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In vitro characterization of *Enterococcus faecium* strain LCM08 as a probiotic.

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Abstract

Background: Under the present situations, where health improvement has become the major concern, food supplements, augmenting health is a matter of interest. Presently, the concept of "functional foods" possessing medicinal attributes has been reborn as probiotics. Acid-tolerant and Gram-positive Lactic acid bacteria (LAB), being the major class of probiotics, possess health-promoting characteristics, provide better health with a robust immune system, and treat various digestive and neurological disorders. Hence, the aim of the present study was the isolation and identification of LAB.

Methodology: The LAB were isolated from various sources, including food products and clinical samples. *Enterococcus faecium* LCM08 being the most potent isolate tested for probiotic traits, including viability under acidic conditions, biological concentrations of bile salts and enzymes.

Results: A total of fifty-four strains were isolated, of which 7.2% showed broad-spectrum inhibition against the tested pathogens. Amongst them, *Enterococcus faecium* LCM08 owing to its broad-spectrum antagonism, when tested for its ability to withstand acidic conditions, displayed 63.58% viability and 72.19% survivability in the presence of bile salts. Moreover, strain sustained in the presence of digestive enzymes including protease (94.65%), trypsin (93.57%) and pancreatin (75.0%) as well as exhibited growth at temperatures ranging between 25˚C and 30˚C where the maximum viable counts recorded as 95% and 96.4%. The strain also produced antimicrobial metabolites at neutral pH. However, elevated temperatures (45˚C-50˚C) inhibited the production of bacteriocin-like inhibitory substances.

Conclusion: The preliminary investigations revealed that *Enterococcus faecium* LCM08 possesses the key attributes pre-requisite for a probiotic candidate.

Keywords

Bacteriocin Like Inhibitory Substances, Bile Salt, *Enterococcus Faecium*, Lactic Acid Bacteria, Probiotics.

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Introduction

Nowadays, people are more concerned about the food they consume; therefore, preference is given to the nutritional ingredients rather than the flavour of the food products[1]. Functional food products containing probiotic bacteria are not less than 10^6 CFU/gm consumed to improve the intestinal microflora[2,3]. In 2002, World Health Organization (WHO)/ Food and Agriculture Organization (FAO) defined probiotics as "live microorganisms which when administered in adequate amount confer health benefits[4]. LAB are Gram-positive cocci or bacilli, facultative anaerobes[5], found in a large number of ecological niches like dairy products[6,7], fruits and vegetables[8], meat sources[9], fermented foods[10], a gut of human and animals[11].

Microbial contamination in food is a real challenge to the modernized world, resulting in a scarcity of food. Bifidobacteria and Lactobacillus assist in milk fermentation, thus prevent bacterial spoilage and influence the taste of milk[2]. Promising probiotic strains belong to the genera of Bifidobacteria and Lactobacillus. Other strains include Enterococcus, Bacillus, Streptococcus and Pediococcus[12].

The essential pre-requisite parameters that characterize LAB as probiotics include survivability under the low gut pH, tolerance to elevated concentrations of bile salts in the small intestine, and must not be the carrier of any antibiotic-resistant gene[11,13]. Moreover, a probiotic should be able to assimilate cholesterol as well as remain viable during food processing. They are well-known for being highly effective immunostimulant, immunosuppressant, antimutagenic and anticarcinogenic agents[14]. They maintain the balance between pathogenic and healthy bacteria present in the gastric habitat and provide defence in Helicobacter pylori infection, Clostridium difficile[15] and Campylobacter jejuni, well as in traveller's diarrhea and bowel syndrome[16]. Other important health attributes include alleviation of β-galactosidase deficiency[17], hypocholesterolemic[18] and antidiabetic potential[19], elevating IgA level, protecting mucosal lining from food allergens, reducing the level of IgE in atopic eczema, preventing allergic rhinitis[20] and counteracting vaginal infections.

Hence, our focus was to explore the probiotic traits of LAB, including survival in acidic medium, bile salts tolerance and enzymatic viability under an in-vitro environment.

Methodology

Sample collection

Samples from indigenous sources such as probiotic sachet (Lactibiane), dairy products, meat and vegetables were gathered from various shops in Karachi, Pakistan. Clinical isolates that included vaginal swabs and infant feces were collected from Dar-ul-Sehat hospital Karachi with the consent of female participants before sample collection.

Isolation of Lactic acid bacteria

Isolation of Lactic acid bacteria was done on de Man's Rogosa Sharpe Agar (MRS) (Oxoid) by Spread plate count method. Kept plates at 37°C for 24 hours, and morphological identification of colonies made. A total of fifty-four Gram-positive, catalase-negative cocci or bacilli were selected for further studies.

Screening of LAB for bacteriocin like inhibitory substances (BLIS)

Isolated LAB strains were screened for their antimicrobial potential against Gram-positive pathogens, including *Staphylococcus aureus*, *Corynebacterium xerosis, Bacillus subtilis, Streptococcus faecalis* and Gram-negative pathogens such as *Escherichia coli, Salmonella typhi, Shigella dysenteriae, Klebsiella pneumonia* and *Pseudomonas aeruginosa.* All isolates were obtained from the Department of Microbiology, University of Karachi. Centrifugation of overnight culture of LAB was done at 3000 rpm for 15 minutes and neutralized using 1N NaOH. Indicator strains (0.5 Macfarland index) were seeded in Nutrient soft agar (0.75%, Oxoid) and overlaid on pre-poured MRS agar. Wells were cut after solidification and filled with 200 µL of supernatant. Plates were refrigerated for diffusion at 4°C for 45 minutes and then kept at 37°C for 24 hours. Inhibitory diameters around the holes were measure in mm[21]. Potent

LAB strains were preserved by supplementing MRS broth with 20% glycerol (Scharlau) and stored at -20°C[6].

Sequence analysis of LCM08

a. Extraction of DNA

Genomic DNA of LCM08 was extracted by suspending 3-4 colonies of LCM08 in 100 µL of nuclease-free water and boiled for 5 minutes at 100°C. Subsequently, the obtained cell lysate was frozen at -20°C and centrifuged at 13,000 rpm for 15 minutes. Finally, the supernatant was separate for DNA amplification[22].

b. Amplification of genomic DNA

Extracted DNA amplified using universal 16S rRNA gene sequence in the volume of 25 µL including 1 µL (100 ng/µL) of template DNA, 1 µL of each 10Mm of 27F' 5'AGAGTTTGATCCTGGCTCAG 3' and 1492 R' 5'GGTTACCTTGTTACGACTT 3' and 12.5 µL Go Taq Green master mix (Promega). Thermocycler (Applied Biosystems, 2720) program was set as initial denaturation for template DNA at 96°C for 10 minutes then denaturation at 94°C for 30 seconds (30 cycles), 20 cycles for annealing at 60°C and 1-minute extension at 72°C and final holding of 10 minutes at 72°C. Electrophoresis of PCR product was done using 1% agarose (Sigma) containing ethidium bromide (Serva) 0.5 µg/mL concentration at 50 mV for 20 minutes[23]. Bands visualized using a UV transilluminator, and a DNA ladder of 100 plus bps was used for the comparison of DNA band with amplified PCR product. Purification of amplicon and sequencing was done from BGI using a forward primer.

Conditions simulating gastric transient

a. Tolerance to varied temperatures

Enterococcus faecium strain LCM08 incubated at different temperatures, i.e., 25°C, 30°C, 37°C, 45°C and 50°C. Cell viability was enumerated by Miles and Misra[24], while absorbance was recorded at 650 nm. Furthermore, antagonism of *Enterococcus faecium* strain LCM08 against *Shigella dysenteriae* was also assessed.

b. Tolerance to bile salts

Broths (MRS) incorporated with 0.5%, 1%, and 1.5% of bile salts (ox gall, Sigma) were inoculated with 10^8 CFU/mL *Enterococcus faecium* strain LCM08 at 37°C. Aliquots were drawn for enumeration of viable cell count after a regular interval of 1 hour for 4 hours depicting the time of food digestion. Control MRS Broths without bile salts were also run simultaneously[25].

c. Tolerance to digestive enzymes

Invitro gastric enzymes like protease (3 mg/mL, pH 7; MP Biomedical), trypsin (3 mg/mL, pH 7; Sigma-Aldrich, Germany) and pancreatin (3 mg/mL, pH 8; Fisher scientific) were prepared in PBS buffer and were filter sterilized using Millipore filters. The final volume was adjusted to 2.0 mL containing 1.2 mL PBS, 0.6 mL enzyme solution and 0.2 mL viable cells of *Enterococcus faecium* strain LCM08 (absorbance 2.1), while in control, volume was made up with PBS. After every 1 hour, 0.1 mL of sample was drawn for 4 hours and plated for determination of viable count[26].

d. Tolerance to low pH

Washed cells of *Enterococcus faecium* strain LCM08 inoculated in MRS broth (30 mL), and pH was adjusted to 2, 3, 4, 5 and 6.4 (control). Broths were incubated at 37°C for 4 hours, and samples were obtained after every 1 hour to determine viability. All experiments were run in triplicate for the determination of standard deviation.

The survival percentage of LAB during stress condition can be calculated by given formula[27]:

$$\text{Survival } \% = \log [\text{CFU } (N_1)/(N_o)x100]$$

Where,

N1 = Survival of LAB in stress condition

N0 = Survival of LAB without stress condition

Statistical analysis

Analysis of data was done by using SPSS (IBM SPSS 20). One-way ANOVA and Post hoc Tukey's test were applied, and results were expressed as mean ± standard deviation and a p-value<0.05 was considered as significant.

Results

Isolation and antimicrobial activity of LAB strains

A total of fifty-four strains were isolated from various sources. Amongst them, 53.7% strains were collected from dairy products, 18.5%, 9.3% and 1.8% strain from meat, vegetables and probiotic sachet (Lactibiane), respectively. Clinical samples including vaginal swabs and infant feces contributed 16.7% in making the library of isolates (Table 1). All of them exhibited morphological and biochemical resemblance to Lactic acid bacteria. Based on morphology, 70.37% and 29.62% were cocci and bacilli.

Table 1: Sources of LAB isolates.

Sources	Number of Isolates	Relative Percentage
Dairy products	29	53.7
Vegetables	5	9.3
Meat	10	18.5
Probiotic Sample	1	1.8
Clinical Samples	9	16.7

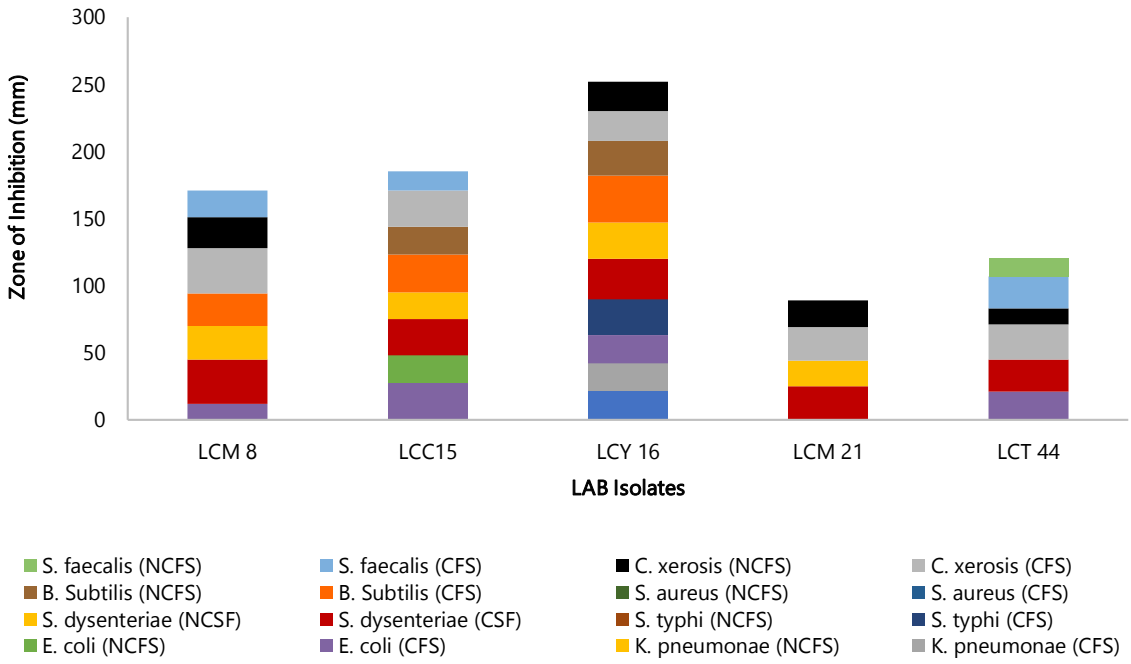

Figure 1: Antimicrobial potential of isolated LAB strains.

Nine out of 54 strains showed BLIS-like inhibitory effects towards pathogenic microorganisms. Strains that exhibited broad-spectrum are LCM08, LCC15, LCY16, LCM21 and LCT44, making a total of 7.4% (Figure 1). The most potent isolate is LCM08 that antagonized *Shigella dysenteriae* exhibiting a zone diameter of 33 mm and 25 mm in CFCS and NCFCS.

Molecular identification

Sequences received from BGI were submitted to the GenBank database for species identification. NCBI assigned accession number as MN636783, and the strain was named *Enterococcus faecium* strain LCM08. When cladogram was constructed, the identified strain showed 100% resemblance to *Enterococcus faecium* MF354917.

Invitro probiotic attributes

a. Tolerance to varied temperatures

At different temperatures, i.e., 25°C, 30°C, 37°C (control), 45°C and 50°C, newly isolated strain Enterococcus faecium strain LCM08 showed 95%, 96.4%, 83% and 79% viability, respectively. There was a significant difference between the growth of Enterococcus faecium strain LCM08 at different temperatures ($p<0.05$).

b. Tolerance to bile salts

Enterococcus faecium strain LCM08 displayed good survivability at different concentrations of bile salts since viability in bile salts is an essential pre-requisite criterion for the probiotic candidate. In presence of 0.5% and 1% bile salts, 78.1% (5.278 ± 0.002 log CFU/mL) and 78.70% (5.165 ± 0.004 log CFU/mL) cells remained viable, while 70.9% (5.135 ± 0.003) cells survived at 1.5% bile salts after 4 hours of incubation ($p<0.05$) (Table 2).

Table 2: Survival percentage of Enterococcus faecium strain LCM08 in the presence of MRS broth supplemented with 0.5%, 1% and 1.5% bile salts.

Time (Hours)	Survival Percentage in the presence of Bile Salts		
	0.5%	1%	1.5%
0	96.0±0.05	94.4±1.00	94.1±0.10
1	96.0±0.08	94.2±1.53	98.0±0.07
2	96.3±0.09	91.9±2.31	84.0±0.05
3	94.1±0.04	90.7±2.08	81.0±0.04
4	78.2±0.02	78.7±1.53	75.0±0.04

Values are given as mean±SD.

c. Tolerance to digestive enzymes

Survivability of *Enterococcus faecium* strain LCM08 in the presence of protease, trypsin and pancreatin was recorded as 94.65%, 93.57% and 75.0%, respectively, after 4 hours (Table 3). At 4-hour a significant difference was observed between the viability of *Enterococcus faecium* LCM08 in medium with and without digestive enzymes ($p<0.05$).

Table 3: Survival percentage of *Enterococcus faecium* strain LCM08 in the presence of digestive enzymes (3 mg/mL) for 4 hours.

Time (Hours)	Survival Percentage in the presence of Enzymes (3 mg/mL)		
	Protease	Trypsin	Pancreatin
0	96.0±4.6	96.0±2.3	97.0±1.5
1	93.2±0.4	99.0±1.8	98.0±1.0
2	93.4± 0.6	99.0±0.5	84.0±0.7
3	94.2±0.6	98.0±2.1	81.0±0.1
4	94.6±0.3	93.6±0.9	75.0±1.1

Values are given as mean±SD.

d. Tolerance to low pH

In this experiment, survivability of *Enterococcus faecium* strain, LCM08, was monitored under the influence of low pH, i.e., from 2 to 5 and 6.4 as a control. At pH 3, 63.58% (4.522 log CFU/mL) of cells remained viable after 4 hours while at pH 4 and pH 5 viable count was 72% (5.204 log CFU/mL) and 99% (8.425 log CFU/mL) (Table 4). There was a significant difference between the viability of Enterococcus faecium strain LCM08 in control (pH 6.4 and test at pH 2, 3 and 4 ($p<0.05$).

Table 4: Survival percentage of *Enterococcus faecium* strain LCM08 at different pH for 4 hours.

Time (Hours)	pH			
	2	3	4	5
0	96.0±1.30	85.4±1.30	93.7±0.08	80.0±2.50
1	0.00±0.00	86.4±1.80	93.8±0.07	90.0±1.70
2	0.00±0.00	69.0±2.20	89.6±0.05	96.0±3.40
3	0.00±0.00	66.3±2.10	81.9±0.02	100.0±1.90
4	0.00±0.00	63.6±3.00	66.1±0.06	99.0±2.00

Values are given as mean±SD.

Discussion

Lactic acid bacteria are predominant microorganisms found in several ecological niches. They are fastidious organisms, therefore, possess complex nutritional requirements for growth and metabolism[28]. In this study majority of the isolates were collected from raw milk, and this correlates with the findings of Masalam, who succeeded in isolating 93 strains from milk origin[29]. In this study, all the Gram-positive cocci and bacilli were preliminarily identified as Lactic acid bacteria. Similarly, Yang et al., 2017 also found LAB having these morphological and biochemical characteristics[28]. In the present investigation, only 9.2% isolates (LCM08, LCC15, LCY16, LCM21 and LCT44) showed inhibition of Gram-positive and Gram-negative pathogens. Similar results were attained when 73 Lactobacillus strains were examined against Gram-positive, and Gram-negative pathogens and only 5.47% of the isolates showed a broad spectrum of activity[30].

Lactic acid bacteria are capable of inhibiting foodborne as well as gut pathogens, and this is due to the release of acids, hydrogen peroxides, fatty acids and bacteriocins. Bacteriocins are less effective against Gram-negative bacteria than Gram-positive pathogen because they possess an additional lipid layer that resists the antagonism[31]. Tolerance to the acidic environment of the gut and resistance to the elevated level of bile salt in the small intestine are the pre-requisite probiotic selection criteria. Hence, *Enterococcus faecium* strain LCM08 survived well at pH 3 for 4 hours and displayed viability of 64%. These results correlate with the survival of *Lactobacillus plantarum* and Lactococcus lactis at optimum acidic condition (pH 3) for 4 hours[27]. In contrast to this, another Lactobacillus strain demonstrated the maximum number of viability at pH 2 for 3 hours[17].

A probiotic must tolerate the physiologic concentration of bile salts, i.e., 0.15% - 0.3%, because bile salts target membrane protein and the lipid bilayer, causing cell lysis. Bile salts hydrolase causes the deconjugation of bile salts and liberates bile acids that aid the strain to survive in the intestine[32]. As the concentration of bile salts within the intestine ranges from 0.2% to 2%[27], *Enterococcus faecium* strain LCM08 exhibited significant viability to all bile salt concentrations demonstrating 70.90% viability at the highest bile salts concentration. Similarly, in another study, distinct viability was noticed in the concentration of 2% bile salts[27]. Moreover, in the presence of 0.3%, bile salts majority of *Lactobacillus plantarum* FH185 cells remained viable[17].

Enterococcus faecium strain LCM08 presented substantial survivability in digestive enzymes, which

correlated with the results reported by Balamurugan et al., 2014 where Lactobacillus species showed significant viability in the presence of pancreatin[33]. Another study suggested that a considerable number of Lactic acid bacteria survived in the presence of 2 g/L trypsin[34]. Pronounced viability of *Enterococcus faecium* strain LCM08 at 45°C and 50°C was recorded as the growth of bacteria at an elevated temperature is considered as a predominant feature which could be interpreted by an increase in the number of cells as well as by acid production[35].

Enterococcus responds to a high temperature by synthesizing heat shock proteins, and this differentiates Enterococcus from other species of Lactic acid bacteria, especially Lactococci[36]. Further studies are required to confirm the potential of the probiotic candidate based on genetic. This gives more understanding of LCM08 as a probiotic so it would be applied in food products.

Conclusion

Our results suggest that the newly identified *Enterococcus faecium* strain LCM08 fulfils essential probiotic eligibility criteria exhibiting viability under a simulated gastric environment. This strain could be used as a promising probiotic and may improve gut health when incorporated in an animal model for analyzing its gastric transient. *Enterococcus faecium* strain LCM08 isolated from milk also displayed significant antagonism against enteropathogens. Therefore, data from the present study would encourage people to consume dairy products because of the presence of healthy bacteria that improves their health status. Further studies will be focused on assessing the immunomodulatory properties of the selected probiotic candidate.

Conflicts of Interest

The authors have declared that no competing interests exist.

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