Functional Probiotic Characterization of *Lacticaseibacillus rhamnosus* S3 & *Limosilactobacillus mucosae* G3

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Abstract

Probiotics are defined as live organisms that when administered in adequate amounts confer health benefits to the host. The functional and physiological action of probiotics is part of a very active field of research and so far, the main health benefits proposed to be associated with probiotics are the improvement of gastrointestinal function, improvement in lactose digestion, reduction of blood cholesterol level, prevention of carcinogenesis and immunomodulation. *Lacticaseibacillus rhamnosus* S3 and *Limosilactobacillus mucosae* FCG3 were isolated in our laboratory from fecal sample of an adult and from an infant fecal sample respectively. The identity of the L. rhamnosus FCS3 and L. mucosa FCG3 was established by 16S rDNA method. In the present we are reporting potential probiotic properties of *L. rhamnosus* FCS3 and *L. mucosa* FCG3. Both the strains exhibited bile salt hydrolase activity, aggregation and coaggregation in the presence of *E. coli* and *Staph. aureus*, antibacterial and antifungal activity. Cell free extract of *L. rhamnosus* FCS3 and *L. mucosa* FCG3 displayed antimicrobial activity against food spoilage organisms and gastrointestinal pathogens. Cells of L. mucosa FCG3 didn't showed inhibition by spot overlay method. Potential probiotic characteristics of *L. rhamnosus* FCS3 and *L. mucosa* FCG3 are strain dependent.

Key words: Probiotics, Lactobacillus, antimicrobial activity,

1. Introduction

Lactobacillus is a genus of Gram-positive bacteria that plays a crucial role in various aspects of human health, are commonly found in the human gut, mouth, and vagina [1-2]. They are known for their ability to produce lactic acid, which creates an acidic

environment that inhibits the growth of harmful bacteria [2]. Lactobacillus species help maintain a healthy balance of gut microbiota, aiding in digestion and nutrient absorption. They can also help alleviate digestive issues like diarrhea and constipation. These bacteria can strengthen the immune system by stimulating the production of antibodies and other immune cells. Lactobacillus species are naturally present in the vagina and help maintain a healthy vaginal pH, preventing infections like bacterial vaginosis [4]. Some strains of Lactobacillus can improve skin conditions such as acne and eczema [5].

Probiotics, often referred to as "friendly bacteria," are live microorganisms that, when consumed in adequate amounts, confer health benefits to the host [6]. These beneficial bacteria primarily reside in the human gut, where they form a complex ecosystem known as the gut microbiota [7]. Probiotics help maintain a healthy gut balance, aid in digestion, boost immunity, and may even alleviate certain digestive disorders [8].

By consuming probiotic-rich foods like yogurt, kefir, and fermented vegetables, or taking probiotic supplements, individuals can support their overall well-being and enhance their gut health [9].

In the present was undertaken to characterise functional properties of Lactobacillus stains with reference to (i) viability of strains under in stress condition, (ii) *Survival during oro-gastro-intestinal transit, (iii) auto aggregation* and coaggregation assay in the presence of pathogen, (vi) antimicrobial activity and other properties like bile salt hydrolase, non-*haemolytic activity, non-biogenic amine production and Dextran production, (vii) Isolation of DNA and amplification using 16s rRNA primer*

2. Materials and Methods

2.1 Bacterial strains

Lactobacillus rhamnosus S3 and *Lactobacillus mucosae* G3 were isolated from the human infant faeces using MRS medium.

2.2 Preparation of lactobacilli cell suspension

Cell suspension was prepared by growing *Lactobacillus* strains in MRS medium at 37°C for 24 h. The cells were pelleted by centrifugation (5000 rpm, 15 min, 4°C), washed twice with phosphate buffer saline (PBS – 0.1 M, pH 7.2, containing 0.85% (w/v) NaCl) and resuspended in phosphate buffer (0.1 M, pH 7) to get cell suspension having A_{600} = 1 and 10^9 cfu/ml.

2.3 Tolerance to pH, bile, NaCl, and phenol

Viability of *Lactobacillus* strains was evaluated as described by Jacobsen et

al.[10]. *Lactobacillus* strains (10^8 cfu/ml) were used to inoculate 2 ml of MRS, MRS modified with bile salt (0.5, 1%), NaCl (2, 4%), pH (2.5, 3) adjusted with 0.1 M HCl, and MRS with phenol (0.4, 0.6% phenol). Samples (0.1 ml) collected from these tubes after 0 and 4 h, were serially diluted, plated on MRS agar, and incubated at 37°C for 48 h for the determination of viability.

2.3.1 Survival during oro-gastro-intestinal transit

Survival of cells in oral cavity was determined following Vizoso-Pinto, Franz, Schillinger and Holzapfel [11]. To mimic the *in vivo* passage in salivary gland, 100 µl of cell suspension was mixed in simulated saliva (SS) solution comprising of sterile electrolytes solution containing lysozyme (100 mg/L). Electrolyte solution, simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were according to Pithva *et al*. [12]. For sequential exposure to SS-SGF-SIF, 100 µl cell suspension was incubated for 10 min with SS. Subsequently, cells pelleted by centrifugation was further evaluated for SS-SGF and SS-SGF-SIF passage tolerance as per the method of Charteris, Kelly, Morelli and Collins [13]. Bacterial suspension without enzymes of SS, SGF and SIF treatment was included as control. Survival (log cful/ml) after each enzymatic exposure i.e lysozyme, pepsin and pancreatin was determined by viable count method.

2.4 Autoaggregation assay

Autoaggregation assay was performed as described by Ambalam et al[14] with certain modifications. Cell suspension (2 ml) was vortexed for 10 s and incubated at 37°C. Aliquot of 0.1 ml collected from the upper surface at regular time interval was mixed with 0.9 ml PBS and its A_{600} was recorded. The autoaggregation (%) is calculated as $[(A_0 - A_t)/A_0] \times 100$, where A_0 is A_{600} at 0 h and A_t represents the A_{600} of cell suspension at different time intervals (2, 4 and 24 h).

2.4.1 Coaggregation assay

Equal volumes of cells suspensions (1 ml=10^9 cfu/ml) of *Lactobacillus* and pathogen strains were mixed, and incubated at 37°C. The control contained 2 ml of pure Lactobacilli or *E. coli* and *Staph. aureus*. A_{600} of these suspensions was measured at predetermined time intervals as described above. The coaggregation (%) was calculated using the equation, $100*[((A_{pat}+A_{Lacto})/2)-(A_{mix})]/[(A_{pat}+A_{Lacto})/2]$, where A_{pat} and A_{Lacto} represent A_{600} of control tubes and A_{mix} represents the A_{600} of the mixture of *Lactobacillus* and pathogen strains at predetermined time intervals [15].

2.5 Bile salt hydrolase activity

Bile salt hydrolase (BSH) activity was evaluated according to Du Toit et al. [16]. MRS agar was supplemented with 0.5% (w/v) bile salt (Himedia, Mumbai, India) and 0.37 g/l of $CaCl_2$. Activated cultures of lactobacilli was streaked on the modified MRS agar plates, and incubated at 37°C for 72 h. The formation of bile acid precipitations around the colony was considered as a positive result.

2. 6 Antibacterial activities

The antibacterial activity of *Lactobacillus* strains was determined by the spot inoculation test [12] with minor modifications. Briefly, 2 µl of *Lactobacillus* cell suspensions were spot-inoculated on the surface of MRS agar plate and incubated for 24 h at 37°C. 100 µl (A_{600} = 0.2) of the indicator strains was mixed with 10 ml of 1% nutrient agar and overlaid on the MRS agar previously spot-inoculated with lactobacilli and incubated further for 24 h at 37°C. The indicator strains are used include *Escherichia coli, Salmonella typhi, Serratia marcescens, Shigella sp., Proteus vulgaris, Klebsiella pneumonia, Enterococcus faecalis, Yersinia enterocolitica, Micrococcus leutus, Listeria monocytogenes, Bacillus subtilis, Bacillus megaterium, Bacillus cereus*. All the strains were cultured in Nutrient broth except Staph. aureus grown in Brain heart infusion broth.

2.6.1 Preparation of cell free culture filtrate of lactobacilli

2% inoculum of lactobacilli cultures were used to inoculate 10 ml of MRS medium (pH 6.8), incubated at 37°C for 24 h. The cultured medium was centrifuged (5000 rpm, 20 min, 4°C) and supernatant was passed through 0.45 µm membrane filter.(Millipore, USA). CFC was used to determine antimicrobial activity by well diffusion assay.

2.6.2 Determination of antimicrobial activity by well-diffusion agar assay

Antimicrobial activity of CFC and EPC was determined by well-diffusion agar assay (British Standard 1974) against the test pathogens. 100 µl of 18 h old cultures of test organisms were inoculated in molten nutrient agar and poured in sterile Petri plates. Wells (7 mm) made using cup borer were loaded with 100 µl of CFC, and pre-incubated at 4°C for 2-3 h before shifting to 37°C and incubated for overnight. Appropriate controls were also included.

2.6.3 Antifungal activity

The antifungal activity of *Lactobacillus* strains was evaluated by overlay assay. Bacterial cell suspension was streaked (2 cm line) on MRS agar plates and incubated for 48 h at 37°C. 10 ml of potato dextrose

agar (1%) inoculated with 0.1 ml of fungal spores (10^5 spores) respectively were poured on the previously inoculated MRS agar plates and incubated at room temperature. The grading of antifungal activity is based on the size of inhibition zone around the bacterial growth.

2.7 Haemolytic activity

MRS agar plates (Himedia, Mumbai, India) containing 5% human blood were streaked with 18 h old *Lactobacillus* cultures growing in MRS medium at 37°C for 48-72 h. The plates were observed for haemolytic reaction.

2.8 Detection of biogenic amine production

The amino acid decarboxylase activity of *Lactobacillus* strains was determined as described by Bover-Cid and Holzapfel [17]. Amino acids used were L-tyrosine disodium salt (Himedia, Mumbai, India) for the detection of decarboxylase activity. Decarboxylase medium not containing amino acid served as negative control. *Enterococcus faecalis* served as positive control.

2.9 Dextran production

Lactobacilli was streaked on MRS media containing 5% sucrose and incubated at 37°C for 72 h. The presence of mucoid colonies indicates dextran production.

2.10 16S rRNA gene amplification

Genomic DNA was obtained from the isolates using the bacterial DNA isolation kit as per the manufacturer's instructions. The DNA quality was checked using agarose gel electrophoresis and the 16S rRNA gene was amplified using universal primers (UNI 8F-5′ AGAGTTTGATCCTGGCTGAG 3′, UNI 1492R-5′GGTTACCTTGTTACGACTT 3′). The conditions for PCR were 98 °C for 2 min, 98 °C for 20 s, 56 °C for 30 s, 72 °C for 80 s, and 72 °C for 5 min.

Each 25 µl PCR reaction mixture contained -Master mix -12.5 µl, Forward primer – 1 µl, Reverse Prime – 1 µl, DNA template – 5 ng, DMSO – 0.25 µl and final volume is made with remaining double distilled water.

3. Results

3.1 Tolerance to pH, bile, NaCl, and phenol

The two Lactobacillus strains, *L. mucosae G3* and *L. rhamnosus S3* showed significant variation in acid, bile, phenol and Nacl. After 4 h *L. rhamnosus S3* showed upto 80% of viability in all conditions (acid, bile phenol and NaCl) compared to cells grown only in MRS (Figure 1A). Similarly, *L. mucosae* G3 showed up to 80% viability in all tested conditions except 0.4% phenol and pH 2.5. (Figure 2B)

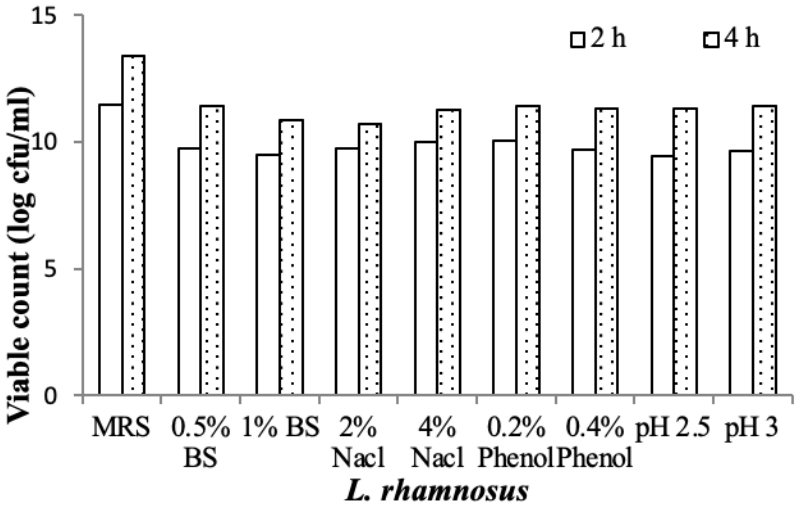


Figure 1A. Growth of *L. rhamnosus S3* in the presence of acid, bile, phenol and NaCl (Viable count is expressed as log cfu/ml).

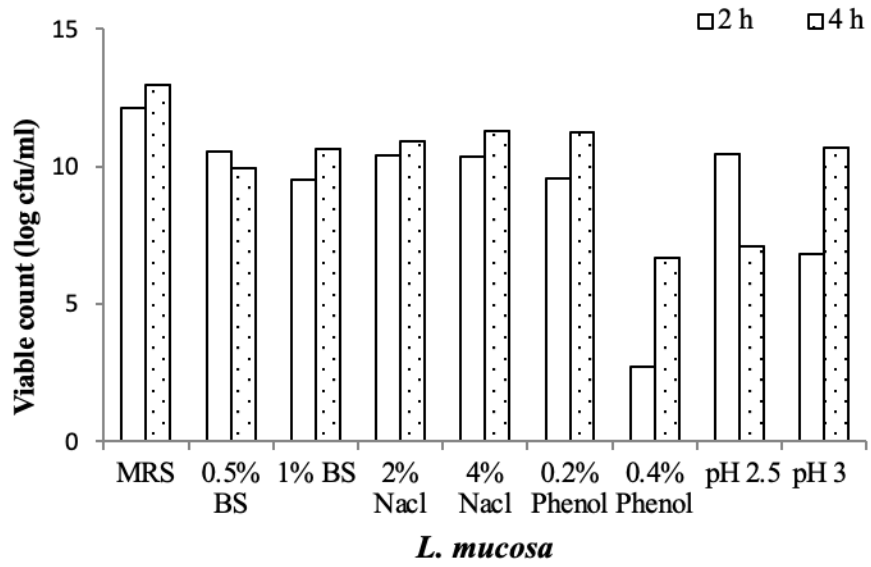


Figure 1B. Growth of *L. mucosae G3* in the presence of acid, bile, phenol and NaCl. (Viable count is expressed as log cfu/ml).

3.1.2 Survival during oro-gastro-intestinal transit

The two strains *L. rhamnosus* S3 and *L. mucosae* G3 were able to survive in the presence of harsh condition tested in SSF, SGF, SIF. However, *L. rhamnosus S3* is more robust than *L. mucosae* G3 as they could survive more under the harsh

condition compared to *L. mucosae G3* (Figure 2).

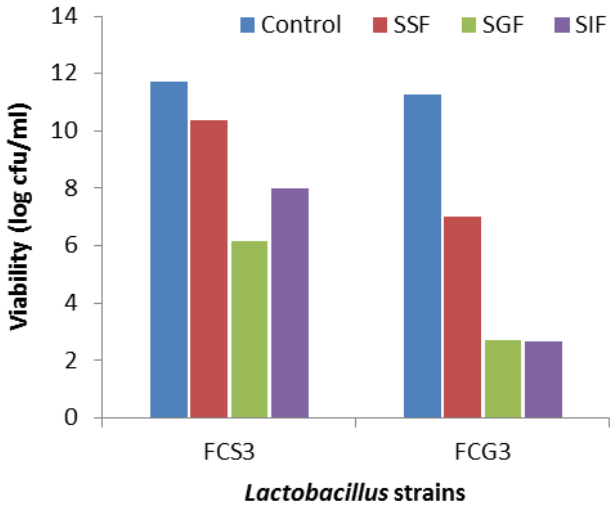


Figure 2. Survival (log cfu/ml) of *L. rhamnosus* G3 and *L. mucosae* S3 strains upon its sequential exposure to simulated saliva fluid, simulated gastric fluid, simulated intestinal fluid as determined by viable plate count method on MRS medium.

3.2 Autoaggregation assay

Auto aggregation increased with incubation time and was higher after 5 h (Figure 3). *L. rhamnosus* S3 showed increased autoaggregation after 2 h and 5 h with and without bile. *L. mucosae* G3 showed less autoaggregation compared to *L. rhamnosus* S3. And also *L. mucosae* G3 decreased in the presence of 0.5% and 1% bile salt.

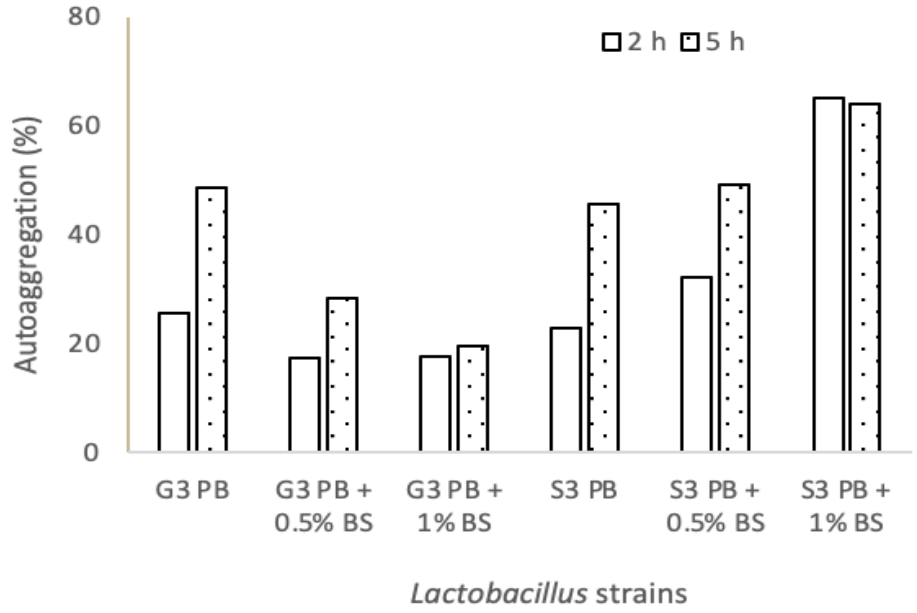


Figure 3 Autoaggregation of *L. rhamnosus S3* and *L. mucosae G3* after 2h and 5h with and without the presence of BS.

3.2.1 Co aggregation assay

The two strains of *Lactobacillus* showed co-aggregation with tested pathogen *E. coli and S. aureus. L. mucosae* G3 exhibited increased co-aggregation with the incubation time and in the presence of bile (Figure 4A). *L. rhamnosus S3* showed

increased co-aggregation with the incubation time and in presence of bile. *L. rhamnosus S3* showed higher co-aggregation compared to *L. mucosae G3* (Figure 4B).

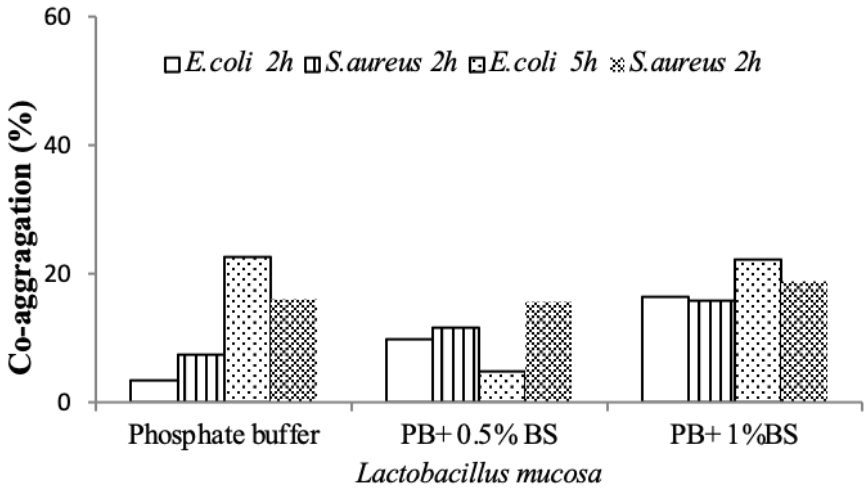


Figure 4A Co-aggregation (%) of *L. mucosae* G3 with *E. coli* and *S. aureus* determined spectrophotometrically after 2 and 4 hr of co-incubation

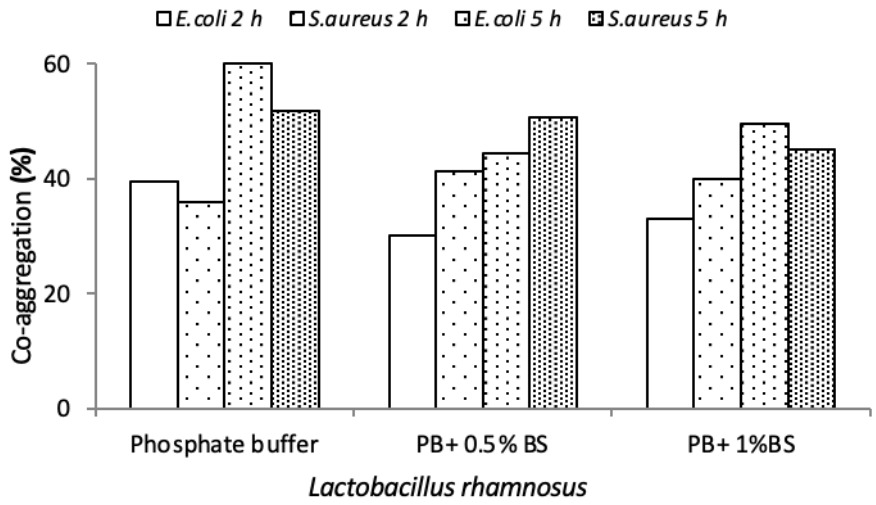


Figure 4B Co-aggregation (%) of *L. rhamnosus S3* with *E. coli* and *S. aureus* determined spectrophotometrically after 2 and 4 h of co-incubation

3.3 Preparation of cell free culture filtrate of lactobacilli

L. rhamnosus S3 showed higher activity against tested pathogen *S. typhi* and they did not show activity against *Sr. marcens, Y. enterocolitica, M. leutus* and *S.aureus* .

L.mucosae G3 did not show any antimicrobial activity against tested pathogens (Figure 5).

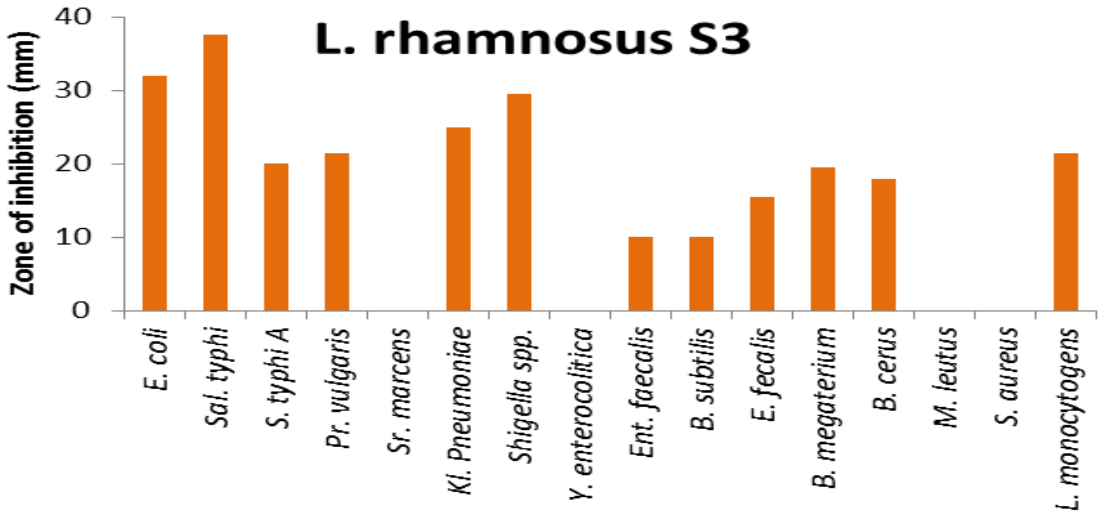


Figure 5 Antimicrobial activity of *L. rhamnosus S3* against test organisms by spot overlay method.

3.3.2 Determination of antimicrobial activity by well-diffusion agar assay

Both the *Lactobacillus* strains *L. rhamnosus S3* and *L. mucosae G3* did not showed antimicrobial activity against *S. typhi A, B. subtilis, S. aureus*, and *L. monocytogens* from all tested pathogens. *L. rhamnosus S3* showed highest activity against *Shigella spp*. And did not show activity against *Kl. pneumoniae. L. mucosae G3* showed highest activity against *B. cereus* and did not show any activity against *S.typhi* (Figure 6)

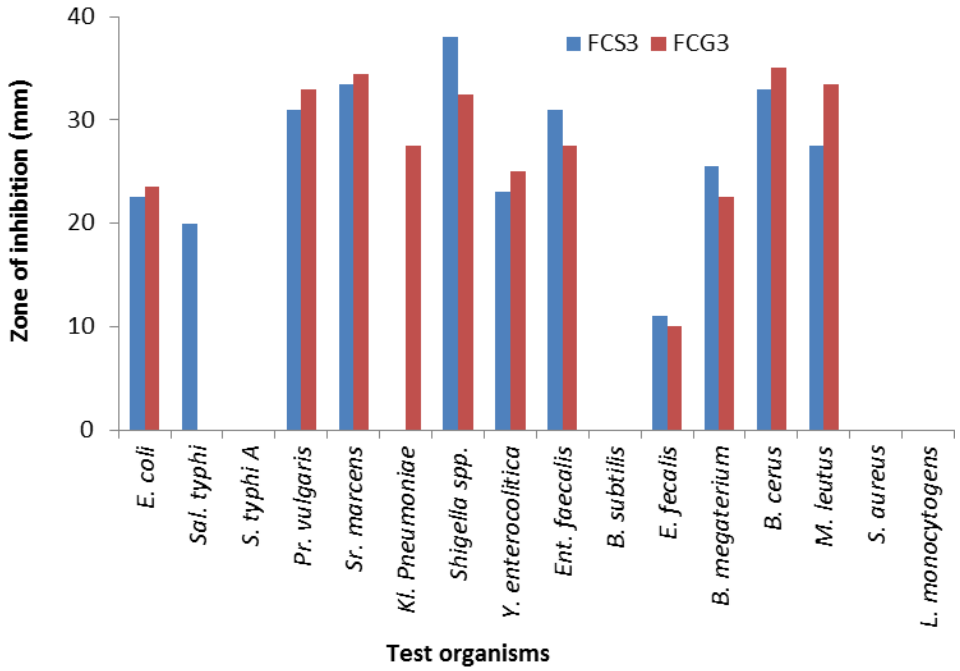


Figure 6 Antimicrobial activity of *L. rhamnosus S3* and *L .mucosae* G3 against all tested pathogens by well diffusion method.

3.4 Other characteristics of L. rhamnosus S3 and L.mucosae G3

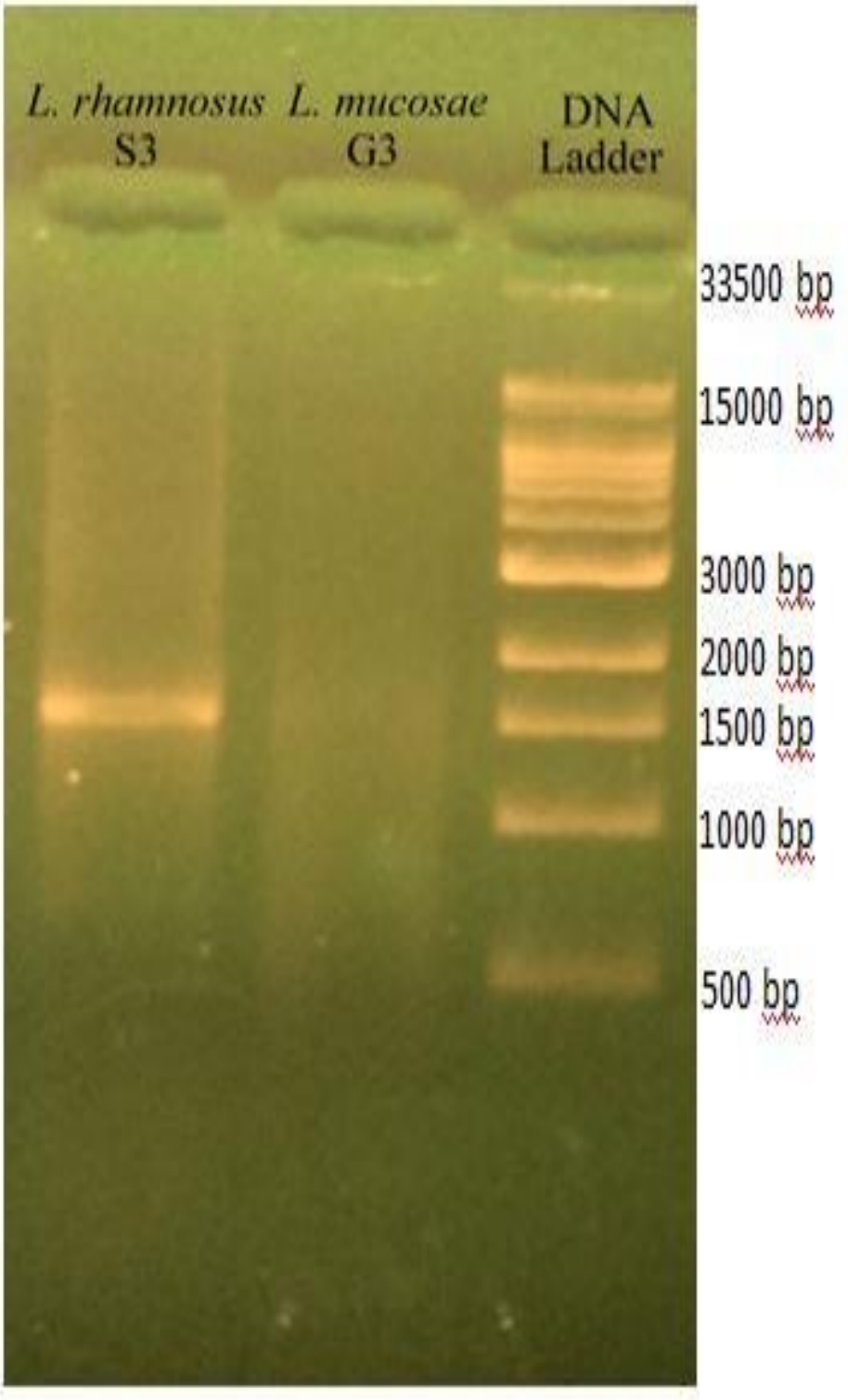
L. rhamnosus S3 did not exhibit antifungal activity, haemolytic activity, biogenic amine production, dextran production and bile hydrolase activity. *L. mucosae* G3 exhibited only antifungal activity (Table 1) Both the strains did not exhibit

Table 1. Other characteristics of *L. rhamnosus* S3 and *L.mucosae* G3

Parameter	***L. rhamnosus***	***L.mucosae***
Antifungal activity	Negative	Positive
Haemolytic activity	Negative	Negative
Biogenic amine production	Negative	Negative
Dextran production	No dextran production	No dextran production
Bile salt hydrolase activity	Negative	Negative

3.5 PCR amplification. Genomic DNA was isolated from the two *Lactobacillus* strains and amplified using 16s rRNA universal primers (Figure 7).

Figure 7. PCR amplification of 16s rRNA gene for *L. rhamnosus* S3 and *L. mucosae* G3, amplified product is around 1.6 Kbp.



4. Discussion

The present study reports the characterization of two human strains of *Lactobacillus* with potential probiotic properties. One of the important parameter to evaluate potential probiotic characteristics is they must be human origin, non-pathogenic, and non-haemolytic, though the lactobacilli have a long history of being safe for humans and been conferred GRAS status. Isolated strain should be robust and must survive and multiply in the harsh conditions prevailing in the GIT. The primary requirement for potential probiotic organisms is to survive during the passage through the acidic (pH 1.5–2.5) environment of the stomach. *L. rhamnosus* S3 and *L. mucosae* G3 survives at pH 2.5 for 4 h, a sufficiently long time for the cells to pass through the stomach and reach their site of action in the intestine[12, 18].

L. rhamnosus S3 and *L. mucosae* G3 possesses the ability to tolerate 4% bile, which is important for survival of probiotics in the GI tract. It is difficult to suggest a precise concentration to which a selected strain should be bile tolerant [10] Different concentrations have been used depending on the type of bile salt used in the studies [19,20]. *L. rhamnosus* S3 and *L. mucosae* G3 possesses the ability to grow in the presence of 0.4% phenol and remained viable in 0.5% phenol, a toxic metabolite produced by intestinal bacteria during putrefaction in the GI tract. Both the strains also possess the ability to grow in the presence of high NaCl concentration [21]. It confers competitive edge over other undesirable organisms during food processing, and together with their antimicrobial activity against food-spoilage organisms, is of advantage for their use in food preservation Both strains are robust in the terms the ability of cells to survive in the presence of bile, NaCl, and phenol can help them to colonize, grow, and elicit the beneficial effect to the host [21].

Autoaggregation and coaggregation abilities enable lactobacilli to form a barrier against colonization of mucosael surfaces by pathogens. This may be attributed to altered microenvironment of the coaggregated cell surface and antimicrobial metabolites of lactobacilli [12]. This property would help Lactobacillus cells to adhere and colonize gastrointestinal surfaces, provide protection in the hostile environment of the GIT and, in turn, extend their homing time in the gut there by helping the host health by affecting the microbial homeostasis towards healthier composition.

Lactobacilli strains exhibit broad antimicrobial spectrum against Gram-positive and Gram-negative organisms, which are major food spoilage organisms and gastrointestinal pathogens [3]. Lactobacillus strains show varying degree of strain-specific antibacterial activity against tested pathogens. *L. rhamnosus* S3 and *L. mucosae* G3 exhibited AMA against test pathogens by well diffusion assay. *L. mucosae* G3 did not show AMA against test pathogens by spot over layer method. It could be possible that activity is growth dependent process [21].

5. Conclusion:

The current study investigated two *Lactobacillus* strains isolated human source for their potential probiotic characteristics. Findings revealed that the two Lactobacillus
strains had great potency to withstand the low pH 3.0, 0.3% bile salts and the in vitro model of gastrointestinal conditions. Moreover, the isolated strains showed varied cell surface properties, including auto-aggregation, hydrophobicity and co-aggregation (even in the presence of BS) with *E. coli* and *S. aureus*, antimicrobial activity against E. *coli* and *S. aureus*. Furthermore, *L. rhamnosus* S3 is more robust strain then *L. mucosae* G3.

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