CHAPTER 4

Production and Characterization of Extracellular Antimicrobial Peptides of *Lactobacillus rhamnosus Fb*

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Introduction

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 (Stanton *et al*., 2001). A probiotic is a 'live microbial food supplements that beneficially affects the host animal by improving the intestinal microbial balance' (Fuller, 1989). 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 (Metchnikoff, 1907). 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 human origin *L. rhamnosus* Fb strain against food-spoilage organisms and human pathogens.

Materials and Methods

Bacterial strain and growth condition

Lactobacillus rhamnosus Fb JX406746 was isolated from the human infant feces using De Man Rogosa and Sharpe (MRS) medium (Himedia, Mumbai, India) at 37°C. Stock culture was maintained in 10% skim milk at 4°C.

Preparation of cell free culture filtrate and extracellular protein concentrate of L. rhamnosus Fb

2% inoculum of *L. rhamnosus* Fb was used to inoculate 1 l 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 (Bradford, 1976).

Antimicrobial activity against food-borne and gastrointestinal pathogens

Determination of antimicrobial activity by well-diffusion agar assay

Antimicrobial activity of CFC and EPC was determined by well-diffusion agar assay (British Standard 1974) against the test pathogens listed in Table. 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 6 h 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% inocula of activated culture of *L. rhamnosus* Fb 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 was 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 L. rhamnosus Fb in skim milk

1 ml of 10 times concentrated biomass was inoculated in 400 ml of skim milk (incubated at 37°C, 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 (Banglore). The gel was stained with Coomassie Brilliant Blue R-250 and destained using methanol:acetic acid: water (40:10:50).

Results

Antimicrobial activity of CFC and EPC of L. rhamnosus Fb

CFC and EPC of *L. rhamnosus* Fb 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*, *Staph. aureus* and *Vibrio cholerae* (Fig. 1a) 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 *Ps. aeruginosa.* Fig. 1b shows the diffuse band of EPC having molecular weight of *ca* 6 kDa.

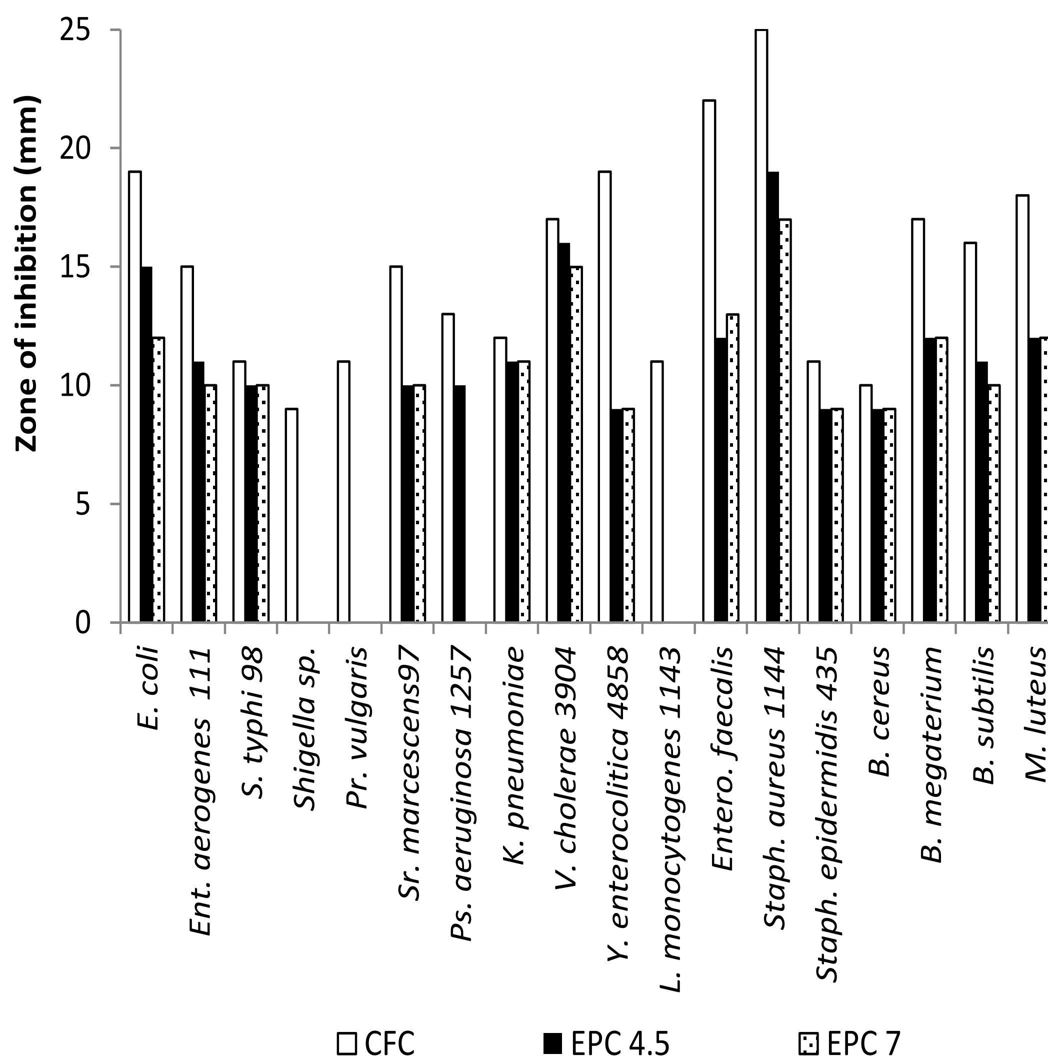

Fig.1a Antimicrobial activity of CFC and EPC (pH 4.5 and 7) prepared from 24 h *L. rhamnosus* Fb growing on MRS medium determined against food-borne and gastrointestinal pathogens by well-diffusion agar assay.

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 (i) synergistic activity against *E. coli* and *Sr. marcescens*, (ii) individual fractions and/or in combination exhibit the activity against *Ent. aerogenes*, *S. typhi, Shigella* sp., *Ps. aeruginosa* and *Staph. aureus,* and (iii) exert activity individually against *Pr. vulgaris* but not in combination with other fractions (Table 1). In certain instances, low concentration of EPC was observed to promote growth but was inhibitory at higher concentration in the case of *Ent. aerogenes* and *S. typhi.* In case of *Shigella*

sp. and *Pr. 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. *Ps. aeruginosa, Sr. marcescens, Staph. aureus* and *B. cereus* growth directly inhibited in the presence of EPC, although growth of *Ps. 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 (Fig. 2a). Various combinations of fractions inhibited the growth of test organisms but in case of *S. typhi* at low concentration, growth remains steady but at higher concentration, the growth decreased (Fig. 2b).

Tricine SDS-PAGE

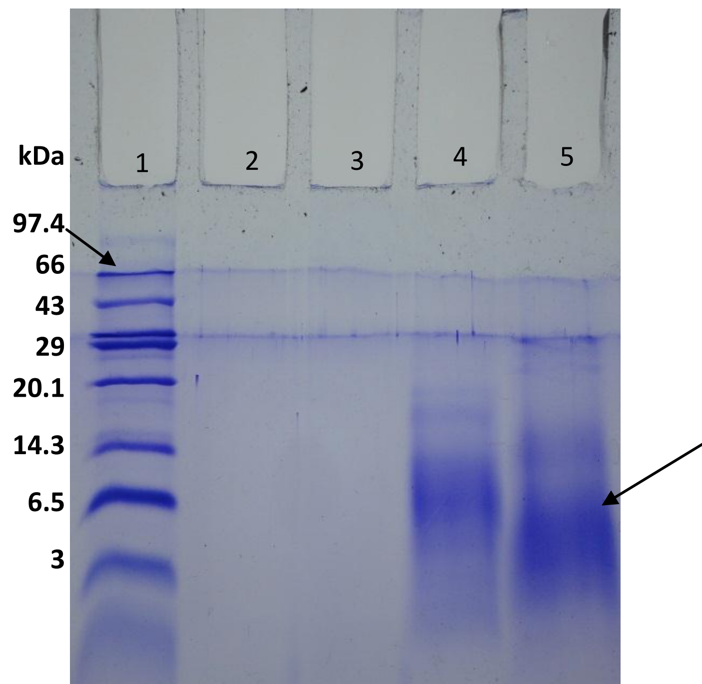

Fig. 1b Tricine-SDS-PAGE of partially purified extracellular peptides of *Lactobacillus rhamnosus* Fb growing on MRS medium. Lane 1. Protein molecular weight standards (Genei, Banglore), lane 2-3 blank, lane 4 EPC after ammonium sulphate precipitation and lane 5 desalted EPC

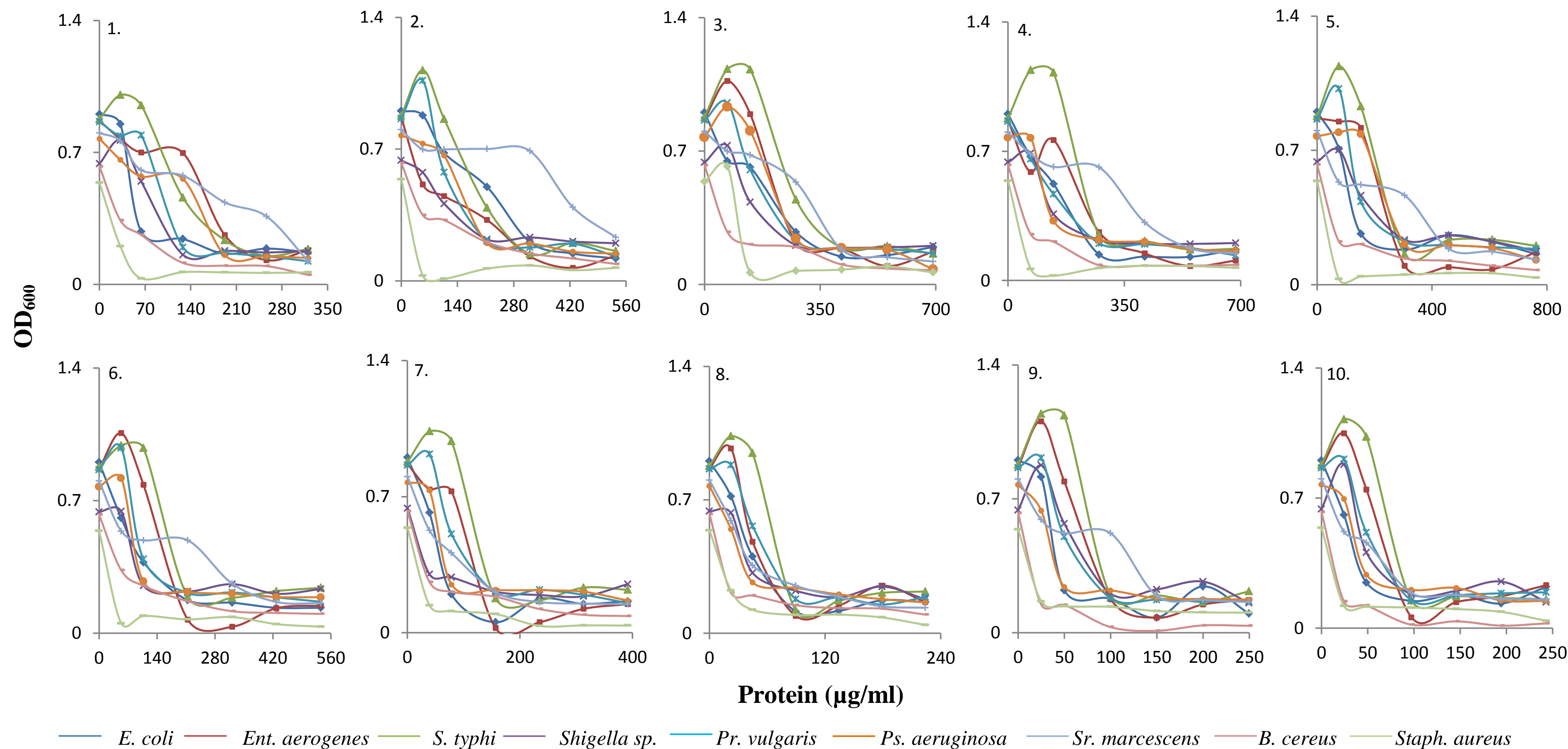

Fig. 2a Antimicrobial activity of fractions (1-10) obtained from gel filtration chromatography using Sephadex G-25 in nutrient medium against food-borne and GIT-pathogens

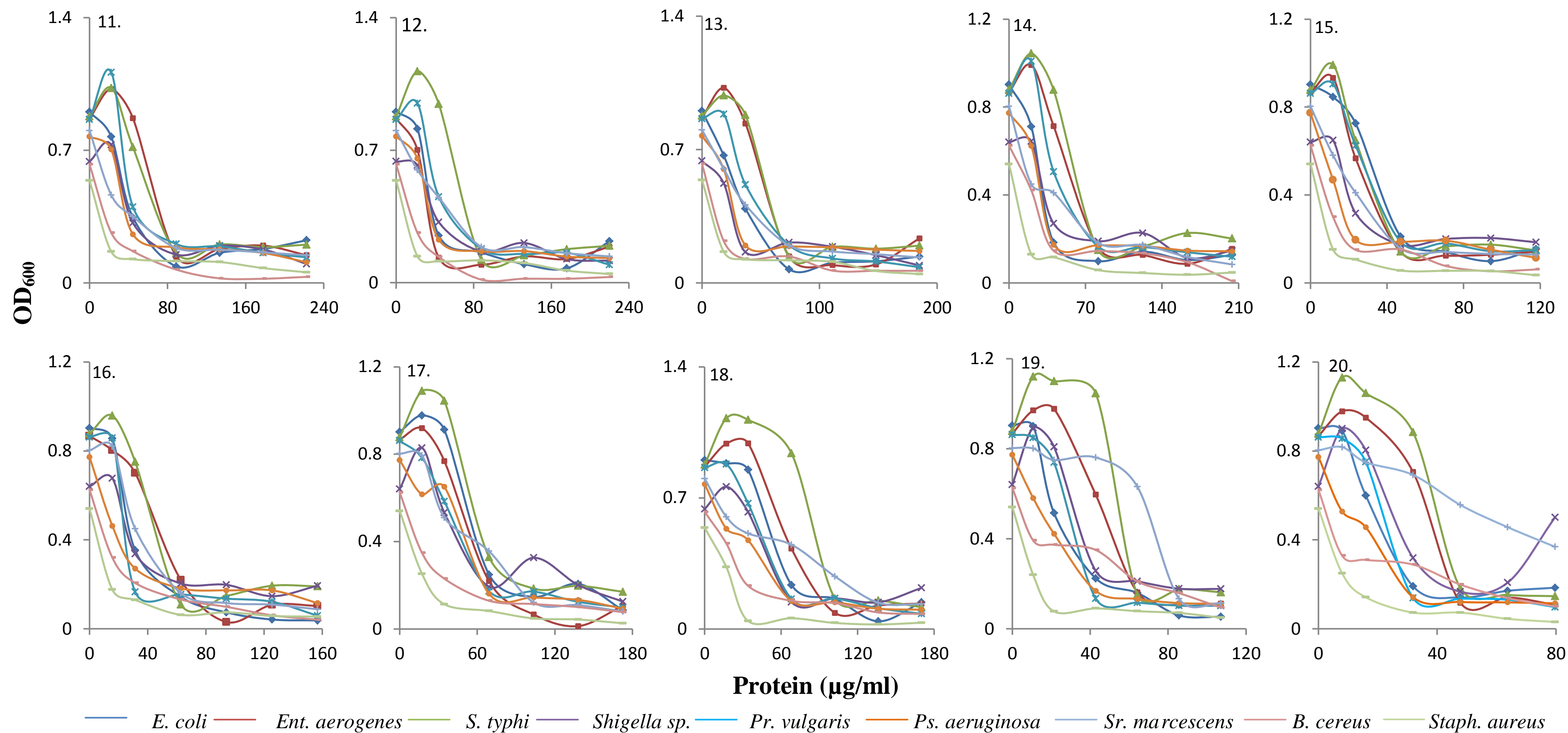

Fig. 2a Antimicrobial activity of fractions (1-20) obtained from gel filtration chromatography using Sephadex G-25 in nutrient medium against food-borne and GIT-pathogens

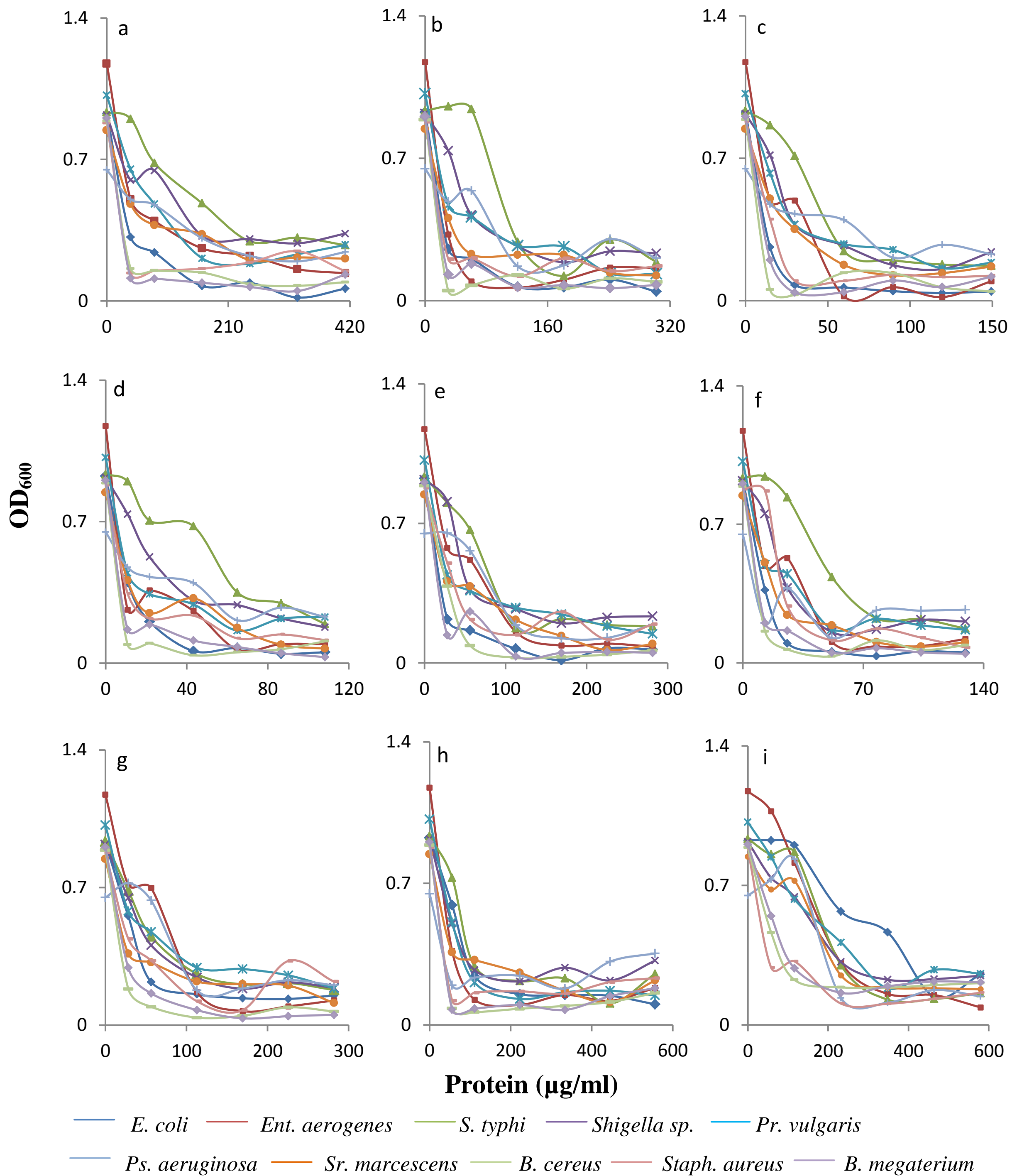

Fig. 2b Antimicrobial activity of various combinations of fractions (a) 1-5, (b) 6-10, (c) 11-15, (d) 16-20, (e) 1-10, (f) 11-20, (g) 1-20, (h) desalted EPC, and (i) EPC obtained from gel filtration chromatography using Sephadex G-25 in nutrient medium against food-borne and GIT-pathogens

Table 1. 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.*
1	320	-	-	-	-	-	-	-	+	-
2	534	-	-	-	-	-	-	-	+	-
3	692	-	-	-	-	-	-	+	+++	-
4	685	-	-	-	+	-	-	+	++++	-
5	762	-	-	-	++	-	-	++	++++	-
6	536	-	-	-	++	-	-	+	++++	-
7	392	-	++	-	++	-	-	++	++++	-
8	224	-	+	-	++	-	-	+++	++++	-
9	250	-	+	-	-	-	-	+++	++++	-
10	243	-	+	-	-	+	-	++	++++	-
11	222	-	++	+	-	+	-	+++	++++	-
12	220	-	+++	+++	+	+	-	+++	++++	-
13	185	-	+++	-	++	++	-	+++	++++	-
14	204	-	+++	-	++	++	-	+++	++++	-
15	118	-	+	-	++	++	-	+++	++++	-
16	157	-	+	-	++	+	-	+++	+++	-
17	173	-	+	-	-	+	-	+++	+	-
18	170	-	-	-	-	-	-	++	+	-
19	107	-	-	-	-	-	-	++	-	-
20	280	-	-	-	-	-	-	+	-	-
*A	412	-	-	-	-	-	-	-	-	-
B	302	-	-	+	-	-	++	-	++++	-
C	149	-	++	+++	+	-	+++	-	++++	-
D	108	-	-	-	-	-	-	-	+++	-
E	282	-	-	-	-	-	-	+	+++	-
F	129	-	-	-	-	-	+	+	++++	-
G	282	-	-	-	-	-	+	+	++++	-
H	557	+	+++	++++	+++	+	+++	+++++	+++++	-
I	590	+	+++	+++	+++	++	++++	++++	+++++	-

- 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 *Ps. aeruginosa* and *Bacillus* spp and reduction in activity against *E. coli, Ent. aerogenes, S. typhi, Shigella* sp. (25%), *Pr. vulgaris* (19%), *Sr. marcescens* (16%) and *Staph. aureus* (33%) (Fig. 3a). 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 *Ps. aeruginosa*, *Staph. aureus* and *Bacillus* spp. (Fig. 3b). EPC exhibited antimicrobial activity over broad pH range (2-9), but the extent of activity varied with test organisms (Table 2). EPC showed activity against *Ent. aerogenes*, *Sr. marcescens* and *Staph. aureus* irrespective of pH (Fig. 4).

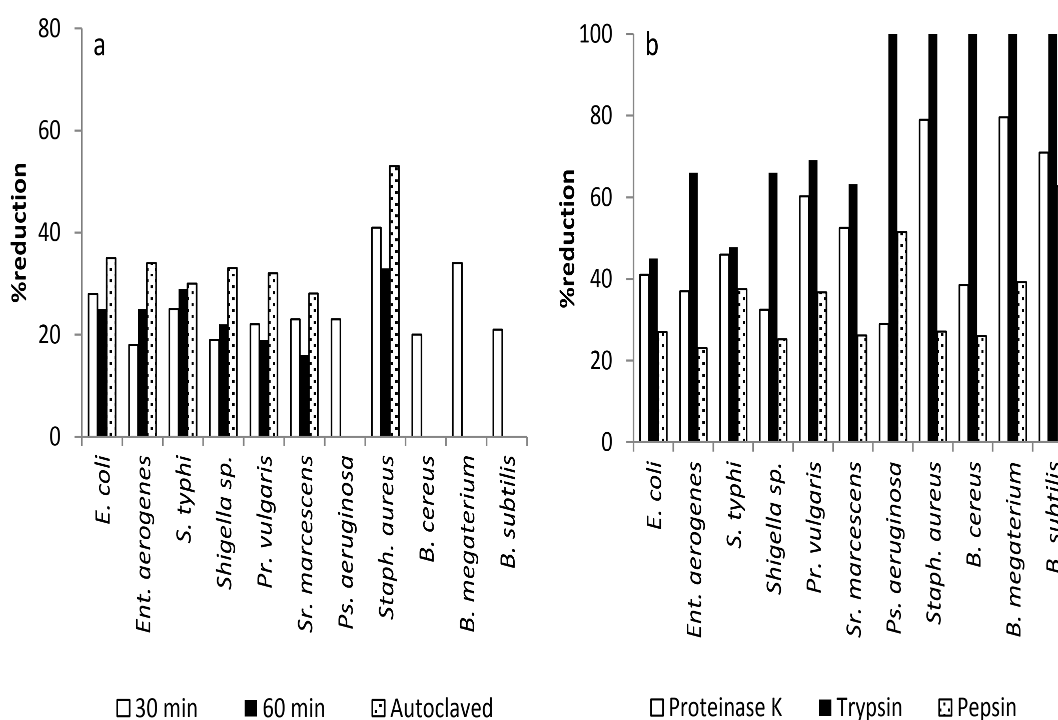

Fig. 3a & b Sensitivity of EPC to heat-treatment and proteolytic enzymes and reduction (%) in the antimicrobial activity.

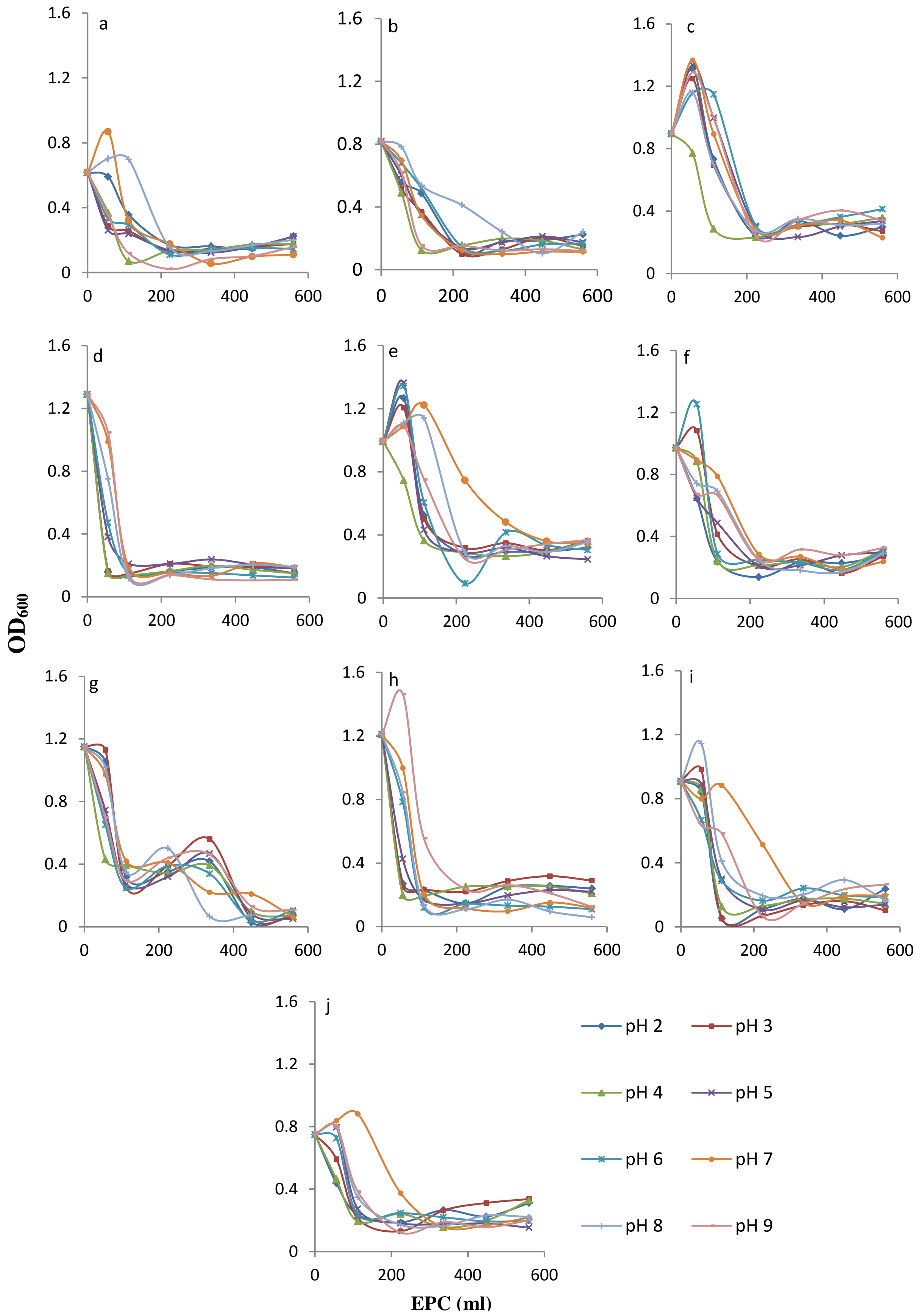

Fig. 4 Influence of pH on the antimicrobial activity of EPC of *L. rhamnosus* Fb against a) *E. coli*, b) *Ent. aerogenes*, c) *S. typhi*, d) *Shigella* sp., e) *Pr. vulgaris*, f) *Sr. marcescens,* g) *Ps. aeruginosa*, h) *Staph. aureus*, i) *B. cereus* and j) *B. megaterium*

Table 2. Antimicrobial activity of EPC determined at pH 2-9 against food-borne and GIT-pathogens in nutrient medium

Test organisms	pH							
	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	+
Sr. marcescens	+++	++++	+++	+++	++++	+	+++	+++
Ps. aeruginosa	+++	s	++	++++	s	s	++++	s
Staph. aureus	+++	+	+	++	++	++	+++	+++
B. megaterium	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

Production of antimicrobial peptides by L. rhamnosus Fb

Antimicrobial activity of Cell Free Culture filtrates (CFC) against the test organisms increased with the culture age of *L. rhamnosus* Fb and became stable when culture reached the stationary phase. A similar antimicrobial spectrum of CFC filtrate was observed against all the test organisms (Fig. 5 b & d). The antimicrobial spectrum of Extracellular Protein Concentrate (EPC) against the test organisms changed with culture age. Antimicrobial activity against *E. coli* was observed to be associated with log phase and it decreased beyond 18 h; i.e. culture in transition phase and it decreases later with increasing culture age (Fig. 5 a & c). Antimicrobial activity against *Ent. aerogenes, B. subtilis, B. megaterium* and *Staph. aureus* appeared after 6 h of growth and increased with culture age and then remained constant in stationary phase. Whereas activity against *Shigella spp., Ps. aeruginosa* and *B. cereus* was observed after 12 h of growth and increased with culture age upto 30 h before decreasing marginally. Antimicrobial activity against *S. typhi* and *P. vulgaris* was observed in stationary phase and it did not change much with the increase in culture age. The antimicrobial extracellular proteins are thus produced during exponential and stationary phases.

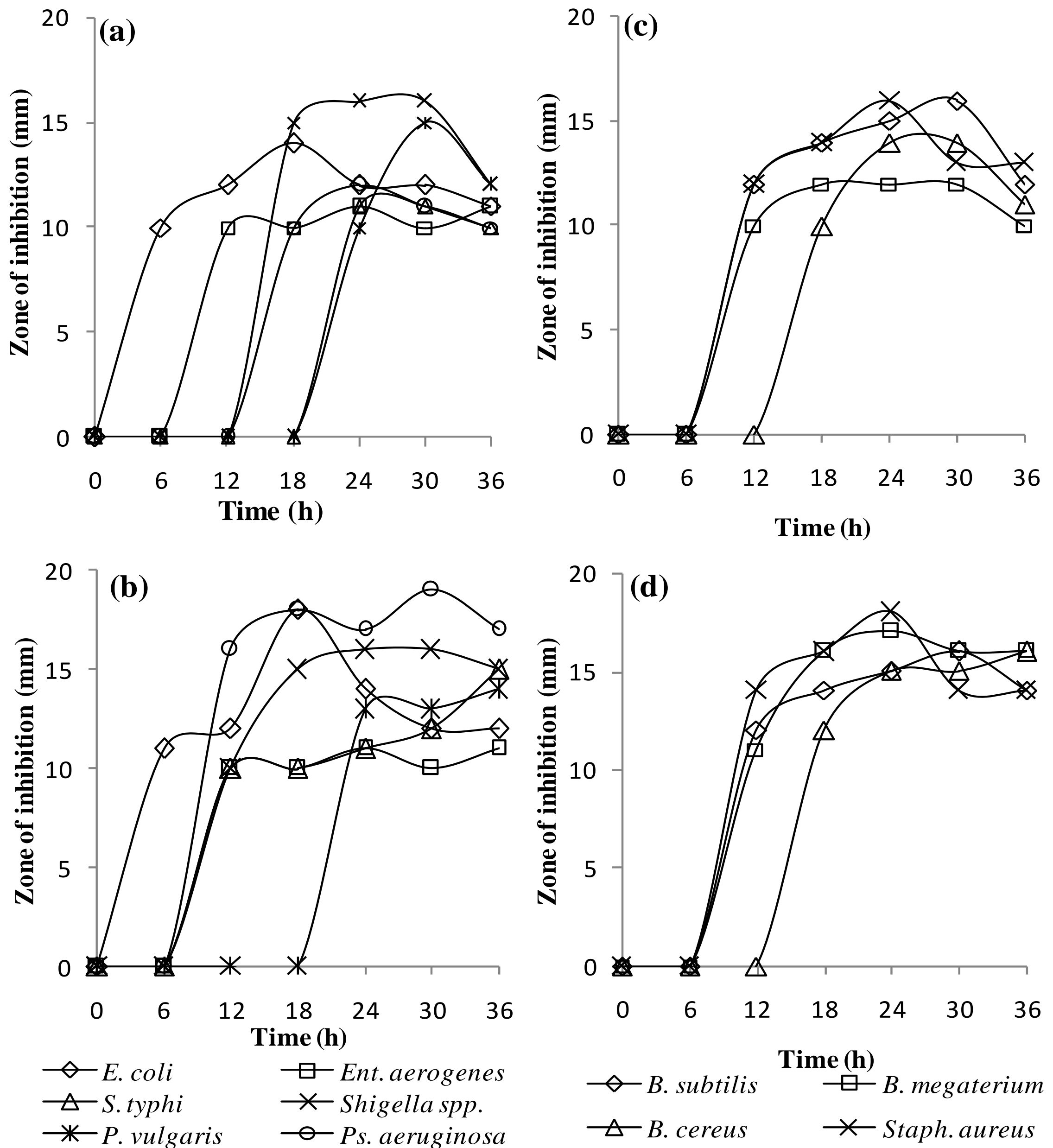

Fig. 5 Association of antimicrobial spectrum of EPC produced *by L. rhamnosus* Fb culture during logarithmic and idiophasic growth, (a) & (c) Antimicrobial activity of Extracellular Protein Concentrate (EPC) and (b & d) Cell Free Culture Filtrate (CFC) of *Lactobacillus rhamnosus* produced at the different growth phases (6, 12, 18, 24, 30 and 36 h) determined by well-diffusion assay against *E. coli, Ent. aerogenes, S. typhi, Shigella sp., Pr. vulgaris, Ps. aeruginosa, B. subtilis, B. megaterium, B. cereus* and *Staph. aureus*

Influence of biofilm grown cells on antimicrobial activity

Surface anchored growth of *L. rhamnosus* Fb was induced by culturing in the presence of glassbeads and used to determine antimicrobial activity along with cells cultured without glassbeads. 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. (Fig. 6). Antimicrobial activity of EPC against *E. coli, Ent. 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 (Table 3) and enhanced the production of EPC.

Table 3. 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*, *Sr. marcescens* and *Staph. aureus* even after 180 days. Antimicrobial activity of EPC lost against *Ent. aerogenes* after 60 days, while *Pr. vulgaris* and *Ps. aeruginosa* lost after 180 days (Table 4 and Fig. 7).

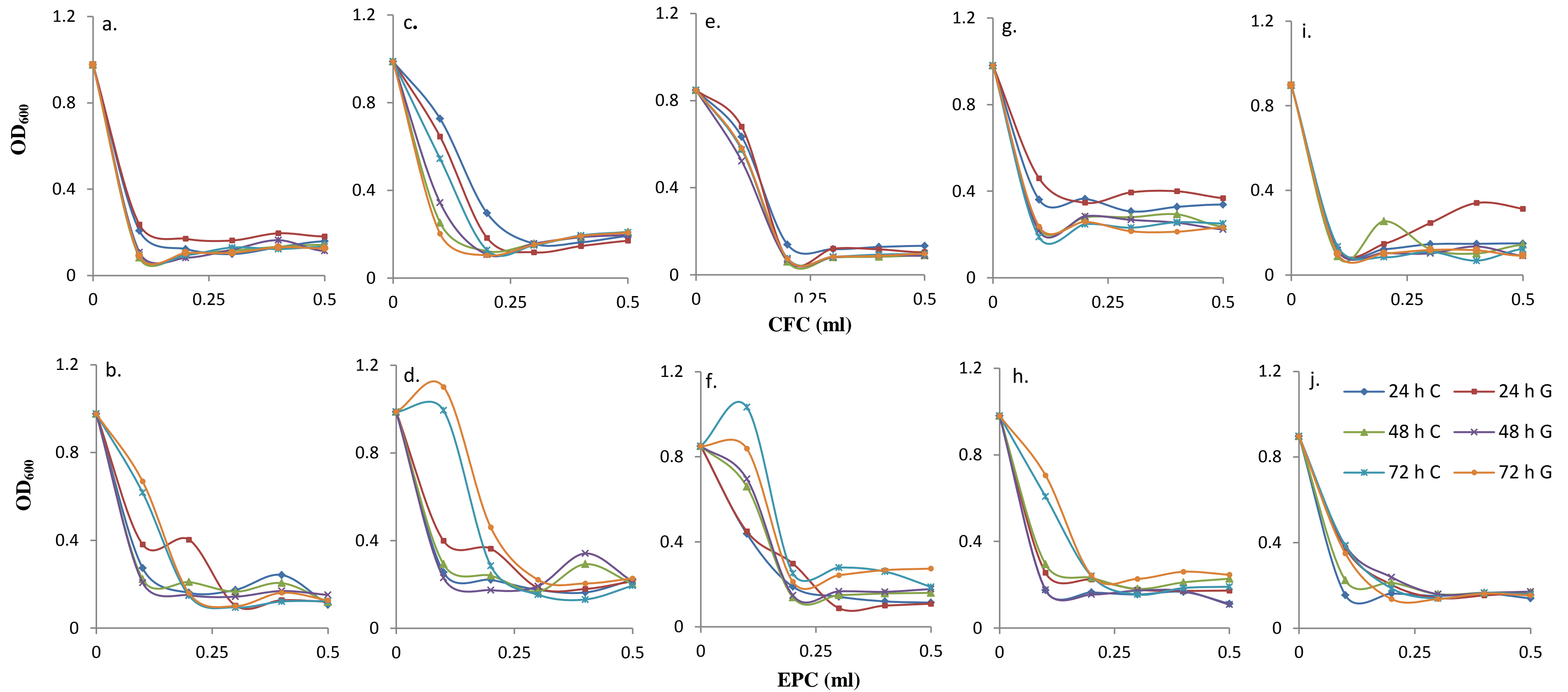

Fig. 6 Influence of incubation time (24, 48 & 72 h) and surface anchored growth with or without glass beads (for biofilm induction) on the antimicrobial activity of CFC and EPC of *L. rhamnosus* Fb against a & b) *E. coli*, c & d) *Ent. aerogenes*, e & f) *S. typhi*, g & h) *Shigella* sp. and i & j) *Staph. aureus*

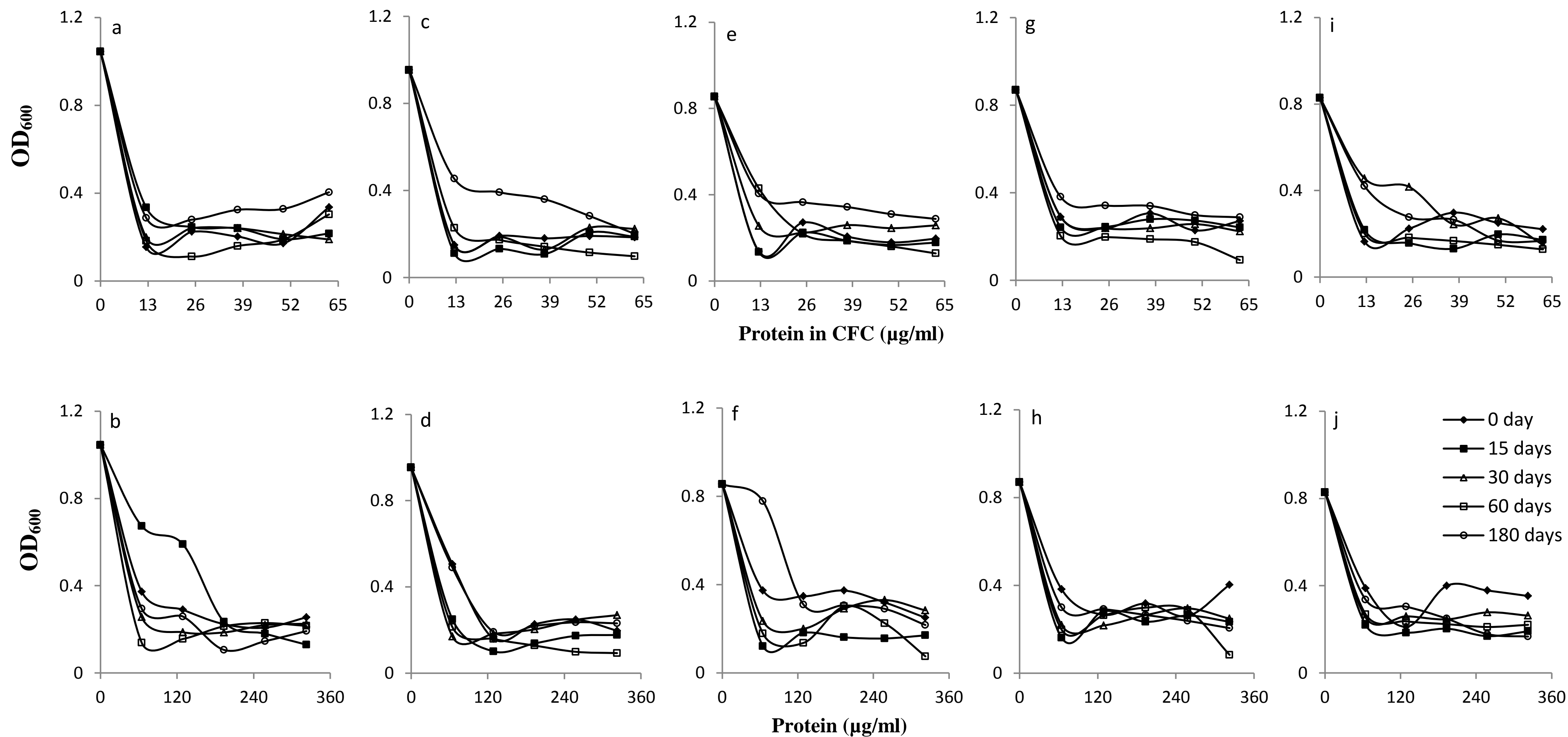

Fig. 7 Antimicrobial activity of CFC and EPC of *L. rhamnosus* Fb during the storage (0-180 days, 4°C) determined in Nutrient medium against a & b) *E. coli*, c & d) *Ent. aerogenes*, e & f) *S. typhi*, g & h) *Shigella* sp. and i & j) *Pr. vulgaris*

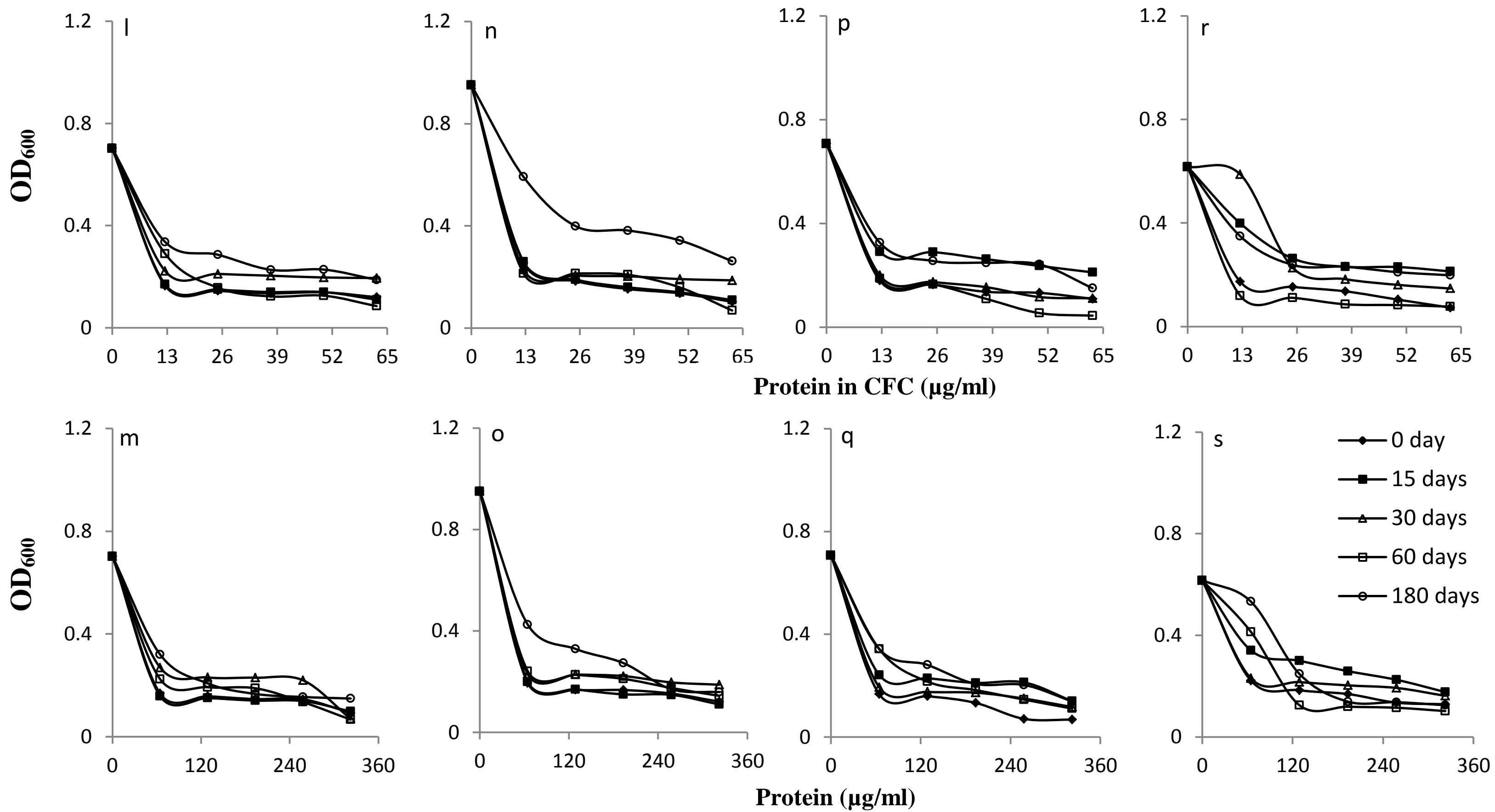

Fig. 7 Antimicrobial activity of CFC and EPC of *L. rhamnosus* Fb during the storage (0-180 days, 4°C) determined in Nutrient medium against l & m) *Ps. aeruginosa*, n & o) *Sr. marcescens*, p & q) *Staph. aureus*, r & s) *B. cereus*

Table 4. 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. aerogenes	+++	++++	+++	++++	+++	++++	s	+++	s	s
S. typhi	++	++++	++	++++	++	++++	++	++	++	++
Shigella sp.	++	++++	++	++++	++	++++	+	s	+	s
Pr. vulgaris	+	+++	+	+++	+	+++	+	+++	s	++
Sr. marcescens	+++	++++	+++	++++	+++	++++	+++	++	+++	s
Ps. aeruginosa	++	++	++	++	++	++	++	++	s	++
Staph. aureus	++++	++	+++	++	+++	++	+++	++	+++	++
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, Ent. aerogenes, S. typhi, Shigella sp., Ps. aeruginosa* and *Staph. aureus*.

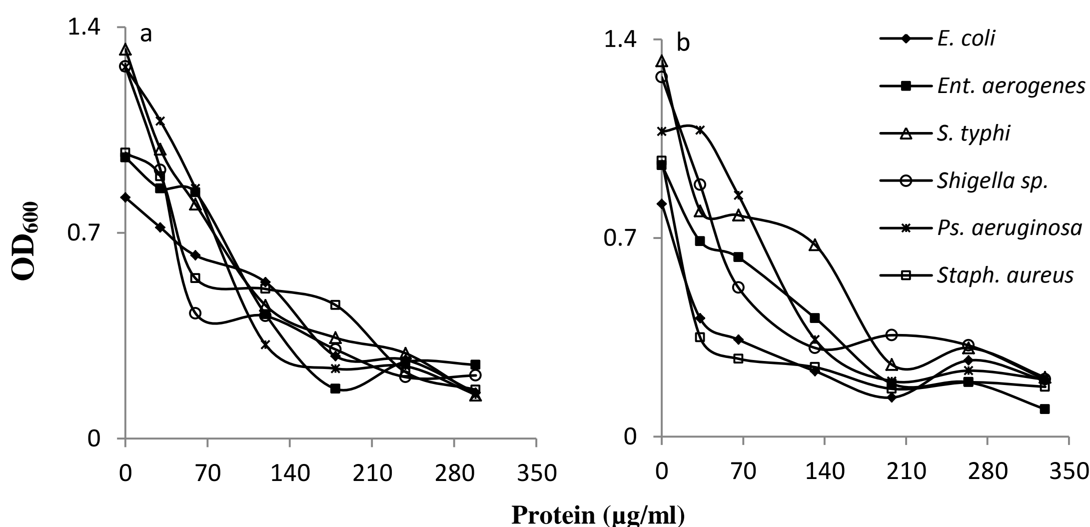

Fig. 8 Antimicrobial activity of a) CFC and b) EPC obtained from *L. rhamnosus* Fb growing skim milk medium

EPC exert bactericidal activity against *Shigella* sp., *Ps. aeruginosa* and *Staph. aureus* (Table 5). Growth of test organisms gradually decreased in the presence of increasing concentration of CFC and EPC (Fig. 8)

Table 5. Antimicrobial activity of CFC and EPC of *L. rhamnosus* Fb growing in skim milk

Protein (μg/ml)	*E. coli*	*Ent. aerogenes*	*S. typhi*	*Shigella*	*Ps. aeruginosa*	*Staph. 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

s- bacteriostatic; c-bactericidal inhibition

Discussion

Human origin *Lactobacillus rhamnosus* Fb JX406746 was 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 (Pithva *et al*., 2014). 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 have not been characterized especially from *Lactobacillus rhamnosus* strains therefore present study emphasized on the characterization of extracellular antimicrobial proteins.

Lu *et al*. (2009) identified and characterized seven small peptides of which one active thermostable peptide (<1000 Da) from *L. rhamnosus* GG exhibited antibacterial activity against both Gram-negative (*Escherichia coli* EAEC 042 and *Salmonella typhi*) and with less potency against Gram-positive (*Staphylococcus aureus*) bacteria. Ambalam et al. (2009) characterized mixture of low molecular weight glycopeptides (4 kDa) of *L. rhamnosus* 231 that exert activity against *E. coli, Ent. aerogenes,*

Salmonella spp., Ps. aeruginosa, Staph. aureus, Helicobacter pylori, Campylobacter jejuni, B. cereus, B. megaterium and *Listeria monocytogenes.* In this context, we have characterized the extracellular low molecular weight peptides of *L. rhamnosus* Fb active against food-borne and human pathogens such as *E. coli, Ent. aerogenes, S. typhi, Sr. marcescens, Ps. aeruginosa, K. pneumoniae*, *Y. enterocolitica, V. cholera, Entero. faecalis, Staph. aureus, Bacillus spp.* and *M. luteus*. *L. rhamnosus* Fb produces other antimicrobial metabolites as evidenced from the antimicrobial activity of CFC was higher in comparison to EPC. This evidence suggests the multifactorial nature of antimicrobial activity and synergistic action. Characterization of other antimicrobial metabolites remains to be identified.

L. rhamnosus Fb produced extracellularly a mixture of low molecular weight peptides, was evidenced from the experimental evidences data described below. Variable sensitivity of antimicrobial activity of EPC against test organisms implies the presence of a more than one antimicrobial peptides active against different test organisms. Gel filtration chromatography (Sephadex G-25) of EPC provides additional evidence of inhibitory protein to be low molecular weight. Gel electrophoresis (Tricine-SDS-PAGE) of EPC shows that proteins present in EPC resolve into diffuse band, diffuse band representing low molecular weight peptides *ca.* 6 kDa (Schagger and Von Jagow, 1987).

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 *Ps. 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 indicates 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. The predominant site of

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 *Ps. 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.

Antimicrobial spectrum of the EPC changes with culture age, indicates that the antimicrobial activity is attributed to mixture of antimicrobial peptides. EPC exhibited antimicrobial activity against test organisms at different culture age *i.e*., at the culture age of 6 h against *E. coli,* 12 h against *Ent. aerogenes, B. subtilis, B. megaterium* and *Staph. aureus,* 18 h against *Shigella* sp., *Ps. aeruginosa* and *B. cereus,* and 24 h against *S. typhi and Pr. vulgaris.* Thus, EPC is a dynamic mixture of antimicrobial peptides, since the antimicrobial spectrum of the EPC is intimately associated with the growth phase. The following experimental evidences related to heat stability, sensitivity to proteolytic enzymes, and gel permeation chromatography further implicate the presence and involvement of more than one antimicrobial peptides in the EPC. Moreover, biofilm-grown cells exert intensely higher inhibitory activity perhaps associated with the higher protein production in the presence of glassbeads. Storage of the EPC at 4°C for ≥180 days remains unchanged except against *Ent. aerogenes*, *Ps. aeruginosa* and *Pr. vulgaris*.

Sevin (2004) pointed the existence of the least characterized fourth class of complex bacteriocin. Bacteriocins are proteinaceous antimicrobial compounds, produced by LAB, that exhibit a bactericidal activity against taxonomically closely related organisms. A diffuse band of low molecular weight bacteriocins, having narrow spectrum of antimicrobial activity has been reported with *L. acidophilus* (Deraz *et al*., 2005). Antimicrobial peptides of *L. rhamnosus* Fb possessing activity against Gram-positive as well as Gram-negative bacteria are therefore are very different from *L. acidophilus* bacteriocins. *L. rhamnosus* GG has been reported to secrete a low

molecular weight, heat-stable, inhibitory substance distinct from lactic and acetic acids, that is active against a wide range of Gram-positive and Gram-negative bacteria (Silva *et al*., 1987).

Probiotic strain *L. rhamnosus* Fb intended for the food application must exert the functional activities as in the complex MRS medium. Fermented milk with *L. rhamnosus* Fb provides functional benefits as it exhibits broad antimicrobial spectrum against gastrointestinal and food-borne pathogens.

In conclusion, antimicrobial activity of *L. rhamnosus* Fb against food-borne and gastrointestinal pathogens is partially attributed to thermostable low molecular weight peptides. Purification and characterization of antimicrobial peptides of *Lactobacillus rhamnosus* strain provides novel approach as anti-infective drug. It has also potential as food additive, treatment of antibiotic resistant organisms. Probiotic formulations derived from this culture can prove to be useful in gastrointestinal problems including various forms of dysbacteriosis. Further studies are being undertaken for the identification, characterization and sequencing of antimicrobial peptides. *In vivo* experimentations are underway to investigate the possibility of using these novel antimicrobial peptides as anti-infective agent.