FINAL REPORT

UGC major Research project (no. 42-478/ 2013 SR)

In vitro evaluation of anticancer and antimicrobial properties of human lactic acid bacteria

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Summary of the project

Project No. and title : 42-478/2013 (SR); *In vitro* evaluation of anticancer and antimicrobial properties of human lactic acid bacteria

The commensal microflora in the gut, mouth and vagina in humans contain lactic acid bacteria (LAB) that are known to exist in harmonal relationship with the respective ecosystems. Some of the genera such as *Lactobacillus* spp, *Pediococcus* spp. are known to perform variety of functions that benefit the host. One of the important function of LAB is to secrete lactic acid and other antimicrobial compounds such as bacteriocins that inhibit the pathogens. Most of the bacteriocins secreted by LAB are known to inhibit only closely related Gram-positive bacteria. But the therapeutic potential of broad-spectrum bacteriocins that inhibit both Gram-positive and Gram-negative pathogens is believed to be superior. Thus, herein, we screened the LAB from saliva (87 isolates), vaginal swab (92 isolates) and fecal samples (110 isolates) of healthy local Indian population for the isolation of LAB that were screened for broad spectrum antimicrobial activities against both Gram-positive and Gram-negative pathogens. All the saliva swab sample isolates inhibited only closely related LAB and none of the human pathogenic strain, whereas, 40% (37 isolates) of the vaginal isolates had antimicrobial activities against at least one pathogenic indicator strains. One vaginal *Lactobacillus crispatus* 3b and 3 vaginal enterococcal isolates *E. faecium* 12a and L12b, and *E. hirae* 20c showed broad-spectrum antimicrobial activities due to proteinaceous metabolites and thus, were selected for the purification of bacteriocins. Seven of the fecal lactobacilli isolated L13, L14, L18, L32, S30, S45 and S49 had broad-spectrum antimicrobial activities against human pathogens but the antimicrobial activities were due to lactic acid secretion and not due to bacteriocins. The L14 was identified as *L. plantarum*, L32 as *L. fermentum* and S45 as *L. pentosus*. The rest of the isolates did not matched the known lactobacilli species. The type of interaction between the CS of the bacteriocin-containing CS of *L. crispatus* and *E. faecium* 12a showed that they synergized the antimicrobial potential of number of first line of antibiotics against *Pseudomonas aeruginosa* and *Salmonella enteric*.

Further, we purified and characterized the bacteriocin from the CS of *E. faecium* 12a by using chromatography techniques and SDS polyacrylamide gel electrophoresis. The bacteriocin of 12a appears to be a high molecular weight antimicrobial protein of molecular weight 65 kDa. Further

the anticancer activities of the CS of *E. faecium* 12a, L12b and *E. hirae* 20c were evaluated by MTT assay. The secreted metabolites of three enterococcal strains, *E. hirae* 20c, *E. faecium* 12a and L12b, selectively inhibited the *in vitro* proliferation of various human cancer cell lines in a dose-dependent manner but had no activity against normal human peripheral blood monocytes. Further, proteinase K-treatment of the CS of all the three enterococci abrogated their anti-proliferative abilities, thereby showing the proteinaceous nature of the secreted metabolites in the CS. The microscopic examination of the cell lines showed that CS-treatment induced apoptosis-like morphological changes in the cancer cells.

In conclusion, this work has identified the promising indigeneous *Lactobacillus* isolates having broad-spectrum antimicrobial activities from the local population that can be further developed as probiotic strains after safety evaluation. Further, a novel high molecular weight bacteriocin from *E. faecium* has been identified that have broad spectrum antimicrobial and anticancer properties. Thus, this work highlights the important functional role of the commensal vaginal gut and vaginal strains. The bacteriocin should be further tested for in vivo anticancer potential in the animal models.

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c. Report of the work done:

i. Brief objective of the project

1. Isolation of lactic acid bacteria (LAB) from different body parts (oral, gastrointestinal and vaginal) of healthy individuals.
2. Screening of isolated LAB bacteriocins against various human bacterial and fungal pathogens.
3. Physico-chemical characterisation of selected broad spectrum bacteriocins
4. Determination of MIC and time kill studies of broad spectrum bacteriocins
5. Purification of selected bacteriocins
6. Chequerboard titrations to determine the interaction of broad spectrum bacteriocins with conventional antibiotics
7. *In vitro* testing of cytotoxicity of bacteriocins (anticancer properties) against various human cancer cell lines.

ii) Work done so far and results achieved and publications

1.1 Isolation of LAB from saliva of healthy humans

Eighty seven isolates of LAB were obtained from the saliva samples of 35 healthy individuals , out of which 11 were rods and 76 were cocci. Rods were identified as belonging to the genera *Lactobacillus* by Gram-staining and catalase test. Physico-chemical characterisation of coccus-shaped LAB were done by using various tests such as growth at temperatures 10°C and 45°C; growth in 6.5% sodium chloride containing De Man Rogosa and Shrape medium, bile-esculin hydrolysis, vancomycin resistance and haemolysis of sheep red blood cells. The cocci LAB belonged to the Genera streptococci (33 isolates), pediococci (22 isolates), enterococci (9) and Leuconostoc (8). Thus, culturable oral microflora that appeared to be dominated by cocci and had 40% streptococci, 26% pediococci, 11% enterococci, 10% leuconostoc/Weissela and 13% Lactobacilli (Figure 1).

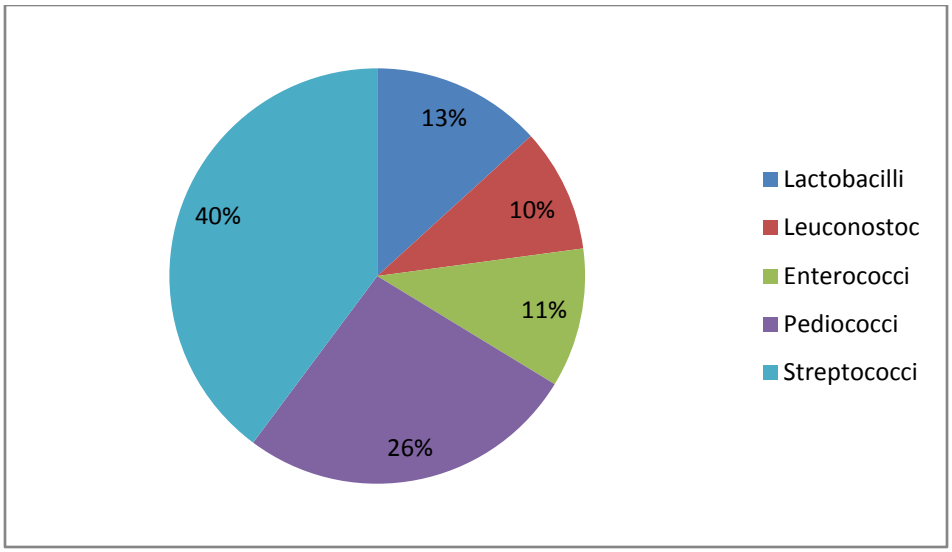

Figure 1. Percentage of various genera of LAB isolated from human saliva from healthy individuals belonging to the age group 20 to 30 yrs.

1.2 Isolation of LAB from vaginal swab samples

A total of 92 LAB isolates were obtained from vaginal swab samples of 48 healthy human females on the MRS media. Twenty two isolates belonged to *Lactobacillus* spp. and the rest were cocci. Among the isolated LAB strains, 40 percent (37 isolates) had antimicrobial activities against at least one pathogenic indicator strains (Table 2). The three *Enterococcal* strains 12a, 20c and L12b and one lactobacilli strain 3b having broad spectrum antimicrobial activities were further characterized by 16S rRNA gene sequencing method. All 16S rDNA sequences were submitted to NCBI GenBank with accession numbers (Table 1).

Table 1. The GenBank accession numbers of fecal lactobacilli isolates characterized by using 16S rDNA sequencing

Isolate	**Genus species**	**NCBI Accession no.**
3b	*L. crispatus*	KM396956
12a	*E. faecium*	MG515327
L12b	*E. faecium*	KY785374
20c	*E. hirae*	KY785319

1.3. Isolation of LAB from fecal samples of healthy humans

One hundred and ten LAB isolates were isolated by using MRS media from the fecal samples of 32 healthy children of age group 2-12 years. The *Lactobacillus* spp. was characterized based on the Gram-staining and physico-chemical characterization. Further, 7 lactobacilli isolates having broad-spectrum antimicrobial activities were characterized by 16s rRNA gene sequencing method and their GenBank accession numbers are listed in Table 2.

Table 2. The GenBank accession numbers of faecal lactobacilli isolates characterized by using 16S rDNA sequencing

Isolate	Genus species	Accession no.
L13	*Lactobacillus spp.*	KY780504
L14	*Lactobacillus plantarum*	KY582835
L18	*Lactobacillus spp.*	KY770976
L32	*Lactobacillus fermentum*	KY770983
S30	*Lactobacillus spp.*	KY780503
S45	*Lactobacillus pentosus*	KY780505
S49	*Lactobacillus spp.*	KY770966

2. Objective 2: Antimicrobial activities of LAB isolates

2.1 Oral LAB isolates

Out of 87 isolates, the CS of 33 isolates exhibited narrow-spectrum antimicrobial activities as they inhibited only closely related LAB isolates (Table 3) but had no activities against pathogenic Gram-negative and Gram-positive bacteria. One of the isolate S. no. 10, appear to inhibit both *Pediococci and Enterococci*, whereas others were selectively inhibiting only one of the tested indicator strains. None of these isolates inhibited pathogenic Gram negative bacteria.

Table 3.The antimicrobial activities of CS of oral LAB isolates against various LAB indicator strains

S.No	Isolate	Zone of inhibition (cm) against below listed Test bacteria		
		Streptococcus	*Pediococcus*	*Enterococcus*
1.	9TF 10^2a	-	11±0.2	-
2.	17T10^3b	-	9±0.3	-
3.	6TFc	-	18±0.1	-
4.	19T10^2a	-	18±0.2	-
5.	11	-	17±0.2	-
6.	13$10^2$a	-	19±0.4	-
7.	9TF10^3b	-	12±0.2	-
8.	16TFb	-	-	11±0.1
9.	19T10^3b	-	-	15±0.2
10.	10T10^2c	-	10±0.1	9±0.3
11.	12T10^2c	-	-	12±0.3
12.	6T10^2a	-	-	6±0.2
13.	15T10^2a	-	13±0.1	-
14.	6T10^3	-	13±0.2	-
15.	12T10^2b	-	15±0.3	-
16.	18TFa	-	-	11±0.2
17.	8TF10^3a	-	11±0.4	
18.	16	-	-	14±0.3
19.	16TFb	-	-	14±0.3
20.	13T10^2b	-	10±0.2	-
21.	15TF10^2	-	12±0.1	-
22.	15TFc	-	14±0.2	-
23.	4TFb	13±0.2	-	-
24.	30	12±0.2	-	-
25.	21	15±0.2	-	-
26.	31	-	-	9±0.3
27.	3	-	11±0.4	-
28.	15T10^4a	-	11±0.2	-
29.	9TF10^3b	-	15±0.5	-
30.	7	-	12±0.3	-
31.	14	-	9±0.2	-
32.	6T10^3	-	15±0.1	-
33.	20	-	13±0.2	-

2.2 Antimicrobial activities of vaginal LAB

Among the isolated LAB strains, 40 percent (37 isolates) had bacteriocin-like antimicrobial activities (activity of the CS was not inhibited by adjusting to pH to 6.5 and by treatment with catalase; however it was completely inactivated on the treatment with proteolytic enzyme) against at least one human pathogenic strain. The *Lactobacillus* isolate 3b and *Enterococcal* isolates 12a, 20c, and L12b exhibited broad-spectrum activity against both Gram-positive (*S. epidermidis)* and Gram-negative strains (*S.enterica, S. flexneri, V. cholera* and *K. pnueumoniae*; Table 4*).* Therefore all the four isolates 3b, 12a, L12b and 20c were selected for further studies. *S. enterica* and *P. aeruginosa* were used as indicator strains for conducting further experiments.

Table 4.The antimicrobial activities of CS of vaginal LAB against various pathogenic bacteria

S.No	Isolate	*S. enterica*	*S. flexneri*	*V. cholera*	*S. epidermidis*	*S. aureus*	*K. pnueumoniae*	*E.coli*	*P. aeruginosa*
1	**3b**	-	20±0.2	-	21±0.3	18±0.2	-	14±0.4	20±0.2
2	**12a**	15±0.1	14±0.1	25±0.1	13±0.2	-	11±0.5	-	-
3	**L12b**	15±0.1	12±0.1	-	-	12±0.1		14±0.1	-
4	**20c**	16±0.1	13±0.1	11±0.1	-	-	-	16±0.1	-
3	P15h	14±0.2	13±0.3	-	-	-	-	-	-
4	7b	-	13±0.2	-	-	-	-	-	-
5	30Lb	-	20±0.1	20±0.3	-	-	14±0.1	-	-
6	21La	16±0.3	13±0.1	-	-	-	12±0.2	13±0.1	-
7	P17f	19±0.1	-	13±0.1	-	-	-	-	-
8	J9d	17±0.2	-	-	-	-	13±0.4	-	-
9	18Lb	16±0.2	12±0.4	-	-	-	-	-	-
10	J11b	16±0.4	-	-	-	22±0.2	-	-	-
11	L11d	12±0.2	16±0.1	13±0.3	-	-	-	-	-
12	LS2a	15±0.1	-	-	-	-	-	-	-
13	J11c	16±0.3	-	-	20±0.1	-	-	-	-
14	30Ld	16±0.4	-	-	-	-	-	-	-
15	P16c	16±0.1	-	-	-	-	-	-	-
16	J9d	16±0.2	12±0.3	14±0.2	-	-	14±0.1	-	-
17	L3a	17±0.1	16±0.2	-	-	-	-	-	-
18	L1a	20±0.2	18±0.2	-	-	30±0.2	-	-	-

19	P9c	-	17±0.1	-	-	18±0.1	-	-	-
20	L10b	16±0.2	-	-	-	-	-	-	-
21	PS14a	-	14±0.1	-	-	-	-	-	-
22	L2e	-	14±0.3	-	-	12±0.4	-	-	-
23	P5a	-	16±0.1	-	-	13±0.1	-	-	-
24	L15a	16±0.2	15±0.2	16±0.2	-	-	-	-	-
25	P9h	-	13±0.2	-	-	12±0.3	13±0.2	-	-
27	38La	12±0.3	-	-	-	18±0.1	-	-	-
28	L10c	13±0.3	12±0.3	-	-	15±0.1	-	-	-
29	7b	14±0.1	12±0.2	12±0.1	-	-	-	-	-
30	P16g	15±0.5	-	-	-	-	-	-	-
31	J7a	13±0.4	11±0.4	-	-	-	-	-	-
32	30Le	-	-	13±0	-	-	13±0.2	-	
33	35Lc	13±0.1	1.2±0.1	-	-	-	-	-	
34	20b	18±0.2	13±0.1	11±0.4	-	-	-	-	
35	J5b	17±0.4	13±0.3	-	-	-	-	-	
36	JS7c	15±0.1	-	18±0.1	-	26±0.4	-	-	
37	P2b	21±031	-	-	-	17±0.1	-	-	

Zones of inhibitions (mm) of CS of vaginal LAB isolates were measured by using agar gel diffusion assay. The pH of CS was neutralized to 6.5 by using NaOH and the effect of H_2O_2 was abrogated by treatment with catalase enzyme (1 mg/ml) before testing them for their inhibitory activities. The results are the mean ± SD of three replicate experiments.

2.3 Antimicrobial activities of fecal Lactobacilli was due to low pH of CS

The CS of 7 lactobacilli isolates (L13, L14, L18, L32, S30, S45 and S49) had broad spectrum antimicrobial activities but on neutralizing the pH of the CS (Table 5), the antimicrobial activities was completely abrogated that showed that the low pH due to organic acids produced in the CS inhibited the pathogens. Thus, the antimicrobial activities was not due to any bacteriocin-like substance.

Table 5. The antagonistic activities of the cell-free CS of fecal lactobacilli against various pathogenic and commensal indicator strains

Indicator strains	Zones of inhibition (mm) ±SD						
	L13	**L14**	**L18**	**L32**	**S30**	**S45**	**S49**
V. cholerae	20±0.3	16±0.1	24±0.3	18±0.2	26±0.2	19±0.2	18±0.2
S. enterica	11±0.1	11±0.2	6±0.2	12±0.1	9±0.1	8±0.2	9±0.1
E. coli	11±0.2	12±0.2	9±0.1	14±0.3	12±0.2	6±0.1	9±0.2
St. aureus	-	-	-	-	20±0.2	22±0.2	19±0.1
L. monocytogenes	24±0.3	-	25±0.2	-	18±0.1	23±0.3	17±0.1
Sh. flexeri	10±0.1	14±0.2	0.8±0.1	-	10±0.2	-	-
V. parahaemolyticus	-	-	-	-	-	-	-
Candida spp.	-	-	-	-	-	-	-
Lactobacillus L13	ND	-	-	-	-	-	-
Lactobacillus L14	-	ND	-	-	-	-	-
Lactobacillus L18	-	-	ND	-	-	-	-
Lactobacillus L32	-	-	-	ND	-	-	-
Lactobacillus S30	-	-	-	-	ND	-	-
Lactobacillus S45	-	-	-	-	-	ND	-
Lactobacillus S49	-	-	-	-	-	-	ND

ND: Not Done

- : No zone of inhibition

The CS of lactobacilli isolates without pH neutralization were tested for the antimicrobial activities by using agar-gel diffusion assay. The experiment was performed three times in triplicate. The results are expressed as the means±standard deviations.

Objective 4: Physico-chemical characterization of antimicrobial component in the CS of vaginal LAB

4.1 The effects of different enzymes on the antimicrobial activities of CS of 3b, 12a, L12b and 20c

Treatment of CS of vaginal LAB *L. crispatus* 3b, and enterococci 12a, L12b and 20c with proteolytic enzymes (papain, pepsin, trypsin and proteinase-K) completely abrogated the antimicrobial effect of CS of all the 4 cultures except 20c in the case of treatment with pepsin Table 6). Catalase and lipase enzyme treatment of CS had no effect on the antimicrobial activity

of the CS thereby showing that the activity was not due to hydrogen peroxide and lipoproteins in the CS. Thus, this data clearly indicates the proteinaceous nature of the inhibitory compound.

Table 6. Effect of various enzymes on the inhibitory activity of bacteriocin produced by *L. crispatus* against *P. aeruginosa*

Enzyme	Concentration (mg/ml)	Zone of inhibition (mm)			
		***L. crispatus* 3b**	***E. faecium* 12a**	***E. faecium* L12b**	***E. hirae* 20c**
Catalase	Control	20±0.1	16±0.2	15±0.3	16±0.1
	0.5	20±0.3	16±0.1	16±0.3	16±0.2
	0.1	20±0.2	16±0.3	16±0.2	16±0.3
	2.0	20±0.2	16±0.3	16±0.2	16±0.2
Pepsin	1.0	-*	-	-	12±0.2
Trypsin	1.0	-	-	-	-
Proteinase K	1.0	-	-	-	-
Papain	1.0	-	-	-	-
Lipase	1.0	19±0.1	12±0.1	14±0.1	15±0.1

*- indicates that no zone of inhibition was obtained. The CS after pH neutralization to 6.0 was treated with different concentrations of various enzymes for 1 h and then heat treated to inactivate the enzymes before evaluating their antimicrobial activities by agar gel diffusion assay. The control CS was not treated with any enzyme. The results are the mean±SD of three replicate experiments.

4.2. The effects of temperature on the antimicrobial activities of CS of 3b, 12a, L12b and 20c

The antimicrobial activity of CS of all the four isolates was stable at temperatures 60, 80 and 100°C for up to 60 min (Table 7). However, there was a complete loss in the activities of the CS of 12a and 20c on autoclaving. The CS of 3b and L12b were stable even after autoclaving the CS

Table. 7 Effect of temperature on the antimicrobial activity of CS of *L. crispatus* 3b and *Enterococcus* spp. 12a, L12b and 20c

Temp (°C)	Time (min)											
	15				30				60			
	3b	**12a**	**L12b**	**20c**	**3b**	**12a**	**L12b**	**20c**	**3b**	**12a**	**L12b**	**20c**
60	21±0.3	13±0.1	15±0.2	16±0.3	20±0.1	13±0.1	15±0.2	15±0.1	19±0.1	13±0.1	15±0.2	15±0.1
80	20±0.1	14±0.1	14±0.1	15±0.1	19±0.3	14±0.1	14±0.1	14±0.1	19±0.4	13±0.1	14±0.1	14±0.1
100	10±0.4	12±0.2	14±0.1	13±0.1	10±0.4	12±0.2	12±0.2	12±0.2	15±0.3	12±0.3	12±0.2	12±0.2
121	10±0.2	-	14±0.1	-	ND	ND	ND	ND	ND	ND	ND	ND

4.3 Effect of pH on the antimicrobial activity of CS of *L. crispatus* 3b and *Enterococcus* spp. 12a, L12b and 20c

The effect of pH on the antimicrobial activity of bacteriocin of *L. crispatus* was determined by adjusting the pH of the CS to the various pH values and incubating at 37°C for 3 h before readjusting the pH to 6.5 and determining its antimicrobial activity. The antimicrobial activity of *L. crispatus* 3b was stable at pH ranging from 2 to 6 but it was completely inactivated at and above pH 8 (Table 8). On the other hand, the antimicrobial activities of the CS of enterococcal isolates 12a, L12b and 20c remained stable at pH ranging from 2 to 8.

Table. 8 Effect of pH on the antimicrobial activity of CS of *L. crispatus* 3b and *Enterococcus* spp. 12a, L12b and 20c

pH	Zone of inhibition (mm)			
	3b	12a	L12b	20c
2	19±0.2	13±0.1	15±0.2	16±0.3
4	19±0.1	14±0.1	14±0.1	15±0.1
6	20±0.3	12±0.2	14±0.1	13±0.1
8	-	12±0.2	14±0.1	14±0.1
10	-	-	-	-

4.4.The effect of solvents on the antimicrobial activities of CS

The CS of various isolates were treated with 50% v/v of different solvents to study the effect of the treatment on the antimicrobial activities. It was observed that chloroform treatment decreased the antimicrobial activity of CS only in the case of 12a and 20c. Methanol, acetone and isopropanol had no effect on the activity of CS of all the four isolates (Table 9).

Table 9. The effect of solvents on the antimicrobial activities of CS

Solvents	Concentration (%v/v)	Zone Of inhibition (mm)			
		3b	12a	L12b	20c
Methanol	50	18±0.1	16±0.4	15±0.2	15±0.3
Chloroform	50	16±0.4	13±0.2	16±0.1	10±0.1
Acetone	50	18±0.1	16±0.2	15±0.2	14±0.2
Isopropanol	50	20±0.3	17±0.2	20±0.2	18±0.3

Kinetics of bacteriocin production by *L. crispatus* 13a and 20c

The bacteriocins are all primary metabolites Thus, the kinetics of the production of bacteriocin in the CS were determined only in case of *L. crispatus* 3a and *E. faecium* 12a. The bacteriocin appeared in the CS of 3a after 8 h and peaked at 16 h (Figure 2). It remained constant till 26 h, after which it showed slight decline by 20%. Bacteriocin production appears to be growth-associated as it first appeared in the mid-log phase and its production plateaued in the stationary phase of the bacterial growth. The pH of the culture medium dropped consistently after 4 h till the final pH of 3.0 was obtained at 32 h (Figure 2). Similarly, in the case of 12a the increase in the bacteriocin concentration was observed 4 h after the inoculation of the bacteria in MRS. The bacteriocin concentration peaked by 8 h and by 22 h it decreased by 50 % (Figure 3). The pH of the MRS showed consistent decrease till 14 h after which it stabilized to 3.0.

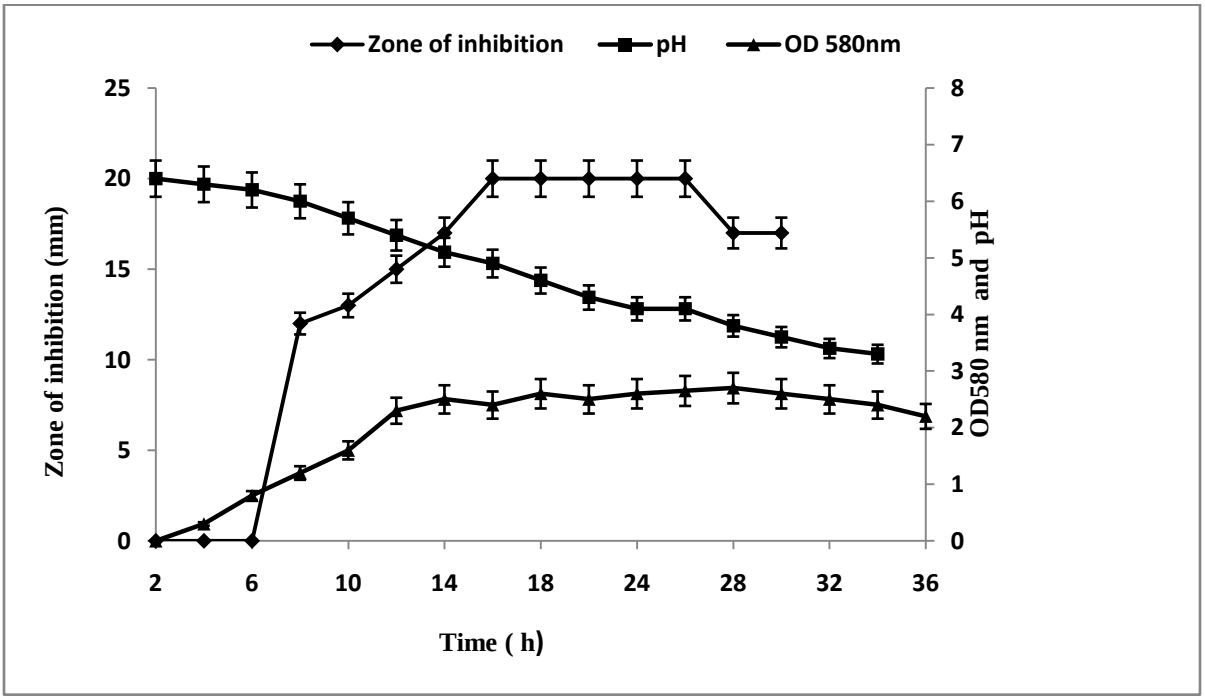

Figure 2 Kinetics of growth, pH and bacteriocin production by *L. crispatus* 3a. *L. crispatus* was grown in MRS medium at 37 °C; 5 % CO_2 under static conditions, and a part of the sample was removed from the cultures every 2 h and analysed for optical density at 580 nm, bacteriocin activity and pH. Bacteriocin activity was measured by using agar gel diffusion method. The results are the mean ± s.d. of three replicate experiments.

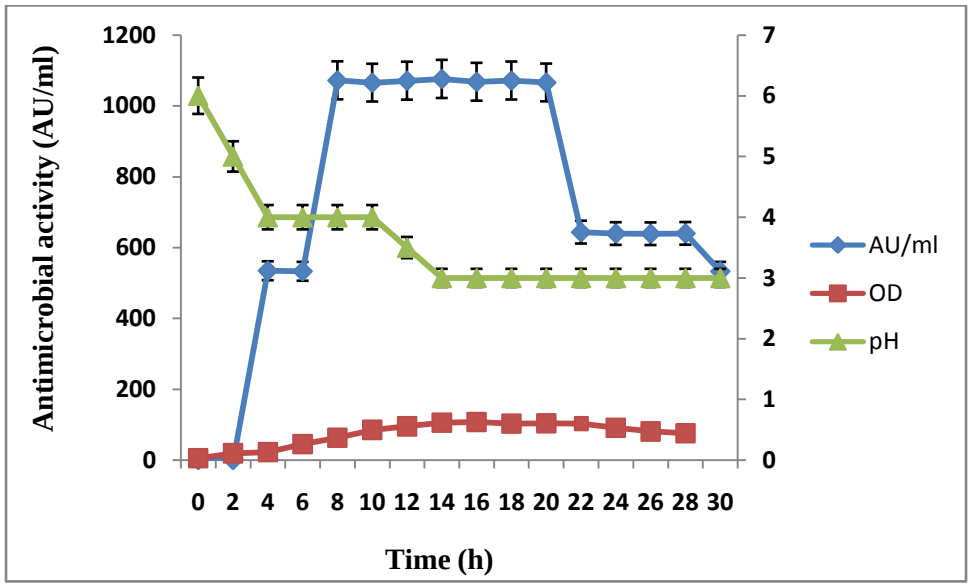

Fig 3. Growth kinetics, bacteriocin production and changes in pH of the medium by *E. faecium* 12a at different time intervals.

Objective 3:Determination of MIC and time kill studies

The minimum inhibitory concentrations (MIC) of the CS of both *L. crispatus* and *E. faecium* 12a was determined against *P. aeruginosa* and *S. enterica*, respectively. The titre of the antimicrobial activity of the CS was defined as the reciprocal of the highest dilution (2*n*) that resulted in inhibition of the indicator strain. Thus, the titre of antibacterial activity of *L. crispatus* against *P. aeruginosa* was 5120 AU/ml; whereas titre of *E. faecium* against *S. enterica* = 1280 AU/ml (Table 10; Figure 4)

Table.10 Titre of antibacterial activities of CS of *L. crispatus* and *E. faecium*

Dilution (Fold)	Zone of inhibition (mm)	
	L. crispatus	***E. faecium***
No dilution	20±0.3	18±0.2
2	20±0.2	17±0.1
4	21±0.2	16±0.1
8	18±0.2	15±0.3
16	16±0.2	14±0.4

Figure 4. The petridish showing zones of inhibitions of different dilutions of CS of *E. faecium* 12a against *S. enterica*

32	16±0.2	13±0.2
64	15±0.2	12±0.1
128	14±0.2	11±0.2
256	13±0.2	-
512	12±0.2	-
1024	-	-

5. Fifth Objective: Purification of selected bacteriocin from *E. faecium* 12a

The production of bacteriocin by L. crispatus was lost over a period of 2 years of subculturing probably because the bacteriocin genes may be present on the unstable plasmids; however, the production of bacteriocin remained consistent from *E. faecium* 12a and thus it was purified and characterized. The novel high molecular weight bacteriocin from the enterococcal isolate 12a was purified from the CS by using various techniques such as ammonium sulphate, cation-exchange column and high performance liquid chromatography and the results showed that the final yield after reverse phase chromatography was 30% (Table 11). A single peak at the retention time 1.72 min was obtained as shown in the HPLC chromatogram (Figure 5). The molecular weight of the bacteriocin was 65 kDa as determined by sodium dodecyl chromatography-polyacrylamide gel chromatography (Figure 6, lane 3). The agar gel overlay of the unstained SDA-PAGE gel further confirmed that the single band so obtained showed zone of inhibition after plating against *S. enterica* cells (Figure 6, lane 4)..

Table 11: Purification steps of bacteriocin from isolate 12a

Purification step	**Total volume**	**Total Protein (µg/ml)**	**Activity (AU/ml)**	**Specific activity (AU/µg)**	**Fold Purification (per step)**	**Yield (%)**
Cell-free supernatant	1 L	101	1066	10.6	1	100
Ammonium sulphate ppt.	5 ml	51	640	12.5	1.1	60

Cation exchange fraction	2.5 ml	9	266	29.5	2.3	41
Reverse phase HPLC	25ml	1	80	80	2.7	30

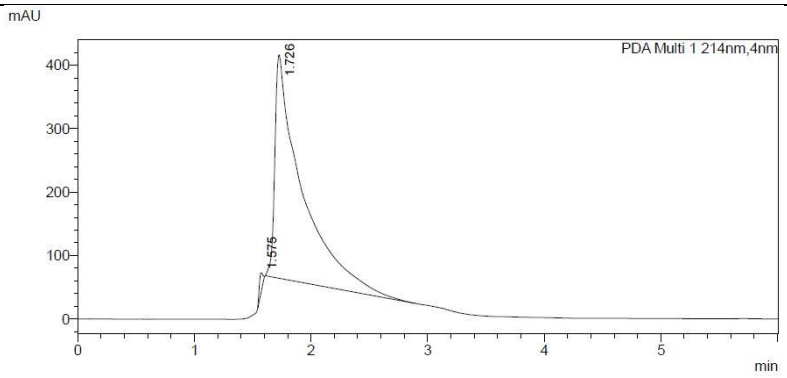

Figure 5: Chromatogram of the purified bacteriocins on semi preparative HPLC

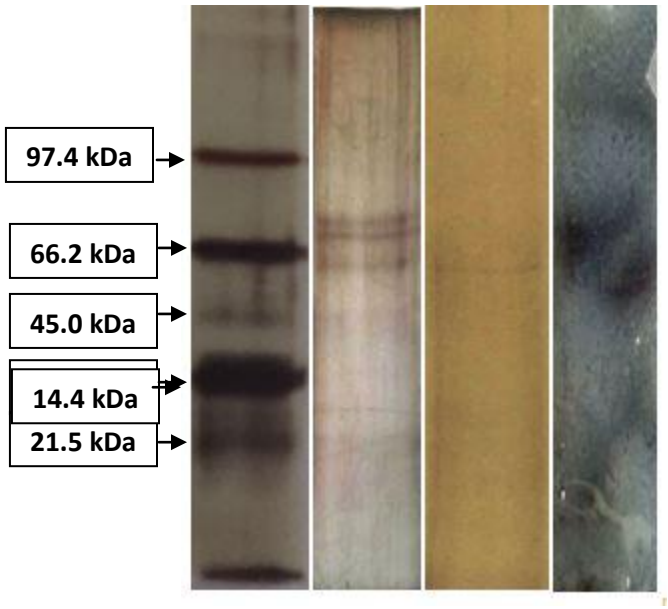

Figure 6. SDS-PAGE of purified bacteriocin of 12a and gel overlay with *S. enterica* (MTCC 733). Lane 1: molecular weight marker. Lane 2: semi-purified bacteriocin. Lane 3: purified bacteriocin . Lane 4: Inhibition zone by bacteriocin on the overlay gel.

6. Objective Chequerboard titrations to determine the interaction of broad spectrum bacteriocins with conventional antibiotics

Agar gel method and chequerboard titrations was carried out to determine the type of interaction (synergistic, additive antagonistic or no interaction) of bacteriocins-containing CS of *L. crispatus* 3a and *E. faecium* 12a with conventional antibiotics. Table 12 and 13 shows that ciprofloxacin, moxifloxacin and streptomycin had synergistic effect with the bacteriocin-containing CS of 3a, whereas, kanamycin, azithromycin and rifampicin had no synergistic interaction with the CS.

Similarly various antibiotics such as gentamycin, streptomycin, moxifloxacin, gatifloxacin, methicillin, linezolid, amikacin, colistin and clavulanic acid synergized with the bacteriocin containing-CS of *E. faecium* 12a for the bactericidal action against *S. enterica* (Table 14).

Table.12 Interaction between conventional antibiotic and bacteriocin of *L. crispatus* 3a against *P. aeruginosa*

Antibiotic	Concentration of antibiotic (µg)	Average zone of inhibition (mm)			Nature of Interaction
		Antibiotic alone	bacteriocin	Antibiotic+ bacteriocin	
Ciprofloxacin	10	24±0.2	11±0.5	30±0.4	Synergistic
Moxifloxacin	5	22±0.5	11±0.7	27±0.4	Synergistic
Streptomycin	150	23±0.7	11±0.3	31±0.5	Synergistic
Kanamycin	30	10±0.5	11±0.2	10±0.3	No Interaction
Azithromycin	15	28±0.3	11±0.5	28±0.6	No Interaction
Rifampicin	30	10±0.4	11±0.7	11±0.5	No Interaction

Agar well diffusion assay was performed and zone of inhibitions were measured in the presence of various antibiotics and bacteriocin at suboptimal concentration (80 AU/ml) separately, and in combination. bacteriocin-inactivated in the presence of pepsin (1mg/ml) was used as negative control. To inactivate bacteriocin, it was treated with 1mg/ml of pepsin and incubated at 37 °C for 2 h before performing agar well diffusion assay. The interaction was regarded as synergistic

if the inhibition zones were at least > 2mm compared with individual zone of inhibitions for antibiotic and bacteriocin. The values are mean + s.d. of experiments performed in triplicate.

Table 13 MIC of antibiotic alone and antibiotic/ bacteriocin of *L. crispatus* 3a against *P. aeruginosa*

Antibiotic	MIC (µg/ml or AU/ml)			[b] Σ FIC of antibiotic+ bacteriocin	Σ FIC of antibiotic+ bacteriocin - inactivated with pepsin
	Antibiotic	Antibiotic/ bacteriocin	[a]Antibiotic/ BLIS-inactivated with pepsin		
Ciprofloxacin	4	0.13/40	4/80	0.28	1.5
Moxifloxacin	4	0.5/40	4/80	0.38	1.5
Streptomycin	128	16 /40	64/160	0.38	1.5

The MIC of bacteriocin alone against *P. aeruginosa* is 160 AU/ml. The interaction was interpreted as follows: synergy, FIC ≤ 0.5; indifference, 0.5<FIC<2; antagonism, FIC>2. The values are mean + s.d. of experiments performed in triplicate.

[a] Negative control experiment was performed in the presence of bacteriocin-inactivated with pepsin. To inactivate bacteriocin, it was treated with 1mg/ml of pepsin and incubated at 37 °C for 2 h before performing chequer board titration assay.

[b] Σ Fractional Inhibitory Concentration = $FIC_{antibiotic}$ + $FIC_{bacteriocin}$= (MIC of antibiotic in combination/MIC of antibiotic alone) + (MIC of bacteriocin in combination/MIC of bacteriocin alone).

Table.14 Interaction between conventional antibiotic and bacteriocin of isolate 12a against *S. enterica*

Antibiotic (conc in µg)	Bacteriocin 3a	Antibiotic Alone	Antibiotic+ bacteriocin	Interaction
		Zone of inhibition (mm)		
Colistin	18±0.2	-	23±0.3	Synergistic
Gentamicin	18±0.2	20±0.3	22±0.2	Synergistic
Streptomycin	18±0.2	18±0.2	21±0.2	Synergistic
Gatifloxacin	18±0.2	18±0.1	20±0.3	Synergistic
Clavulanic Acid	18±0.2	18±0.3	22±0.2	Synergistic
Amikacin	18±0.2	22±0.2	27±0.2	Synergistic
Methicillin	18±0.2	-	23±0.2	Synergistic
Linezolid	18±0.2	46±0.2	51±0.1	Synergistic
Moxifloxacin	18±0.2	-	22±0.3	Synergistic
Oxacillin	18±0.2	-	17±0.1	No Interaction
Ampicillin	18±0.2	40±0.2	39±0.3	No Interaction
Nalidixic Acid	18±0.2	-	18±0.2	No Interaction
Ciprofloxacin	18±0.2	-	18±0.4	No Interaction
Ofloxacin	18±0.2	16±0.5	18±0.2	No Interaction
Erythromycin	18±0.2	-	19±0.2	No Interaction
Kanamycin	18±0.2	-	18±0.1	No Interaction
Norfloxacin	18±0.2	-	19±0.2	No Interaction
Clarithromycin	18±0.2	-	18±0.5	No Interaction
Sparfloxacin	18±0.2	-	21±0.2	No Interaction
Co-Trimoxazole	18±0.2	-	20±0.3	No Interaction
Cefexin	18±0.2	31±0.2	20±0.2	No Interaction
Novobiocin	18±0.2	29±0.1	19±0.2	Antagonistic
Cephalothin	18±0.2	32±0.3	22±0.3	Antagonistic

7 Objective: *In vitro* testing of cytotoxicity of bacteriocins against various human cancer cell lines.

Evaluation of anti-proliferative activities of the CS of enterococcal isolates on the cancer cell lines HeLa, A549 and HCT-15 was done. CS of all the three enterococcal isolates had dose-dependent anti-proliferative activity against all the cancer cell lines tested. CS of isolate 12a, 20c and L12b at the highest dose of 50 µg/ml resulted in 8.4, 9.7 and 16.4% reduction in the viabilities of HeLa, respectively (Figure 7A) The 50% inhibitory concentration (IC_{50}) values of the CS of 12a, 20c and L12b for HeLa was calculated as 12.5, 7.7 and 15.9 µg/ml, respectively.

Similarly, the secretory metabolites all the three enterococcal isolates at the dose of 50 µg/ml resulted in maximum inhibition of the growth of HCT-15. The viability of HCT-15 was reduced to 13.7, 14.4 and 15.8% following treatment with the CS (50 µg/ml) of 12a, 20c and L12b, respectively (Figure 7B). The IC_{50} values calculated for the CS of 12a, 20c and L12b were 9.9, 14.4 and 15.3 µg/ml, respectively.

Further, 24 h treatment of the cell line A549 with the CS of the enterococcal isolates12a, 20c and L12b at the concentration 50 µg/ml, reduced the viabilities to 17.5, 21.3 and 18.7%, respectively (Figure 7C). The IC_{50} values calculated for the CS of 12a, 20c and L12b were 14.01, 21.3 and 11.7 µg/ml, respectively.

The antiproliferative effect of CS of all the three enterococcal isolates were also determined against normal human PBMCs. The residual viabilities of PBMC after treatment with the CS of 12a, L12b and 20c were 74.5, 76.5 and 81.5 %, respectively (Figure 7D).

Proteinase K treatment of CS of all the three enterococcal isolates resulted in abrogation of the anti-proliferative effect of CS (Figure 8). The viabilities of HCT-15 was reduced only by 18.7, 22.9 and 26.8% with proteinase K-treated CS of 12a, 20c and L12b, respectively. Thus, the anti-

proliferative activity of CS of all the isolates is due to some proteinaceous substance present in the CS.

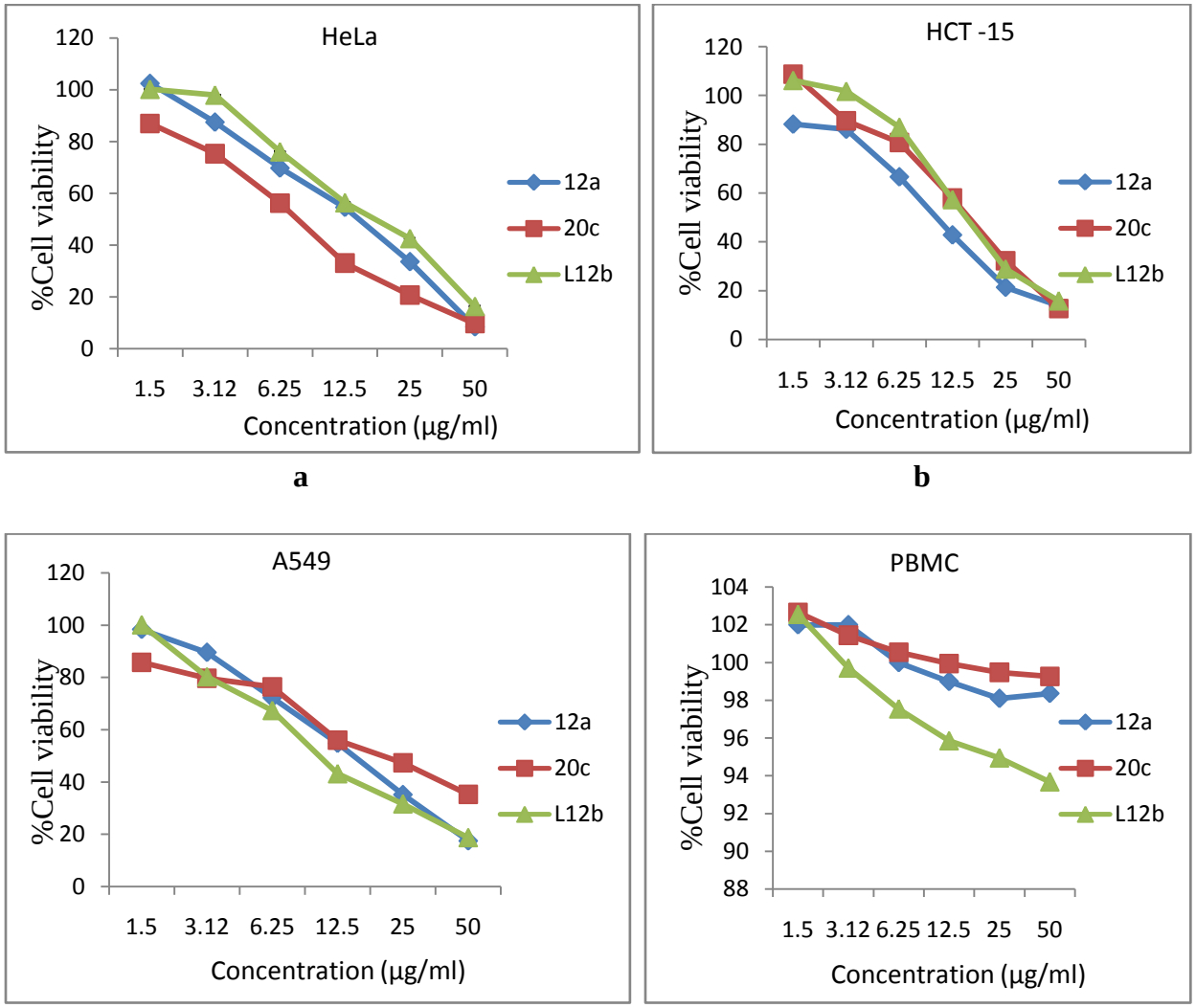

Figure 7. Dose-dependent effect of the secreted metabolites of the CS of *Enterococcal* isolates 12d, 20c and L12b on the viability of (a) HeLa (b) HCT 15 (c) A549 (d) Human PBMCs after 24 h treatment. Error bars represent standard deviation of the mean values.

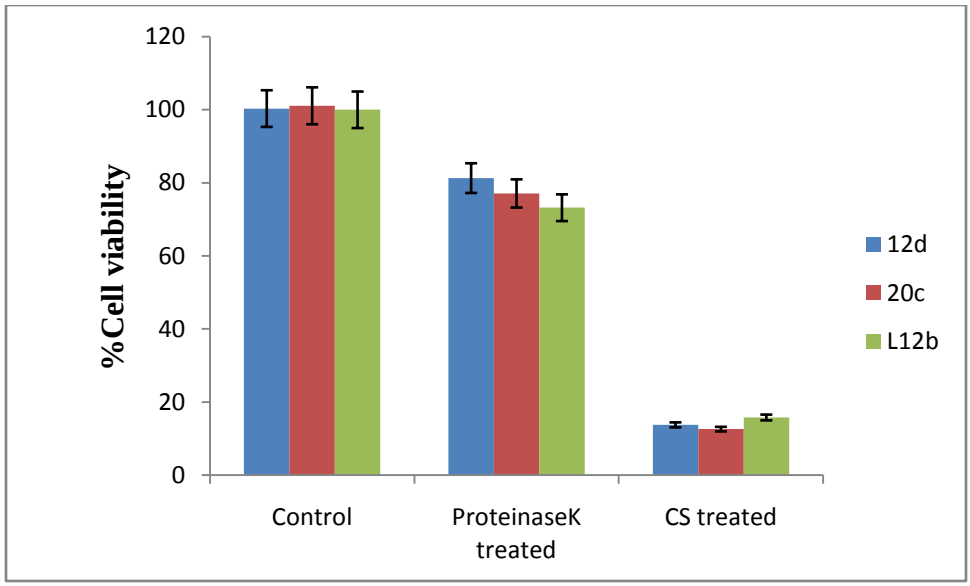

Figure 8. Anti-proliferative effect of CS (50µg/ml) of *Enterococcal* isolates, with and without proteinase K(1µg/ml) treatment on HCT-15 cell line. Untreated CS was used as control.

3.3 Microscopic detection of morphological changes in the CS-treated cell lines

Apoptosis of eukaryotic cells is associated with certain morphological changes in their cell membranes and DNA that could be studied microscopically. To study the effect of CS on the morphology of HCT-15 cells, they were treated with 50 µg/ml of CS and then stained with dyes, Hoechst 33342, propidium iodide (PI), and Giemsa (Merck, Darmstadt, Germany). Further, to study the mechanism of the anti-proliferative activity of CS, the morphological changes of CS-treated cancer cell line HCT-15 cells were studied by bright field and fluorescent microscopy. Apoptosis also known as programmed cell death involves microscopically visible morphological changes such as chromatin condensation and margination, cell shrinkage, membrane blebbing and formation of apoptotic bodies. Most of the cells in the control sample (treated with MRS) remained viable with normal cell morphology and, clear outline of cell membrane and nucleus (Figure 9; Lanes A1, B1, C1). On the other hand, the cells treated with the CS of enterococcal isolates 12a and 20c showed morphological changes typical of apoptosis (Figure 9). The CS-treated and Geimsa-stained cells (Lanes A2 and A3) showed cell shrinkage, nuclear

condensation and nuclear fragmentation. Whereas, CS-treated and fluorescent-stained (Lanes B2, B3, C2 and C3) cells showed nuclear condensation and fragmentation.

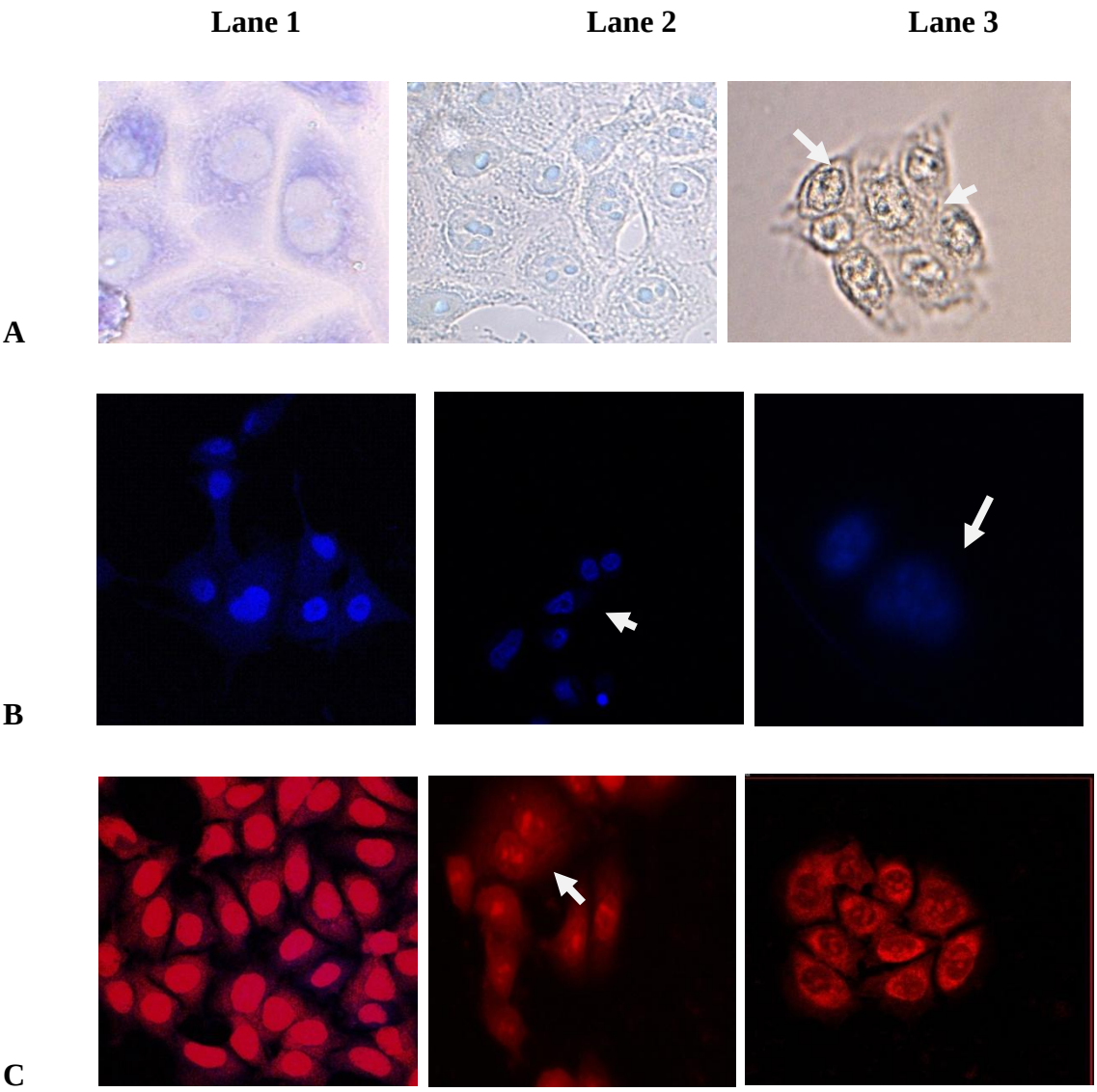

Figure 9. Morphological changes induced in HCT-15 following 24 h treatment with CS of *Enterococcal* isolates. (A) Giemsa-stained (B) Hoechst 33342 (C) PI stained HCT-15 cells.
Lane 1: untreated HCT-15 cells; Lane 2: HCT-15 cells treated with CS of 12d and Lane 3: HCT-15 cells treated with CS of 20c.

List of publications:

1. Sharma P, Kaur S, Kaur R, Kaur M and **Kaur** S. 2018. Proteinaceous secretory metabolites of probiotic human commensal *Enterococcus hirae* 20c, *E. faecium* 12a and L12b as antiproliferative agents against cancer cell lines. Front. Microbiol. (In Press) doi: 10.3389/fmicb.2018.00948. IF: 4.1
2. Kaur S, Sharma P, Kalia N, Singh J, **Kaur S**. 2018. Anti-biofilm Properties of the Fecal probiotic lactobacilli against *Vibrio* spp. *Frontiers in cellular and infection microbiology.* (In Press) https://doi.org/10.3389/fcimb.2018.00120. IF.: 4.4
3. Kaur S and **Kaur S**. Bacteriocins as Potential Anticancer Agents. *Frontiers in Pharmacology*. 2015;6:272. doi:10.3389/fphar.2015.00272. IF: 4.4
4. **Kaur S**, and Sharma P (2015) Protease-sensitive inhibitory activity of cell-free supernatant of *Lactobacillus crispatus* 156 synergizes with ciprofloxacin, moxifloxacin and streptomycin against *Pseudomonas aeruginosa*: an i*n vitro* study. Probiotic and Antimicrobial Protein. doi: 10.1007/s12602-015-9188-4. ISSN: 1867-1306. IF.: 1.8.
5. Kaur S and **Kaur S**. 2016. Isolation and characterization of oral microflora from triclosan-free and triclosan–containing toothpaste users. International Journal of Applied Research 2016; 2(3): 778-783.

Annexure – XI

Final Report Assessment / Evaluation Certificate

(Two Members Expert Committee Not Belonging to the Institute of Principal Investigator)

(to be submitted with the final report)

It is certified that the final report of Major Research Project (42-478/2013 (SR) titled "*In vitro* evaluation of anticancer and antimicrobial properties of human lactic acid bacteria", by Dr. Sukhraj Kaur, Assistant Professor, Department of Microbiology, Guru Nanak Dev University, Amritsar, has been assessed by the committee consisting the following members for final submission of the report to the UGC, New Delhi under the scheme of Major Research Project.

Comments/Suggestions of the Expert Committee:-

Name & Signatures of Experts with Date:-

Name of Expert with Date	University/College name	Signature
1.		
2.		

It is certified that the final report has been uploaded on UGC-MRP portal on ----------

It is also certified that final report, Executive summary of the report, Research documents, monograph academic papers provided under Major Research Project have been posted on the website of the University/College.

(Registrar/Principal)