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Screening for Probiotic Potential of *Lactobacillus Rhamnosus* Strain CRD4

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Abstract: Lactic acid bacteria are the foremost used probiotic worldwide. Its successful application in manufacturing dairy products and probiotic foods makes it a promising industrial prerequisite. The objective of the present investigation was to isolate, identify, and molecularly characterize Lactic acid bacteria from local dairy samples of Odisha state of India and explores its probiotic traits. One potential strain was isolated using a selective *Lactobacillus*-MRS agar medium. The biochemical studies illustrated the bacteria were gram-positive, catalyze (-ve), and non-motile. The taxonomical diversity of the bacterium was analyzed by 16s RNA sequencing and classified as *Lactobacillus rhamnosus* strain CRD4 with gene bank accession no [MG573074]. Further, the selected strain was screened for its probiotic competence of lower acid and bile tolerance. The result confirmed that *lactobacillus* strain successfully defended the low pH and bile stress and acclaimed 70% cell surface hydrophobicity. Antibiotic studies obtained confirmed the possible resistance of the strain. The maximum zone of inhibition was expressed in diameter 42mm against Ciprofloxacin. In conclusion, based upon the above results, *Lactobacillus rhamnosus* can be a profound probiotic candidate.

Keywords: *Lactobacillus rhamnosus*; Probiotics; Acid-Bile tolerance; Antibiotic Resistance.

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1. Introduction

Lactic acid bacteria (LAB) are the foremost industrial prerequisite for the sustainable production of wide-ranging dairy foods, probiotic centered foods, processed functional foods, and fermented foods across the globe [1]. Currently, their application as health-promoting microorganisms makes it more vivacious as compared to other microbes. Many food industries are cognizant of the fact that the diet preferences among the world community have been shifting towards products with desirable health aid [2, 3]. Although microorganisms find their crucial application in environmental bioremediation biosensors and pathogen detection, metal detoxification, however, the pharmaceutical implication of probiotic bacteria, reported from traditional foods, plays a major role in influenza infection with additional health benefits [4]. Different scientific investigations are conducted on several probiotic isolates from ancient dairy foods with enhanced antimicrobial activities. At present, food has a significant role in preventing acute infectious diseases and protecting human health [5]. Microorganisms with probiotic potential are reported to prevent several diseases, including cancer, skin irritation,

allergies, stomach infections, etc. [6-10]. The mechanism underlying the beneficial effects of probiotics includes modulation of the gut microbiome, blocking of infectious pathogens, host immunity alteration, prevention, and diagnosis of different diseases like urinary tract infections, non-alcoholic fatty liver diseases, cancers, and other factors such as bio generation of organic acids [11-13].

Despite the probiotic activity, the microorganism should able to sustain in GIT to boost human health. Probiotic supplements act as vital compounds for foods containing microorganisms, showing enhanced human health and well beings [14]. Thereby the essential constituents of probiotic-centered foods are to modulate host immunity and health. Currently, a number of microorganisms are underuse as a probiotic supplement in fermented foods having a nano-science and medicinal application, which includes probiotic nanoparticles, baby foods, dairy products, beverages, and fruit juices [15-18].

Lactic acid bacteria are mostly used starter strains due to their high viability and growth rate. Currently, reported LAB starter strains are *Lactobacillus brevis, Lactobacillus delbrueckii, Lactobacillus plantarum,* and *Lactobacillus Casei* [19-21]. At present, Food industries are aiming to use LAB in the manufacturing of probiotic-based commercial products, including cereals, vegetables, and milk products [14, 15]. Another application of probiotics for the preservation of a wide spectrum of foods, likely functional foods, fermented foods, and vegetables, to protect them from microbial spoilage prolonging their self-life is considered as a new developmental trend in food science [14-17].

Thus, edible products from plant origin are presently in high demand for development [22]. It was reported that various plant materials like chitosan, whey proteins, and probiotics like *Bifidobactria animalis*, *Lactobacillus plantarum*, have been inducted as an edible coating material to safeguards the food products by synthesizing antimicrobial agents naturally [22]. However, now peoples are holding no desire for junk foods; as a result, tilting their focus to natural food-based products comprising probiotic bacteria is a safer substitute [23]. As per US-FDA and EFSA, probiotics are generally regarded as safe (GRAS) and can be used in the manufacturing of food products [24, 25].

The primary purpose of the investigation is a. To isolate *Lactobacilli* from collected dairy samples b. Molecular identification of the isolated bacterial strain, c. Screening for probiotic potential. However, no such screening and investigations on probiotics from dairy samples have been addressed in state Odisha, India; neither is there a report on potential applications of probiotics in the industrial field for the development of fermented foods and functional foods.

2. Materials and Methods

2.1. Sample collection.

Dairy product has a vital economic contribution in many developed and developing countries worldwide. According to the international farm comparison network (IFCN), the Odisha region has been a potential economic contributor in the dairy production system, which equals Haryana state as the highest dairy producer of India [26]. Likewise, according to the previous reports, almost 80% of ruminants in Odisha are kept on farms. So, we aseptically collected 50 ml curd samples from different dairy farms (20.2961°N latitude and 85.8245°E longitude) of the Khurda district of Odisha state of India using sterile 100 ml polypropylene

tubes (Tarson, India). All the samples were shifted to the laboratory in an icebox and kept separately for further experiments.

2.2. Isolation & characterization.

The curd bacteria were isolated and characterized by a nutrient-agar medium [27]. Prior to isolation, an aliquot of 100 µl, from 10^{-7} fold serially diluted curd, was spread onto nutrient agar Petri-plate and incubated overnight at 37°C. A prominently grown and morphologically distinct bacterial colony was sub-cultured onto MRS agar plate, incubated overnight at 37°C 24hr. Further, the isolated bacterium was subjected to biochemical characterizations such as catalase enzyme production, motility analysis, fructose-glycerol fermentation analysis, and gram staining, etc. Bacterial morphology was examined using Microscope- Olympus BX43. The strain was kept in MRS agar slant at 4°C for further analysis.

2.3. DNA extraction and gene amplification.

The chromosomal DNA was extracted from freshly grown bacterial culture following the phenol-chloroform extraction protocol [21]. The 16S rRNA genes were amplified using the universal primer set of 27F (5′-GCCTACGGGNGGCWGCAG-3′) and 1492 R (5′-ACTACHVGGGTATCTAATCC-3′). The reaction was carried out for 35 cycles with the following standard PCR cycle parameters: DNA Pre-heating at 94ºC for 3min; denaturation at 94ºC 1min for 35 cycles, annealing at 55ºC for 45s, 72ºC for 1min of primer extension, and elongation step at 72ºC for 10 min. To analyze PCR products, 2 µl of reaction mix (10 mm TE buffer and 2 µl) was electrophoresed at 120v for about sixty minutes to get the existing DNA fragments [27]. Latterly, the purity analysis of DNA was conducted comparing the A260/280 ratio.

2.4. Sequence data analysis and phylogenetic study.

The amplified rRNA sequence was translated using the Sangers' Sequencing technique [21]. The sequence was processed for quality analysis, subsequently submitted to the Gene-Bank database. Post-submission, the sequence was tallied with the available nucleotides from the NCBI-database using the blast algorithm. The phylogenetic tree was constructed by adjoining ten sequence matches with the query sequence using the CLC sequence viewer.

2.5. Screening for probiotic activity.

2.5.1. Acid and bile tolerance.

Acid and bile tolerance is the most widely used screening model for probiotic bacteria. The screening was conducted as described by Guo *et al*., 2016 with little modifications [28]. The inoculum was prepared separately by overnight incubation at 37°C. Then 600 ml of freshly made MRS broth medium was randomly divided into 6 groups that have supplemented with OX-bile of four different concentrations OXB-0.3, OXB-0.5, OXB-0.75, and OXB-1.0% (w/v). The pH has been adjusted to 1.5, 2.5, 3.0, and 4.0 using 1M HCl. 100 µl of starter culture were tipped to the all bile enriched and pH adjusted mediums and incubated for 24 hr. at 37°C. Following centrifugation for 10 min at 8000× g at 4°C, bacterial cells of each group were individually recovered, and cell viability was determined in MRS agar petri dish following serial dilution.

2.5.2. *In-vitro* Growth response pattern *Lactobacillus rhamnosus* with prebiotic.

It has been demonstrated that prebiotics can well tolerate the digestive tract and fast stimulates the genera *Lactobacillus* in the colon. So, we examined the strain *Lactobacillus rhamnosus* for the growth proficiency in the presence of inulin prebiotic and FOS as per Onal *et al*., 2018 [29]. Three groups of MRS broth (50 mL) mediums were prepared (Group-1) 2% inulin and (Group-2) 2% FOS; however medium with glucose (group-3) was a control. 100 µl from overnight grown lactobacillus suspensions was poured into enriched media in two different flasks and incubated 24 hr at 37°C. Post-incubation, to determine the effect of inulin with *Lactobacillus* strain CRD4, 0.1mL of 24hr. grown cell suspension was tipped and spread onto MRS agar plates. Similarly, plates were incubated 24 hr. at 37°C to determine the viable cell count [21].

2.5.3. Cell surface hydrophobicity.

Cell adherence to the hydrocarbons was examined using toluene, xylene, and chloroform. The isolated strain was grown for 24 hr in the MRS broth medium. The obtained biomass was centrifuged at 8000× g for 20min at 4ºC. However, the cell pellet was washed twice with PBS and re-suspended with the same buffer. To determine strain tolerance to the different concentration of hydrocarbons, the absorbance of three groups were pre-measured at ($OD_{600nm,\ BEFORE}$) and then 1ml of each solvent was transferred into 4ml of cell suspensions of three solvent groups and allowed to settle for 10min. To visualize the binary phase separation, the solvent-bacterial mix was vortexed individually [30]. Latterly, the watery part was defenestrated, and the absorbance was measured at (OD 600nm, AFTER), followed by spectrophotometric analysis.

The degree of hydrophobicity was determined using subsequent equation: Hydrophobicity (%) = [OD_{600} after – OD_{600} at 0min / OD_{600} after] ×100

2.5.4. Bacterial antibiotic resistance pattern analysis.

Probiotic bacteria with innate antibiotic resistance or possibly because of gene mutation could be helpful in countering harmful strains in the intestinal reservoir and restoring it. We examined antibiotic resistance of the isolated strain following Kirby Bauer's disc diffusion method, Clinical and Laboratory Standards Institute [31]. Seven groups include beta-lactams, aminoglycosides, macrolides, quinolones, carbapenems, penicillin, and sulphonamides, were analyzed for *Lactobacillus rhamnosus* strain isolated from curd. Firstly, using the spread plate technique, 100 µl of overnight grown *Lactobacillus rhamnosus* cell suspension were spread into each trial plates and air-dried for 10 min. Subsequently, antibiotic discs were positioned, followed by 24 hr. incubation at 37ºC. [32]. The zone of inhibitions was mapped individually in the measurement scale.

2.6. Chemicals and instruments used.

All the laboratory glassware and chemicals for experimental use were analytical grades. Nutrient agar, *Lactobacillus* MRS Agar, Phenol red glycerol broth, and MRS Broth were purchased from Hi-Media, Mumbai, India. Hydrochloric acid (ACS reagent, Sigma-Aldrich, India), and OX-bile, antibiotic discs were also purchased from hi-media for investigating the tolerance proficiency. PCR biosystem was used for 16S rRNA gene amplification, whereas a spectroscope and incubator were obtained for cell growth analysis.

3. Results and Discussion

3.1. Isolation and characterization.

One potential strain with relatively high growth was isolated using a nutrient agar medium (24hr incubation at 37°C) and repeatedly streaked in MRS agar plates to get the purest culture. The growth proficiency in the *Lactobacillus*-MRS agar medium was found to be substantial after 24hr of incubation at 37°C [21]. Bacteria showed a yellowish-white, 0.5-0.8mm diameter round colonies on MRS agar plates. Biochemical analysis confirmed the cells were non-motile, gram-positive, and catalase-negative Table 1. Further, the glycerol fermentation assay was results positive; the color of phenol red glycerol broth has changed from red to yellowish after 24hr. incubation at 37°C.

Table 1. Biochemical analysis isolated probiotic strain.

Serial no.	Biochemical tests	Strain CRD4
1.	Gram staining	+
2.	Catalase	-
3.	Oxidase	+
4.	Glycerol fermentation	+
5.	Fructose fermentation	+
6.	Physiological appearance	Round shape
7.	Color	Yellowish white
8.	Motility	-
9.	Diameter	0.8

3.2. Molecular identification and phylogenetic analysis.

Previously, bacterial diversity analysis has been limited to isolation using selective agar medium and identification followed by biochemical investigation and morphology analysis. However, highly complex and time-consuming, thereby most of the scientific communities are relying on molecular approaches like the polymer chain reaction (PCR) technique for better precision analysis [27]. The isolate was speciated by the PCR technique using 16S rRNA Figure 2.

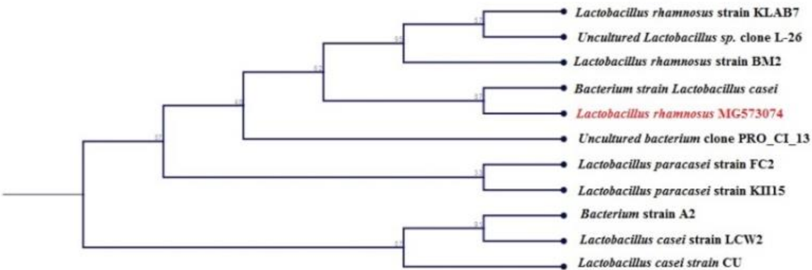

Figure 3. Phylogenetic tree of *Lactobacillus rhamnosus* strain CRD4 [MG573074] constructed with CLC-Sequence viewer 7.6. program using 16S rRNA gene sequences.

Then, the retrieved 16S rRNA gene sequence was submitted to Genbank and obtained the accession number MG573074. Using the BLAST search, the percentage of similarity of the strain was evaluated. Lower E-value and greater query coverage indicated a relatively better percentage of matches of the isolated strain CRD4- MG573074 were found to be 99% homologous to *Lactobacillus rhamnosus* of the database. The Genbank report of the strain CRD4 revealed that it belongs to family Lactobacillaceae and Genus *Lactobacillus* with 708bp of the gene sequence. The closest phylogenetic hierarchies of the *Lactobacillus rhamnosus*

strain CRD4 are *Lactobacillus paracasei* strain KII 15 and Bacterium strain *Lactobacillus casei* with 99% of sequence match Figure 3.

3.3. Bacterial characterization for acid and bile tolerance.

Lactobacillus rhamnosus showed substantial cell viability to the critical pH and OX-bile. Above 50% of growth, proficiency was observed in case of bile tolerance at concentrations OXB-0.5% and OXB-0.75%; the maximum growth was obtained 6.29 log_{10} CFU/ ml^{-1} at 0.3% of ox-bile. The cell count at concentration OXB-0.5% and OXB-0.75% were 5.13 log_{10} CFU/ml^{-1} and 5.07 log_{10} CFU/ ml^{-1} respectively, whereas isolated strain was found to be more sensitive to OXB-1.0% ox-bile with 3.54 log_{10} CFU/ ml^{-1}. However, the pH of the culture medium was found relatively low with increasing incubation time. Post incubation, the pH of the tested medium was altered from pH 6.91 to pH 6.59 in the presence of bile. The survivability of probiotic strain at pH 2.5 is the critical acidic concentration to screen the bacterial tolerance strength, the cell viability at pH 2.5 was 5.15 log_{10} CFU/ ml^{-1}. The maximum colony forming units (CFU/mL) was 6.75 log_{10} CFU/ ml^{-1} observed at pH 3.0, whereas at pH 1, the growth was likely to negligible with cell viability 1.13 log_{10} CFU/ ml^{-1}. After 6hr. and 8hr of incubation, the cell viability was varied between 3.97 log_{10} CFU/ ml^{-1} to 4.16 log_{10} CFU/ ml^{-1} with lower pH 2.5, which is significant. As a result, isolated LAB strain was successfully able to survive the critical pH, which is more than 50%.

3.4. Cell surface hydrophobicity.

Cells with a strong affinity to the hydrocarbon are entitled hydrophobic [33, 34]. A study was conducted to evaluate the affinity pattern of isolated strains to hydrocarbons revealed remarkable results with toluene, xylene, and chloroform. The mixing of cell suspension with hydrocarbons provides a pathway of interaction between microbial systems and the hydrocarbon phase. *Lactobacillus rhamnosus* strain CRD4 had shown a strong affinity to the xylene (70%± 1.10) and toluene (70%± 1.23), respectively, whereas relatively less affinity was found with chloroform, that is, (57%± 1.0).

*3.5. In-vitro Growth response pattern Lactobacillus rhamnosus CRD4 with prebiotic***.**

The effect of adding prebiotic to the MRS agar medium successfully modulated the growth of *Lactobacillus rhamnosus* strain CRD4. The bacterial population density with inulin and FOS were found to be more than 50%. Carbon is a prime source of enrichment for the number of microbes. So, using 2% of inulin and FOS, the maximum cell count of 7.91 log_{10}CFU/ml^{-1} and 7.33 log_{10}CFU/ml^{-1} were obtained. However, the control medium has shown 9.58 log_{10} CFU/ ml^{-1} cell viability. When compared with medium holding glucose, both inulin and FOS showed significant cell growth.

3.6. Antibiotic susceptibility tests.

The isolated bacterial strain showed highly resistant against the tested groups of antibiotic quinolones, tetracyclines, penicillins, aminoglycosides, nitrofuran, cephems, macrolides, tetracycles, beta-lactams, and fluoroquinolones. However, only six tested antibiotics, including Amoxicillin/Clavulanic, Tetracycline, Ciprofloxacin, Amikacin, Chloramphenicol, and Ampicillin/Cloxacillin, were susceptible to *Lactobacillus rhamnosus* strain CRD4 Figure 4. Similarly, results have been reported previously [35]. The maximum

zone of inhibition was listed, such as (42 mm) to Ciprofloxacin, chloramphenicol (38 mm), amoxicillin/clavulanic acid (44mm) and Ampicillin/cloxacillin (40mm), tetracycline (30mm) and amikacin (35 mm), respectively Table 2.

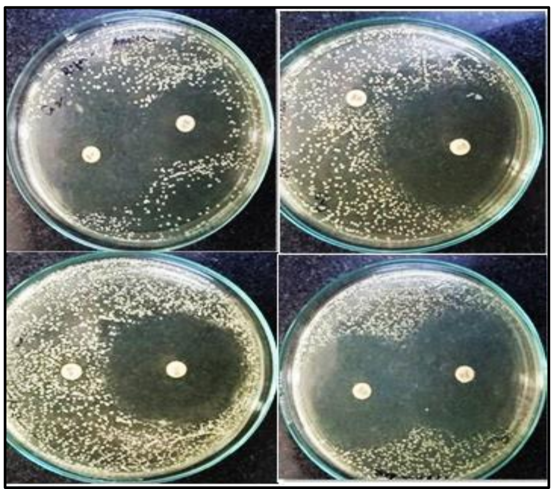

Figure 4. Antibiotic susceptibility and resistance pattern analysis of *Lactobacillus rhamnosus* strain CRD4, The zone of inhibition were listed such as (42 mm) to Ciprofloxacin, chloramphenicol (38 mm), amoxicillin/clavulanic acid (44mm) and Ampicillin/cloxacillin (40mm), tetracycline (30mm) and amikacin (35 mm), respectively

Table 2. Antibiotic resistance and susceptibility.

Antibiotic Discs	Resistance (R) and susceptible (S)
Erythromycin	**R**
Penicillin G	**R**
Gentamicin	**R**
Nalidixic acid	**R**
Co-trimoxazole	**R**
Amoxicillin/clavulanic acid	**S**
Cefoperazone	**R**
Chloramphenicol	**S**
Levofloxacin	**R**
Ceftazidime	**R**
Ciprofloxacin	**S**
Amikacin	**S**
Tobramycin	**R**
Ampicillin/cloxacillin	**S**
Tetracycline	**S**

3.7. Discussion.

In most of the studies, alteration of gut microflora by using probiotics and prebiotics has been getting considerable attention due to associated human health benefits [36]. Lactobacillus with beneficial sway can be selectively stimulated by prebiotics in the gastrointestinal tract. The present study has evaluated the probiotic activity of *Lactobacillus rhamnosus* strain CRD4 from a curd sample and contributed a deep understanding of the symbiotic impact of prebiotic and probiotic on the human gastrointestinal system. Furthermore, the cell surface hydrophobicity, antibiotic resistance, glycerol fermentation, acid, and bile tolerance has been investigated.

Initial pH is highly harmful to bacterial cell growth, thereby affecting probiotic concentration in the human colon [37]. Resistance to the acidic environment has been linked with the lower gastric pH.2.5 sustainability. The acid tolerance assay was performed and found positive for the growth of *Lactobacillus rhamnosus* strain CRD4 in the harsh acidic condition. The growth modulating the strength of the isolated strain CRD4 has strongly contributed to its

cell multiplications pattern in acidic medium, h^+ extrusion mechanism regulates the pH that allows a tolerance of bacteria [38]. The pH of the GIT is near about 1.5. So, we studied four variable pH concentrations 1.5, 2.5, 3.0, and 4.0. The cell count was 5.15 log_{10} CFU/ ml^{-1} at critical pH 2.5 with maximum viability of 6.75 log_{10} CFU/ ml^{-1} at pH 3.0.

Bile salt alters membrane permeability leads to bacterial cell death [38]. So we investigated four different ox-bile concentrations, the maximum growth was found 6.29 log_{10} CFU/ ml^{-1} at 0.3% of bile. The cell viability of concentration OXB-0.5% and OXB-0.75% were 5.13 log_{10}CFU/ml^{-1} and 5.07 log_{10}CFU/ml^{-1} respectively, however, strain CRD4 was found to be more susceptible to OXB-1.0%, and the cell count was 3.54 log_{10} CFU/ ml^{-1}.

Electrostatic interactions are the key factor in cell surface hydrophobicity [34]. Microbial adhesion to hydrocarbons emphasizes through the phase separation of the water and hydrocarbons layer. The surface hydrophobicity of the isolated strain varied from 50% to 70% in response to change in a growth phase that signifies the alteration in surface retention time in the gastrointestinal system. Thus, boosts the affinity between host and microorganisms. Surface hydrophobicity was observed with xylene and toluene was (70%) with strain CRD4 as compare to chloroform (64%). A similar percentage of hydrophobicity has been reported earlier [33].

Despite being pathogen-specific, some antibiotics can destroy commensal microbes in GIT [32]. But, the rise in the consumption of probiotic products demands a necessary evaluation of resistance patterns. As per US-FDA and EFSA, antibiotic resistance is the decisive safety norm. However, knowledge regarding antibiotic exposure of probiotic bacteria is still limited. We examined the susceptibility and resistance patterns using different groups of antibiotics. *Lactobacillus rhamnosus* strain CRD4 was susceptible to Ciprofloxacin, chloramphenicol, amoxicillin/clavulanic acid, Ampicillin/cloxacillin, tetracycline, and amikacin. A similar result has been reported previously [30]. Whereas Anamycin, Cefoperazone, Ceftazidime, Erythromycin, Penicillin G, Gentamicin, Imipenem, Piperacillin, Cefoperazone, Sulphatriad, Tobramycin, Nitrofurantoin, Levofloxacin, Cefepime, Nalidixic Acid, Ticarcillinmeropenem were found to be resistance to strain CRD4 Figure 4. However, *mef* and *erm*-class genes are the predominant mechanisms accounting for the antibiotic resistance among all antibiotics [35]. Point mutation and the presence of methylases may be the key genetic factor; involved in *Lactobacillus rhamnosus* strain, CRD4 showed strong resistance to macrolides antibiotics.

Complex sugars can modulate in intestinal microbiota using carbon as a sole source [36]. Inulin is natural prebiotics and can easily pass through the small bowel and ferment the large bowel [23]. Strain CRD4 showed the highest cell growth in the presence of (Group-1) inulin 7.91 log_{10} CFU/ $ml^{-1,}$ whereas 7.33 log_{10} CFU/ ml^{-1} were obtained with (Group-2) FOS. So higher cell growth is directly proportional to the concentration of FOS and inulin prebiotics. Thus, the rise in cell density of *Lactobacillus rhamnosus* strain CRD4 with prebiotics can boost the gut microbiome with rapid fermentation of large bowl and are highly beneficial to human health.

Similarly, glycerol is a significant by-product of alcoholic fermentation besides ethanol and hydrogen. However, our analysis for glycerol fermentation found positive; the color has changed from red to yellowish, indicates a more acidic medium in the presence of *Lactobacillus rhamnosus* strain CRD4. Thus, the fermentative capacity of strain CRD4 in the presence of glycerol can change the gut fermentation pattern by producing acetic acid, propionic acid, and butyric acid that naturally modulate the gut microbiome [39]. *dha-* regulation is the principal mechanism that enables the LAB to grow by using glycerol as a sole source of carbon [39, 40].

4. Conclusions

In the process of evolution consumer's choice of diet-pattern has been transformed. The gap between customers and their food habits has been occupied by probiotic-based foods and beverages as a new trend of the 21st century. From a technological standpoint, probiotics have shown a bold prospect to humanity due to its potential to modulate several disease conditions. The growing market shares probiotic foods; lots of industries are also implementing the different applications of probiotics in food products. However, the purpose of the present investigation was to isolate *Lactobacillus* from the dairy samples and screen it for possible probiotics. Our investigational outcomes recommend the strain CRD4 can be a potential probiotic. Furthermore, the human digestive tract has a complex environment and often harsh for many microbial ecologies. Probiotics consumption alone or with antibiotics has a vital role in determining human gastric health stability. The isolated strain CRD4 had shown stronger tolerance at pH 2.5 and 2% ox-bile. The growth in the presence of proven prebiotics like inulin and FOS was found high with cell count 7.91 $\log_{10}$ CFU/ml^{-1} and 7.33 $\log_{10}$CFU/ml^{-1}. Similarly, the use of the molecular approach assisted in the deep understanding of probiotic diversity, mechanism of action, and antibiotic resistance pattern can be a future prospect for medical research.

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Conflicts of Interest

All the authors have certified that there is no conflict of interest associated with this manuscript.

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