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Original Research Article

AN ANALYTICAL STUDY ON PRODUCTION AND CHARACTERISATION OF EXTRACELLULAR ANTIMICROBIAL PEPTIDES OF LACTOBACILLUS

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ABSTRACT

Probiotics provide several health benefits that transcend basic nutritional requirements of our daily meal. The focus of this study is to evaluate the antimicrobial capability of probiotic bacteria isolated from commercially available yoghurts in Indore city. Three cans of different branded yoghurts were purchased from local supermarkets and prepared using standard procedures. The samples were transferred into De Man, Rogosa and Sharpe (MRS) broth and incubated anaerobically for 24 hrs. The isolates were identified using biochemical tests. The antibacterial activity was evaluated using agar diffusion assay. The effects of pH and temperature on the growth of isolated Lactobacillus spp. as well as their ability to tolerate bile salt were assayed. All isolated lactobacillus spp. has inhibitory effect on E. coli, S. aureus and P. aeruginosa. Bile salts incorporated in the bacteria cultures were effective on bacterial viability. The present study also focused on the antimicrobial activity of cell free culture filtrate and extracellular protein concentrate of Lactobacillus strain against food-spoilage organisms and human pathogens. Though both bacteria and fungi that have beneficial health effects are categorized as probiotics, Lactobacillus spp. have been well characterized as prominent members of the probiotic group. The probiotic bacteria isolated in commercially available yoghurts have health beneficial effects, which include antibacterial activities.

Keywords: Mycobacterium tuberculosis, Tuberculosis, Aspartate pathway, Metabolomics, Persistent infection, Aspartate kinase, Feedback inhibition, Combination therapy, Multi-drug resistant tuberculosis (MDR-TB)

INTRODUCTION

Yoghurt is fermented milk which is widely consumed as a probiotic worldwide because of their several health benefits. Milk is known to contain vitamins, magnesium, protein, potassium and calcium. It plays an important role in prevention and treatment of high blood pressure osteoporosis, prevent vaginal infection and restore microbiota balance in the gut thereby reducing the risk of gastrointestinal disorder. Outside milk, bacterial culture is one of the major components of yoghurt. These are special strains of bacterial called probiotics because of the numerous health benefits they confer on humans when taken in enough. Probiotic bacteria are known to inhibit the growth of dangerous

pathogens in the gut, prevent respiratory tract infection in the elderly, children and immunocompromised patients, prevent development and pro-liferation of monoclonal cells, lower blood cholesterol and boost immune defense against related infectious diseases.

In the last two decades, antimicrobial peptides have been gaining attention as antimicrobial alternatives to chemical food preservatives and commonly used antibiotics due to the emergence of multi-drug resistant bacteria. Under such conditions, Lactic acid bacteria and their metabolites are good alternatives as a source of antimicrobial agents. Lactobacillus and Bifidobacterium species are normal inhabitants of the human gastrointestinal tract and are found primarily in dairy products and fermented foods. There organisms are currently attracting keen interest as probiotic health supplements from both consumers and researchers because of heightened awareness of the beneficial links between health, nutrition and diet. A probiotic is a 'live microbial food supplements that beneficially affects the host animal by improving the intestinal microbial balance. The treatment of gastrointestinal disorders with probiotics is a widely used remedy for intestinal complications in humans. There is a concern that industry will no longer be able to develop effective antibiotics at a rate sufficient to compete with the development of microbial resistance to old antibiotics. These factors have renewed interest in the possibility of deliberately feeding beneficial microorganisms to humans as an alternative to antibiotic therapy especially in the case of gastrointestinal disorders. The principle of using harmless bacteria for conquering pathogens has been recognized for many years. In fact, probiotics have been used for as long as people have eaten fermented foods. However, it was Metchnikoff at the turn of the 20th century who first suggested that ingested bacteria could have a positive influence on the normal microbial flora of the intestinal tract. He hypothesized that Lactobacilli were important for human health, and longevity and therefore promoted yogurt and other fermented foods as healthy. Probiotics are usually targeted for use in intestinal disorders in which specific factors (such as antibiotics, medication, diet or surgery) disrupt the normal flora of the gastrointestinal tract, making the host susceptible to disease(s). Examples of such diseases include antibiotic associated diarrhoea, inflammatory bowel diseases, etc. The goal of probiotic therapy is to increase the numbers and activities of those microorganisms suggested to possess health-promoting properties until such time that the normal flora can be re-established.

The present study focused on the antimicrobial activity of cell free culture filtrate and extracellular protein concentrate of Lactobacillus strain against food-spoilage organisms and human pathogens. Though both bacteria and fungi that have beneficial health effects are categorized as probiotics, Lactobacillus spp. have been well characterized as prominent members of the probiotic group. Studies have reported that several strains of Lactobacillus player a significant role in boosting host immune responses, maintaining the ecosystem of the intestine and inhibiting the growth of several pathogenic bacteria in the gut. The ability of probiotics to influence the immune responses of the specialized cells in the body is gaining attention from pharmaceutical and medical researchers. Understanding the mechanisms behind the influence of probiotic on adaptive immunity could open opportunities in prevention medicine. The focus of this study was to ascertain the presence of probiotic bacteria in commercially available yoghurts in and to scientifically investigate some of their claimed putative health benefits.

MATERIALS AND METHODS

Yogurt samples: Three (3) samples of yogurt were collected from different areas of Indore city.

Isolation and Identification of Microbial Isolates

A volume of 100µl of each sample of yoghurt was transferred into a flask containing MRS Broth (Hi Media Laboratories Pvt. Ltd, Mumbai, India) and incubated at 37°C for 24 hours. Thereafter, 100µl of the broth samples were spread on MRS agar (Hi Media Laboratories Pvt. Ltd, Mumbai, India) and further incubated at 37°C anaerobically for 48 hours. Bacterial cultures were sub-cultured to obtain pure isolates. The isolates were identified using the following biochemical tests: Gram staining, lactose fermentation, Indole test, Catalase test, Coagulase test, Oxidase test, Urease test, Citrate test, Starch hydrolysis and Lysine hydrolysis.

Evaluation of 0.3 % Bile Salt Tolerance

Saturated bile solution made from powdered bile extract was prepared. The saturated bile salt at 0.3% concentration was added to an overnight MRS broth culture containing the test isolates. Pour plate counts method using 10-fold serial dilutions was used to enumerative viable cells of Lactobacillus spp.

Resistance to Low pH

The pH of an overnight MRS broth culture containing 0.1mL of aliquot was adjusted. Each sample was taken every hour and the viable cells were enumerated using pour plate counts method.

Preparation of Cell Free Culture Filtrate and Extracellular Protein Concentrate of Lactobacillus

2% inoculum of Lactobacillus was used to inoculate 1L of MRS medium (pH 6.8), incubated at 37°C for 24 h. The cultured medium was centrifuged (5000 rpm, 20 min, 4°C) and supernatant was passed through 0.45 µm Millipore filter. Total protein in CFC filtrate was precipitated by ammonium sulphate (80% saturation) precipitation and kept overnight at 4°C. The precipitates were separated by centrifugation (10000 rpm, 20 min, 4°C) and dissolved in a minimum amount of acetate buffer (pH 4.5, 10 mM). Clear supernatant obtained upon centrifugation (10000 rpm, 20 min, 4°C) was used as extracellular protein concentrate (EPC). CFC and desalted EPC (using PD-10 column, sephadex G-25, Pharmacia) were used for the determination of antimicrobial activity in all the experiments. Protein content of CFC and EPC was determined according to Bradford assay.

Determination of Antimicrobial Activity by Well-Diffusion Agar Assay

Antimicrobial activity of CFC and EPC was determined by well-diffusion agar assay. 100 µl of 18 h old cultures of test organisms were inoculated in molten nutrient agar and poured in sterile petri plates. Wells (7 mm) made using cup borer were loaded with 100

µl of CFC or EPC (pH 4.5, and 7) and pre-incubated at 4°C for 2-3 h before shifting to 37°C and incubated for overnight. Appropriate controls were also included.

Antimicrobial Activity in Nutrient Medium

Nutrient broth (10x) containing aliquots of CFC or EPC (0.1-0.5 ml) in the final volume of 1 ml were inoculated with test pathogens (OD_{600} = 0.2) and incubated at 37°C for 24 h. OD_{600} was recorded and aliquots of 0.1 ml were used to re-inoculate fresh N-broth to determine the bacteriostatic or bactericidal activity.

Sensitivity of EPC to Heat, Proteolytic Enzymes and pH

The antimicrobial activity of EPC was evaluated after treatment with heat, proteolytic enzymes, and at pH (2-9). The EPC was heated at 100°C for 30 and 60 min and 121°C for 15 min. Aliquot of 1 ml EPC was treated with 1 mg/ml of trypsin, pepsin and proteinase K solutions (Himedia, India), incubated at 37°C for 4 h, followed by boiling the mixture for 5 min to inactivate enzyme. Inactivated buffered enzyme solution was used as control. Antimicrobial activity of the EPC at various pH values was evaluated using EPC prepared in buffers as described above. The residual antimicrobial activity was determined by liquid assay.

Influence of Culture Age on the Production of Antimicrobial Peptides

2 ml of activated culture of L. rhamnosus Fb was inoculated in 100 ml MRS medium and incubated at 37°C. The entire content of the flasks was harvested at an interval of 6h and analyzed for biomass and pH. CFC and EPC were prepared as described above and their antimicrobial activities were determined.

Influence of Biofilm Grown Cells on Antimicrobial Activity

Antimicrobial activity of Erlenmeyer flasks (500 ml) containing 300 ml MRS medium with or without 300 g of glass beads were autoclaved and inoculated with 2% inoculam of activated culture of Lactobacillus and incubated at 37°C. Flasks with or without glass beads were harvested at 24, 48 and 72 h of incubation. The CFC and EPC were prepared as described above and their antimicrobial activity was determined.

Influence of Storage on the Antimicrobial Activity of EPC

Antimicrobial activity of EPC stored at 4°C was determined at 0, 15, 30, 60 and 180 days using Nutrient broth.

Production of Antimicrobial Peptides of Lactobacillus in Skimmed Milk

1 ml of 10 times concentrated biomass was inoculated in 400 ml of skimmed milk (incubated at 37 , 24 h), supernatant was separated by centrifugation (10000 rpm, 20 min, 4°C). Total protein in CFC filtrate was precipitated by ammonium sulphate (80% saturation) precipitation and kept overnight at 4°C. The precipitates were separated by centrifugation (10000 rpm, 20 min, 4°C) and dissolved in a minimum amount of phosphate buffer (pH 7.0, 10 mM). Clear supernatant obtained upon centrifugation (10000 rpm, 20 min, 4°C) was used as extracellular protein concentrate (EPC).

Tricine-SDS-PAGE

SDS-PAGE was performed as described by Schagger and Von Jagow (1987). Two gels, each composed of 4% acrylamide and 0.5% bisacrylamide in the stacking gel and 16.5% acrylamide and 0.5% bisacrylamide in the separating gel, were prepared. Electrophoresis was operated at constant current (10 mA in the stacking gel and at 21 mA during the rest of separation). 30 µl aliquot of EPC was mixed with equal volume of sample buffer and heated at 100°C for 10 min. Molecular mass standard was from Genei (Bangalore). The gel was stained with Coomassie Brilliant Blue R-250 and destained using methanol: acetic acid: water (40:10:50).

RESULT AND DISCUSSION

Microbial Isolation

The initial step on isolation and identification of the test isolates was based on the colony morphology. The round creamy colonies of the Lactobacillus spp. on MRS agar were observed as shown in Figure 1.

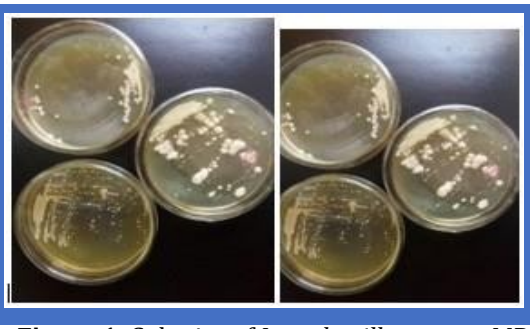

Figure 1: Colonies of *Lactobacillus* spp on MRS Agar

Biochemical Confirmation Test of the Isolate

The biochemical tests showed that the test isolates were generally Gram positive, indole negative, oxidase negative, catalase negative and non-motile bacilli. The isolates were able to fermentation glucose, fructose, D-mannitol and sucrose. The antibacterial activity of all the isolated Lactobacillus has inhibitory effect on E. coli, S .aureus and P. aeruginosa. Bile tolerance test revealed that the isolates could also survive bile salt.

Tolerance to Acid, Bile Salt and Temperature by Lactobacilli

At pH < 3, the number of bacteria in the medium decreased because of the loss of viability. At pH ≤ 2.0, no viable bacterial cells were detected after the first hour suggesting that most isolates were killed by severe pH. Also, all the isolates were able to grow in the presence of bile salt. Though the isolates were able to grow at temperature ranges from 25-40°C, the optimal growth was observed at 37°C.

Table 1: Effect of pH and Temperature on The Growth of The Test Isolates

pH	1	2	3	4	5	5.5	6	6.5	7	7.5
Growth	NG	NG	+	+	++	++	++	++	++	+
Temperature (ºC)	20	25	30	37	42	45				
Growth	+	+	+	+	+	+				

+ = Low Growth, ++ = Moderate Growth, +++ = High Growth, while NG = No Growth

Antimicrobial Activity of CFC and EPC of Lactobacillus

CFC and EPC of Lactobacillus exhibited broad antimicrobial spectrum against Gram-positive and Gram-negative food-borne and human pathogens. Total protein in the CFC filtrate and EPC having pH 4.5 and pH 7 was 430, 1045, and 1238 µg/ml respectively. Antimicrobial activity of CFC filtrate was higher against Serratia marcescens, Enterococcus faecalis, Staphylococcus aureus, Bacillus megaterium, Bacillus cereus, Micrococcus luteus, and Yersinia enterocolitica in comparison to EPC. Moreover, EPC was not active against Shigella sp., Proteus vulgaris and Listeria monocytogenes. Antimicrobial activity of EPC was higher against Escherichia coli, Staphylococcus aureus and Vibrio cholerae than other test organisms. Antimicrobial activity of EPC against E. coli was higher at pH 4.5 than at pH 7. EPC pH 7 did not exhibit activity against P. aeruginosa. Fig. 3 shows the diffuse band of EPC having molecular weight of ca 6 kDa.

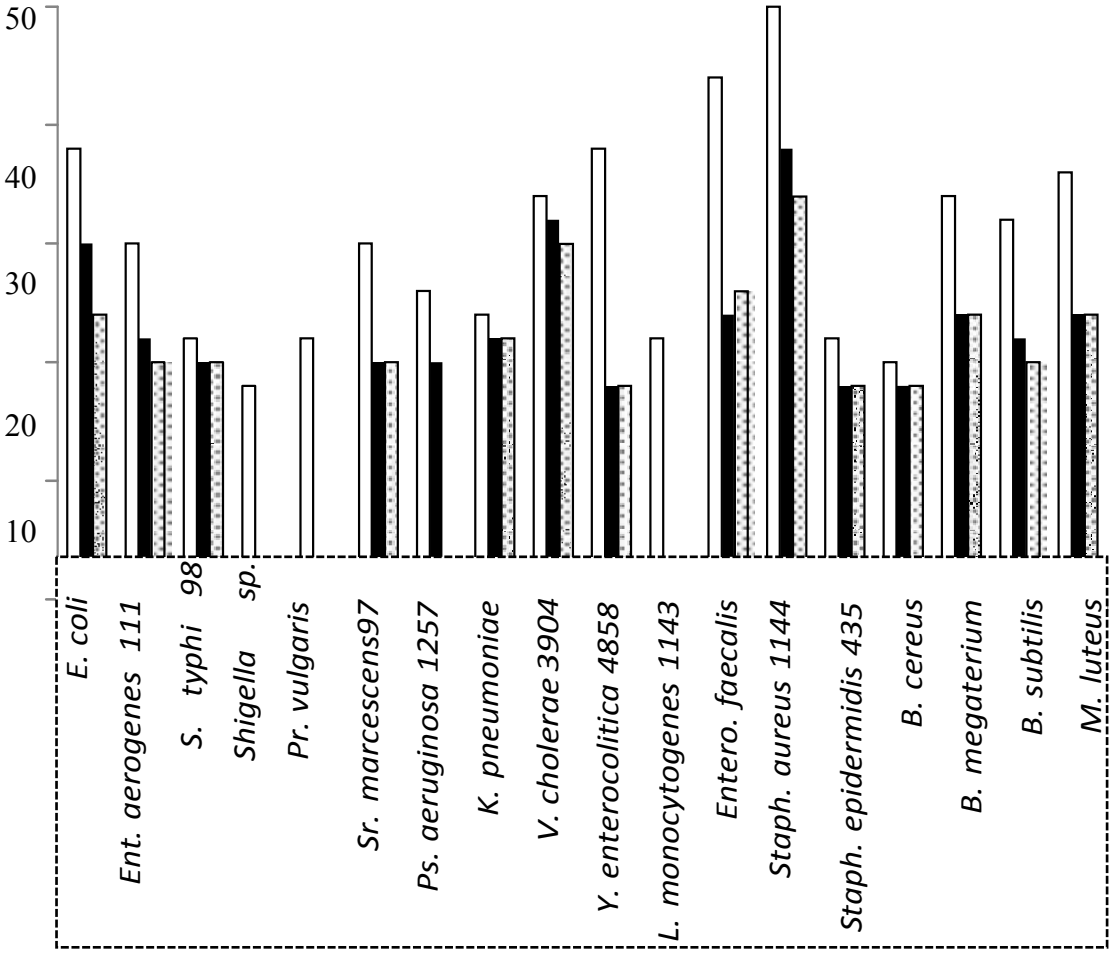

Figure 2: Antimicrobial Activity of CFC and EPC (pH 4.5 and 7) Prepared from 24 h Lactobacillus Growing on MRS Medium Determined against Food-Borne and Gastrointestinal Pathogens by Well Diffusion Agar Assay. Fractionation of EPC using PD-10

Fractionation of EPC using PD-10

EPC was fractionated by Sephadex G-25 gel filtration chromatography and fractions exhibited strain-specific antimicrobial activity. According to their bactericidal inhibition, fractions were categorized in three groups

- Synergistic activity against E. coli and S. marcescens,
- Individual fractions and/or in combination exhibit the activity against Enterococcus aerogenes, S. typhi, Shigella sp., Pseudomonas aeruginosa and Staphylococcus aureus, and
- Exert activity individually against P. vulgaris but not in combination with other fractions

In certain instances, low concentration of EPC was observed to promote growth but was inhibitory at higher concentration in the case of Enterococcus aerogenes and S. typhi. In case of Shigella sp. and Proteus vulgaris the growth increased at low concentration but only in the presence of certain EPC fractions 1, 3-12, 16-20 and 2, 3, 5-15 respectively. Pseudomonas aeruginosa, Streptococcus marcescens, Staphyloccus aureus and Bacillus cereus growth directly inhibited in the presence of EPC, although growth of Pseudomonas aeruginosa was promoted in the presence of fractions 3 and 6. At lower concentrations, the inhibition is bacteriostatic while at higher concentrations the inhibition is bactericidal. Various combinations of fractions inhibited the growth of test organisms but in case of Salmonella typhi at low concentration, growth remains steady but at higher concentration, the growth decreased.

Tricine SDS-PAGE

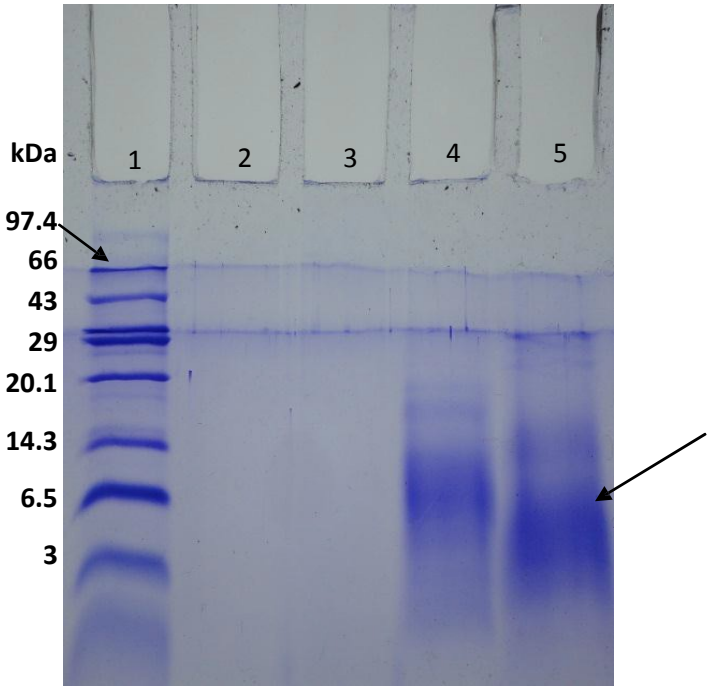

Figure 3: Tricine-SDS-PAGE of Partially Purified Extracellular Peptides of Lactobacillus rhamnosus Fb Growing on MRS Medium. Lane 1. Protein Molecular Weight Standards (Genei, Banglore), Lane 2-3 Blank, Lane 4 EPC after Ammonium Sulphate Precipitation and Lane 5 Desalted EPC

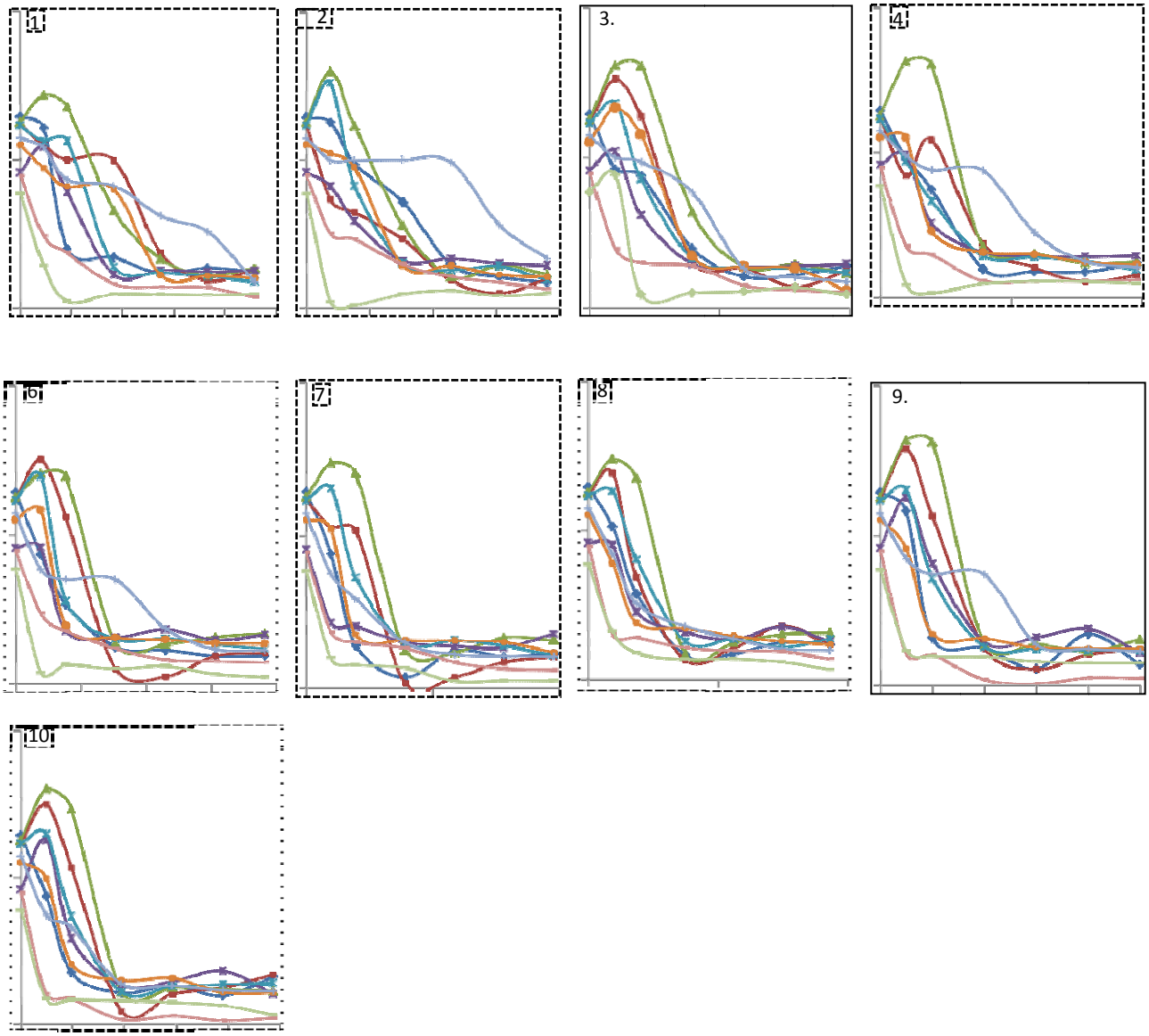

Figure 4: Antimicrobial Activity of Fractions 1-10 Obtained From Gel Filtration Chromatography Using Sephadex-25 in Nutrient Medium Against Food Borne and GIT Pathogens.

Table 2: Antimicrobial Activity of Fractions (1-20) Obtained From Gel Filtration Chromatography Using Sephadex G-25 in Nutrient Medium Against Food-Borne and GIT-Pathogens.

Fraction no.	Protein (µg/ml)	E.coli	Ent. aerogenes	S. typhi	Shigella sp.	Pr. vulgaris	Sr. marcescens	Ps. aeruginsa	Staph. aureus	Bacillus spp.
01	3	-	-	-	-	-	-	-	+	-
02	5	-	-	-	-	-	-	-	+	-
03	6	-	-	-	-	-	-	+	++	-
04	6	-	-	-	+	-	-	+	+++	-
05	7	-	-	-	+	-	-	++	+++	-
06	5	-	-	-	+	-	-	+	+++	-
07	3	-	+	-	+	-	-	++	+++	-
08	2	-	+	-	+	-	-	++	+++	-
09	2	-	+	-	-	-	-	++	+++	-
10	2	-	+	-	-	+	-	++	+++	-
11	2	-	+	+	-	+	-	++	+++	-
12	2	-	+	++	+	+	-	++	+++	-
13	1	-	+	-	+	+	-	++	+++	-
14	2	-	+	-	+	+	-	++	+++	-
15	1	-	+	-	+	+	-	++	+++	-
16	1	-	+	-	+	+	-	++	++	-
17	1	-	+	-	-	+	-	++	+	-
18	1	-	-	-	-	-	-	++	+	-
19	1	-	-	-	-	-	-	++	-	-
20	2	-	-	-	-	-	-	+	-	-
A	4	-	-	-	-	-	-	-	-	-
B	3	-	-	+	-	-	+	-	+++	-
C	1	-	+	++	+	-	++	-	+++	-
D	10	-	-	-	-	-	-	-	++	-
E	2	-	-	-	-	-	-	+	++	-
F	1	-	-	-	-	-	+	+	+++	-
G	2	-	-	-	-	-	+	+	+++	-
H	5	+	+	++	++	+	++	+++	+++	-
I	5	+	+	++	++	+	++	+++	+++	-

- bacteriostatic inhibition, + cidal (1 ml), ++ cidal (0.8 & 1 ml), +++ cidal (0.6, 0.8 & 1 ml), ++++ cidal (0.4, 0.6, 0.8, & 1 ml), *combination of fractions A: 1-5, B:6-10, C: 11-15, D: 16-20, E: 1- 10, F: 11-20, G: 1-20, H: desalted protein and I: EPC (without desalted)

Sensitivity of EPC to Heat, Proteolytic Enzymes and pH

Thermostability of EPC was observed to be strain-specific. Heat treatment of the EPC caused complete loss of activity against Pseudomonas aeruginosa and Bacillus sp and reduction in activity against E. coli, Enterococcus aerogenes, S. typhi, Shigella sp. (25%), Proteus vulgaris (19%), Streptococcus marcescens (16%) and Staphylococcus aureus (33%). Sensitivity of EPC to proteolytic enzymes like Proteinase K, Trypsin and Pepsin also showed strain specificity, it varied with test organisms further indicating the proteinaceous nature of the active agent. EPC treated with Proteinase K caused reduction in antimicrobial activity against all the test organisms. Trypsin treatment totally abolished the activity against P. aeruginosa, Staphyloccus aureus and Bacillus spp. EPC exhibited antimicrobial activity over broad pH range (2-9), but the extent of activity varied with test organisms. EPC showed activity against Enterococcus aerogenes, Streptococcus. marcescens and Staphylococcus aureus irrespective of pH.

Table 3: Antimicrobial Activity of EPC Determined at pH 2-9 Against Food-Borne and GIT-Pathogens in Nutrient Medium

	pH							
Test	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
E. coli	+	++	++	++	s	s	++	**s**
Ent. aerogenes	++ +	++ +	++	+	+	+ +	++	**+++**
S. typhi	+++ +	+++ +	+++ +	++ +	++ +	s	++ +	**s**
Shigella sp.	++	++ +	++	++	+	s	+	**+++**
Pr. vulgaris	+++ +	++	++ +	++	++	s	s	**+**
S. marcescens	++ +	+++ +	++ +	++ +	+++ +	+	++ +	**+++**
P. aeruginosa	++ +	s	++	+++ +	s	s	+++ +	**s**
S. aureus	++ +	+	+	++	++	+ +	++ +	**+++**
B.	s	s	s	s	s	s	s	**s**
B. cereus	s	s	s	s	s	s	s	**s**

+ bactericidal inhibition at 560 µg/ml; ++ ≥448 µg/ml; +++ ≥336 µg/ml; ++++ ≥224 µg/ml; s- bacteriostatic inhibition

Influence of Biofilm Grown Cells on Antimicrobial Activity

Surface anchored growth of L. rhamnosus Fb was induced by culturing in the presence of glass beads and used to determine antimicrobial activity along with cells cultured without glass beads. Antimicrobial activity of CFC and EPC was determined against Escherichia coli, Enterobacter aerogenes, Salmonella typhi, Shigella sp. and Staphylococcus aureus in nutrient broth. The growth of test organisms decreased with the increases in the amount of CFC and EPC in the culture medium, at higher concentration CFC and EPC exhibited bactericidal activity. Antimicrobial activity of EPC against E. coli, Enterobacter aerogenes, S. Typhi and Shigella sp., switch over to bactericidal activity from bacteriostatic inhibition in the biofilm grown cells. The bactericidal activity increases with the culture age and enhanced the production of EPC.

Table 4: Influence of Biofilm Grown Cells on the Antimicrobial Activity of EPC

Protein (µg/ml)	Incubation Conditions	E. coli	Ent. aerogenes	S. typhi	Shigella sp.	Staph. aureus
260	24 h Control	s	s	+	+	+
490	24 h Glassbeads	s	s	+	s	+
540	48 h Control	s	+	++	+	+
650	48 h Glassbeads	++	+++	+++	++	++
950	72 h Control	s	s	+	++	+
1026	72 h Glassbeads	+	+++	+++	++	++

s-bacteriostatic inhibition; +, ++, +++ extent of bactericidal inhibition from higher to lower protein concentrations

Influence of Storage on the Antimicrobial Activity of EPC and CFC

The storage stability of EPC and CFC at 4°C was evaluated in liquid medium at an interval of 0, 15, 30, 60 and 180 days. The activity of EPC remained unchanged against S. typhi, Streptococcus marcescens and Staphylococcus aureus even after 180 days. Antimicrobial activity of EPC lost against Enterobacter aerogenes after 60 days, while Proteus vulgaris and Pseudomonas aeruginosa lost after 180 days.

Table 5: Antimicrobial Activity of EPC and CFC During Storage at 4° C

Test Organisms	Time (Days)									
	0		15		30		60		180	
	EPC	CFC	EPC	CFC	EPC	CFC	EPC	CFC	EPC	CFC
E. coli	s	+	s	+	s	+	s	+	s	+
Ent.	++	+++	++	+++	++	+++	s	++	s	s
S. typhi	++	+++	++	+++	++	+++	+	+	+	+
Shigella sp.	++	+++	++	+++	++	+++	+	s	+	s
Pr. vulgaris	+	++	+	++	+	++	+	++	s	+
S.	++	+++	++	+++	++	+++	++	+	++	s
P.	++	++	++	++	++	++	+	+	s	+
Staph.	+++	++	++	++	++	++	++	+	++	+
B. cereus	s	s	s	s	s	s	s	s	s	**s**

s-bacteriostatic inhibition, +, ++, +++, ++++ indicates extent of bactericidal inhibition from higher to lower protein concentration.

Production of Antimicrobial Peptides in Skim Milk

10% skim milk medium was selected for the determination of antimicrobial activity and production of antimicrobial peptides. Following 24 h of incubation total protein in CFC and EPC was 298 and 330 µg/ml and exhibit antimicrobial activity against E. coli, Enterobacter aerogenes, S. typhi, Shigella sp., Pseudomonas aeruginosa and Staphylococcus aureus. EPC exert bactericidal activity against Shigella sp., Pseudomonas aeruginosa and Staphylococcus aureus (Table 5). Growth of test organisms gradually decreased in the presence of increasing concentration of CFC and EPC.

Table 6: Antimicrobial Activity of CFC and EPC of Lactobacillus spp. Growing in Skim Milk.

Protein (µg/ml	E. coli	Ent. aerogenes	S. typhi	Shigella	P. aeruginosa	S. aureus
132	s	s	s	s	s	**s**
198	s	s	s	s	s	**s**
264	s	s	s	s	c	**s**
330	**s**	**s**	**s**	**c**	**c**	**c**

DISCUSSION

Lactobacillus is shown to be a promising probiotic strain with regard to its survival properties viz. acid, bile, NaCl and phenol tolerance and conditions mimicking the main obstacles of the GI-tract. In addition, it has aggregation, coaggregation ability with

enteropathogens and Candida spp., and broad antimicrobial spectrum against food-borne and human pathogens; a fact that improves the potential of the probiotic bacteria as a food additive, as well as due to broad antimicrobial spectrum can aid in its establishment and colonization in the gut. Intestinal Lactobacilli in humans are closely associated with the host's health because they are an important biodefense factor in preventing colonization and subsequent proliferation of pathogenic bacteria in the intestine. The nature of the inhibitory substances has not been characterized therefore present study emphasized on the characterization of extracellular antimicrobial proteins.

Antimicrobial activity of EPC was thermostable (60 min at 100°C), thermostability was evidenced from the bactericidal activity of heat treated EPC, heat treatment caused complete loss of activity against P. aeruginosa and Bacillus spp. Heat stability of antimicrobial proteins has been suggested to be the major feature of low molecular weight bacteriocins and arises from complex pattern of disulphide intramolecular bonds that stabilize secondary structures by reducing the number of possible unfolded structures (Cintas et al., 1995). Currently we do not know the reasons for the stability of antimicrobial peptides but the work is in progress to further characterize the structure and functions of EPC. Sensitivity of EPC to proteolytic enzymes like Proteinase K, Trypsin and Pepsin shows strain specificity, it varies with test organisms which further indicate the proteinaceous nature of the active agent. Proteinase K exhibits broad substrate specificity. Proteinase K degrades many proteins in the native state even in the presence of detergent. Cleavage is the peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids, which block alpha amino group (Ebeling et al., 1974). Trypsin cleaves peptide chains mainly at the carboxyl site of the amino acids lysine or arginine, except when either is followed by proline. Pepsin is most efficient in cleaving peptide bonds between hydrophobic and preferably aromatic amino acids such as phenylalanine, tryptophan, and tyrosine (Dunn, 2001). EPC is less sensitive to pepsin except against P. aeruginosa and B. subtilis. Antimicrobial activity of EPC shows activity over broad pH range (2-9) but the activity varies with test organisms. Moreover, activity of EPC at neutral and alkaline pH suggests that the antimicrobial activity of peptides is not because of low pH.

CONCLUSION

The purpose of the study was to screen human origin probiotic Lactobacillus strains, as the gut microbiota varies among individuals, depending on factors such as diet, lifestyle and genetic factors. Lactobacilli is a diverse group of bacteria, to which we are exposed in our day-to-day life as they are commonly present in foods such as fruits, vegetables, and fermented foods. Some Lactobacillus species that reside in the gastrointestinal tract of mammals and vagina of humans and animals are associated with the well-being of the host. There are numerous probiotic strains available for commercial use, but isolation and characterization of novel strains is still a fascinating research area particularly in India. Therefore, one of the aims of the work was to characterize autochthonous human origin Lactobacillus strains, for their probiotic properties and evaluation with reference to well-documented standard strains Lactobacillus rhamnosus GG. There are many strains among lactobacilli with documented probiotic ability, thus they have a more application in prevention of infection. During the evaluation of acid and bile tolerance, the growth abilities of the isolated strains showed that all the isolates. In this study could not tolerate the pH $\leq$ 2 but could grow in the presence of bile salt.

Similar findings were reported previously and showed that the protective effect of food matrix prevents bacteria from bile exposure and hence, gives rise to the increased bile resistance of microbial strains. Acid and bile salt tolerance have been demonstrated as important virulence mechanisms for pathogenic microorganisms.

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