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Research Article

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Determination of Probiotic Properties of *L. fermentum* MT308789 and *L.oris* MT308790 Isolated from Mother Breast Milk

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Abstract: Mother milk is a complete food for the child. It contains vital nutrients and biomolecules required for the growth and development of a child. It mainly contains carbohydrates, proteins, fats and minerals. Recently, it was also found that it contains certain different microorganisms which play a vital role in the body. Most of the microorganism of milk belongs to the *Lactobacilli* family. Knowing the importance of *Lactobacilli* in health, certain food industries have focused on these microorganisms to be used as a health supplement nowadays called probiotics. Here in this study, *Lactobacilli* were isolated from mother breast milk and their probiotic characteristics were studied to determine their potential for probiotic. Strains were isolated using MRS medium and analyzed for their biochemical properties, pH resistance, bile salt tolerance, NaCl concentration and antimicrobial activity. Results of the study have shown that out of two strains, one strain was *L. fermentum* MT308789 and other was *L oris* MT308790. Both the species have shown good resistance to acidic pH and bile salt. They were also having good antimicrobial activity. Based on the study it was found that both strains have potential to be used as probiotic.

Keywords: *Lactobacilli,* probiotic, mother breast milk, pH resistance, bile salt tolerance, antimicrobial activity

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1. INTRODUCTION

Several species of *Lactobacilli* and *Bifidobacterium* can be obtained from varieties of sources but not all of them can be used as probiotics. [1,2] Strain which can be used as probiotic must possess a certain minimum characteristic. These characteristics may be called probiotic properties of the microorganism. These probiotics properties are nothing but various metabolic activities of microorganisms which enhance the ability of microorganisms for better health benefits. Probiotic properties include antibacterial-antifungal activities, bile salt hydrolase activity, ability to survive at low pH and high salt concentration, adhesion assay, production of gamma amino butyric acid (GABA), β-galactosidase assay, hemolytic activity, mucin degradation etc. [1–6] Various methods are used for determination of these properties. Selection of method is highly dependent on the character and type of microorganism. Hence it's become very essential to identify the microorganism upto species level first.[7–9] For determination of antimicrobial activity, zone of inhibition is one of the most widely used and accepted methods. [10,11] Certain microorganisms like *E.coli, Enterobacteria, Salmonella typhi, Shigella* sp*, Proteus vulgaris, S. marcescens*, *Bacilli* sp, *Candida* sp, *Aspergillus* sp, *Penicillium* and *Rhizoctonia* are known to be pathogenic and their consumption may lead to various diseases. Probiotics are known to have good antimicrobial activity. Hence consumption of probiotic strains as food ingredients or as supplements will improve health.[10–12] Bile salt hydrolase (BSH) enzyme plays a vital role in removal of cholesterol. BSH positive strain is considered more potential as compared to BSH negative. BSH activity can be determined by using spectrophotometer, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). In each method extraction of lipids from the cell is a common step which is done using various chemical and physical methods. [13–16] Probiotic strain must also be capable of surviving at low pH. As the digestion of food takes place in the stomach where pH is highly acidic and high salt may be present. If the microorganism cannot tolerate these conditions, then it cannot survive. Tolerance capability can be easily determined by changing the pH and salt concentration in the growth media. [17–20] Adhesion assay determines the ability of microorganism to adhere on the intestinal epithelial cells or mucosal surface. This is considered as one of the most significant properties of probiotic strains. If the strains are not able to adhere to the cells, then it will be washed out and may be destroyed. Adhesion assay also includes biofilm formation ability. Biofilm allows the probiotic strains to form colonies in the gut and other parts of the gastrointestinal tract which ensures the stability of probiotic strains in the body. Cell adhesion assay is generally carried out using animal cell lines like Caco2 and HT-29. [21–23] GABA is a non protein amino acid which is widely distributed in nature and well known for its several physiological functions like induction of hypotension, diuretic effect etc. Many probiotics strains are known to produce GABA in sufficient quantities. TLC is one of the most common and efficient method for qualitative and quantitative determination of GABA. [24–26] β-galactosidase performs three major function, it hydrolyses lactose to galactose and glucose, it catalyses transgalactosylation of lactose to allolactose and it converts the allolactose to monosaccharides. Activity of β-galactosidase can be determined by spectrophotometric method using o-nitrophenyl- β-D-galactopyranoside as substrate. [27–29] In addition to all these properties, probiotic strain must be non hemolytic and should not possess any DNAse activity.[25,30] Hemolytic activity can be determined by incubating the media containing plates with blood for optimum period of time and temperature. Clear zone indicates hemolytic activity.[25,31] DNAse activity can be determined using DNAse agar medium plates.[30,32] Mucin is a protective layer of gastrointestinal tract and any damage in this layer can affect the defense system of the person. An ideal probiotic strain must not degrade this mucin layer. Mucin degradation ability can be determined by spectrophotometer and SDS PAGE.[21,33] In this study few important parameters like resistance to pH, tolerance to bile salts, salt resistance and antimicrobial activity were analyzed and obtained results were discussed in detail.

2. MATERIALS AND METHODS

2.1 Isolation of Microorganism

Probiotic microorganism from mother breast milk was isolated using a spread plate method after enrichment and serial dilution. For collection of sample, breast was cleaned with soap solution and hot water just before the collection. Breast milk was collect in a sterile container by manual expression. Precautions were taken to avoid contamination while collecting the samples. Collected samples were immediately processed for experiments. In the first step, bacteria present in the milk were enriched by cultivating in de Man, Rogosa & Sharpe (MRS) broth for 24 hrs at 37°C. After enrichment, serial dilutions were made and 100µL of each dilution were spread on MRS agar. Plates were incubated under anaerobic condition at 37 °C until the visible colonies were seen. After incubation few colonies were selected based on the colony morphology, gram staining and motility test. Selected colonies were further purified on MRS agar and stored at low temperature for future applications. [34–36]

2.2 Identification of Microorganisms

Selected bacteria were identified using 16S rRNA sequencing method. [9,37] For this, in a clean sterile PCR amplification tube, 5 µl of pure DNA was taken and mixed with 20 µl of PCR master mix was added. To that 1.5 µl of forward primer and reverse primers were added vortexed gently. Tube was placed in PCR machine and sample were amplified as follow: initial denaturation at 94.0 °C for 3.0 minutes, Denaturation at 94.0 °C for 0.5 minute, annealing at 50.0 °C for 1.0 minute, extension at 72.0 °C for 1.0 minute, final extension at 72.0 °C for 10.0 minute (only once) for 40 cycles. Once the PCR is completed the product is collected and purified with Montage PCR clean up kit (Millipore). Product was stored at 4.0 °C. DNA sequencing was done on ABI 3730xl as per standard procedure by using universal primers and four different fluorescent labeled dNTPs.

2.3 Determination of resistance of pH

For determination of resistance of pH by selected strains, PBS with difference pH were prepared as per standard protocol using dilute HCl. MRS broth was prepared and incubated for 24 hrs to check the presence of any contamination. Inoculum for the experiment was prepared by growing the microbial strain in MRS broth for overnight at 37°C under anaerobic condition. 1.0 % of freshly grown overnight culture was inoculated in 10mL of sterile PBS of various pH and allowed to grow incubate for 0 hr, 1 hrs, 2

hrs and 3 hrs at 37 °C. After desired time of incubation, 100 µL mixture was inoculated in 100 mL sterile MRS broths and allowed to incubate at 37 °C under anaerobic condition. After incubation, microbial growth was determined by spectrophotometer. Turbidity was measured at 620 nm.[17,18,38]

2.4 Determination of bile salt tolerance

For determination of bile salt tolerance by probiotics strains, MRS broths with difference bile salt concentration were prepared as per standard protocol. Prepared media were incubated for 24 hrs to check the presence of any contamination. Inoculum for the experiment was prepared by growing the microbial strain in MRS broth for overnight at 37 °C under anaerobic condition. 1.0 % of freshly grown overnight culture was inoculated in 100 mL of MRS broth of various bile salt concentrations and allowed to grow for 24 hrs at 37 °C under anaerobic conditions. After incubation, microbial growth was determined by spectrophotometer. Turbidity was measured at 620 nm.[13,39]

2.5 Determination of growth at different Salt (NaCl) concentrations

For determination of bile salt tolerance by probiotics strains, MRS broths with different salt concentration were prepared as per standard protocol. Prepared media were incubated for 24 hrs to check the presence of any contamination. Inoculum for the experiment was prepared by growing the microbial strain in MRS broth for overnight at 37°C under anaerobic condition. 1.0% of freshly grown overnight culture was inoculated in 100mL of MRS broth of various salt (NaCl) concentrations and allowed to grow for 24 hrs at 37°C under anaerobic conditions. After incubation, microbial growth was determined by spectrophotometer. Turbidity was measured at 620 nm.[40,41]

2.6 Determination of antimicrobial activity

Antimicrobial activity was determined by agar well diffusion method. For antimicrobial study, four microbial strains containing two gram positive and two gram negative strains were used. MRS broth was prepared as per standard protocol. Prepared media were incubated for 24 hrs to check the presence of any contamination. Simultaneously, nutrient agar plates were also prepared as per standard procedure and incubated at 37 °C for 24 hrs to check the presence of any contamination. After 24 hrs, selected strains were inoculated and allowed to grow overnight at 37 °C under anaerobic condition. Active culture of standard strains was prepared by inoculating culture in N-broth and incubating for overnight. To precede with agar well diffusion methods, nutrient agar plates were first spread with an active culture of standard strains. Followed by spreading, wells were made using sterile cup borers. To the wells 50 µL of cell free supernatants of probiotic culture media were filled. Plates were allowed to incubate 24 hrs at 37 °C. After incubation, plates were observed and zones of inhibitions were measured.[20,25,42,4] All the experiments in this study were carried out in triplicate and average values were taken under consideration, result and conclusion.

3. STATISTICAL ANALYSIS

Mean values of all the data were statistically analyzed by t-test and P value ≤0.05 were considered as significant.

4. RESULTS

4.1 Identification of bacteria

Based on the16S rRNA sequences, it was found that strains which were coded as M2P1 and S1 were *L. fermentum* and L. *oris* respectively. Both the sequences were submitted to GenBank NCBI with accession number MT308789 and MT308790 respectively.(Figure 1 and 2). Pure colonies of isolated strains are shown in the figure 3 and 4.

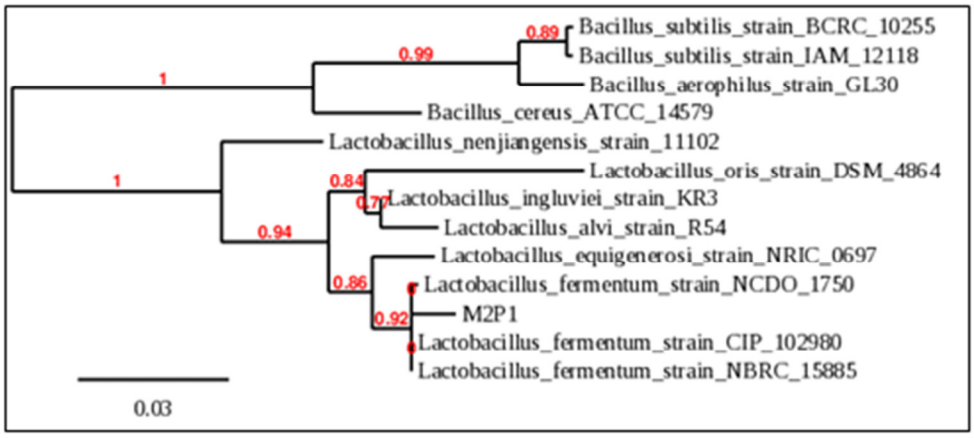

Fig 1. Phylogenetic tree for M2P1 *L. fermentum* MT308789

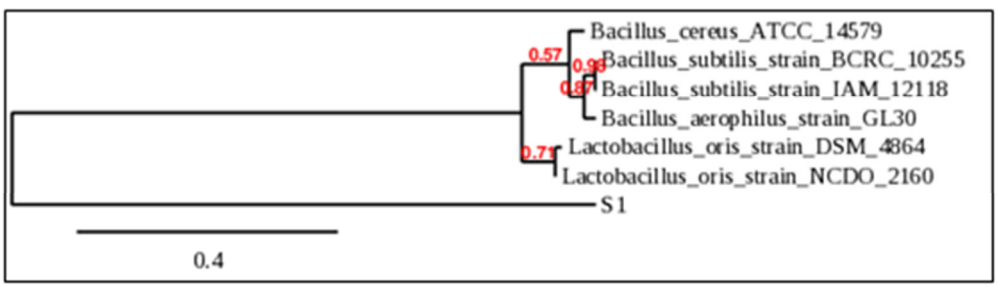

Fig 2. Phylogenetic tree for S1 *L. oris* MT308790

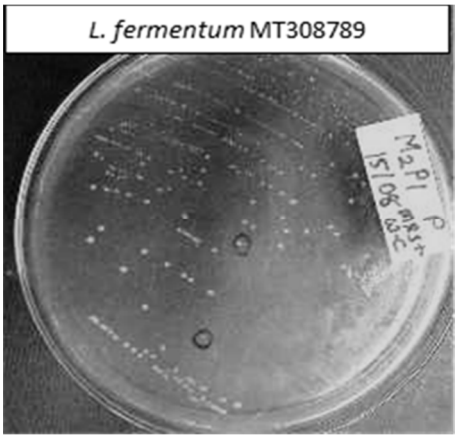

Fig 3. Pure culture of *L. fermentum* MT308789

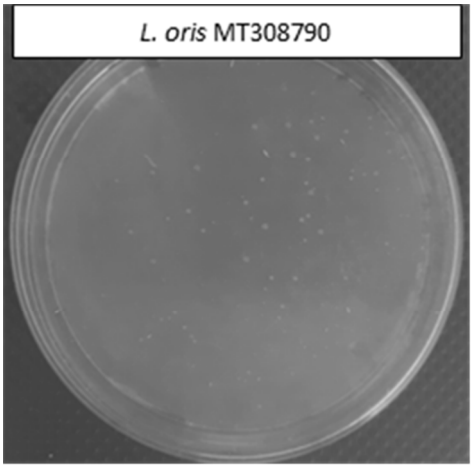

Fig 4. Pure culture of *L. oris* MT308790

4.2 *Determination of resistance of pH*

As mentioned in the methods, 4 MRS broths containing the microorganisms incubated at various pHs for different time intervals were analyzed for microbial density using spectrophotometer at 620nm. Below tables and graphs provide details of results (Table 1, 2 and Figure 5, 6).

Table 1. Resistance of pH by *L. fermentum* MT308789 (incubation time in Hrs)

L. fermentum MT308789				
Incubation Time	**pH of PBS buffer**			
	2	**2.5**	**3**	**3.5**
0 hr	0.81±0.03	1.03±0.04	1.21±0.05	1.42±0.06
1 hr	0.45±0.02	0.51±0.02	0.62±0.02	0.81±0.03
2 hrs	0.25±0.01	0.37±0.01	0.41±0.02	0.52±0.02
3 hrs	0.17±0.01*	0.26±0.01*	0.27±0.01*	0.33±0.01*
4 hrs	0.12±0.00**	0.23±0.01*	0.24±0.01*	0.31±0.01*

*Values are Mean±SD (n=3), *p<0.05 as compared to 0 hrs, **p<0.01 as compared to 0 hrs*

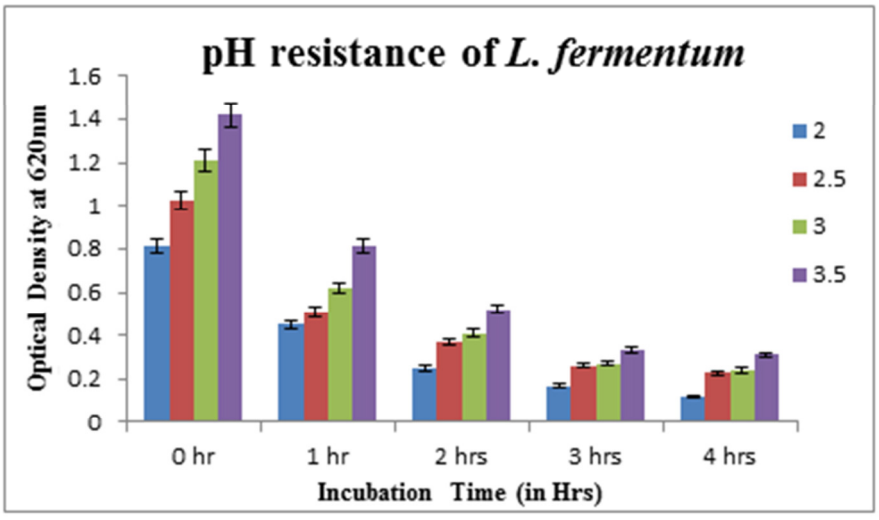

Fig 5. Resistance of pH by *L. fermentum* MT308789 (incubation time in Hrs)

Table 2. Resistance of pH by *L. oris* MT308790 (incubation time in Hrs)

L. oris MT308789				
Incubation Time	**pH of PBS buffer**			
	2	2.5	3	3.5
0 hr	0.78±0.03	0.93±0.04	1.14±0.05	1.36±0.05
1 hr	0.47±0.02	0.53±0.02	0.59±0.02	0.91±0.04
2 hrs	0.24±0.01	0.36±0.01	0.46±0.02	0.58±0.02
3 hrs	0.16±0.01*	0.21±0.01*	0.26±0.01*	0.36±0.01
4 hrs	0.10±0.01**	0.20±0.01*	0.21±0.01*	0.23±0.01*

*Values are Mean±SD (n=6), *p<0.05 as compared to 0 hrs, **p<0.01 as compared to 0 hrs*

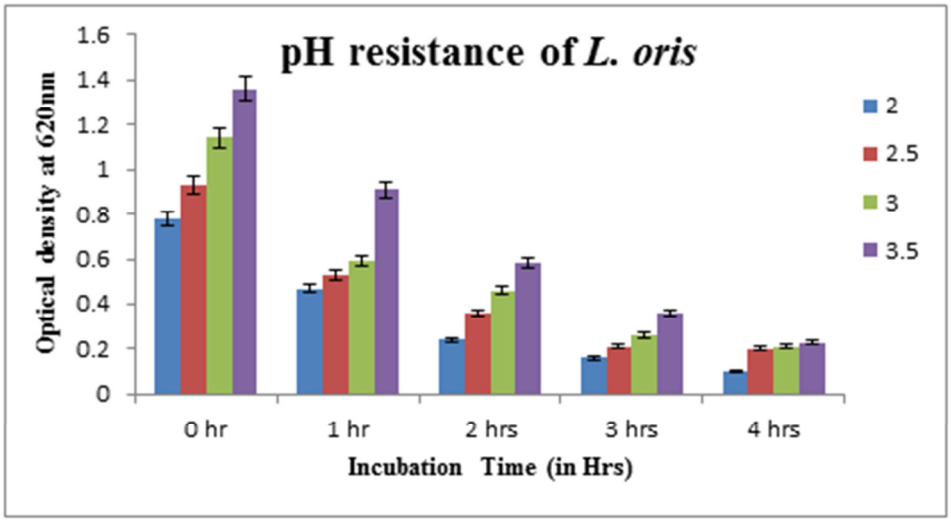

Fig 6. Resistance of pH by *L. oris* MT308790 (incubation time in Hrs)

Based on the obtained results it was found that both probiotic strains can grow at low pH upto 2.0. However, as the pH moves toward acidic range and time of incubation increases microbial growth decreases. It was also observed that incubation time for about 4 hrs drastically reduces the microbial population. Reduction may be upto 25.0 % or less than that. pH 3.5 has favored the growth of microorganism as compared to pH 2.0. From the literature it was noted that the optimum pH for the growth of *Lactobacilli* range between 5.5 to 6.5. [44,45]

4.3 Determination of bile salt tolerance

Three different concentrations of bile salt were taken to determine the tolerance of probiotic strain. Obtained data of this experiment are as below (Table 3 and Figure 7).

Table 3. Resistance of bile salt by *L. fermentum* MT308789 and *L. oris* MT308790

Bile Salt Conc. (in %)	*L. fermentum* MT308789	*L. oris* MT308790
0.00	2.41±0.32	2.62±0.41
0.50	2.20±0.31	2.30±0.35
1.00	1.07±0.09*	1.03±0.10*
1.50	0.06±0.01**	0.08±0.02**

Values are Mean±SD (n=6), *p<0.05 as compared to 0 hrs, **p<0.01 as compared to 0 hrs

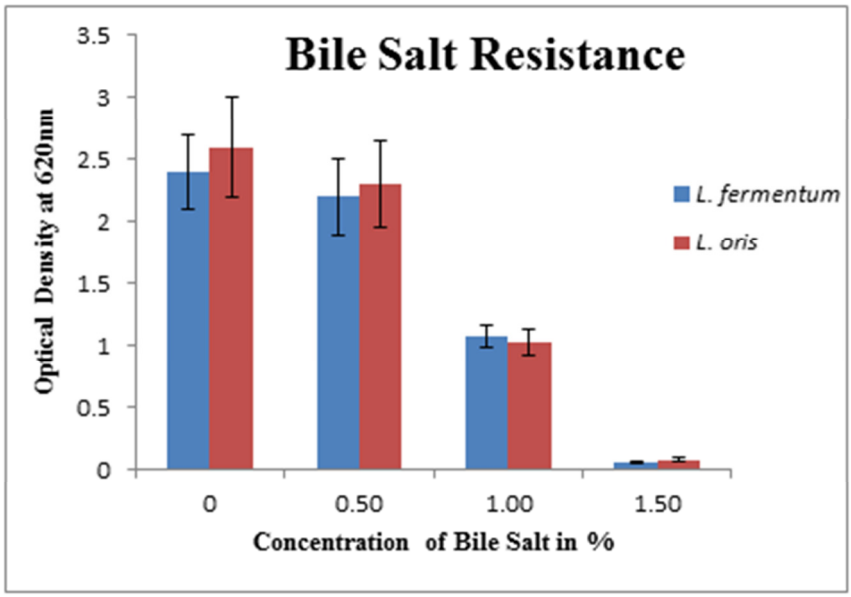

Fig 7. Resistance of bile salt by *L. fermentum* MT308789 and *L. oris* MT308790

From the results, it was noted that both strains are not able to resist very high concentration of bile salt i.e. 1.0% or above. However, both have shown very good growth with 0.5% bile salt concentration. Around 90.0% of viability is seen as compared to the control where bile salts were not added. According to literature if a strain can tolerate 0.3% of bile salt than it can be considered as a good probiotic. [13–15,44,46]

4.4 Determination of growth at different Salt (NaCl) concentrations

NaCl is generally required by all microorganisms for their growth. Probiotic microorganisms should be capable of resistance of higher salt concentration. This makes them ideal for various applications as probiotics. Here in this study the effect of NaCl was determined up to 6.0% and obtained results are mentioned as below. (Table 4)

Table 4. Resistance of NaCl salt by *L. fermentum* and *L. oris*

	Strains	
Salt Concentration (in %)	***L. fermentum***	***L. oris***
0.5	+++	+++
1.0	+++	+++
2.0	+++	+++
3.0	++	++
4.5	++	++
5.0	+	+
6.0	+	T

(+++ = excellent growth, ++ = good growth, + = less growth, T = trace

From the result it was observed that moderate concentration of salt i.e. upto 2.0% did not affect the microbial growth significantly. As the concentration of salt increases, growth of the microbes decreases. Beyond 6.0 % may be both strains may not be able to survive. Since both the strains can tolerate upto 4.5% of NaCl concentration, they can be used in the preparation of many probiotic products. This is because most probiotic products have salt concentrations less than 4.5%. [19]

4.5 Determination of antimicrobial activity

As mentioned in the method, two gram positive and two gram negative strains were taken for determination of antimicrobial activity. Zone of inhibition were measured in mm and noted in a tabular form (Table 5).

Table 5. Zone of Inhibition by *L. fermentum* and *L. oris*

	Zone of Inhibition (in mm)			
	Gram Positive		Gram Negative	
Strain	***B. subtilis***	***S. aureus***	***P. aeruginosa***	***E. coli***
L. fermentum	22.0±3.0	18.0±1.0	15.0±1.0	25.0±2.0
L.oris	18.0±2.0	15.0±2.0	17.0±1.0	24.0±3.0

Values are Mean±SD (n=6), *p<0.05

Both the strains *L. fermentum* and *L. oris* have shown antimicrobial activity against all the four standard microorganisms. Maximum inhibition was observed against *E. coli* followed by *B. subtilis*. Since both strains have some antimicrobial activity, it is a good sign for potential probiotic strains.

5. DISCUSSION

Preparation of commercial probiotics needs to optimize certain parameters. Among them all pH resistance, bile salt resistance, NaCl tolerance and antimicrobial activity are important criteria. When we consume any food, it goes to the gastrointestinal tract. Human gastrointestinal tract has a highly acidic pH near about 2.0 which helps in digestion of food. One must keep this in mind while preparing probiotics. If the microbial species selected for the probiotics cannot have resistance against low pH then it dies immediately and will not play any important role. Once the probiotic crosses the stomach, it will go to the intestine and can grow there and make the colonies. In a study carried out by Mehanna *et al*, they have studied 13 *Lactobacilli* and 5 cocci for pH resistance. They have found that most of the *Lactobacilli* can survive at pH 2 and above for more than 3 hrs of incubation. [43] In a study carried out by Ashraf *et al*, they have found that species like *L. acidophilus, L. paracasei, L. salivarius* are highly resistant till pH 4.0 but are not able to survive at pH 2.0. [47] *Lactobacilli* strains isolated by Melia *et al* from buffalo milk have shown that around 79.0 % - 97.0 % survival rate at pH 2.0 when incubated for 90 minutes. This rate was reduced to 55.0 % - 73.0 % when the incubation time extended up to 180 minutes.[48] When 58 various strains were studies for the probiotic properties, Santos *et al* found that *L. acidophilus* CYC 10051 and *L. kefiranofaciens* CYC 10058 have good adhesion capability, good resistance to pH and bile salt and can inhibit the growth of certain pathogenic microorganisms. [10] Vaidya *et al* in their study have shown that out of 27 strains 12 are highly resistance to low pH. But their viability decreases as the incubation time increases. [49] Similar kinds of results were obtained in our study too. Similar to pH resistance, bile salt resistance is also considered as one of the significant criteria. It is thought that bile salt affects the cell morphology and cell viability in different ways. It affects the adhesion efficiency of microorganisms. There are several mechanisms proposed for these. In contradiction to all these, theories are in place which tell about the mechanism of cholesterol removal by probiotics. Anandharaj *et al* have noted that as the concentration of bile salt increases, the resistance ability of microorganisms against diseases. However the resistance ability and reduction in resistance ability highly depend on the type of microbial strain. [1] In the study of Zhang *et al*, out of 29 lactobacilli two strains have shown resistance ability. Six strains were bile salt tolerant, 13 strains were weakly tolerant and remaining were sensitive. They have done the tolerance test using 0.3% bile salt only. [50] Many studies have suggested using upto 0.3% bile salt to determine the resistance as at a particular time stomach cannot have bile salt concentration above 0.3%. But still there are studies in which higher concentrations upto 2.0% of bile salts have been taken for analysis. [15,43,44,51] Owusu-Kwarteng *et al* have conducted their study on 16 various species of *L. fermentum*. They have observed that most of the species are

sensitive to higher concentration of bile salt. Concentration about 0.3% drastically reduces the resistance against bile salt. [52] Horackova *et al* have conducted their experiment using bile salt concentration upto 1.0 % bile salt and 24 hrs of incubation time. They have found that around 40.0 % -50.0 % microorganisms die because of higher concentration of bile salt and longer incubation time. [53] Jose *et al* have shown that most of the probiotic species isolated from food dairy and animal rumen can survive at 2.0 % bile salt concentration upto 6 hrs on incubation. [54] Sodium chloride (NaCl) is an inhibitory substance which inhibits the growth of certain microorganisms. It was observed that around 1.0 % NaCl concentration highly favours the growth of organisms. But as the concentration increases the viability reduces except in case of halophiles. Diba *et al* in their study have shown that certain Lactobacilli can survive upto 7.0 % of NaCl concentration. About that they cannot grow. [41] Similar observations were made by Chowdhury while he was working on *Lactobacilli* isolated from buffalo yoghurt. [55] In many other studies the same results were obtained. Previous studies have also emphasized on antimicrobial properties of probiotics strains. According to them, antimicrobial activity makes the probiotics strain more potential. Each strain has a different antimicrobial profile. In a study carried out by Santos *et al* all the 58 *Lactobacilli* strains have shown different antimicrobial profiles. Among the 58 strains, *Lactobacilli* CYC 10058 have shown best antimicrobial activity against *E. coli, L. monocytogenes, S. typhi, S. flexneri, Y. enterocolitica, S. enteritidis*. [10] Beasley has noted that antimicrobial compounds produced by LABs are natural preservatives. Their natural preservative can increase the shelf life and safety of processed food. They have also noted that LABs have better antimicrobial activity against gram positive than gram negative. [56] The principle compound which is produced by lactic acid bacteria is bacteriocin. Bacteriocins are peptides produced by the ribosomes which work against closely resemble microorganisms.[11,57] In our study, both the isolated *Lactobacilli* have shown good inhibition of all the four standard strains.

6. CONCLUSION

Based on the study, it was concluded that both the stains *L. fermentum* MT308789 and *L. oris* MT308790 have high tolerance against acidic pH and bile salt. Both are having good antimicrobial activity and capable of surviving with high NaCl concentration. All these properties reflect that both the strains can be used as potential probiotic strains.

7. AUTHORS CONTRIBUTION STATEMENT

Ms. Shradhdha Gondaliya has carried out all the experimental studies required for this study and Dr. Vimal Ramani has given direction for the planning and execution of work. Obtained data were analyzed by both the authors. Both the authors have contributed equally for preparation of the manuscript.

8. CONFLICT OF INTEREST

Conflict of interest declared none.

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