ORIGINAL ARTICLE

Evaluation of the antimicrobial attribute of bioactive peptides derived from colostrum whey fermented by *Lactobacillus* against diarrheagenic *E. coli* strains

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Abstract Colostrum known as "liquid gold" contains approximately 60–80% of whey proteins that can be a great source of bioactive peptide production. Therefore, this study aimed to perform a 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 cutoff 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). In addition, ST-1 and LT-1 genes of *E. coli* strains were also confirmed using PCR which is responsible for the diarrheagenic property. Further, the interaction of potent peptides against *E. coli* strains was also observed by scanning electron microscope. Hence, the significance of the present study emphasized that these bioactive peptides generated from fermented colostrum whey can be used as ingredients in functional food against diarrhoea.

Keywords Colostrum whey · A bioactive peptide · Fermentation · Antimicrobial activity

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Abbreviations

E. coli	*Escherichia coli*
L. rhamnosus	*Lacticaseibacillus rhamnosus*
L. casei	*Lacticaseibacillus casei*
WHO	World Health Organisation
SEM	Scanning Electron Microscopy
MTCC	Microbial Type Culture Collection
ATCC	American Type Culture Collection
NDRI	National Dairy Research Institute
ETEC	Enterotoxigenic *E. coli*
cAMP	Cyclic Adenosine Monophosphate
MIC	Minimum Inhibitory Concentration
PCR	Polymerase Chain Reaction
LT	Heat-labile Toxin
ST	Heat-stable Toxin

Introduction

Diarrhoea caused by enterotoxigenic *E. coli* is one of the leading causes of death especially in developing countries. Diarrhoea generally infects people of all age groups while people children and old age people are observed mainly infected. Recently, diarrhoea is recognized as the most commonly occurring symptom in COVID-19 infected patients mainly in old age patients (Huang et al. 2020). In children, 1 out of 5 (under the age of 5) was found to be suffering from diarrhoea which accounts for nearly 5000 children's death per year across the world (WHO 2021). Two key toxin-producing genes ST-1 and LT-1 are mainly responsible for the enterotoxigenic (ETEC) nature of *Escherichia coli* and present only on Ent plasmid. As per their mode of action, the LT-1 gene is mainly responsible for the entry of *E. coli* into the host cell the and ST-1 gene synthesizes a large amount of cAMP (cyclic adenosine monophosphate) that resulting in increases more

water and ion content loss and leads to diarrhoea (Kipkirui et al. 2021). Continued feeding with nutritional supplements is one of the widely accepted approaches to cure childhood diarrhoea, but except continued feeding, there are always some arguments that remain regarding the optimal proper diet or dietary ingredients for maintaining and recovering nutritional status in children suffering from diarrhoea (Gaffey et al. 2013). Bioactive-peptide derived from milk and milk products have been of great research interest over the past few decades (Georgiev 2008). In addition to this, peptide obtained from colostrum has become a new concept because colostrum is now very well known as an excellent source of bioactive protein fraction (Georgiev 2008).

It contains 60–80% of whey proteins of the total protein that differ greatly from mature milk (Playford et al. 2020), The foremost protein in colostrum are immunoglobulins (IgG, IgM, IgA) followed by lactoferrin (LF), α-lactalbumin (α-la), β-lactoglobulin (β-lg), enzymes lactoperoxidase (LP), lysozyme (LZM), and other proline-rich polypeptides minor components which make generated bioactive peptide more functional.

Bioactive peptides are small (usually 2–20 amino acids) sequences generally liberated from native protein (Stelwagen et al. 2009). There are many ways to produce bioactive peptides such as enzymatic hydrolysis by gastrointestinal enzymes (pepsin, trypsin), fermentation by *Lactobacillus,* etc (Singh et al. 2017). Among these, fermentation is one of the most suitable means for the liberation of the peptide as it increases health benefits and improves the overall quality of fermented products (Shahidi and Zhong 2008). Korhonen and Pihlanto (2006) found that microbial fermentation by *Lacticaseibacillus helveticus* increases bio functionality like cheese ripening. Bioactive peptides also play an important role in metabolic functions and promote health benefits properties of living organisms. Therefore, they can classify on basis of their mode of action such as antimicrobial, antithrombotic, anti-hypertensive, opioid, immunomodulatory, mineral binding, and antioxidative peptides (Shahidi and Zhong 2008). In the present study, we mainly emphasized the antimicrobial activity of generated bioactive peptides as these peptides inhibit the growth of diarrhoea-causing pathogens and also increase immunity in immune-compromised persons. Keeping these points in mind, this study aimed to evaluate the antimicrobial activity of bioactive peptides against diarrheagenic *E. coli* generated from colostrum whey by the fermentation process.

Material and methods

Chemicals and bacterial strain

Pathogenic indicator bacterial strains including *Escherichia coli* MTCC723, *Escherichia coli* MTCC725, and *Escherichia coli* MTCC724 were obtained from Microbial Type Culture Collection (MTCC)-Chandigarh, India. Another, *Escherichia coli* ATCC25922 was procured from American Type Culture Collection (ATCC)-Manassas, USA. All reagents and chemicals used in the present study were of analytical grade and purchased from Sigma Aldrich Corporation (Missouri, USA) and Hi-Media (Mumbai, India).

Bioactive peptide isolation

Collection and preparation of buffalo colostrum whey

Colostrum from buffalo was procured from the Livestock Research Centre (LRC), ICAR-NDRI, Karnal, India. Buffalo colostrum of the first three milking was pooled, stored at 4 °C, and clarified using a muslin cloth to remove dust particles. Cleared colostrum was diluted to a 1:2 (colostrum: water) ratio and stored at − 20 °C until use. Further, colostrum whey was prepared using collected cleared colostrum. In brief, colostrum was initially diluted with Milli-Q water in a 1:2 ratio followed by defatting by centrifuged at 6000 rpm for 20 min at 4 °C, and whey was separated by adding rennet (0.1%). After separation, it was filtered by Millipore (0.22 μm) filtration unit and stored at − 20 °C for further use.

Fermentation of colostrum whey and peptide purification

Colostrum whey was fermented using *Lactobacillus* cultures *(Lacticaseibacillus rhamnosus* C25, *Lacticaseibacillus rhamnosus* C6, and *Lacticaseibacillus casei* NCDC17*)* to collect generated bioactive peptides. Briefly, all cultures were activated in De-Man Rogosa Sharpe (MRS) broth for 24 h at 37 °C and after 3 to 4 subculturing, 4×10^8 CFU/mL activated inoculum of each strain was transferred to 1 L of colostrum whey and kept at 37 °C for 48 h for fermentation. After fermentation, the supernatant was separated by centrifugation at 6000 rpm for 15 min at 4 °C, and pH was adjusted to 7 with 0.1 N NaOH. Peptide fractions of > 10 kDa, 5–10 kDa, 3–5 kDa, and < 3 kDa molecular weight were fractionated from fermented colostrum whey using different molecular weights cut-off (MWCO) membrane filters (Himedia, Mumbai, India). The peptides fractions of < 10 kDa were passed through a 5 kDa MWCO membrane and peptide fractions of 5 kDa permeate were further fractionated using a 3 kDa MWCO membrane. The different peptide fractions (> 10 kDa, 5–10 kDa, 5–3 kDa, and < 3 kDa) were collected from permeate and retentate fractions of 10 kDa, 5 kDa, and 3 kDa and all the samples were lyophilized (Labconco, Terra Universal, USA), and stored at − 20 °C for analyzing their antimicrobial and immunomodulatory activities.

Peptide content determination

The peptides present in each fraction were determined by using the ortho-o-phthaldiadehyde (OPA) method (Church et al. 1983). In brief, 250 µL of each fraction (> 10 kDa, 5–10 kDa, 5–3 kDa, and < 3 kDa) obtained from colostrum whey fermented with different *Lactobacillus* cultures was mixed with a 500 µL solution of trichloroacetic acid (0.75%) and incubated at room temperature for 10 min. After incubation, the reaction mixture was filtered through Whatman-filter paper number 42 (2.5 µm) (Whatman, California, USA) and then 150 µL of the filtrate was mixed with 3 mL OPA (o-Phthaldialdehyde) reagent (Sigma, Missouri, USA) and kept at room temperature for 3 min. Absorbance was recorded spectrophotometrically at 340 nm (Shimadzu, UV-1800) followed by obtained optical density calculated with the standard curve of lysine (1 mg/mL).

Antimicrobial activity

The antimicrobial activity of all collected fractions was confirmed using the agar-well diffusion method (Schillinger 1989) against diarrheagenic strains (*E. coli* MTCC724, *E. coli* MTCC723, *E. coli* MTCC725, and *E. coli* ATCC25922) with slight modifications. Briefly, nutrient hard agar (13% nutrient broth and 15% agar) plates were prepared aseptically and overlaid with 7 mL soft agar (13% nutrient broth and 7.5% agar) inoculated with 1×10^4 CFU/mL activated diarrheagenic pathogens. After solidification of agar, wells were prepared using a sterile well borer, and 100 µL from each collected fraction (> 10 kDa, < 10 kDa, 5–10 kDa, 5–3 kDa, 3–5 kDa, and < 3 kDa) were poured into the wells. Plates were stored at 4 °C for 10 min for diffusion then incubated at 37 °C for 2–5 h. A clear zone of inhibition of 1 mm or greater diameters was considered as positive inhibition against indicator diarrheagenic strains.

Minimum inhibitory concentration (MIC) determination

MIC of peptide fractions with good antimicrobial activity was determined by spot-on lawn assay as protocol reported by Rath and Padhy (2013). In brief, samples from each fraction were serially diluted in the concentration of 100, 50, 25, 12.5, 6.25, 3.12 mg/mL and 20 µL of activated pathogen culture (1×10^4 CFU/mL) were added into each dilution. 5 µL of 0.5% of 2,3,5-triphenyl tetrazolium chloride was also added to each dilution and incubated at 37 °C for 18 h. After incubation, optical density was recorded spectrophotometrically at 450 nm (Shimadzu, UV-1800).

Pathogenic gene expression evaluation

Enterotoxigenic (ETEC) property of all diarrheagenic strains (*E. coli* MTCC724, *E. coli* MTCC723, *E. coli* MTCC725, and *E. coli* ATCC25922) was confirmed using a standard PCR method.

Plasmid isolation

All *E. coli* strains (*E. coli* MTCC 723, *E. coli* MTCC 724, *E. coli* MTCC 725, and *E. coli* ATCC 25922) were assessed for ST and LT genes (responsible for diarrhoea). To assess each gene, the plasmid was isolated by a three-step (resuspension, denaturation, and neutralization) method (Feliciello and Chinali 1993). Briefly, all strains of *E. coli* were cultured overnight in Luria–Bertani (LB) broth (Hi-media, Mumbai, India) and cells were isolated from activated inoculum by centrifugation at 5000 RPM for 10 min. Cells were resuspended to 100 µL resuspension buffer containing Tris HCl (25 mM, pH 8.0), glucose (50 mM), and EDTA (10 Mm) and vertex gently. Then, 200 µL of denaturation (lysis) buffer containing sodium dodecyl sulphate (SDS) (1%) and sodium hydroxide (NaOH) solution (0.2 N) was added to pellets and incubated on ice for 10 min. After incubation, 150 µL of renaturation solution containing potassium acetate (5 M) and 11.5 mL glacial acetic acid was added to the pellets and incubated on ice for 5 min with proper inversion. The supernatant was collected by centrifuging the mixture at $12,000 \times g$ for 5 min and 350 µL of isopropanol was added to it. Finally, plasmid pellets were harvested with the addition of 100% ethanol by centrifuging at $13,000 \times g/20$ min.

PCR analysis

LT-1 and ST-1 toxin genes in diarrheagenic pathogen *E. coli* strains were confirmed by PCR analysis. The Primers such as *LT-1F-5'ACGTTCCGGAGGTCTTATGC-3' and LTR-5' AGCCGGTTTGTGTTCCTCTC-3'; ST-1F-5'CGTGAA ACAACATGACGGGAG-3'* and *ST1-R-5' CAGTTGACC TGACTAAAAGAGGGGA-3'* were used for LT-1 and ST-1 toxin genes respectively were designed using nucleotide sequence obtained from NCBI-Gene bank (Accession number- S60731.1) and used for PCR analysis. A PCR reaction mixture of 20 µL was prepared for each pathogen and analysis was carried out with Mycycler™ thermal cycler (Biorad, California, USA). The reaction mixture was prepared using 0.2 µL of HotstartTaq® DNA polymerase, 2 µL primer, 1 µL PCR buffer, and 2 µL of DNA sample. PCR reaction was run through activation step (95 °C/15 min), followed by denaturation step 35 cycles of (95 °C/15 min), annealing (55 °C/45 s), extension (68 °C/2 min), and final elongation (72 °C/5 min). PCR amplified products were subjected to electrophoresis using agarose gel (1.0% w/v). After the run,

PCR product size and bands were examined under UV tans-illuminator (Foto/UV-21, Fotodyne Inc., USA) and photographed using BioRad Gel Doc system (Bio-rad, Hercues, CA, USA) in correspondence with DNA ladder (Bangalore Genei, Bangalore, India) of 100 bp.

Scanning electron microscopy (SEM)

Examination of pathogenic *E. coli* strains treatment with and without collected peptide fraction of < 10 kDa was carried out by SEM. Briefly, 1×10^8 cells of each *E. coli* strain (*E. coli* MTCC 723, *E. coli* MTCC 724, *E. coli* MTCC 725, and *E. coli* ATCC 25922) were inoculated with 100 μL of peptide fractions and incubated at 37 °C for 4 h. After incubation, treated cells were harvested by washing three times with phosphate buffer saline (PBS) (0.1 M, pH 7.2). *E. coli* cells were prepared in three steps (fixation, dehydration, and coating) for SEM examination. Initially, harvested cells were immersed in 3% glutaraldehyde (Hi-media, Mumbai, India) at room temperature for 4 h then washed with PBS (0.1 M, pH 7.2) and centrifuge at $7500 \times g$ for 10 min. After washing, the cell was subjected to dehydration sequentially in 30%, 50%, 70%, 80%, and 90% ethanol graded solution for 5–15 min with centrifugation at $7500 \times g$ for 10 min. Finally, cells were kept in 100% ethanol solution for dehydration. Further, the dehydrated sample was air dried and mounted on a stub followed by Tungsten. The SEM examined was done at the Sophisticated Analytical Instrumentation Facility (SAIF), Punjab University (PU), Chandigarh, India up to 10 μm depth.

Statistical analysis

Data acquired in the present study were analyzed statistically using GraphPad Prism (GraphPad Software-5.01, CA, USA). The standard error mean differences were analyzed to obtain a significance of *P* value using One Way Analysis of Variance (ANOVA) for each experiment performed in triplicate and values were calculated as mean ± SEM with the significance difference $p < 0.05$.

Result and discussion

Peptide content obtained from fermented colostrum whey

Colostrum whey was embedded with many bioactive peptides that can inhibit a broad spectrum of microorganisms (Ruiz-Diaz et al. 2019). As per the literature, many studies have reported the antimicrobial activity of these isolated proteins, but few studies were reported on peptides derived from the fermentation of colostrum whey along with their antimicrobial activity. Peptides obtained from *L. rhamnosus* C25 fermented colostrum whey showed maximum peptide content within a range from 4.35 ± 0.03 to 8.1 ± 0.04 mg/mL (Table 1). Peptide content was within the range of 4.52 ± 0.03 to 6.55 ± 0.05 mg/mL of peptide fraction obtained from *L. rhamnosus* C6 fermented colostrum whey. It was found that peptide content was < 10 kDa (6.49 ± 0.03[d]), 5–10 kDa (6.49 ± 0.03[d]), < 5 kDa (6.45 ± 0.03[d]). Whereas, in 3–5 kDa (5.55 ± 0.02) and < 3 kDa (4.52 ± 0.04). A similar decreasing trend in peptide content was observed in fractions obtained from *L. rhamnosus* C6 fermented colostrum as found with *L. rhamnosus* C25 (Table 1). Further, peptide content of fractions obtained from *L. casei* NCDC17 fermented colostrum whey, showed were within the range of 3.52 ± 0.03 to 5.89 ± 0.05 mg/mL, and lower peptide content was observed in peptide content of all the fractions as compared to peptide content of fraction obtained from *L. rhamnosus* C25 and *L. rhamnosus* C6 fermented colostrum whey. This may be due to the low proteolytic activity of *L. casei* NCDC 17 which resulted in low peptide content.

Moreover, it was also observed that in all three different fermented colostrum whey, a similar decrease in peptide content was observed as peptide fraction seize decreased

Table 1 Total peptide content present in the fractions obtained from fermented colostrum whey

|	Peptide content (mg lysine/mL)					
	> 10 kDa	< 10 kDa	5–10 kDa	< 5 kDa	3–5 kDa	< 3 kDa
L. rhamnosus C25 fermented whey	8.1 ± 0.04[b]	7.92 ± 0.01[c]	7.32 ± 0.03[d]	6.35 ± 0.03[d]	5.32 ± 0.03[d]	4.35 ± 0.03[d]
L. rhamnosus C6 fermented whey	7.09 ± 0.04[b]	6.55 ± 0.01[c]	6.49 ± 0.03[d]	6.45 ± 0.03[d]	5.55 ± 0.03[d]	4.52 ± 0.03[d]
L. casei NCDC 17 fermented whey	6.09 ± 0.04[b]	5.89 ± 0.01[c]	5.02 ± 0.03[d]	4.52 ± 0.03[d]	4.02 ± 0.03[d]	3.52 ± 0.03[d]

Values are mean ± S.E.M. of triplicate

Values [a, b, c, d] are significantly different at $P \leq 0$

L. rhamnosus C25, *Lacticaseibacillus rhamnosus* C25; *L. rhamnosus* C6, *Lacticaseibacillus rhamnosus* C6; *L. casei* NCDC17, *Lacticaseibacillus casei* NCDC17

from < 10 to < 3 kDa. This large variation maybe was been due to sequential passing permeate to the smaller membrane from 5–10 kDa to 5 kDa MWCO membrane and 3–5 kDa to 3 kDa that decreased the peptide content. Recently, Fajardo-Espinoza et al. (2020) reported a similar study, where colostrum whey was hydrolyzed by pepsin, and separated hydrolysates were separated and evaluated with ACE inhibitory and anti-oxidative activity. It was found that maximum antioxidant activity (934.9 µMol/L) and ACE inhibitory (261.56 µMol/L) were achieved in hydrolysate obtained after 36 h of fermentation. A similar decreasing trend was observed by Singh et al. (2020) when bioactive peptides were separated using a similar fraction technique from soybean fermented milk using 10 kDa, 5 kDa, and 3 kDa molecular weight cut-off membranes, and a decreasing trend in peptide content was found in fraction obtained as maximum in 10 kDa in comparison to 5 kDa and < 3 kDa fractions. This decrease in Cut-Off from 10 to 3 kDa sizes allowed only small peptides to pass through a membrane which led to decreases peptide content (Puchalska et al. 2014).

Antimicrobial activity of ultra-filtered fractions

Peptide fractions obtained after fractionation were evaluated for antimicrobial activity against diarrheagenic *E. coli* pathogens and a higher zone of inhibition was shown by the fractions of > 10 kDa in comparison to < 10 kDa. A similar pattern was observed for all fractions and noticed that *E. coli* MTCC 723 (15 ± 0.07 mm) was found more resistant against all collected fractions as compared to *E. coli* MTCC 725 (18 ± 0.07 mm), *E. coli* MTCC 724 (17 ± 0.07 mm), and *E. coli* ATCC 25922 (16 ± 0.04 mm) as shown in Fig. 1D. In addition, peptide fractions (< 10 kDa, > 10 kDa, 5–10 kDa, and, < 5 kDa) obtained from *L. rhamnosus* C25 fermented colostrum whey fractions showed a significant ($p < 0.01$) zone of inhibition against all diarrheagenic strains (*E. coli* MTCC 724, *E. coli* MTCC 723, and *E. coli* MTCC 725) as shown in Fig. 1 A. Whereas, intermediate and low zone of inhibition was shown by 5–10 kDa, 5 kDa i.e., within the range of 8.7 ± 0.07 to 10 ± 0.03 mm, and 3–5 kDa and < 3 kDa within a range of 6 ± 0.04 to 7 ± 0.05 mm respectively. The antimicrobial activity of peptide fractions (> 10 kDa, to < 5 kDa) obtained from *L.*
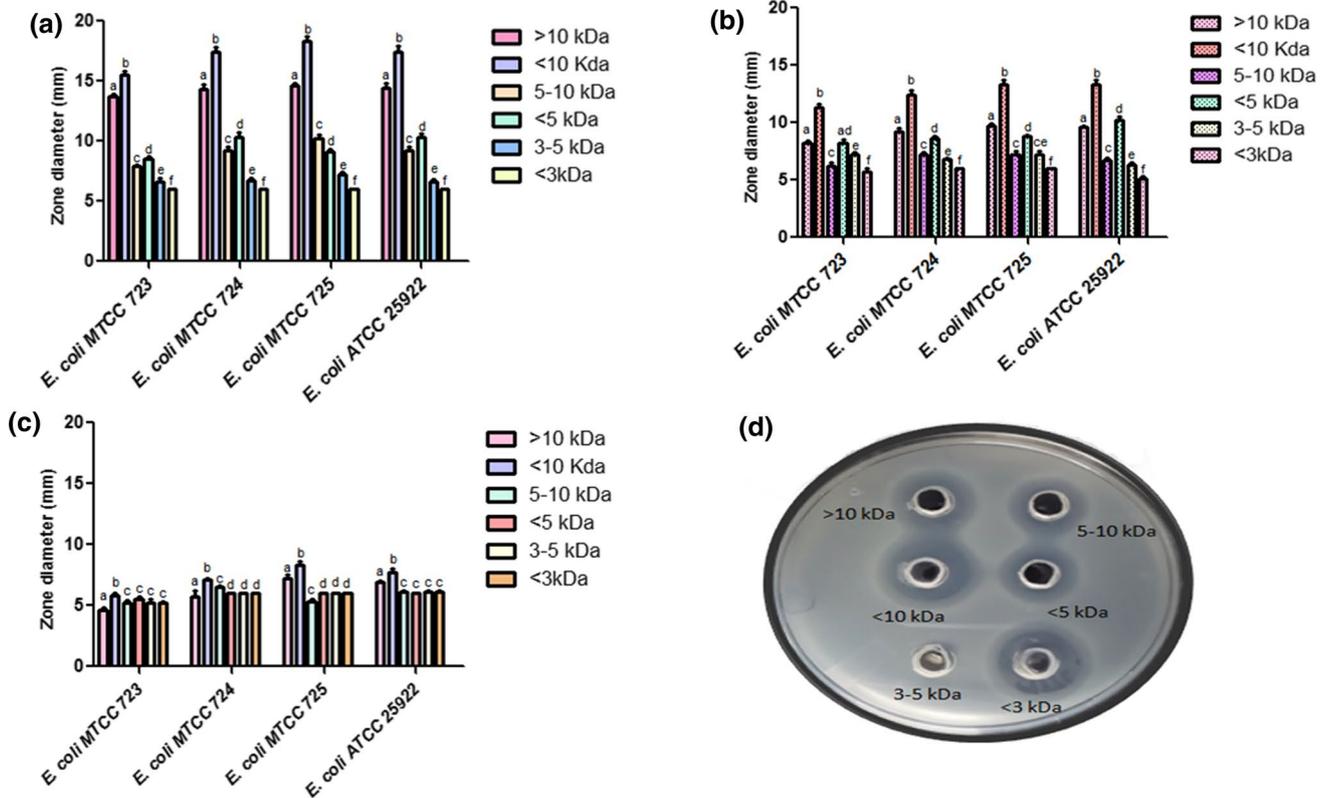
Fig. 1 Antimicrobial activity of bioactive peptide fraction obtained from **a** *L. rhamnosus* C25 fermented colostrum whey; **b** *L. rhamnosus* C6 fermented colostrum whey; **c** *L. casei* NCDC17 fermented colostrum whey against *E. coli* stains. **d** Plate represents antimicrobial activity of ultrafiltered fraction obtained from *L. rhamnosus* C25 fermented colostrum whey against *E. coli* MTCC 723. Values are mean $\pm$ S.E.M. of triplicate. Values a, b, c, d, e, f are significantly different at $P \leq 0.05$. All zone of inhibition including 6 mm of well diameter*

rhamnosus C6 fermented colostrum whey showed antimicrobial activity within the range of 7 ± 0.01 to 13 ± 0.04 and maximum antimicrobial activity (Fig. 1B) was observed in fraction < 10 kDa within a range from 18 ± 0.05 mm to 15 ± 0.07 mm. Whereas, < 5 kDa and < 3 kDa showed moderate (13.04 ± 0.6 to 11.4 ± 0.8) and weaker inhibition (8 ± 0.04 to 7.5 ± 0.05).

This difference in the activity may be due to the number of antimicrobial peptides present in the peptide fractions. Ultra-filtered fractions obtained from *L. casei* NCDC17 fermented colostrum whey showed weak inhibition (Fig. 1C) against less sensitive stains within the range of 7.9 ± 0.64 to 6.5 ± 0.4 comparedpare to antimicrobial activity obtained from fermented colostrum whey obtained from *L. rhamnosus* C25 and *L. rhamnosus* C6. The mechanism behind the antimicrobial peptide may involve electrostatic interactions between antimicrobial peptides and *E. coli* cell wall that result in disintegration of the lipid bilayer and lead to disruption of cell. Mostly, peptides obtained from colostrum were found amphipathic in nature and overall net charge decided the interaction with bacteria (Bahar and Ren 2013).

Recently, Kashyap et al. (2022) reported phagocytosis activity of colostrum-derived peptide fraction (< 10 kDa) in vitro, where previously bioactive peptides were generated from *L. rhamnosus* C25 fermented. After evaluation, the fraction was characterized by SDS-PAGE and HPLC and identified by LCMS/MS. It was found that a total of 56 novel peptides were recognized after LCMS/MS analysis where 16 peptides sequences were identified with antimicrobial activity and 40 peptides were immunomodulatory potential.

Singh and Vij (2017), reported antimicrobial activity of fermentate obtained from soy milk and found that soy milk fermented with different *Lactobacillus* strains like *L. plantarum* C6, *L. rhamnosus* C8, *L. rhamnosus* C25, and *L. rhamnosus* NCDC 288 for 24 h exhibited significant antimicrobial activity against pathogenic organisms. A strong zone of inhibition was observed against *E. coli* ATCC 25922 (15–20 nm), *S. aureus* MTCC 1144 (11–14 mm), *B. cereus* ATCC 14579 (11–14 mm), whereas minimum inhibition (8–10 mm) was found against *S. enteric* NCTC 6017 and *S. dysenteriae* NCDC 107. In the same tune of the above-discussed results in the present study, peptide fraction of < 10 kDa showed higher inhibition (18 ± 0.05) compared to fermentate (10 ± 0.05 mm).

Recently, Halavach et al. (2020) also reported a similar study where colostrum whey was hydrolyzed by alcalase and neutrase enzymes and after hydrolysis, maximum inhibition (82%) was observed by neutrase-hydrolysate colostrum whey against *E. coli* ATCC 8739. This was due to neutrase treatment generating more peptides as compared to alcalase. Similarly in this study, the least peptide contents were noticed in *L. casei* NCDC 17 fermented whey in comparison to *L. Plantarum* C6 and *L. rhamnosus* C25. Singh et al.

(2016) reported antimicrobial activity of a total of 61 *Lactobacilli* strains isolated from 110 food samples and found strong inhibition against *S. aureus* and *Aspergillus*. The maximum zone of inhibition (up to 28 mm) was 59% isolates against *Aspergillus*. With this support in the present study also, antimicrobial activity of collected fractions isolated from fermented whey was confirmed which showed a zone of inhibition within a range from 18 to 7.5 mm.

Screening of *Lactobacillus* cultures based on antimicrobial activity

Fractions that showed moderate to strong inhibition were screened and divided into three categories i.e., high zone of inhibition (13–20 mm), intermediate zone of inhibition (6–12 mm), and low zone of inhibition (< 6 mm including zone diameter). As shown in Table 2, it was found that among all 21 fractions, only 8 fractions including < 10 kDa, 5–10 kDa, and < 5 kDa were selected from *L. rhamnosus* C25 and *L. rhamnosus* C6 fermented colostrum whey which showed zone of inhibition within range of 18 ± 0.05 to 7.5 ± 0.04 against all *E. coli* pathogenic strains. While peptide fraction obtained from *L. casei* NCDC 17 did not show any significant zone of inhibition. Similar, results were reported by Singh et al. (2020), where ACE inhibitory activity was found significantly ($p < 0.05$) higher (65.52 ± 1.40) for *L. rhamnosus* C25 and *L. rhamnosus* C8 (62.90 ± 0.52) as compared to the reference strains (56.50 ± 1.22). Moreover, after isolation and characterization of the *Lactobacillus* strain by PCR method, among 61 isolates, *L. Plantarum* showed moderate to strong inhibition against *S. aureus* and *Aspergillus* (up to 28 mm) whereas no zone of inhibition was observed against *E. coli* (Singh et al. 2016).

MIC of bioactive peptide fractions

MIC of peptide fractions with the highest antimicrobial activity was calculated and Table 3 depicted that < 10 kDa fraction showed minimum IC_{50} concentration to inhibit *E. coli* strains within the range of 0.49 ± 0.4 to 0.03 ± 0.7 mg/mL. Whereas, other fractions such as > 10 kDa and 5–10 kDa, < 5 kDa required higher peptide concentrations within the range of 2.12 ± 0.4 to 0.26 ± 0.3 and most sensitive strains required less concentration for inhibition as *E. coli* MTCC 725 (0.03 ± 0.7), and *E. coli* ATCC 25922 (0.06 ± 0.1 mg/mL). On other hand, most pathogenic strains such as *E. coli* MTCC 723 required higher (49 ± 0.4 mg/mL) peptide concentration. Recently, García-Borjas et al. (2021) reported a minimum inhibitory concentration of lactoferrin proteins isolated from bovine colostrum and found 0.8 μM against *E. coli* and *L. monocytogenes*. Similarly in the present study, < 10 kDa fraction showed maximum inhibition at higher concentrations of 0.49 ± 0.4 to 0.03 ± 0.7 mg/

Table 2 Antimicrobial activity of bioactive peptide obtained after ultra-filtered fractions against diarrhoeagenic *E. coli* strains

Peptide fractions obtained from	kDa	*E. coli* MTCC 723	*E. coli* MTCC 724	*E. coli* MTCC 725	*E. coli* ATCC 25922
L. rhamnosus C25 fermented colostrum whey	>10	13±0.03[b]	14±0.03[b]	14±0.03[b]	14±0.03[b]
	<10	15±0.07[c]	17±0.07[c]	18±0.07[c]	17±0.07[c]
	5–10	8±0.03[d]	9±0.03[d]	10±0.03[d]	9±0.03[d]
	<5	8.7±0.07[d]	10±0.07[e]	9.5±0.07[e]	10±0.07[e]
	3–5	6.5±0.05[e]	6.5±0.05[f]	7±0.05[f]	6.5±0.05[f]
	<3	6±0.04[e]	6±0.04[f]	6±0.04[f]	6±0.04[f]
L. rhamnosus C6 fermented colostrum whey	>10	8.4±0.04[b]	9±0.04[b]	9.5±0.04[b]	9.5±0.04[b]
	<10	11±0.04[b]	12±0.04[b]	13±0.04[b]	13±0.04[b]
	5–10	6.5±0.05[a]	7±0.05[a]	7.5±0.05[a]	6.9±0.05[a]
	<5	8±0.04[b]	7.5±0.04[b]	7.7±0.04[b]	8±0.04[b]
	3–5	6.4±0.01[c]	6.7±0.01[c]	7±0.01[c]	6.5±0.01[c]
	<3	6±0.03[d]	6±0.03[d]	6±0.03[d]	6±0.03[d]
L. casei NCDC17 fermented colostrum whey	>10	6±0.05[a]	6±0.05[a]	8±0.05[a]	6±0.05[a]
	<10	6±0.04[b]	6.7±0.04[b]	7.5±0.04[b]	6±0.04[b]
	5–10	6±0.01[c]	6±0.01[c]	7±0.01[c]	6±0.01[c]
	<5	6±0.03[d]	6±0.03[d]	6±0.03[d]	6±0.03[d]
	3–5	6±0.07[e]	6±0.07[e]	6±0.07[e]	6±0.07[e]
	<3	6±0.05[a]	6±0.05[a]	6±0.05[a]	6±0.05[a]

Values are mean±S.E.M. of triplicate

Values [a, b, c, d,e] are significantly different at $P \leq 0.05$

All zone of inhibition including 6 mm of well diameter

L. rhamnosus C25, *Lacticaseibacillus rhamnosus* C25; *L. rhamnosus* C6, *Lacticaseibacillus rhamnosus* C6; *L. casei* NCDC17, *Lacticaseibacillus casei* NCDC17

Table 3 MIC of the bioactive peptide with highest antimicrobial activity derived from colostrum whey fermented with *L. rhamnosus* C25 and *L. rhamnosus* C6

IC_{50} (mg/ml)

Peptide fractions obtained from	kDa	*E. coli* MTCC 723	*E. coli* MTCC 724	*E. coli* MTCC 725	*E. coli* MTCC 25922
L. rhamnosus C25 fermented colostrum whey	>10	2.12±0.4[a]	0.53±0.3[b]	0.13±0.7[c]	0.26±0.3[d]
	<10	0.49±0.4[a]	0.06±0.1[b]	0.03±0.7[c]	0.06±0.003[d]
	5–10	3.65±0.6[a]	0.91±0.8[b]	0.45±0.3[c]	0.91±0.7[d]
	<5	3.175±0.9[a]	0.79±0.4[b]	0.39±0.1[c]	0.79±0.3[d]
L. rhamnosus C6 fermented colostrum whey	>10	1.86±0.5[a]	0.46±0.4[b]	0.23±0.1[c]	0.46±0.3[d]
	<10	0.81±0.5[a]	0.20±0.4[b]	0.10±0.1[c]	0.20±0.3[d]
	5–10	3.75±0.7[a]	1.86±0.3[b]	0.931±0.6[c]	1.86±0.8[d]
	<5	3.26±0.5[a]	1.63±0.6[b]	1.63±0.2[c]	1.63±0.3[d]

Values are mean±S.E.M. of triplicate

Values [a, b, c, d] are significantly different at $P \leq 0.05$

L. rhamnosus C25, *Lacticaseibacillus rhamnosus* C25; *L. rhamnosus* C6, *Lacticaseibacillus rhamnosus* C6; *L. casei* NCDC17, *Lacticaseibacillus casei* NCDC17

mL which was due to interference of antimicrobial activity of peptides generated from lactoferrin and other protein. In this report, it was also observed that exposure to lower concentration only results in inhibition of most sensitive strain bacteria, whereas, resistant strains required higher concentration.

Hilpert et al. (2005) also reported MIC of synthetic peptide "bactenecin" within the range of 2 µg/mL against *E.*

coli and *S. aureus*. Similarly, IC$_{50}$ concentrations of two antimicrobial peptides casidin A and casidin B was again noticed at 2 mg/mL and 1 mg/mL respectively against *E. coli* O157:H7, while a low dose of 0.5 mg/mL showed no significant inhibition (McDonnell et al. 2012). With the same tune, similar patterns were obtained in the present study, and MIC concentration of < 10 kDa fraction obtained from *L. rhamnosus* C25 fermented colostrum whey showed lower MIC in comparison to other fractions.

Genotypic characterization

ST and LT toxin-producing genes are mainly responsible for the enterotoxigenic (ETEC) nature of *E. coli* and present on Ent plasmid. Therefore, LT-1 (110 bp) and ST-1 (154 bp) genes were detected in the plasmid of all diarrheagenic E. *coli* strains and as shown in Fig. 2, where all *E. coli* strains express both LT-1 and ST-1 genes. This PCR-based expression confirmation of both genes indicated that selected *E. coli* strains were satisfactory diarrheagenic pathogens to investigate the antimicrobial property of generated peptides. In support of our finding, Patel et al. (2011) reported both ST-1 and LT-1 bands present in *E. coli* MTCC 723 in potable water by using the PCR method. A similar study was again conducted by Shabana et al. (2017) where they also targeted similar genes among 60 isolates of different faecal samples collected from cattle, goats, and lambs. Ram et al. (2007) also reported that nearly 75% of *E. coli* isolates from the Indian River Ganga and 67% from the Saryu River contained both ST-1 and LT-1 genes which justified the enterotoxigenic

property and diarrheagenic nature of these isolates and this finding also support our outcomes.

Mode of action of antimicrobial bioactive peptides

Peptide fraction of < 10 kDa obtained from *L. rhamnosus* C25 fermented colostrum whey was treated with *E. coli* strains and investigated by SEM examination. During the microscopic examination, clear disruptions in the cells of *E. coli* MTCC 724, *E. coli* MTCC 723, *E. coli* MTCC 725, and *E. coli* ATCC 25922 as presented observed in Fig. 3B–E (treatment) in comparison to control (Fig. 3A). This cell disruption may indicate that bioactive peptides liberated from fermented colostrum whey had a potential antimicrobial property that resulted in the cell death of the pathogens. Forex, lactoferricin peptide liberated from lactoferrin that wide range of antimicrobial activity against many pathogens. Our outcomes presented in this study directly supported the finding of Li et al. (2015) where *E. coli* cells were treated with a novel peptide (P7) obtained from *L. crustorum* MN047 and its impact on cells was analyzed by SEM examination. They noticed that *E. coli* cells showed wrinkled cell surface of the cell after 30 min of treatment and complete cell lysis was observed due to pore formation in the cell membrane after 2 h.

Similarly, Mishra et al. (2013) also reported a study where peptide WFRKQLKW (lactoferrin isolated peptide) with MIC concentration (4 μg/mL) was incubated with ESBL *E. coli* for 15 min and 30 min. During SEM images, blabbing was noticed on the cells within 15 min, and complete lysed cell debris was observed after 30 min of incubation. The

Fig. 2 Confirmation of **a** ST-1 at110 bp, **b** LT-1 gene at 154 bp in *E. coli* enterotoxigenic strains, where Lane 1 *E. coli* MTCC 723, Lane 2 *E. coli* MTCC 724, Lane 3 *E. coli* MTCC 725 and Lane 4 *E. coli* ATCC 25922. Lane 1 Marker, Lane 2 (*E. coli* MTCC 723, Lane 3 *E. coli* MTC 724, Lane 4 *E. coli* MTCC 724 and Lane 5 *E. coli* ATCC 25922
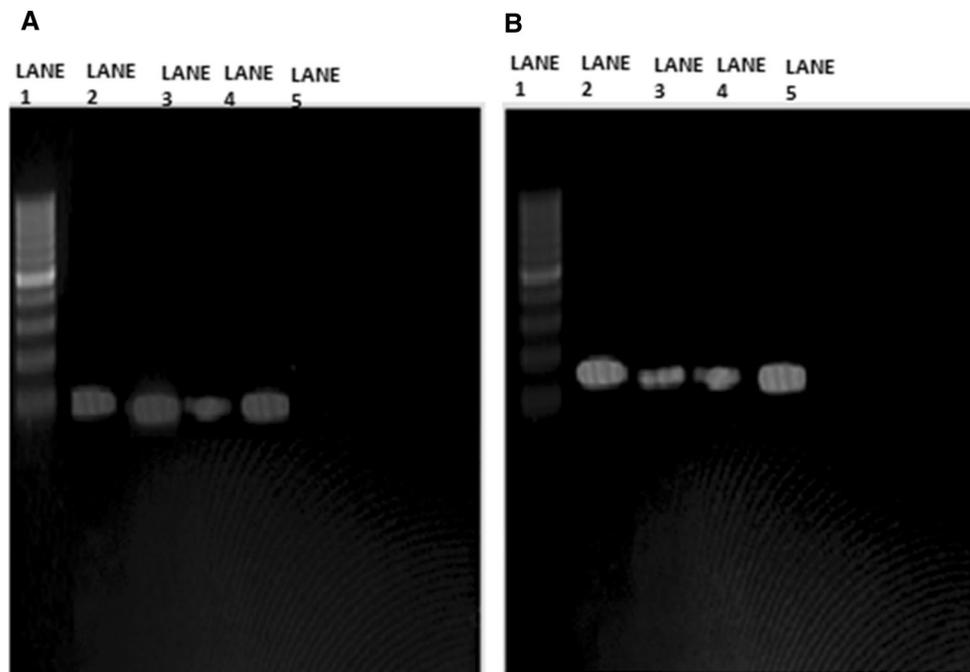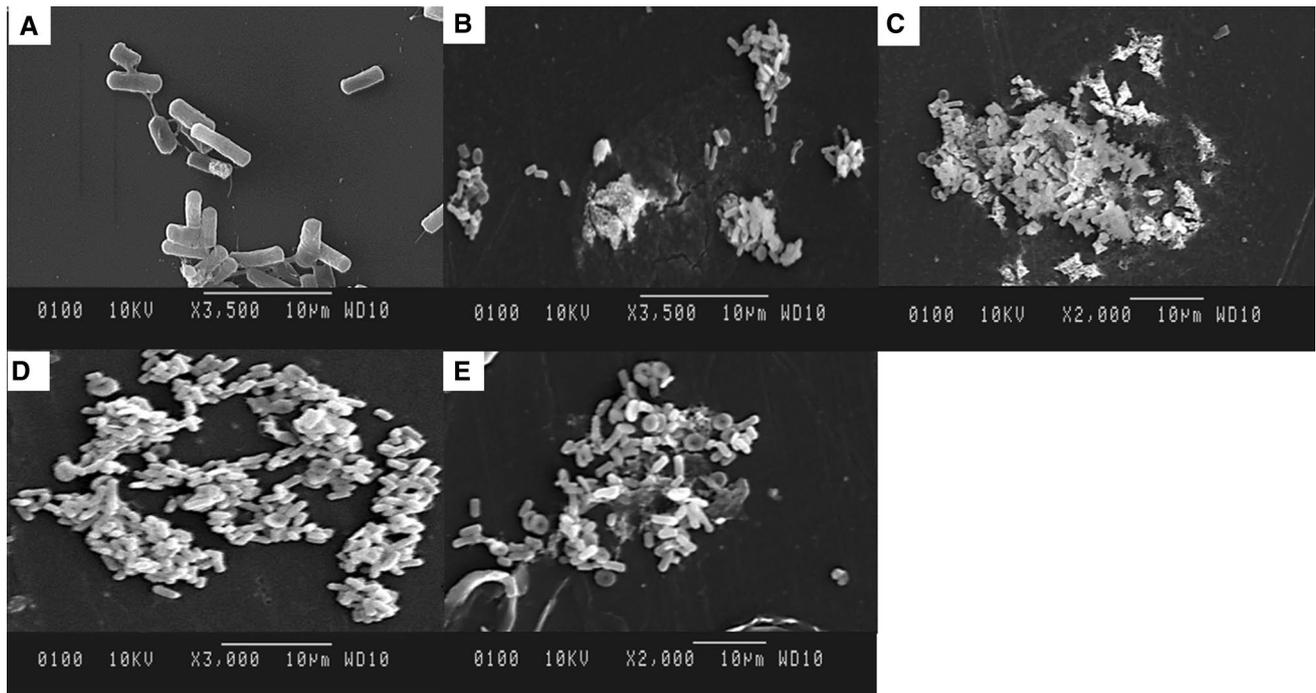
Fig. 3 Scanning electron microscope view of antimicrobial activity against *E. coli* MTCC 723 **a** control, **b** *E. coli* MTCC 723 + < 10 kDa fraction, **c** *E. coli* MTCC 724 + < 10 kDa fraction, **d** *E. coli* MTCC 725 + < 10 kDa fraction, **e** *E. coli* ATCC 25922 + 10 kDa fraction

above-discussed finding directly supports our finding that more lysed cellular disruption and debris were noticed after 4 h of incubation.

Conclusion

Several studies have been reported previously about the production of the bioactive peptides from milk matrix but to the best of our knowledge, this is the first reporting the production of bioactive peptides by fermentation of protein-rich colostrum whey using three different strains (*L. rhamnosus* C25, *L. rhamnosus* C6, and *L. casei* NCDC17). In this study, fermentation of whey by *L. rhamnosus* C25 was significantly ($p < 0.001$) emphasized in comparison to other *Lactobacillus* strains, and bioactive peptides of < 10 kDa with significant ($p < 0.001$) antimicrobial attribute were separated from *L. rhamnosus* C25 fermented colostrum whey also showed higher peptide content as compared to other two fermented colostrum whey. A decreasing peptide content was observed in the fractions from < 10 to < 3 kDa because of the sequential passing of permeate to the smaller membrane. Further, antimicrobial activity was analyzed against diarrheagenic *E. coli* strains and found that among all fractions, < 10 kDa fraction obtained from *L. rhamnosus* C25 fermented whey showed significantly ($p < 0.001$) higher antimicrobial activity in comparison to all collected fractions. The mode of

action was further confirmed by SEM examination that showed in clear disruption of *E. coli* cells due to the presence of antimicrobial peptides. Therefore, the significance of this study emphasizes that colostrum whey-derived bioactive peptides can be used as a potential therapeutic antimicrobial agent in various functional foods against many diarrheagenic food-borne pathogens.

Declarations

References

Bahar AA, Ren D (2013) Antimicrobial Peptides Pharmaceu 6(12):1543–1575. https://doi.org/10.3390/ph6121543

Church FC, Swaisgood HE, Porter DH, Catignani GL (1983) Spectrophotometric assay using o-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. J Dairy Sci 66(6):12194–21227. https://doi.org/10.3168/jds.S0022-0302(83)81926-2

Fajardo-Espinoza FS, Romero-Rojas A, Hernández-Sánchez H (2020) Production of bioactive peptides from bovine colostrum whey using enzymatic hydrolysis. Rev Mex Ing Quím 19(1):1–9. https://doi.org/10.24275/rmiq/Alim525

Feliciello I, Chinali G (1993) A modified alkaline lysis method for the preparation of highly purified plasmid DNA from *Escherichia coli*. Anal Biochem 212(2):394–401. https://doi.org/10.1006/abio.1993.1346

Gaffey MF, Wazny K, Bassani DG, Bhutta ZA (2013) Dietary management of childhood diarrhea in low- and middle-income countries: a systematic review. BMC Public Health 13(S17):1–16

García-Borjas KA, Ceballos-Olvera I, Luna-Castro S, Peña-Avelino Y (2021) Bovine Lactoferrin can decrease the *in vitro* biofilm production and show synergy with antibiotics against *Listeria monocytogenes* and *Escherichia coli* isolates. Protein Pept Lett 28(1):101–107. https://doi.org/10.2174/0929866527666200403111743

Georgiev IP (2008) Differences in chemical composition between cow colostrum and milk. Bulg J Vet Med 11(1):3–12

Halavach TM, Dudchik NV, Tarun EI, Zhygankov VG, Kurchenko VP, Romanovich RV, Asafov VA (2020) Biologically active properties of hydrolyzed and fermented milk proteins. J Microbiol Biotechnol 9(4):714–720. https://doi.org/10.1186/s40794-021-00157-z

Hilpert K, Volkmer-Engert R, Walter T, Hancock RE (2005) High-throughput generation of small antibacterial peptides with improved activity. Nat Biotechnol 23(8):1008–1012. https://doi.org/10.1038/nbt1113

Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Cao B (2020) Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet 395(10223):497–506. https://doi.org/10.1016/S0140-6736(20)30183-5

Kashyap R, Narayan KS, Vij S (2022) Identification of antibacterial and immunomodulatory bioactive peptides generated from buffalo (Bubalus bubalis) colostrum whey fermented by Lactobacillus rhamnosus C25: LC-MS/MS-based analysis. J Funct Foods 95:105158. https://doi.org/10.1016/j.jff.2022.105158

Kipkirui E, Koech M, Ombogo A, Kirera R, Ndonye J, Kipkemoi N, Kirui M, Philip C, Roth A, Flynn A, Odundo E, Kombich J, Daud I (2021) Molecular characterization of enterotoxigenic *Escherichia coli* toxins and colonization factors in children under five years with acute diarrhea attending Kisii Teaching and Referral Hospital, Kenya. Trop Dis Travel Med Vaccines 7:31. https://doi.org/10.1186/s40794-021-00157-z

Korhonen H, Pihlanto A (2006) Bioactive peptides: production and functionality. Int Dairy J 16(9):9454–9960. https://doi.org/10.1016/j.idairyj.2005.10.012

Li L, Shi Y, Cheng X, Xia S, Cheserek MJ, Le G (2015) A cell-penetrating peptide analog, P7, exerts antimicrobial activity against *Escherichia coli* ATCC25922 via penetrating cell membrane and targeting intracellular DNA. Food Chem 166:231–239. https://doi.org/10.1016/j.foodchem.2014.05.113

McDonnell MJ, Rivas L, Burgess CM, Fanning S, Duffy G (2012) Inhibition of verocytotoxigenic *Escherichia coli* by antimicrobial peptides casein A and B and the factors affecting their antimicrobial activities. Int J Food Microbiol 153(3):260–268. https://doi.org/10.1016/j.ijfoodmicro.2011.10.001

Mishra B, Leishangthem GD, Gill K, Singh AK, Das S, Singh K (1828) Dey S (2013) A novel antimicrobial peptide derived from modified N-terminal domain of bovine lactoferrin: design, synthesis, activity against multidrug-resistant bacteria and Candida. Bioch Biophy Acta (BBA) Biomembr 2:677–686. https://doi.org/10.1016/j.bbamem.2012.09.021

Patel CB, Vajpayee P, Singh G, Upadhyay RS, Shanker R (2011) Contamination of potable water by enterotoxigenic *Escherichia coli:* qPCR based culture-free detection and quantification. Ecotoxicol Environ Saf 74(8):2292–2298. https://doi.org/10.1016/j.ecoenv.2011.07.022

Playford RJ, Garbowsky M, Marchbank T (2020) Pasteurized chicken egg powder stimulates proliferation and migration of AGS, RIE1, and Caco-2 cells and reduces NSAID-induced injury in mice and colitis in rats. J Nutr 150(6):1434–1442. https://doi.org/10.1093/jn/nxaa083

Puchalska P, Marina ML, García MC (2014) Isolation and identification of antioxidant peptides from commercial soybean-based infant formulas. Food Chem 148:147–154. https://doi.org/10.1016/j.foodchem.2013.10.030

Ram S, Vajpayee P, Shanker R (2007) Prevalence of multi-antimicrobial-agent resistant, Shiga toxin and enterotoxin producing *Escherichia coli* in surface waters of river Ganga. Environ Sci Technol 41(21):7383–7388. https://doi.org/10.1021/es0712266

Rath S, Padhy RN (2013) Monitoring in vitro antibacterial efficacy of *Terminalia alata* Heyne ex. Roth, against MDR enteropathogenic bacteria isolated from clinical samples. J Acute Med 3(3):93–102. https://doi.org/10.1016/j.jacme.2013.06.002

Ruiz-Diaz MD, Argüello A, Padilla D, Earley B, Castro N (2019) Influence of treatment and refrigeration time on antimicrobial activity of goat and sheep colostrum. J Dairy Res 86(4):450–453. https://doi.org/10.1017/S0022029919000657

Schillinger U (1989) Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl Environ Microbiol 55(8):19014–21906. https://doi.org/10.1128/aem.55.8.1901-1906.1989

Shabana II, Bouqellah NA, Zaraket H (2017) Investigation of viral and bacterial enteropathogens of diarrheic sheep and goats in Medina, Saudi Arabia. Trop Biomed 34(4):944–955. https://doi.org/10.1016/j.sjbs.2020.01.009

Shahidi F, Zhong Y (2008) Bioactive peptides. J AOAC Int 91(4):914–931. https://doi.org/10.1093/jaoac/91.4.914

Singh BP, Vij S (2017) Growth and bioactive peptides production potential of *Lactobacillus plantarum* strain C2 in soy milk: a LC-MS/MS-based revelation for peptides biofunctionality. LWT 86:293–301. https://doi.org/10.1016/j.lwt.2017.08.013

Singh P, Saini P, Sachan S, Dubey S (2016) Characterization, antimicrobial activity, and antibiotic susceptibility of lactic acid bacteria isolated from food samples. Int J Curr Microbial 5(7):901–911. https://doi.org/10.20546/ijcmas.2016.507.102

Singh TP, Kaur G, Kapila S, Malik RK (2017) Antagonistic activity of *Lactobacillus reuteri* strains on the adhesion characteristics of selected pathogens. Front Microbiol 8:486. https://doi.org/10.3389/fmicb.2017.00486

Singh BP, Bhushan B, Vij S (2020) Antioxidative, ACE inhibitory and antibacterial activities of soy milk fermented by indigenous strains of lactobacilli. Legume Sci 2(4):e54. https://doi.org/10.1002/leg3.54

Stelwagen K, Carpenter E, Haigh B, Hodgkinson A, Wheeler TT (2009) Immune components of bovine colostrum and milk. J Anim Sci 87(13):3–9. https://doi.org/10.2527/jas.20081377

WHO (2021) Pneumonia and diarrhoea progress report. https://ourworldindata.org/diarrheal-diseases