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Effect of in vitro simulated gastrointestinal digestion on the antibacterial properties of bovine lactoferrin

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Research Article

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Abstract

The aim of this research was to investigate the ability of an in vitro simulated gastrointestinal digestion (SGID) to generate peptides from bovine lactoferrin (LF) that possess antibacterial activity. *Escherichia coli* was examined as the target pathogen due to its prevalence in foods and the well-documented antibacterial effect of both LF and LF peptides against this organism. Results showed that *in-vitro* digested LF, specifically gastric LF digesta, exhibited significant antibacterial activity at low concentrations against *E. coli* compared to its undigested counterpart. Additionally, the highest antibacterial activity in the gastric digesta was associated with a relatively high molecular weight fraction of >30 kDa obtained within the first 30 min of the SGID. This demonstrates that the digestive process can result in the generation of antibacterial LF peptides and contribute to improving the antimicrobial properties of LF exhibited in its undigested state, making it a suitable dairy food additive to potentially provide protection against bacterial pathogens within the gastrointestinal system.

Lactoferrin (LF) is an iron binding glycoprotein found in the milk of all mammals that acts as a protective factor in milk, and is thought to provide antimicrobial activity to the infant (Chandan *et al.*, 2015). Bovine LF is a large protein of circa 80 kDa, depending on the degree of glycosylation (Korhonen and Marnila, 2011). It is comprised of a simple polypeptide chain folded into two symmetrical lobes, the N- and C-lobes (García-Montoya *et al.*, 2012), and contains around 690 amino acids, with a considerable proportion of them being branched chain amino acids of leucine, isoleucine and valine at 18.6% combined. The quantity of LF ranges from 20 to 200 mg/l in bovine milk, with fluctuations due to the stage of lactation and with the highest quantity recorded in colostrum, the milk produced in the first few days of lactation (Cheng *et al.*, 2008).

The antimicrobial activity of LF has been extensively reviewed (Superti, 2020; Gruden and Poklar Ulrih, 2021; Rascón-Cruz *et al.*, 2021) including its effectiveness against viruses (Ammendolia *et al.*, 2012; Redwan *et al.*, 2014) and potential efficiency against SARS-CoV-2 or Covid-19 (Kell *et al.*, 2020; Wang *et al.*, 2020). Both LF and the antimicrobial peptide lactoferricin (LFcin) have been shown to exhibit host-protective effects to the gastrointestinal tract when consumed orally in various *in-vivo* animal and human studies, as reviewed by Tomita *et al.* (2002). A more recent review reported the positive effect of LF on the gut microbiome, detailing how LF can affect the growth of intestinal bacteria by promoting the growth of selected probiotic strains (Vega-Bautista *et al.*, 2019).

The generation of bioactive peptides from LF after *in-vitro* simulated digestion has shown ACE-inhibitory activity (Wada and Lönnerdal, 2015; Tu *et al.*, 2021) as well as anticoagulation properties (Tu *et al.*, 2021) and antioxidation activity (Wada and Lönnerdal, 2015). LF has also been shown to exhibit antimicrobial activity in its native state against a range of pathogenic bacteria (Tian *et al.*, 2010; Murata *et al.*, 2013) including *Escherichia coli* (Yen *et al.*, 2011) and *Cronobacter sakazakii* (Harouna *et al.*, 2015). However, LF antibacterial bioactivity is mainly associated with peptides generated by hydrolysis (Tomita *et al.*, 1991). For example, an *in-vitro* simulation of human digestion produced antibacterial peptides from LF that were affective against *C. sakazakii* (Abad *et al.*, 2023). Much investigation has gone into the antibacterial activity of small LF peptides generated by pepsin hydrolysis, located in the N1-domain of the LF molecule including LFcin, f(17–41) (Bellamy *et al.*, 1992b) and lactoferricin B (LFcin B), f(17–30) (Hwang *et al.*, 1998). Also located in the N1-domain is the LF peptide lactoferrampin (LFampin) f(265–284) which has shown antifungal activity (van der Kraan *et al.*, 2004). These well characterised peptides have also been shown to be active against a wide range of bacteria including *Staphylococcus aureus* and *E. coli* (Flores-Villaseñor *et al.*, 2010, Huertas *et al.*, 2017), including *E. coli* O157:H7 (Haiwen *et al.*, 2019), *Listeria monocytongenes*

(Longhi *et al*., 2005), *Bacillus subtilis* and *Pseudomonas aeruginosa* (van der Kraan *et al*., 2004) and *Pseudomonas syringae* (Kim *et al*., 2016). The C-terminal end of LF is reportedly more resistant to hydrolysis, however, Rastogi *et al*. (2014b) successfully used trypsin to partially hydrolyse this region, and reported large fragments of approximately 21, 38 and 45 kDa which were shown to be antibacterial against the Gram-negative bacteria *E. coli* and *Yersinia enterocolitica* (Rastogi *et al*., 2014a).

Reviews on the specific mechanisms of the antibacterial activity of LF such as iron binding (Kell *et al*., 2020), and specific antibacterial activity against *E. coli* (Yen *et al*., 2011), including against the Shiga-like toxin producing *Escherichia coli* O157:H7 which causes severe intestinal infections in humans (Rybarczyk *et al*., 2017), have been recently examined. A well-documented antibacterial mechanism is the transferrin ability of LF to bind iron and, therefore, reduce iron availability, which is an essential nutrient for bacterial growth, resulting in a bacteriostatic activity (Chandan *et al*., 2015; Kell *et al*., 2020; Gruden and Poklar Ulrih, 2021). LF is the only transferrin with the ability to bind iron over a wide range of pH, with iron ions still strongly attached at pH 3 whereas other transferrins dissociate the attached ferric ions at pH 5 (Korhonen and Marnila, 2011). This iron binding ability has also been associated with the prevention of biofilm formation for example against the pathogen *Pseudomonas aeruginosa* (Singh *et al*., 2002). Another antibacterial mechanism of LF is direct interaction with bacterial cell membranes, particularly Gram-negative bacterial cell membranes which contain lipopolysaccharides, as LF was found to bind to the lipid part A of bacterial cell wall lipopolysaccharides characteristic of Gram-negative bacteria including *E. coli* (Orsi, 2004). This disruption to the bacterial cell walls increases the membrane permeability and decreases the membrane integrity resulting in a bactericidal activity (Rybarczyk *et al*., 2017; Gruden and Poklar Ulrih, 2021). Examples of this include direct bactericidal activity of the peptide LFcin recorded as a result of lipopolysaccharide membrane damage (Yamauchi *et al*., 1993). Cell wall disruption was also observed by Flores-Villaseñor *et al*. (2010) for the peptides LFcin B, LFampin and particularly by a chimaeric construct of both peptides against *E. coli* O157:H7 at low concentrations. Additionally, bovine LF was recorded to reduce adhesion of *E. coli* O157:H7 to human Caco-2 cells (Atef Yekta *et al*., 2010). Other factors that contribute to the antibacterial activity of LF include environmental factors of pH, temperature and the food matrix composition which can reportedly lead to variability in antibacterial activity within *in-vitro* studies (Rybarczyk *et al*., 2017).

The antimicrobial effect of LF fragments generated after hydrolysis with protease enzymes naturally found within the digestive system has been established. The digestive enzymes pepsin (Tomita *et al*., 1991; Bellamy *et al*., 1992a, 1992b; Yamauchi *et al*., 1993; Jones *et al*., 1994; Murata *et al*., 2013; Kim *et al*., 2016), and trypsin (Tomita *et al*., 1991; Rastogi *et al*., 2014a, 2014b) have been commonly used. The enzymes are used at their optimum hydrolysis conditions rather than those that would simulate digestion, therefore the effect of digestive processes and natural conditions of these enzymes within a gastrointestinal simulation is less well characterised, as only very recent studies have examined *in-vitro* digestion of dairy products on either *Staphylococcus aureus* or *Cronobacter sakazakii* (Abad *et al*., 2023, Abad *et al*., 2024).

Due to its relevance in clinical microbiology, *E. coli,* a Gram-negative rod-shaped bacterium, is one of the most characterised bacteria of the Enterobacteriaceae family. Some strains are pathogenic and are associated with gastrointestinal infections, fevers and diarrhoeal diseases, while other strains naturally inhabit the intestinal tracts of human and animals and are important for facilitating nutrition by synthesising vitamins particularly vitamin K (Madigan *et al*., 2019). Recent outbreaks of pathogenic enterohaemorrhagic *E. coli* O157:H7 have occurred in the UK (Gobin *et al*., 2018), while outbreaks of the Shiga toxin-producing *E. coli* are increasingly common (European Food Safety Authority *et al*., 2021).

Therefore, the aim of the present work was to use the simulated gastro-intestinal digestion protocol (SGID) developed by the COST INFOGEST Network (Brodkorb *et al*., 2019) to generate digesta at several time points and to examine their antibacterial activity against the common food pathogen *E. coli* using the broth microdilution assay. This method allowed for the study of the microbial growth curve throughout a 24-h period to determine both the extent of a lag phase and the microbial load after 24 h.

Materials and methods

Materials

Lactoferrin (Lactoferrin, Bioferrin 2000, ≥95% protein containing 15 mg Iron/100 g), was kindly donated by Glanbia Nutritionals. The digestive enzymes used were of porcine origin, pepsin (EC 3.4.23.1) and trypsin (EC 3.4.21.4) obtained from Sigma-Aldrich (Ireland), supplied as powders, and stored at −20°C. The salts (KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2(H_2O)_6$, $(NH_4)_2CO_3$, $CaCl_2(H_2O)_2$,) used in the simulated digestive fluids were of general-purpose reagent grade and obtained from Sigma-Aldrich (Ireland), along with HCl and NaOH. The bacterial strain used was *Escherichia coli* (Migula) Castellani and Chalmers (ATCC 8739™). Müeller-Hinton (MH) broth, maximum recovery diluent (MRD) and plate count agar (PCA) were obtained from Sparks Laboratory Supplies (Ireland). Sterile consumables were obtained from VWR International. Simulated salivary (SSF), gastric (SGF) and intestinal digestive fluids (SIF) were prepared prior to the digestion according to Brodkorb *et al*. (2019) and were used in the procedure at a ratio of 1:1 wt/wt sample/digestive fluid. The enzyme preparations of pepsin at 2000 Units/ml and trypsin at 100 Units/ml of final volume were prepared on the day of digestion according to Brodkorb *et al*. (2019).

In-vitro digestion of LF

LF was digested using the *in-vitro* simulated gastrointestinal digestion (SGID) method of Brodkorb *et al*. (2019) with modifications. As the study was designed to examine the digestion of a sample containing ≥95% protein, protease enzymes were used exclusively with no lipase or bile salts (Minekus *et al*., 2014). LF was prepared at 25 g/l in deionised water. SSF was added, and the mixture incubated for 2 min at 37°C in a shaking water bath at 160 movements/min, to simulate the oral phase. For the gastric phase, after addition of the SGF the pH was reduced to pH 3 ± 0.05 using 1 M HCl. Pepsin at 2000 U/ml final digesta volume was added and the sample was incubated for two hours. Aliquots were removed at different time points, at 30, 60 and 120 min, labelled LF G_{30}, G_{60} and G_{120} respectively and the pH increased to pH 8 using 1 M NaOH to terminate the gastric digestion of these aliquots. For the intestinal phase, SIF was added to the remaining digesta, and the pH adjusted to pH 7 ± 0.05

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using 1 M NaOH. Trypsin at 100 U/ml of final digesta volume was then added and the sample incubated for another two hours. Aliquots were removed at the end of the 2-h intestinal phase (240 min in total, labelled LF GI_{240}). Undigested LF samples were prepared at 25 g/l containing the relevant digestive fluids of SSF and SGF to ensure that ion concentration was maintained, with no enzyme added. All digesta were stored at 4°C until use.

Fractionation of LF digesta

The digesta sample obtained after 30 min of gastric digestion (G_{30}) was fractionated by ultrafiltration (UF) using different molecular weight cut off (MWCO) filter units (Amicon® Ultra-15 Centrifugal Filters) and a Hettich Rotofix 32A centrifuge. An aliquot of digesta (15 ml) was placed in the upper chamber of an UF unit fitted with a 30 kDa MWCO membrane and centrifuged at 4000 rpm for 20 min. The permeate fraction was collected and subjected to further fractionation using a 10 kDa MWCO filter. The respective retentates and filtrates were collected and yielded the following large (Mw > 30 kDa), intermediate (30 < Mw kDa < 10), and small (Mw < 10 kDa) peptide fractions. The protein content of each fraction was determined by measuring absorbance at 280 nm using UV spectroscopy and the samples were appropriately diluted to 1 mg/ml in MRD before antibacterial activity was assessed.

Antibacterial activity

Antibacterial activity of undigested LF and LF digesta samples were examined using spectrophotometric analysis (Rautenbach *et al.*, 2006), involving standardised sterile techniques used in microbiology. The test strain, *Escherichia coli* ATCC 8739, was cultured in sterile MH broth at 37°C for 30 h under aerobic conditions (i.e., stationary phase), resulting in a cell density of approximately 10^9 CFU/ml. The bacterial culture was then diluted in sterilised MH to achieve approximately 10^5 CFU/ml, with enumerations completed using a standard plate count technique and PCA agar. This was done on each experimental day to validate the bacterial concentration (CFU/ml) of the culture. The antimicrobial activity of samples of undigested LF G_0 and LF digesta G_{30}, G_{60}, G_{120}, GI_{240}, at a pH of 7 ± 0.5, were evaluated by appropriately diluting them in sterile MRD to achieve different protein concentrations (8, 4, 2, 1, 0.5, 0.25, 0.125 mg/ml), and sterilised by filtration through sterile Whatman 0.2 µm membrane PES filters (Sigma-Aldrich). 100 µl of each LF test sample and concentration were placed into individual wells on a PCR 96-well plate followed by 100 µl of 10^5 CFU/ml bacterial cell suspension in MH, obtaining a final bacterial concentration of approximately 5×10^4 CFU/ml. Relevant growth controls including a positive control of *E. coli* (+Ctrl) and negative growth controls (sterilised LF in medium, and sterilised medium) were included in each plate. Using a Thermo Scientific™ Multiskan™ FC Microplate Photometer, Absorbance values at 620 nm (Abs_{620}) over 24-h, at 37°C were recorded every 20 min after prior minor shaking. Antibacterial activity of the samples was determined on separate days to give at least three replicates.

Antibacterial measurements

Antibacterial activity was determined by plotting bacterial growth curves and examining the effect of LF against *E. coli* by plotting Abs_{620} values recorded over a 24-h period. Two measures of antibacterial activity were determined using the bacterial growth curves. The lag phase (LP), which is the phase prior to the start of exponential growth (Madigan *et al.*, 2019), was measured as the period of time during which the Abs_{620} value did not increase by more than 5% from the value immediately post inoculation (Peleg and Corradini, 2011). A significant increase in LP may be described as a delay to bacterial growth or a delay to the exponential phase and would indicate bacteriostatic activity of the relative LF sample (Fig. 1). If no increase from the initial Abs_{620} value was observed over 24-h, samples were assigned a LP value of 25-h for data analysis purposes and antibacterial activity was assumed to be bactericidal due to no recorded bacterial growth. A second measure of antibacterial activity was evaluated as % inhibition, which was calculated as a percentage difference between the final recorded Abs_{620} value after 24-h of the *E. coli* + Ctrl *vs.* the test LF sample, taking into consideration the background absorbance values of each. Significant % inhibition would indicate that the bacterial load of *E. coli* had been reduced, indicating a bacteriostatic effect of the sample (Fig. 1).

Statistical analysis

Results presented are the mean of triplicate measurements for each sample. Statistical analysis was carried out using IBM SPSS 27 Statistics software. Multiple comparison tests were carried out using one-way analysis of variance (ANOVA), with least-significant difference (LSD) used post-hoc. Values of $P < 0.05$ were significant, and values of $P < 0.001$ were very significant. Results are expressed as mean ± standard deviation (SD) of $n = 3$ unless stated otherwise.

Results and discussion

All samples examined exhibited typical microbial growth curves characterised by a lag phase (LP), followed by an exponential growth phase where the absorbance of the medium increased to a plateau after 24-h, characteristic of the stationary phase. The LP for the positive control *E. coli* at initial inoculation level of 5×10^4 CFU/ml was 3.8 ± 0.3 h ($n = 12$). As readings were taken every 20 min, or every 0.3 h, the growth of the bacterial strain was shown to be highly reproducible across different plates and different days.

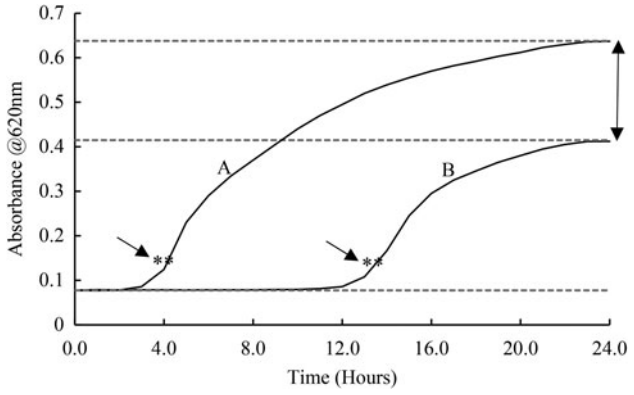

Figure 1. Idealised dose–response curves. Predicted bacterial growth curves plotting absorbance at 620 nm recorded every 20 min, over 24 h of (A) *E. coli*, with no LF, and (B) response of *E. coli* with LF. (**) 5% increase from initial Abs indicating exponential growth. ↕: % inhibition of LF sample compared to the +Ctrl.

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Table 1. Antibacterial effect of undigested LF examining LP and % inhibition, as measured by Abs_{620nm} after 24 h

Undigested LF (mg/ml)	Lag Phase (hrs)	% Inhibition
+Ctrl	3.8 ± 0.3^{a}	0^{a}
1	4.3 ± 0.7^{a}	35 ± 6^{c}
2	5.0 ± 0^{a}	27 ± 1^{b}
4	5.0 ± 0^{a}	39 ± 12^{c}
8	8.1 ± 2.9^{b}	36 ± 8^{bc}

LP, lag phase
[a–d]Mean values with different letter indices indicate significant difference at $P < 0.05$.

Antibacterial activity of undigested LF

Firstly, the antibacterial activity of native LF before undergoing SGID digestion was determined. Results displayed in Table 1 show the effect of undigested LF concentration on both LP and % inhibition.

Undigested LF did not significantly increase LP at concentrations up to 4 mg/ml inclusive (Table 1). However, at a concentration of 8 mg/ml, LP doubled to 8.1 ± 3 h, with this concentration being the only one of statistical significance, showing that high concentrations of undigested LF greater than 4 mg/ml can delay *E. coli* growth significantly. This effect was assumed to be bacteriostatic as bacterial growth was delayed rather than totally suppressed. For % inhibition, undigested LF significantly reduced bacterial load after 24 h at all examined concentrations (1 to 8 mg/ml), as evident from Table 1. These results demonstrate that to delay the time until exponential phase, a concentration greater than 4 mg/ml of undigested LF is required, whereas to reduce bacterial load, lower concentrations of 1 mg/ml can be significant.

Concentrations of intact bovine LF greater than 5 mg/ml have been similarly observed to alter bacterial growth by Tian *et al.* (2010) against both Gram-positive and Gram-negative food pathogens including *E. coli.* This occurred in a dose-dependent manner when concentrations of up to 40 mg/ml were examined. LF concentrations below 5 mg/ml have been shown to be effective, as LF showed bacterial inhibition against the pathogenic strain *E. coli* O111 with an MIC of 2 mg/ml recorded (Tomita *et al.*, 1991). In addition, activity has been shown against non-pathogenic *E. coli* strains such as *E. coli* K-12 (where 2 mg/ml reduced colony forming units two-fold: Murata *et al.*, 2013) and *E. coli* O157:H7 strain (when a lethal concentration 50 (LC_{50}) of LF at 1.9 mg/ml was recorded: Rastogi *et al.*, 2014a). As shown in Table 1, LF at 1 mg/ml exhibited a significant bacterial inhibition of 35%. Similar inhibition scores were recorded by Flores-Villaseñor *et al.* (2010) for intact LF against three different *E. coli* strains at this same concentration, where a minimum inhibition of 36.7% was recorded. A maximum inhibition of 81% against the enterohaemorrhagic *E. coli* O157:H7 strain was observed (Flores-Villaseñor *et al.*, 2010). In an exposure-based study on *E. coli*, the same concentration of LF at 1 mg/ml resulted in a bactericidal activity, as a result of bacterial membrane breakdown observed using scanning electron microscopy (Yen *et al.*, 2011). Extended exposure time from 2 to 4 h resulted in more significant membrane damage than the 2 h exposure which was still significant (Yen *et al.*, 2011). Our study supports the bacteriostatic properties of intact LF against Gram-negative bacteria as seen by an extended lag phase and decreased bacterial load (at 24 h) observed at low concentrations.

Effect of in-vitro digestion of LF on antibacterial activity

After undergoing SGID, the antibacterial properties of the various LF digesta were tested and compared to the undigested LF. All concentrations greater than 1 mg/ml showed significant antibacterial activity in terms of both LP and % inhibition for LF gastric digesta (2 mg/ml, 4 mg/ml). The LP effect of SGID LF digesta (Fig. 2) and the % inhibition (Table 2) are shown at a concentration of 1 mg/ml. Digested LF significantly increased LP compared to its undigested counterpart, indicating an increase of LF bacteriostatic activity upon SGID.

All LF digesta at 1 mg/ml (G_{30}, G_{60}, G_{120}) resulted in significantly increased LP, as shown in Fig. 2. A maximum LP of 18.7 ± 6 h was observed in the initial digestion stage after 30 min of gastric digestion (G_{30}) which thereafter declined over time. This LP effect was lost when LF SGID samples were exposed to the intestinal digestion stage (GI_{240}), as these samples showed no significant difference from the positive control. This transient antibacterial effect was equally observed at higher LF concentrations of 2 mg/ml and 4 mg/ml, with LP likewise peaking for the LF G_{30} sample and decreasing with increased digestion time until no longer statistically significant for the GI_{240} samples (results not shown). These results demonstrate the effectiveness of gastric generated LF peptides in delaying growth of *E. coli* at

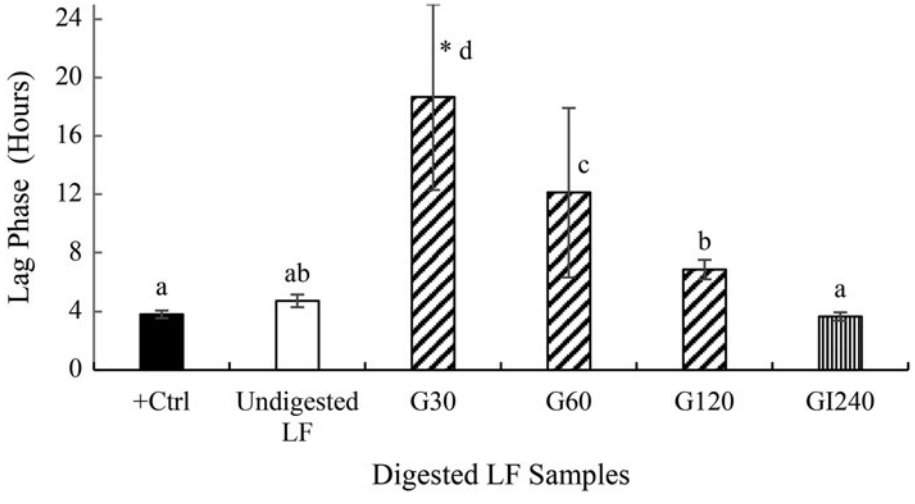

Figure 2. The effect of LF after undergoing SGID on the LP of *E. coli*, presented at 1 mg/ml, showing differences with digestion stage and time points within. Black: *E. coli* + Ctrl, White: Undigested LF, Diagonal Stripes: Gastric stage, Vertical Stripes: Intestinal stage. *No change in absorbance recorded after 24 h for some replicates, assigned a value of 25 h for data analysis purposes. [a–d]Mean values with different letter indices indicate significant difference at $P < 0.05$. LP, lag phase.

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Table 2. % inhibition by LF digesta samples detailing undigested LF and SGID LF samples at 1 mg/ml after 24 h, with negative values indicating an increase above the +Ctrl

LF Digesta	% Inhibition
+Ctrl	0^{a}
Undigested LF	33.4 ± 14.4^{b}
G_{30}	65.7 ± 33.5^{cd}
G_{60}	48.3 ± 21.2^{bc}
G_{120}	18.2 ± 11.1^{b}
GI_{240}	-4.5 ± 8.4^{a}

[a–d]Mean values with different letter indices indicate significant difference at $P < 0.05$.

concentrations of 1 mg/ml and greater, albeit that this antibacterial activity decreases on extended digestion.

As can also be seen in Fig. 2, the exposure of the LF samples to a 2-h intestinal phase as part of the SGID protocol resulted in the loss of the extension of LP observed on gastric digestion. The time at which this antibacterial activity was lost during the intestinal stage was further investigated by removing additional aliquots at 15, 30 and 60 min within the intestinal digestion stage. After 15 min of *in-vitro* intestinal digestion, no antibacterial LP effect was observed, with all subsequent aliquots similarly exhibiting no LP effect (results not shown). This indicates that the stage of digestion can influence LF antibacterial activity, as only gastric samples altered the LP of *E. coli* to a significant level.

In Table 2 the effect of digestion phase on % inhibition is shown. Significant inhibition after 24-h was observed for all LF gastric digesta (G_{30}, G_{60}, G_{120}) at 1 mg/ml. A twofold increase was observed when compared to the intact LF, as a maximal inhibition of 65.7% was observed for the LF G_{30} sample which decreased thereafter. A significant % inhibition was not observed for LF GI_{240}, suggesting that any antimicrobial effect observed for both intact LF or any additional effect generated within the gastric stage were lost upon sample exposure to intestinal digestion conditions. This may be due to the exposure to a second hydrolysis step upon addition of the trypsin enzyme within the SGID. Results showing antibacterial activity of LF increasing after hydrolysis with a single enzyme have been previously reported. Pepsin has been used to generate antimicrobial peptides from LF over the last few decades, with the peptide LFcin B (generated by pepsin hydrolysis of bovine LF: Yamauchi *et al.*, 1993; Tomita *et al.*, 2002) showing strong antibacterial activity first being recorded against the food pathogen *E. coli* O111 (Tomita *et al.*, 1991). LFcin was found to bind to lipopolysaccharides on the outer membrane of Gram-negative cells and affect bacterial growth *via* this direct interaction (Yamauchi *et al.*, 1993). Further investigation into this peptide shows that LFcin B has a molecular mass of 6.6 kDa (Jones *et al.*, 1994) and has effective antibacterial activity against a wide range of both Gram-positive and -negative bacteria (Bellamy *et al.*, 1992a, 1992b; Jones *et al.*, 1994). LFcin B is thought to exhibit bactericidal activity against *E. coli* by interacting with the lipopolysaccharides and causing depolarisation of the bacterial membranes (Ulvatne *et al.*, 2001, Ulvatne and Vorland, 2001). In a recent animal study, Haiwen *et al.* (2019) examined LFcin B against *E. coli* O157:H7, showing that intestinal damage caused by this enterohaemorrhagic bacteria was attenuated in the animals consuming this LF peptide at a dose of 0.5 mg/kg body weight. Another Gram-negative bacteria, *Pseudomonas syringae,* has also been shown to be inhibited by bovine LF hydrolysates generated by pepsin at pH 3 and 37°C, with this antibacterial activity shown to be associated with small peptides generated from the N-terminal region of the bovine LF molecule (Kim *et al.*, 2016). When trypsin was used to hydrolyse LF, the tryptic hydrolysates of LF were more effective than the intact LF counterpart against the Gram-negative bacteria *E. coli* and *Yersinia enterocolitica* (Rastogi *et al.*, 2014a).

The outcome seen here of LF digesta exhibiting maximal antibacterial effect in a time-dependent manner, with a short 30 min digesta sample exhibiting the highest activity when compared to 60 and 120 min digestion, is novel. Tomita *et al.* (1991) examined the antibacterial activity of peptic digests of LF against *E. coli* O111. Similar to the findings of the present study, the authors found that antibacterial activity was generated after only 30 min of digestion. However, in contrast to the present findings, they found that the activity persisted after 4 h of pepsin hydrolysis, with no transient decrease observed. More recent investigations have examined only one time point of the relevant LF hydrolysis for the generation of antibacterial LF peptides. For example, in a recent study examining a lactoferrin supplemented milk fat globule membrane, gastric digesta showed bacteriostatic activity against a *Cronobacter sakazakii* strain, although only an aliquot at the end of the 120 min gastric phase was examined (Abad *et al.*, 2023). The duration of the hydrolysis varies dramatically between experiments, from 30 min with human gastric juices (Furlund *et al.*, 2013), 90 min with trypsin (Rastogi *et al.*, 2014a, 2014b), 240 min with pepsin (Kim *et al.*, 2016) and even 5hrs when the enzymes Proteinase K, thermolysin, trypsin, and chymotrypsin were used (Salami *et al.*, 2010), with intermediate aliquots not examined. Hence, the observed transient change in LF antibacterial activity seen within this SGID experiment has not been indicated before.

When Furlund *et al.* (2013) used human gastric juice followed by human duodenal juices in an *in-vitro* digestion system to digest bovine LF, large peptides were produced after 30 min of gastric digestion step which were degraded to smaller peptides following the 30 min intestinal digestion step. This could explain the loss of antibacterial activity of the LF digesta observed for the gastric sample in this experiment, as upon entering the intestinal SGID phase (LF GI_{240}), the peptides with antibacterial activity may have been degraded in such a manner that the potency reduced over time, as observed with the maximal LF G_{30} sample which decreased in a transient manner and terminated after the SGID intestinal phase. Therefore, the complete loss of antibacterial activity in the LF digesta samples after exposure to SGID intestinal conditions may be as a result of tryptic degradation of the antibacterial peptides produced during the gastric stage of the SGID. This result is in contrast to a recent study that showed that the intestinal digesta of a lactoferrin supplemented milk fat globule membrane resulted in a greater antibacterial effect than the gastric digesta (Abad *et al.*, 2024), although this was seen against *S. aureus* which is a Gram-positive bacteria. As seen in this study, digestion of LF resulted in significantly increased LP and % inhibition compared to its undigested counterpart, indicating an increase of LF bacteriostatic activity due to the generation of antibacterial peptides from bovine LF hydrolysis during SGID. Notably, this antibacterial effect was observed at a high inoculation level of $<10^4$ CFU/ml, showing the potential of LF to provide antibacterial activity to the host due to the generation of antimicrobial peptides during the digestive process.

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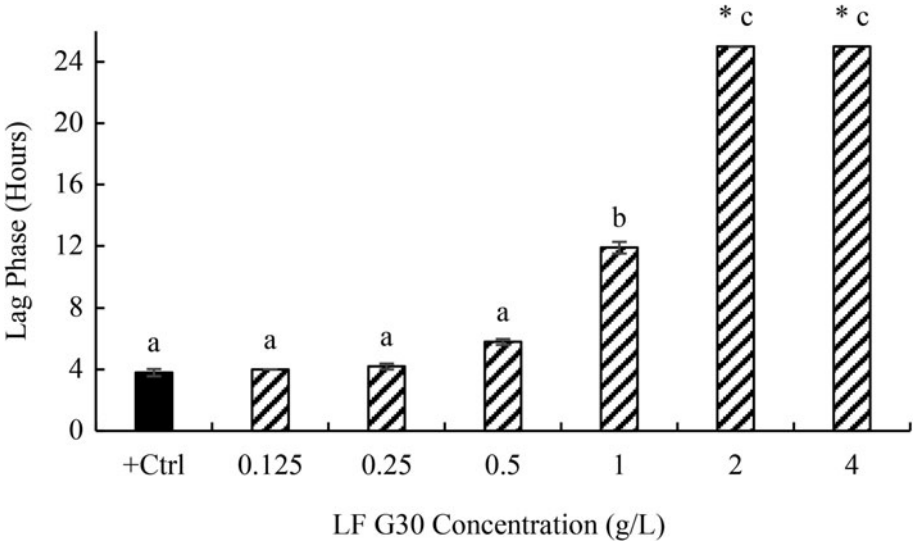

Figure 3. Effect of LF G30 concentration on LP of *E. coli*. [a–c]Mean values with different letter indices indicate significant difference at $P < 0.05$.
*Included replicates of 25 h. LP, lag phase.

Table 3. The effect of LF G30 concentration on the % inhibition of *E. coli* over 24 h

LF G_{30} Digesta (mg/ml)	% Inhibition
+Ctrl	0[a]
0.125	22.1 ± 4.8[ab]
0.25	28.6 ± 4.9[b]
0.5	27.4 ± 11[b]
1	63.7 ± 39.2[c]
2	92.1 ± 18.3[d]
4	99.6 ± 0.09[d]

[a–d]Mean values with different letter indices indicate significant difference at $P < 0.05$.

LF G_{30} concentration effect

Since the maximum antibacterial effect was observed for the LF G_{30} digesta sample, a more detailed analysis was carried out to establish if a dose–response relationship existed (Fig. 4) and an attempt to identify the fraction responsible for the antibacterial activity within this sample was also completed. Figure 3 shows a classic dose response on the effect of increasing concentration of LF G_{30} on the LP, where a direct relationship between the increase in concentration and the increase in the antibacterial effect was observed. Concentrations less than 0.5 mg/ml did not significantly alter LP, while all concentrations of 1 mg/ml or greater showed significant and substantial delay to *E. coli* growth. At concentrations of 2 mg/ml and above, *E. coli* failed to grow over the 24-h incubation period. Whether these observed antibacterial effects at these higher concentrations were bacteriostatic or bactericidal was investigated by re-plating in fresh media. Results were not consistent as some re-plated replicates exhibited bactericidal activity (no growth observed), while others exhibited bacteriostatic activity only, therefore, further investigation is needed to fully establish the true nature of antibacterial activity.

Table 3 reveals a similar concentration-dependent effect for LF G_{30} on % inhibition of *E. coli*. A concentration as low as 0.25 mg/ml showed a significant ($P < 0.05$) % inhibition of 28.6% ± 4.9, observing a maximum % inhibition of 99.6% ± 0.09 at the highest concentration tested (4 mg/ml). These results highlight that LF after 30 min of gastric digestion can delay the onset of bacterial growth at LF concentrations over 1 mg/ml, while significantly reducing the final bacterial load at much lower concentrations as an IC50 (Rautenbach *et al.*, 2006) which was achieved at 1 mg/ml.

Fractionation of antibacterial digesta by ultrafiltration (UF)

An attempt was made to isolate the peptide fraction responsible for the increased antibacterial activity seen in the LF G_{30} digesta using filtration of digesta through 30 and 10 kDa MWCO filters

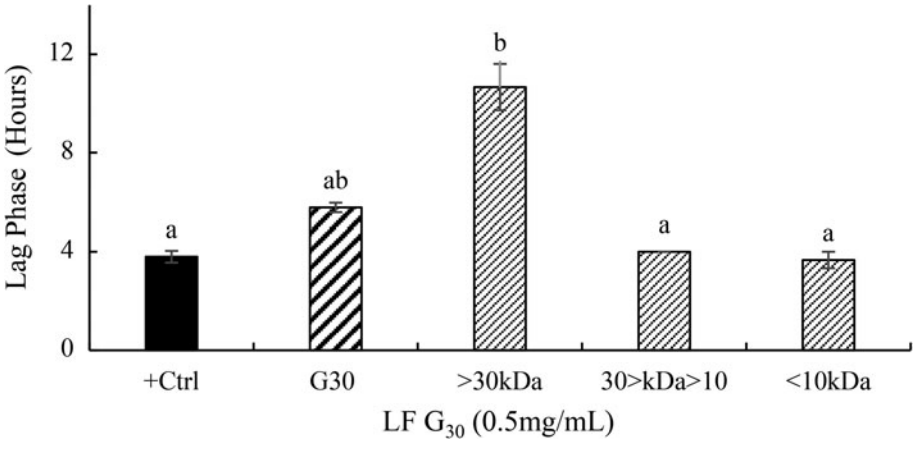

Figure 4. Size fractionation of LF G_{30} sample using MWCO units and the effect on LP, to identify the size of the fraction associated with antibacterial activity within this gastric digested sample. Different letter indices indicate significant difference at $P < 0.05$. MWCO, molecular weight cut off; LP, lag phase.

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(Amicon® Ultra Centrifugal Filters). The following peptide fractions were isolated: A retentate with large molecular weight, Mw > 30 kDa, an intermediate fraction between 30 and 10 kDa, and a small molecular weight fraction in the filtrate Mw < 10 kDa. All three fractions were tested for antibacterial activity examining LP effect (Fig. 4) and % inhibition (data not shown).

The large molecular weight peptide fraction at 0.5 mg/ml was the only isolated peptide fraction to increase LP significantly to 10.7 h ± 0.9 ($P < 0.001$). The two other two peptide fractions of lower molecular weight showed no significant effect on LP at the concentration studied, as seen in Fig. 4. In terms of % inhibition, none of the three isolated peptide fractions reduced the bacterial load after 24 h, with no significant % inhibition observed. These results might suggest that LF peptides within the large peptide fraction >30 kDa at the concentration tests were mostly responsible for the antibacterial activity of the LF G_{30} sample. The antibacterial activity of this sample was observed at half the concentration (0.5 mg/ml) at which the non-fractionated LF G_{30} showed significant antibacterial activity (1 mg/ml). Another instance where peptides of Mw ~30 kDa, derived from LF hydrolysis, showed antibacterial activity against *E. coli* was when tryptic derived bovine LF fractions 21, 38 and 45 kDa exhibited a significant reduction in *E. coli* viability at 1.5 mg (Rastogi *et al.*, 2014a). Thus, it appears that the >30 kDa fraction within this study was effective at much lower concentrations (0.5 mg/ml), than those previously reported.

Our antibacterial activity against *E. coli* was only observed in the high molecular weight fraction of >30 kDa. This contrasts with much of the reported literature in which antimicrobial activity of LF hydrolysates against both Gram-negative and -positive bacteria is largely attributed to relatively small peptides such as LFcin f(17–41) (Bellamy *et al.*, 1992b), LFcin B f(17–30) (Hwang *et al.*, 1998), and LFampin f(265–284))(van der Kraan *et al.*, 2004). These would be expected to have been found within the smallest peptide fraction of <10 kDa in the present study. In addition to this, previous studies report that the antibacterial activity of hydrolysed LF was due to the presence of small peptides generated from the N-terminal region of the bovine LF molecule (Kim *et al.*, 2016). The expectation of finding previously characterised antibacterial LF peptides within samples after undergoing digestive hydrolysis was similarly not observed by Furlund *et al.* (2013) who could not identify LFcin f(17–41) during either *in-vitro* or *in-vivo* digestion. Therefore, these results suggest that there may be a large peptide fraction generated from LF by hydrolysis within an *in-vitro* digestion system that can exhibit significant antibacterial activity against a Gram-negative bacterium, which has yet to be fully characterised.

In conclusion, bovine LF significantly inhibited growth of *E. coli* at high concentrations. This microbial inhibitory potential significantly increased upon exposure to gastric digestive conditions for short periods of 30 min but declined on continued SGID, disappearing completely upon exposure to intestinal conditions. LF G_{30} digesta showed concentration dependent effects on both LP and % inhibition. The largest peptide fraction isolated from the LF G_{30} sample of Mw > 30 kDa appeared to be chiefly responsible for the observed antibacterial effect, particularly on the extension of LP, which was significant at a low concentration of 0.5 mg/ml. These results point towards a transient generation of large antibacterial LF peptides within the digestive process that peaks in the early gastric stage and need further characterisation. These findings suggest that a small concentration of intact LF within a contaminated food could provide antibacterial protection to the host acting as a bacteriostatic agent.

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