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PROBIOTIC POTENTIAL AND STRESS TOLERANCE IN LACTOBACILLUS MU1008 ISOLATED FROM CHILLED YOGURT SAMPLES OF MAJMAAH

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ABSTRACT: In recent years, scientific research has confirmed the presence of large amounts of probiotic *Lactobacillus sp.* in fermented dairy products, which shows a positive impact on human health. This study was designed to isolate Lactobacillus with probiotic potential from local and commercial samples. 40 Lactobacilli strained were isolated from commercial and local yogurt samples by tenfold dilution plating method and plated on Man-Rogosa-Sharp agar medium and were screened for their use as potential probiotics. The isolates were tested for their ability to survive at pH 2.0, pH 3.0, in the presence of 0.3% bile salt and antimicrobial activity; sensitivity against 10 specific antibiotics (ciprofloxacin, erythromycin, tetracycline, penicillin G, ampicillin, streptomycin, polymyxin B, vancomycin, chloramphenicol and rifampicin) using filter paper disc diffusion method and two-fold serial dilution methods. Out of the 40 strains, 24 strains (42%) had survival rates above 90% after 2 h of incubation at pH values of 2.0 or 3.0 pH. Further screening performed on the above 40 isolates indicated that 24 strains show to 0.3% bile salt. Lactobacilli strains exhibited inhibitory activity against *Salmonella strains* and *Candida albicans*. Moreover, all of the strains were resistant to vancomycin and streptomycin. The 24 strains that were found suitable for Potential probiotic activity including strains *Lactobacillus casei* (95%), *Lactobacillus salivarius* (25%), *Lactobacillus plantarum* (75%), and *Lactobacillus buchneri* (72%).

INTRODUCTION: From the group of lactic acid-producing bacteria also known as LAB, are among the largest group whose members are found in intestinal tract of vertebrates, including humans, that are involved inz process of fermentation of different food products, resulting in improving the quality of food and safety, including the health status and comfortableness of the consumers.

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These characteristic in a microorganism make them safe for use as food and as medication in the form of probiotics [1]. The other probiotic strains also include *Pediococcus*, *Bifidobacterium*, *Lactobacillus* and *Enterococcus* [2].

Probiotics are characterized as living micro-organisms which provide good effects on the organism, and can change as per the host body's micro-ecological scale, modify intestinal functions, also affect digestion and immune response. *Lactobacillus sp.* was among the earlier discovered probiotics out of the three types of probiotics, which include *Bifidobacterium* and Gram-positive cocci [3]. A lot of species of *Lactobacillus* are accepted as safe for intake, so, are often used in

food products [4]. Members of species of *Lactobacillus*, which include *Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei, Lactobacillus paracasei*, *Lactobacillus johnsonii*, *Lactobacillus reuteri*, and *Lactobacillus rhamnosus*, are used as probiotics [5]. A lot of recent years research has confirmed the existence of large number of probiotic *Lactobacillus* sp. in fermented food and dairy production which shows positive effect on human health. More emphasis has been given to the probiotic potential of *Lactobacillus*.

Different elements should be considered while screening the potential probiotic lactic acid bacterial strains in chilled samples which has to be used for consumption. The more advanced studies show that when probiotics are to be used for gut health, the bacterial strains shall survive the passage through the gastrointestinal tract [5, 6]. Taking probiotic as food or with food are good way to get its benefits and yogurt and ice cream are commonly consumed by human which are easy transporting vehicles for these beneficial lactic acid bacteria [7].

This study was designed to identify the best potential probiotic Lactobacillus isolates from household and commercially available 40 chilled yogurt samples collected from Majmaah. The *in-vitro* probiotic properties like Bile tolerance, pH, acid tolerance, antimicrobial activity, antibiotic susceptibility, auto and co-aggregation property and cell surface hydrophobicity of the selected isolates were studied.

MATERIALS AND METHODS:

Sampling: Forty samples of yogurt were collected of different commercial brands. Isolation was based on methods Ananthanarayan, R [8]. *Lactobacillus* sp were isolated on Man-Rogosa-Sharp (MRS) agar medium and stored at 37 ºC for 24 to 48 h for further phenotypic and genotypic identification [9].

Phenotypic and Genotypic Identification of Stains: Forty strains were isolated from the from commercial and yogurt samples by tenfold dilution plating method and plated on MRS Medium. All the isolates were first identified using conventional method of Gram staining, catalase activity and gas production in presence of glucose followed by carbohydrate fermentation. 16Sr RNA gene sequence analysis technique was used to identify the species. For this process genomic DNA was isolated using Genomic DNA isolation kit (HiPurA™, HiMedia). The amplification of 16SrRNA was done using thermal cycler and using prokaryotic 16S ribosomal DNA universal primer pair BSF8/27 (5′-AGAGTTTGATCCTGGCTC AG-3′) and BSR1492/20 (5′-GGTTACCTTGTTA CGACTT-3′) [10, 11]. All the sequences obtained are then compared using BLAST to the Gene Bank database. Resulting in the identification of 31 *Lactobacillus* isolates.

Acid Tolerance: Acid tolerance test was done as per the method given by Chung *et al.*, [9] with little changes. In in this method, 10 µL of overnight stored bacterial samples in MRS broth were inoculated into 1 mL of broth of MRS with pH 2.0, and pH 3.0, and pH 6.4 as control.

Study of Culture Characters: The enumeration of culture characters and morphology was carried out according to the method described by Banson, H.J [10].

To study these aspects, streak plate method was performed. Individual colonies grown on MRS agar plates were carefully studied with respect to size, color, opacity, form rise and margin.

Proteolytic Activity: To study proteolytic activity of lactic acid bacteria, MRS agar with additional 10% skimmed milk was introduced, left for solidification and then dry. Whatman paper discs sterilized and kept on the surface of MRS agar. 20µl young culture were given to each paper disk. Incubated at 37 ºC for 24 hour, after which proteolytic activity was measured as diameter of clear zones around the discs [11].

Lipolytic Activity: To measure Lipolytic activity, all strains were introduced on agar having spot in Tween 80 with different concentrations as 1%, 3% and 5% [12]. Incubated for 72 h at 25 ºC. The strains which showed an opaque area around the spot because of esters formation with calcium which produce fatty acids are recorded as positive[13]. Diameter of lytic zone were measured to study the lipolytic activity.

Cell Surface Hydrophobicity: To study the bacterial adherence with hydrocarbons was done as per Mishra and Prasad. Lactobacillus sp. were

harvested after incubation for 18 h at 37 °C precede by centrifuging the sample at 5000 rpm for 15 min. The cells collected were then washed two times with Phosphate Urea Magnesium Sulphate (PUM) buffer and then kept on an individual basis in PUM buffer at 10^8 cfu/ml level. The spectrophotometer reading of the suspension were recorded at 600 nm (A). From each cell suspension an amount of 5 ml was blended in 1 ml of hydrocarbon including ethyl acetate, xylene, toluene and chloroform. The mixture obtained was then vortexed for 1 min and the phases were separate after one hours at 37 °C. The aqueous stage was cautiously separated using a sterilized Pasteur pipette and the absorbency (A0) was read at 600 nm to compute cell surface hydrophobicity [14].

Medium: LS broth with 0.4%- 1% bile salt (Sodium taurochlate).

Procedure: LS broth was prepared with 0.4%, 0.6%, 0.8% and 1% sodium taurochlate and dispended to test tubes in appropriate proportion. The tubes were inoculated by loop inoculation process and then incubated at 37 ºC for 24 h along with a control. The results were analyzed after 24 hours of incubation spectrophotometrically at 600nm.

Test for Anti-microbial Activity: Probiotic strains have been demonstrated to inhibit the growth of many enteric pathogens. There are several metabolic compounds produced by probiotic bacteria such as organic acids, fatty acids, hydrogen peroxide, diacetyl, bacteriocin and proteinaceous substances, which have antimicrobial effect.

Medium: Soft nutrient agar with 6g/L of agar (pH-7.2).

Procedure: Soft nutrient agar was prepared with 6g/L of agar (pH-7.2), the agar medium was cooled to 45 ºC. The medium was then poured into four sets of sterile petri plates aseptically and allowed to set. After cooling, 100µL of inoculums of three different pathogenic strains were pipetted out separately with the help of micropipettes are poured aseptically to the center of the agar plates of each set. With the help of sterile paper discs (about 8mm thick) were placed aseptically on the center of the inoculation plates. These paper discs were then inoculation at 37 ºC for 24 h. After 24 h of inoculation the plates were observed for zone of inhibition. The four pathogenic strains were *Salmonella typhi. E. coli*, *Staphylococcus aureus* and *Candida albicans* and the test isolate were *lactobacillus* species.

Antibiotic Susceptibility Test: To test the sensitivity of bacteria to different antibiotics disk diffusion method [14] was performed using dodeca discs (Hi Media, India) of different antibiotics like Ciprofloxian, Erythromycin, Tetracycline, Penicillin, Ampicillin, Streptomycin, Vancomycin, Azithromycin, Chloramphenicol, Gentamycin, Neomycin and Oxacillin. The active cultures were poured into the plate with a base layer on Muller Hinton agar plates. The inhibition zone diameters were recorded after 24 h of incubation at 37 °C.

Statistical Analysis: Statistical analyses were done using SPSS 14.0 software (SPSS Inc.; Chicago, IL, USA). Significant differences among treatments were tested by ANOVA developed by Ronald Fisher in 1923 with a level of significance at $\alpha = 0.05$. Data were expressed as Mean Values ± Standard Deviation (SD). All experiments were performed in duplicate and repeated three times.

RESULTS AND DISCUSSION:

Morphological and Colony Characteristics: Morphological and colony characteristics of the isolated bacterial culture from yogurt samples were observed carried out. All together four types of different bacterial colonies with distinct morphological characters were isolated.

Identification by Molecular Biology Method: Out of 40 stains, 34 were found Gram-positive, rod-shaped, and catalase-negative using the conventional method of identification, whereas by using molecular biology technique, isolated stains were found to be *Lactobacillus casei* (28 strains), *Lactobacillus salivarius* (2 strains), *Lactobacillus plantarum* (3 strains) and *Lactobacillus* buchneri (1 strain).

Result of Agitation: The growth in the incubator shaker was less than compared to the ordinary incubator; thus, it confirms that the isolates are all facultative anaerobes. The spectrophotometric analysis was shown in graphical representation **Table 1**.

Acid Tolerance: For a potential probiotic bacteria to work efficiently, it must have the capacity to survive in the gastrointestinal tract. In many studies, the acid resistance for Lactobacillus at pH 2.0 and 3.0 was studied with MRS broth [14]. In our study, 34 strains of Lactobacillus were studied for acid tolerance in this study. Acidic pH affects the growth of *Lactobacillus* a lot. It was found that 24 strains had good resistance to low pH, as shown in **Table 3**. It shows that the survival rate of 10 strains after 2 h incubation compared with the control. *Lactobacillus* 1004, 2014, 3026, 3030, 3035, 4036, 4038, 4039, 1008, and 4037 were found to be ≥ 90% at pH 3.0 and *Lactobacillus strains* 1004, 1024, 3026, 3030, 3032, 3035, 4036, 5039 and 1008 were found to be ≥ 90% at pH 2.0. Strain 3032 was the most acid-tolerant at pH 2.0, with survival rates of 98%. Out of 24 strains, eleven strains showed a survival rate ≥ 90% at pH, as shown in **Table 1**.

TABLE 1: RESULT OF VIABILITY (LOG CFU/mL) AT DIFFERENT pH, SURVIVAL PERCENTAGE AND AGITATION AT 600 nm OF DIFFERENT *LACTOBACILLI* STRAINS

Isolates	Absorbency at 600nm (without agitation)	Absorbency at 600nm (with agitation)	pH 6.2* Viable count (log CFU/mL)	pH 3.0 Viable count (log CFU/mL)	% survival (%)	pH 2.0 Viable count (log CFU/mL)	% survival (%)
L. casei 1004	0.056	0.022	9.12±0.01	8.72±0.04	95	8.70±0.02	95
L. casei 1006	0.055	0.024	8.91±0.08	7.61±0.10	85	7.11±0.07	79
L. casei 1007	0.038	0.03	9.79±0.02	8.09±0.02	82	8.01±0.09	82
L. casei 1010	0.042	0.051	8.92±0.10	7.62±0.09	85	7.50±0.04	84
L. casei 1021	0.039	0.024	8.61±0.12	7.51±0.10	87	7.22±0.40	83
L. casei 2012	0.058	0.045	9.06±0.04	7.86±0.24	86	7.36±0.32	82
L. casei 2014	0.061	0.038	9.00±0.22	8.10±0.24	90	8.90±0.28	91
L. casei 2017	0.044	0.052	7.92±0.07	6.52±0.07	82	6.12±0.70	77
L. casei 2019	0.065	0.033	8.78±0.33	7.86±0.33	89	7.36±0.41	84
L. casei 2022	0.084	0.053	8.45±0.09	7.95±0.03	92	7.25±0.08	85
L. casei 3015	0.072	0.047	8.58±0.19	7.53±0.10	88	7.43±0.21	87
L. casei 3026	0.083	0.026	8.82±0.11	8.21±0.10	93	8.01±0.29	92
L. casei 3030	0.047	0.027	8.66±0.04	8.22±0.15	95	8.09±0.34	93
L. casei 3033	0.088	0.051	8.75±0.02	7.50±0.12	86	7.12±0.18	81
L. casei 3035	0.053	0.031	8.96±0.23	8.16±0.23	92	8.94±0.12	91
L. casei 4036	0.064	0.059	9.21±0.45	8.91±0.45	96	8.34±0.40	90
L. casei 4038	0.031	0.02	7.54±0.12	6.94±0.12	93	6.44±0.22	85
L. casei 4039	0.049	0.021	7.49±0.13	6.99±0.13	92	6.89±0.23	91
L. casei 4040	0.069	0.039	8.37±0.35	6.17±0.25	73	5.98±0.20	71
Lactobacillus salivarius 1008	0.059	0.023	9.07±0.31	8.77±0.21	96	8.47±0.09	92
Lactobacillus salivarius 3031	0.087	0.061	7.46±0.04	6.26±0.14	84	5.96±0.21	78
Lactobacillus plantarum 2011	0.066	0.041	8.90±0.29	7.70±0.20	86	7.20±0.11	80
Lactobacillus plantarum 3032	0.082	0.066	7.76±0.45	6.60±1.25	86	8.40±1.01	98
Lactobacillus buchneri 4037	0.037	0.024	7.26±0.02	6.96±0.12	94	5.96±0.07	81

Bile Salt Tolerance: Bile salt concentration 0.4% - 1% show different degrees of inhibition on 24 strains. The result obtained was analyzed using Gilliland *et al*., (1984) [15].

Two strains Lactobacillus 3032 and 1004 were considered to be resistant (d≤ 15 min); Out of 24 strains, 4 strains were found to be tolerant strains (1008, 1024, 3030 and 3035; 15 < d ≤ 40 min); 11 strains (Lactobacillus 1007, 2011, 2012, 2014, 2022, 3026, 4036, 4038, 4039, 4037 and 5039; 40<d<60) were found to be weakly tolerant strains as shown in **Fig. 1**.

Antibacterial Activity: The antimicrobial activity against four pathogenic strains were examined on 10 Lactobacilli strains that were found good in screening tests. Lactobacillus strains 1008, 1024, 3030, 3035, 2022, 3026, 4036, 4038, and 5039 showed an inhibitory effect against *E. coli* GU128 with an inhibition zone 12.60-24.50 mm in diameter. All the 24 strains showed positive results against all the four pathogens (with inhibition zones 12.60-30.50 mm in diameter). *Lactobacillus plantarum* 3032 shows zone of inhibition from 24.60- 30.50 mm in diameter **Table 2**.

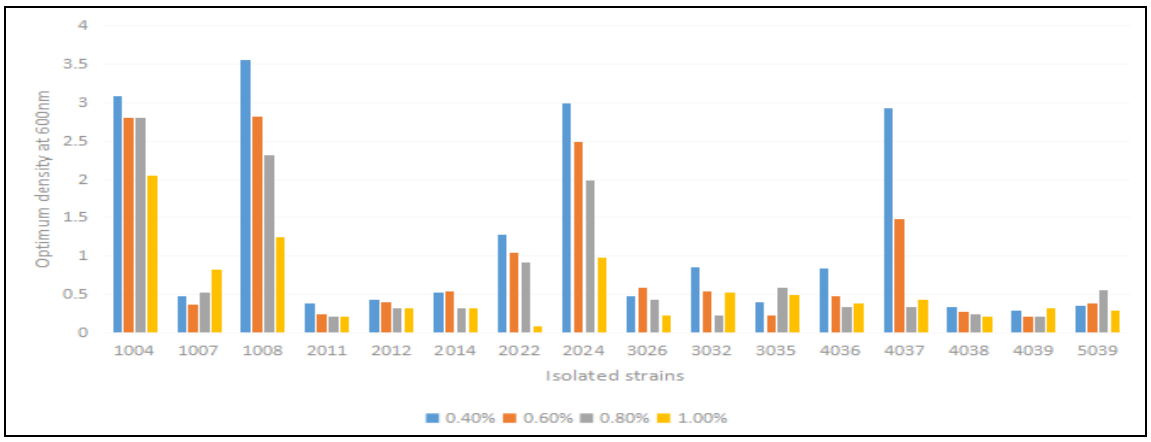

FIG. 1: BILE ACID TOLERANCE (0.4% TO 1.0%) OF DIFFERENT STRAINS AT OPTIMUM DENSITY OF 600 nm

TABLE 2: ANTIMICROBIAL ACTIVITY OF DIFFERENT LACTOBACILLI STRAIN AGAINST DIFFERENT *E. COLI, STAPHYLOCOCCUS AUREUS, SALMONELLA TYPHI* AND *CANDIDA ALBICANS*

S. no.	Strains	*Salmonella typhi*	*Staphylococcus aureus*	*E. coli*	*Candida albicans*
1	*L. casei*1004	++	+++	++	++++
2	*L. casei*1006	++	+++	+++	++++
3	*L. casei*1007	++	+++	+++	++++
4	*L. casei*1010	++	+++	++	+++
5	*L. casei*1021	+	+++	++	++++
6	*L. casei*2012	+++	++++	++	+++
7	*L. casei*2014	+++	++++	++	+++
8	*L. casei*2017	+++	++++	++	+++
9	*L. casei*2019	+++	++++	++	+++
10	*L. casei*2022	++	++	+++	+++
11	*L. casei*3015	++++	++++	++++	+++
12	*L. casei*3026	++	++	+++	+++
13	*L. casei*3030	++++	+++	+++	++++
14	*L. casei*3033	+++	++++	++++	+++
15	*L. casei*3035	++++	++	+++	++++
16	*L. casei*4036	++++	+	+++	+++
17	*L. casei*4038	++++	+++	+++	++++
18	*L. casei*4039	+++	++	++	+++
19	*L. casei*4040	+++	+++	++++	+++
20	*Lactobacillus salivarius* 1008	++	+	+++	+++
21	*Lactobacillus salivarius* 3031	++++	++++	++	+++
22	*Lactobacillus plantarum* 2011	++++	++++	++	+++
23	*Lactobacillus plantarum* 3032	++++	++++	+++	+++
24	*Lactobacillus buchneri* 4037	++++	++	++	+++

*+, Diameter of inhibition zone: 8-12 mm; ++, 12 to 16mm; +++, 16 to 20mm; ++++, more than 20mm

Antibiotic Susceptibility Test: To study the resistance of different 20 lactobacilli strains against 12 different antibiotics susceptibility test was carried out and the result are shown in **Table 3**.

TABLE 3: ANTIBIOTIC SUSCEPTIBILITY TEST FOR LACTOBACILLUS ISOLATES AGAINST DIFFERENT ANTIBIOTICS

S. no.	Antibiotics used	No. of Resistant strains	No. of Sensitive strains	No. of Intermediate strains
1	Ciprofloxacin	7 (35%)	12 (60%)	-
2	Erythromycin	5 (25%)	15 (75%)	-
3	Tetracycline	8 (40%)	10 (50%)	-
4	Penicillin	9 (45%)	11 (55%)	-
5	Ampicillin	17(85%)	2 ((10%)	1 (5%)
6	Streptomycin	5(25%)	16(66%)	3 (15%)
7	Vancomycin	9 (45%)	6 (30%)	-
8	Azithromycin	7 (35%)	9 (45%)	2 (10%)
9	Chloramphenicol	6 (30%)	10 (50%)	-
10	Gentamycin	5 (25%)	13 (65%)	1 (5%)
11	Neomycin	8 (40%)	6 (30%)	-
12	Oxacillin	15 (75%)	-	2 (10%)

Cell Surface Hydrophobicity: The cell surface hydrophobicity of 24 different strains of lactobacilli were studied. The hydrophobicity of these strains was found significant ($p<0.05$; $p<0.01$), and the percentage of hydrophobicity was found between 10% to 97%. It was found that the hydrophobicity of five strains was more than 60%: 3031 (96%), 3015(92%), 4038 (87%), 4040 (73%), and 2019 (68%). Result as shown in **Table 4**.

TABLE 4: PERCENTAGE OF CELL SURFACE HYDROPHOBICITY OF SEVEN STRAINS WITH HIGH HYDROPHOBICITY

Selected strains	Cell-surface hydrophobicity (%)
Lactobacillus salivarius 3031	96%
*L. casei*3015	92%
*L. casei*4038	87%
*L. casei*4040	73%
*L. casei*2019	68%
Lactobacillus salivarius 3031	58%
*L. casei*3033	54%

The bacterial species isolated from curd samples were subjected to general bacteriological isolation techniques. Among the biochemical test, the important results showed by the isolates were catalase-negative, lactic acid production from glucose, and heavy growth on Tomato juice agar. All the isolates give pigmentation, which ranges from orange to brown color. These results have brought the isolates under Lactobacillus genus. 16Sr RNA gene sequence analysis technique was used to identify the species. The growth of isolates on MRS agar plates at pH 6.5 also confirms the lactobacillus sp. [16]. For probiotic bacteria to work efficiently, it must have the capacity to survive in the gastrointestinal tract. In many studies, the acid resistance for Lactobacillus at pH 2.0 and 3.0 was studied with MRS broth. In a study on ewe's milk, the strains showed favorable resistance at pH 2.0 of 72% from 50 lactobacillus strains [17].

In the present study, more than 40% strains showed the survival rate of at pH 3.0, whereas 37.5% of the strains showed the survival rate at pH 2.0 **Table 1**. In the human gut, NaCl is an inhibitory substance that can inhibit the growth of the microbe so, the isolates were grown in a medium with NaCl concentration ranging between 0.4-1% bile concentration, and it was found that all 24 strains were able to survive at 0.5% bile salt, but only 6 were found to be tolerant to all concentrations between 0.4-1% whereas the rest were found to be weakly tolerant **Fig. 1**. All the bacterial strains to be used for probiotics must not have any antibiotic-resistant gene present [18]; for this reason, all the strains were assessed for antibiotic resistance test. The result, as shown in **Table 4,** all the strains were found susceptible to all antibiotics tested except oxacillin and ampicillin, which shows the strains are safe to be used for probiotic potential. The most important use of probiotic bacteria is to protect the organism from pathogenic bacteria within the intestinal eco-system [19].

In the present study, all 24 strains showed different levels of inhibition against *E. coli*, *Staphylococcus aureus, Salmonella typhi,* and *Candida albicans*. A similar result was reported in many studies against gram-positive and gram-negative bacteria [20]. In comparison to a study by Osuntoki *et al*., [21] where the Lactobacillus spp. showed antimicrobial activity only against some clinically important pathogens like *E. coli* (4.2 mm), *Salmonella typhi* (4.3 mm), and *Listeria monocytogenes* (5.0 mm).

Cell surface hydrophobicity is an important character of probiotic bacteria to adhere to the intestinal mucus. The results of the present study **Table 4** shows that the lactobacillus strains isolated from the curd sample of Majmaah have very high cell surface hydrophobicity 96%, which is higher than the study by Zhang *et al*., [22] which was reported on 92.15%.

CONCLUSION: In conclusion, out of 40 strains, 24 strains were selected as appropriate probiotic potential strains which can be used for promoting hosts intestinal health and to maintain healthy natural micro-flora during antibiotic treatment. Also need for in vivo study is there to verify the effectiveness of selected strains.

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