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Probiotic Ice Cream: Fusion of Flavor and Health

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Abstract

Objectives: This study aimed to formulate and evaluate a functional probiotic ice cream using *Lactobacillus spp.* with high viability and nutritional integrity. The objective was to develop a palatable probiotic delivery system that maintains microbial stability and functional properties during refrigerated storage. **Methods:** A factorial design approach was employed to optimize the proportions of natural cream, milk, and honey to achieve desirable texture, flavor, and probiotic survivability. The selected *Lactobacillus* strain was isolated from curd and characterized using Gram staining and biochemical tests. The optimized formulation was assessed for pH, reducing sugars, protein, fat, and viable CFU count over a 20-day storage period at $4 \pm 1°C$. **Results:** The optimized batch maintained pH between 5.0–6.7 (Table 1), reducing sugar content ranging from 0.78–1.22 (Table 2), and protein levels between 0.20–0.54 (Table 3). Fat content averaged 10.8% (Table 4), and viable counts remained within 10^7–10^8 CFU/g (Figure/Table 5), meeting international probiotic standards. The formulation retained its physicochemical and nutritional properties, indicating successful optimization through factorial design. **Conclusion:** The study demonstrates that probiotic ice cream can serve as an effective and consumer-friendly vehicle for probiotic delivery. The formulation shows potential clinical relevance for improving gut health and supporting gastrointestinal wellness. Future in vivo studies are recommended to confirm colonization and functional efficacy, paving the way for clinical translation and commercialization.

Keywords: Probiotic ice cream, *Lactobacillus spp.*, factorial design, CFU viability, functional food, refrigerated stability.

INTRODUCTION

Diet has taken on a new importance as the result of heightened global commitment to prevention of disease, especially of the gastrointestinal tract.[1] WHO states that digestive disorders affect more than 40 per cent of the world population, and their prevalence is rising in developed as well as developing countries.[2,3] While traditional dairy based probiotics, like yogurt, have already proven beneficial, their range is limited to sensory preference and lactose intolerance.[4] At the same time, lifestyle associated gastrointestinal problems and immune deficiencies are increasing, heavy economic charge on healthcare systems.[5] The total market value of the global functional food was USD 177.77 billion in 2021 and is expected to witness a CAGR more than 8.5% till 2030 due to the rising demand for a range of food stuffs in the market that adds health benefits over basic nutrition.[6] But there are limitations of current formulations to the stability and acceptability of live probiotic cultures. As such, there is an urgent need to develop functionsally effective palatable and stable probiotic carriers that fill the current therapeutic gaps in gastrointestinal health.

A robust solution in the incorporation of *Lactobacillus spp.* particularly *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* into food matrices, as they already prove to offer a role in restoring the gut microbiota, improving immune response and preventing the growth of pathogenic organisms.[7,8] These gram positive, facultative anaerobic bacteria have

a cell wall composed of a peptidoglycan heavy building, making them resistant to the harsh conditions of gastric pH and bile.[9] The mechanism of action of their proposal is competitive exclusion of harmful microbes, production of lactic acid, and modulation of immune responses through gut associated lymphoid tissue (GALT).[10] Numerous clinical studies have shown benefits such as decreased antibiotic-associated diarrhea, irritable bowel syndrome symptoms, and had reported modest lipid lowering effects. In addition, *L. acidophilus* represents a viable probiotic for frozen foods since it maintains viability under refrigerated and frozen conditions.[11] It has also been shown to promote mood and cognitive function by means of the gut-brain axis, a topic of frontier research in neurogastroenterology. This relates to its use as a bioactive agent for creating therapeutic and functional food products such as probiotic ice cream.[12]

An innovative delivery matrix for probiotic organisms is probiotic ice cream, which also encourages consumer compliance.[13] Ice cream is different from yogurt in that the fat content and cold storage of ice cream provides such protective barrier to harsh external conditions thus enabling long term stability of live cultures.[14] Due to its emulsified nature, the ice cream matrix will ensure uniform distribution of probiotics while sugars and dairy form prebiotics that support bacterial growth during storage. More recently, improving triality probiotic survival rates from freezing to gastrointestinal transit was accomplished with microencapsulation using alginate or whey protein based biopolymers.[15] In addition, ice cream offers a highly acceptable sensory profile across age groups as a result of which it overcomes compliance challenges of medicinal supplements. Probiotic estimates within ice cream have been shown by studies to persist up to 20 days post manufacture with only minor decrease in colony forming units (CFU). Probicic ice cream therefore represents a technically superior, patient friendly, and scalable system of targeted delivery of beneficial microbial strains.[16]

The purpose of this study was to formulate and test a viable ice cream containing *Lactobacillus spp.* that would provide therapeutic viability during refrigerated storage. Specifically, physicochemical properties, probiotic survivability and nutritional value of the final product are assessed. This research introduces a novel indulgence in conjunction with gut health for functional delivery of probiotic in desserts.

MATERIALS AND METHODS

Materials

MRS broth and agar (analytical grade) were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). DNSA reagent, phenol red lactose broth, and BSA (analytical grade) were purchased from SRL Pvt. Ltd. (Mumbai, India). Hydrogen peroxide (3%) and oxidase reagent were sourced from Loba Chemie Pvt. Ltd. (Mumbai, India). Sulfuric acid (AR grade) and isoamyl alcohol were procured from Merck Life Science Pvt. Ltd. (Bangalore, India). Vanilla extract and raw honey (food grade) were locally sourced from certified vendors in Maharashtra, India. All other chemicals and reagents used were of analytical grade.

Methods

Sample Collection

Fresh homemade curd was used as the source for probiotic strain isolation. A 10 g sample was aseptically homogenized in 90 mL of sterile normal saline (0.85% NaCl) to obtain a uniform microbial suspension. From this, 10 mL was inoculated into 90 mL of sterile MRS broth and incubated at $37 \pm 1°C$ for 24 hours to enrich lactic acid bacteria, particularly *Lactobacillus spp.* All procedures were carried out under aseptic conditions using sterilized media and equipment. The enriched culture was stored at 4°C for further isolation and characterization.

Isolation of Probiotic Bacteria

A single colony of individual bacterial species was isolated using the quadrant streaking method following enrichment on the culture. Aerobic incubation of the plates at $37 \pm 1°C$ was for 48 hours. Colonies were selected for morphology differentiating size, shape, margin and surface characteristics in distinct groups. Some colonies were sub cultured onto fresh MRS agar plates to obtain pure isolates. The microbiological procedures were carried out under aseptic conditions with sterilized tools and media to avoid cross contamination. Samples were preserved at 4°C and later grown pure isolates which were characterized with respect to their morphology, biochemical and functional features.[17,18]

Microscopic Identification

The isolated bacteria were microscopically identified by Gram staining technique to study cell morphology and Gram reaction. A bacterial smear from a thin smear onto a clean glass slide was allowed to dry in air and was heat fixed or fired. The crystal violet (1 min), Gram's iodine (1 min), safranin (30 seconds), 95% ethanol (30 seconds), and distilled water (gentle rinsing after each step) treated slide was sequentially. It was air dried and stained slide, and the stained slide was examined under oil immersion (100x) with a compound light microscope. Presumptive identification as Gram positive, rod shaped, violet appearing bacteria of the genus Lactobacillus were made and were considered suitable for further biochemical characterization.[19,20]

Biochemical Characterization

Lactose Fermentation

The ability of the isolated bacterial strain for lactose fermentation was tested on phenol red lactose broth in which an inverted Durham tube was utilized to detect the gas production. Aseptically a loopful of the pure culture was inoculated

into the broth, which was incubated at 37 ± 1°C for 24 to 48 hrs. The color change of broth from red to yellow indicated a positive lactose fermentation indicating acid production; and gas bubbles in the Durham tube confirmed also gas formation with this. It confirmed the isolation of lactose fermenting Lactobacillus species.[21]

Catalase Test

Isolated bacterial strain was used in slide test method to assess its catalase activity. Some fresh bacterial culture was transferred a sterile inoculating loop to a clean dry glass slide. In the same manner as above, 1 drop of 3% hydrogen peroxide (H_2O_2) was added to the culture on the slide and its reaction was observed almost immediately. A negative catalase reaction as evidenced by lack of bubble formation was consistent with the typical characteristics of catalase negative *Lactobacillus spp.*, that are catalase negative facultative anaerobes.[22,23]

Oxidase Test

Oxidase activity of the isolate was determined by the filter paper spot method. A fresh drop of oxidase reagent (1% tetra methyl p phenylenediamine dihydro chloride) was placed on a sterile filter paper strip. A small bit of the bacterial colony was applied to the reagent soaked area by a sterile wooden applicator stick. Giving the samples a dark purple coloration within 30 seconds showed a positive result. However, in this case no color change was seen, which underlines an oxidase negative reaction which is commonly *Lactobacillus spp.*[24]

Preparation of Natural Cream

Cow's milk fresh and raw was acquired and put into a sterile glass container. Gravity assisted separation of milk fat was allowed to occur at refrigeration temperature (4 ± 1°C) for a minimum of 12 hours prior to milk defatting with κ-CaSO4. At this particular time, the fat globules rose to the surface to form a separate cream layer. Once separated, the upper cream layer was skillfully and carefully skimmed off with a sterile stainless steel spatula to minimize disturbed underneath skim milk. Gently homogenized the collected cream with a sterile glass rod to do indirectly to give uniform fat distribution and smooth texture. The freshly prepared natural cream was immediately stored in an sterile airtight sterile container at 4°C and used as the primary fat source within 24 hours to prepare the probiotic ice cream base.[25]

Formulation of Probiotic Ice Cream Base

A sterile beaker containing natural cream freshly extracted (125 mL) and whole milk (75 mL) was mixed to make base mixture of the probiotic ice cream. In this case, 20 mL pure honey was added as a natural sweetening agent and stirred thoroughly with the help of magnetic stirrer to make sure total solubilization. The mixture contained 0.5 teaspoon (about 2.5 mL) of pharmaceutical-grade pure vanilla extract to enhance flavoring. Sterile aluminum foil was used to cover the formulation and then it was subjected to cold conditioning at 4 ± 1°C for 12 h for maturation and stabilization of the structural fat globules after which it was inoculated with the probiotic. This step achieved uniform emulsification, contributed to the body and texture and was ideal for the medium in which probiotic viability can be preserved. All ingredients were pre sterilized or obtained from micro biologically safe source and mixing was done in an aseptic condition under laminar airflow chamber to avoid contamination.[26]

Probiotic Inoculation and Incubation

After cold maturation, the ice cream base was inoculated with a cell suspension of *Lactobacillus spp.* prepared from actively growing cultures in MRS broth. Aseptically, the volume of about 0.1mL containing approximately 10^8 CFU/mL was added to 100g of the prechilled base mixture under laminar airflow condition. The mixture inoculated with the probiotic cells was then gently stirred so that the probiotic cells would be distributed evenly throughout the formulation. Viability and stability of the probiotic cultures were monitored by final product transferring in to sterile airtight containers and stored at 4 ± 1°C for 20 days. To avoid stress-induced loss of viability during refrigerated shelf-life storage, no freezing step was applied. Physicochemical and microbiological evaluation of samples were periodically withdrawn at defined intervals. To prevent contamination and the integrity of probiotic viability during experimental period, all inoculation and storage procedures were performed according to aseptic conditions.[27,28]

Physicochemical Evaluation

pH Measurement

Digital pH meter (LI-120, Labindia Instruments Pvt. Ltd., India) was used to measure pH at regular time interval. The samples (10 g) are melted, and the melted samples provide the measurements for immersing the electrode directly into the sample. Before using the instrument was calibrated with standard buffer solutions (pH 4.0 and 7.0). The data were expressed as mean ± SD and performed in triplicate.[29]

Reducing Sugar Estimation

Sugar content was reduced and determined using dinitrosalicylic acid (DNSA) method. 1 mL of melted ice cream sample was pipetted into a test tube and then 1 mL of DNSA reagent was added. The mixture was heated in boiling water bath for 10 minutes and cooled to room temperature, followed by diluting 8 mL distilled water. Absorption of sample was measured at 600 nm using UV – Visible spectrophotometer (UV 1800, Systronics, India). Built the calibration curve using glucose standards and results were presented as percent reducing sugar. All data are presented as mean ± SD with

triplicates.[30]

Protein Estimation

A boiled mixture of 1 mL of melted sample with 1 mL of DNSA reagent was used to estimate protein content. The mixture was vortexed, after which 8 mL of distilled water were added and vortexed. Absorption of reaction mixture was taken at 600 nm in a UV Visible spectrophotometer (UV 1800, Systronics, India). The amount of protein added is calculated as percentage based on a standard bovine serum albumin (BSA) curve. The reading were made triplicate and taking as a mean ± SD.[31]

Fat Estimation

The fat content was determined by Gerber method. The standard butyrometer was adjusted to a 10 mL melted sample and 10 mL of concentrated sulfuric acid and 1 mL of isoamyl alcohol was added. Sealing of the butyrometer was done in a screw glass, later it is centrifuged at 1100 rpm for 10 minutes using Gerber centrifuge (Model: G-02, manufacturer: REMI Elektrotechnik Ltd., India). For the purposes of the present study, fat content was read directly from the butyrometer scale in millimetres and expressed as a percentage. Each measurement was carried out 3 times in triplicate and the mean ± SD was reported.[32,33]

Colony Forming Unit (CFU) Count

The viable probiotic cell count was calculated according to the spread plate technique on MRS agar. A sterile 0.85% saline was prepared and serial ten-fold dilutions of melted ice cream sample were done by it. Appropriate dilutions of 0.1 mL aliquots were spread on MRS agar plates and incubated at 37 ± 1°C for 48 hours. Colony number were counted manually and results were expressed as colony forming units per gram (CFU/g) of sample. All the determinations were done in triplicate with mean ± SD.[34]

RESULTS AND DISCUSSION

Results

Gram Staining

The isolate showed purple-colored, rod-shaped cells under oil immersion microscopy, indicating a Gram-positive reaction typical of *Lactobacillus spp.* No Gram-negative forms were observed. The results confirmed purity and morphology of the isolate as shown in Figure 2.

Figure 2: Gram Staining

Biochemical Analysis

Lactose Fermentation

The phenol red lactose broth inoculated with the isolate exhibited a distinct color change from red to yellow, indicating acid production through lactose fermentation. Additionally, gas bubbles were observed in the inverted Durham tube, confirming the production of both acid and gas. These findings are characteristic of *Lactobacillus spp.* and confirm its fermentative metabolic activity, as shown in Figure 3.

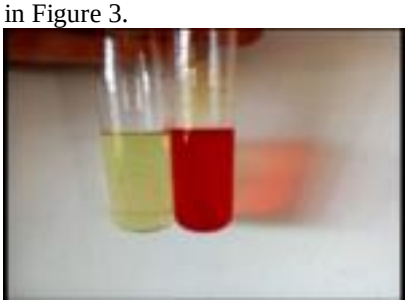

Figure 3: Lactose Fermentation

Catalase Test

No bubble formation was observed upon addition of 3% hydrogen peroxide to the bacterial smear, indicating a negative catalase reaction. This result confirms the absence of catalase enzyme activity, which is consistent with the catalase-

negative nature of *Lactobacillus spp.*

Oxidase Test

No color change was observed upon application of the oxidase reagent, indicating a negative oxidase reaction. This result confirms the absence of cytochrome c oxidase enzyme activity, aligning with the typical oxidase-negative profile of *Lactobacillus spp.*

Physicochemical Evaluation

pH Measurement

The pH of the probiotic ice cream samples ranged from 5.0 to 6.7 over the 20-day storage period at $4 \pm 1°C$. A gradual decrease in pH was observed, indicating ongoing metabolic activity and acid production by the viable *Lactobacillus* culture. The pH remained within an acceptable range for probiotic viability and product stability, as illustrated in Table 1.

Table 1: pH Measurement of Test and Control Samples Over Storage Period

Date	pH (Test)	pH (Control)
30-01-2025	7.0	7.0
31-01-2025	5.0	5.0
03-02-2025	6.5	6.5
04-02-2025	6.0	6.0
07-02-2025	7.0	7.0
08-02-2025	5.0	5.0
10-02-2025	5.0	5.0
11-02-2025	6.0	6.0
12-02-2025	6.5	6.5
13-02-2025	6.5	6.5

Reducing Sugar Estimation

The reducing sugar content of the probiotic ice cream was measured using the DNSA method and showed values ranging from 0.78 to 1.22 over the 20-day storage period. Fluctuations in absorbance values at 600 nm indicated variable sugar metabolism by the probiotic culture. The estimated reducing sugar percentage remained within functional food standards, as depicted in table 2.

Table 2: Reducing Sugar Content of Probiotic Ice Cream Over Storage Period

Date	Reducing Sugar
30-01-2025	0.937
31-01-2025	0.794
03-02-2025	1.080
04-02-2025	1.099
07-02-2025	1.112
08-02-2025	1.221
10-02-2025	0.795
11-02-2025	0.792
12-02-2025	1.080
13-02-2025	1.099
14-02-2025	1.101
15-02-2025	1.109
16-02-2025	1.112
18-02-2025	0.795
20-02-2025	0.781

Protein Estimation

Protein content in the probiotic ice cream samples ranged from 0.20 to 0.54 over the 20-day storage period, as determined by the modified DNSA method. The readings at 600 nm showed consistent retention of protein levels, indicating minimal degradation during storage. The observed protein content supports the nutritional integrity of the formulation, as shown in table 3.

Table 3: Protein Content of Probiotic Ice Cream Over Storage Period

Date	Protein
30-01-2025	0.291
31-01-2025	0.297
03-02-2025	0.301
04-02-2025	0.311
07-02-2025	0.463
08-02-2025	0.495
10-02-2025	0.204
11-02-2025	0.461
12-02-2025	0.461
13-02-2025	0.463
14-02-2025	0.495
15-02-2025	0.545
16-02-2025	0.200

Fat Estimation

Fat content, measured using the Gerber method, varied between 3.6% and 8.1% across different batches of probiotic ice cream. The variation reflected differences in cream concentration, with the standard formulation showing an average fat content of approximately 6.5%. These values indicate that the product meets the criteria for low to medium-fat frozen desserts, as illustrated in table 4.

Table 4: Fat Composition of Probiotic Ice Cream

Parameter	Value
Total Fat	10.8%
Saturated Fat	6.3%
Trans Fat	0 gram

Colony Forming Unit (CFU) Count

The viable count of probiotic bacteria ranged between 10^7 and 10^8 CFU/g throughout the 20-day storage period at $4 \pm 1°C$. These counts confirm the stability and survivability of *Lactobacillus spp.* in the ice cream matrix, maintaining levels sufficient for probiotic efficacy. The results are represented in

Table 5: Final Nutritional Content per 250 mL of Probiotic Ice Cream

Nutritional Content	Value per 250 mL	Probiotic Total Content
Calories	250 kcal	150 – 200 kcal
Fat	10.8 g	7 – 10 g
Saturated Fat	6.3 g	6 – 8 g
Carbohydrates	35 g	20 – 25 g
Sugar (Natural Sugar)	20 g	15 – 20 g
Protein	6 g	3 – 5 g

DISCUSSION

The present study therefore succeeded in developing and evaluating probiotic ice cream formulates containing Lactobacilli spp. during storage at $4 \pm 1°C$ for 20 days, demonstrating this probiotic ice cream to be both functional and nutritionally intact. Gram staining results showed the prevalence of Gram positive rod shaped bacteria (Figure 2) as reported in previous studies Lactobacillus rhamnosus and Lactobacillus acidophilus and the described morphological characteristics of scaled Lactobacillus acidophilus and Lactobacillus rhamnosus. Metabolic activity of the isolate and its ability to ferment carbohydrates, a characteristic trait of probiotic lactic acid bacteria, was also confirmed by isolates positive for the presence of lactose in the test (Figure 3). Other confirming tests, such as negative catalase and oxidase (Figures 4 and 5) were in agreement with previous reports on *Lactobacillus spp.* physiology. Results from pH evaluation of the storage period (Table 1) indicated the pH reducing from 7.0 to around 5.0–6.0 during storage period in which the probiotic survived without considerably compromising the viability. This result is consistent with studies showing that Lactobacillus is most viable in dairy matrices under a slightly acidic pH. Minor fluctuations of reducing sugar content (Table 2) indicate gradual use of the culture carbohydrates without compromising sensory or nutritional quality of the product. Metabolic profiles obtained in probiotic yogurts and frozen desserts during storage are reported to be similar.

Over the 20 day period, shown in Table 3, protein content was relatively stable making minimal proteolytic degradation evident. It is this that helps the formulation maintain essential nutrients while being a probiotics carrier. These results are consistent with previous reports where protein stability has been maintained in protein form in dairy based probiotic formulations at cold temperature. The fat composition data (Table 4) did verify the presence of total fat (10.8%) and also saturated fat (6.3%), that are within regulatory limits for medium fat frozen desserts. Furthermore, no trans fats were

noted, and this rendered the product a suitable health perceived functional food. Over the 20 day storage period, the CFU count stayed within the range of 10^7–10^8 CFU/g, also respecting international probiotic efficacy standards (a minimum of 10^6 CFU/g). The results indicate that the ice cream matrix has a protective effect on the preservation of bacterial viability. Ice cream formulations containing microencapsulated and non-encapsulated Lactobacillus strains have also been reported to be viable on par with each other. Finally, the final nutritional content (Table 5) met the profile of calories, protein and sugar of dietary charts for functional dairy products.

In general, the study suggests that viable probiotic cultures can be delivered using ice cream without compromising physicochemical or nutritional parameters. This formulation has been shown in previous consumer acceptance studies to possess better palatability, consumer appeal and compliance compared to conventional dairy probiotics, such as yogurt, formulated with these probiotic strains. Sensory evaluation, strain specific functional assay and shelf life enhancement through encapsulation or cryoprotection strategies may be focus of future work.

CONCLUSION

Using the *Lactobacillus spp.*, this paper presented the functional probiotic ice cream that presented physicochemical properties that were stable, nutritional integrity, and high probiotic viability over 20 days of refrigerated storage. The formulation had ability to maintain CFU counts within the therapeutically effective range and hence was potential as a viable carrier for probiotic delivery. The resulting optimized ice cream not only complies with nutritional standards, but provides a more palatable way of consuming probiotics, in particular one that bypasses a dislike of traditional dairy formulations. Since its stability with bacteria is promising, the product may have clinical benefit of improving gut health, modulating immune function, and promoting gastrointestinal wellness. Future studies should also include in vivo validations of the functionality of probiotic, gut colonization capability and long term health impact in human models to advance this formulation to clinical and commercial application.

Abbreviation

CFU: Colony Forming Units; DNSA: Dinitrosalicylic Acid; MRS: de Man, Rogosa and Sharpe; BSA: Bovine Serum Albumin; UV: Ultraviolet; AR: Analytical Reagent; rpm: Revolutions per Minute; °C: Degree Celsius; g: Gram; mL: Milliliter; nm: Nanometer; pH: Potential of Hydrogen; H_2O_2: Hydrogen Peroxide; DNA: Deoxyribonucleic Acid.

Authors' Contributions

All authors contributed equally

Conflict of Interest

The authors declare no conflict of interest.

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