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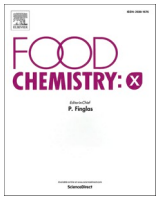

Development and health impact assessment of a novel functional probiotic beverage by fermenting hibiscus tea with coconut water☆

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ABSTRACT

This study developed a probiotic beverage by fermenting hibiscus tea with coconut water using *Lactobacillus acidophilus* (10307). The strain demonstrated survival at pH-3.0 and pH-2.0, along with significant auto-aggregation (40.20 %), co-aggregation (23.0–11.60 %) and moderate hydrophobicity (1.38–38.38 %). It showed antimicrobial activity against pathogens and lacked hemolytic activity, confirming its safety. Fermentation induced significant physicochemical changes, reducing pH and increasing titratable acidity due to organic acid production. Phytochemical analysis revealed enhanced total phenolic content (7.80 to 14.0 mg GAE/100 mL) and flavonoid content (26.50 to 87.70 mg QE/100 mL). FTIR analysis indicated biomolecular alterations and sensory evaluation scored the beverage 6.90 on a 9.0-point hedonic scale. Additionally, the beverage exhibited strong antioxidant activities, scavenging DPPH and H_2O_2 radicals effectively. These findings highlight the potential of this fermented hibiscus tea and coconut water beverage as a functional drink with probiotic and antioxidant benefits.

1. Introduction

Probiotics are live microorganisms that, when consumed in appropriate amounts, confer health benefits to the host. These include antimicrobial activity, immune system modulation, enhanced lactose digestion, reduced blood cholesterol levels, anti-carcinogenic and antimutagenic effects, diarrhea alleviation and improvements in inflammatory bowel disease (Shah, 2007). Fermented foods contain bioactive components that influence the gut microbiota, contributing to overall health. Therefore, considering the availability of functional bioactive compounds in beverages is reasonable for regulating and determining the gut microbiota composition (Kandylis et al., 2016).

Scientific studies highlight the vital role of gut microbiota in various physiological processes, including neurodevelopment, mood and behaviour of the host through the intricate gut-brain axis (Dahiya & Nigam, 2022). Studies has proven that the gut microbiome can be enhanced through the consumption of functional foods containing probiotics (Amara & Shibl, 2015). Probiotics and probiotic-fortified foods are generally regarded as safe and are commonly utilized. Probiotic strains are readily accessible and widely employed in food production and as supplements in human and animal food (Dahiya & Nigam, 2022).

The traditional approach to delivering probiotic microorganisms to the gut involves consuming dairy-based fermented products, typically made from milk. Popular fermented beverage options globally include yogurt smoothies, fermented milk and kefir. Dairy products often contain added probiotic cultures and consumers are familiar with the presence of microorganisms in such items (Terpou et al., 2019). However, the consumption of such products may not be suitable for lactose-intolerant individuals, those with a milk protein allergy, or those adhering to a vegan diet. The intake of dairy products is also declining due to concerns over their cholesterol content and their potential association with gastroesophageal reflux disease (GERD). Lactose-

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intolerance is highly prevalent, affecting approximately 75 % of the global population (Panghal et al., 2018). As a result, lactose-free dairy alternatives are increasingly utilized in food and beverage production to cater to lactose-intolerant individuals. Non-dairy products are becoming more popular as replacements for milk-based preparations due to their numerous health advantages and widespread commercial acceptance. The demand for non-dairy alternatives is growing everyday due to factors such as changing lifestyles, increased health awareness, the use of natural nutritional supplements, and a rise in daily allergy cases. Consequently, the market for dairy substitutes is expanding its focus to include extracts from soybeans, rice, and coconuts as primary matrices for developing probiotic fruit and vegetable drinks (Rasika et al., 2021). Fruits, corn, vegetables, and legumes are among the fundamental raw materials that can be used to produce probiotic beverages.

Using fruits and vegetables in beverage preparation offers the advantage of avoiding issues associated with lactose and cholesterol in dairy products, making non-dairy beverages accessible to a diverse range of consumers. Lactic acid bacteria (LAB) are commonly used to ferment fruits, vegetables and cereals, enhancing taste and generating bioactive components beneficial for various health conditions such as inflammation, compromised immunity, glycaemic imbalance and fatigue (Kim et al., 2002). Various plants and their derivatives have long been used as herbs or spices in food, serving as natural preservation, flavor enhancers and even treatments for common human diseases. These medicinal properties primarily stem from the bioactive compounds present in plants. Utilizing natural plant-derived antimicrobials can effectively reduce reliance on antibiotics, lower the risk of resistance to antibiotics, and help prevent cross-contamination (Voon et al., 2012). Furthermore, in addition to their antibacterial and antioxidant properties, plants or their extracts can serve as naturally occurring food coloring agents, which are generally considered harmless and non-toxic to humans (Rasika et al., 2021).

Hibiscus rosa-sinensis L., with its red flowers, is well-suited for these applications, as it is extensively used in traditional cuisine as a flavor enhancer and in local medicine for its various medicinal properties, including antipyretic, analgesic, anti-asthmatic and anti-inflammatory effects attributed to its secondary metabolites (Sivaraman & Saju, 2021). Coconut water, derived from green coconuts (*Cocos nucifera* L.), is also highly valued as one of the most widely available fruit-based liquids worldwide (Lima et al., 2015). It boasts a rich composition of electrolytes, vitamins, minerals, cytokines and proteins. In addition to its medicinal properties, coconut water is high in fiber, acts as a diuretic and laxative, aids in natural hydration, enhances energy levels, possesses antimicrobial properties and contributes to anti-aging efforts (Lima et al., 2015). Due to its unique chemical composition including vitamins, sodium, free amino acids, phosphorus, chloride, potassium, magnesium, carbohydrates, proteins, and growth-promoting elements, coconut water is particularly beneficial for treating human diarrhea in underdeveloped regions, hydrating individuals, and protecting the digestive tract from infections (Prado et al., 2008). Besides being consumed by humans, it is also serves as a suitable growth-enhancing medium for different kinds of beneficial microorganisms. Given these advantages, hibiscus and coconut water have received a significant attention in functional food preparation.

The primary goal of this study was to develop a functional probiotic beverage containing lactic acid bacteria by combining hibiscus tea with coconut water. Furthermore, the fermented mixture of hibiscus tea and coconut water was evaluated for its sensory acceptability, antioxidant activity, phytochemical components and biological and microbiological properties. This unique non-dairy functional beverage is particularly well-suited for lactose-intolerant individuals and vegans, as it is formulated with a blend of coconut water and hibiscus tea.

2. Materials and methods

2.1. Collection of coconut water

Fresh green coconuts were procured from a local market in Vadodara, Gujarat, India. To ensure surface sterilization, the coconuts were exposed to UV light for two complete cycles. Following sterilization, they were aseptically opened using a sterile knife to extract the coconut water. The collected coconut water was sequentially filtered using a 0.65-μm polyether sulfone filter (Sigma-Aldrich), followed by a 0.45-μm filter paper before being used for fermentation (Prado et al., 2008).

2.2. Preparation of red hibiscus extract

Red hibiscus tea extract was prepared using fresh or dried red hibiscus flowers. Briefly, fresh or dried hibiscus flowers were added to 100 mL of distilled water in a conical flask and heated to 90 °C for 15–20 min. After allowing the mixture to steep for a few minutes, the extract was strained and subsequently filtered using a 0.45-μm filter paper. The filtered extract was then used for further experiments (Anomohanran, 2014).

2.3. Lactic acid bacteria

The lactic acid bacterial strain, *Lactobacillus acidophilus* (MTCC 10307) was obtained from the Microbial Type Culture Collection (MTCC) Chandigarh, India. The strain was cultured on MRS agar media (De Man, Rogosa and Sharp) at 37 °C for 72 h and subsequently stored at 4 °C for future use.

2.4. Probiotic screening assay

2.4.1. Acid tolerance

The acid tolerance of *L. acidophilus* was assessed following the procedure described by (Kumari et al., 2022). Briefly, bacterial cell pellets were collected from an overnight-grown culture and resuspended in the phosphate buffer saline (pH - 7.2). The cell suspension, containing approximately 10^9 CFU/mL, was exposed to different acidic conditions (pH - 1.0, 2.0 and 3.0) in MRS broth and incubated for 5 h at 37 °C. MRS broth (pH - 6.5) was used as a control. After incubation, the samples were serially diluted and plated on MRS agar to determine the viable count (CFU/mL).

2.4.2. Cell surface hydrophobicity

The cell surface hydrophobicity (H%) of *L. acidophilus* was determined using a modified version of the method described by Rosenberg et al. (1980). An overnight-grown culture of *L. acidophilus* was inoculated into freshly prepared MRS broth and incubated for 14 h at 37 °C. The bacterial cells were harvested by centrifugation, washed twice with 1.0 M PBS solution (pH - 7.4) and resuspended in PBS. The cell suspension was then adjusted to an absorbance of 0.90 at 600 nm (A_0), corresponding to a bacterial concentration of 10^7 CFU/mL. Subsequently, 1 mL of an organic solvent (toluene, chloroform and ethyl acetate) was added to 3 mL of the bacterial suspension. The mixture was vortexed for 30 s and incubated at 37 °C for 10 min to allow for temperature equilibration. Following a second vortexing step, the samples were incubated for 1 h at 37 °C for phase separation. After carefully removing the aqueous phase, the absorbance at 600 nm (A_t) was recorded. The percentage of cell surface hydrophobicity (H%) was calculated using the following formula:

$$H\% = A_0 - A_t/A_0 \times 100$$

2.4.3. Auto-aggregation assay

The auto-aggregation assay was performed following the method described by Solieri et al. (2014) with slight modification. An overnight-

grown culture of *L. acidophilus* was inoculated into freshly prepared MRS broth and incubated at 37 °C for 14 h. The bacterial cells were harvested by centrifugation, washed twice with 1.0 M PBS solution (pH - 7.4) and resuspended in PBS. A, 4 mL aliquot of the bacterial suspension (10^9 CFU/mL) was vortexed and left undisturbed at room temperature for 5 h. After incubation, an aliquot from the upper layer of the suspension was carefully withdrawn and its absorbance at 600 nm (A_{Final}) was measured. The auto-aggregation percentage (A%) was calculated based on the decrease in absorbance relative to the initial absorbance ($A_{Initial}$) using the following equation:

$$\text{Auto} - \text{aggregation}\ (A\%) = 1 - (A_{Final}/A_{Initial}) \times 100$$

2.4.4. Co-aggregation assay

The co-aggregation ability (Co-A%) was evaluated following the method described by Solieri et al. (2014). The pathogenic bacterial strains *S. marcescens*, *E. coli*, *C. violaceum* and *P. aeruginosa* were used for the assay. The bacterial cell suspensions were prepared as described previously. Equal volumes of *L. acidophilus* and the pathogenic strain suspensions were mixed, vortexed for 10 s and incubated for 5 h at room temperature. At the end of incubation, absorbance of the test strain (Ax), pathogenic strain (Ay) and the mixed suspension [A (x + y)] were recorded at 600 nm using a UV–Vis spectrophotometer. The cell suspensions of individual strains served as controls. The co-aggregation percentage (Co-A%) was calculated using the following equation:

$$\text{Co} - \text{A\%} = [(\text{Ax} + \text{Ay})/2 - \text{A(x} + \text{y})/(\text{Ax} + \text{Ay}/2)] \times 100$$

Where, Ax - represents absorbance of *L. acidophilus*, Ay - represents absorbance of the pathogens; A (x + y) - represents absorbance of the mixture of both.

2.4.5. Hemolytic activity

The hemolytic activity of *L. acidophilus* was assessed following the method described by Casarotti et al., 2017, with slight modifications. The bacterial strain was streaked onto MRS blood agar plates and incubated for 48 h at 37 °C. After incubation, hemolysis was evaluated based on the appearance of distinct zones around the bacterial colonies. Beta hemolysis (a clear zone around the colony), alpha hemolysis (a green zone surrounding the colony) and gamma hemolysis (no clear zone surrounding colonies) were observed for the culture.

2.5. Antibacterial activity

The antibacterial activity of the *L. acidophilus* culture supernatants was evaluated using the agar cup-diffusion method, as described by Valgas et al. (2007), with slight modifications. The cell-free supernatant was collected and filtered using a 0.45-μM Millipore filter before use. Different bacterial strains such as, *B. cereus*, *S. marcescens*, *S. aureus* and *E. coli* were used as bacterial pathogens. The overnight grown cultures of these pathogens were spread evenly on MHB agar plate using a sterile swab. Wells were then created on the agar plates using a sterile cork borer and cell-free supernatant of *L. acidophilus* was introduced into each well. The plates were incubated at 37 °C for 24 h, after which the antibacterial activity was assessed by measuring the diameter of the inhibition zones around each well. MRS broth served as the negative control, while Streptomycin was used as the positive control.

2.6. Preparation of probiotic beverage

A mixture of hibiscus extract and coconut water (1:3 ratio) was prepared and 15 % honey was incorporated as a natural sugar source. Subsequently, 1 mL of activated *L. acidophilus* (7.8×10^9 CFU/mL) was inoculated into 150 mL of the mixture in a sterilized glass bottle. The fermentation process was carried out by incubating the bottle at 37 °C for 24–48 h to develop the probiotic beverage.

2.6.1. Determination of pH and acidity of probiotic beverage

The total acidity of the probiotic beverage was determined by titration, using phenolphthalein as an indicator and 0.01 N NaOH as the titrant. The acidity was expressed as a percentage (*w/v*) of lactic acid. Additionally, the pH of the probiotic beverage was measured using a pre-calibrated pH meter, following the procedure described by Liew et al. (2022).

2.6.2. Viable cell count

The viability of *L. acidophilus* in the probiotic beverage was determined using the standard plate count method, following the method described by Marnpae et al. (2022). The samples were plated on MRS agar medium and incubated at 37 °C for 48 h. After incubation, the cell viability was expressed as log CFU/mL of the sample.

2.7. Phytochemical analysis of probiotic beverage

2.7.1. Determination of total sugar and reducing sugar

The total and reducing sugar contents of the probiotic beverage were determined following the method described by Marnpae et al. (2022). The probiotic beverage was first centrifuged at 10,000 rpm for 10 min at 4 °C, and the supernatant was collected for analysis. Reducing sugar content was measured using the 3,5-dinitrosalicylic acid (DNS) method. A 1 mL sample of the probiotic beverage was mixed with 1 mL of DNS reagent, which contained 1 % 3,5-dinitrosalicylic acid, 0.2 % phenol, 0.05 % sodium sulfate, and 1 % sodium hydroxide. The mixture was then heated at 100 °C for 20 min to stabilize the color and subsequently combined with 1 mL of a 40 % potassium sodium tartrate solution. After cooling to room temperature, the absorbance was measured at 540 nm using a UV–Vis spectrophotometer. For total sugar determination, 400 μLof the sample was mixed with 10 μL of 80 g/100.0 mL phenol and 1 mL of sulfuric acid. The reaction mixture was incubated at 37 °C for 10 min, followed by an additional 10 min of cooling at room temperature. The sample was then diluted five times with distilled water and absorbance was recorded at 490 nm using a microplate reader. Glucose solution was used as the standard in both assays.

2.7.2. Determination of total phenolic content

The total phenolic content of the probiotic beverage was determined following the method described by Johari and Khong (2019), with slight modifications. A 100 μL sample of the probiotic beverage was mixed with 500 μL of Folin-Ciocalteu reagent and 6 mL of distilled water in a test tube for dilution. The mixture was allowed to stand at room temperature for 5 min. Then, 1.5 mL of 20 % sodium carbonate (Na_2CO_3) was added and the test tube was gently shaken to ensure thorough mixing. After 90 min, the absorbance of the mixture was measured at 725 nm using a UV–Vis spectrophotometer. Gallic acid used as the standard reference for generating the calibration curve.

2.7.3. Determination of total flavonoid content

The total flavonoid content of the probiotic beverage was determined using the aluminum chloride colorimetric method, with slight modifications as described by Chang et al. (2002). Quercetin was used as the standard to generate the calibration curve. A stock solution was prepared by dissolving 1 mg of quercetin in methanol, which was then diluted to obtain final concentrations of 5, 25, 50, 75 and 100 μg/mL. For the assay, 1.0 mL of each standard solution or sample was mixed with 3 mL of methanol, 0.2 mL of 10 % aluminum chloride, and 0.2 mL of 1 M potassium acetate. The mixture was incubated at room temperature for 40 min, after which the absorbance was measured at 430 nm using a UV–Vis spectrophotometer.

2.7.4. Determination of total tannins

The tannin content of the probiotic beverage was determined using the Folin-Ciocalteu method, as described by Kavitha Chandran and Indira (2016), with slight modifications. A 0.1 mL sample extract was

transferred into a 10 mL volumetric flask, followed by the addition of 0.5 mL of Folin-Ciocalteu reagent. After 1 min, 1.25 mL of 20 % sodium carbonate solution was added, and the mixture was thoroughly mixed. The solution was then incubated at room temperature for 30 min. The absorbance was measured at 765 nm using a UV–Visible spectrophotometer, with gallic acid as the standard reference for generating the calibration curve.

2.8. Antioxidant activity of probiotic beverage

2.8.1. DPPH radical scavenging activity

The antioxidant capacity of the probiotic beverage was assessed using the DPPH assay, following the method described by Gulcin and Alwasel (2023) with slight modifications. A 1 mL sample of the probiotic beverage was mixed with 3 mL of a 0.2 mM DPPH solution in methanol at varying concentrations. The mixture was then incubated for 1 h at 37 °C. After incubation, the decrease in absorbance was measured at 517 nm using a UV–visible spectrophotometer. The DPPH radical scavenging activity was calculated using a standard curve of ascorbic acid and determined using the following formula:

$$\text{Scavenging effect (\%)} = [(\text{control absorbance} - \text{sample absorbance}) / (\text{control absorbance})] \times 100$$

2.8.2. H_2O_2 scavenging activity

The H_2O_2 scavenging activity of the probiotic beverage was determined using the method described by Khan et al. (2012) with slight modifications. A 40 mM hydrogen peroxide solution was prepared in phosphate buffer (pH - 7.4). Different concentrations of the probiotic beverage (250, 500, and 1000 μg/mL) were added to 0.6 mL of the hydrogen peroxide solution (40 mM). The mixture was incubated for 10 min, after which the absorbance was measured at 230 nm using a UV–Visible spectrophotometer. A blank solution containing only phosphate buffer without H_2O_2 was used as a reference. The percentage of H_2O_2 scavenging activity was determined using the following formula:

$$\text{Scavenging effect (\%)} = [(\text{control absorbance} - \text{sample absorbance}) / (\text{control absorbance})] \times 100$$

2.9. FTIR analysis of probiotic beverage

The FTIR spectra were recorded using a Bruker FTIR spectrometer equipped with an ATR accessory and a DTGS detector, operating at a resolution of 4 cm^{-1}. A small amount of each sample was carefully applied to the ATR crystal surface, ensuring an optimal signal-to-noise ratios. Each spectrum was obtained by scanning against the background spectrum of a clean ATR crystal and was presented in absorbance mode. After each measurement, the ATR crystal was cleaned with demineralized water and dried with soft filter paper before proceeding with the next sample analysis (Sitorus, 2019).

2.10. Microbiological analysis

2.10.1. Viability assay

The total viable count (TVC) was determined using the standard plate count method, as described by Islam et al. (2022). Initially, MRS agar media and a sterile saline solution (0.9 % NaCl, *w/v*) were prepared. A 1 mL sample of the probiotic beverage was aseptically transferred to 9 mL of sterile saline solution to create a 10^{-1} dilution. Serial tenfold dilutions were performed up to 10^{-6}. For each dilution, 1 mL of the diluted sample was transferred into a sterile Petri dish, and 10–15 mL of molten, tempered (45–50 °C) MRS agar medium was poured into the dish. The plates were gently swirled to ensure uniform distribution of the inoculum and allowed to solidify at room temperature. The plates were then inverted and incubated at 32 °C for 48 h. Following incubation, colonies were enumerated manually. Plates containing 30–300 colony forming units (CFU) were selected for counting to ensure statistical reliability. The TVC was calculated using the following formula:

$$\text{TVC (CFU/mL)} = (\text{Number of colonies} \times \text{Dilution factor}) / \text{Volume plated (mL)}$$

Duplicate plates were prepared and analyzed for each dilution to minimize experimental error and enhance the accuracy of the results. The final TVC was expressed as the mean ± standard deviation of the duplicate counts.

2.11. Sensory analysis

After refrigeration, samples of the fermented probiotic beverage were assessed for sensory attributes, including texture and body, appearance and color, flavor, acidity, and overall acceptability. The nine-point Hedonic scale was used for this assessment, where one for extreme dislike, two for very much dislike, three for moderate dislike, four for mild dislike, five for neither like nor dislike, six for like slightly, seven for like moderately, eight for like very much, and nine for like extremely—were used to conduct this evaluation. The sensory panel consisted of untrained judges (discriminative and communicative) from the Food Technology faculty at Parul University, Vadodara, India. The nine-point Hedonic scale was selected due to its widespread applicability in assessing product acceptability and consumer preference (Šertović et al., 2019).

2.12. Statistical analysis

The results are presented as mean ± SD of the number of experiments performed. The significance of the results was determined for the treatments using an ordinary one-way ANOVA followed by Bonferroni's multiple comparisons test at $p < 0.05$. The analyses were carried out using the Graph Pad Prism software 8.0.

3. Results

3.1. Probiotic potential of L. acidophilus

3.1.1. Acid tolerance

The ability of *L. acidophilus* to survive under highly acidic conditions was evaluated to determine its probiotic potential. The bacterial culture was exposed to pH levels of 1.0, 2.0, 3.0 and 7.0 (control) for 24 h. The results indicated that *L. acidophilus* did not survive at pH 1.0 after 24 h of incubation. However, the bacteria exhibited significant viability at pH 2.0, 3.0 and 7.0 (control), as shown in Fig. 1.

3.1.2. Cell surface hydrophobicity

The adhesion ability of *L. acidophilus* to organic compounds was evaluated using bacterial adhesion to hydrocarbons. Higher adhesion to hydrocarbons indicates greater cell surface hydrophobicity. Fig. 2 illustrates the percentage adhesion of *L. acidophilus* to different hydrocarbons. Among the three tested hydrocarbons, the highest adhesion was observed with chloroform (38.38 %), followed by ethyl acetate (34.33 %), while toluene exhibited the lowest adhesion (1.38 %).

3.1.3. Auto-aggregation and co-aggregation activity

The auto-aggregation ability of *L. acidophilus* was assessed by incubating the bacterial suspension for 5 h at 37 °C. The percentage of auto-aggregation was calculated by comparing the absorbance of the initial and final suspensions. The results indicated that *L. acidophilus* exhibited a strong auto-aggregating phenotype, with an aggregation rate of 40.20 % after 5 h (Fig. 3). This suggests its potential to form stable multicellular aggregates, which may enhance colonization and adhesion in the gastrointestinal tract.

The co-aggregation activity of *L. acidophilus* was evaluated by mixing

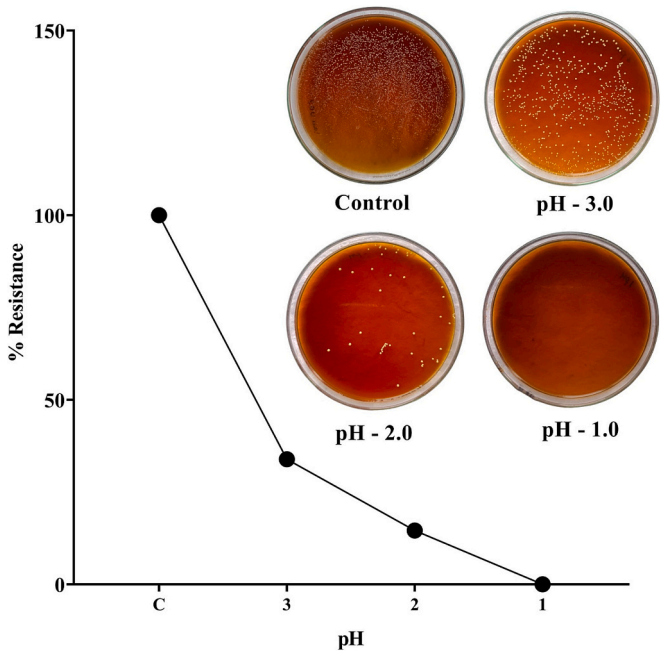

Fig. 1. Effect of various pH conditions (pH 1, pH 2, pH 3 and control) on the viability of *L. acidophilus*.

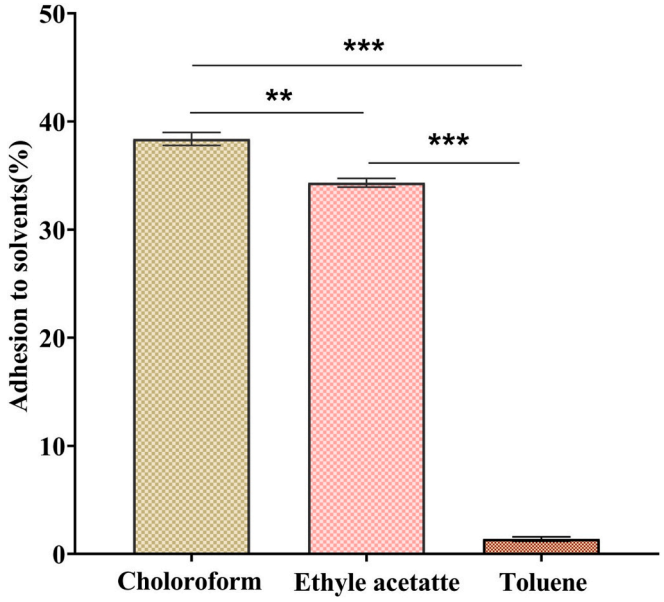

Fig. 2. Effect of different solvent systems (toluene, ethyl acetate and chloroform) on cell surface hydrophobicity of *L. acidophilus*.

the bacterial suspension with different pathogenic strains, including *S. marcescens*, *E. coli*, *C. violaceum*, and *P. aeruginosa*, followed by incubation for 24 h at 37 °C. The percentage of co-aggregation was determined by comparing the optical densities of the initial and final suspensions. The results demonstrated that *L. acidophilus* exhibited the highest co-aggregation with *E. coli* (23 %), followed by *C. violaceum* (15 %), *S. marcescens* (12 %) and *P. aeruginosa* (11.6 %) (Fig. 3). These findings highlight the potential of *L. acidophilus* to interact with pathogenic bacteria, potentially limiting their adhesion and colonization in the gastrointestinal tract.

3.1.4. Hemolytic assessment

No distinct transparent (β-hemolysis) or greenish (α-hemolysis) zones were observed around the colonies of *L. acidophilus* on blood agar plates, confirming its γ-hemolytic or non-hemolytic nature.

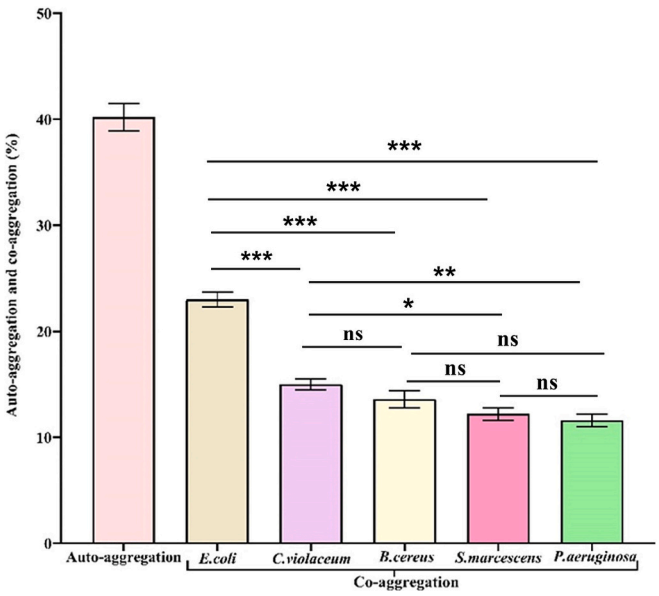

Fig. 3. Assessment of *L. acidophilus* co-aggregation and Auto-aggregation percentage via spectrophotometry.

3.2. Antimicrobial activity

The inhibitory effect of *L. acidophilus* cell-free supernatant (CSF) against human and food-borne pathogens was assessed using the agar well diffusion assay. Streptomycin served as the positive control, while MRS broth was used as the negative control. As shown in Fig. 4, *L. acidophilus* exhibited the highest inhibitory activity against *S. aureus*, followed by *S. marcescens* and *E. coli*, with the least activity observed against *B. cereus*.

3.3. Development of mixture of hibiscus tea and coconut water fermentation product

After confirming the probiotic potential of *L. acidophilus*, it was incorporated into the formulation of a novel fermented probiotic beverage combining Hibiscus tea and coconut water (Fig. 5). The beverage was evaluated for probiotic viability, pH, acidity and phytochemical composition, as summarized in Table 1. Additionally, its

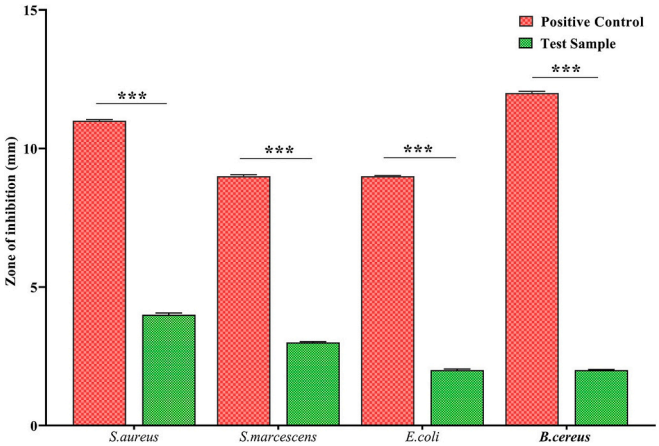

Fig. 4. Antimicrobial Activity of *L. acidophilus* against various bacterial pathogens.

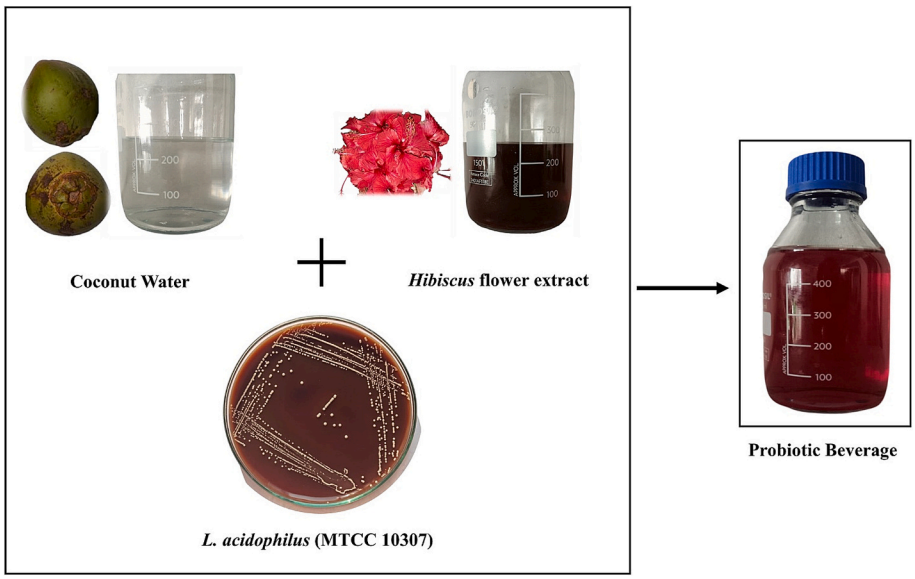

Fig. 5. Development of a novel probiotic beverage combining hibiscus tea, coconut water and *L. acidophilus*.

Table 1
Determination of pH, cell viability and acidity of the probiotic drink containing *L. acidophilus*.

Parameters	Before fermentation (0 h)	After fermentation (48 h)	After storage (7 days)	After Storage (14 days)	After Storage (21 days)	After Storage (28 days)
pH	5.741 ± 0.20	4.23 ± 0.10	3.58 ± 0.10	3.28 ± 0.20	2.91 ± 0.20	2.65 ± 0.20
Cell viability (CFU/mL)	4.70×10^6	3.01×10^8	3.28×10^8	12.80×10^8	6.0×10^7	2.10×10^6
Titratable acidity	0 ± 0.10	0.37 ± 0.10	0.59 ± 0.20	1.14 ± 0.90	1.26 ± 0.40	1.34 ± 0.20

antioxidant activity was assessed, and FTIR analysis was conducted to characterize its biochemical properties.

3.4. Viable cell count and physicochemical changes during fermentation

The viability of *L. acidophilus* in the fermented probiotic beverage was monitored at the beginning (time 0) and after 28 days of fermentation using the standard plating technique. Initially, the bacterial count was 4.70×10^6 CFU/mL, which decreased to 2.10×10^6 CFU/mL by the end of the fermentation cycle. Concurrently, the pH dropped to 2.65 ± 0.20, indicating successful fermentation and organic acid production by *L. acidophilus*. The fermented product also exhibited a titratable acidity of 1.34 ± 0.20 % of lactic acid per 100.0 mL (Table 1).

3.5. Changes in cell viability, pH and titratable acidity during storage

After 28 days of cold storage at 4 °C, the viability of *L. acidophilus* showed a moderate decline, stabilizing at 2.10×10^6 CFU/mL. The pH of the fermented probiotic beverage continued to decrease, reaching a minimum of 2.65 ± 0.23. In contrast, titratable acidity increased over the storage period, reaching 1.34 ± 0.20 g of lactic acid per 100 mL (Table 1).

3.6. Total phenolic compounds, flavonoids, and tannin in fermented probiotic beverage

During fermentation, the combination of hibiscus tea and coconut water inoculated with *L. acidophilus* showed an increase in total phenolic, flavonoid and tannin concentrations. The total phenolic contents of the probiotic beverage with added sugar (honey) and without added sugar (honey) ranged from 11.63 ± 0.60 to 14.63 ± 0.62 mg GAE/100.0 mL and 7.801 ± 0.68 to 10.50 ± 0.24 mg GAE/100.0 mL

Table 2
- Phytochemical analysis of the probiotic beverage.

Parameter	Total phenolic content (mg GAE/ 100 mL)	Total flavonoid content (mg QE/ 100 mL)	Total tannin content (mg GAE/ 100 mL)	Total sugar (glucose mg/100 mL)	Total sugar (glucose mg/100 mL)
Non-fermented (0 h)	7.80 ± 0.60	26.50 ± 0.70	22.02 ± 1.10	59.10 ± 1.21	9.647 ± 0.90
Fermented (48 h)	10.50 ± 0.20	48.40 ± 3.80	31.10 ± 1.50	21.16 ± 2.80	5.68 ± 0.70
Non-fermented with added sugar	11.60 ± 0.60	40.20 ± 1.39	38.10 ± 0.60	79.46 ± 0.60	10.70 ± 0.60
Fermented with added sugar	14.60 ± 0.40	87.70 ± 1.46	59.30 ± 0.40	69.35 ± 2.40	7.60 ± 0.70

(Table 2). The flavonoid levels in the probiotic beverage with added sugar (honey) increased from 40.20 ± 1.39 to 87.70 ± 1.46 mg/100 mL, while those without added sugar (honey) increased from 26.50 ± 7.20 to 48.54 ± 3.80 mg/100 mL (Table 2). Similarly, the total tannin content of the probiotic beverage with added sugar (honey) varied from 38.10 ± 0.65 to 59.20 ± 0.40 mg GAE/100 mL and for the beverage without added sugar (honey), it ranged from 22.13 ± 1.10 to 31.10 ± 1.50 mg GAE/100 mL (Table 2).

3.7. Total sugar and reducing sugar, in fermented probiotic beverage

Table 2 summarizes the total and reducing sugar content of the Hibiscus tea and coconut water mixture before and after fermentation. A significant reduction in both total and reducing sugar content was observed post-fermentation, indicating microbial utilization of sugars during the fermentation process.

3.8. Sensory acceptability

The sensory attributes of the Hibiscus tea and coconut water mixture fermented with *L. acidophilus* were evaluated using a 9.0-point hedonic rating scale, where 1.0 represents "strongly dislike" and 9.0 represents "strongly like". Evaluated parameters included appearance, color, aroma, taste, texture and overall acceptability. The results are summarized in Table 3.

3.9. Antioxidant potential of fermented probiotic beverage

The antioxidative potential of the fermented probiotic beverage was assessed using the DPPH• radical scavenging assay and H_2O_2 scavenging assay. Both assays measured the reduction in absorbance of DPPH• and H_2O_2 free radicals due to interactions with the beverage. The results indicated significant antioxidant activity, with the beverage effectively scavenging both radicals, leading to a decrease in absorbance values. Notably, the antioxidative capacity increased with higher concentrations of the beverage, as shown in Fig. 6A & B.

3.10. FTIR analysis of the probiotic beverage

FTIR spectroscopy was employed to analyze the vibrational characteristics of functional groups within the fermented probiotic beverage, highlighting structural modifications after inoculation with *L. acidophilus* in the hibiscus tea and coconut water mixture. The FTIR spectra (Fig. 7A & B) displayed distinct peaks in the 3500.0–500.0 cm^{-1} range, revealing notable biochemical changes. The fermented probiotic beverage spectrum revealed characteristic peaks that allowed for the differentiation of glucose, fructose and sucrose within the 1400.0 to 950.0 cm^{-1} range. Specifically, fructose bonds were observed at 919 cm^{-1} and sucrose exhibited a peak at 1242.0 cm^{-1}. The band at 3421.0 cm^{-1} corresponded to the stretching vibration of the -OH functional group, while the 2936.0 cm^{-1} band represented the stretching vibration of C—H aliphatic compounds. Additionally, the presence of carboxylic acid was indicated by the band at 2369.51 cm^{-1}. The band at 1385.11 cm^{-1} was attributed to O—H in-plane deformation in polyphenols and the band at 1637.0 cm^{-1} represented the stretching vibration of the C═C double bond in the carboxyl region. While several functional groups were identified, some bands overlapped, limiting precise assignment.

4. Discussion

The integration of probiotics into food products has become a major focus in the realm of functional foods, given their numerous health benefits, including anti-inflammatory, anti-cancer, anti-diabetic, anti-obesity, anti-pathogenicity, and anti-allergic effects (Kerry et al., 2018). Traditionally, probiotics are associated with fermented dairy products such as milk and yogurt. However, the increasing preference for plant-based alternatives in developed countries, coupled with concerns over lactose intolerance and cholesterol content in dairy products, has driven the demand for non-dairy probiotic options (Kerry et al., 2018).

Coconut water is widely recognized for its role in oral rehydration and treatment of childhood diarrhea, gastroenteritis and cholera. It contains essential organic and inorganic compounds that support the body's antioxidant system. Similarly, *Hibiscus rosa-sinensis*, native to tropical Asia, is commonly consumed in tea and used as an herbal remedy for managing hypertension, cholesterol levels and cancer progression (Goldberg et al., 2017).

In this study, we developed an innovative probiotic beverage by combining *L. acidophilus* with hibiscus tea and coconut water. A comprehensive evaluation of *L. acidophilus* was conducted to assess its probiotic potential. Acid tolerance, a key factor for probiotic efficacy, revealed that *L. acidophilus* could survive at very low pH levels (2.0 and 3.0), consistent with previous studies (Nyabako et al., 2020). Hydrophobicity, an important criterion for probiotic selection, was measured and our results were comparable to those reported by (Guan et al., 2020). The auto-aggregation ability of *L. acidophilus*, essential for bacterial persistence in the gut, aligned with the studies of Farid et al. (2021), confirming no correlation between adherence ability and auto-aggregation. Co-aggregation, which plays a role in pathogen removal from the gastrointestinal tract, varied across microorganisms, as previously reported by Tuo et al. (2013). The antibacterial activity of *L. acidophilus* against four pathogenic bacteria further validated its probiotic efficacy, supporting findings by Giri et al. (2018).

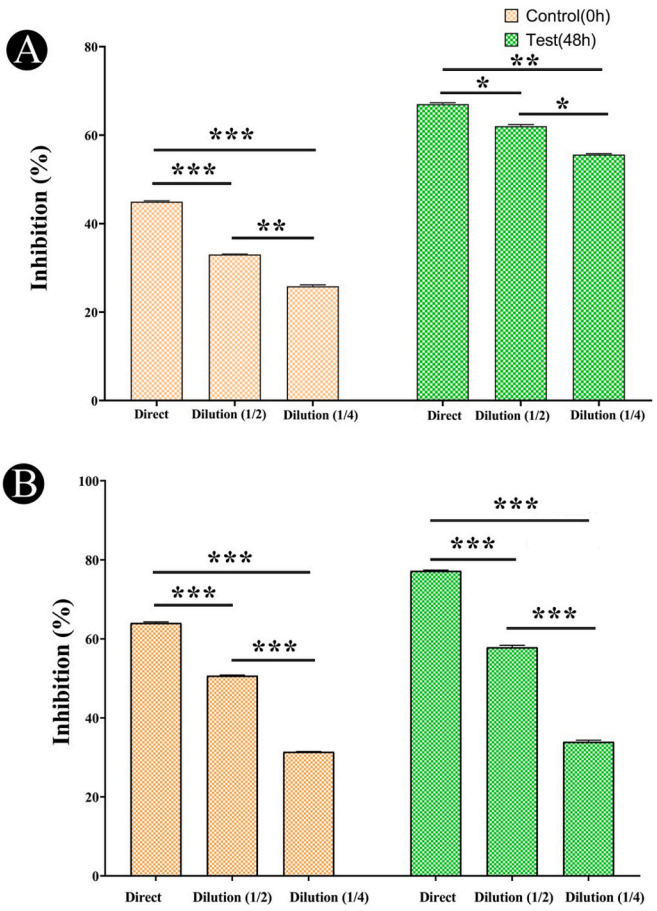

Fig. 6. Impact of varying concentrations of fermented probiotic beverage extract on DPPH (A) and H_2O_2 (B) radical scavenging activity.

Table 3
Sensory evaluation of the prepared probiotic beverage.

Parameters	Before fermentation (0 h)	After Storage (28 days)
Appearance	8.0 ± 1.10	7.80 ± 0.90
Flavor	7.30 ± 0.80	6.44 ± 1.20
Smell	6.60 ± 0.60	5.50 ± 0.60
Texture	7.10 ± 0.60	7.0 ± 0.30
Overall Acceptance	7.27 ± 0.03	6.66 ± 0.40

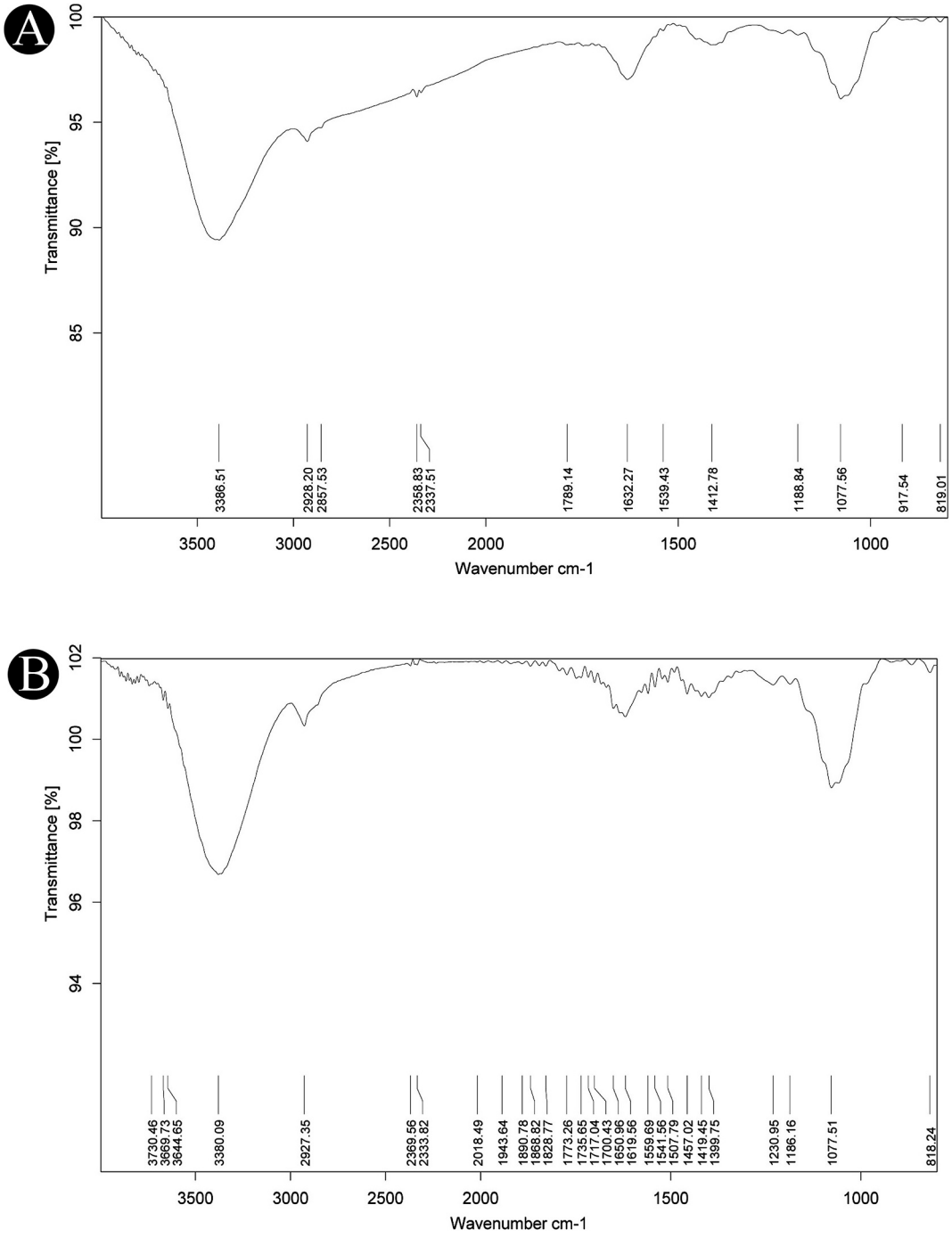

Fig. 7. FTIR analysis of unfermented (A) and fermented (B) probiotic beverages.

Additionally, safety assessments confirmed the absence of hemolysis, in agreement with Halder et al. (2017).

Following the screening of *L. acidophilus* for its probiotic characteristics, we prepared the probiotic beverage and monitored various physicochemical properties, including pH, acidity and viability. The substantial decrease in pH after 48 h of fermentation supports its inhibitory effect on undesirable microorganisms, as reported by Rahman (2020). Viability studies confirmed that *L. acidophilus* was able to produce lactic acid and proliferate in the coconut water and hibiscus tea mixture, consistent with the findings of Malik et al. (2019) on *L. casei* fermented beetroot and carrot juice. The antioxidant potential of the developed probiotic beverage was comparable to that reported by Kantachote et al. (2017). Phytochemical analysis revealed a significant increase in total phenolic and flavonoid content post-fermentation, aligning with previous studies on *Lactobacilli*-fermented coconut water (Kantachote et al., 2017). Furthermore, the total and reducing sugar content decreased notably, indicating the consumption of glucose and fructose by *L. acidophilus*, as observed by Minervini et al. (2018). Additionally, total tannin content increased significantly post-fermentation.

The modern food and beverage industry is increasingly driven by consumer demand for safer, non-GMO, gluten-free and organic options. However, sensory analysis indicated that the control sample, which lacked probiotic lactic acid bacteria, was preferred over the probiotic-containing sample. This discrepancy may be attributed to the production of secondary metabolites, particularly organic acids, which can

alter the sensory characteristics of the probiotic beverage.

Overall, this study presents a novel probiotic beverage combining *L. acidophilus*, hibiscus tea and coconut water. The beverage demonstrated promising probiotic properties, safety and beneficial effects on physicochemical and phytochemical parameters. It also exhibited significant antioxidant activity and high levels of phytoconstituents including flavonoids, phenols, and tannins, which may contribute to potential health benefits, supporting the body in combating various metabolic diseases.

5. Conclusion

This study highlights the development of a novel fermented probiotic beverage using hibiscus tea and coconut water inoculated with *L. acidophilus*. The fermentation process significantly enhanced the total phenolic and flavonoid content, leading to improved antioxidant activity, including effective scavenging of DPPH• and hydroxyl free radicals. Additionally, the probiotic beverage demonstrated good sensory acceptance. These findings highlight the potential of lactic acid bacteria as starter cultures for producing functional probiotic drinks with notable health benefits. Moreover, medicinal plant's flower and fruit based fermented beverage presents a promising alternative for individuals with lactose intolerance, expanding options for probiotic-rich formulations. The results of present study provide a strong foundation for the future development and commercialization of plant-based probiotic beverages.

Author contribution

Ashutosh Aher, Mitesh Patel and Mohd Adnan contributed to study concept and design, collected/analyzed data, project administration and drafted the original manuscript. Reem Binsuwaidan, Nawaf Alshammari and Angum M. M. Ibrahim, contributed to methodology, data curation, investigation, visualization, analysis, review and editing. All authors read and approved the final manuscript.

CRediT authorship contribution statement

Ashutosh Aher: Writing – original draft, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Reem Binsuwaidan:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Mohd Adnan:** Writing – original draft, Project administration, Formal analysis, Data curation, Conceptualization. **Nawaf Alshammari:** Writing – review & editing, Visualization, Methodology, Investigation, Data curation. **Angum M.M. Ibrahim:** Writing – review & editing, Visualization, Methodology, Formal analysis, Data curation. **Mitesh Patel:** Writing – review & editing, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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