Identification and Isolation of Lactobacillus spp. from Curd and Its Application in Probiotic Chocolate

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***Abstract*—Probiotics are live microorganisms that provide health benefits to the host when consumed in adequate amounts. Among the various probiotic microorganisms, Lactobacillus species are widely recognized for their role in maintaining intestinal microbial balance, improving digestion, and enhancing immune function. The present study was undertaken to isolate and identify Lactobacillus spp. from curd and evaluate its application in the preparation of probiotic chocolate.**

Fresh curd samples were collected and subjected to serial dilution followed by cultivation on de Man, Rogosa and Sharpe (MRS) agar medium for the isolation of Lactobacillus spp. The obtained isolates were characterized using morphological and biochemical methods including Gram staining, motility determination, and catalase testing. The isolates exhibited Gram-positive rod-shaped morphology, non-motile behavior, and catalase-negative reaction, indicating characteristics consistent with Lactobacillus spp.

The identified bacterial culture was propagated, preserved, and incorporated into a chocolate formulation prepared using milk powder, chocolate compound, and chocolate essence. The prepared probiotic chocolate showed acceptable physical characteristics and demonstrated the feasibility of using chocolate as a carrier matrix for probiotic microorganisms. The study highlights the potential of curd as an economical source of beneficial Lactobacillus spp. and supports the development of probiotic chocolate as a functional food product with potential health-promoting properties.

***Index Terms*—Lactobacillus spp., Probiotics, Curd, Probiotic Chocolate, Functional Food, Lactobacillus acidophilus**

I. INTRODUCTION

The human gastrointestinal tract contains a complex and diverse population of microorganisms that play an important role in maintaining health and normal physiological functions. These microorganisms contribute to digestion, nutrient absorption, immune system regulation, and protection against pathogenic bacteria. Disturbances in the natural microbial balance of the intestine may result in various gastrointestinal disorders and reduced immune function. Consequently, increasing attention has been directed toward the use of probiotics as a natural approach to maintain and restore intestinal microbial balance.

Probiotics are defined as live microorganisms that confer health benefits to the host when administered in adequate amounts. Among the different probiotic microorganisms, species belonging to the genus Lactobacillus are the most widely studied and commonly utilized due to their safety, stability, and beneficial physiological effects. These bacteria produce lactic acid during carbohydrate fermentation, thereby creating an unfavourable environment for the growth of harmful microorganisms and contributing to intestinal health. [1]

Fermented dairy products are considered one of the most important natural sources of probiotic microorganisms. Curd, a traditional fermented milk product, contains a variety of beneficial lactic acid bacteria and is widely consumed due to its nutritional and therapeutic properties. The naturally occurring Lactobacillus species present in curd contribute to improved digestion, enhanced nutrient utilization, and maintenance of healthy gut microflora.

Isolation of probiotic bacteria from natural food sources has become an important area of research in food microbiology and biotechnology. Identification and characterization of these microorganisms provide

valuable information regarding their potential application in the development of functional foods and nutraceutical products. Morphological and biochemical characterization remain important preliminary methods for identifying probiotic bacterial isolates obtained from fermented foods. [2]

Functional foods are foods that provide health benefits beyond their basic nutritional value. In recent years, there has been a growing demand for functional food products enriched with probiotics due to increasing consumer awareness regarding preventive healthcare and nutritional wellness. Traditionally, probiotic cultures have been incorporated into yogurt, fermented milk, and dietary supplements. However, researchers are continuously exploring alternative food matrices capable of improving probiotic stability and consumer acceptance.

Chocolate has emerged as a promising carrier for probiotic microorganisms because of its pleasant taste, widespread consumer acceptance, and protective composition. In addition to its sensory appeal, chocolate contains several bioactive compounds including flavonoids, polyphenols, and antioxidants that contribute to human health. The low moisture content and high fat composition of chocolate may help protect probiotic bacteria during storage and gastrointestinal transit, thereby enhancing their survival and effectiveness. [3]

Several studies have demonstrated that probiotic cultures can be successfully incorporated into chocolate without significantly affecting its quality or acceptability. The combination of probiotics and chocolate provides an innovative approach for developing a functional food product that is both nutritious and appealing to consumers. Such products may serve as convenient alternatives to conventional probiotic supplements while delivering additional nutritional benefits.

Research Gap

Although several studies have reported the incorporation of probiotics into dairy products and fermented foods, limited information is available regarding the isolation of Lactobacillus spp. from locally available curd and its direct utilization in probiotic chocolate preparation. Therefore, there is a need to explore the potential of curd-derived probiotic bacteria for the development of novel functional food products. [4]

Objectives of the Study

1. To isolate Lactobacillus spp. from curd.
2. To identify the isolated bacteria using morphological and biochemical methods.
3. To prepare probiotic chocolate using the isolated bacterial culture.
4. To evaluate the feasibility of chocolate as a carrier matrix for probiotic microorganisms.

The present study was therefore undertaken to isolate and identify Lactobacillus spp. from curd and investigate its application in the preparation of probiotic chocolate as a functional food product.

II. MATERIALS AND METHODS

2.1 Study Area

The present study was carried out in the Department of Microbiology, sitabai Thite college of Pharmacy Shirur dist pune during the academic year2025-2026. All microbiological experiments were conducted under aseptic laboratory conditions using standard microbiological techniques.

2.2 Collection of Sample

Fresh curd samples were collected from a local dairy source under hygienic conditions and transported to the laboratory in sterile containers. The collected samples were processed immediately after collection to minimize contamination and maintain bacterial viability. Prior to experimentation, all glassware, media, and laboratory instruments were sterilized according to standard microbiological procedures.

2.3 Materials Used

The materials used in the study included fresh curd, MRS (de Man, Rogosa and Sharpe) agar medium, MRS broth, sterile distilled water, Gram staining reagents, hydrogen peroxide (3%), milk powder, chocolate compound, chocolate essence, Petri plates, test tubes, conical flasks, micropipettes, incubator, microscope, and other standard laboratory equipment.

2.4 Isolation of Lactobacillus spp.

Isolation of Lactobacillus spp. was performed using the serial dilution and spread plate technique. One gram of curd sample was aseptically transferred into 9 mL of sterile distilled water and mixed thoroughly to obtain the primary suspension. Serial dilutions were subsequently prepared up to 10^{-6} dilution.

From each dilution, 0.1 mL aliquots were inoculated onto MRS agar plates using the spread plate method. The inoculated plates were incubated at 37°C for 24–48 hours. Following incubation, colonies exhibiting characteristics typical of Lactobacillus species were selected and purified by repeated streaking on fresh MRS agar plates to obtain pure cultures.

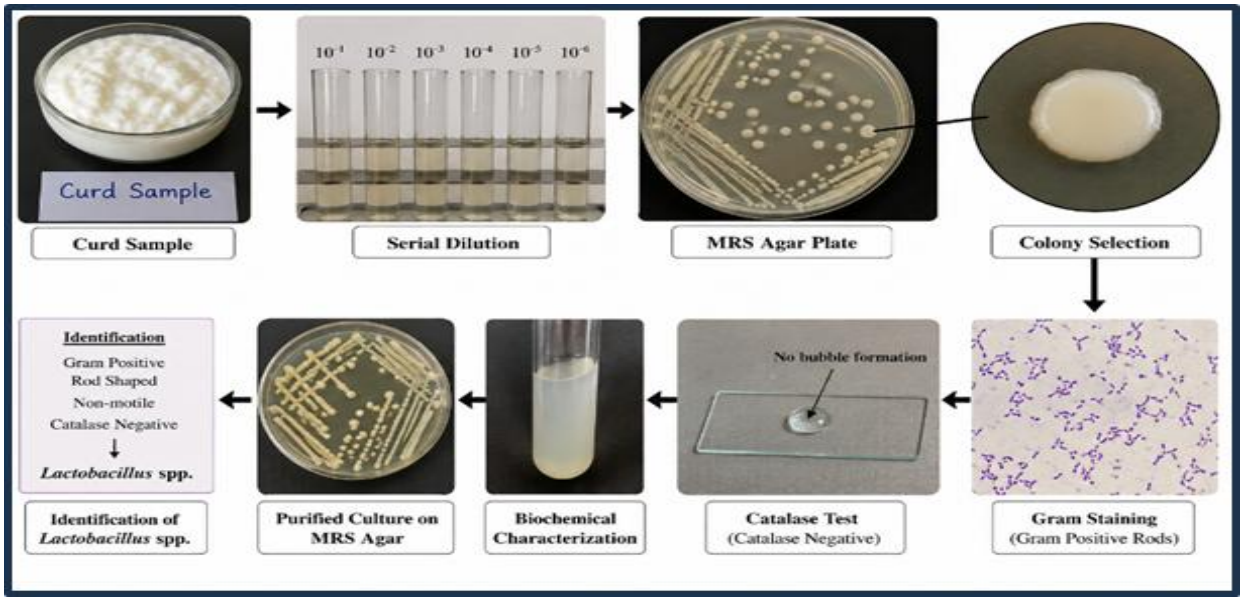

Figure 1. Isolation and Identification of Lactobacillus spp.

Pure cultures obtained after purification were maintained on MRS agar slants and stored under refrigerated conditions for further characterization.

2.5 Identification of Lactobacillus spp.

The purified isolates were characterized using morphological and biochemical methods. Colony morphology was observed based on colony size, shape, color, margin, surface texture, and elevation.

Microscopic examination was carried out using Gram staining. Bacterial smears were prepared on clean glass slides, heat-fixed, and stained using crystal violet, iodine solution, alcohol decolorization, and safranin. The stained slides were examined under an oil immersion microscope.

Gram-positive rod-shaped bacterial cells were considered presumptive Lactobacillus isolates.

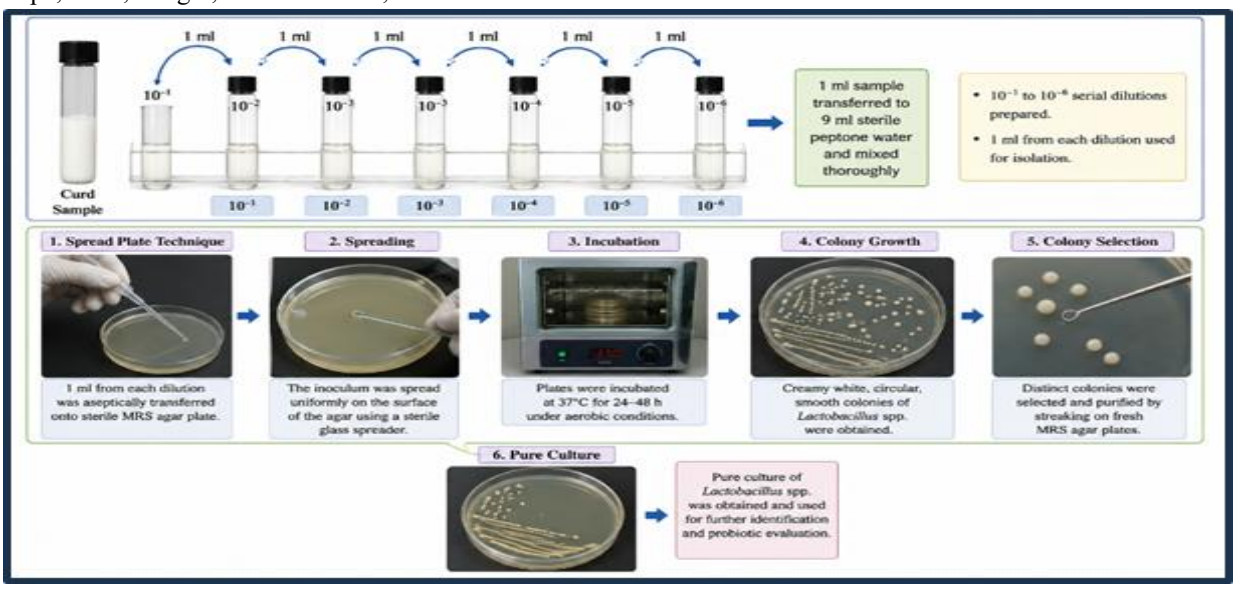

Figure 2. Serial Dilution and Isolation of Lactobacillus spp.

2.6 Motility Test

Motility of the bacterial isolates was determined using the hanging drop method. A drop of actively growing culture was placed on a coverslip and observed under a microscope. The absence of directional movement indicated a non-motile nature, which is characteristic of Lactobacillus species.

2.7 Catalase Test

The catalase test was performed to differentiate Lactobacillus species from catalase-positive bacteria. A small amount of bacterial culture was transferred onto a clean glass slide and a drop of 3% hydrogen peroxide solution was added.

The reaction was observed immediately for bubble formation. Absence of oxygen bubbles indicated a catalase-negative reaction, which is a characteristic feature of Lactobacillus spp.

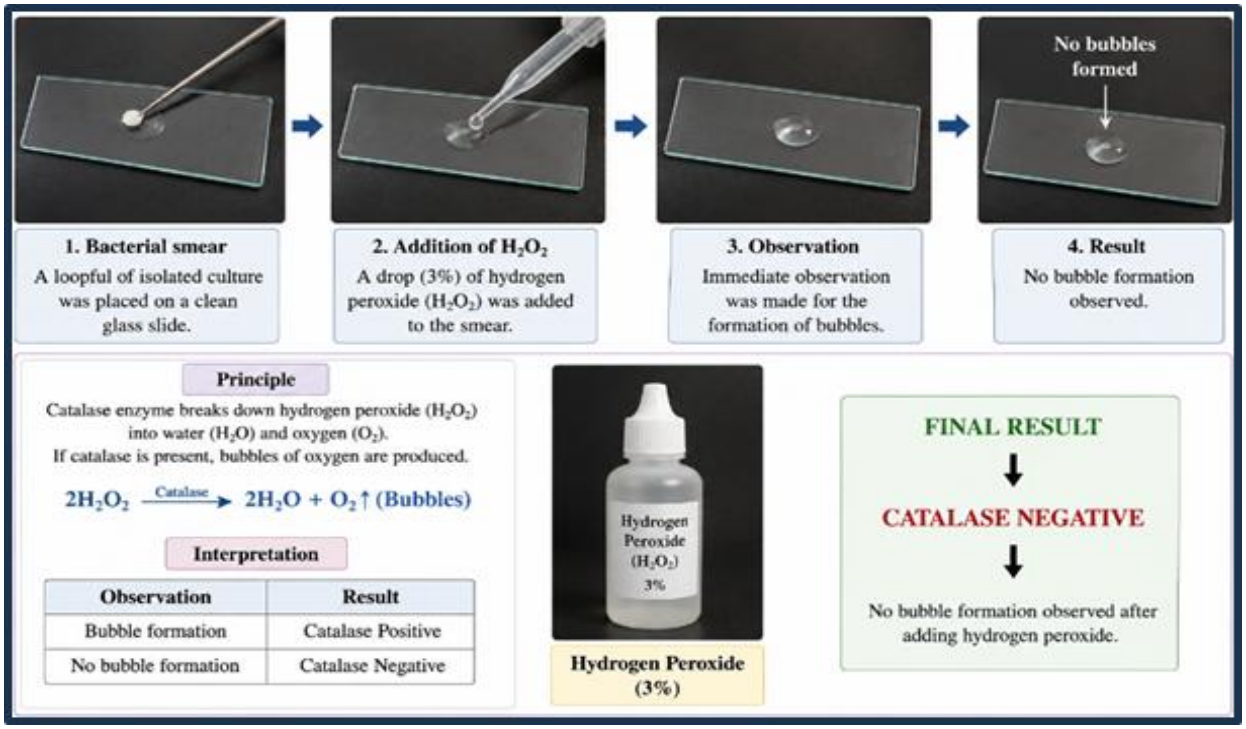

Figure 3. Catalase Test Showing Negative Reaction

2.8 Preparation of Probiotic Chocolate

Probiotic chocolate was prepared using milk powder, chocolate compound, milk, and chocolate essence. Approximately 200 g of milk powder and 40 g of chocolate compound were heated gently until complete melting occurred. A small quantity of milk was added to obtain the desired consistency.

Chocolate essence was incorporated to improve flavor and aroma. The chocolate mixture was then allowed to cool to room temperature before the addition of the probiotic culture. The isolated Lactobacillus culture was incorporated into the cooled chocolate mixture and mixed thoroughly to ensure uniform distribution.

The prepared chocolate mixture was poured into molds and refrigerated until solidification occurred. The solidified chocolate was removed from the molds, wrapped in aluminum foil, and stored under refrigerated conditions.

Figure 4. Prepared Probiotic Chocolate

2.9 Experimental Workflow

The overall experimental procedure involved sample collection, isolation of Lactobacillus spp., morphological and biochemical characterization, preparation of probiotic culture, and incorporation of the culture into chocolate to develop a probiotic functional food product.

III. RESULTS

The present study was conducted to isolate and identify Lactobacillus spp. from curd and evaluate its application in the preparation of probiotic chocolate. The results obtained from isolation, characterization, probiotic viability assessment, and product development are presented below.

3.1 Isolation of Lactobacillus spp.

Following incubation of diluted curd samples on MRS agar medium, distinct bacterial colonies were observed. The colonies appeared creamy white, circular, smooth, and convex in appearance. Repeated sub-culturing on fresh MRS agar plates resulted in the successful isolation of pure bacterial cultures.

[Insert Figure 1 Here]

Figure 1. Isolation and Identification of Lactobacillus spp.

The isolated colonies exhibited characteristics generally associated with Lactobacillus species and were selected for further microscopic and biochemical characterization.

3.2 Colony Morphology

Macroscopic examination of the purified colonies was performed and the observations are presented in Table 1.

Table 1. Colony Morphology of Isolated Bacteria

Parameter	Observation
Colour	Creamy white
Shape	Circular
Margin	Entire
Elevation	Convex
Surface	Smooth

The colony morphology was found to be consistent with characteristics commonly reported for Lactobacillus spp. isolated from fermented dairy products. [5]

3.3 Microscopic Characteristics

Gram staining was performed to determine the cellular morphology and Gram reaction of the isolate. Microscopic examination revealed Gram-positive rod-shaped bacterial cells occurring singly and occasionally in short chains.

Table 2. Microscopic Characteristics of Isolated Bacteria

Parameter	Observation
Gram Reaction	Positive
Cell Shape	Rod-shaped
Cell Arrangement	Single and short chains

The microscopic observations indicated that the isolate belonged to the genus Lactobacillus.

3.4 Biochemical Characteristics

The bacterial isolate was subjected to biochemical characterization using motility and catalase tests. The isolate showed no directional movement during microscopic examination, indicating a non-motile nature. Similarly, no bubble formation was observed following the addition of hydrogen peroxide, confirming a catalase-negative reaction.

Table 3. Biochemical Characteristics of Isolated Bacteria

Test	Observation	Result
Motility Test	No directional movement	Non-motile
Catalase Test	No bubble formation	Negative

The biochemical properties observed during the study supported the preliminary identification of the isolate as Lactobacillus spp. [6]

3.5 Summary and Identification of Lactobacillus spp.

The combined results obtained from colony morphology, Gram staining, motility testing, and catalase testing were used for identification of the isolate.

Table 4. Summary of Identification of Lactobacillus spp.

Characteristic	Observation
Gram Reaction	Positive
Cell Shape	Rod-shaped
Motility	Negative
Catalase Activity	Negative
Probable Identification	Lactobacillus spp.

Table 5. Comparison of Observed Characteristics with Standard Lactobacillus Characteristics

Characteristic	Observed Result	Standard Lactobacillus Characteristics
Gram Reaction	Positive	Positive
Cell Shape	Rod-shaped	Rod-shaped
Motility	Negative	Negative
Catalase Activity	Negative	Negative

The observed characteristics showed complete agreement with the standard characteristics of Lactobacillus spp., thereby supporting the identification of the isolate.

3.6 Preparation of Probiotic Chocolate

The isolated probiotic culture was successfully incorporated into the chocolate formulation. The prepared probiotic chocolate exhibited uniform texture, desirable appearance, and pleasant aroma. No visible defects such as cracking, separation, or discoloration were observed.

Table 6. Composition of Probiotic Chocolate

Ingredient	Quantity
Milk Powder	200 g
Chocolate Compound	40 g
Milk	Required Quantity
Chocolate Essence	Few Drops
Lactobacillus Culture	Required Quantity

The successful incorporation of the probiotic culture demonstrated the feasibility of utilizing chocolate as a carrier matrix for Lactobacillus spp. [7]

3.7 Viability of Lactobacillus spp. During Storage

The survival of Lactobacillus spp. in probiotic chocolate was evaluated during storage. A gradual reduction in viable bacterial count was observed with increasing storage duration. However, the bacterial population remained above the recommended minimum probiotic level throughout the storage period.

Table 7. Viability of Lactobacillus spp. in Probiotic Chocolate During Storage

Storage Period (Days)	Viable Count (CFU/g)
0	1.2×10^8
15	9.8×10^7
30	8.1×10^7
45	6.5×10^7
60	5.2×10^7

The results indicate that the chocolate matrix provided a protective environment that supported the survival of probiotic bacteria during storage.

3.8 Sensory Evaluation of Probiotic Chocolate

The developed probiotic chocolate was evaluated using a 9-point hedonic scale. The product received

favorable scores for appearance, aroma, taste, texture, and overall acceptability.

Table 8. Sensory Evaluation of Probiotic Chocolate

Parameter	Mean Score
Appearance	8.4
Aroma	8.2
Taste	8.6
Texture	8.3
Overall Acceptability	8.4

Table 9. Overall Quality Assessment of Probiotic Chocolate

Quality Parameter	Observation
Colour	Good
Aroma	Pleasant
Texture	Smooth
Taste	Acceptable
Overall Quality	Excellent

The sensory evaluation results demonstrated good consumer acceptability of the probiotic chocolate formulation and indicated its suitability as a functional food product. [8]

3.9 Overall Findings

The study successfully isolated Lactobacillus spp. from curd and identified the isolate through morphological and biochemical characterization. The probiotic culture was effectively incorporated into chocolate and maintained satisfactory viability during storage. Furthermore, the prepared probiotic chocolate exhibited good sensory characteristics and high overall acceptability.

These findings suggest that curd-derived Lactobacillus spp. can be successfully utilized in the development of probiotic chocolate as a functional food product with potential health-promoting benefits.

IV. DISCUSSION

The present study successfully demonstrated the isolation and identification of Lactobacillus spp. from curd and its subsequent application in the development of probiotic chocolate. Fermented dairy products are widely recognized as natural reservoirs of beneficial lactic acid bacteria, particularly members of the genus Lactobacillus. The successful recovery of Lactobacillus spp. from curd in the present investigation supports previous reports indicating that fermented milk products serve as important sources of probiotic microorganisms with potential health-promoting properties. [9]

The use of MRS agar medium facilitated the selective growth and isolation of the bacterial cultures. Colonies obtained on the medium were creamy white, circular, smooth, and convex in appearance. Similar colony morphology has been reported by several researchers during the isolation of Lactobacillus spp. from fermented dairy products. The successful growth of the isolate on MRS medium indicates the suitability of this medium for the cultivation and maintenance of lactic acid bacteria. [10]

Microscopic examination revealed Gram-positive rod-shaped bacterial cells arranged singly and occasionally in short chains. Gram-positive rod morphology is one of the characteristic features commonly associated with Lactobacillus species. The observed microscopic characteristics were consistent with standard descriptions reported in Bergey's Manual of Determinative Bacteriology and other microbiological studies involving lactic acid bacteria. [11]

Biochemical characterization further supported the identification of the isolate. The isolate was found to be non-motile and catalase-negative. The absence of motility and catalase activity is considered an important distinguishing feature of Lactobacillus spp. Catalase-negative bacteria are unable to decompose hydrogen peroxide into water and oxygen through catalase enzyme activity, a characteristic frequently observed among lactic acid bacteria. The agreement between the observed characteristics and standard identification criteria confirmed the probable identity of the isolate as Lactobacillus spp. [12]

One of the major objectives of the present study was to evaluate the possibility of utilizing the isolated probiotic culture in the preparation of chocolate. The successful incorporation of Lactobacillus spp. into chocolate demonstrated that chocolate can function as an effective carrier matrix for probiotic microorganisms. The prepared chocolate maintained desirable physical characteristics including uniform texture, pleasant aroma, and acceptable appearance, indicating that incorporation of the probiotic culture did not adversely affect product quality. [13]

The viability study revealed a gradual decline in the viable count of Lactobacillus spp. during storage. Such a reduction is expected due to environmental stress

and storage-related factors. However, the bacterial population remained above the minimum recommended level for probiotic effectiveness throughout the storage period. This observation suggests that the chocolate matrix provided a protective environment capable of supporting bacterial survival. Similar findings have been reported in studies investigating the use of chocolate as a delivery system for probiotic microorganisms. [14]

Sensory evaluation of the probiotic chocolate demonstrated high scores for appearance, aroma, taste, texture, and overall acceptability. The favorable sensory characteristics observed during the study indicate that probiotic incorporation did not negatively influence consumer acceptance. In fact, the combination of probiotic cultures with chocolate may improve the attractiveness of functional foods, particularly among children and consumers who prefer food-based probiotic delivery systems rather than conventional supplements. [15]

The increasing demand for functional foods has created opportunities for the development of innovative probiotic products with enhanced nutritional and therapeutic value. Probiotic chocolate combines the health benefits associated with Lactobacillus spp. with the antioxidant and sensory properties of chocolate. Such products may contribute to improved gastrointestinal health while providing an enjoyable and convenient method of probiotic consumption. [16]

Overall, the findings of the present investigation indicate that curd is a suitable and economical source of Lactobacillus spp., while chocolate serves as an effective carrier matrix for probiotic delivery. The successful isolation, identification, viability maintenance, and sensory acceptability observed in this study support the potential application of probiotic chocolate as a functional food product. Further investigations involving molecular characterization, shelf-life evaluation, and large-scale production may provide additional information regarding its commercial and therapeutic potential.

V. CONCLUSION

The present study successfully isolated and identified Lactobacillus spp. from curd using conventional microbiological and biochemical techniques. The isolate exhibited characteristic features including Gram-positive rod-shaped morphology, non-motile behavior, and a catalase-negative reaction, which are consistent with the standard characteristics of Lactobacillus species.

The isolated probiotic culture was successfully incorporated into a chocolate formulation, resulting in the development of probiotic chocolate with acceptable physical and sensory properties. The viability study indicated that the chocolate matrix was capable of supporting the survival of probiotic bacteria during storage, while sensory evaluation demonstrated good consumer acceptability.

The findings of the study suggest that curd can serve as an economical and readily available source of probiotic Lactobacillus spp. Furthermore, chocolate may be effectively utilized as a carrier matrix for probiotic delivery, thereby contributing to the development of functional foods with potential health-promoting benefits. The study highlights the potential application of probiotic chocolate as a novel functional food product and provides a foundation for future research in this field.

VI. LIMITATIONS OF THE STUDY

Although the present study yielded promising results, certain limitations were encountered:

1. Identification of the isolate was based only on morphological and biochemical characterization.
2. Molecular identification techniques such as 16S rRNA sequencing were not performed.
3. The study was conducted on a laboratory scale with limited sample size.
4. Long-term shelf-life evaluation was not carried out.
5. Antimicrobial activity and detailed probiotic characterization were not investigated.

VII. FUTURE SCOPE

Future studies may focus on molecular identification and characterization of the isolated Lactobacillus strain using advanced molecular techniques. Investigation of probiotic stability during extended storage periods, antimicrobial activity against pathogenic microorganisms, and large-scale production of probiotic chocolate may further strengthen its commercial applicability.

Additional studies involving consumer acceptance surveys, nutritional analysis, and clinical evaluation of health benefits may provide valuable information regarding the therapeutic potential of probiotic chocolate as a functional food product.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this research work.

Funding Statement

The authors declare that no external funding was received for conducting this research work.

Ethical Statement

The present study did not involve human participants or experimental animals. Therefore, ethical committee approval was not required.

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