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Probiotic Characteristics of *Pediococcus pentosaceus* and *Apilactobacillus kunkeei* Strains: The lactic Acid Bacteria Isolated from Bangladeshi Natural Honey

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Abstract

Background and Objective: Lactic acid bacteria are known for their strong probiotic effects on the hosts. The probiotic characterization of lactic acid bacteria from Bangladeshi natural honey is limited. The objectives of this study included isolation and assessment of the probiotic and safety characteristics of lactic acid bacteria in Bangladeshi honey.

Materials and Methods: Spread and streak plate techniques were used for the bacterial isolation and purification. Isolates were identified using 16S rRNA gene sequence analysis. Agar well diffusion and poisoned food methods were used for antibacterial and antifungal assessments, respectively. Antioxidant activity was carried out based on the microbial free-radical scavenging ability. Microbial autoaggregation, coaggregation and adhesion were assessed using cell sedimentation assay. Blood-agar was used in the hemolytic assay. Antibiotic susceptibility assay was carried out using disc diffusion method.

Results and Conclusion: From a total of 25 strains isolated from honey, ten Gram-positive, catalase-negative non-spore-forming isolates were selected and used in agar well diffusion assay. Three of the isolates showed prominent antimicrobial effects with large inhibition zones against all the pathogenic strains, including *Bacillus cereus*, *Staphylococcus aureus*, *Vibrio cholerae*, *Salmonella typhi* and *Candida albicans*. Extensive characterization of these isolates was carried out, which revealed their growth and biochemical characteristics and carbohydrate fermentation abilities. Moreover, 16S rRNA gene sequence analysis suggested that the isolates belonged to *Pediococcus pentosaceus* (two strains) and *Apilactobacillus kunkeei*. Their tolerance to simulated gastric conditions was assessed *in vitro* wherein the isolates showed significant survival in low pH, bile salts and phenol. They showed good adhesion ability to ethyl acetate, chloroform and xylene as well as high autoaggregation and coaggregation characteristics. Moreover, free-radical scavenging activity of the isolates suggested the presence of considerable antioxidant effects. In safety assessment, the isolates did not show hemolytic activities and were resistant to several antibiotics. Therefore, these results indicate that honey can be an important source of beneficial lactic acid bacteria species providing several probiotic advantages.

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1. Introduction

Probiotics represent live microorganisms, which confer one or more health benefits to their hosts when administered in adequate quantities [1,2]. The most important group of probiotic microbes are lactic acid bacteria (LAB), which are known for their significant ability of producing beneficial cellular metabolites in the gastrointestinal tract (GIT) of their host thereby improving the host's health status [3,4]. The LAB are ubiquitous in nature and isolated from a wide range of habitats, including fermented and non-fermented food products [1,5,6], vegetables [7], fruits and honey [8-10]. The extent of their probiotic activities can be divided into nutritional, physiological, immunomodulatory and antimicrobial effects [11]. Naturally, LAB produce various beneficial metabolites such as organic acids, antioxidants and antimicrobial peptides [12,13] that contribute to improved gut microbial balance [14], better digestion [15], alleviation of constipation symptoms [16] and strengthening of the mucosal barrier [17]. Probiotic LAB isolates may possess anticancer, anti-allergic or antidiabetic effects as well [18,19]. One of their most prominent health benefits is their ability to inhibit pathogenic and spoilage microorganisms by a variety of mechanisms including adherence to epithelial cells, secretion of antimicrobial compounds and modulation of the immune system [1,20]. This justifies their use in biopreservation of foods, therapeutics and starter culture under controlled conditions. Therefore, bacteria of the LAB group have been recognized within the best probiotic candidates. Indeed, a few organisms of this group are already used as feed additives or alternative to antibiotics [1,3]. Their isolation and characterization from indigenous natural sources or traditional foods such as fruits and vegetables have attracted increasing research interests in recent years [21].

In addition to having beneficial health effects, a probiotic candidate must also possess characteristics of intestinal competence such as ability to survive and colonize in various gut conditions [14]. For example, the candidate probiotic should be able to resist bile salts and low pH, and adhere to epithelial cells [22,23]. Hence, bacteria that have been shown to possess health promoting activities must be assessed for the above probiotic competence prior to their use in foods or medicines. The present study was carried out to isolate novel LAB species from the locally available natural honey and assess the isolates' probiotic characteristics. Honey itself is known to include several health benefits and has been used as a traditional medicine. It contains a wide variety of beneficial molecules including vitamins such as riboflavin, niacin, pantothenic acid and ascorbic acid; minerals such as copper, calcium, manganese, magnesium, iron, potassium, phosphorus and zinc; and natural products such as carotenoids, flavonoids and phenolic compounds which can provide the consumers with several health promoting effects such as antimicrobial, antidiabetic, antioxidant, and anti-inflammatory effects [24]. Moreover, recent studies have suggested that honey contains beneficial bacteria with various probiotic effects [8,25,26]. These bacteria have been reported to produce compounds with effective antibacterial and antioxidant activities [8,10,27]. However, information on the isolation of LAB and their possible roles as probiotics in the Bangladeshi natural honey is limited. Despite being a promising source, LAB in the local Bangladeshi honey and their beneficial or safety issues have remained largely unknown. Therefore, this study was carried out to assess probiotic potentials of the LAB strains isolated from natural honey collected locally in the Chattogram City, Bangladesh. Extensive morphological, biochemical and taxonomic characterizations of the strains were carried out. Moreover, antimicrobial activity of the selected isolates against bacterial and fungal pathogens, their antioxidant capacity, autoaggregation and coaggregation characteristics, cell surface hydrophobicity and safety assessment with respect to hemolytic activity and antibiotic resistance were assessed.

2. Materials and Methods

2.1. Isolation of honey associated bacteria

Ripe honey is often collected with the honey combs by local honey collectors and immediately sold locally near the Chittagong University Campus without further processing. Five different samples of ripe honey were locally collected under hygienic conditions and transported to the laboratory using cold box. One ml of the honey sample was enriched in de Man, Rogosa and Sharpe (MRS) broth at 37 °C for 24 h. Then, 10^{-1} to 10^{-6} serial dilutions of the enriched culture were prepared using phosphate buffer saline (PBS) and 100 µl from each dilution was transferred onto MRS agar media and incubated aerobically at 37 °C for 24 h. Morphologically discrete colonies were further subcultured onto MRS agar plates and stored as slant cultures at 4 °C or as glycerol stocks at -20 °C [28,29].

2.2. Culture conditions

All isolates were routinely maintained on MRS media at 37 °C. Cultures were revived from the slants or glycerol stocks and incubated overnight. Activated overnight cultures were transferred into fresh MRS media using 1% (v v^{-1}) inoculum, cultured until the optical density (OD) reached ~1.5 at 600 nm and used for further analyses.

2.3. Assessment of the cultural and biochemical characteristics

Primary characterization of the isolated LAB strains was based on their morphological, cultural and biochemical characteristics as previously described [7,30]. Morphological and biochemical analyses included Gram staining, and catalase, oxidase, casein hydrolysis, indole, deep glucose agar, nitrate reduction, citrate utilization, gelatin liquefaction, methyl-red (MR), Voges Proskauer (VP), starch hydrolysis and carbohydrate fermentation tests. Growth of the isolates at various temperatures and NaCl concentrations was assessed as well [3].

2.4. Assessment of antimicrobial activity

Antimicrobial activity of the isolates was assessed using agar well diffusion method [31]. Each test strain (100 µl, adjusted to 0.5 McFarland turbidity standard) was mixed with soft agar and overlaid on Muller-Hinton agar (MHA). Then, wells were created in the media using sterile gel cutter. One drop of the melted agar was transferred into each well and allowed to solidify. Supernatant of the LAB cultures (~1.5 OD_{600}) was collected using centrifugation at 8000 rpm for 10 min at 4ºC. Then, 100 µl of the culture filtrate was poured into each well and incubated at 4 °C for 2-3 h. Following further incubation at 37 °C for 24 h, diameter of the clear zones was measured and recorded (n = 3).

2.5. Test strains

Pathogens used for the assessment of antimicrobial assay included *Bacillus cereus* ATCC 14574, *Staphylococcus aureus* ATCC 6538, *Vibrio cholerae* ATCC 14035, *Salmonella typhi* ATCC 14028 and *Candida albicans* ATCC 10231. In coaggregation assay, *Escherichia coli* ATCC 8739 was used.

2.6. Polymerase chain reaction and sequencing of 16S rRNA gene

For the amplification of 16S rRNA gene, genomic DNA was extracted as previously described in [32] and polymerase chain reaction (PCR) was carried out using the universal 16S rRNA gene-specific primers 27F (5´-AGAGTTTGATCMTGGCTCAG-3´) and 1492R (5´-CGGTTACCTTGTTACGACTT-3´) [33]. The PCR products were then purified using Wizard SV Gel and PCR Clean-Up System (Promega, USA) based on the manufacturer's protocols. Purified PCR products were sequenced using Applied Biosystems Big Dye Terminators v3.1 (Thermo Fisher Scientific, USA). Sequences were submitted to National Center for Biotechnology Information (NCBI) GenBank Database under the following accession numbers: ON831368 to ON831370.

2.7. Sequence analysis

Sequence similarity was carried out using NCBI BLAST optimized for 'Highly similar sequences' (MEGABLAST) [34,35]. Taxonomic assignment was based on the number of hits and maximum score [36].

2.8. Acid, bile salt and phenol tolerance assays

Ability of the LAB isolates to tolerate acid, bile salt and phenol was assessed as previously described by Li et al. [3] with some modifications. To assess acid tolerance, pH of the MRS broth was adjusted to 2.0 or 6.5 with 1 M HCl. Briefly, 100 µl of the overnight culture of each LAB was added to 10 ml of the media followed by incubation at 37 °C for 120 and 240 min. Growth was estimated by measuring OD of the culture at 600 nm. For the assessment of bile salt tolerance, MRS broth added with 0 or 0.5% of bile salt was inoculated with 1% culture of each isolate, incubated for 120 min and 240 min at 37 °C and measured at OD_{600} nm. To assess phenol tolerance, 1% overnight culture of the LAB isolates was inoculated into MRS broth supplemented with 0, 0.4, 0.5 or 0.6% (v v^{-1}) phenol and growth was estimated by reading absorbance at 600 nm after 24 h of incubation at 37ºC [37]. Each experiment was repeated twice in triplicate and the growth rate was calculated using the Eq. 1:

$$\text{Percent (\%) of growth} = \frac{\text{Growth in test media}}{\text{Growth in control media}} \times 100 \qquad \text{Eq. 1}$$

2.9. Autoaggregation assay

Autoaggregation assay was carried out using a method described by Zommiti et al. [38] with some modifications. Briefly, each isolate was cultured in MRS broth at 37ºC for 18 h and cells were harvested by centrifugation at 8,000 rpm for 10 min at 4 ˚C. Cells were washed three times with sterile phosphate buffered saline (PBS) and suspended in 2 ml of PBS to an OD_{600} adjusted to 0.25 ±0.05. The cell suspension was incubated at 37ºC and OD_{600} was measured at 0, 2, 6, 12 and 24 h. The autoaggregation rate was calculated using the Eq. 2:

$$\text{Autoaggregation (\%)} = \frac{(Ax - Ay)}{Ax} \times 100 \qquad \text{Eq. 2}$$

Where, Ax refers to OD_{600} at time (t) = 0 and Ay refers to OD_{600} at t = 2, 4, 6, 12 or 24 h. All experiments were carried out in triplicates.

2.10. Coaggregation assay

Coaggregation assay was carried out using slightly modified protocol described by Prabhurajeshwar et al. [39]. The LAB isolates and the indicator test organism *Escherichia (E.) coli* were cultured separately at 37 ºC for 24 h in MRS and LB media, respectively. Equal volumes of each LAB culture and *E. coli* were mixed (1:1 v v^{-1}) and incubated at 37 ºC under static culture conditions. Absorbance of the mixed culture was measured at 0, 2, 6, 12 and 24 h and coaggregation (%) was calculated by Eq. 3:

$$\text{Coaggregation (\%)} = \frac{[(Apro + Apat) - Amix]}{Apro + Apat} \times 100 \quad \text{Eq. 3}$$

Where, A_{pro} and A_{pat} refer to the absorbance of the mixed culture at 0 h and A_{mix} refers to the absorbance at other time points. A triplicate analysis was carried out for each sample.

2.11. Assessment of cell surface hydrophobicity

Assessment of the cell surface hydrophobicity of the isolates was based on their adhesion to various hydrocarbons according to the method described by Rokana et al. [40]. Each isolate was cultured overnight in MRS broth and cells were harvested by centrifugation at 8,000 rpm for 10 min at 4 °C, washed twice with sterile PBS buffer and resuspended in PBS. Cell density was measured at 600 nm (A_0). Then, 3 ml of the cell suspension was mixed with 1 ml of ethyl acetate, chloroform or xylene and incubated at 37°C for 1 h under static conditions to allow separation of the aqueous phase from the organic phase. 1 ml from the upper aqueous phase was removed and absorbance (A_1) was measured at 600 nm. Hydrophobicity (%) was estimated using the Eq. 4:

$$\text{\% cell surface hydrophobicity (\%)} = \left(1 - \frac{A1}{A0}\right) \times 100 \quad \text{Eq. 4}$$

2.12. Assessment of antifungal activity

Isolates were screened for the presence of antifungal activity using poisoned food technique [41]. Briefly, *C. albicans* was used as the test fungus cultured on potato dextrose agar at 25ºC for 5-6 d. Then, 0.5 ml of the filtered cell-free supernatants of the LAB isolates was transferred to a sterile Petri dish. Sterile nutrient agar was added to the dish, mixed well and set to solidify. Then, a 5-mm well was created at the center of the plate, placed with 5-mm fungal block and incubated at 25ºC. Nutrient agar plate without culture extract was used in the control experiment. Diameter (mm) of the fungal radial growth was measured after 5 d of incubation and proportion of inhibition was estimated using the Eq. 5:

$$\text{Inhibition of mycelial growth (\%)} = \frac{C - T}{C} \times 100 \quad \text{Eq. 5}$$

Where, C was diameter of the fungal colony in the control and T was diameter of the fungal colony in the treatment.

2.13. Antioxidant assay

Antioxidant activity was assessed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay as described by Yin J-Y et al. [42] with some modifications. Overnight LAB culture was inoculated to 10 ml MRS broth and incubated at 37 ºC for 24-48 h. Incubated broth was centrifuged at 8000 rpm for 10 min at 4ºC. Supernatant was collected and stored as 1 ml aliquot at 20ºC. Furthermore, 1.0 ml of the supernatant was added to 1.0 ml of ethanolic DPPH radical solution, mixed well and stored at room temperature for 30 min in dark. The ethanolic DPPH with distilled water (1:1) was used as control and ethanol with supernatant (1:1) was used as blank. After incubation, discoloration of the solution was spectrophotometrically measured at 517 nm in triplicate. Ascorbic acid was used as positive control. The radical scavenging activity was calculated using the Eq. 6:

$$\text{Scavenging ability (\%)} = \lfloor 1 - (A_{\text{sample}} - A_{\text{blank}} / A_{\text{control}}) \rfloor \times 100 \quad \text{Eq. 6}$$

Where, A_{sample}, A_{blank} and $A_{control}$ represented absorbance of the sample, blank and control, respectively.

2.14. Hemolytic activity analysis

Analysis of the hemolytic activity was carried out using a method described by Yadav et al. [43]. Each isolate was streaked onto blood agar plates (Oxoid, Germany) supplemented with 5% (w v^{-1}) sheep blood. After incubation at 37ºC for 48 h, microbial hemolytic activity was assessed based on the formation of clear zones. *Staphylococcus aureus* ATCC 6538 was used as positive control [44].

2.15. Assessment of antibiotic susceptibility

Kirby-Bauer disc diffusion method was used for the antibiotic susceptibility assay of the isolates [45]. Antibiotics (Oxoid, UK) used in the assay include (µg/disc) amoxycillin 30, clindamycin 2, azithromycin 30, ciprofloxacin 5, tetracycline 30, ofloxacin 5, erythromycin 15, chloramphenicol 30, doxycycline hydrochloride 30, ampicillin 20, penicillin 10 and gentamycin 10. Overnight culture of each isolate was spread on MRS agar plates and set to dry. Discs containing the antibiotics were transferred onto the inoculated agar and incubated at 37 ºC for 24 h. Diameter of the inhibition zones was measured and the results were interpreted as resistant (R), moderately sensitive (M) or sensible (S) based on the guidelines by Clinical and Laboratory Standards Institute (CLSI, 2014) [46]. Each experiment was carried out in triplicate.

2.16. Statistical Analysis

All quantitative analyses were carried out in triplicate and data were presented as mean ±SD (standard deviation). Groups were compared and analyzed using GraphPad Prism Software v.9.0 and one-way ANOVA with Tukey's. Statistical significance was reported at $p \leq 0.05$.

3. Results

3.1. Isolation, selection and characterization of the honey associated bacteria

A total of 25 bacterial isolates were initially collected from various honey samples and subjected to preliminary biochemical analysis. Of these isolates, ten catalase-negative, oxidase-negative, Gram-positive non-spore-forming isolates were selected and assessed for antimicrobial activity against Gram-positive and Gram-negative test strains using agar diffusion method (Table 1). Three of the isolates (H5, H4 and H2) that showed antagonistic activity against all the test strains were selected for subsequent analysis. A detailed biochemical characterization of the three isolates was carried out, which revealed

that the strains were non-motile facultative anaerobes (Table 2). The H5 and H4 were cocci, whereas H2 was bacillus. The isolates showed various abilities in sugar fermentation as all isolates were able to ferment glucose, fructose and galactose but none of them could ferment mannitol, lactose, starch and sucrose. Additionally, H5 fermented raffinose, rhamnose and maltose, H4 fermented xylose, rhamnose and maltose and H2 fermented xylose and raffinose. The microbial growth was assessed at various temperatures and salinity (Table 2). Although the isolates were able to grow at all salt concentrations from 0 to 7% NaCl; however, a better growth was mostly observed at 1% NaCl. Moreover, isolates grew well at 27 and 37 °C. However, while H5 and H4 could grow at 45 °C, H2 could not survive at this temperature. A 16S rRNA gene sequence based taxonomic analysis was further carried out; in which, H5 and H4 showed the highest sequence similarity to *Pediococcus* strains and H2 to *Apilactobacillus* strains; therefore, indicating their taxonomic affiliations to the respective genera (Table 2).

3.2. Antibacterial and antifungal activities

Relative antimicrobial activity of the isolates was assessed against two Gram-positive and two Gram-negative pathogenic bacteria and the fungal pathogenic strain *C. albicans*. All isolates showed strong antibacterial activities in agar well diffusion assay. The *P. pentosaceus* strain H5 was the most potent antagonistic strain producing the largest inhibition zones against all pathogens (Table 3). *S. aureus* and *S. typhi* were the most affected test strains. Significant inhibition of fungal growth was demonstrated by the isolates, indicating their wide antagonistic spectrum (Table 3).

3.3. Antioxidant activity

Generally, LAB species can alleviate oxidative stresses by providing antioxidant effects. Antioxidant activity of the isolates' cell-free supernatant was assessed based on the DPPH free radical scavenging capacity (Table 4). Isolates demonstrated high antioxidant activities with the two *Pediococcus* (*P.) pentosaceus* strains showing relatively better activities (~61 and 88% in H5 and H4, respectively) than those *Apilactobacillus (A.) kunkeei* H2 did (~60%).

3.4. Acid, bile salt and phenol tolerances

Tolerance to the harsh gastrointestinal environment such as acid, bile salt and phenol is an important selection criterion for probiotic candidates. The LAB isolates were therefore assessed for their viability at low-pH, bile salt and phenol (Fig. 1). The three isolates showed various degrees of survival ability at simulated gastric conditions (Fig. 1A). The *A. kunkeei* strain H2 exhibited a better survival rate (~37%) within the first 2 h of exposure to pH 2 and decreased survival (~15%) upon longer exposure. In contrast, survival of the two *P. pentosaceus* strains increased from 2 to 4 h of exposure to low pH conditions. The two *P. pentosaceus* isolates showed strong survival abilities in the presence of 0.5% bile salt (Fig. 1B). However, viability decreased with longer exposures. Assessment of their phenol tolerance showed moderate survival rates (~19-28%) at 0.4% phenol but lower survival rates at higher phenol concentrations (Fig. 1C). Generally, *P. pentosaceus* isolates seemed slightly more tolerant to the above conditions than the *A. kunkeei* isolate.

3.5. Autoaggregation and coaggregation abilities

Autoaggregation and coaggregation abilities are important for epithelial adhesion of the probiotic microbes and their pathogen antagonism. In the present study, autoaggregation and coaggregation with *E. coli* varied across the LAB isolates (Table 4).

Table 1. Primary screening of the isolates for antimicrobial activity.

Isolates	Test strains			
	Vibrio cholerae	*Salmonella typhi*	*Bacillus cereus*	*Staphylococcus aureus*
H5	++	++	+	++
H4	++	+	++	++
H2	++	+	++	+
H3	-	+	-	-
H6	-	-	+	-
H10	-	-	-	+
H12	+	-	-	-
H15	-	-	+	-
H19	-	+	-	+
H21	-	-	+	-

– = no inhibition; + = inhibition zone < 9.0 mm; ++ = inhibition zone > 9.0 mm

Table 2. Biochemical characteristics and 16S rRNA gene based taxonomy of the selected isolates

Features	H5	H4	H2
Colony and cell morphology			
Colony characteristics	Circular, medium, smooth, opaque, raised, whitish, even	Circular, medium, smooth, opaque, raised, whitish, even	Circular, small, smooth, opaque, raised, whitish, even
Gram staining	Gram +ve	Gram +ve	Gram +ve
Cell shape	cocci (1.9 µm in diameter)	cocci (1.9 µm in diameter)	short rod (2.93-3.38 in length and 1.56-1.95 in width)
Cellular arrangement	single	single	single and paired
Biochemical tests[#1]			
Oxidase test	-	-	-
Catalase test	-	-	-
Casein hydrolysis test	-	-	-
Indole test	-	-	-
Deep glucose agar test	Facultative anaerobes	Facultative anaerobes	Facultative anaerobes
Nitrate reduction test	-	-	+
Citrate utilization test	Turbid	Turbid	Turbid
Gelatin liquefaction test	-	-	-
MR test	-	-	-
VP test	+	+	+
Starch hydrolysis test	-	-	-
Motility test	-	-	-
Fermentation of carbohydrates[#1]			
Fructose	+	+	+
Galactose	+	+	+
Glucose	+	+	+
Xylose	-	+	+
Raffinose	+	-	+
Mannitol	-	-	-
Lactose	-	-	-
Rhamnose	+	+	-
Starch	-	-	-
Sucrose	-	-	-
Maltose	+	+	-
Growth at different salt concentrations (%)[#2]			
0	++	++	++
1	+++	+++	++++
3	++	++	+
7	+	+	-
Growth at different temperatures (°C)[#2]			
27	+++	+++	++
37	+++	+++	+++
45	++	++	-
16S rRNA gene sequence based taxonomy			
Taxonomy	*Pediococcus pentosaceus*	*Pediococcus pentosaceus*	*Apilactobacillus kunkeei*
% identity	99.42%	99.59%	98.72%
Accession no.	ON831370	ON831369	ON831368

[#1] - = negative, + = positive[#2] - = no growth, + = scanty, ++ = moderate, +++ = good

Table 3. Antimicrobial and antifungal activities of the isolates

Isolates	Inhibition Zone (mm)				Inhibition (%)
	Vibrio cholerae	*Salmonella typhi*	*Bacillus cereus*	*Staphylococcus aureus*	*Candida albicans*
Pediococcus pentosaceus H5	12.10±0.05[a]	15.00±0.05[a]	15.00±0.05[a]	15.03±0.06[a]	66.79±0.12[a]
Pediococcus pentosaceus H4	10.03±0.08[b]	12.03±0.06[b]	12.00±0.57[b]	13.00±0.05[b]	66.67±0.21[a]
Apilactobacillus kunkeei H	12.03±0.06[a]	13.00±0.10[b]	10.10±0.05[b]	13.00±0.05[b]	48.27±0.12[b]

Values are means of triplicate experiments; ± indicates standard error of the mean. Different letters represent significant difference; $p \leq 0.05$.

Although all isolates showed significant autoaggregation and coaggregation abilities, the maximum level of autoaggregation was demonstrated by *A. kunkeei* strain at 12 h (26%) and 24 h (49%). In contrast, *P. pentosaceus* strain H4 showed the highest coaggregation ability. For all isolates, however, autoaggregation and coaggregation increased from 12 to 24 h.

3.6. Cell surface hydrophobicity

Cell surface hydrophobicity is an estimate of cell adhesion to the hydrocarbons and a high hydrophobicity usually indicates strong interactions with mucosal cells. Cell surface hydrophobicity of the isolated LAB was assessed against three various hydrocarbons, including ethyl acetate, chloroform and xylene; in which, isolates exhibited moderate to high adhesion abilities (Table 4).

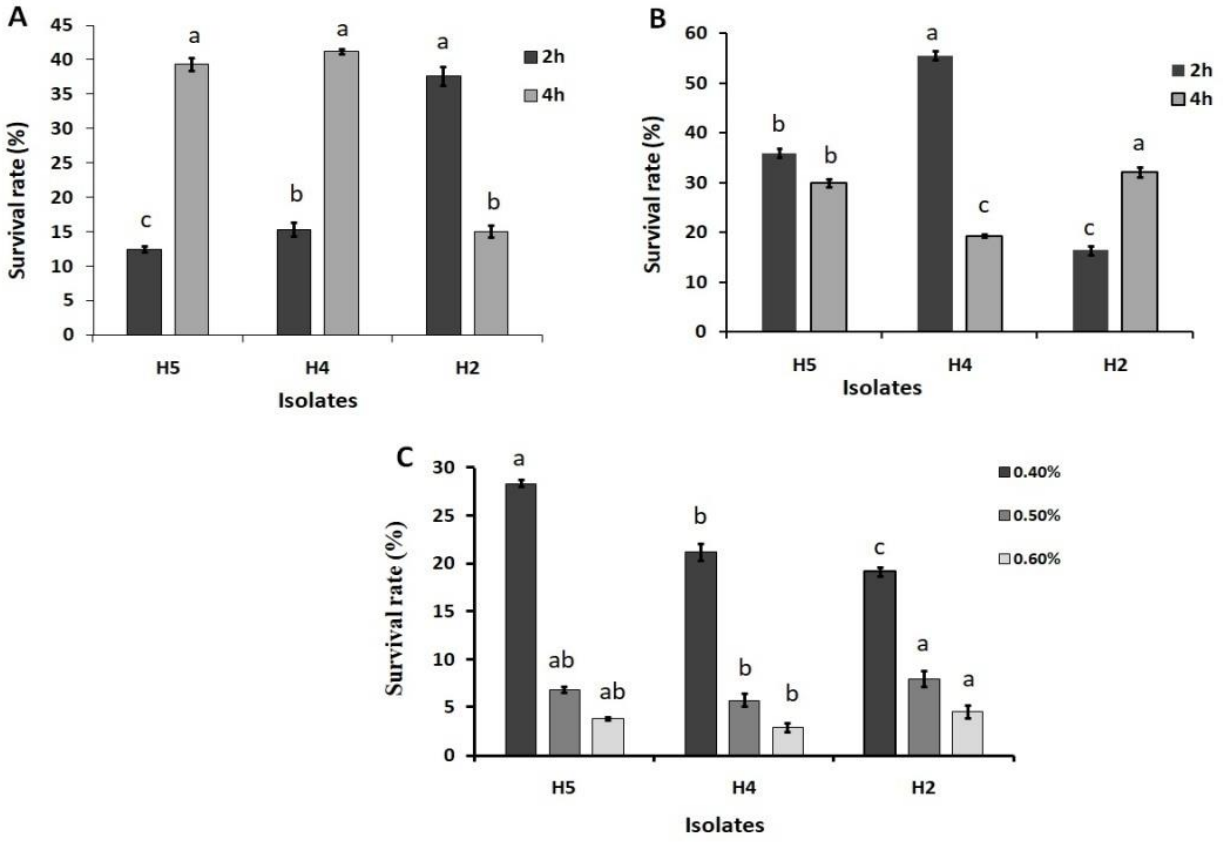

Figure 1. Tolerance of the LAB strains to (A) simulated gastric juice (pH 2); (B) 0.5% bile salt and (C) 0.4–0.6% phenol. Values are mean of triplicate experiments; ± indicates standard error from the mean. The letters indicate significant difference at $p \leq 0.05$

Table 4. Autoaggregation, coaggregation, adhesion and antioxidant capacities of the isolates

Isolates		*Pediococcus pentosaceus* H5	*Pediococcus pentosaceus* H4	*Apilactobacillus kunkeei* H2
DPPH scavenging (%)		61.14± 1.04^{b}	88.12±0.46^{a}	57.99±0.91^{c}
Autoaggregation (%)	Time			
	12h	11.06±0.40^{c}	20.60±0.26^{b}	25.99±0.44^{a}
	24h	34.20±0.67^{b}	31.40±0.34^{b}	49.27±0.34^{a}
Coaggregation (%)	Time			
	12h	22.41±0.99^{b}	35.36±0.59^{a}	18.74±0.79^{b}
	24h	40.04±0.61^{b}	51.92±0.99^{a}	35.47±0.56^{b}
Hydrophobicity (%)	Hydrocarbon			
	Ethyl acetate	20.95±1.01^{a}	14.05±0.55^{b}	20.86±0.65^{a}
	Chloroform	12.39±0.19^{b}	14.06±1.03^{b}	32.86±0.74^{a}
	Xylene	35.22±1.06^{b}	45.71±0.80^{a}	37.65±0.92^{b}

Values are means of replicate experiments; ± indicates standard deviation from the mean. Different letters represent significant difference; $p \leq 0.05$.

Isolates showed maximum hydrophobicity rates to xylene which were ~35, ~45 and ~37% for H5, H4 and H2, respectively. Hydrophobicity to ethyl acetate ranged 14-20%, and to chloroform ranged 12–32%. In general, *A. kunkeei* strain showed a better cell surface hydrophobicity to hydrocarbons followed by the *P. pentosaceus* H4 strain.

3.7. Safety assessment

Safety assessment of the isolates was based on their hemolytic activity and antibiotic susceptibility profiles. None of the isolated strains showed hemolytic activity, indicating that the isolates could be considered safe. In antibiotic susceptibility assays against 12 common antibiotics, *P. pentosaceus* H5 strain was resistant to seven antibiotics, *P. pentosaceus* H4 was resistant to six antibiotics and *A. kunkeei* H2 was resistant to five antibiotics (Table 5). All isolates demonstrated resistance to ofloxacin, amoxicillin, penicillin and ciprofloxacin and full or moderate sensitivity to chloramphenicol, clindamycin, gentamycin, ampicillin and doxycycline hydrochloride.

Table 5. Antibiotic susceptibility profiles of the isolates in disc diffusion method

Isolates	Antibiotic susceptibility pattern											
	AZM	TE	OF	C	CD	AMX	E	AMP	P	CIP	GEN	DO
Pediococcus pentosaceus H5	R	R	R	S	S	R	R	M	R	R	S	M
Pediococcus pentosaceus H4	R	M	R	S	S	R	R	M	R	R	S	M
Apilactobacillus kunkeei H2	S	R	R	S	S	R	S	S	R	R	S	S

R = Resistance, S = Sensitive, M = Moderately Sensitive. AZM = Azithromycin, TE = Tetracycline, O = Ofloxacin, C = Chloramphenicol, CD = Clindamycin, AMX = Amoxycillin, E = Erythromycin, AMP = Ampicillin, P = Penicillin, CIP = Ciprofloxacin, GEN = Gentamycin, DO = Doxycycline hydrochloride.

4. Discussion

This study presents an extensive characterization of the probiotic features of three LAB strains from honey. Of the strains, two strains were *P. pentosaceus* and one strain was *A. kunkeei*. All isolates included characteristics that were beneficial to health, including antagonistic activity against bacterial and fungal pathogens and free radical scavenging capacity. Antagonistic activity against pathogens is considered one of the most important characteristics of probiotic microbes to avoid gastrointestinal infections [20]. Moreover, it may help prevent spoilage by the microorganisms in foods. Indeed, all probiotic microbes used currently in functional foods or medicines include this activity. Previously, LAB species isolated from natural honey have been reported to include antimicrobial effects. Hasali *et al.* reported five strains of *Lactobacillus* spp. isolated from Meliponine honey, which showed antimicrobial activity against *S. aureus*, *S. epidermidis*, *B. subtilis*, *E. coli*, *Pseudomonas aeruginosa*, *S. typhimurium* and *Listeria monocytogenes* [8]. Several other strains of *Lactobacillus* spp. isolated from honey could resist the growth of one or more foodborne pathogens, including *S. enteritidis*, *Listeria monocytogenes*, *S. aureus*, *Shigella flexneri*, *B. cereus* and enteropathogenic *E. coli* [9]. Bulgasem *et al.* reported isolation of 25 LAB from the honey samples of Libya, Malaysia, Saudi Arabia and Yemen. Four strains of the LAB, *L. curvatus* HH from Al-Hanon honey, *L. plantarum* HS from Al-Seder honey, *P. pentosaceus* HM from Al-Maray and *P. acidilactici* HC from Tualang honey, could inhibit pathogenic *Candida* spp. [25]. The LAB from honey could therefore be a good source of antimicrobial activity providing protection from bacterial and fungal pathogens. Additionally, the three isolates showed strong antioxidant effects providing further benefits to the consumers. Consumption of natural antioxidants with the foods can particularly be beneficial to human health [47]. Antioxidant activity of the LAB is due to several bioactive compounds, as demonstrated by several studies [12,48–50]. In presence of insufficient quantities of antioxidants in human body, free radicals can cause cellular damages as well as degenerative or pathological processes such as aging and cancer [50]. Bioactive compounds produced by LAB include significant roles in decreasing effects of free radical reactive oxygen species. Results presented in this study have indicated high antioxidant activities in the isolates, which are similar to those in previous studies [48,51,52].

In addition to providing beneficial health effects, it is important for the LAB to survive and colonize the GIT. Therefore, the LAB isolates were assessed for their tolerance capacity to simulated gastric juice, bile salts and phenol. Resistance to low pH is critical for selecting appropriate bacterial strains for probiotic uses. Unfavorable acidic conditions with pH of the host GIT varying nearly 2 make it particularly difficult for the microorganisms to inhabit the organ [53]. Findings of the present study suggested that the isolated LAB strains included relatively high survival rates (39-42%) within 4 h of exposure to pH 2, while food travels along the human gut for maximum 3 h. Although pH of the human stomach can be milder than 2 depending on the feeding state and due to the buffer action of dietary ingredients, resistance of the isolates to the pH better ensures their possible survival in the GIT. However,

not all the LAB strains can survive at pH 2. Investigations suggested that survival of LAB significantly varied across the strains at pH 2 or 3 [54]. However, several *in vitro* assessments have described LAB isolates of food, human and animal origin, which could retain their viability when subjected to pH 2-4 [12,55]. In contrast, other studies reported severe decreases in growth of *Lactobacillus* spp. at pH 2.5 and only survival at pH 5, 6 and 7 [12]. The three LAB strains of this study demonstrated resistance to bile salts and phenol, a major requirement for probiotic selection. Assessment of the bile salt tolerance is important in understanding of the candidate probiotics' metabolic status and colonization capacity in the GIT [1]. The three isolates showed strong to moderate survival to 0.5% bile salts, which suggested that they could tolerate such conditions. Additionally, they could survive exposure to phenol, a toxic compound produced from the deamination of certain amino acids (AA) by gut microbiota [56]. It is expected from the probiotic candidates to tolerate toxic effects produced by phenol.

The honey associated isolates further proved their probiotic potentials by demonstrating significant autoaggregation, coaggregation and adhesion abilities. Autoaggregation and coaggregation abilities help LAB form barriers to prevent pathogens from mucosal colonization in the gut [57]. Furthermore, autoaggregation and coaggregation abilities of the isolates reveal their capacities to adhere to mucosal surfaces and epithelial cells. Previous studies reported probiotic LAB showing good aggregation characteristics [1,12]. In the present study, the isolates' ability to autoaggregate and coaggregate increased with time, similar to other studies [3,12]. The LAB isolates were shown with high to moderate adhesion activities to hydrocarbons, including ethyl acetate, chloroform and xylene. Surface hydrophobicity is another key factor for the selection of functional probiotics. It is addressed within the key physicochemical characteristics to support the first contact between the bacteria and host tissues. Moreover, strains with higher adhesion abilities were demonstrated with higher aggregation abilities in studies, suggesting positive correlations between adhesion and aggregation which was reported in the present study [1,3].

The LAB isolates considered for probiotic development must be assessed for their safety for consumption. Hence, isolates of this study were subjected to hemolytic and antibiotic sensitivity assays. The assessed strains did not show hemolytic activities on sheep blood agar, which indicated that the isolates could be considered safe. Similar to this finding, Coelho et al. reported the absence of hemolytic activity in LAB and yeast strains isolated from a honey based beverage [58,59]. Similarly, *Lactobacillus* spp. isolated from the gut of the worker honey bee L. *Apismellifera*, including *L. brevis* HBE2 and *L. casei* HBE5 [60], or probiotic *Bacillus* spp. isolated from honey and honey bee did not show hemolytic activity [61,62]. Antibiotic susceptibility assay of the LAB strains revealed their resistance to five to seven out of 12 antibiotics. The 12 antibiotics used in this study covered all the seven most common groups of antibiotics, including penicillin (penicillin, amoxicillin and ampicillin), aminoglycosides (gentamycin), lincosamide (clindamycin), macrolide (azithromycin and erythromycin), quinolone (ciprofloxacin and ofloxacin), chloramphenicol and tetracycline (tetracycline and doxycycline hydrochloride). The LAB isolates were mostly resistant to two antibiotics of the penicillin group used in the present study such as penicillin and amoxicillin and one antibiotic from the quinolone group such as ofloxacin. In fact, resistance of LAB to a few antibiotics is generally common and may be attributed to non-transferable intrinsic resistance [63]. Previously, LAB isolates from a number of foods were resistant to several antibiotics such as those isolated from honey [64], broilers [58], coconut palm nectar [1], fermented fish and chicken [65], fermented meats [66] and dairy products [67]. Technically, presence of natural resistance to antibiotics in LAB is considered beneficial since it may protect the bacteria against antibiotic therapy [38]. Moreover, it helps simultaneous use of antibiotics and probiotic LAB when used for therapeutic and preventive purposes.

5. Conclusion

To the best of the authors' knowledge, this is the first report of extensive probiotic characterization of LAB present in natural Bangladeshi honey. The isolated LAB strains demonstrated a variety of health promoting effects such as antibacterial, antifungal and antioxidant effects, as well as probiotic competences such as tolerance to extreme gut conditions and autoaggregation, coaggrergation and adhesion abilities. Hence, the LAB isolates can be addressed as potential sources of antimicrobial and antioxidant compounds having additional advantages of critical gut endurance capacity. Further studies particularly *in vivo* evidence of their health benefits and adaptation to gut environments are needed for the establishment of these isolates as functional probiotics.

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7. Conflict of Interest

The authors report no conflict of interest.

8. Authors Contributions

JF and TC contributed to conception, design and supervision of the study; SP and SI carried out laboratory experiments and analyzed and interpreted all data. JF wrote and prepared the first draft; FA reviewed and helped in manuscript writing and TJH extensively revised, edited and finalized the manuscript. All authors read and approved the final manuscript

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ویژگی‌های پروبیوتیکی باکتری‌های اسید لاکتیک *پدیکوکوس پنتوزاسه‌اوس* و *آپیلاکتوباسیلوس کانکه‌ای* جدا شده از عسل طبیعی بنگلادش

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چکیده

سابقه و هدف: باکتری‌های لاکتیک اسید به‌دلیل اثرات زیست‌یاری[۱] قوی بر میزبان شناخته شده‌اند. مشخصات زیست‌یاری باکتری‌های لاکتیک اسید موجود در عسل طبیعی بنگلادش محدود است. اهداف این مطالعه شامل جداسازی و ارزیابی ویژگی‌های زیست‌یاری و ایمنی باکتری‌های لاکتیک اسید در عسل بنگلادشی بود.

مواد و روش ها: برای جداسازی و خالص‌سازی باکتری از روش‌های کشت خطی و پخش کردن بر سطح بشقابک استفاده شد. جدایه‌ها با استفاده از تجزیه و تحلیل توالی ژن S rRNA۱۶ شناسایی شدند. برای ارزیابی خواص ضد باکتریایی و ضد قارچی، به‌ترتیب از روش انتشار چاهک آگار و غذای مسموم استفاده شد. فعالیت ضداکسایشی[۲] براساس توانایی مهار رادیکال آزاد میکروبی انجام شد. خودتجمعی میکروبی، انعقاد و چسبندگی با استفاده از روش رسوب سلولی مورد بررسی قرار گرفت. آگار خون‌دار[۳] در سنجش همولیتیک استفاده شد. سنجش حساسیت ضدبیوتیکی با استفاده از روش انتشار صفحه انجام شد.

یافته‌ها و نتیجه‌گیری: از مجموع ۲۵ سویه جدا شده از عسل، ۱۰ ایزوله گرم مثبت غیر اسپورزا کاتالاز منفی انتخاب و در روش انتشار چاهک آگار استفاده شد. سه مورد از جدایه ها اثرات ضد میکروبی متمایزی با ایجاد مناطق بازدارنده وسیع در برابر همه سویه‌های بیماری زا از جمله *باسیلوس سرئوس*، *استافیلوکوکوس اورئوس*، *ویبریوکلرا*، *سالمونلا تیفی* و *کاندیدا آلبیکنس* نشان دادند. ویژگی‌های جامع‌تر این سه جدایه بررسی شد تا ویژگی های مربوط به رشد، خواص بیوشیمیایی و توانایی تخمیر کربوهیدرات آنها تعیین شود. علاوه بر این، تجزیه و تحلیل توالی ژن S rRNA۱۶ نشان داد که جدایه‌ها متعلق به *پدیکوکوس پنتوزاسه اوس* (دو سویه) و *آپیلاکتوباسیلوس کانکه‌ای* هستند. میزان تحمل آنها در شرایط شبیه‌سازی شده معده در شرایط برون‌تنی[۴] ارزیابی شد، نتایج نشان داد جدایه ها به‌طور قابل توجهی در pH پایین، نمک‌های صفراوی و فنل زنده ماندند. این باکتری‌ها توانایی خوبی در چسبندگی به اتیل‌استات، کلروفرم و زایلن و همچنین ویژگی‌های خودتجمعی و انعقادی برجسته‌ای داشتند. علاوه بر این، فعالیت مهار رادیکال آزاد هیدروکسیل جدایه‌ها بر ویژگی ضداکسایشی قابل توجه باکتری دلالت می کرد. در ارزیابی ایمنی، ایزوله ها فعالیت همولیتیک نشان ندادند و به چندین ضدبیوتیک مقاوم بودند. بنابراین، نتایج نشان می‌دهد که عسل می‌تواند منبع مهمی از گونه‌های مفید باکتری لاکتیک اسید با چندین مزیت زیست‌یاری باشد.

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[۱] Probiotic effects
[۲] Antioxidant activity
[۳] Blood-agar
[۴] *in vitro*