International Dairy Journal 153 (2024) 105899

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Gastrointestinal digestion and technological treatments modify the antibacterial activity of lactoferrin supplemented dairy matrices against *Staphylococcus aureus*

I. Abad [a, b], A. Bailac [a, c], M.D. Pérez [a, b], J.J. Carramiñana [a, b], M. Calvo [a, b], L. Sánchez [a, b, *]

[a] Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Universidad de Zaragoza, Zaragoza, Spain
[b] Instituto Agroalimentario de Aragón IA2 (UNIZAR-CITA), Zaragoza, Spain
[c] Universidad de Lleida, Lleida, Spain

ARTICLE INFO

Article history:
Received 30 November 2023
Received in revised form
30 January 2024
Accepted 30 January 2024
Available online 8 February 2024

ABSTRACT

Milk contains antimicrobial proteins, such as lactoferrin from whey or proteins from the milk fat globule membrane (MFGM), which can be used in functional foods to strengthen children and adult defenses. Foodborne bacteria, such as *Staphylococcus aureus*, can contaminate dairy products causing intoxication. The aim of this study was to evaluate the antibacterial activity of lactoferrin, free and in dairy matrices, against *S. aureus* before and after gastrointestinal digestion. Six dairy formulas, supplemented with lactoferrin and MFGM, were subjected to technological treatments and their antibacterial effect was analyzed after in vitro digestion. Intact lactoferrin slightly reduced *S. aureus* growth, but its digests lost this activity. Gastric digests of non-treated or homogenized formulas reduced significantly the bacterial growth, probably due to the antimicrobial peptides generated by pepsin, while pasteurization decreased such activity. Intestinal digests showed the greatest antibacterial effect, probably due to the action of intestinal enzymes and the generated peptides.
© 2024 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY-NC license (http://creativecommons.org/licenses/by-nc/4.0/).

1. Introduction

Milk is a good source of bioactive components that can be used to elaborate functional foods designed to improve health and prevent certain pathologies (Roberfroid, 2000). In the process to transform milk into products, the dairy industry generates some by-products, such as whey and buttermilk (Svanborg, Johansen, Abrahamsen, & Skeie, 2015).

Whey is a dairy by-product that results of the separation of precipitated casein during cheese manufacture. It is a greenish yellow liquid whose physicochemical composition varies depending on the type of milk (bovine, ovine, etc.), the season, the phase of lactation, and the cheese manufacturing method used (rennet or acid coagulation) (Pires, Marnotes, Rubio, Garcia, & Pereira, 2021). Whey consists of lactose, proteins, fat and mineral salts (Walzem, Dillard, & German, 2002). Whey is a magnificent raw material for obtaining a wide variety of compounds with technological interest. Specifically, whey proteins have functional properties that are very useful in the food area (Barukčić, Jakopović, & Božanić, 2019) and some of these proteins have interesting biological properties. Among the latter, lactoferrin (LF) is a multifunctional iron-binding glycoprotein with a molecular weight of 80 kDa. LF is expressed and secreted by epithelial cells in exocrine secretions (Telang, 2018). This protein belongs to the transferrin family and has numerous protective effects such as anti-inflammatory, antimicrobial, antioxidant and immunomodulatory activity (García-Montoya, Cendón, Arévalo-Gallegos, & Rascón-Cruz, 2012). LF is essential in the newborn diet and plays an important role in protecting babies against infections and promoting the maturation of their innate and adaptative immune system (Superti, 2020). Some infant formulas are supplemented with LF, with the aim of preventing infections in premature newborns. In Europe, bovine LF was allowed as a food ingredient in 2012 and in the Regulation (EC) N^{o} 258/97 the levels at which it can be used were established depending on the food category (European Commission, 2012). LF has bacteriostatic and bactericidal activity against a multitude of bacteria, and this is due to two different mechanisms. LF binds and sequesters free iron from the medium, preventing microorganisms

* Corresponding author.
E-mail address: lousanchez@unizar.es (L. Sánchez).

https://doi.org/10.1016/j.idairyj.2024.105899
0958-6946/© 2024 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY-NC license (http://creativecommons.org/licenses/by-nc/4.0/).

from obtaining this substrate necessary for their growth. Additionally, bactericidal activity involves a direct interaction of LF with the pathogenic agent, destabilizing the bacterial membrane and altering its permeability and viability (Superti, 2020).

Buttermilk is another by-product form dairy industry. It is a yellowish white liquid product obtained in butter manufacture. Buttermilk has a relatively high content of phospholipids from the milk fat globule membrane (MFGM), which also have some bioactive proteins that are beneficial to health due to their various antibacterial and antiviral activities (Ali, 2019). One of the main functions of this membrane is to protect fat from enzymatic degradation. However, the MFGM could be affected after being subjected to some technological treatments carried out on milk, such as heat treatment, refrigeration or spray-drying (Singh, 2006).

In addition to the impact of technological treatments, dairy proteins and peptides are also subjected to multiple modifications during gastrointestinal (GIT) digestion. Protein hydrolysis occurs by proteinases, such as gastric pepsin and pancreatic trypsin and chymotrypsin, giving rise to a variety of peptides, some of which are bioactive. Likewise, peptides can be hydrolyzed by peptidases from pancreatic secretions, turning them into small peptides and free amino acids that will be absorbed at the intestine passing into blood (Mohanty, Mohapatra, Misra, & Sahu, 2016). For maintaining the activity of bioactive peptides, it is necessary that they survive the proteolysis generated in the GIT tract. The stability of several bioactive peptides from proteins, such as LF and epidermal growth factor, has been reported (Jahan-Mihan, Luhovyy, El Khoury, & Anderson, 2011). The proteolysis of LF with pepsin generates a bioactive peptide with demonstrated antibacterial effect, lactoferricin. Furthermore, lactoferrampin, another peptide with antibacterial potential that is located near lactoferricin in the three-dimensional structure of the protein, is also generated in LF digestion (Furlund et al., 2013).

Stahpylococcus aureus is a facultative anaerobic and Gram-positive coccus with a size of 0.5–1 μm of diameter, which produces coagulase and catalase. It is immobile, does not form spores and can grow at temperatures between 18 and 40 °C. *S. aureus* belongs to the Staphylococcaceae family. The genus *Staphylococcus* is made up of 52 species, being *S. aureus* one of the species most regularly associated with pathologies in humans (Pasachova, Ramirez, & Muñoz, 2019). *S. aureus* mainly causes nosocomial infections that have been associated with diseases such as pneumonia and other respiratory and cardiovascular infections (Cheung, Bae, & Otto, 2021). Multidrug-resistant *S. aureus* infections represent a significant threat to global human health. The spread of antibiotic resistance arises in bacterial pathogens through the conjugative transfer of plasmid DNA, which encodes resistance genes. The molecular basis for resistance transmission by the nicking enzyme in *S. aureus* (NES) is necessary for conjugative transfer. NES imitates and terminates the transfer of plasmids that confer resistance to a variety of drugs, such as vancomycin, gentamicin and mupirocin (Edwards et al., 2013). Some strains of *S. aureus* produce enterotoxins that cause staphylococcal food poisoning. The foods mainly involved in food poisoning of this type are meat products, bakery products, milk and dairy products. Poisoning occurs due to the ingestion of enterotoxins produced in food, due to improper handling and storage at high temperatures (Argudín, Mendoza, & Rodicio, 2010). The incidence of staphylococcal poisoning according to European data is 0.06 cases per 100,000 inhabitants, which mean a low prevalence (EFSA-ECDC, 2017). Infant powdered milk formula are not sterile products, and can be contaminated with pathogens, such as *Cronobacter sakazakii* or *S. aureus*, due to mishandling or improper storage (Wang et al., 2012). For this reason, some clinical cases of foodborne illness in children are related to the consumption of infant formula contaminated with pathogens, especially *C. sakazakii*, *Salmonella enterica* and *S. aureus* (Cho et al., 2019). In fact, *S. aureus*, according to 2020 European Union reports, was the cause of 43 outbreaks of foodborne illnesses, 402 cases of human illnesses and 32 hospitalizations (EFSA-ECDC, 2021). Hence, the use of natural antibacterial compounds in milk formula, such as LF, is not only convenient for infant intestinal health, but also as an strategy of hurdle technology to control the proliferation of bacterial contaminants.

Therefore, the main objective of this study was to analyze the effect of in vitro digestion on bovine LF alone and as a supplement of dairy formulas, focusing on its antimicrobial effect against *S. aureus*. Furthermore, the effect of different technological treatments on the antibacterial activity of these dairy formulas was determined. For this, six dairy formulas, based on whey or buttermilk and supplemented with LF and MFGM, were subjected to homogenization or pasteurization and their antibacterial effect against *S. aureus* was analyzed after the different steps of an in vitro digestion.

2. Material and methods

2.1. Obtaining dairy fractions

Raw bovine milk was supplied by the dairy company Villacorona (El Burgo de Ebro, Spain). It was processed at the Food Science and Technology Pilot Plant of the University of Zaragoza, located at the Veterinary Faculty. The quality of milk was assured by measuring the pH, acidity, fat percentage, and alkaline phosphatase and lactoperoxidase activities. Milk was heated and skimmed as explained in our previous study (Abad et al., 2022a).

Skim milk was heated at 35 °C in a 25 L cheese vat and 30% $CaCl_2$ was added at dilution 1:8000 (v/v). Next, bovine rennet was added to milk at dilution 1:15,000 (v/v) and incubated for 1 h. After achieving casein coagulation, the curd was cut with a lyre obtaining whey, which was filtered through cheesecloth and glass wool, lyophilized and kept at −20 °C for later use.

To obtain buttermilk, the cream (43% of fat) obtained in the previous skimming phase was used. The cream was cooled and subjected to mechanical stirring with a Phillips Cucina mixer (Philips, Amsterdam, The Netherlands). This process was carried out until phase inversion took place, thus obtaining butter grains, which were formed by the agglomeration of milk fat globules, allowing the release of buttermilk. The obtained buttermilk was filtered through cheesecloth and glass wool, lyophilized and kept at −20 °C. A part of buttermilk was subjected to one-phase homogenization at 250 bar, using the Panda model homogenizer (GEA Niro Saovi, Parma, Italy).

MFGM was obtained by centrifuging buttermilk, homogenized or not, at 40,000 *g* for 30 min at 4 °C. The pellet obtained after centrifugation contained the MFGM (Ripollés et al., 2018).

The amount of protein present in dairy fractions was analyzed by performing a bicinchoninic acid test, obtaining values of 153.8 mg protein per g of whey, 136.7 mg protein per g of buttermilk, and 97.7 mg protein per g of MFGM.

Commercial bovine LF was donated by the company Tatua Nutritionals (Morrinsville, New Zealand). Its iron saturation was below 10% and its purity was higher than 90%. The lipopolysaccharide level of this LF was determined (Abad et al., 2022b) and it was considered minimal, so it did not influence the results.

2.2. Preparation of samples and dairy formulas

Six dairy formulas (F1–F6) were prepared to be subjected to static in vitro GIT digestion and to subsequent evaluation of their antimicrobial activity against *S. aureus*. All the formulas were based

on whey or buttermilk and supplemented with bovine LF (10 mg mL^{-1}) and MFGM (the pellet obtained in the centrifugation of a volume of buttermilk in 1:1 ratio with the base of the formula). Two formulas (F3 and F4) were made with homogenized buttermilk and/or MFGM, and two other formulas (F5 and F6) were subjected to a pasteurization heat treatment at 72 °C for 20 s (Table 1).

For thermal treatment, F5 and F6 were aliquoted into 1 mL vials and a thermal probe connected to a data logger (Almeno 2409, Ahlborn, Ilmenau, Germany) was placed inside one vial for temperature control. First, two water baths (Unitronic 200 and Precisterm S-138, both from J.P. Selecta, Barcelona, Spain) were tempered at 60 °C and 72 °C, respectively. The samples were introduced into the first bath at 60 °C and, upon reaching that temperature, they were transferred to the bath at 72 °C. Once they reached 72 °C, they were held for 20 s. After pasteurization, samples were immediately cooled down immersing them into ice and stored at −20 °C until use.

2.3. *In vitro gastrointestinal digestion*

The digestion process used followed the InfoGest Consensus Method and it was based on the protocol by Mackie and Rigby (2015) and Brodkorb et al. (2019) with modifications.

A static in vitro digestion of LF and dairy formulas was performed. This process consists of three consecutive phases: salivary, gastric and intestinal phase. First, the digestion solutions were prepared according to the concentrations of salts detailed in Abad et al. (2022a): simulated salivary solution (SSS) at pH 7, simulated gastric solution (SGS) at pH 3 and simulated intestinal solution (SIS) at pH 7.

For digestion, 4 mL of the corresponding sample was taken, to which 3.2 mL of SSS, 20 μL of $CaCl_2(H_2O)_2$ and 780 μL of Milli-Q water were added, giving rise to a final volume of 8 mL that was adjusted to pH 7. The mixture was incubated under agitation for 2 min at 37 °C. After this time, a 4 mL aliquot of this sample was taken, which was called salivary digest (SD) and it was frozen in liquid nitrogen. The remaining volume was subjected to the next phase of the digestion.

To carry out gastric digestion, 3 mL of SGS, 2 μL of $CaCl_2(H_2O)_2$, 0.8 mL of porcine gastric pepsin (Sigma Aldrich, St Louis, MO, USA) at a concentration of 2000 U mL^{-1} and 118 μL of Milli-Q water were added to the salivary digest. The mixture was adjusted to pH 3 and incubated for 2 h at 37 °C under agitation. After incubation, a 4 mL aliquot of this sample was removed and frozen in liquid nitrogen to inactivate the effect of the enzymes. This aliquot was called gastric digest (GD). The remaining volume was subjected to the last stage of digestion.

For intestinal digestion, 2.2 mL of SIS, 8 μL of $CaCl_2(H_2O)_2$, 1 mL of pancreatin (Sigma Aldrich, 8 × USP) to achieve 100 U mL^{-1} of trypsin activity in the final mixture, 0.5 mL of 10 mM porcine bile (Sigma Aldrich) and 262 μL of Milli-Q water were added. The mixture was adjusted to pH 7 and incubated for 2 h at 37 °C under agitation. At the end of the incubation, the entire sample was frozen in liquid nitrogen. This fraction was called intestinal digest (ID).

The three frozen digests were lyophilized and subsequently resuspended with the adequate volume of Milli-Q water to obtain a final LF concentration of 5 mg mL^{-1}. The digests and the original undigested LF and formulas were filter sterilized using a 2 μm prefilter and a 0.45 μm low binding protein filter for later use in the assays.

2.4. *Culture of* Staphylococcus aureus

The bacterial strain used in this study was *S. aureus* CECT 435, supplied by the Spanish Type Culture Collection (CECT, Valencia, Spain), which corresponds with the strain ATCC 25923 of the American Type Culture Collection. This strain of *S. aureus* is a human clinical isolate, so the results of this study may have relevance in practice. *S. aureus* ATCC 25923 is used as a standard laboratory testing control strain. It is sensitive to a variety of antibiotics, including methicillin. The *S. aureus* ATCC 25923 was chosen because it is recommended as reference strain by international quality standards, since 2003 until nowadays (ISO, 2003, 2023).

The bacteria were fixed to porous rings and stored in cryovials at −80 °C for the reference stock. To cultivate *S. aureus*, a porous ring was transferred to a tube with 10 mL of trypticase soy broth (TSB) (Merck, Darmstad, Germany) supplemented with yeast extract (YE) (Oxoid, Basingstoke, UK) at 0.6% (v/v). It was incubated for 24 h at 37 °C in aerobiosis. The culture was seeded by depletion on a plate of trypticase soy agar (TSA) (Merck) with 0.6% YE, and the plate was incubated for 24 h at 37 °C to obtain isolated colonies for the assays.

A single colony of *S. aureus* was incubated at 37 °C in 10 mL of TSB with YE for 8 h (exponential phase) or for 18–20 h (stationary phase). Serial dilutions were made with 1% (w/v) bacteriological peptone water (Oxoid) to reach an approximate concentration of 10^5 colony forming units (cfu) mL^{-1}.

Both the culture of *S. aureus* and the following assays were carried out in a sterile environment in a Telstar laminar flow hood model PV-30/70 (ThermoFisher Scientific, Rockford, IL, USA).

2.5. *Antibacterial activity of lactoferrin against* S. aureus

S. aureus culture obtained from exponential and stationary phase were used to analyze the effect of bovine LF on bacterial viability depending on the growth phase. Native LF at different

Table 1
Composition of the six dairy formulas (F1-F6) elaborated on a base of whey or buttermilk and subjected to homogenization or pasteurization. LF: lactoferrin at 10 mg mL^{-1}. MFGM: milk fat globule membrane.

Formula	Base	Supplement	Treatment
Formula 1 (F1)	Whey	LF MFGM	–
Formula 2 (F2)	Buttermilk	LF MFGM	–
Formula 3 (F3)	Whey	LF Homogenized MFGM	Homogenization
Formula 4 (F4)	Buttermilk (Homogenized)	LF Homogenized MFGM	Homogenization
Formula 5 (F5)	Whey	LF MFGM	Pasteurization
Formula 6 (F6)	Buttermilk	LF MFGM	Pasteurization

concentrations (0.5, 1, 2, 5 and 10 mg mL^{-1}) was mixed with the bacterial suspension at 10^5 cfu mL^{-1} in a 1:1 ratio (v/v). Samples were incubated at 37 °C for 4 or 24 h, to determine the effect of LF depending on incubation time, and then were seeded in TSA with YE plates. After an incubation of 24 h at 37 °C, colonies were counted. A control sample, with bacterial suspension and peptone water instead of LF was included. All samples were analyzed in duplicate in three independent experiments.

2.6. Antibacterial activity of digests against S. aureus

The same procedure detailed in section 2.5. was carried out to determine the antibacterial activity of LF and dairy formulas, before and after the different stages of digestion, against *S. aureus*. This analysis was performed only at stationary phase of growth. All samples were evaluated in duplicate in three independent experiments. Two different incubation times, 4 and 24 h at 37 °C, were tested.

2.7. Statistical analysis

Results are presented as the mean ± standard deviation. Their statistical analysis was performed using the statistical software GraphPad Prism v8.0.2 (GraphPad Software, San Diego, CA, USA). The normality of data was verified with the Shapiro–Wilk test. For data that followed a normal distribution, analysis of variance (ANOVA) was used to compare the means of three or more unpaired groups, and Dunnet's test was used as a multiple comparison test. Data that did not follow a normal distribution were subjected to the non-parametric Kruskal–Wallis test followed by Dunn's test as a multiple comparison test. Differences with a p-value ≤ 0.05 were considered statistically significant.

3. Results and discussion

3.1. Antibacterial activity of lactoferrin against S. aureus

The antibacterial activity of bovine LF against *S. aureus* was evaluated in different stages of bacterial growth. The results obtained in the exponential (8 h of incubation) and in the stationary (18–20 h of incubation) phases after 4 and 24 h of incubation with different concentrations of LF are shown in Fig. 1. The *S. aureus* culture was quite resistant to the effect of LF during the exponential phase (Fig. 1A); while the stationary phase culture was slightly sensitive to this protein (Fig. 1B).

LF did not show any antibacterial effect against *S. aureus* in exponential phase; none of the concentrations of this dairy protein decreased the growth of the bacteria (Fig. 1A). These results agree with those of Bhimani, Vendrov, and Furmanski (1999), who demonstrated that *S. aureus* (ATCC 6538) at exponential phase was weakly sensitive to human and bovine LF. It is known that *S. aureus* is able to produce siderophores (Perry et al., 2019), which are responsible for the absorption of iron present in the medium and bound to LF; avoiding the effect of this protein and maintaining the growth of the bacteria (Hussan et al., 2022). When *S. aureus* and LF coexist in an environment that does not have enough iron, as our culture medium, a competition occurs between the bacteria and the protein to capture the iron from the medium. It has been reported that when *S. aureus* is in the exponential phase of growth, it produces siderophores that can uptake iron bound to transferrin, an iron-binding protein very similar to LF (Lindsay, Riley, & Mee, 1995). Furthermore, it has been shown in a study by Aguila et al. (2001) that the addition of iron to the culture medium favoured the growth of *S. aureus*, decreasing the antibacterial effect of human LF.

In contrast to our results, in the study by Padrão et al. (2016), in which the function of a composite of bacterial cellulose and LF as edible antimicrobial packaging was evaluated, they verified that LF had an effect against *S. aureus* in the exponential phase. All the LF concentrations tested in that study (0.25, 0.5, 1, 2.5, 5 and 10 mg mL^{-1}) reduced the specific growth rate of *S. aureus*, although this effect was not dose dependent, since the antibacterial activity was very similar at concentrations between 0.5 and 10 mg mL^{-1}. However, in this referenced study the strain of *S. aureus* used was not specified, so a strain more sensitive to the action of LF could have been used.

In the study by Bai et al. (2010) several segments of recombinant bovine LF were expressed in *Pichia pastoris* and their antibacterial activity evaluated against *S. aureus* (the same strain used in our study). The results showed that the protein including the inter-lobe region and the N-lobe of LF exerted higher antibacterial activity against *S. aureus* in exponential phase than the N-lobe alone or the whole recombinant LF at 5 mg mL^{-1}.

In our study, the incubation of the bacteria with a concentration of 5 mg mL^{-1} of LF for 4 h began to inhibit the growth of *S. aureus* in stationary phase. However, after 24 h of incubation, a lower amount

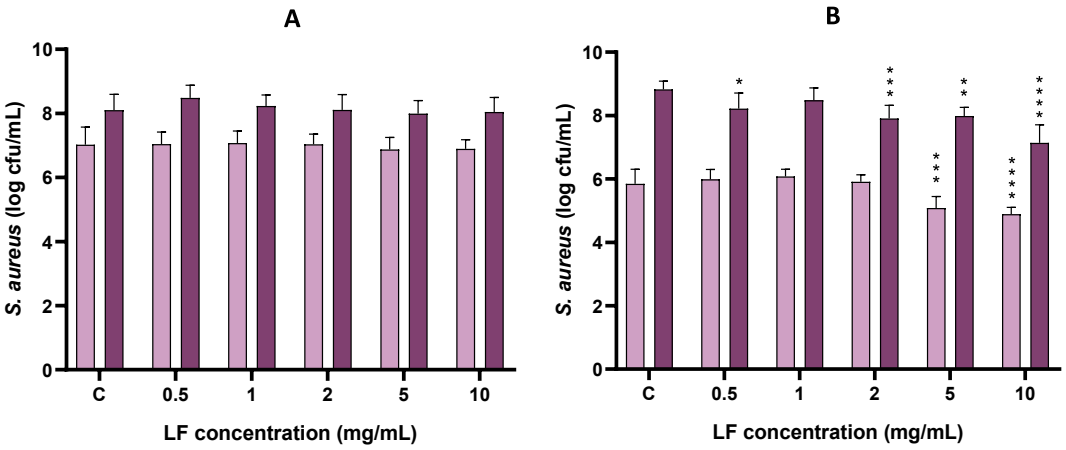

Fig. 1. Antibacterial activity of bovine LF at different concentrations against *S. aureus* at (**A**) exponential phase and (**B**) stationary phase after an incubation of 4 h (□) and 24 h (■). C: control of bacteria with peptone water without LF. The values represent the mean ± standard deviation of two replicates in three independent experiments (n = 6). Asterisks indicate significant differences respect to control (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

of LF was necessary to start inhibiting bacterial growth (Fig. 1B). Kutila, Pyörälä, Saloniemi, and Kaartinen (2003) obtained similar results to ours. In their study, LF at 1.67 and 2.67 mg mL^{-1} significantly slowed the growth of *S. aureus*; and LF at 2.67 mg mL^{-1} decreased the maximum growth after 20 h of incubation. Therefore, it can be stated that the activity of LF against *S. aureus* is dose dependent and varies depending on the incubation time.

3.2. Antibacterial activity of simulated digestion solutions

Before analyzing the effect of LF, dairy formulas and their respective digests, we tested whether the simulated digestion solutions (SSS, SGS and SIS) had an antibacterial activity against *S. aureus* by themselves (Fig. 2). Both salivary and gastric solutions did not present antibacterial effect, allowing the normal growth of the bacteria. However, the SIS significantly decreased the growth of *S. aureus* after an incubation of 4 and 24 h, reducing the amount of the bacteria in 3 and 4 logarithmic units (u.log), respectively. Previous studies have demonstrated that pancreatin, present in SIS, has antibacterial effect against *S. aureus* (Banerjee et al., 2020), and that bile acids also decrease the growth of certain bacteria such as *Listeria monocytogenes* and *Salmonella typhimurium* (Akritidou et al., 2022). Therefore, it is important to consider these results in order to analyze correctly the effect of intestinal digests of LF and dairy formulas.

3.3. Antibacterial activity of lactoferrin and its digests against S. aureus

The analysis of the antibacterial effect of LF and its digests is illustrated in Fig. 3. Salivary and gastric digests did not show an antibacterial activity against *S. aureus*. The only sample that significantly inhibited the growth of the bacteria was the ID. However, it could be expected that the effect of this digest after 4 h of incubation was mainly due to the components of the SIS, since the decrease in the number of bacteria was similar to that obtained only with the solution (Fig. 2). At 24 h of incubation, the effect shown by the ID was slightly greater than that obtained with SIS, which could be due to the long-term effect of the LF peptides generated in the intestinal phase.

In the study by Dionysius and Milne (1997), LF peptides were obtained by digestion of LF with pepsin and, once purified, their antibacterial activity against some bacteria, including *S. aureus* (ATCC 9144), was analyzed. Although the conditions used in that study were different from ours, since no simulated digestion solution was used and times and temperatures were not the same, *S. aureus* showed relative resistance to these peptides generated after digestion with pepsin.

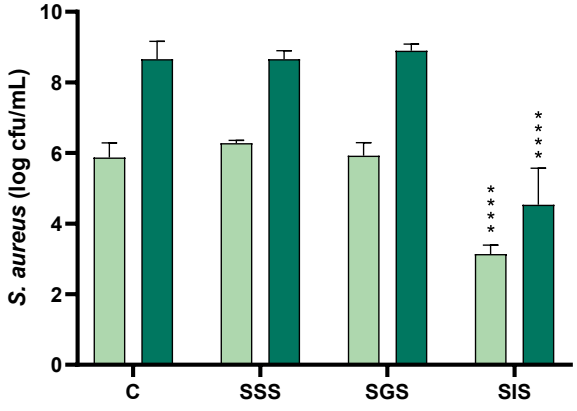

Fig. 2. Antibacterial activity of simulated digestion solutions with digestive enzymes against *S. aureus* after an incubation of 4 h (□) or 24 h (■). C: control of bacteria with peptone water, SSS: simulated salivary solution, SGS: simulated gastric solution, SIS: simulated intestinal solution. The values represent the mean ± standard deviation of two replicates in three independent experiments (n = 6). Asterisks indicate significant differences respect to control (****p < 0.0001).

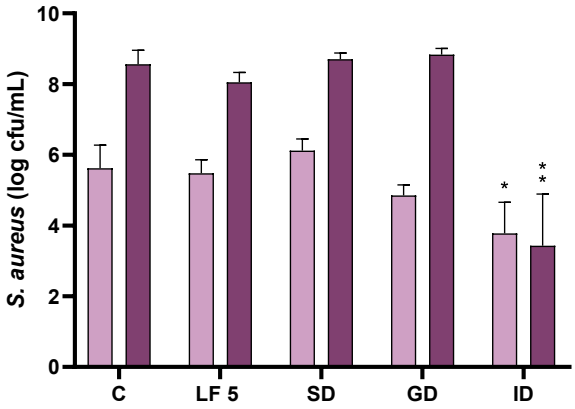

Fig. 3. Antibacterial activity of LF and its digests against *S. aureus* after an incubation of 4 h (□) or 24 h (■). C: control of bacteria with peptone water without LF, LF 5: LF at 5 mg mL^{-1}, SD: salivary digest, GD: gastric digest, ID: intestinal digest. The values represent the mean ± standard deviation of two replicates in three independent experiments (n = 6). Asterisks indicate significant differences respect to control (*p < 0.05, **p < 0.01).

Flores-Villaseñor et al. (2010) analyzed the antibacterial effect of synthetic LF peptides against *Escherichia coli* and *S. aureus* (ATCC 25923). In that study, lactoferricin and lactoferrampin inhibited the growth of *S. aureus* by more than 85%, which is not consistent with our results. However, it should be noted that in the study by Flores-Villaseñor et al. (2010) the peptides used were synthetic and purified, and not a digest of LF with more complex composition, as in our case.

In the study carried out by Aguila et al. (2001), the effect of LF and its peptides generated by acid proteolysis was analyzed on laboratory strains of *S. aureus* and on clinical isolates of this bacterium. Furthermore, they evaluated the influence of the culture medium and its composition on the antibacterial activity of LF and its peptides. When LF was added to a culture of *S. aureus* in a medium without iron, the growth of the laboratory strains decreased in a dose-dependent manner. By adding iron to this culture, the action of LF was reversed, increasing the bacterial growth. On the other hand, the LF peptides did not show antibacterial effect against *S. aureus* when added to growth-supportive media, which coincide in some way with our results. They concluded that when culture conditions are optimal for bacterial growth, *S. aureus* exhibits the ability to effectively counteract the bactericidal mechanisms exerted by LF-derived peptides, probably by repairing the cell wall damage that these peptides may have caused. Finally, in the study by Aguila et al. (2001), the clinical isolates of *S. aureus*, with a strain similar to that used in our study, showed higher resistance to LF than laboratory strains.

3.4. Antibacterial activity of dairy formulas and its digests against S. aureus

Finally, the effect of different dairy formulas containing LF and MFGM, and some of them subjected to technological treatments, and their digests against *S. aureus* was analyzed (Fig. 4). Mostly, dairy formulas before digestion did not produce a decrease in the growth of *S. aureus*; however, their effect against this bacterium

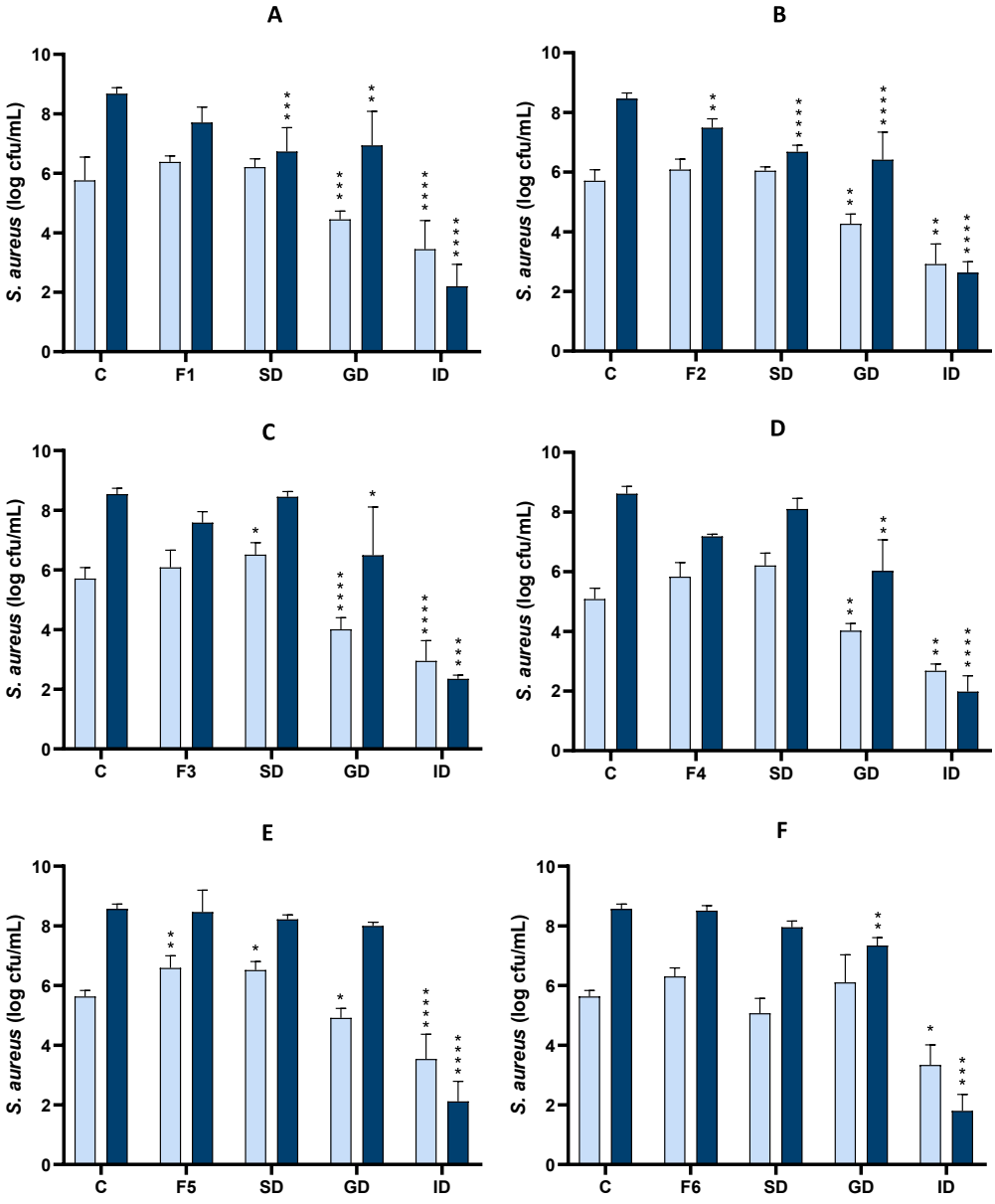

Fig. 4. Antibacterial activity against *S. aureus* of (**A**) F1 and its digests, (**B**) F2 and its digests, (**C**) F3 and its digests, (**D**) F4 and its digests, (**E**) F5 and its digests and (**F**) F6 and its digests after an incubation of 4 h (□) or 24 h (■). C: control of bacteria with peptone water, SD: salivary digest, GD: gastric digest, ID: intestinal digest. The values represent the mean ± standard deviation of two replicates in three independent experiments (n = 6). Asterisks indicate significant differences respect to control (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

increased with the different stages of digestion. In general, the IDs were the samples with the highest antibacterial activity, followed by GDs. After 24 h of incubation, the decrease in the number of bacteria with the ID was not exclusively due to the effect of the digestive enzymes present in the SIS, which had produced a decrease of 4 u.log (Fig. 2). Possibly, the antimicrobial peptides (AMPs) generated in the digestion exerted some effect, reducing the number of bacteria up to 6 u.log (Fig. 4).

Some studies have affirmed that milk-derived peptides present antibacterial effect against *S. aureus*. Folliero et al. (2022) analyzed the activity of AMPs derived from casein of kashk, an Iranian dairy product similar to whey, against *S. aureus* in wound healing. They concluded that the peptide fraction obtained was effective, reducing the skin colonization rate of *S. aureus* with a dose-dependent effect.

Furthermore, several AMPs have been evaluated for their potential to inhibit pathogens in different food matrices. A novel antimicrobial peptide from the whey acidic protein of *Larimichthys crocea* (LCWAP) presented a mechanism of action against *S. aureus*, with a minimal inhibitory concentration (MIC) of 15.6 μg mL^{-1} (Yang et al., 2020). The MIC of peptide LCWAP was lower than the MIC of the entire protein (184.5 μg mL^{-1}). This showed that LCWAP has a strong inhibitory effect against *S. aureus*, and this effect is dose-dependent. Furthermore, LCWAP reduces biofilm formation by *S. aureus* in a manner directly proportional to the peptide concentration (Yang et al., 2020).

BCp12, another novel AMP isolated from buffalo casein hydrolyzated, also showed antibacterial activity, damaging the *S. aureus* wall and causing pores in it (Shi, Li, Yang, Wei, & Huang, 2023), and an inhibitory effect on biofilm formation by *S. aureus* (Li et al., 2022).

In our study, the effect of the first four formulas, F1 and F2 without treatment and F3 and F4 subjected to homogenization, presented a very similar activity in all their digests. However, the last two formulas, F5 and F6, subjected to a pasteurization treatment, suffered a loss of activity, especially in the digests obtained after gastric digestion (Fig. 4).

Although more antibacterial effect was observed with the F2 (Fig. 4B), based on buttermilk, than with the F1 (Fig. 4A), based on whey, the differences in antibacterial activity against *S. aureus* between these two dairy matrices were minimal.

When LF is added to a matrix, it can interact with other proteins and components, such as the casein micelles (Anema & De Kruif, 2011), thus reducing LF activity. This would explain the low antibacterial activity against *S. aureus* of the undigested formulas compared to the digested formula.

It has been reported that homogenization modifies the interaction between LF and caseins (Lee & Sherbon, 2002), and favours the digestibility of proteins, improving the release of bioactive peptides (Tunick et al., 2016). However, dairy formulas subjected to a thermal treatment are more susceptible to pepsin hydrolysis, and this treatment could denature some proteins, causing the loss of their activity (Halabi, Croguennec, Bouhallab, Dupont, & Deglaire, 2020). Furthermore, thermal treatments, such as pasteurization or ultra-high-temperature, increase fat and protein aggregation due to the breakdown of MFGM (Tunick et al., 2016); which could explain the loss of activity in the GDs of F5 and F6, being the proteins aggregated, preventing a correct digestion and peptide liberation.

The Commission Regulation (EC) N° 2073/2005 of 15 November 2005 on microbiological criteria for foodstuff established limits on the count of *S. aureus* to avoid the production of toxins. The maximum limit in whey was set to 10^2 ufc g^{-1} (European Commission, 2005). Therefore, although some of the tested samples decreased the count of *S. aureus*, it did not ensure inhibition of enterotoxin production. Only those samples that decreased the count by 6 u.log (IDs) could be considered effective against the production of toxins by *S. aureus*.

4. Conclusions

In the last decade, the addition of MFGM to infant formulas has attracted great interest. Some proteins present in MFGM have a protective role due to its ability to inhibit infections by bacteria and to generate bioactive peptides. Furthermore, supplementation of infant formulas with bovine LF has also increased in recent years, contributing to potentiate the protective capacity of formulas against various pathogens and also to contribute to the development of a healthy microbiota in the infant.

The results obtained in this study show that native bovine LF and its digests do not appear to have a clear effect on their own against *S. aureus*; but when it is added to a dairy formula as supplement together with MFGM, the antibacterial effect increases. Furthermore, it could be considered that the production of enterotoxins by *S. aureus* was inhibited by the IDs of the formulas, since they decreased the colony counts to 2 u.log.

In addition, gastric digests of dairy formulas reduce the growth of *S. aureus* both at 4 and 24 h of incubation, possibly due to the AMPs generated during the GIT digestion. Technological treatments, such as homogenization or pasteurization, modifies the effect of digestion on proteins and, consequently, the antibacterial activity against *S. aureus*. In our results, pasteurization treatment of dairy formulas altered the digestion process, reducing the antibacterial activity of gastric digests compared to those of non-thermally treated formulas. However, pasteurization does not affect the intestinal digests, which had an effect against *S. aureus* similar to that of the untreated formulas.

Furthermore, it is also likely that *S. aureus* adapted to LF during incubation and developed resistance mechanisms, such as the expression of siderophores.

All these results allow us to deepen our knowledge of the activity of milk proteins and peptides in complex environments such as the GIT tract and thus enhance their use as supplements in dairy formulas, and revalue the by-products generated in the dairy industry.

Funding sources

This work was funded by grants from the Spanish Ministry of Economy, Industry and Competitiveness and the European Regional Development Fund (ERDF/FEDER) (AGL2017-82987 and PID2022-139104OB-I00 projects), the European Social Found (ESF) and the Aragon Regional Government (A20_23R).

CRediT authorship contribution statement

I. Abad: Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation, Data curation, Conceptualization. **A. Bailac:** Software, Methodology, Investigation, Data curation. **M.D. Pérez:** Writing – review & editing, Methodology. **J.J. Carramiñana:** Writing – review & editing, Methodology. **M. Calvo:** Supervision, Funding acquisition, Conceptualization. **L. Sánchez:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The authors would like to acknowledge the use of Servicio General de Apoyo a la Investigación (SAI), Universidad de Zaragoza (Spain).

References

Abad, I., Serrano, L., Graikini, D., Pérez, M. D., Grasa, L., & Sánchez, L. (2022a). Effect of in vitro gastrointestinal digestion on the antibacterial activity of bioactive dairy formulas supplemented with lactoferrin against *Cronobacter sakazakii*. *Biometals, 36*(3), 667–681. https://doi.org/10.1007/s10534-022-00459-5

Abad, I., Sangüesa, A., Ubieto, M., Carramiñana, J. J., Pérez, M. D., Buey, B., et al. (2022b). Protective effect of bovine lactoferrin against *Cronobacter sakazakii* in human intestinal Caco-2/TC7 cells. *International Dairy Journal, 133*, Article 105428. https://doi.org/10.1016/j.idairyj.2022.105428

Aguila, A., Herrera, A. G., Morrison, D., Cosgrove, B., Perojo, A., Montesinos, I., et al. (2001). Bacteriostatic activity of human lactoferrin against *Staphylococcus aureus* is a function of its iron-binding properties and is not influenced by antibiotic resistance. *FEMS Immunology and Medical Microbiology, 31*(2), 145–152. https://doi.org/10.1111/j.1574-695X.2001.tb00511.x

Akritidou, T., Akkermans, S., Gaspari, S., Azraini, N. D., Smet, C., Van de Wiele, T., et al. (2022). Effect of gastric pH and bile acids on the survival of *Listeria monocytogenes* and *Salmonella Typhimurium* during simulated gastrointestinal digestion. *Innovative Food Science & Emerging Technologies, 82*, Article 103161. https://doi.org/10.1016/j.ifset.2022.103161

Ali, A. H. (2019). Current knowledge of buttermilk: Composition, applications in the food industry, nutritional and beneficial health characteristics. *International*

Journal of Dairy Technology, 72(2), 169–182. https://doi.org/10.1111/1471-0307.12572
Anema, S. G., & De Kruif, C. G. (2011). Interaction of lactoferrin and lysozyme with casein micelles. *Biomacromolecules, 12*(11), 3970–3976. https://doi.org/10.1021/bm200978k
Argudín, M.Á., Mendoza, M. C., & Rodicio, M. R. (2010). Food poisoning and *Staphylococcus aureus* enterotoxins. *Toxins, 2*(7), 1751–1773. https://doi.org/10.3390/toxins2071751
Bai, X., Teng, D., Tian, Z., Zhu, Y., Yang, Y., & Wang, J. (2010). Contribution of bovine lactoferrin inter-lobe region to iron binding stability and antimicrobial activity against *Staphylococcus aureus*. *Biometals, 23*, 431–439. https://doi.org/10.1007/s10534-010-9300-x
Banerjee, S., Vishakha, K., Das, S., Dutta, M., Mukherjee, D., Mondal, J., et al. (2020). Antibacterial, anti-biofilm activity and mechanism of action of pancreatin doped zinc oxide nanoparticles against methicillin resistant *Staphylococcus aureus*. *Colloids and Surfaces B: Biointerfaces, 190*, Article 110921. https://doi.org/10.1016/j.colsurfb.2020.110921
Barukčić, I., Jakopović, K. L., & Božanić, R. (2019). Valorisation of whey and buttermilk for production of functional beverages—An overview of current possibilities. *Food Technology and Biotechnology, 57*, 448–460. https://doi.org/10.17113/ftb.57.04.19.6460
Bhimani, R. S., Vendrov, Y., & Furmanski, P. (1999). Influence of lactoferrin feeding and injection against systemic staphylococcal infections in mice. *Journal of Applied Microbiology, 86*(1), 135–144. https://doi.org/10.1046/j.1365-2672.1999.00644.x
Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., et al. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols, 14*(4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1
Cheung, G. Y. C., Bae, J. S., & Otto, M. (2021). Pathogenicity and virulence of *Staphylococcus aureus*. *Virulence, 12*(1), 547–569. https://doi.org/10.1080/21505594.2021.1878688
Cho, T. J., Hwang, J. Y., Kim, H. W., Kim, Y. K., Il Kwon, J., Kim, Y. J., et al. (2019). Underestimated risks of infantile infectious disease from the caregiver's typical handling practices of infant formula. *Scientific Reports, 9*(1), 9799. https://doi.org/10.1038/s41598-019-46181-0
Dionysius, D. A., & Milne, J. M. (1997). Antibacterial peptides of bovine lactoferrin: Purification and characterization. *Journal of Dairy Science, 80*(4), 667–674. https://doi.org/10.3168/jds.S0022-0302(97)75985-X
Edwards, J. S., Betts, L., Frazier, M. L., Pollet, R. M., Kwong, S. M., Walton, W. G., et al. (2013). Molecular basis of antibiotic multiresistance transfer in *Staphylococcus aureus*. *Proceedings of the National Academy of Sciences, 110*(8), 2804–2809. https://doi.org/10.1073/pnas.1219701110
European Commission. (2005). Commission Decision of 15 November 2005 on microbiological criteria for foodstuffs under Regulation (EC) N° 2073/2005 of The European Parliament and of the Council. *Official Journal of the European Union, 338*, 1–26.
European Commission. (2012). Commission Decision of 22 November 2012 authorising the placing on the market of bovine lactoferrin as a novel food ingredient under Regulation (EC) N° 258/97 of The European Parliament and of the Council. *Official Journal of the European Union, 327*, 46–48.
European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA-ECDC). (2017). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. *EFSA Journal, 15*(12), Article e05077. https://doi.org/10.2903/j.efsa.2017.5077
European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA-ECDC). (2021). The European Union One Health 2020 Zoonoses Report. *EFSA Journal, 19*(12), 6971. https://doi.org/10.2903/j.efsa.2021.6971
Flores-Villaseñor, H., Canizalez-Román, A., Reyes-Lopez, M., Nazmi, K., de la Garza, M., Zazueta-Beltrán, J., et al. (2010). Bactericidal effect of bovine lactoferrin, LFcin, LFampin and LFchimera on antibiotic-resistant *Staphylococcus aureus* and *Escherichia coli*. *Biometals, 23*, 569–578. https://doi.org/10.1007/s10534-010-9306-4
Folliero, V., Lama, S., Franci, G., Giugliano, R., D'Auria, G., Ferranti, P., et al. (2022). Casein-derived peptides from the dairy product kashk exhibit wound healing properties and antibacterial activity against *Staphylococcus aureus*: Structural and functional characterization. *Food Research International, 153*, Article 110949. https://doi.org/10.1016/j.foodres.2022.110949
Furlund, C. B., Ulleberg, E. K., Devold, T. G., Flengsrud, R., Jacobsen, M., Sekse, C., et al. (2013). Identification of lactoferrin peptides generated by digestion with human gastrointestinal enzymes. *Journal of Dairy Science, 96*(1), 75–88. https://doi.org/10.3168/jds.2012-5946
García-Montoya, I. A., Cendón, T. S., Arévalo-Gallegos, S., & Rascón-Cruz, Q. (2012). Lactoferrin a multiple bioactive protein: An overview. *Biochimica et Biophysica Acta, 1820*(3), 226–236. https://doi.org/10.1016/j.bbagen.2011.06.018
Halabi, A., Croguennec, T., Bouhallab, S., Dupont, D., & Deglaire, A. (2020). Modification of protein structures by altering the whey protein profile and heat treatment affects in vitro static digestion of model infant milk formulas. *Food & Function, 11*(8), 6933–6945. https://doi.org/10.1039/D0FO01362E
Hussan, J. R., Irwin, S. G., Mathews, B., Swift, S., Williams, D. L., & Cornish, J. (2022). Optimal dose of lactoferrin reduces the resilience of in vitro *Staphylococcus aureus* colonies. *PLoS One, 17*(8), Article e0273088. https://doi.org/10.1371/journal.pone.0273088
International Organization for Standardization (ISO). (2003). *ISO 6888-3:2003. Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus *and other species). Part 3: Detection and MPN technique for low numbers*. Geneva, Switzerland: International Organization for Standardization.
International Organization for Standardization (ISO). (2023). *ISO 6888-1:2021/Amd1:2023. Microbiology of the food chain. Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus *and other species). Part 1: Method using Baird-Parker agar medium - Amendment 1*. Geneva, Switzerland: International Organization for Standardization.
Jahan-Mihan, A., Luhovyy, B. L., El Khoury, D., & Anderson, G. H. (2011). Dietary proteins as determinants of metabolic and physiologic functions of the gastrointestinal tract. *Nutrients, 3*(5), 574–603. https://doi.org/10.3390/nu3050574
Kutila, T., Pyörälä, S., Saloniemi, H., & Kaartinen, L. (2003). Antibacterial effect of bovine lactoferrin against udder pathogens. *Acta Veterinaria Scandinavica, 44*(1), 1–8. https://doi.org/10.1186/1751-0147-44-35
Lee, S. J., & Sherbon, J. W. (2002). Chemical changes in bovine milk fat globule membrane caused by heat treatment and homogenization of whole milk. *Journal of Dairy Research, 69*(4), 555–567. https://doi.org/10.1017/S002202990200571X
Li, Y., Li, S., Yang, K., Guo, R., Zhu, X., Shi, Y., et al. (2022). Antibiofilm mechanism of a novel milk-derived antimicrobial peptide against *Staphylococcus aureus* by downregulating agr quorum sensing system. *Journal of Applied Microbiology, 133*(4), 2198–2209. https://doi.org/10.1111/jam.15653
Lindsay, J. A., Riley, T. V., & Mee, B. J. (1995). *Staphylococcus aureus* but not *Staphylococcus epidermidis* can acquire iron from transferrin. *Microbiology, 141*(1), 197–203. https://doi.org/10.1099/00221287-141-1-197
Mackie, A., & Rigby, N. (2015). InfoGest Consensus Method. In K. Verhoeckx, P. Cotter, I. López-Expósito, C. Kleiveland, T. Lea, A. Mackie, et al. (Eds.), *The impact of food-bioactives on gut health* (pp. 13–22). New York, USA: Springer International Publishing. https://doi.org/10.1007/978-3-319-16104-4_2.
Mohanty, D. P., Mohapatra, S., Misra, S., & Sahu, P. S. (2016). Milk derived bioactive peptides and their impact on human health–A review. *Saudi Journal of Biological Sciences, 23*(5), 577–583. https://doi.org/10.1016/j.sjbs.2015.06.005
Padrão, J., Gonçalves, S., Silva, J. P., Sencadas, V., Lanceros-Méndez, S., Pinheiro, A. C., et al. (2016). Bacterial cellulose-lactoferrin as an antimicrobial edible packaging. *Food Hydrocolloids, 58*, 126–140. https://doi.org/10.1016/j.foodhyd.2016.02.019
Pasachova, J., Ramirez, S., & Muñoz, L. (2019). Staphylococcus aureus. *Nova, 17*(32), 25–38. https://doi.org/10.22490/24629448.3631
Perry, W. J., Spraggins, J. M., Sheldon, J. R., Grunenwald, C. M., Heinrichs, D. E., Cassat, J. E., et al. (2019). *Staphylococcus aureus* exhibits heterogeneous siderophore production within the vertebrate host. *Proceedings of the National Academy of Sciences, 116*(44), 21980–21982. https://doi.org/10.1073/pnas.1913991116
Pires, A. F., Marnotes, N. G., Rubio, O. D., Garcia, A. C., & Pereira, C. D. (2021). Dairy by-products: A review on the valorization of whey and second cheese whey. *Foods, 10*(5), 1067. https://doi.org/10.3390/foods10051067
Ripollés, D., Parrón, J. A., Fraguas, J., Calvo, M., Pérez, M. D., & Sánchez, L. (2018). Determination of lactadherin concentration in dairy by-products by ELISA: Effect of heat treatment and hydrolysis. *Journal of Dairy Science, 101*(2), 912–923. https://doi.org/10.3168/jds.2017-13608
Roberfroid, M. B. (2000). Concepts and strategy of functional food science: The European perspective. *The American Journal of Clinical Nutrition, 71*(6), 1660S–1664S. https://doi.org/10.1093/ajcn/71.6.1660s
Shi, Y., Li, Y., Yang, K., Wei, G., & Huang, A. (2023). A novel milk-derived peptide effectively inhibits *Staphylococcus aureus*: Interferes with cell wall synthesis, peptidoglycan biosynthesis disruption reaction mechanism, and its application in real milk system. *Food Control, 144*, Article 109374. https://doi.org/10.1016/j.foodcont.2022.109374
Singh, H. (2006). The milk fat globule membrane—A biophysical system for food applications. *Current Opinion in Colloid & Interface Science, 11*(2–3), 154–163. https://doi.org/10.1016/j.cocis.2005.11.002
Superti, F. (2020). Lactoferrin from bovine milk: A protective companion for life. *Nutrients, 12*(9), 2562. https://doi.org/10.3390/nu12092562
Svanborg, S., Johansen, A. G., Abrahamsen, R. K., & Skeie, S. B. (2015). The composition and functional properties of whey protein concentrates produced from buttermilk are comparable with those of whey protein concentrates produced from skimmed milk. *Journal of Dairy Science, 98*, 5829–5840. https://doi.org/10.3168/jds.2014-9039
Telang, S. (2018). Lactoferrin: A critical player in neonatal host defense. *Nutrients, 10*(9), 1228. https://doi.org/10.3390/nu10091228
Tunick, M. H., Ren, D. X., Van Hekken, D. L., Bonnaillie, L., Paul, M., Kwoczak, R., et al. (2016). Effect of heat and homogenization on in vitro digestion of milk. *Journal of Dairy Science, 99*(6), 4124–4139. https://doi.org/10.3168/jds.2015-10474
Walzem, R. L., Dillard, C. J., & German, J. B. (2002). Whey components: Millennia of evolution create functionalities for mammalian nutrition: What we know and what we may be overlooking. *Critical Reviews in Food Science and Nutrition, 42*(4), 353–375. https://doi.org/10.1080/10408690290825574
Wang, X., Meng, J., Zhang, J., Zhou, T., Zhang, Y., Yang, B., et al. (2012). Characterization of *Staphylococcus aureus* isolated from powdered infant formula milk and infant rice cereal in China. *International Journal of Food Microbiology, 153*(1–2), 142–147. https://doi.org/10.1016/j.ijfoodmicro.2011.10.030
Yang, S., Li, J., Aweya, J. J., Yuan, Z., Weng, W., Zhang, Y., et al. (2020). Antimicrobial mechanism of Larimichthys crocea whey acidic protein-derived peptide (LCWAP) against *Staphylococcus aureus* and its application in milk. *International Journal of Food Microbiology, 335*, Article 108891. https://doi.org/10.1016/j.ijfoodmicro.2020.108891