

## Isolation and functional characterisation of honey bee gut bacteria with probiotic, antimicrobial, and antioxidant properties

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Probiotics are live microorganisms that confer health benefits to the host, particularly by modulating the gut microbiota. Honey bees possess a unique gut microbiome that may harbour novel probiotic strains with potential therapeutic applications. This study isolated and characterised probiotic bacteria from the gut of two honey bee species (*Apis mellifera* and *Tetragonula iridipennis*). *Lactobacillus casei* Shirota (Yakult) was used as a probiotic positive control. Out of the total isolates, 12 were analysed for probiotic traits, including bile and acid tolerance, starch and arginine hydrolysis, antimicrobial activity, beta-galactosidase activity, antioxidant activity, and adhesion ability. All isolates were negative for amylase and arginine dihydrolase but positive for Bile Salt hydrolase. Every isolate showed antibacterial activity, with three of them being more promising. Only 7 isolates demonstrated antifungal activity. Ten isolates were positive for  $\beta$ -galactosidase. Four isolates exhibited antioxidant activity comparable to that of the *Lactobacillus casei* Shirota. The control strain exhibited a hydrophobicity of 73.54%, while the highest hydrophobicity of 43.80% was observed in one of the gut isolates. Nine isolates exhibited apparent inhibitory effects against *E. coli* in non-neutralised supernatant, confirming that lactic acid is a potential antimicrobial substance. The isolates demonstrate potential probiotic properties based on *in vitro* evaluations, comparable to those of *Lactobacillus casei* Shirota.

**Keywords:** *Apis mellifera*, Honey bee gut, Lactic acid bacteria, Probiotic properties, Probiotics, *Tetragonula iridipennis*

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### Introduction

Probiotics are living microorganisms that, when administered in adequate amounts, contribute to improved health and well-being. Probiotic species typically belong to groups such as *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Bacillus*, and *Enterococcus*. Some yeast strains, such as *Saccharomyces boulardii*, are also recognised for their probiotic properties. Among these, *Lactobacillus* and *Bifidobacterium* are the most widely studied, as they naturally inhabit the gastrointestinal tract and play a vital role in regulating gut microbiota and enhancing the immune system<sup>1</sup>.

Honey bees are key pollinators that can produce and store honey and other beneficial substances. Honey bee gut plays an important role in honey bee health and the potential for their microbiomes to influence human health. Its gut bacteria help with digestion, nutrient absorption, and immune system function, and studies are exploring their potential for human probiotic

application<sup>2</sup>. The honey bee's gut microbiota is necessary for maintaining its health, providing nutrition and protection against pathogens. The unique microbial composition of honey, influenced by the bee gut microbiota and environment, holds potential prebiotic and probiotic benefits for human health<sup>3</sup>.

Probiotics contribute to maintaining a balanced intestinal environment, especially when the microbiota is disturbed by infections, antibiotics, or poor dietary habits. They support gut health by outcompeting harmful bacteria for nutrients and attachment sites, secreting antimicrobial compounds such as organic acids and bacteriocins, and modulating the body's immune response<sup>4</sup>. Certain strains also produce the enzyme  $\beta$ -galactosidase, which helps digest lactose and alleviates symptoms of lactose intolerance.

Their benefits extend beyond digestion. Probiotics help absorb nutrients such as calcium, iron, and magnesium, and may reduce allergic responses by balancing immune functions. Recent research has also highlighted their role in the gut-brain axis, where they may influence mental well-being by affecting neurotransmitter production and reducing inflammation<sup>5</sup>.

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In addition to lactic acid bacteria, spore-forming strains like *Bacillus coagulans* and *Bacillus clausii* offer unique advantages due to their high resistance to heat, acidity, and environmental stress<sup>6</sup>. These qualities make them suitable for incorporation into dietary supplements and livestock feed. Yeast-based probiotics, particularly *Saccharomyces cerevisiae* and *S. boulardii*, are effective against gastrointestinal disorders and do not carry the risk of transferring antibiotic resistance, making them ideal for clinical use<sup>7</sup>.

A crucial factor for probiotic effectiveness is their ability to survive and establish themselves in the gastrointestinal tract. This requires resilience to stomach acid and bile, followed by adherence to the intestinal mucosa. Adhesion is influenced by microbial surface structures like proteins, lipoteichoic acids, and exopolysaccharides. Strong adhesion supports colonisation, helps displace pathogens, and fosters interactions with host cells that reinforce intestinal barrier function and immune regulation<sup>8</sup>.

Probiotics are now incorporated not only in food products but also in medical and clinical applications. They are prescribed for managing conditions such as antibiotic-associated diarrhoea, irritable bowel syndrome (IBS), inflammatory bowel diseases (IBD), and certain neonatal gastrointestinal disorders. However, the effectiveness of probiotics depends on multiple factors, including the specific strain, dosage, duration of use, and the individual's health condition. For this reason, strain-specific research and tailored probiotic therapy are essential for ensuring optimal results<sup>9</sup>.

Insect-associated bacteria have received relatively little attention as potential sources of probiotic candidates, despite the fact that probiotics derived from dairy products and the human gastrointestinal tract have been the subject of much investigation. Though its potential as a reservoir of functionally relevant probiotic strains for wider applications has not been thoroughly explored, the gut microbiome of honey bees has been mainly studied in relation to bee health, disease resistance, and ecological adaptation. Specifically, there are few studies that thoroughly assess several probiotic characteristics in isolates derived from various honey bee species, including bile salt hydrolase activity, antimicrobial qualities,  $\beta$ -galactosidase production, antioxidant capacity, and adhesion-related traits. Additionally, there is a dearth of comparative evaluations of isolates from *Tetragonula iridipennis* and *Apis mellifera*. Therefore, to ascertain the intestinal bacteria of honey bees and to broaden the

variety of possible strains outside traditional sources, a systematic in vitro study of these bacteria is required.

In all, probiotics serve as a bridge between ancient dietary wisdom and modern biomedical science. Their broad range of health benefits, from digestive support to immune enhancement and possible mental health improvement, has positioned them as valuable components of preventive and therapeutic strategies. With the ongoing advancements in probiotic research, their use in functional foods and medicine is likely to expand significantly, offering new opportunities for promoting human health.

Thus, the aim of this study was to isolate, characterise and identify Probiotic organisms from the Honey bee gut, performing various tests that confirm probiotic characteristics and also checking the antioxidant potential of the isolated organisms.

## Materials and Methods

### Collection of samples

Honeybee samples of *A. mellifera* and *T. iridipennis* were collected from the Central Bee Research and Training Institute, Pune.

### Isolation of honey bee gut microflora

The bee guts were aseptically dissected, homogenised in sterile saline, and serially diluted from  $10^{-1}$  to  $10^{-5}$ . 100  $\mu$ L of each dilution was spread on de Man, Rogosa and Sharpe (MRS) agar and Luria agar (LA) plates, incubated at 37°C for 48 hours under anaerobic conditions<sup>10,11</sup>. Colonies with distinct morphology were selected to check their probiotic properties. *Lactobacillus casei* Shirota (Yakult) was used as a probiotic positive control.

### Characterisation and identification of isolated bacteria

Colony morphology, Gram staining, motility test, catalase, and oxidase tests were performed according to standard protocols. Furthermore, biochemical tests, including IMViC, starch and cellulose hydrolysis, sugar fermentation, and the Urea Utilisation Test, were also conducted<sup>12,13</sup>. Bergey's manual of determinative Bacteriology was used to identify the genus and its tentative species.

### Evaluation of probiotic properties of isolated bacteria

#### Bile Salt Hydrolase (BSH) Test

A bacterial suspension was spot-inoculated onto MRS agar supplemented with bile salt and  $\text{CaCl}_2$ , and incubated anaerobically at 37°C for 48 hours. After incubation, the plates were flooded with Gram's

iodine; a white opaque zone around the colony indicated bile salt deconjugation<sup>12,13</sup>.

#### **Arginine Hydrolysis Test**

Isolates were inoculated in arginine dihydrolase broth containing bromocresol purple and incubated anaerobically at 37°C for 48 hours. Yellow to purple colour change of the broth indicated a positive result<sup>12,13</sup>.

#### **Antimicrobial activity of the isolates**

Antimicrobial activity was evaluated using the double agar diffusion method against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella sp.*, and *Candida albicans*. Isolates were spot-inoculated onto sterile MRS agar plates and incubated anaerobically at 37°C for 48 hours. Thereafter, 100 µL of log-phase cultures of test organisms were added to sterile 0.8 per cent molten agar media, mixed thoroughly, and carefully poured onto the inoculated bacterial growth on the MRS plates. Plates were incubated at 37°C for 24 hours; diameters of the zone of inhibition were measured<sup>14</sup>.

#### **Characterisation of antimicrobial substance**

The isolates were examined for the production of antimicrobial substances, such as organic acids, using the agar well-diffusion technique with slight modification. Isolates were cultured in MRS broth and incubated anaerobically for 48 hours. Cell-free supernatants were obtained via centrifugation, for organic acids like lactic acid, 5 mL of supernatant was adjusted to pH 6.5±0.1 using 1 N NaOH. Exactly 100 µL was added to 8 mm wells on *E. coli* spread nutrient agar plates. Plates were incubated at 37°C for 24 hours, and zones of inhibition were measured. The same procedure was repeated using non-neutralised supernatants<sup>15</sup>.

#### **Determination of β-Galactosidase activity of isolated organisms**

The substrate hydrolysis method was used to ascertain the production of beta-galactosidase by the isolates. Briefly, isolates were spot-inoculated (four per plate) on sterile cystine–lactose–electrolyte-deficient CLED agar media plates. Plates were incubated anaerobically for 24 hours at 37°C in an anaerobic jar and compared with an uninoculated control. Bromothymol blue, an indicator included in the medium, turns yellow in acidic pH environments and blue in alkaline pH environments. Since lactose is a fermentable sugar, it will be hydrolysed if the test strain produces beta-galactosidase. Bacterial growth

turns yellow as the pH is lowered by the lactic acid produced by this enzymatic action. Thus, yellow colonies indicated lactose fermenters, while blue colonies indicated non-fermenters<sup>16</sup>.

#### **Assessment of antioxidant properties**

Antioxidant activity was evaluated using the FRAP assay. This technique relies on the reduction of ferric ion (Fe<sup>3+</sup>), and the absorbance was used to measure the reduction of Fe<sup>3+</sup> to Fe<sup>2+</sup>. Exactly 100 µL of supernatant was combined with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide, followed by a 20-minute incubation period at 50°C. Following the addition of 2.5 mL of 10% trichloroacetic acid, the mixture was centrifuged for 10 minutes at 3000 rpm. After being collected in a new tube, the supernatant was combined with 0.5 mL of 1% ferric chloride and 2.5 mL of distilled water. After 10 minutes of incubation at room temperature, the absorbance was measured at 700 nm. A higher reaction mixture absorbance suggests that the extracts have reductive potential. A positive control was ascorbic acid<sup>17</sup>.

#### **Cell surface hydrophobicity by the BATH (Bacterial Adhesion to Hydrocarbon) assay**

The bacterial adherence to hydrocarbons (BATH) assay was used to assess the cell surface hydrophobicity of the isolates. In summary, 48-hour-old MRS broth cultures were centrifuged at 4000 rpm for 5 minutes, washed twice with PBS, and resuspended in 1 mL PBS. OD was measured at 600 nm, and this was the initial OD<sub>i</sub>. 1 mL of hydrocarbon, hexane, was combined with 1 mL of cell suspension. The mixture was then incubated at 37°C for 10 minutes, vortexed for 15 seconds, and left undisturbed for 30 minutes at 37°C for phase separation. OD at 600 was measured as OD<sub>t</sub> after the bottom aqueous phase was meticulously recovered. The formula per cent Hydrophobicity = (OD<sub>i</sub> – OD<sub>t</sub>/OD<sub>i</sub>) × 100 was used to determine the percentage hydrophobicity, or the adhesion of cells to hydrocarbons; <20% = hydrophilic, 20–50% = moderately hydrophobic, >50% = highly hydrophobic<sup>16</sup>.

## **Results**

#### **Isolation of honey bee gut microflora**

Honey bees of the species *A. mellifera* and *T. iridipennis* were collected, and the dissected gut was used to isolate bacteria from it. 12 morphologically different colonies were selected and labelled as C0,

Table 1 — Colony characters of isolated bacteria

Isolate	Size	Shape	Colour	Margin	Elevation	Opacity	Consistency	Gram's character	Motility
C0	Pinpoint	Circular	Off white	Entire	Flat	Opaque	Smooth	Gram-positive cocci	Non motile
C1	Pinpoint	Circular	Off white/ cream	Entire	Flat	Translucent	Sticky	Gram-positive bacillococci	Motile
C2	Pinpoint	Circular	Cream	Entire	Flat	Translucent	Sticky	Gram-positive rods	Non Motile
C4	Pinpoint	Circular	Off white	Entire	Flat	Translucent	Sticky	Gram-positive bacillococci	Non Motile
C6	1 mm	Circular	Cream	Entire	Flat	Translucent	Sticky	Gram-positive rods	Motile
C7	3 mm	Irregular	Off white	Entire	Raised	Translucent	Sticky	Gram-positive bacillococci	Motile
C8	1 mm	Circular	Cream	Entire	Raised	Translucent	Sticky	Gram-positive bacillococci	Non-motile
C10	4 mm	Irregular	Cream	Entire	Flat	Opaque	Sticky	Gram-positive rods	Motile
C11	2 mm	Circular	Cream	Entire	Flat	Opaque	Sticky	Gram-positive bacillococci	Non-motile
C12	Pinpoint	Circular	Off white	Entire	Flat	Translucent	Sticky	Gram-positive bacillococci	Non-motile
C15	1mm	Circular	Cream	Entire	Raised	Translucent	Sticky	Gram-positive bacillococci	Non Motile
C16	Pinpoint	Circular	Off white	Entire	Flat	Translucent	Smooth	Gram-positive bacillococci	Non Motile

C1, C2, C4, C6, C7, C8, C10, C11, C12, C15, and C16 from dilutions of crushed gut spread on sterile MRS agar and LA plates. *L. casei* Shirota, which was used as a positive control in all further assays, was labelled as C.

#### Characterisation and identification of bacteria

The isolates were characterised by colony and Gram staining characteristics, as well as motility. These results are tabulated in Table 1. All isolates were Gram-positive with different shapes and motility.

#### Catalase, oxidase, and urease test

Colonies C2, C6, and C10 were catalase-positive, as indicated by bubble formation, while the remaining isolates, including the positive control C, were catalase-negative, as they did not form bubbles. These results are depicted in Table 2.

The urease test was conducted to determine the ability of the bacterial isolates to produce the enzyme urease, which catalyses the hydrolysis of urea into ammonia and carbon dioxide. The production of ammonia increases the pH of the medium, leading to a colour change from yellow or light orange to pink in the presence of phenol red, indicating a positive result. No colour change in the medium characterised a negative result. The control strain (C) tested negative for urease activity. All tested isolates, including C0, C1, C2, C4, C6, C7, C8, C10, C11, C12, C15, and C16, exhibited negative urease activity, as no colour change was observed in the medium. The negative results across all isolates suggest that urease enzyme activity is absent or not expressed under the test conditions.

#### Sugar fermentation test

The sugar fermentation test showed that the bacterial isolates (C to C16) have different abilities to ferment various sugars. All isolates could ferment

Table 2 — Catalase, oxidase, and urease test for all isolates

Colony	Catalase test	Oxidase test	Urease test
C	-	-	-
C0	-	-	-
C1	-	-	-
C2	+	-	-
C4	-	-	-
C6	+	-	-
C7	-	-	-
C8	-	-	-
C10	+	-	-
C11	-	-	-
C12	-	-	-
C15	-	-	-
C16	-	-	-

\*Note: (-): Negative, (+): Positive

lactose, and most could also ferment glucose, though some, like C0, C1, and C2, did not produce gas. C, C0, C1, C2, C4, C6, C7, C8, C10, C12, and C16 isolates fermented sucrose. C, C0, C2, C6, C7, C8, and C10 isolate fermented mannitol. C, C0, C2, C4, C6, C10, and C11 isolates fermented fructose; some showed little or no activity. Fewer isolates fermented xylose and galactose, while arabinose was the least fermented. Cellobiose and maltose were fermented by all isolates except C11 and C16, suggesting these sugars are more easily used. Some isolates, such as C, C0, and C1, could ferment almost all the sugars tested and produce both acid and gas, while others, such as C11, C12, and C16, showed limited fermentation. C2, C6, and C10 showed results similar to the control organism. These results are depicted in Table 3.

#### IMViC test

Control (C) showed negative results for the indole test. All tested organisms showed negative results for the indole test. No colour change indicates bacteria do not possess the enzyme tryptophanase, which can

Table 3 — Sugar fermentation test for all the isolates

Sugars	C		C0		C1		C2		C4		C6		C7		C8		C10		C11		C12		C15		C16		
	A	G	A	G	A	G	A	G	A	G	A	G	A	G	A	G	A	G	A	G	A	G	A	G	A	G	A
Lactose	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-
Glucose	+	-	+	-	+	+	+	-	+	-	+	+	+	-	+	+	+	-	-	-	+	-	+	+	+	+	-
Sucrose	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	-	+	-	+	-	+	-	+	+
Mannitol	+	+	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+	-	-	-	-	+	+	-	-	-	-
Xylose	+	+	+	+	+	-	+	-	-	-	+	+	-	-	-	-	+	+	-	-	-	-	+	+	+	+	-
Galactose	+	+	-	-	-	-	+	+	-	-	+	+	-	-	-	-	+	+	+	+	+	-	+	-	-	-	-
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	+	+	+	+	+	-	-	-	-
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	-
Arabinose	+	-	-	-	-	-	+	+	+	+	+	+	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-
Cellobiose	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	-

\*Note: ('-': Negative), ('+': Positive), ('A': Acid), ('G': Gas)

break down tryptophan into indole, ammonia, and pyruvic acid. Control (C) showed positive results for the methyl red test. All organisms showed positive results for the Methyl red test. The pH of the broth drops below 4.5, turning the methyl red indicator red, indicating that the bacteria utilise the mixed acid pathway and produce stable acidic end products. Control (C) showed negative results in the Voges-Proskauer test. C2, C6, and C10 showed positive results for citrate utilisation, as evidenced by a change in agar colour from green to blue. All remaining isolates (C0, C1, C4, C7, C8, C11, C12, C15, C16) showed negative results, as indicated by the medium remaining green. C0, C1, C4, C7, C8, C11, C12, C15, and C16 isolates showed similar results for IMViC, like the control (C) strain and these results are depicted in Table 4.

**Evaluation of probiotic properties of isolated bacteria**

**Starch hydrolysis, Cellulase production, and Bile Salt Hydrolase (BSH) for all isolates**

A starch hydrolysis test was carried out to determine the ability of bacterial isolates to produce extracellular amylase, an enzyme responsible for breaking down starch into simpler sugars. None of the isolates, including the control strain (C), demonstrated a positive result for starch hydrolysis, indicated by the absence of clear zones around the colonies after iodine application.

The control strain (C) showed positive results for cellulase activity. Of the 12 colonies tested, three isolates (C4, C6, and C8) exhibited positive cellulase activity, as evidenced by clear zones around the colonies. This indicates their ability to secrete extracellular cellulase enzymes capable of degrading cellulose. The remaining isolates, including C0, C1, C2, C7, C10, C11, C12, C15, and C16, showed no

Table 4 — IMViC test for all isolates

Colony	Indole test	Methyl Red test	Voges Proskauer test
C	-	+	-
C0	-	+	-
C1	-	+	-
C2	-	+	-
C4	-	+	-
C6	-	+	-
C7	-	+	-
C8	-	+	-
C10	-	+	-
C11	-	+	-
C12	-	+	-
C15	-	+	-
C16	-	+	-

\*Note: (-): Negative, (+): Positive

zone of clearance, suggesting an absence of cellulase under the given conditions.

All isolates tested, including the control strain (C), showed positive Bile Salt Hydrolase (BSH) activity, indicating their ability to hydrolyse bile salts. This trait is significant as it suggests potential for bile tolerance, a key probiotic property necessary for survival in the gastrointestinal tract. The presence of BSH activity suggests potential involvement in bile tolerance and may indicate relevance to cholesterol metabolism and gut health; however, its direct impact was not assessed in this study.

C4, C6, and C8 showed results similar to those of the Control (C) strain for Starch hydrolysis, Cellulase production, Bile Salt Hydrolase (BSH).

The results of all three assays are presented in Table 5.

**Arginine hydrolysis test for all isolates**

The arginine hydrolysis test was conducted to assess the ability of bacterial isolates to hydrolyse

arginine via the arginine dihydrolase enzyme. The test organisms possessing arginine dihydrolase hydrolysed arginine in the broth, as indicated by bromocresol purple as a pH indicator; a colour change from purple to yellow indicated a positive result due to acid production after hydrolysis. In contrast, no colour change (remaining purple) indicates a negative result. The control (C) strain showed a negative reaction. All isolates tested, including C0, C1, C2, C4, C6, C7, C8, C10, C11, C12, C15, and C16, showed negative results, with the broth colour remaining purple throughout the testing period (Table 6).

#### Identification of isolated organisms

A total of 12 bacterial isolates were identified at the genus level based on colony characters, Gram staining, and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology. The isolates belonged to a diverse group of genera

Table 5 — Starch hydrolysis, Cellulase production, and Bile Salt Hydrolase (BSH) test for all isolates

Colony	Starch hydrolysis test	Cellulase test	Bile Salt Hydrolase test
C	-	+	+
C0	-	-	+
C1	-	-	+
C2	-	-	+
C4	-	+	+
C6	-	+	+
C7	-	-	+
C8	-	+	+
C10	-	-	+
C11	-	-	+
C12	-	-	+
C15	-	-	+
C16	-	-	+

\*Note: (-): Negative, (+): Positive

Table 6 — Arginine hydrolysis test for all isolates

Colony	Arginine hydrolysis test
C	-
C0	-
C1	-
C2	-
C4	-
C6	-
C7	-
C8	-
C10	-
C11	-
C12	-
C15	-
C16	-

\*Note: (-): Negative, (+): Positive

(Table 7). These included: *Lactococcus*: 1 isolate (C0) may belong to the *lactis* species. *Lactobacillus*: 5 isolates (C1, C2, C7, C12, C15), may belong to species including *agilis*, *plantarum*, *rhamnosus*, *acidophilus*, and *reuteri*. *Streptococcus*: 1 isolate (C4) may belong to the *thermophilus* species. *Bacillus*: 2 isolates (C6, C10) may belong to the *subtilis* and *licheniformis* species. *Enterococcus*: 1 isolate (C8) may belong to the *faecium* species. *Bifidobacterium*: 2 isolates (C11, C16) may belong to the *infantis* and *adolescentis* species.

These identifications are provisional and based on phenotypic characterisation; molecular confirmation would be required for precise species confirmation.

Members of these genera have been reported in the literature to include strains with probiotic traits, particularly within *Lactobacillus* and *Bifidobacterium*. Certain species of *Bacillus* and *Enterococcus* have also been explored for probiotic applications. However, probiotic functionality is strain-specific, and the present findings only indicate that the isolates belong to genera that have previously been associated with probiotic potential. Further molecular characterisation and safety assessment would be necessary before drawing definitive conclusions regarding their probiotic suitability.

#### Antimicrobial activity of the isolates

The double agar assay evaluated the antimicrobial activity of isolated organisms against *S. aureus*. The control strain (C) showed the highest inhibition zone of 35 mm. Among the isolates, C11, C12, and C0 exhibited notable antibacterial activity, followed by C2, C8, and C7. Inhibition by C1, C4, C6, C10, C15, and C16 was observed to a lesser extent. The least inhibition was recorded for C15.

Table 7 — Tentative identification of isolated organisms based on colony characters, Gram staining and biochemical tests

Isolates	May be identified as
C0	<i>Lactococcus lactis</i>
C1	<i>Lactobacillus agilis</i>
C2	<i>Lactobacillus plantarum</i>
C4	<i>Streptococcus thermophilus</i>
C6	<i>Bacillus subtilis</i>
C7	<i>Lactobacillus rhamnosus</i>
C8	<i>Enterococcus faecium</i>
C10	<i>Bacillus licheniformis</i>
C11	<i>Bifidobacterium infantis</i>
C12	<i>Lactobacillus acidophilus</i>
C15	<i>Lactobacillus reuteri</i>
C16	<i>Bifidobacterium adolescentis</i>

\*Note: Need to confirm the species of the isolates by 16S rRNA sequencing

The double agar layer assay evaluated the antimicrobial activity of various microbial isolates against *E. coli*. The control strain (C) exhibited an inhibition zone of 14 mm. Among the tested isolates, C7 displayed the highest inhibitory activity, followed by C8 and C4. Isolates C0, C2, C12, C1, C6, C10, C11, and C15 showed inhibition to a lesser extent. Isolate C16 showed the least activity.

The antimicrobial activity of the isolated organisms against *Klebsiella spp.* was assessed using the double agar layer method. The control strain (C) had an inhibition zone of 30 mm. Isolates C2, C4, C7, C10, C11, and C12 exhibited significant zones of inhibition showing antibacterial activity against *Klebsiella spp.* Isolates C6, C8, C0, C15, and C1 exhibited smaller inhibition zones. Among all the test isolates, C16 showed the least inhibition zone.

The antimicrobial activity of various microbial isolates against *Candida spp.* was evaluated using the double agar layer assay. The assay results, measured as zones of inhibition in millimetres, revealed that several isolates demonstrated varying degrees of antifungal activity. The control strain (C) produced a zone of 13 mm, validating the assay's effectiveness. Among the test isolates, C11 exhibited the highest activity. Inhibition was observed in isolates C1, C6, C10, C12, and C15 with smaller zones. C16 showed the smallest inhibition zone. No antimicrobial activity was observed in isolates C0, C2, C4, C7, and C8, as indicated by the absence of visible zones. The diameter of zone of inhibition for all the test organisms are mentioned in the Table 8.

**Characterisation of antimicrobial substance**

The supernatants from microbial growth were either neutralised or left unneutralised to assess their antimicrobial activity against *E. coli* using the agar well diffusion method. In this assay, a positive result with non-neutralised supernatant, reflecting the presence of a visible zone of inhibition around the well, suggested that the isolate produces lactic acid as a possible antimicrobial compound. On neutralisation, the lactic acid was neutralised and showed no inhibitory action, as seen in Table 9. Of the 13 organisms tested, nine (C, C0, C1, C4, C8, C10, C11, C15, and C16) showed apparent inhibitory effects against *E. coli* in the non-neutralised supernatant, confirming that lactic acid is a potential antimicrobial substance.

**Beta-galactosidase activity of isolated organisms**

The β-galactosidase activity of all bacterial isolates was assessed using Cystine Lactose Electrolyte

Deficient (CLED) agar and the results are depicted in Table 10. CLED is a differential medium, commonly used to cultivate and differentiate urinary pathogens. CLED agar supports the detection of lactose fermentation, which is facilitated by the enzyme β-galactosidase. Positive β-galactosidase activity was indicated by a colour change in the media from greenish blue to yellow due to acid production from lactose fermentation. A clear zone around the colonies accompanied it due to a pH shift. The control isolate (C) displayed a positive reaction, producing a visible yellow colouration, confirming its ability to ferment lactose via β-galactosidase activity. Among the test isolates, C1, C4, C6, C7, C8, C10, C11, C12, C15, and C16 all tested positive, showing the formation of a zone with colour change, indicative of active

Table 8 — Antimicrobial activity of isolated organisms against test organisms

Isolates	Zone of inhibition diameter (mm)			
	<i>S. aureus</i>	<i>E. coli</i>	<i>Klebsiella spp.</i>	<i>Candida spp.</i>
C	35±0.198	14±0.221	30±0.219	13±0.126
C0	22±0.201	13±0.217	13±0.243	-
C1	10±0.256	12±0.232	09±0.271	08±0.243
C2	21±0.187	13±0.202	19±0.223	-
C4	18±0.214	15±0.244	19±0.208	-
C6	11±0.192	12±0.199	17±0.213	08±0.194
C7	20±0.249	17±0.225	23±0.242	-
C8	21±0.231	16±0.257	14±0.228	-
C10	17±0.237	12±0.231	22±0.234	10±0.212
C11	25±0.277	11±0.229	18±0.250	18±0.237
C12	23±0.162	14±0.181	23±0.122	11±0.166
C15	08±0.196	10±0.164	10±0.138	11±0.172
C16	11±0.273	08±0.218	08±0.294	06±0.258

\*Note: (-): Negative, organism was resistant to the compound at that concentration

Table 9 — Antimicrobial nature of supernatant of all isolates against *E. coli*

Isolates	With non-neutralised supernatant	With Neutralised supernatant
C	+	-
C0	+	-
C1	-	-
C2	-	-
C4	+	-
C6	+	-
C7	-	-
C8	+	-
C10	+	-
C11	+	-
C12	-	-
C15	+	-
C16	+	-

\*Note: (-): Negative, (+): Positive

Table 10 — Beta-galactosidase activity of all isolates

Isolates	$\beta$ -galactosidase activity
C	Positive
C0	Negative
C1	Positive
C2	Negative
C4	Positive
C6	Positive
C7	Positive
C8	Positive
C10	Positive
C11	Positive
C12	Positive
C15	Positive
C16	Positive

$\beta$ -galactosidase expression and lactose metabolism. In contrast, isolates C0 and C2 were negative for  $\beta$ -galactosidase activity, showing no colour change or zone formation, suggesting an inability to utilise lactose under the conditions tested.

#### Antioxidant property of the isolates

The antioxidant capacities of various isolates were evaluated using the Ferric Reducing Antioxidant Power (FRAP) assay, which quantifies the ability of antioxidants to reduce ferric ( $\text{Fe}^{3+}$ ) to ferrous ( $\text{Fe}^{2+}$ ) ions, indicated by an increase in absorbance at 700 nm. FRAP values obtained in this assay represent comparative *in vitro* reducing capacity under standardised culture conditions rather than normalised antioxidant efficiency per unit biomass.

The positive control demonstrated the highest reducing activity, followed by C and isolates C8, C12, C15, and C6, all of which exhibited comparatively higher ferric-reducing capacities among the test isolates.

The results stated in Table 11, suggest that these isolates possess measurable reducing potential under the assay conditions. Moderate reducing activity was observed in isolates C1, C7, C11, and C16, while isolate C0 showed the lowest activity.

#### Cell surface hydrophobicity

The Bacterial Adherence to Hydrocarbons (BATH) assay was performed to evaluate the cell surface hydrophobicity of various bacterial isolates. The hydrophobicity percentage was calculated based on the reduction in optical density (OD) at 600 nm after incubation with a hydrocarbon phase and is presented in Table 12. The control strain (C) exhibited a hydrophobicity of 73.54%, establishing a baseline for comparison. Among the tested isolates, C15 showed the highest hydrophobicity, followed by C16 and C2.

Table 11 — Reducing antioxidant power of all isolates

Isolates	OD at 700 nm
C	1.091 $\pm$ 0.42
C0	0.398 $\pm$ 0.36
C1	0.752 $\pm$ 0.28
C2	0.658 $\pm$ 0.34
C4	0.818 $\pm$ 0.33
C6	0.845 $\pm$ 0.40
C7	0.782 $\pm$ 0.39
C8	0.956 $\pm$ 0.32
C10	0.675 $\pm$ 0.36
C11	0.759 $\pm$ 0.40
C12	0.884 $\pm$ 0.41
C15	0.859 $\pm$ 0.38
C16	0.747 $\pm$ 0.32
Ascorbic acid	2.890 $\pm$ 0.27

Table 12 — Per cent Hydrophobicity of isolated organisms

Isolates	Hydrophobicity percentage
C	73.54 $\pm$ 0.12
C0	04.67 $\pm$ 0.23
C1	18.76 $\pm$ 0.20
C2	25.90 $\pm$ 0.22
C4	18.60 $\pm$ 0.27
C6	19.50 $\pm$ 0.21
C7	15.68 $\pm$ 0.25
C8	16.29 $\pm$ 0.25
C10	15.08 $\pm$ 0.26
C11	13.44 $\pm$ 0.22
C12	22.97 $\pm$ 0.23
C15	43.80 $\pm$ 0.21
C16	35.59 $\pm$ 0.23

Moderate hydrophobicity was observed in isolates C12, C6, C1, and C4. Isolates such as C7, C8, C10 and C11 displayed comparatively lower values, whereas C0 had the lowest hydrophobicity.

#### Discussion

The present study isolated and characterised Lactic acid bacteria (LAB) from Honey bee gut samples using MRS and Luria agar. The isolates, primarily Gram-positive and homofermentative, were assessed via morphological and biochemical tests. Comparisons with previous studies<sup>18-20</sup>, revealed core LAB traits across sources but also highlighted differences in enzyme activity and fermentation profiles due to sample origin. These findings emphasise the influence of the host environment on microbial diversity and LAB characteristics.

In the present study, the double agar diffusion method was employed to evaluate the antimicrobial activity of the isolates against three bacteria (*E. coli*, *S. aureus*, and *Klebsiella* spp.) and one yeast (*C. albicans*). All isolates demonstrated activity against

*S. aureus* and *Klebsiella* spp. but only C, C0, C1, C8, C10, and C11 were active against *E. coli*. Antifungal testing against *C. albicans* revealed C11 as the most effective, with minimal or no activity observed in several isolates. In comparison, a study<sup>14</sup> assessed LAB from Kashk samples against *E. coli*, *S. aureus*, *P. aeruginosa*, and *S. typhimurium*, identifying KK4 and KF3 (*Pediococcus pentosaceus*) as the most effective isolates. Their study did not include an antifungal assay, which was incorporated in the present study.

In a study<sup>15</sup>, *Lactobacillus* isolates Y9, Y10, and Y13 were evaluated for their antimicrobial activity and the nature of the inhibitory compounds produced. Using a modified agar well-diffusion method, the study differentiated the roles of bacteriocins, organic acids, and hydrogen peroxide. When the supernatants were neutralised to pH 6.5, no inhibition was observed, confirming that the antimicrobial activity was due to organic acids. In the present study, supernatants from various microbial isolates were neutralised and assessed against *E. coli* using the agar well diffusion method. The absence of inhibition zones post-neutralisation indicated that organic acids were the primary antimicrobial agents. Eight isolates—C0, C4, C6, C8, C10, C11, C15, and C16—exhibited positive antimicrobial activity attributed to organic acid production. This comparative analysis reinforces the significance of organic acids as key antimicrobial agents in probiotic isolates and highlights the variability in antimicrobial mechanisms across strains.

In this study,  $\beta$ -galactosidase activity of bacterial isolates was assessed using CLED agar, where a colour change from greenish-blue to yellow indicated lactose fermentation. All isolates, excluding C0 and C2, were positive for  $\beta$ -galactosidase activity. These findings align with those who<sup>16,21</sup> observed  $\beta$ -galactosidase activity in *Streptococcus thermophilus* MCC0200 and in commercial probiotic supplements, attributing lactose utilisation.

In the present study, LAB isolates from honeybee guts showed moderate reducing power by the FRAP antioxidant assay. These results align with findings from a study<sup>22</sup>, which reported increased FRAP values in *C. difficile*-infected faecal water treated with probiotics, highlighting strain-specific antioxidant potential. Similarly, another study<sup>23</sup>, reported FRAP values of 0.25–2.81 mM/ $\mu$ g protein in kefir-based *Lactobacillus* strains. The higher antioxidant values in kefir isolates are likely due to a richer microbial environment compared to the selective honey bee gut, along with

strain-specific genetic traits. Variation in reducing power may be due to microbial source, assay expression formats, and environmental adaptation.

In the present study, the Bacterial Adherence to Hydrocarbons (BATH) assay was used to assess the cell surface hydrophobicity of bacterial isolates. Hydrophobicity was measured by the decrease in OD after mixing with hydrocarbons. The control strain (C) showed 73.54% hydrophobicity. Among the isolates, C15 had the highest hydrophobicity, while C0 had the least. In comparison, a study<sup>16</sup> evaluated cell surface hydrophobicity of *Streptococcus thermophilus* MCC0200 using the BATH assay with various solvents (chloroform, ethyl acetate, xylene). MCC0200 showed a broad hydrophobicity range (8.08–80.4%), with the highest adherence to chloroform (80.4%), indicating strong cell surface hydrophobicity and adhesion potential to intestinal epithelial cells (HT-29 line).

## Conclusion

We successfully isolated and characterised gut bacteria from two honey bee species, *A. mellifera* and *T. iridipennis*, with a focus on identifying potential probiotic strains. The identified genera included: *Lactobacillus*, *Lactococcus*, *Bacillus*, *Streptococcus*, *Enterococcus*, and *Bifidobacterium*. The preliminary screening results showed the presence of probiotic traits, including bile and acid tolerance, lactose fermentation, adhesion ability, antimicrobial, and antioxidant activity. Further *in vivo* studies, safety validation, molecular characterisation, and clinical investigations are required before any therapeutic or nutraceutical application can be considered; once these are completed, these strains can be employed for the development of probiotic supplements for both human and bee health.

## Conflict of interest

The authors declare that they have no conflict of interest.

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