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Evaluation of Antibacterial Activity of Milk Peptides Released during the Growth of Lactobacilli in Milk

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The present study is carried out to evaluate the antibacterial peptides released during the growth of lactobacilli in milk. Among 8 isolates of lactobacilli and yoghurt cultures used for study, the yoghurt culture released the maximum quantity of peptides (90 ± 1.25 µg/ml). When tested for antibacterial activity only yoghurt culture filtrate showed the antibacterial potential. Further, yoghurt culture lysate was prepared, which was used to treat the milk to bring in proteolysis. The supernatant obtained after centrifugation of this treated milk was used to partially purify peptides by gel filtration using Sephadex G-50. One of the three fractions (F2) showed antibacterial activity against test organisms, *E. coli*, *Salmonella* sp. and *Shigella dysentriae.* The peptide fraction was incorporated

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into curd samples inoculated with different lactic cultures and spiked with *E. coli* to determine antibacterial activity. It was found that peptide showed a bacteriostatic effect on *E. coli*, and the viable count of it was almost reduced by two log cycles when compared with the control.

Keywords: Antibacterial peptides; lactobacilli; yoghurt culture; fermented milk; bioactive peptides; purification; enteric pathogens.

1. INTRODUCTION

Microbial fermentation to obtain bioactive peptides is gaining recognition due to being a natural, safe, and cost-effective strategy. Lactic acid bacteria (LAB) have developed the ability to hydrolyze proteins to compensate for their amino acid requirement. Lactic acid bacteria exhibit antibacterial activity against many bacteria including food pathogens. These days food processors are aiming at natural, effective, safe, and low-cost substitutes for enhancing the shelf life of food products. Antimicrobial resistance is prevalent across both species and geographical boundaries due to the food chain web, and relaxation in international trade barriers. To overcome the antibiotic resistance and ill effects of chemical preservatives through the application of bio-preservatives and antimicrobial peptides from milk.

Vijayalakshmi et al., (2001) studied the immunoenhancing effect of bioactive peptides in milk. The sodium caseinate was incubated with trypsin at 37 ºC for various periods. After completion of the incubation period, the activity of the enzyme was stopped and centrifuged at 2000 rpm for 15 minutes. The protein content of the supernatant was determined by the Lowry method.

Peptides for whey protein fermentation (PWPF) were preliminarily isolated by macroporous resin D101. PWPF (20 mL, 150 mg/mL) were loaded on macroporous resin D101 column (25 mm×460 mm), and stepwise eluted with a series of gradient ethanol solutions at a flow rate of 2.5 mL/min. Automatic collectors were used to collect the eluate (2 min/tube). To obtain the elution curve of the sample solution, the absorbance of the fractions was measured at 214 nm, 254 nm, and 280 nm. Besides, the peptide content and ABTS radical clearance rate were measured in every three tubes. The absorption peaks were collected, pooled, rotary evaporation and lyophilized. The purified fractions, labeled F1, F2, F3, and F4, were stored at –20℃ until needed. A Sephadex G-15 chromatography was used to purify the fraction with strong radical scavenging activity. The column was pre-equilibrated with deionized water. The purified fraction was formulated into a 0.7 mg/mL solution with deionized water, and then the peptide solution (4 mL) was loaded onto the column (16 mm×100 cm). Deionized water was used to elute the column at a volume of 4 mL/tube, with absorbance monitoring at 214 nm, 254 nm, and 280 nm. The fractions were lyophilized for further use (Guo 2023).

A study on comparative antimicrobial evaluation of the bioactive peptide generated from *L. rhamnosus* C25*, L. rhamnosus* C6, and *L. casei* NCDC17 fermented colostrum whey. Peptide fractions 10 kDa, 5 kDa, and 3 kDa were isolated using their respective molecular weight cut-off membranes and antimicrobial activity was evaluated against diarrheagenic *E. coli* strains. The higher inhibition was shown by < 10 kDa peptide fractions from *L. rhamnosus* C25 fermented colostrum whey and the zone of inhibition was 15 ± 0.06 (*E. coli* MTCC 723), 17 ± 0.04 (*E. coli* MTCC 724), 18 ± 0.05 (*E. coli* MTCC 725), and 17 ± 0.02 (*E. coli* ATCC 25922) (Kashyap et al., 2022).

The proteolytic activity of LABs is exerted in a species- and strain-dependent manner. To name a few LABs*, Lactobacillus helveticus, Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis*/d*iacetylactis*, and *Lb.delbrueckii* subsp. *lactis/cremoris* display effective proteolytic activity for milk protein hydrolysis. *Lactobacillus acidophilus*-generated peptides (IKHQGLPQE, VLNENLLR, and SDIPNPIGSENSEK) displayed antibacterial activity against pathogenic *Enterobacter sakazakii* and *E. coli* (Singh et al., 2023) AMP in milk 23. The present study is aimed at partial purification of peptides in milk treated with lactic cultures and yoghurt culture, further incorporating them into milk inoculated with enteric pathogen *E. coli* to study their antibacterial activity. These peptides can be used as biopreservatives in dairy foods to improve food safety.

2. MATERIALS AND METHODS

2.1 Material Required

Different lactic cultures screened for antibacterial activity, Lyophilizer, Sephadex G-50 from Merck, Coloumn chromatography set up, Trichloro acetic acid, Sonicator and other required glasswares for antibacterial activity.

2.1.1 Quantification of peptides in filtrates of fermented milk

Lactic cultures and yoghurt cultures screened for antibacterial activity against *E.coli* were selected and inoculated in sterilized skim milk at the rate of 1-2 % and incubated at 37 ºC for 24-48 h or until the curd is set. The curd was subjected to centrifugation at 10,000 rpm for 20 min. and the supernatant was collected. (modified method of Yamamtoto et al, (1999). The sterilized skim milk acidified with 2% sterile lactic acid served as control. The peptides or proteins above 50 kDa were precipitated by addition of 10 % Tri Choloro Acetic (TCA) and kept at 4ºC to settle down the proteins (Scopes et al., 1986). The clear supernatant was filtered using Whatman No. 1 filter paper and peptide content in it was determined using Lowry method using bovine serum albumin standard curve. The quantity of peptides was expressed in terms of µg per ml.

2.2 Determination of Antibacterial Activity of Milk Cultures

The cell-free supernatant obtained after centrifugation was freeze-dried. The powder was reconstituted at a 10X rate. This freeze-dried concentrate (FDC) was used for testing antibacterial activity using the agar well assay technique (Prabha et al., 1984).

2.3 Preparation of Yoghurt Culture Cell Lysate

The active yoghurt culture was grown in YG broth inoculated at 2% (v/v) and incubated anaerobically at 37ºC for 48 h. Later, after growth broth was subjected to centrifugation at 5000 rpm for 10 min. After centrifugation, the supernatant was decanted and only cell pellets were harvested. These cells were resuspended into a phosphate buffer of pH 6.7 and mixed properly. The suspended cells were transferred to a stainless steel sonicator, with glass beads at ratio of 1:1. The tightly closed sonicator was subjected to oscillation in a cell homogenizer for 1.5 h at a low temperature of 2-10ºC by repeated blasting with nitrogen gas. After homogenization, the contents were transferred to a sterile centrifuge tube and centrifuged at 5000 rpm for 10 min. After that, the supernatant was decanted into the sterile test tube and stored in the deep freezer, using a culture lysate.

2.4 Preparation of Milk Hydrolysate

The cell lysate obtained in 2.3 was added to sterilized skim milk at 1 % (v/v) and incubated for 15 h, later rennet was added at the rate of 18 mg per litre of milk and incubated at 37 ºC, until the coagulum was formed. This coagulum was subjected to centrifugation at 10000 rpm for 20 min and the supernatant obtained was freeze-dried and used for the antibacterial assay.

2.5 Partial Purification of Peptides by Size Exclusion Chromatography

The peptides in freeze-dried supernatant were separated by employing size exclusion chromatography with Sephadex G-50 using a modified method of Parker et al (1984). The sample was loaded into the Sephadex column at the rate of 2 % of the bed volume and peptides were eluted employing the eluent 0.01 M Tris-HCl, pH 7.0 at the rate of 1 ml per minute. The eluted peptides were collected and freeze-dried. The antibacterial activity was checked by agar well assay technique with a 4mm diameter well and 20 µl sample.

2.6 Effect of Antibacterial Peptides on the Growth of Added *E. coli* in Milk Fermented with Lactic Cultures

Partially purified peptide fraction obtained in 2.5 was added to 10 ml of reconstituted skim milk at different concentrations. About 100 ml of reconstituted skim milk was heated to momentary boiling, cooled to room temperature and inoculated with different lactic cultures – *Lb. acidophilus*, *Lb. lactis* ssp. *lactis*, *Lb*. *lactis* ssp *cremoris*, *Lb. lactis* ssp *lactis* var. *diacetylactis* and *Leuconostoc sp.* at the rate of 1 % (v/v). This inoculated milk was distributed into different sterile test tubes (10 ml each). To all these samples, *E. coli* culture was added to have 10^4 cells per ml. The peptide fraction was added at different levels (50 µl to 300 µl) to different test tubes having spiked with *E. coli*. The contents were mixed well for proper distribution of peptide fraction and incubated at optimum temperature

(30/37ºC). At intervals, pH was checked, and once it reached 5.5 or nearer the samples were examined for viable count of *E. coli* and titratable acidity.

3. RESULTS AND DISCUSSION

3.1 Quantification of Peptides in Milk Fermented with Different Lactic Cultures

The selected cultures of Lactobacillus and yoghurt culture were inoculated in sterilized skim milk at the rate of 1-2% and incubated at 37 C /24-48h or until the coagulum was set. This was subjected to centrifugation at 10,000 rpm for 20 minutes and supernatant was examined for peptide concentration. The quantity of peptides released during the growth of cultures is shown in Table 1 and Fig. 1, the maximum concentration was observed in case of yoghurt culture (90 ± 1.25 µg/ml). Among the single lactobacilli cultures, *Lb. acidophilus* C1 liberated highest quantity of peptides (70 ± 1.25 µg/ml), while *Lb. animalis* D5 produced the lowest (55 ± 1.25µg/ml), In Similar studies conducted by Yang Yu et al., (2021) the highest concentration of five bioactive peptides was 28.44 µg/ml after incubation for 8 h.

3.2 Antibacterial Activity of Yoghurt Culture Filtrate

The volume of yoghurt culture filtrate was freeze-concentrated to 1/10th of its original volume and concentrated culture filtrate was subsequently diluted with water. As shown in Table 2, and Fig. 2, freeze-concentrated filtrate failed to show inhibitory activity when mixed with water to give 10 % solution. However, when the solution concentration increased to 30 – 100 % activity gradually increased from 40.05 ± 0.90 mm^2 to 117.52 ± 0.75 mm^2. In a similar study conducted by Varadaraj et al., (1993) Neutralized extracellular culture filtrate of the lactic cultures added at a level of 10% in sterile, 10% reconstituted non-fat dry milk was able to either suppress or retard the growth of selected bacterial cultures when incubated at 37°C for 24 h using agar incorporation method.

Table 1. Quantity of peptides released by different lactic cultures in fermented milk

Name of culture	Isolate No	Qty of peptide (µg/ml)
Control	C	42 ± 1.70
Str.thermophilus + *Lb. bulgaricus* (yoghurt culture)	Yg 1	90 ± 1.25
Lb. acidophilus	C1	70 ± 1.25
Lb. delbrueckii ssp. *delbrueckii*	G1	63 ± 1.63
Lb. delbrueckii ssp. *bulgaricus*	C10	63 ± 0.82
Lb. fermentum	C6	61 ± 1.25
Lb. delbrueckii ssp. *lactis*	G2	59 ± 1.25
Lb. animalis	D5	55 ± 1.25
Lb. brevis	C5	56 ± 1.70

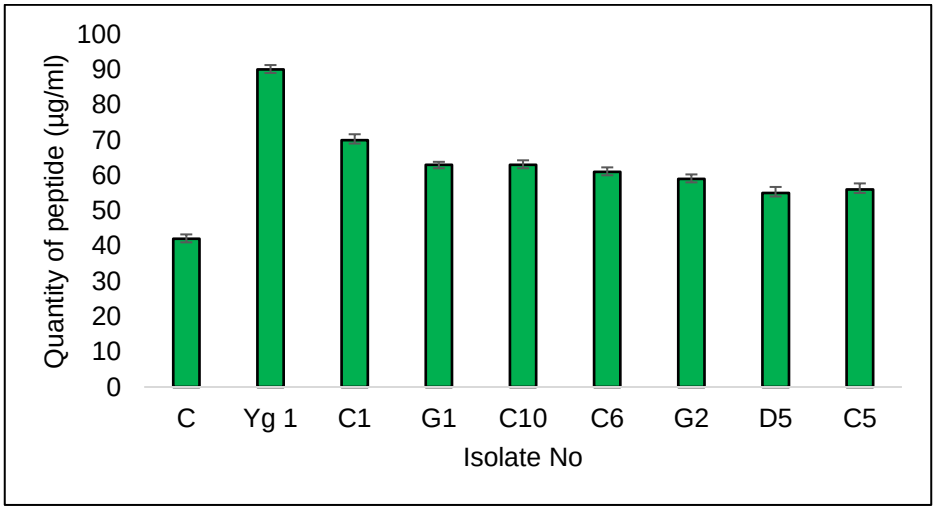

Fig. 1. Quantity of peptides released by different lactic cultures in fermented milk

Table 2. Antibacterial activity of freeze-concentrated yoghurt culture filtrate against *E. coli*

Concentration of yogurt culture filtrate	Antibacterial activity (Sq.mm)
Control (0 % solution)	0
10 % Solution	0
30 % Solution	40.05 ± 0.90
50 % Solution	74.50 ± 0.71
70 % Solution	93.65 ± 0.76
90 % Solution	113.25 ±1.24
100 % Solution	117.52 ± 0.75

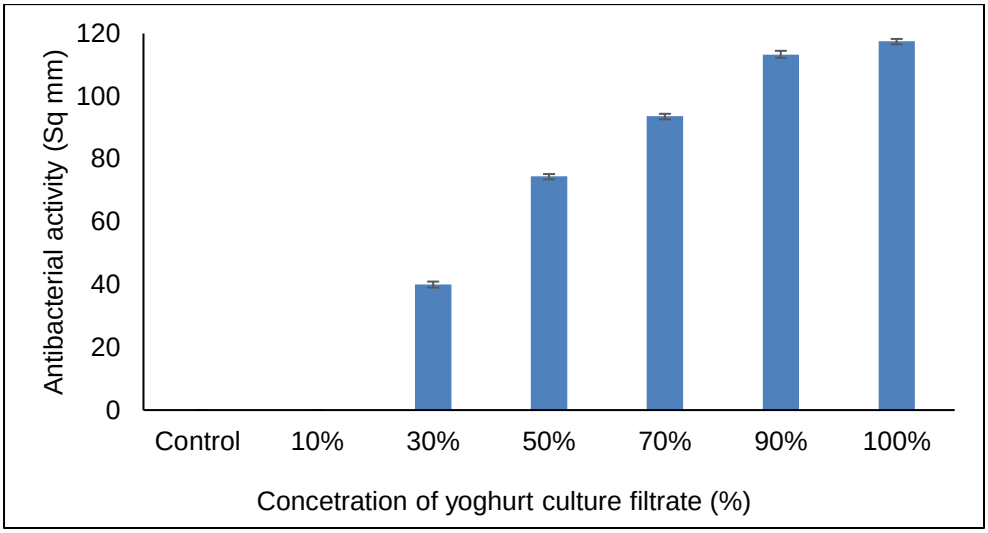

Fig. 2. Antibacterial activity of freeze-concentrated yoghurt culture filtrate against *E. coli*

3.3 Antibacterial Activity of Yoghurt Culture Lysate-treated Milk

Milk treated with yoghurt culture cell lysate for different periods at 37 ºC was examined for antibacterial activity, against different pathogens. The milk was hydrolyzed for different periods and was examined for the presence of antibacterial activity. It may be observed in Table 3 and Fig. 3 that, milk treated for 6 h showed 39.50 ± 0.61 sq. mm, while it increased to 115.5 ± 0.90 sq. mm after 15 h treatment concerning *E. coli.* Antibacterial activity against *Salmonella* sp. ranged from 32.25 ± 0.58 sq. mm to 95.52 ± 0.36 sq. mm. For *Shigella dysentriae* the range was from 41.25 ± 0.58 sq. mm to 85.24 ± 1.05 sq. mm. Similar study was conducted for antioxidant activity and antibacterial activity of cultures *Lb. acidophilus* (T2) and *Lb. helveticus* (T3) in combination with yoghurt culture (1:1) in yoghurt made from buffalo milk. Water soluble protein extract of yoghurt displayed antibacterial activity against *E. coli* which was in accordance with Sah et al. (2020). Also, positive antibacterial activity was shown against S. *aureus, S. typhimurium* and *B. cereus* (Taha et al., 2017).

Table 3. Antibacterial activity of milk treated with yogurt culture cell lysate

Duration of treatment of milk with culture cell lysate (h)	Antibacterial activity (Sq mm)		
	E. coli	***Salmonella* sp**	***Shigella dysentriae***
3	0	0	0
6	39.50 ± 0.61	32.25 ± 0.58	41.25 ± 0.58
9	64.25 ± 0.86	48.08 ± 0.32	40.87 ± 0.49
12	73.89 ± 0.43	56.52 ± 1.38	48.08 ± 0.26
15	115.5 ± 0.90	94.20 ± 0.53	85.24 ± 1.05
18	115.2 ± 0.78	95.52 ± 0.36	85.17 ± 0.34

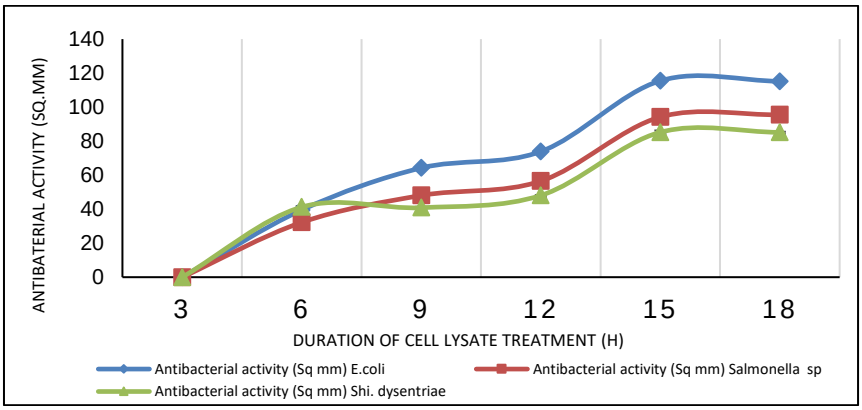

Fig. 3. Antibacterial activity of milk treated with yogurt culture cell lysate

3.4 Antibacterial Activity of Peptide Fraction Incorporated into Milk Cultures

The peptide fraction F2 exhibited antibacterial activity and was incorporated into curd prepared by different cultures and observed for its inhibitory action against incorporated *E. coli*. In the case of *L. cremoris* curd, the viable count in control i.e. milk without peptide, increased from 4.0 $\log_{10}$ cfu/ml initially to 7.6 $\log_{10}$ cfu/ml, after incubation for 8-12 h at 30ºC.

Whereas in the sample with 50 µl peptide, the viable count of *E. coli* increased from 4.0 $\log_{10}$ cfu/ml to 7.1 $\log_{10}$ cfu/ml indicating the least effect. However, antibacterial activity improved when peptide concentration was increased. At a peptide concentration of 300 µl, the viable count of *E. coli* increased from 4.0 $\log_{10}$ cfu/ml to 5.3 $\log_{10}$ cfu/ml, indicating the bacteriostatic effect. In the study was carried out for partial purification of antimicrobial peptides from fermented Iraqi camel's milk by *Streptococcus thermophilus* and *Lactobacillus delbrueckii* sp. *bulgaricus*. The second and the third fractions purified with gel filtration showed high inhibition activity against *E. coli*, the diameters of the inhibition zone were between 10-24 mm after 24 h of incubation at 37°C (Algaboory et al., 2017).

In the case of *L. lactis* curd the viable count increased from 4.0 $\log_{10}$ cfu/ml initially to 6.8 $\log_{10}$ cfu/ml in control. But at 300 µl peptide concentration, it increased from 4.0 $\log_{10}$ cfu/ml to 5.1 $\log_{10}$ cfu/ml, showing the bacteriostatic effect. Ordóñez AA and co-authors (Ordóñez et al., 2013) showed that, the milk-derived Alpha s2-casein(183–207)peptide to its antibacterial activity against the food-borne pathogens *Listeria monocytogenes* and *Cronobacter sakazakii*. Also showed that the simultaneous replacement of various positively charged amino acids was linked to a loss of bactericidal activity. On the other hand, the replacement of Pro residues resulted in a significantly increased antibacterial potency. In all the cases, the titratable acidity ranged from 0.39 to 0.42 % lactic acid.

Table 4. Inhibitory action of antibacterial peptide against *E.coli* in milk cultured with different lactic cultures

Amount of antibacterial peptide added to milk (µl)*	Antibacterial activity against *E. coli* on milk cultured with			
	L. cremoris	***L. lactis***	***Lb. acidophilus* D2**	***L. diacetylactis* + *Leuconostoc* sp**
Control**	7.6 ± 0.09	6.8 ± 0.08	6.8 ± 0.08	7.3 ± 0.08
50	7.1 ± 0.08	6.8 ± 0.08	6.7 ± 0.05	6.8 ± 0.08
100	7 ± 0.08	6.4 ± 0.05	6.6 ± 0.05	6.7 ± 0.08
150	6.7 ± 0.05	6.0 ± 0.08	6.0 ± 0.08	6.2 ± 0.05
200	6.3 ± 0.05	5.7 ± 0.05	5.7 ± 0.05	6.0 ± 0.08
250	5.8 ± 0.08	5.5 ± 0.05	5.4 ± 0.05	5.8 ± 0.05
300	5.3 ± 0.05	5.1 ± 0.54	5.1 ± 0.05	5.5 ± 0.08

**The fraction F2 was added to 10 ml milk at different levels, inoculated with lactic cultures (1%) and E.coli (4 $\log_{10}$ cfu/ml) simultaneously and incubated at 30-37 ºC for 18-20 h. After the curd was set, the sample was drawn and examined for the number of survivors of E. coli.*

*** A control sample prepared without incorporating peptide fraction served as a control.*

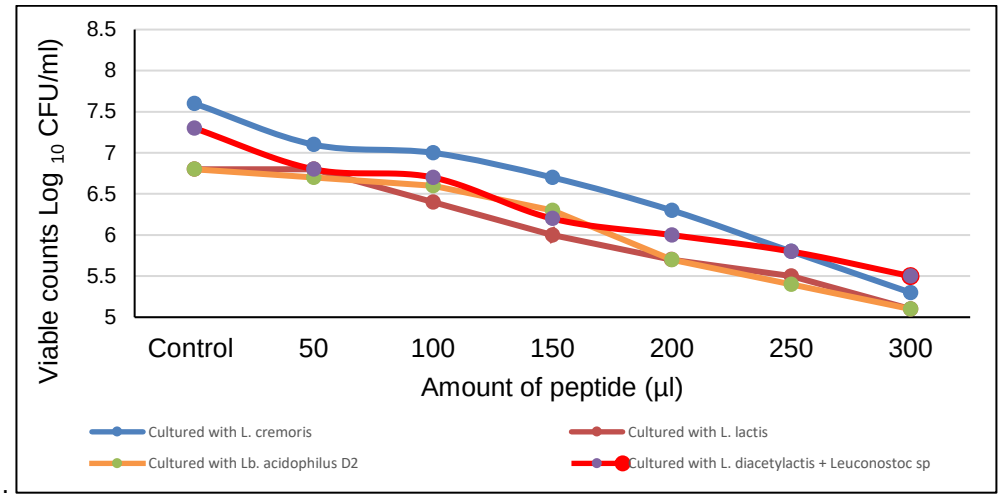

Fig. 4. Inhibitory action of the antibacterial peptide against pathogens in milk cultured with different lactic cultures

In the case of *Lb. acidophilus* D2 curd, the viable count of *E.coli* increased from 4.0 log_{10} cfu/ml initially to 6.8 log_{10} cfu/ml in control. However, it showed comparatively less increase from 4.0 log_{10} cfu/ml initially to 5.1 log_{10} cfu/ml at peptide concentration of 300 µl. Similarly, curd cultured with *Lb. diacetylactis* and *Leuconostoc* sp. showed a similar trend. In this case, the count of *E.coli* decreased by one log cycle compared to the control. In general, it may state that the peptide incorporated was bacteriostatic in nature. In addition, in all cases, the viable count of *E.coli* decreased by two log cycles, except in the case of curd cultured with *Lb. diacetylactis* and *Leuconostoc* sp. where only one log cycle reduction was observed.

4. CONCLUSION

The fermentation of milk by lactobacilli and yoghurt culture leads to the release of several peptides and some of them are known to possess antibacterial activity. By selectively using cultures, which produce large quantities of antibacterial peptides it may be possible to produce the fermented milk products having high inhibitory activity against enteropathogens. The antibacterial peptide identified in this study would further enhance the probiotic properties of the cultures used. By developing suitable methods to produce and purify these antibacterial peptides in a large scale, one can improve the food safety of a variety of foods against enteric pathogens. Further, the antibacterial peptides isolated can be further studied, like amino acid sequencing, purification and quantification, and incorporation of them in other foods for checking their efficiency as antibacterial peptides.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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