larvae* highlights its potential as a candidate biocontrol agent for managing American foulbrood (AFB) disease, warranting further *in vivo* validation.

KEYWORDS: *Apis cerana indica*, *Enterococcus faecalis* strains (Shac and Kac), antibiotic susceptibility test, biochemical profile, antagonistic potential, *Paenibacillus larvae*

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INTRODUCTION

Honeybees are vital to the function of the global ecosystem by providing the service of pollinating wild plants and agricultural crops, and thus helping sustain biodiversity and food security (Motta & Moran, 2024). Over the last several decades, a decline in the population of honeybees has led to increased investigations of the causes of bee mortality, including the gut microbiome and how it is structured and functions (Yang *et al.*, 2023). The honeybee gut is a unique microbial ecosystem that serves important functions in host nutrition, immune function, development and defence against pathogens (Koch *et al.*, 2013; Engel & Moran, 2013).

Both social transmission and environmental exposure influence the gut microbiota of *A. cerana indica*, a eusocial insect arranged into intricate colonies. A variety of ecological niches are encountered by foraging worker bees, which makes it easier for them to pick up microorganisms and environmental pollutants such as pesticides, heavy metals, and bacteria that are resistant to antibiotics (Cilia, 2019). The honeybee gut microbiome is highly specialised and dominated by a conserved group of core bacterial taxa, including *Bifidobacterium, Gilliamella, Snodgrassella*, and lactic acid bacteria like *Lactobacillus* spp., despite its very simple composition (Zheng *et al.*, 2019; Kwong & Moran, 2016). Colony fitness and host physiology depend on these microbial populations.

Because of their probiotic potential and antibacterial action, Lactic Acid Bacteria (LAB), which include members of the genus *Enterococcus*, have drawn attention.

A variety of bioactive substances, including organic acids, hydrogen peroxide, and bacteriocins, are produced by LAB. These substances inhibit pathogenic microorganisms, such as *P. larvae*, which is the cause of American Foulbrood Disease (AFB), a fatal disease that affects honeybee broods (Ye *et al.*, 2023; Kacaniova *et al.*, 2018). However, the use of antibiotics in apiculture, especially tylosin and oxytetracycline, has disrupted the native gut microbiota and led to the emergence of resistant strains of the gut bacteria of honeybees, raising concerns about the spread of antimicrobial resistance (Raymann *et al.*, 2017).

Despite being known for its antibacterial qualities and its probiotic uses, *E. faecalis* shows strain - dependent variation in functional characteristics and may possibly contain genes that confer resistance to antibiotics. Therefore, assessing its usefulness in bee health management requires an awareness of its dual role as a potential repository of resistance determinants and a beneficial symbiont.

Enterococcus faecalis strain-level functional variation is still poorly understood despite a wealth of research on the gut microbiota of honeybees, especially with regard to antibiotic resistance and antagonistic potential against *P. larvae* in *A. cerana indica*. With little emphasis on connecting phenotypic traits like biochemical characteristics, antimicrobial resistance profiles, and biocontrol efficacy, the majority of current research has either concentrated on the composition of microbial communities using short-read sequencing or on the general probiotic properties of lactic acid bacteria. Additionally, there is still a lack of research on how ecological factors affect strain-level diversity and functionality in region-specific comparative analyses, particularly in India's Western Ghats.

In order to close this gap, the current study evaluates *E. faecalis* isolates from two ecologically diverse locations (Shivamogga and Kodagu) using biochemical characterisation, antibiotic susceptibility profiling, and antagonistic tests. A thorough foundation for comprehending strain-level diversity is provided by the combination of functional phenotypic studies with full-length 16S rRNA sequencing for accurate taxonomic resolution. Significant differences in antagonistic activity and resistance patterns between isolates are noteworthy in this investigation, indicating their potential as biocontrol agents against *P. larvae*. These results offer fresh perspectives on the dual function of honeybee-associated bacteria as potential candidates for long-term illness control in apiculture as well as reservoirs of antibiotic resistance. The goal of this work is to describe the antibiotic resistance profiles of *P. larvae* and *E. faecalis* isolates from the gut of *A. cerana indica* from two ecologically different locations (Shivamogga and Kodagu). Therefore, a detailed study was undertaken to determine the biocontrol potential of these isolates against American foulbrood disease in bees and also to assess their antagonistic capability against *P. larvae*. We hypothesised that *E. faecalis* strains isolated from geographically distinct populations of *A. cerana indica* exhibit strain - dependent variation in biochemical characteristics, antibiotic susceptibility profiles and antagonistic activity against *P. larvae* due to ecological and environmental differences between locations.

MATERIALS AND PROCEDURES

Honeybee sample collection and location details

The gut microbiome of *A. cerana indica* was examined by collecting approximately 100 adult worker bees from active and healthy colonies. The hives are situated in Shivamogga and Kodagu, districts in Karnataka, India, that are rich in biodiversity and are part of the Western Ghats. Bhadravathi (13.8276° N, 75.7064° E), Gajanore (12.3769° N, 77.1118° E), and Harakere (13.9077° N, 75.5611° E) were among the Shivamogga sampling sites, whereas Ponnampet (12.1515° N, 75.9430° E), B. Shettigere (13.104° N, 77.3959° E), and Chettalli (12.3702° N, 75.8309° E) were among the Kodagu sampling sites. A number of parameters, such as active foraging habit, the existence of nearby crops, balanced population dynamics, healthy brood patterns, the lack of pests and diseases, and sufficient food storage, were taken into consideration while choosing colonies (Joseph *et al.*, 2025).

A total of 300 bees were sampled; 30 adult forager bees were taken from three apiaries, each with three colonies, and deposited in sterile 50-mL conical tubes. RNAlater solution (Thermo Fisher Scientific, Waltham, MA, USA) was used to stabilise nucleic acids. Samples were transported to the Division of Genomic Resources, ICAR-NBAIR, Bengaluru, refrigerated with iceboxes and stored at −80°C for downstream processing.

Genomic DNA extraction and sample preparation

The cytochrome c oxidase subunit I (COI) gene was molecularly identified using COI-F (5′-GGTCAACAAATCATAAAGATATTGG-3′) and COI-R (5′- TAAACTTCAGGGTGACCAAAAATCA - 3′) as standard barcoding primers to verify bee species identity.

One representative bee from each replication was used for this purpose. Following BLASTn verification and sequencing of the resultant amplicons, the sequences were submitted to GenBank and assigned accession numbers (Districts of Shivamogga: Bhadravathi (OP763646), Gajanore (OP727527), and Harakere (OP782568); District of Kodagu: Ponnampet (OP782572), B. Shettigere (OP782573), and Chettalli (OP763749).

Bees were submerged in 70% ethanol for one minute, then rinsed twice for two minutes each in sterile distilled water and double-distilled water to get rid of surface impurities. Using sterile instruments and a UV-sterilised laminar airflow bench, the intestine was carefully dissected from the ventriculus to the rectum (Joseph *et al.*, 2025). Fifty dissected bee guts collected across three hives per apiary were pooled and homogenised in 1 mL of sterile Phosphate-Buffered Saline (PBS) in 1.5 mL Eppendorf tubes using sterile micropestles. To sediment coarse debris, the homogenates were briefly spun using a Tarsons Spinwin microcentrifuge. The supernatant was then used for culture-dependent bacterial isolation.

Isolation and culture of gut-associated bacteria

In accordance with the standard procedure of (Engel *et al.*, 2012), gut homogenates were serially diluted and plated on a variety of media, including Nutrient Agar (NA) M001-500G, Trypticase Soy Agar (TSA) M1968-500G, Luria Bertani Agar (LBA) GM2120-500G, Brain Heart Infusion agar (BHI) M211V-500G, Tomato Juice Agar (TJA) M048-500G, Rogosa Agar (RA) M1899-500G, and Man-Rogosa-Sharpe agar (MRS) M1923-500G. After preparing 1 L of media, they were autoclaved for 20 minutes at 121°C and then transferred into sterile Petri dishes. 50 µL aliquots of each dilution (10^0 to 10^{-10}) were plated and disseminated using sterile spreaders. Repeated sub-culturing was performed to procure pure cultures.

Sub-culturing of *Paenibacillus larvae*

From the microbial type culture collection (Chandigarh, India), *P. larvae* strain MTCC-7660 was procured. The procured strain was cultured on (Muller - Hinton broth, Yeast extract, Potassium phosphate, Glucose and Pyruvate) MYPGP agar plates to check the purity (Alippi & Reynaldi, 2006a) at 37 ˚C.

Isolation and identification of Lactic Acid Bacteria (LAB)

To isolate the Lactic acid bacteria, we followed Gouda *et al.*, 2024 procedure with minor modifications. The gut homogenates were plated on MRS agar supplemented with L-cysteine and CaCO3; cycloheximide was added to inhibit the growth of the fungi. The plates were incubated for a period of 72 h in an anaerobic chamber at 30 ± 2°C. The obtained LAB were stored at - 80°C in LB broth supplemented with 30% glycerol.

Microscopic analysis and gram staining of bacterial isolates

Colonies were chosen based on morphological traits and verified by Gram staining after plates were incubated for 24 to 48 h at 37°C. The procedure for Gram staining is as follows: in a sterile setting, bacterial isolates were applied to a sanitised glass slide and heat-fixed. For staining, crystal violet was applied for 30 s, followed by a rinse with distilled water. Gram's iodine (Sigma-Aldrich, USA) was applied for 32 s, and for the second time, a smooth wash with slow tap water was performed. 95% acetone alcohol was applied to a slide for 5 - 7 s in order to decolourise it. Following washing, 45 s of counterstain safranin was applied to the slide, followed by a final rinse with tap water. The slide was air-dried and observed under a microscope at 100X magnification.

Extraction of bacterial DNA and subsequent PCR amplification

Single colonies were grown in their separate broths at 37°C for 24 h. A modified CTAB technique was utilised to extract DNA from bacterial pellets generated by centrifugation at 13,000 rpm using the Msangosoko *et al.*, 2020 protocol. Concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA), and DNA quality was assessed using 1.2% agarose gel electrophoresis. Primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-AAGGAGGTGATCCAGCCGCA-3′) were used to amplify the 16S rRNA gene for each isolate. A 25 µL PCR reaction was performed using 5 µL of DNA, 12.5 µL of Emerald Master Mix (Takara Bio India), 1.5 µL of each primer (10 µM), and RNase-free water. The PCR cycling conditions included an initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 10 min. Amplicons were evident on 2% EtBr-stained agarose gels using the Bio-Rad Gel Doc Go imaging instrument.

Assessment of antibiotic susceptibility of bacterial isolates

Antimicrobial resistance assay using the *E. faecalis* strains (Shac) and (Kac) were performed against Cefalexin

(30 mcg), Vancomycin (30 mcg), Norfloxacin (10 mcg), Cefazolin (30 mcg), Erythromycin (15 mcg), Tetracycline (30 mcg), Gentamycin (10 mcg), Chloramphenicol (30 mcg), Ampicillin (10 mcg), Enrofloxacin (10 mcg), Lincomycin (2 mcg), and Rifampicin (5 mcg) (Oxoid, Basingstoke, UK). After being cultivated on Muller-Hinton agar for 24 h at 37°C, the bacterial strains were suspended in sterile distilled water and tested to match the cells to about 10^5 cells/mL (A620 = 0.388, equivalent to a McFarland standard). The bacterial suspension was uniformly spread on the sterilized Muller-Hinton agar plates onto which different antibiotics disc was placed using sterile forceps. Following incubation, the diameter of the inhibitory zones was assessed. For every isolate strain, three replicates were examined. (Al-Waili *et al.*, 2012).

The antimicrobial activity of the tested antibiotics was evaluated based on the measured inhibition zone diameters. Since standardised EUCAST interpretive criteria are not available for all antibiotics and bacterial species included in this study—particularly *Paenibacillus larvae* and several antibiotics tested against *Enterococcus faecalis*—the results were interpreted as a comparative assessment of the relative antimicrobial sensitivity of the isolates rather than assigning susceptibility categories (Susceptible/Intermediate/ Resistant; S/I/R). (Matuschek *et al.*, 2014).

Antagonistic activity testing

The suspension of *P. larvae* (vegetative cells) is normally adjusted to 0.5 McFarland turbidity and evenly dispersed over an MRS agar plate. A sterilised cork borer (about 5 - 8 mm in diameter) is used to punch holes in the inoculated agar. The bored wells are filled with 100 µL of *E. faecalis* (Shac and Kac) suspension adjusted to 6.0×10^8 CFU/mL (2.0 according to the McFarland Standard). Three replicates of each isolate strain were investigated. The plates are incubated at 35 - 37°C for 24 - 48 h to allow the antimicrobial chemicals to diffuse into the agar and suppress the bacteria. The diameter of the transparent halo surrounding the well is measured in millimetres (Sabate *et al.*, 2009).

RESULTS

Growth and cultivation of Lactic Acid Bacteria (LAB)

Morphological characterisation of the culturable LAB communities isolated from the gut of *A. cerana indica* revealed notable variations in colony texture and colony colour. Subsequent 16S rRNA gene sequencing identified the isolates as members of the genus *Enterococcus*, specifically *Enterococcus faecalis*, a LAB associated with honeybee gut microbiota from Shivamogga, depicted as *Enterococcus faecalis* Shac, where the colony morphology is as follows: Elevation - Raised; Colony Texture - Shiny; Edge - Smooth; Colony colour - White; Gram nature - Gram positive; Accession Number - PQ120881. And Kodagu, depicted as *Enterococcus faecalis* Kac, where the colony morphology is as follows: Elevation - Raised; Colony Texture - Coarse; Edge - Smooth; Colony colour - Creamy; Gram nature - Gram positive; Accession Number - PQ236994. At the phylum level, the isolates were classified under *Bacillota* (formerly *Firmicutes*), class *Bacilli*, order *Lactobacillales*, family *Enterococcaceae*, and genus *Enterococcus*.

Biochemical characterisation of the bacterial isolates

Biochemical characterisation of the isolates was performed to determine their metabolic and enzymatic properties, which are essential for accurate identification and differentiation of microorganisms. These tests provide information on the ability of the isolates to utilise specific substrates and produce characteristic enzymes, thereby helping to distinguish closely related species. Furthermore, biochemical profiling supports the confirmation of preliminary identification and contributes to the taxonomic classification and functional assessment of the isolate. The biochemical test results for the following isolates are as follows:

***Enterococcus faecalis* Shac**

The biochemical characterisation of *E. faecalis* Shac was performed using standard biochemical assays to confirm its metabolic profile. The isolate tested negative for indole and citrate utilisation. Whereas, positive reactions for methyl red and Voges-Proskauer tests. In the case of carbohydrate fermentation, tests revealed that *E. faecalis* Shac was capable of fermenting glucose, adonitol, lactose, mannitol, rhamnose and sucrose, as indicated by positive reactions. In the case of fermentation of arabinose and sorbitol, there were negative results for these sugars. The overall biochemical profile (Table 1; Figure 1) of the Shac strain confirms its metabolic versatility and aligns with the established phenotypic characteristics of *E. faecalis*, confirming the identity of the isolate as *E. faecalis* Shac.

***Enterococcus faecalis* Kac**

The biochemical characterisation of *E. faecalis* Kac was carried out using a series of standard biochemical assays to confirm its metabolic profile. The isolate tested negative for indole and citrate utilisation. Whereas, in the case of both methyl red and Voges-Proskauer tests, it was tested

to be positive. In the case of carbohydrate fermentation tests, *E. faecalis* Kac was capable of fermenting glucose, adonitol, lactose, sorbitol, mannitol, rhamnose, and sucrose, as indicated by positive reactions. In contrast, arabinose fermentation was negative, suggesting the strain's inability to metabolise this carbohydrate. The overall biochemical

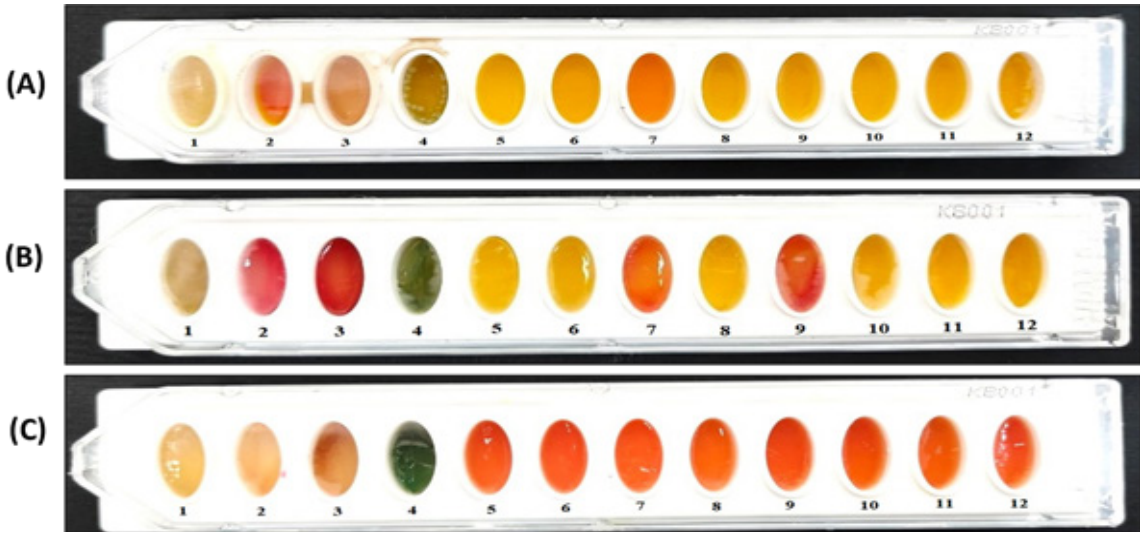


Figure 1. Biochemical identification strip showing results of 12 standard tests for bacterial characterisation. Tests include **(1)** Indole, **(2)** Methyl Red, **(3)** Voges-Proskauer, **(4)** Citrate utilisation, and carbohydrate fermentation assays comprising **(5)** glucose, **(6)** adonitol, **(7)** arabinose, **(8)** lactose, **(9)** sorbitol, **(10)** mannitol, **(11)** rhamnose, and **(12)** sucrose. Colour changes indicate positive reactions according to standard biochemical interpretation. **(a)** Biochemical profile of *E. faecalis* Kac, **(b)** Biochemical profile of *E. faecalis* Shac, **(c)** Biochemical profile of *P. larvae*.

Table 1. Phenotypic characterisation of bacterial isolates (*E. faecalis* Shac, *E. faecalis* Kac, and *P. larvae*) based on IMViC and carbohydrate fermentation profiles

S. No.	Biochemical test	*E. faecalis* Shac strain		*E. faecalis* Kac strain		*Paenibacillus larvae*	
		Positive	Negative	Positive	Negative	Positive	Negative
1	Indole		✓		✓		✓
2	Methyl red	✓		✓			✓
3	Voges Proskauer	✓		✓			✓
4	Citrate		✓		✓		✓
5	Glucose	✓		✓			✓
6	Adonitol	✓		✓			✓
7	Arabinose		✓		✓		✓
8	Lactose	✓		✓			✓
9	Sorbitol		✓	✓			✓
10	Mannitol	✓		✓			✓
11	Rhamnose	✓		✓			✓
12	Sucrose	✓		✓			✓

profile (Table 1; Figure 1) is consistent with the typical biochemical characteristics of *E. faecalis*, confirming the identity of the isolate as *E. faecalis* Kac.

Paenibacillus larvae

The biochemical characterisation of the isolated *P. larvae* strain revealed a uniform pattern of negative reactions across all tests performed. The isolate tested negative for indole, methyl Red, Voges-Proskauer, and citrate utilisation, indicating the absence of indole production, mixed-acid fermentation, acetoin formation and citrate metabolism. In the carbohydrate fermentation assays, the isolate did not utilise any of the tested sugars, including glucose, adonitol, arabinose, lactose, sorbitol, mannitol, rhamnose, and sucrose. The lack of carbohydrate metabolism suggests a metabolically restricted profile for the strain. The overall biochemical profile is presented in (Table 1; Figure 1).

Antibiotic resistance of the bacterial isolates

***Enterococcus faecalis** Shac*

The antibiotic susceptibility profile of *E. faecalis* Shac, isolated from the gut of *A. cerana indica* collected in Shivamogga, was evaluated using the disc diffusion assay (Raymann *et al.*, 2018). The isolate exhibited a differential response to the twelve antibiotics tested. The highest zones of inhibition were observed for Norfloxacin (25.33 ± 0.57 mm), followed by Chloramphenicol (23.66 ± 1.52 mm), Enrofloxacin (22.00 ± 1.00 mm), and Tetracycline (21.66 ± 0.57 mm), indicating strong antibacterial susceptibility. Moderate sensitivity was recorded for Gentamycin (18.33 ± 1.15 mm) and Cefazolin (15.33 ± 1.52 mm). In contrast, no inhibition was observed for Cefalexin, Ampicillin, Lincomycin, Rifampicin, Vancomycin, and Erythromycin, indicating complete resistance to these antibiotics. Overall, the susceptibility profile (Table 2) demonstrates that *E. faecalis* Shac is highly sensitive to fluoroquinolones and phenicol, moderately susceptible to aminoglycosides and cephalosporins, and resistant to β-lactams, macrolides, and glycopeptides.

***Enterococcus faecalis** Kac*

The antibiotic susceptibility profile of *E. faecalis* Kac, isolated from the gut of *A. cerana indica* collected in Kodagu, was evaluated using the disc diffusion assay against twelve antibiotics. The isolate exhibited variable levels of susceptibility across the tested antibiotics. The highest zones of inhibition were observed for Erythromycin (22.66 ± 0.57 mm), followed by Cefalexin (20.66 ± 0.57 mm), Chloramphenicol (20.66 ± 0.57 mm), and Enrofloxacin (20.66 ± 0.57 mm), indicating strong antibacterial susceptibility. Moderate inhibition was recorded for Lincomycin (18.66 ± 0.57 mm), Gentamycin (18.33 ± 0.57 mm), Norfloxacin (18.33 ± 0.57 mm), and Cefazolin (16.66 ± 0.57 mm). Reduced susceptibility was observed for Ampicillin (11.33 ± 0.57 mm) and Tetracycline (11.33 ± 0.57 mm), while Vancomycin exhibited minimal inhibitory activity (9.33 ± 1.15 mm). No zone of inhibition was observed for Rifampicin, indicating complete resistance. Overall, the susceptibility profile (Table 2) demonstrates that *E. faecalis* Kac is highly sensitive to macrolides, fluoroquinolones, phenicols, and certain β-lactam antibiotics (*e.g.*, Cefalexin), while resistance is evident toward Rifampicin and reduced susceptibility toward Vancomycin, Ampicillin, and Tetracycline.

Paenibacillus larvae

The antibiotic susceptibility profile of *P. larvae* was determined using the disc diffusion assay against twelve antibiotics. The bacterium exhibited variable levels of susceptibility across the tested antibiotics. The highest zones of inhibition were observed for Enrofloxacin (22.6 ± 0.57 mm) and Erythromycin (22.00 ± 1.00 mm), indicating strong antibacterial susceptibility. Cefalexin (22.69 ± 0.57 mm) and Gentamycin (22.66 ± 1.15 mm) also demonstrated pronounced inhibitory effects. Moderate susceptibility was recorded for Vancomycin (19.00 ± 1.00 mm), Chloramphenicol (18.66 ± 0.57 mm), Norfloxacin (19.35 ± 0.57 mm), and Tetracycline (16.66 ± 0.57 mm). Reduced sensitivity was observed for Ampicillin (16.33 ± 1.15 mm), Rifampicin (10.66 ± 0.57 mm), and Cefazolin (9.33 ± 1.15 mm). No zone of inhibition was observed for Lincomycin, indicating complete resistance. Overall, the susceptibility profile (Table 2) demonstrates that *P. larvae* is highly sensitive to fluoroquinolones and macrolides, moderately susceptible to aminoglycosides, glycopeptides, and phenicols, and exhibits resistance to certain β-lactam antibiotics (*e.g.*, Ampicillin and Cefazolin), with complete resistance to Lincomycin.

Antimicrobial activity of the Lactic Acid Bacteria (LAB) against *P. larvae*

Antimicrobial activity of two *E. faecalis* isolates (Shac and Kac) against *P. larvae* was quantified as the diameter of inhibition zones (mean ± SD, n = 3). *E. faecalis* Kac produced a substantially larger inhibition zone (28.67 ± 0.58) than *E. faecalis* Shac (19.67 ± 0.58). On performing Welch's t-test to compare the mean inhibition zones between the two strains, *E. faecalis* Kac showed a significantly greater antagonistic effect than *E.*

Table 2. Comparative antibiotic susceptibility profiles of *E. faecalis* Shac, *E. faecalis* Kac, and *P. larvae* based on Kirby-Bauer disk diffusion assay (zone of inhibition, Mean ± SD, mm)

S. No.	Antibiotic	Inhibition zone (in mm) of *E. faecalis* Shac	Inhibition zone (in mm) of *E. faecalis* Kac	Inhibition zone (in mm) of *P. larvae*
1	Cefalexin CN	0.00 (ND)	20.66 ± 0.57 (ND)	20.69 ± 0.57 (ND)
2	Ampicillin AMP	0.00 (R)	11.33 ± 0.57 (R)	16.33 ± 1.15 (ND)
3	Norfloxacin NX	25.33 ± 0.57 (ND)	18.33 ± 0.57 (ND)	19.35 ± 0.57 (ND)
4	Chloramphenicol C	23.66 ± 1.52 (ND)	20.66 ± 0.57 (ND)	18.66 ± 0.57 (ND)
5	Tetracycline TE	21.66 ± 0.57 (ND)	11.33 ± 0.57 (ND)	16.66 ± 0.57 (ND)
6	Gentamycin GEN	18.33 ± 1.15 (ND)	18.33 ± 0.57 (ND)	22.66 ± 1.15 (ND)
7	Lincomycin L	0.00 (ND)	18.66 ± 0.57 (ND)	0.00 (ND)
8	Cefazolin CZ	15.33 ± 1.52 (ND)	16.66 ± 0.57 (ND)	9.33 ± 1.15 (ND)
9	Enrofloxacin EX	22.00 ± 1.00 (ND)	20.66 ± 0.57 (ND)	22.6±0.57 (ND)
10	Rifampicin RIF	0.00 (ND)	0.00 (ND)	10.66 ± 0.57 (ND)
11	Vancomycin VA	0.00 (R)	9.33 ± 1.15 (R)	19.00 ± 1.00 (ND)
12	Erythromycin E	0.00 (ND)	22.66 ± 0.57 (ND)	22.0 ± 1.00 (ND)

Note: R-Resistant; ND- Not Defined by EUCAST

Table 3. Antagonistic activity of *Enterococcus faecalis* isolates (Shac) & (Kac) against *P. larvae* determined by agar diffusion assay. Values are expressed as mean inhibition zone diameter ± standard deviation (mm)

S. No.	Beneficial bacterial Isolate	Inhibition zone against *P. larvae* (mm)
1	*Enterococcus faecalis* Shac	19.67 ± 0.58
2	*Enterococcus faecalis* Kac	28.67 ± 0.58

faecalis Shac (Table 3). The standardised effect size was large (Cohen's $d \approx 16$), indicating a pronounced difference in antagonistic activity between the strains. These results indicate that *E. faecalis* Kac (Figure 3) is markedly more antagonistic to *P. larvae* than *E. faecalis* Shac (Figure 2) under the conditions tested, where Welch's two-tailed t-test demonstrated a highly significant difference between the two isolates ($t = 19.005$, $df = 4.00$, $p = 0.000045$), with a mean difference of 9.00 mm (95% CI: 7.69-10.31 mm).

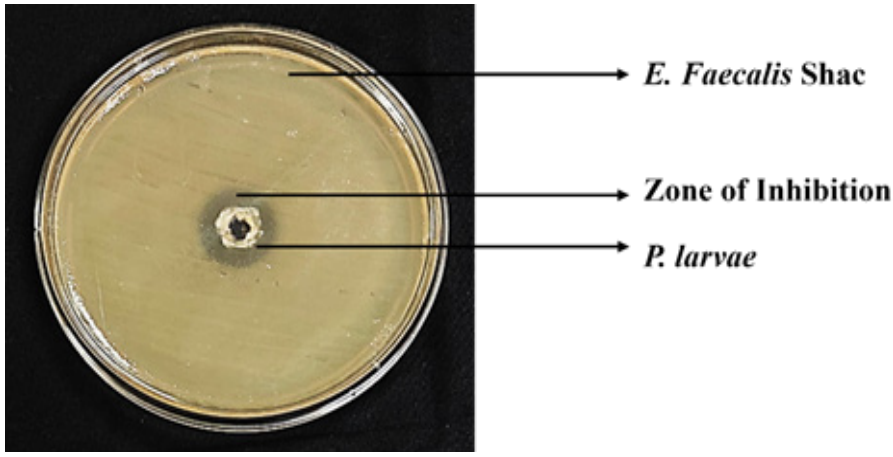


Figure 2. Antimicrobial activity of *E. faecalis* Shac isolated from the gut of *A. cerana indica* against *P. larvae.* A clear zone of inhibition surrounding the central well indicates the suppression of *P. larvae* growth due to antimicrobial compounds produced by the isolate. The diameter of the inhibition zone was measured to evaluate the antagonistic potential.

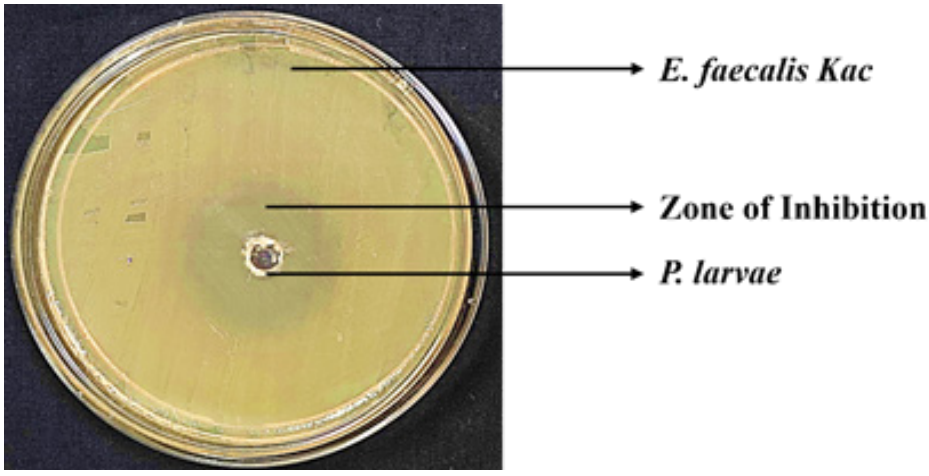


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DISCUSSION

The present study provides a detailed functional characterisation of culturable gut-associated bacteria from *A. cerana indica*, with emphasis on strain-level variability in biochemical properties, antibiotic susceptibility, and antagonistic potential against *P. larvae*, the etiological agent of AFB. American Foul Brood is the most lethal and contagious bacterial illness that affects honeybees worldwide. It is caused by the spore-forming bacterium *P. larvae* and is harmful since the spores can last for decades. AFB is recognised as a highly serious illness that endangers apiculture and worldwide agriculture due to the unavoidable colony collapse (Stephan *et al.*, 2020).

The obtained results advance our knowledge of the gut microbiota of honeybees as a possible source of antibiotic resistance as well as a storehouse of advantageous symbionts.

The isolation of LAB from the *Enterococcus* genus, specifically *E. faecalis*, is consistent with prior research characterising LAB as important constituents of the

honeybee gut microbiome (Engel & Moran, 2013; Kwong & Moran, 2016). These bacteria are recognised to have important roles in host feeding, immunological regulation, and pathogen defence. The biochemical profiles of both isolates (Shac and Kac), which include positive methyl red and Voges-Proskauer reactions as well as broad carbohydrate fermentation capabilities, are consistent with the known phenotypic characteristics of *E. faecalis*. However, reasonable changes in carbohydrate utilisation, such as sorbitol fermentation, reveal strain-level heterogeneity, which is common among *Enterococcus* species and reflects underlying genetic and metabolic diversity (Hollenbeck & Rice, 2012).

In contrast, *P. larvae* exhibited a metabolically restricted biochemical profile, with negative reactions across all tested assays. This observation is consistent with earlier studies describing *P. larvae* as a specialised pathogen with limited metabolic flexibility, adapting itself to the nutrient environment of the honeybee larval gut (Genersch, 2010). The absence of carbohydrate fermentation further supports its pathogenic lifestyle rather than a symbiotic role.

The antibiotic susceptibility profiles showed marked differences not only between species but also between *E. faecalis* strains procured from different geographical locations. *E. faecalis* Shac exhibited high sensitivity to fluoroquinolones (Norfloxacin, Enrofloxacin) and phenicol (Chloramphenicol), while demonstrating complete resistance to β-lactams, macrolides, and glycopeptides. In contrast, *E. faecalis* Kac showed broader susceptibility, including sensitivity to macrolides (Erythromycin) and certain β-lactams (Cefalexin). These findings highlight significant strain-level variability, which may arise due to differences in environmental exposure, selective pressures, and horizontal gene transfer of resistance determinants. Such variability in antibiotic response among *Enterococcus* strains has been widely documented and is often attributed to intrinsic resistance mechanisms, including altered penicillin-binding proteins, efflux pumps, and acquisition of resistance genes (Hollenbeck & Rice, 2012; Andersson & Hughes, 2014).

The antibiotic susceptibility pattern of *P. larvae* observed in this study further supports its sensitivity to fluoroquinolones and macrolides, while exhibiting resistance to lincomycin and reduced susceptibility to β-lactam antibiotics. These results are in agreement with previous studies reporting variable antibiotic sensitivity in *P. larvae* and raising concerns about emerging resistance due to prolonged antibiotic usage in apiculture (Alippi & Reynaldi, 2006). The widespread use of antibiotics such as oxytetracycline and tylosin has been shown to disrupt the native gut microbiota and promote the development of resistant strains, posing a significant challenge for sustainable disease management (Raymann *et al.*, 2017).

A key finding of the present study is the prominent antagonistic activity of *E. faecalis* isolates against *P. larvae*, with *E. faecalis* Kac exhibiting significantly greater inhibitory activity compared to Shac. This difference, supported by a large effect size (Cohen's $d \approx 16$), indicates strong functional divergence between the strains. The enhanced antagonistic potential of Kac may be attributed to increased production of antimicrobial compounds such as bacteriocins, organic acids, or hydrogen peroxide, which are characteristic of LAB and have been previously reported to inhibit *P. larvae* (Forsgren *et al.*, 2010; Daisley *et al.*, 2020). These findings reinforce the concept that not all strains within a species exhibit equal probiotic or biocontrol potential.

The observed differences between *E. faecalis* Shac and Kac isolates highlight the role of environmental and ecological factors in influencing microbial function. The two isolates were from geographically separate locations (Shivamogga and Kodagu) of the Western Ghats, with varying meteorological conditions, vegetation, and anthropogenic impacts such as pesticide exposure. Such environmental differences could have operated as selective forces, driving adaptability, metabolic diversity, and resistance acquisition in gut-associated bacteria.

Collectively, the results highlight the dual role of honeybee gut microbiota as both beneficial symbionts and potential reservoirs of antibiotic resistance. While *E. faecalis* Kac demonstrates antagonistic activity against *P. larvae*, its application as a probiotic agent requires careful evaluation due to the known opportunistic pathogenicity and resistance potential of *Enterococcus* species. Therefore, further studies involving whole-genome sequencing, identification of antimicrobial compounds, and *in vivo* validation in honeybee colonies are essential to assess safety, efficacy, and practical applicability.

To summarise, this study emphasises the significance of strain-level functional characterisation in understanding the ecological and applied roles of honeybee gut bacteria. The discovery of *E. faecalis* Kac as an antagonist against *P. larvae* opens up new opportunities for the development

of long-term, microbiome-based strategies for controlling American foulbrood in apiculture.

The present study evaluated only two culturable *E. faecalis* isolates recovered from geographically distinct locations against *P. larvae* of *A. cerana indica*. The analysis was restricted to phenotypic characterisation, antibiotic susceptibility testing, and *in vitro* antagonistic assays. Whole genome sequencing, characterisation of antimicrobial metabolites, and colony-level validation under field conditions were beyond the scope of the study. Therefore, the probiotic and biocontrol potential of the isolates should be interpreted cautiously until further genomic and *in vivo* investigations are conducted.

CONCLUSION

This study provides comprehensive insights into the biochemical characteristics, antibiotic resistance profiles, and antagonistic potential of gut-associated bacteria isolated from *A. cerana indica*. The identification of *E. faecalis* strains (Shac and Kac) and *P. larvae* was strongly supported by phenotypic and metabolic analyses, highlighting the functional diversity of honeybee gut microbiota. Notably, both *E. faecalis* isolates exhibited Multidrug-Resistant (MDR) traits, underscoring the role of environmental and anthropogenic pressures in shaping the resistome of bee-associated bacteria.

Importantly, the superior antagonistic activity of *E. faecalis* Kac against *P. larvae* demonstrates its potential as a promising biocontrol candidate for the management of AFB. However, the detection of resistance traits, including possible vancomycin resistance, raises concerns regarding the safe application of such strains. The observed strain-level variability between isolates from Shivamogga and Kodagu further emphasises the influence of ecological factors on microbial adaptation and functional traits.

Overall, these findings highlight the dual role of honeybee gut microbiota as both a reservoir of antimicrobial resistance and a source of beneficial probiotic candidates. Future studies integrating genomic, functional, and *in vivo* validation approaches are essential to evaluate the safety, stability, and efficacy of these isolates before their application in sustainable apicultural disease management strategies.

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AUTHOR CONTRIBUTION

Jenifer Sheeba Joseph: Conceptualisation; Writing - original draft; Methodology; Validation; Visualisation; Software; Formal analysis; Data curation; Investigation; T. C. Suman: Methodology; Formal analysis; Software; Gandhi Gracy Ramasamy: Supervision; Resources; Gopalakrishnan Menon: Supervision; Visualisation. Venkatesan Thiruvengadam: Conceptualisation; Investigation; Project administration; Writing - review and editing; Resources; Supervision.