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Evaluation of Probiotic Properties and Antimicrobial Activity of *Enterococcus faecium* BM10 KY788342 and *Lactobacillus casei* GM10 KY794586

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Abstract: *Enterococcus faecium* BM10 and *Lactobacillus casei* GM10 isolated from buffalo milk and goat milk, respectively, and identified as Gram-positive, catalase negative, non-motile and non-sporing in nature. 16s rRNA sequencing was performed for molecular identification of isolates. Both isolates evaluated for probiotic properties; acid and bile salt tolerance and showed good survival index of acid and bile salt tolerance. Bacteriocins of *E. faecium* BM10 and *L. Case* GM10 showed a wide range of antimicrobial activity against pathogenic bacteria and fungi. Bacteriocins activity was eliminated after treatment with proteinase-K indicates their proteineous nature and not fluctuated by treatment with catalase. Bacteriocin activity of *L. casei* GM10 stable at all tested pH 2,3 and 7 but bacteriocin activity of *E. faecium* BM10 not stable at lowest pH 2. Moreover, bacteriocin activity of *E. faecium* BM10 was more sensitive at high temperature than *L. casei* GM10. Results of study revealed that both isolates proved as novel and potent probiotics as well as antimicrobial agents.

Key words: Probiotic, Bacteriocin, Antimicrobial, Acid and bile tolerance.

Introduction

The word 'probiotic' originates from Greek verbal 'pro bios' which means 'for life' contrasting to 'antibiotics' which means 'against life'. Probiotics have recently emerged as the most powerful food grade microbial agents comprise the ability to express numerous health promoting functions of considerable commercial value and therapeutic potential. These are live microorganisms, when administered in adequate amounts confer several health benefits on the host [1] which includes improving cholesterol assimilation [2], lactose tolerance [3], anti-inflammatory [4], anti-oxidative [5], anti-cancer [6], anti-diarrheal [7], anti-allergic [8], anti-diabetic [9], anti-hypertensive effects [10], controlling obesity [11] and also helps in mineral absorption [12]. Probiotics received generally-regarded-as-safe (GRAS) status, due to its safety profile. Lactic acid bacteria (LAB) also enhance the stability and nutritional value of food products by preventing the growth of pathogenic and spoilage microbes [13]. General mechanism of probiotics include inhibition of pathogens via compete for nutrients and adhesive sites [14]. LAB produces compound like bacteriocins which causes direct antagonism [15] and boost up the immune system [16]. The prerequisite conditions for a probiotic strain to establish in market are: must be compete with gut microflora, acid tolerant and bile salt tolerant as during transit from gut, these have to survive in high acidic environment and bile salt concentration and must have adherent potential. In the present study, the potent probiotic strains isolated from milk and identified on the basis of morphological, physiological and molecular methods. Isolates evaluated for probiotic potential as well as antibacterial and antifungal activities against pathogenic strains. Effect of various factors: enzymes,

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surfactants, pH and temperature on antimicrobial activity of bacteriocins of both isolates also examined for characterization.

Materials and methods

Bacterial isolation and identification

Lactic Acid Bacteria (LAB) were isolated by 10 fold dilution method. 1 ml of sample mixed with 10 ml 0.1 % peptone salt solution, stirred well and poured on de Man Rogosa Sharpe (MRS) medium plates. Plates were incubated at 37°C for 24 h under anaerobic condition. After incubation, colonies were picked randomly and streaked on MRS plates for pure isolates. Pure isolates were maintained in MRS broth and stored in 20 % glycerol at -20°C. LAB isolates were examined for gram staining, catalase test, motility test and spore test. The molecular identification of *Enterococcus faecium* BM10 *and Lactobacillus casei* GM10 were carried out by PCR amplification and 16s rRNA gene sequencing.

Acid tolerance and bile salt tolerance

LAB were grown for 6 h in MRS broth at 37°C. An aliquot of 1ml of the 6 h old culture was inoculated into 100 ml MRS broth and pH was adjusted to 2, 3 or 7 using 1N HCL or NaOH. Bacterial growth was monitored by determination of optical density at 620 nm after incubation of 6 and 24 h at 37°C. Surviving percentage was calculated according to Sieladie *et al.*[17].

A modified method given by Pereira and Gibson [18] was used for estimation of bile salt tolerance. LAB isolate was grown for 6h in MRS broth at 37°C. An aliquot of 1ml of the 6 h old culture was inoculated into 100 ml MRS broth with 0.2 or 0.4 % (w/v) bile salts (Sigma, USA). Bacterial growth was monitored by determination of optical density at 650nm after 6 and 24 h at 37°C. The percent difference between the variation of optical density (OD) of culture without bile salts (ΔOD0 % BS) and 0.2 or 0.4% bile salts (ΔOD0.2 or 0.4% BS) would give a survival index of isolates.

Preparation of partial purified bacteriocin

10 µl LAB culture was inoculated in 150 ml MRS broth and incubated at 37°C in water-bath for 3 days. Then cell free supernatant (CFS) was prepared by centrifugation (10,000 rpm for 20 min, at 4°C) and pH was adjusted to 7 by 1M NaOH to exclude antimicrobial effect of organic acid. The culture supernatant subjected to ultrafiltration using a 10 kDa cutoff membrane cartridge filter, since the bacteriocin was assumed to be below this molecular mass. The proteinaceous substance in CFS was precipitated with 80 % of saturated ammonium sulphate and kept overnight at 4°C. The precipitated protein was collected by centrifugation (10,000 g, 20 min, 4°C) and resuspended in a minimal quantity of 10 mM phosphate buffer (pH 6.0) and dialyzed (18 h, 4°C) in the same buffer using 1 kDa cut off pore size dialysis membrane (Himedia, LA387). The dialyzed samples were collected and studied for characterization.

Antibacterial and antifungal assay

Antibacterial activity of the partial purified bacteriocin of isolates were examined against Gram-positive pathogenic bacteria: *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 740), *Enterobacter aerogenes* (MTCC 7661) and Gram-negative pathogenic bacteria: *Salmonella enterica* (MTCC 735), *Klebsiella pneumonia* (MTCC 432), *Escherichia coli* (MTCC 443), *Pseudomonas aeruginosa* (MTCC 424), *Clostridium perfringens* (MTCC 450), *Proteus vulgaris* (MTCC 1771) and *Vibrio cholerae* (MTCC 3906) using agar well diffusion method. Test (indicator) microorganisms were grown in a nutrient broth at 37°C for 24 h. Streptomycin was used as standard.

Fungal strains of *Aspergillus niger* (MTCC 1881), *Fusarium oxysporum* (MTCC 284), *Rhizopus stolonifer* (MTCC 2591), *Penicillium chrysogenum* (MTCC 160) and *Candida albicans* (MTCC 7315) were grown in potato dextrose broth medium at 25°C for 48 h. A 10 µl fungal suspensions (~10^5 spores/ml) swabbed on PDA plates and antifungal activity of bacteriocin was determined by agar well diffusion method. Gentamycin was used as standard.

Effect of enzymes, surfactants, pH and temperature on bacteriocin activity

The enzymes (Trypsin, catalase, lysozyme, α-amylase and proteinase-K) were prepared in 50 mM phosphate buffer (pH 6.5) at a final concen-

tration of 1 mg/ml and Proteinase K was prepared from 1N NaOH (pH 6.5). The bacteriocin (500 µl) incubated with enzyme preparations for 1 hr at 30°C.

The various surfactants such as non-ionic (Tween 80 and Tween 20), anionic-sodium dodecyl sulphate (SDS), and ethylene-diamine tetra acetic acid (EDTA) at 1 % final concentration were used to study their effect on bacteriocin activity. These preparations were incubated for 60 min at 30°C.

To determine the effect of pH on bacteriocin activity, the pH was adjusted to 2, 3 and 7 using 2N HCl or 2N NaOH and MRS broth with same volume used as control. Samples were incubated for 2 h at 30°C.

Thermo-stability of bacteriocins of isolates was checked by heating at various temperatures such as 40°, 75°, 100° and 121°C for 90, 60, 30 and 10 min, respectively.

After all treatments, the residual activity was examined using the agar well diffusion method against indicator pathogenic strain: *Escherichia coli* (MTCC 443) and compared with the activity of the corresponding controls. All the experiments were conducted in triplicates.

Statistical analysis

Values were expressed as the mean ± S.D., n=3 and statistical analysis were carried out employing one-way ANOVA (Completely randomized design) by using Graph pad prism 7 (Inc., San Diego, CA, USA). Differences between the data were considered significant at $p<0.05$.

Results

Isolation and identification of LAB strains

Enterococcus faecium BM10 and *Lactobacillus casei* GM10 isolated from buffalo milk and goat milk respectively. Isolates were gram positive, catalase negative, non-sporing and non-motile in nature. On the basis of 16s rRNA gene sequence similarities, the isolated strains BM10 and GM10 identified as *Enterococcus faecium* and *Lactobacillus casei* and deposited in the gene bank database under accession number KY788342 and KY794586.

Acid tolerance and bile salt tolerance

The survival (%) of *E. faecium* BM10 and *L. casei* GM10 were 59.11±0.44 and 54.26±0.51 respectively, after 6 h at pH 2 although isolates did not survive at pH 2 after 24 h as the survival percentages were below 50 % (46.76±0.88 and 45.84±1.02 respectively). The survival percentage (%) of *E. faecium* BM10 and *L. casei* GM10 were 85.40±0.75 and 81.54±0.60 at pH 3 after 6 h (Fig. 1) and 71.54±0.98 and 69.43±0.80 respec-

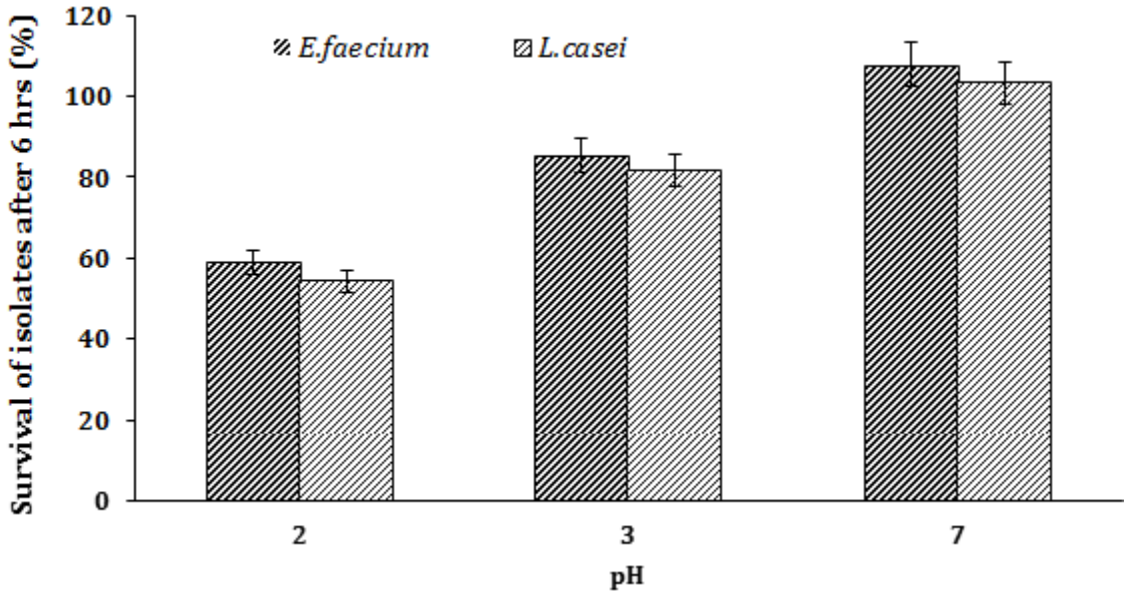

Fig. 1. Survival percentages (%) of *E. faecium* and *L. casei* after incubation of 6 h at different pH

tively after 24 h (Fig. 2). Thus, both isolates showed very good tolerance at pH 2 and 3. The survival percentage (%) of *E. faecium* BM10 and *L. casei* GM10 were increased to 107.79±0.97 and 107.21±0.68 at pH 7 after 6 h and 120.5±1.33 and 112.2±1.82 respectively after 24 h due to optimum pH growth conditions.

LAB isolates were grown in MRS broth containing 0, 0.2 and 0.4 % bile salt (BS) concentrations. After incubation of 6 h at 0.4 % BS, the survival percentage (%) of *E. faecium* BM10 and *L. casei* GM10 were 61.47±0.48 and 58.5±0.55, respectively, although after 24 h at the same concentration, the survival percentage (%) decreased to 45.31±0.86 and 45.90±0.80. The decrease in survival percentage (%) showed the intolerance ability of LAB isolates at higher concentration of BS, therefore the viability of isolates also decreased. At 0.2 % BS, the survival percentage (%) of *E. faecium* BM10 and *L. casei* GM10 were 71.4±0.51 and 69.3±0.68, respectively, after incubation of 6 h, the survival percentage (%) were decreased to 56.04±0.99 and 57.11±1.03 after incubation of 24 h. Survival percentages (%) were not decreased below 50 %, showed very good tolerance to bile salt concentration according to classification criteria. At 0 % BS, the survival rate of *E. faecium* BM10 and *L. casei* GM10 increased to 98.1±0.70 and 96±0.82 respectively, after 6 h of incubation (Fig. 3). The survival percentage (%) of *E. faecium* BM10 and *L. casei* GM10 increased to 110±0.75 and 115.1±0.95 respectively at the same concentration after 24 h of incubation (Fig. 4).

Antimicrobial activity

Antimicrobial activity of partially purified bacteriocins of *E. faecium* BM10 and *L. casei* GM10 were checked against five Gram-positive bacteria, seven Gram-negative bacteria and five fungal pathogenic strains. Both isolates showed a different antimicrobial spectrum against pathogenic strains. *E. faecium* BM10 exhibited maximum inhibition zone against *E.coli* (>15 mm) whereas *L. casei* GM10 exhibited maximum inhibition zone against *S. aureus* (>15 mm). *E. faecium* BM10 exert inhibiting activity against all tested Gram-positive bacteria, including Lactobacilli strains and also against Gram-negative bacteria: *S. enterica*, *E. coli*, *P. aeruginosa*, *V. cholarae*; no inhibition zone was recorded against *K. pneumonia* and *P. vulgaris*. *L. casei* GM10 exert strong inhibitory activity (>15 mm) against *S. aureus*, *S. enterica*, and *E. coli;* weak (zone of inhibition: <5 mm) against *E. aerogenes, K. pneumonia, P. vulgaris* and *V. cholerae* and no

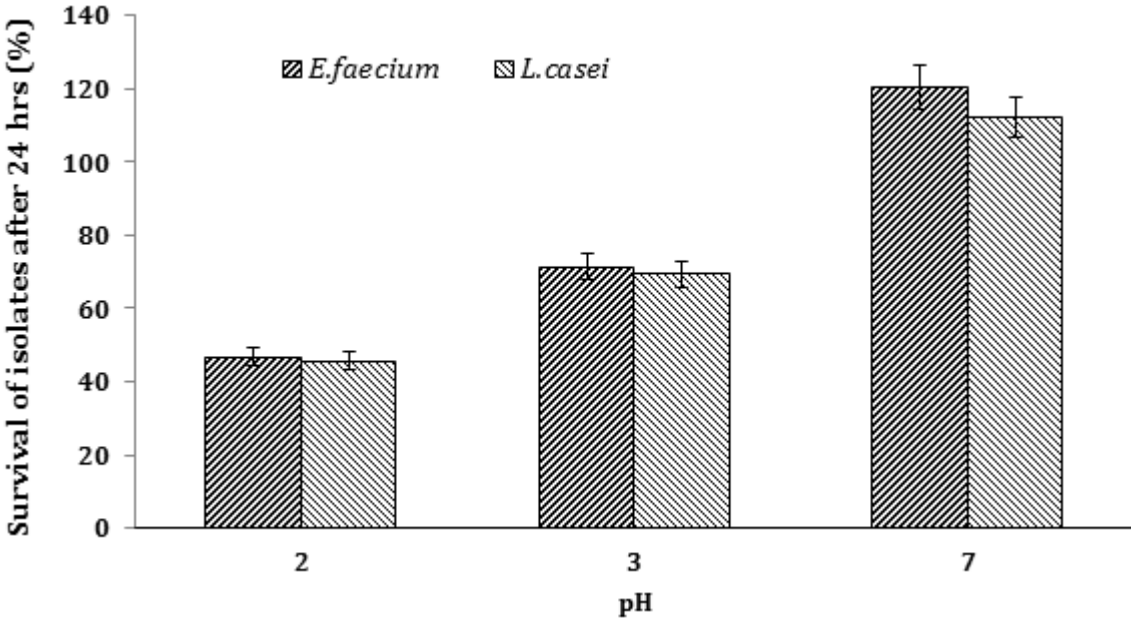

Fig. 2. Survival percentages (%) of *E. faecium* and *L. casei* after incubation of 24 h at different pH.

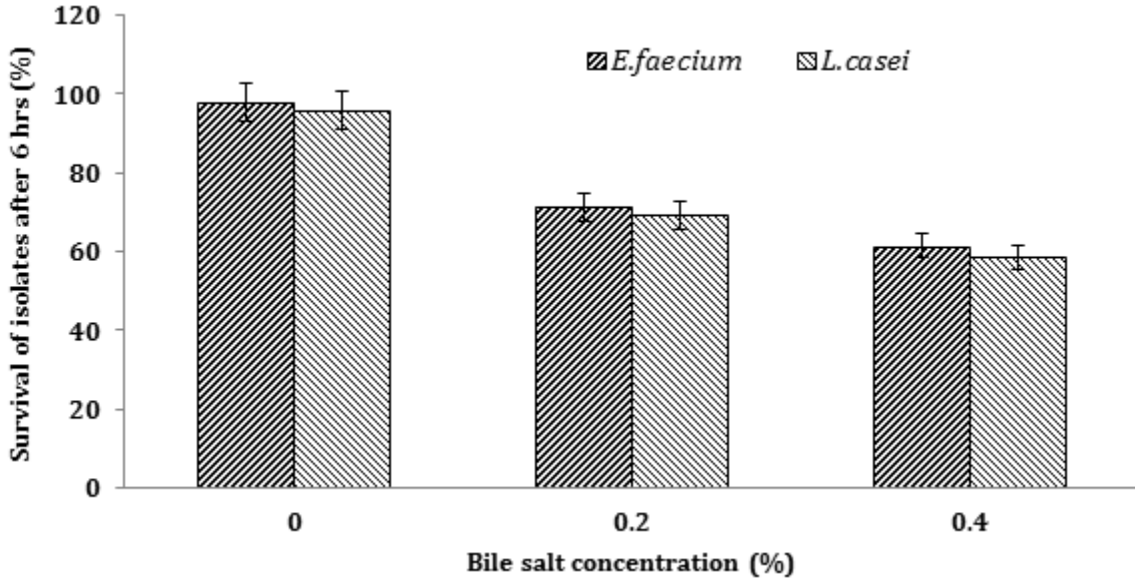

Fig. 3. Survival percentages (%) of *E. faecium* and *L. casei* after incubation of 6 h at different bile salt concentrations

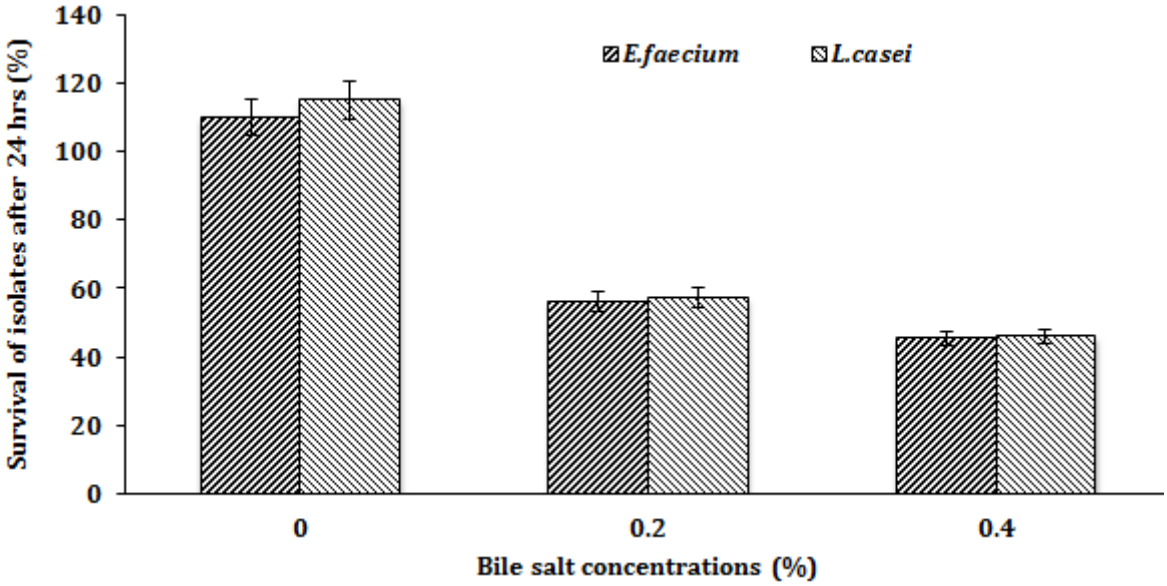

Fig. 4. Survival percentages (%) of *E. faecium* and *L. casei* after incubation of 24 h at different bile salt concentrations

inhibitory activity against lactobacilli strains *P. aeruginosa*.

E. faecium BM10 showed antifungal activity (zone of inhibition: 5-10 mm) against *A.niger, R. stolonifer*; weak activity (<5 mm) against *F. oxysporum* and *C. albicans* and no inhibition against *P. chrysogenum* although *L.casei* GM10 showed (zone of inhibition: 5-10 mm) against *F. oxysporum, C. albicans;* weak (zone of inhibition: <5 mm) against *P. chrysogenum* and no inhibition against *A. niger* and *R. stolonifer* (Table 1).

Effect of enzymes, surfactants, pH and temperature on bacteriocin activity

Results in Table 2 shown that antibacterial activity of bacteriocin of *E. faecium* BM10 remain active after treatment with catalase and lysozyme

Table 1. Antimicrobial activity of *E. faecium* BM10 and *L.casei* GM10 against some bacteria (Gram-positive, Gram-negative) and fungi using agar well diffusion assay

Indicator strains	Medium	Incubation temperature (°C)	Antimicrobial activity[a] of *E. faecium*	Antimicrobial activity[a] of *L. casei*
Gram -positive bacteria				
Bacillus subtilis	NB	37	+++	++
Staphylococcus aureus	NB	37	++	+++
Enterobacter aerogenes	NB	37	+	+
Lactobacillus acidophilus	MRS	37	+	-
Lactobacillus fermentum	MRS	37	+	-
Gram -negative bacteria				
Salmonella enterica	NB	37	++	+++
Klebsiella pneumonia	NB	37	-	+
Escherichia coli	NB	37	+++	+++
Pseudomonas aeruginosa	NB	37	++	_
Clostridium perfringens	NB	37	+	++
Proteus vulgaris	NB	37	-	+
Vibrio cholerae		37	++	+
Fungi				
Aspergillus niger	PDB	28	++	-
Fusarium oxysporum	PDB	28	+	++
Rhizopus stolonifer	PDB	28	++	-
Penicillium chrysogenum	PDB	28	-	+
Candida albicans	PDB	28	+	++

[a] Results of antimicrobial activity were recorded in the diameter of inhibition zones around the wells (8 mm in diameter)

-, no inhibition zone; +, zone < 5 mm; ++, zone < 5–10 mm; +++, zone > 15 mm

NB: Nutrient broth

MRS: de man Rogosa Sharpe

PDB: Potato dextrose broth

whereas trypsin, catalase and α-amylase enzymatic treatment did not fluctuate antibacterial activity of *L. casei* GM10. Proteinase-K treatment completely eliminated antibacterial activity of bacteriocins of both isolates. Treatment with various surfactants showed that bacteriocin activity of *L. casei* GM10 was less sensitive than *E. faecium* BM10. Activity of bacteriocin of *L. casei* GM10 remained active at pH 2, 3 and 7 although at pH 2 bacteriocin activity of *E. faecium* BM10 completely eliminated. Bacteriocin of *L. casei* GM10 also showed good antibacterial activity after high temperature treatments than *E. faecium* BM10.

Discussion

The probiotic strain must tolerate low pH 2-3, which is prevalent in upper gastrointestinal tract, simultaneously should perform their function efficiently in order to exert their beneficial effect [19]. Our findings of acid tolerance showed similar results of a study, reported by Kalui *et al.*[20] that eighteen strains of *L. plantarum* were survived at pH 2.5 after exposure for 3 h and 10 % of these strains could not at pH 2. However, Sirilun *et al.*[21] reported that only 43 out of 114 LAB strains at pH 3 after 2h of incubation survived although at pH 2, surviving percentage was higher than 50 % observed in 27 strains only.

Table 2. Effect of enzymes, surfactants, pH and temperature on antibacterial activity of bacteriocins of *E. faecium* and *L. casei* against *E. coli*

Treatments	Antibacterial activity[a] of bacteriocin of *E. faecium*	Antibacterial activity[a] of bacteriocin of *L. casei*
Enzymes		
Trypsin	-	+
Catalase	+	+
Lysozyme	+	-
á-amylase	-	+
Proteinase-K	-	-
Surfactants		
Tween-80	+	+
Tween-20	-	+
SDS[b]	+	+
EDTA[c]	-	-
pH		
2	-	+
3	+	+
7	+	+
Temperature		
40°C for 90 min	+	+
75°C for 60 min	-	+
100°C for 30 min	-	+
121°C for 10 min	-	-

[a] Antibacterial activity against *Escherichia coli* (MTCC 443)
[b] Sodium dodecyl sulphate
[c] Ethylene-diamine tetra acetic acid
+: Presence of Antibacterial activity
-: Absence of antibacterial activity

Results of bile salt tolerance indicated good bile salt tolerance ability of isolates might be due to presence of bile salt hydrolases (BSH) in isolates. Succi *et al*.[22] isolated sixty three *L. rhamnosus* strains from Parmigiano Reggiano cheese and tested their ability lower pH in presence of different percentages (1.0 %, 1.5 % and 2.0 %) of bile salts, after comparing inhibitory effects of bile salts, three strains of *L. rhamnosus* showed best ability to lower pH in presence of different bile salt concentrations. Kalui *et al*.[19] demonstrated in a similar study that 18 out of the 19 *L. plantarum strains* were able to grow in broth supplemented with 0.3 % bile salts subsequent exposure to pH 2.5. Bile salt synthesized in liver and secreted in small intestine, it acts like detergent and disrupt cell membrane of LAB, which is composed of lipids and fatty acids. Consequently the survivals of LAB in such harsh condition become unendurable. Genomes of several intestinal bacteria, including *Bifidobacterium* and *Lactobacillus* species encodes bile salt hydrolases (*bsh*), which present in distal end of small intestine and colon, break down bile salt in to steroids. These bile salt hydrolases favors survival of bacteria in high bile salt conditions [23]. Results of antimicrobial activity suggested that both isolates can be used as potent antibacterial agent on the other hand weak antifungal activity was exhibited by both strains. Bacteriocin activity of *L.casei* GM10 was more resistant of various treatments of enzymes, temperature, pH and surfactants than *E.*

faecium BM10. El-Ghaish *et al*.[24] found that bacteriocins of *Lactococcus lactis* subsp. lactis A15 and *Enterococcus faecium* A15 remain active at pH 5 and 8 and stable also until 100°C for 10 min. Similarly, Plantaricin TF711 was sensitive to protease activity whereas its activity remain stable at high temperature and at a wide pH range[25]. Inactivation of bacteriocin activity due to proteases showed its proteineous nature.

Conclusion

Probiotic bacteria are beneficial for human health. These can be a perfect replacement of antibiotics. Antibiotics have broad spectrum growth inhibition activity, therefore kill useful flora in gut with the target pathogen while probiotics contain narrow antimicrobial activity. Antibiotics also generate resistance in pathogenic micro-organisms. Research must be proceed to identify and exploitation of new isolates, which fulfill the requirement as probiotic. Keeping ahead a step in this direction to get potent probiotic isolates, in this present study, two isolates *Enterococcus faecium* BM10 and *Lactobacillus casei* GM10 showed maximum antimicrobial potential in primary screening were identified by gram staining, catalase test, motility test, spore test and 16s rRNA sequencing. Both isolates exhibited strong antibacterial activity, but weak antifungal activity and showed good acid and bile tolerance. The results concluded that these isolates fulfill the requirements of probiotic strains and can be further analyzed for clinical and biotherapeutic applications.

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