



Characterization of *Lactobacillus rhamnosus* GG (ATCC 53103) isolated from fermented milk: a probiotic for gut health

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Abstract

Probiotics, particularly lactic acid bacteria (LAB) like *Lactobacillus rhamnosus*, are recognized for their health-promoting properties and ability to prevent gastrointestinal disorders. This study focused on isolating and evaluating the probiotic potential of *Lactobacillus rhamnosus* GG (ATCC 53103), isolated from fermented milk. The bacterium was tested for survival in harsh gastrointestinal conditions, bile salt tolerance, sugar fermentation, and morphological physiological characterization. The isolate exhibited robust growth under a pH range of 4.5–7.5, with no growth at extremely acidic or alkaline conditions. It demonstrated significant ($p < 0.05$) bile salt tolerance, maintaining viability across concentrations of sodium deoxycholate, sodium taurocholate, and cholic acid, particularly favoring conjugated bile salts. Gastric and intestinal fluid tolerance tests revealed a high survival rate (70–90%), indicating its potential to endure gastrointestinal transit. The sugar fermentation profile classified *L. rhamnosus* as heterofermentative, with carbon dioxide production observed alongside lactic acid. The isolate showed no catalase activity, confirming its anaerobic nature. Morphological and cultural analyses revealed colonies as Gram-positive rods and cocci with smooth, creamy characteristics. The findings confirm the probiotic potential of *L. rhamnosus* GG, highlighting its ability to survive under gastrointestinal and bile conditions. This study supports the application of *L. rhamnosus* GG in functional foods and supplements, emphasizing its role in gut health and its adaptability to diverse gastrointestinal challenges. These results provide a foundation for further exploration of *L. rhamnosus* in therapeutic and dietary applications, addressing safety and efficacy for human consumption.

1. Introduction

Probiotics, renowned for improving well-being and potentially preventing gastrointestinal issues, are becoming increasingly popular as dietary supplements and innovative health products. (Wu *et al.*, 2022). Among the vital groups of probiotics, lactic acid bacteria (LAB), which incorporate strains like *Lactobacilli* and *Bifidobacteria* are the most predominant. *Lactobacillus* is an important component of the digestive microbiota, known for different medical advantages (Damodharan *et al.*, 2016).

Certain strains of lactic acid bacteria (LAB) play an essential role in the digestive system by producing substances like bacteriocins, which have antimicrobial properties. These metabolites help inhibit the growth of harmful microorganisms and prevent infections. To be considered effective as probiotics, LAB strains need to meet specific criteria. These include producing beneficial compounds like bacteriocins, surviving acidic conditions and bile salts in the digestive system, adhering to the intestinal lining, and not possessing resistance to antibiotics

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(Son *et al.*, 2018). Many LABs, particularly those in the *Lactobacillus* genus, produce bacteriocins (antimicrobial peptides) that specifically target and inhibit the growth of closely related microorganisms, distinguishing them from traditional antibiotics (Todorov *et al.*, 2020).

Lacticaseibacillus rhamnosus is a flexible, facultative anaerobe known for its capacity to ferment lactic acid. This bacterium is tracked down in a large number of conditions, including conventional fermented food sources, the human body, and animal sources (Peirotten and Landete, 2020). It holds the “Generally Recognized as Safe” (GRAS) designation from the US FDA and is included in the “Qualified Presumption of Safety” (QPS) list by the European Food Safety Authority. Moreover, *L. rhamnosus*, first identified in the human gut, shows notable resistance to digestion and is effective in both lab research and clinical trials (Huang *et al.*, 2020). It offers benefits such as resistance against inflammation in the gut, enhanced lipid metabolism, antioxidation properties, and relief from diarrhea, making it a valuable option for therapeutic and nutritional use (Stavropoulou and Bezirtoglou, 2020).

The safety and effectiveness of probiotics can vary significantly between different strains. If not used correctly, probiotics might cause potential negative effects, such as infections spreading throughout the body, production of harmful substances, overstimulation of the immune system, or the transfer of genes that could lead to antibiotic resistance or increased virulence (Fu *et al.*, 2022; Zhang *et al.*, 2021). Accordingly, it is critical to completely survey the security and possible advantages of recently distinguished probiotic strains before thinking about their utilization in food or drug applications. Analyzing the complete genome of probiotic strains is a reliable approach for evaluating their safety and understanding their characteristics. This method allows for a detailed examination of their genetic makeup, which helps in determining their potential benefits and risks (Saroj and Gupta, 2020).

In this study, *Lactobacillus rhamnosus* GG (ATCC 53103) was isolated from a fermented dairy source and evaluated for its probiotic potential. The strain was assessed for gastric and bile salt resistance, sugar fermentation capabilities, and intestinal fluid tolerance. Additionally, survivability tests were conducted to evaluate the viability of *Lactobacillus rhamnosus* under challenging conditions.

2. Materials and Methods

2.1. Procurement of material

The fermented milk was obtained from the local market of Bahawalpur, Pakistan. Culture media (MRS agar and MRS broth) were purchased from Sigma-Aldrich (SigmaAldrich, Tokyo, Japan) while all other chemical reagents were purchased from Carlo-Erba (Milano, Italy).

2.2. Isolation of bacterial strain

Bacterial strains were isolated from fermented milk. To ensure the integrity of the samples and promote the growth of lactic acid bacteria, the milk was collected in sterile glass bottles containing MRS (de Man, Rogosa, and Sharpe) broth, which is specifically designed for cultivating lactic acid bacteria. The samples were promptly transported to the laboratory under refrigeration to prevent any spoilage or unwanted microbial growth. Once in the lab, the samples were incubated at 37°C overnight. Following this, After enrichment, serial dilutions of the sample were prepared to obtain a suitable concentration for plating. The diluted samples were then poured plated onto MRS agar that was supplemented with 0.05 µg/mL of bromocresol purple, a pH indicator, and L-cysteine hydrochloride, which helps create an anaerobic environment conducive to the growth of these bacteria. The agar plates were then incubated at 37°C for 48 hours under anaerobic conditions (Ahmad *et al.*, 2019).

2.3. Identification of bacterial isolates

Acid-producing colonies were recognized by the formation of a clear zone around each colony, prompting the selection of those with varied morphologies for further purification. Individual pure colonies were isolated and subcultured for additional experiments. The chosen lactic acid bacteria (LAB) isolate was then evaluated for morphology, catalase activity, and Gram reaction. The isolates that exhibited Gram-positive, catalase-negative characteristics and a rod shape were identified as potential lactobacilli and were subsequently stored in an MRSc medium supplemented with 20% glycerol at -80°C for long-term preservation (Rajoka *et al.*, 2017).

2.4. Probiotic properties assessment

2.4.1. pH stability

The tolerance of bacterial isolates to different pH levels was evaluated using MRS broth. Pure isolates were inoculated into MRS broth media with pH levels set at 3, 4.5, 6, 7.5, and 9, following the method described by Hadadji *et al.* (2010). After incubating the cultures for 48 hours at 37°C, growth was assessed.

2.4.2. Bile salt tolerance

The growth of the selected strain (*Lactobacillus rhamnosus* GG ATCC 53103) in the presence of varying bile salt concentrations was evaluated using three specific salts: sodium deoxycholate, sodium taurocholate, and cholic acid (HiMedia), at levels of 0.1%, 0.2%, and 0.3% (w/v), as outlined by Kuda *et al.* (2016). These concentrations were prepared by adding bile salts to 100 mL flasks containing 20 mL of sterile MRS broth, with MRS broth alone serving as the control. The flasks were then inoculated with lactic acid bacteria cultures grown overnight and incubated at 37°C for 15 hours.

2.4.3. Gastric juice and intestinal fluid tolerance

Overnight cultures of *L. rhamnosus* GG were centrifuged at 10,000 g for 5 minutes, washed three times with PBS (pH 7.4), and suspended in artificial gastric juice to achieve a concentration of approximately 108 CFU/mL. The cultures were incubated for 3 hours, and at hourly intervals, 100 µL aliquots were taken for serial dilution, followed by plating on MRS agar to determine viable bacterial counts. Subsequently, the bacteria were centrifuged again, suspended in artificial intestinal juice, and incubated for 5 hours. Live bacterial counts were measured, and the survival rate of *L. rhamnosus* GG was calculated using a specific formula (Ali *et al.*, 2023).

$$\text{Survival (\%)} = \frac{\text{Log CFUN1}}{\text{Log CFUN0}} \times 100$$

2.4.4. NaCl test

The impact of NaCl on bacterial growth was assessed by testing against various concentrations of NaCl. The bacterial culture was inoculated into MRS broth along with different NaCl concentrations i.e., 2.5%, 3.5%, 4.5%, 5.5%, 6.5%, 7% (w/v). After a 48-hour incubation period, the growth of the cultures at each NaCl concentration was observed. Finally, the results were documented (Mahmoudi *et al.*, 2013).

2.4.5. Catalase test

It was utilized to determine whether bacterial isolates produced catalase enzymes or not. The isolates were examined for catalase activity through slide slide-drop method. A microscope slide was put inside the petri dish, but the experiment can be performed without the use of a petri dish. The metal loop was sterilized before use and bacterial colony was picked up from the isolates with the help of this loop and placed on the glass slide. The hydrogen peroxide (H₂O₂, 3%) was poured on a glass slide dropwise. Immediately the petri dish was covered with the lid to reduce the aerosol and record the results (Mahmoudi *et al.*, 2013).

2.4.6. Sugar fermentation

A 10% (w/v) sugar solution was prepared by dissolving various sugars—glucose, maltose, fructose, sorbitol, lactose, sucrose, dextrose, arabinose, trehalose, rhamnose, and mannitol in distilled water. This solution was then sterilized using tentalization at 100°C for 15 minutes. Peptone water was prepared by dissolving 5 g of peptone and 2.5 g of NaCl in distilled water to a final volume of 500 mL. The pH was adjusted to 7.2 with a digital pH meter, followed by sterilization at 121°C for 20 minutes. The bacterial isolates were subsequently cultured in both peptone water and sugar solutions, with observations recorded after incubation at 37°C, according to Hadadji *et al.* (2010).

2.5. Statistical analysis

The data was collected and analyzed by using descriptive statistics using SPSS software.

3. Results and Discussion

3.1. Isolation of *Lactobacillus rhamnosus*

Inoculum (1%) was used from the lyophilized culture and added into the test tube containing MRS broth for the growth of *L. rhamnosus*. Incubation was carried out for 48 hours and after that centrifugation process was carried out for harvesting of bacteria. On the other MRS agar or nutrient agar can also be used for good colony growth. The test tube was filled with distilled water (9 mL) for the introduction of 1% inoculum. The phenomenon of serial dilution was used and ten serial dilutions were made and poured onto agar plates. The incubation was done at 37°C for 48 hours for good colony growth. After incubation gram staining technique was used to determine the cocci and rod-shaped gram-positive bacteria. The microscope was used for the evaluation of colony characteristics of *L. rhamnosus* and bacterial colonies were white, creamy, round, and convex (Table 3.1). The colonies having these characteristics were isolated and transferred to nutrient agar plates for the growth of pure isolates. The results agreed with Rajoka *et al.* (2017) as isolated and characterized the Bifidobacterium. French yoghurt and milk were used for the isolation of bacteria and 10 serial dilutions were made. The isolated samples were grown on MRS agar containing 0.05% cysteine. Verdenelli *et al.* (2009) utilized milk as a potential source of isolation for *L. rhamnosus* and demonstrated that *L. rhamnosus* has the tendency of probiotics and is present in the gut and fermented dairy products.

3.1.2. Morphological characterization of isolates

Colonies of *Lactobacillus rhamnosus* were haphazardly chosen from agar plates and results appeared in Table 1The isolates were examined under the microscope and appeared as cocci and rods. These aspects of isolates are in obedience to Vervedelli *et al.* (2009) who demonstrated that *Lactobacillus* species appeared as gram-positive, cocci, and rods in small chains (S₄). The total ratio of gram-positive bacteria and cocci on MRS agar plates containing 0.05% L-cysteine was 80:20. The isolates of S₄ were further selected for the isolate purification and conformation of *L. rhamnosus*.

Table 1. Cultural and morphological features of bacterial isolates on MRS agar plates

Sample	Morphology of colonies	Characteristics
S ₁	Circular, creamy, and convex	Gram-positive, cocci
S ₂	White, round, and smooth	Gram-positive, rods
S ₃	Large, creamy, and round	Cocci
S ₄	White shiny, dome shape, small	Gram-positive, rods in small chain

S₁, S₂, S₃, S₄ = Samples of bacterial isolates from lyophilized culture

3.1.3. Isolates purification

The purification phenomenon was carried out by the streak plate method. The *Lactobacillus rhamnosus* isolates were transferred on growth media augmented with L-cysteine (0.05%). The incubation was performed at the proper temperature (37°C) for 48 hours. The bacterial colonies were grown on agar plates after the incubation, and they were subjected to a gram staining technique for the conformation of colonies (Gram-positive). The microscopic examination showed that the colonies were white, smooth, and round. The results demonstrated by Yang *et al.* (2017) comply with the present work. According to their demonstration, purified isolates were smooth and rod-shaped and gave positive results with gram staining (stain purple).

3.2. Probiotic potential

3.2.1. pH stability

The bacterial isolates were grown at different pH levels (3, 4.5, 6, 7.5, and 9) to demonstrate the pH effect on bacterial survival. The ideal pH for the growth of *L. rhamnosus* was extended from 4.5-7.5 and no growth was observed above 7.5 (Table 2). The results of bacterial growth regarding pH level are consistent with Li *et al.* (2016) who demonstrated that pH range of *L. rhamnosus* was 4-7.5. The ideal pH for the better development of microbes was in the domain of 6-7.5. These results are also agreed with Liptakova *et al.* (2014). They demonstrated that *L. rhamnosus* was not growing at a low pH (below 4) and bacterial growth seized at this low pH.

3.2.2. Effect of NaCl

Different concentrations of NaCl (2.5%, 3.5%, 4.5%, 5.5%, 6.5%, 7%) were used to demonstrate the salt effect on bacterial survival. Various concentrations of salt were added into the medium of culturing and growth was assessed following 48 hours of incubation. The bacteria were able to grow at all the concentrations of NaCl (Table 3). The present findings completely agreed with Reale *et al.* (2015) as they demonstrated bacterial growth at 2-6.5% NaCl concentration. They also showed that bacterial growth was reduced at concentrations 6.5% and above.

3.2.3. Catalase test

The test is utilized to differentiate between aerobic and obligate anaerobic bacteria along with the presence or absence of enzyme (i.e. catalase). Catalase enzyme initiates the breakdown of hydrogen peroxide into hydrogen and oxygen. Anaerobic bacteria do not show catalase activity while aerobic bacteria do. *Lactobacillus rhamnosus* is an anaerobic bacterium and showed negative results for the catalase test. The slide containing *L. rhamnosus* colony was fed with one droplet of 3% H₂O₂ and observed. No bubble formation was seen which means that there is no liberation of oxygen (Table 4). Our findings are in line with Mahmoudi *et al.* (2013) who also identified the negative

results for *L. rhamnosus* during the catalase test. The results for the catalase test are also in line with Srinivasan *et al.* (2013) who demonstrated that there was no bubbles

Table 2. pH stability of isolates

pH	Results
3.0	-
4.5	+
6.0	+
7.5	+
9.0	-

–ve mean no growth and +ve mean growth of isolates

Table 3. NaCl stability of isolates

NaCl Conc. (%)	Result
2.5	+
3.5	+
4.5	+
5.5	+
6.5	+
7.0	-

+ve means stability of isolates in NaCl solution

Table 4. Catalase test of isolates

Hydrogen peroxide	Bubble formation	Result
3%	No bubble formation	-
3%	bubble formation	+

Where +ve means the presence of catalase enzyme and -ve means the absence of catalase enzyme (*L. rhamnosus*)

formation after adding the drop of H₂O₂ to the bacterial colony.

2.3.4. Gastric and intestinal juice tolerance

L. rhamnosus GG demonstrated remarkable resilience in artificial gastric juice (pH ~2) by maintaining viability with minimal reduction in colony-forming units (CFUs) over the 3-hour incubation period (7.8 log CFU/g). This indicates its potential to withstand harsh acidic conditions similar to those encountered in the stomach. After transfer to artificial intestinal juice, the survival rate further improved (7.90 log CFU/g), with CFUs remaining consistently high during the 5-hour incubation. The strain exhibited robust tolerance to bile salts and alkaline pH (7.5–8.0), reinforcing its suitability for intestinal colonization.

The calculated survival percentage, based on CFU measurements pre- and post-incubation, was significantly high (70–90%) compared to strains reported in the literature.

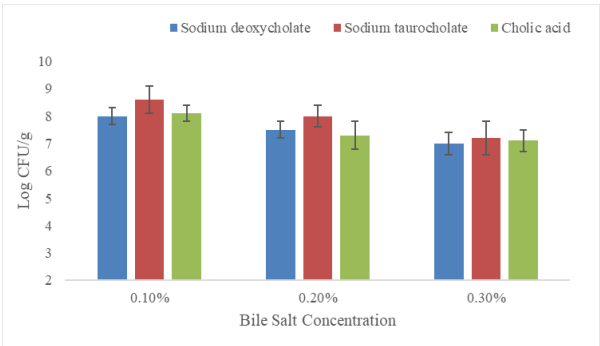


Figure 1. *L. rhamnosus* GG tolerance to bile salts

Studies have shown that survival rates above 70% under simulated gastrointestinal conditions are indicative of excellent probiotic potential. The observed results align with prior research indicating that *L. rhamnosus* GG is among the most resilient probiotic strains, capable of surviving gastric and intestinal transit. Similar studies have reported survival rates of 60–85% for *L. rhamnosus* GG in simulated conditions (Kim *et al.*, 2019; Surve *et al.*, 2022).

3.2.5. Tolerance to Bile Salts

L. rhamnosus GG exhibited significant growth across all tested bile salt concentrations, with a slight reduction in growth observed at higher concentrations (0.3% w/v). Growth was highest in the presence of sodium taurocholate compared to sodium deoxycholate and cholic acid, indicating better adaptation to conjugated bile salts (Figure 1).

Although growth rates in bile-supplemented media were slightly lower than the control (MRS broth alone), the strain maintained robust viability, showcasing its bile tolerance. The results are consistent with previous findings (Kuda *et al.*, 2016), which report *L. rhamnosus* GG as a bile-tolerant probiotic strain capable of surviving gastrointestinal bile concentrations.

Table 5. Carbohydrate fermentation of isolates

Sugar	Acid	Gas
Glucose	+	+
Lactose	+	+
Maltose	+	+
Fructose	+	+
Sorbitol	+	+
Sucrose	+	+
Dextrose	+	+
Arabinose	+	+
Trehalose	+	+
Rhamnose	+	+
Mannitol	+	+

3.2.6. Sugar fermentation profile

Different sugars were utilized for the determination of the carbohydrate fermentation profile of *L. rhamnosus* (Table 5). The sugar fermentation profile of the bacteria was determined to investigate whether the bacteria is homofermentative or heterofermentative. MRS broth was supplemented with 1% of each sugar and 5 mL of peptone water. In peptone water pH indicator, bromocresol purple was also added which turns yellow or purple in acidic and basic pH respectively. A full loop of bacterial culture was transferred into a test tube containing broth and peptone water and carried out the incubation at 37°C for 48 hours. In homofermentative, lactic acid is the principal acid with no production of any gas. The results showed that *L. rhamnosus* was heterofermentative because there was gas production in the test tube. The following results conform to Hedberg *et al.* (2010) as they demonstrated *L. rhamnosus*, a heterofermentative bacterium. They declared that *L. rhamnosus* ferments all the sugars along with the production of carbon dioxide. Senedese *et al.* (2015) also investigated lactic acid production by *L. rhamnosus* and their sugar fermentation profile. They stated that *L. rhamnosus* is heterofermentative and is in compliance with our predictions.

4. Conclusion

In conclusion, *Lactobacillus rhamnosus* demonstrates robust probiotic potential, characterized by high survival rates under harsh gastrointestinal and bile conditions, significant bile salt tolerance, and beneficial fermentation profiles. These findings support its application in functional foods and supplements, promoting gut health and adaptability to diverse gastrointestinal challenges. Further research is warranted to explore its therapeutic and dietary efficacy for human consumption.

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7. Conflict of Interest

The authors declare no conflict of interest.

8. Authors' Contribution

Umair Ali: Conceptualization, Methodology, Writing - Original Draft. Muhammad Shahzad: Data Curation, Formal Analysis, Writing - Review & Editing. Areej Nasir: Investigation, Resources, Validation. Khushbu Riasat: Visualization, Writing the manuscript. Sadia Riaz: Software, Data Analysis, Writing - Review & Editing. Muhammad Asad Ali Khan: Funding Acquisition, Resources, Supervision.

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