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STUDIES ON ANTAGONISTIC ACTIVITY AND PROBIOTIC PROPERTIES OF *ENTEROCOCCUS FAECIUM* AND *LACTOCOCCUS LACTIS*

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ABSTRACT

The present study compared the antagonistic activity and probiotic properties of two Lactic Acid Bacteria (LAB) such as *Enterococcus faecium* NCIM 2605 and *Lactococcus lactis* NCIM 2606. Bacteriocin from both the cultures was produced using MRS broth by shake flask fermentation. In well diffusion method, the cell free supernatant (CFS) of *E. faecium* showed activity against both gram positive and gram negative organisms especially methicillin resistant *S, aureus* (MRSA). Maximum inhibition was observed against *B. subtilis* and *V. parahaemolyticus.* The CFS of *L. lactis* showed activity against only gram-positive organisms, but not against MRSA. Both the isolates produced bacteriocin at maximum quantity during the exponential phase at pH 6 and pH 7 when incubated at 37^0C. The arbitrary unit for *E. faecium* and *L. lactis* found to be 3,200 AU/ml and 800AU/ml, respectively. The antimicrobial substances was retaining the activity after treated upto 121^0 C for 15 minutes, stable at pH values between 2.0 and 5.0 and 2.0 and 9.0 for *E. faecium* and *L. lactis* respectively. Both the substances were found to be sensitive to proteinase K. The tested two LAB survived for 3 hours in the presence of 0.3% w/v bile and pH 3 at 37^0 C. The strain *E. faecium* having a broad spectrum of activity, possess probiotic ability than the *L. lactis* and can be developed as probiotic diary fermented products.

KEY WORDS: **Bacteriocin, LAB, probiotic, Nisin, Enterocin**

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INTRODUCTION

Lactic Acid Bacteria (LAB) are regarded as a major group of probiotic bacteria and general recognized as safe (GRAS) organism with no pathogenic, or virulence properties. The probiotics are capable of boosting immunity against infection and generating antimicrobial substances.LAB synthesize enzymes, vitamins, antioxidants and bacteriocins[1,2]. With these properties, intestinal LAB constitutes an important mechanism for the metabolism and detoxification of foreign substances entering the body[3]. Bacteriocins (Bcn) are antimicrobial substances produced extracellularly released peptides or protein molecules by lactic acid bacteria. It has either bactericidal or bacteriostatic mode of action against closely related species. The inhibitory spectrum of some bacteriocins includes food spoilage and food-borne pathogenic microorganisms[4]. *Lactococcus lactis subsp. lactis* produced nisin, which is widely used for many decades as a food preservative[5]. Nisin also used as an anti-infective agent for bovine mastitis[6]. *Enterococcus faecium* is naturally occurring lactic acid bacterium that grows in human and animal intestinal contents. It has been used as a human probiotic for more than 25 years. During course of antibiotics, the probiotic supplements may reduce the adverse effects of antibiotics in the intestinal environment. The present study compared the antagonistic activity and probiotic properties of two Lactic Acid Bacteria (LAB) such as *Enterococcus faecium* and *Lactococcus lactis*.

MATERIALS AND METHODS

(i) Description of bacterial strains

The LAB cultures such as *Enterococcus faecium* NCIM 2605 and *Lactococcus lactis* NCIM 2606 used in this study were obtained from the National Collection of Industrial Microorganisms (NCIM). Both cultures were subcultured periodically and maintained on nutrient agar slants at 4^{o}C until further use.

(ii) Detection of antibacterial activity of CFS

Antibacterial activity of cell free supernatant (CFS) was determined by agar well diffusion method. Inoculum of *E. faecium* and *L. lactis* was prepared using MRS broth at 37^{0}C for 18 hours of incubation. Ten percent of inoculum was transferred into each 100 ml of the conical flask containing MRS broth. For the production of bacteriocin, the production flasks were kept in a rotary shaker for 24 hours at 37^{0}C. The fermented medium was centrifuged at 10000 RPM for 10 minutes at 4^{0}C. The CFS collected from both the organisms was tested separately against indicator organisms. Freshly prepared bacterial pathogens such as *Staphylococcus aureus*, methicillin resistant *S. aureus, Bacillus subtilis, Lactobacillus sp., Escherichia coli, Salmonella typhi, Shigella dysenteriae, Vibrio cholerae, V. parahaemolyticus, Proteus vulgaris* and *Pseudomonas aeruginosa* were inoculated onto Muller Hinton agar (MHA) separately and spread by using a sterile cotton swab. Well with 5 mm diameter was cut on MHA plates using well cutter. About 50 µl of CFS was loaded into the well. The zone of inhibition was measured after 24 hours of incubation at 37^{0}C and expressed in millimetre in diameter[7].

(iii) Effect of pH on bacteriocin production

To determine the effect of pH on bacteriocin production by *E. faecium* and *L. lactis*, both the cultures were grown on MRS broth at pH 6 and pH7 separately at 37^{0}C for 24 hours. After incubation, the CFS was collected and tested for antibacterial activity by the drop test in which 3 µl of the CFS was placed over the MHA plates inoculated with *Lactobacillus sp*.

(iv) Bacteriocin production and measurement of bacteriocin activity[7]

The inoculum (10%) of *E. faecium* and *L. lactis* was transferred into each 100 ml of MRS broth. Both the flasks were incubated at 37^{0}C. For every 24 hours, each 2 ml of fermentation medium was collected and measured the growth of bacteriocin producers at 610 nm. After measuring the growth, the cell free supernatant was collected and tested for antibacterial activity against *Lactobacillus sp.* by agar well diffusion test.

(v) Measurement of arbitrary unit (AU)

The arbitrary unit was measured by agar well diffusion method and critical dilution assay.

Twenty microliter of two fold diluted CFS was added into 5 mm diameter wells made on nutrient agar plates previously inoculated with the indicator organism *Lactobacillus sp*. Zone of inhibition was measured after 24 hours of incubation at 37^0C.

(vi) Partial purification of bacteriocins

Strains *E. faecium* and *L. lactis* were grown on MRS broth separately by shake flask fermentation in rotary shaker at 37^0C for 24 hours. The fermented medium was taken and adjusted to pH 5.0. The cell free supernatantwas collected by centrifugation at 6000 rpm for 15 minutes at 4^0C. Pellet was collected separately from each organism and resuspended in 100 mM saline (pH 2) and kept at 4^0C. After 30 minutes, the supernatant from each organism was collected by centrifugation at 8000 rpm for 10 minutes at 4^0C. CFS was pooledand mixed with chloroform at 2:1 ratio and poured into separating funnel. After 10-15 hours, the protein precipitate was collected and evaporated the solvent by air dry. The dried sample was used as a crude bacteriocin[8].

(vii) Detection of antibacterial activity of crude bacteriocin

Fifty microlitre of crude bacteriocin from *E. faecium* and *L. lactis* were loaded on to sterile 5mm diameter filter paper disc and air dried. Then the disc was placed over the nutrient agar plates swabbed with *B. subtilis* and incubated at 37^oC. The inhibition zone was observed after 24 hours of incubation.

(viii) Effect of different treatments on bacteriocin[9]

The CFS of *E. faecium* and *L. lactis* was adjusted to pH ranging from 2.0 to 9.0 and another set of CFS was heat treated at 100^0C for 15 and 30 minutes and also at 121^0C for 15 minutes. Twenty microliter of each pH treated and heat treated CFS were tested against *Lactobacillus sp*. by agar well diffusion method. To determine the protein nature of the bacteriocin, 1 mg/ml of proteinase K was added to CFS of both organisms separately and incubated at 37^0C for 3 hours. The residual inhibitory activity was measured by agar well diffusion method as described.

(ix) Estimation of the bacteriocin molecular weight using SDS-PAGE[10]

The molecular weight of the crude bacteriocin from both organisms was determined by using 10% separation gel and 5% stacking gel. Bovine Serum Albumin (45kDa) and were used as molecular mass markers. Electrophoresis were run at a constant voltage (80 V). After electrophoresis, the gel was stained with Silver nitrate.

(x) Protein estimation[11]

Protein concentrationof crude bacteriocins obtained from*E. faecium* and *L. lactis* were estimated by the method described by Lowry et al.,(1951) with bovine serum albumin as a standard.

(xi) Resistance of strains to bile and low pH values[12]

The methods for *in vitro* analysis of probiotic properties, acid and bile tolerance study were used. *E. faecium* and *L. lactis* cell suspension was collected from the cultured MRS broth by centrifugation at 3000 rpm for 10 minutes. The cells were added into each 50 ml of MRS broth and modified MRS broth containing 0.3% (w/v) of OX bile. At intervals of 0, 1, 2, and 3 hours incubation at 37^oC, 100 microlitre of cell suspension from both MRS and modified MRS broth were inoculated into MRS agar. After 24 hours of incubation each plate was counted for number colonies and recorded. In another experiment, the cells of *E. faecium* and *L. lactis* were inoculated into each 50 ml of phosphate buffer saline at pH 3, pH 5 and were incubated at 37^0C. At 0, 1, 2 and 3 hours of incubation, was taken and plated on MRS agar for viable counting.

RESULTS AND DISCUSSION

The antibacterial activity crude bacteriocins (CFS) produced from *E. faecium* and *L. lactis* was given in table 1.

Table 1
Antibacterial activity of crude bacteriocins produced from E. faecium and L. Lactis

Test pathogens	*E. faecium CFS*	*L. lactis CFS*
Gram positive bacteria		
Lactobacillus sp.	13	13
Bacillus subtilis	11	12
Staphylococcus aureus	9	10
Methicillin resistant *S. aureus*	9	-
Gram negative bacteria		
Proteus vulgaris	9	-
Escherichia coli	9	-
Shigella dysenteriae	10	-
Salmonella typhi	10	-
Pseudomonas aeruginosa	11	-
Vibrio cholerae	10	-
V. parahaemolyticus	13	-

The results showed that CFS from *E. faecium* inhibited both gram positive and Gram negative bacteria such as *Lactobacillus sp., B. subtilis, S. aureus,* Methicillin resistant *S. aureus* (MRSA) and *P. vulgaris, E. coli, Shigella dysenteriae, Salmonella typhi, P. aeruginosa, V. cholerae, V. parahaemolyticus*. Similar results were recorded for bacteriocin produced *E. faecium* GM-1 have broad spectrum of activity against *Escherichia coli, Staphylococcus aureus, Vibrio spp., Salmonella typhimurium, Listeria monocytogenes, Lactobacillus acidophilus,* and *Streptococcus thermophilus*[13]. The CFS from *L. lactis* inhibited only the gram-positive bacteria except MRSA. This result is similar to that of Izildinha Moreno et al, who found that the strain *L. lactis* subsp. *lactis* ITAL 383 produced a bacteriocin, which inhibited closely related species and other Gram-positive microorganisms including *L. monocytogenes* and *S. aureus* (author was not tested against MRSA) but no activity was found against Gram-negative bacteria tested. In our study, bacteriocin activity was observed during the early logarithmic phase (6-7 h of growth) suggesting that the peptide is a primary metabolite. It was maximal at 18 h of incubation during the early stationary phase. During extended stationary phase incubation the activity of bacteriocin decreased considerably. The loss of activity has been ascribed to proteolytic degradation by endogenous extracellular proteases induced during this growth phase, protein aggregation and adsorption to cell surfaces and feedback regulation[14].The arbitrary unit (AU) of *E. faecium* CFS and *L. lactis* CFS were found to be 3,200 AU/ml and 800AU/ml, respectively. Bellei B et al. (2011) reported that *E. faecium* E86 bacteriocin AU was found that 2,560 AU/ ml[15]. Didem Sahingil *et al*. observed that Lactococcin BZ bacteriocin production was 400 AU/mL[16](fig 1 and 2).

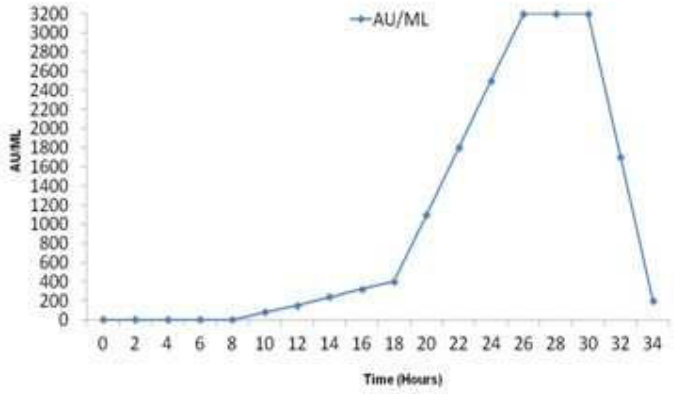

Figure 1
The rate of bacteriocin production by E. Feacium

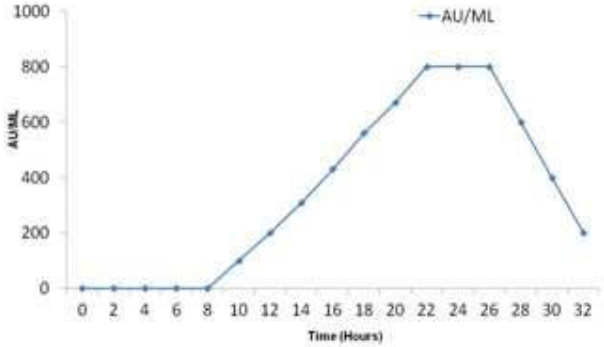

Figure 2
The rate of bacteriocin production by L.lactis

The effect of pH on the CFS of *E. faecium* showed activity in pH range from 2 to 5 whereas *L. lactis* CFS retained its activity against indicator organism in the pH range of 2 to 9, which indicating that variations in pH values did not affect their antimicrobial activity (Table 2).

Table 2
Stability of crude bacteriocins at different temperature ranges

pH range	*E. faecium CFS*	*L. lactis CFS*
2	+	+
3	+	+
4	+	+
5	+	+
6	-	+
7	-	+
8	-	+
9	-	+

Perin et al. (2013) have reported that antimicrobial activity after the pH was adjusted at distinct values[17]. The nisin antimicrobial activity maintains at a variety of pH values and exhibits higher inhibitory activity at low pH[18]. CFS produced from *E. faecium* and *L. lactis* were found to be stable at 100^0C for 30 minutes and 121^0C for 30 minutes (Table3).

Table 3
Sensitivity of CFS of E. faecium and L. lactis to heat treatment

Heat treatment	*E. faecium*	*L.lactis*
100°C for 15 minutes	+	+
100°C for 30 minutes	+	+
121°C for 15 minutes	+	+

The enterocin retained antibacterial activity even following heating to 121°C for 15 min[19]. In contrary, another study reported that the antimicrobial activity of the *L. lactis* Lc08 CFS against *L. monocytogenes* was lost after autoclave treatment at 121°C for 15min[17,20]. The maintenance of antimicrobial activity at distinct pH values and heat treatments supports the application of Lc08 or their bacteriocin as biopreservatives in processed dairy product. The antimicrobial property of both *E. faecium* and *L. lactis* was lost when incubated with proteinase K. The same results were observed in various study[17,19,20]. The partially purified bacteriocins were found to inhibit *B. subtilis*. Partially purified bacteriocins obtained from both organisms were electrophoresed on SDS-PAGE, the result showed that single diffusible band was migrated behind the egg albumin standard protein (45 KDa). The amount protein found in the partially purified bacteriocins was 98µg/ml and 32 µg/ml of *E. faecium* and *L. lactis* bacteriocins, respectively. Bile tolerance is an important characteristic of bacteria to survive in small intestine. Bile resistance of some strains is related to specific enzyme activity, bile salt hydrolase which helps to hydrolyse conjugated bile, thus reducing its toxic effect[21]. The 0.3% bile concentration is considered as an average intestinal bile concentration of the human gastrointestinal tract[22]. In our study, about 94% and 88% of the tested *E. faecium* and *L. lactis* respectively showed survival after 0.3% bile treatment (Fig 3,4). Another study reported that *E. faecium, L. lactis subsp. cremoris* and *L. plantarum* isolates showed a good stability at 0.1% concentration indicated by the increase in the optical density. At 0.3% concentration, none of the isolates showed a marked increase in the optical density but they could survive at this concentration for 4 hours[23].

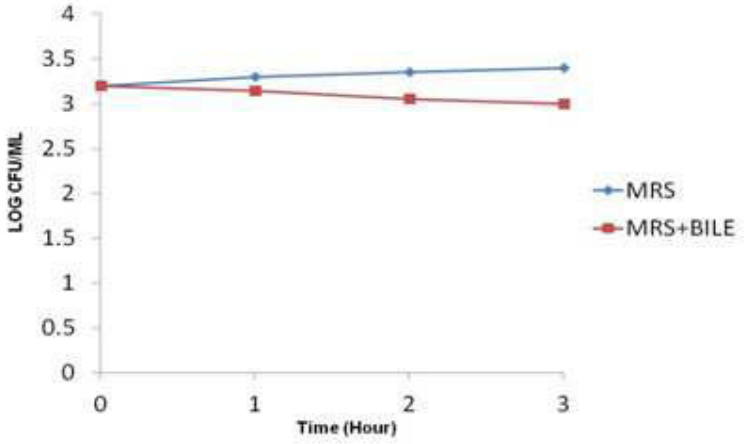

Figure 3
Survival of E. faecium in MRS broth and MRS broth+0.3%bile

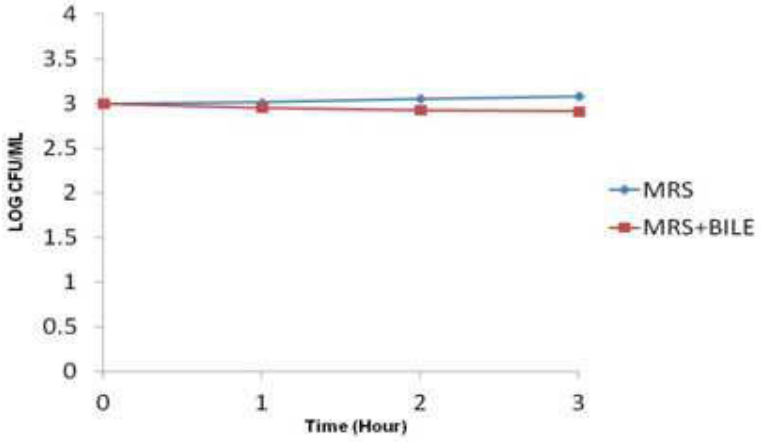

Figure 4
Survival of L .lactis in MRS broth and MRS broth+0.3%bile

Resistance property of LAB to low pH is one of the major selection criteria for probiotic strains[24,25]. In *in vitro* assay, pH 3.0 has been preferred, due to the fact that a significant decrease in the viability of strains is often observed at pH 2.0 and below. Our study showed that 94% of *E. faecium* was survived at pH 3 upto 3 hour but at pH 5, the organism growth was increased. Whereas about 91% of *L. lactis* were survived at pH3, and also increased growth was observed at pH 5 (Fig 5,6). Another studyreported that, 63.6% of *E. faecium* were survived in pH 3.0 for 3 hours. *L. lactis* subsp. c*remoris* were more sensitive to low pH[23].

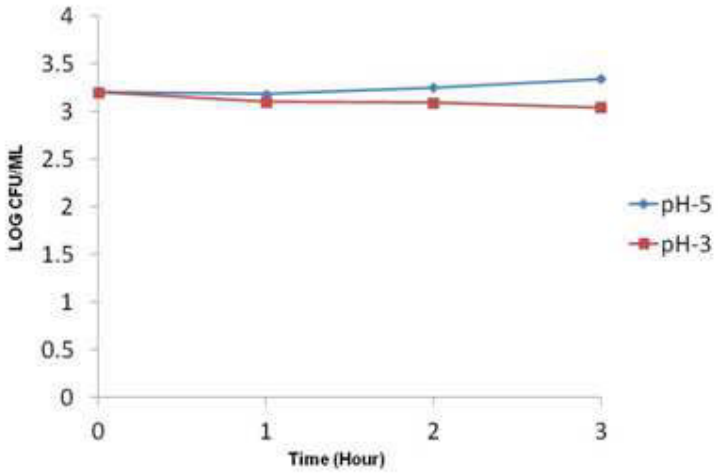

Figure 5
Survival of E. faecium in phosphate buffered saline

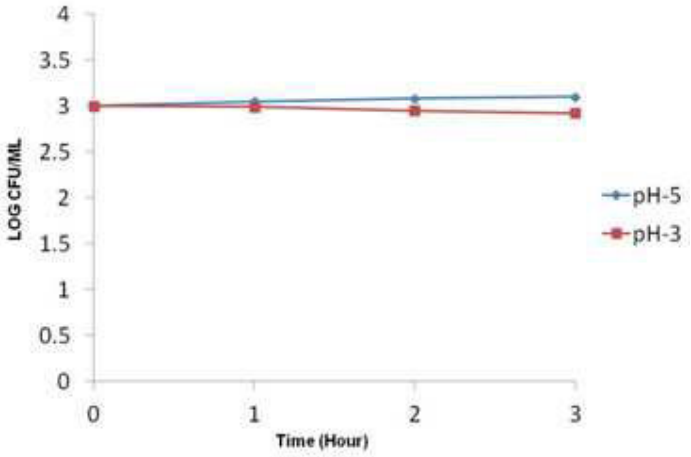

Figure 6
Survival of L. lactis in phosphate buffered saline

CONCLUSION

In conclusion, the results obtained from both organisms for antimicrobial activity and probiotic potential were revealed that *E. faecium* having a broad spectrum of activity include anti-MRSA and possess potential probiotic ability than the *L. lactis.* Further study is needed to assess the antibiotic resistance, adhesion to epithelium tissue and fermentation of dairy products.

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