Journal of Functional Foods 112 (2024) 105936

Contents lists available at ScienceDirect

Journal of Functional Foods

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

Does lactoferrin, free, encapsulated or in dairy matrices, maintain its antibacterial activity after in vitro digestion?

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ARTICLE INFO

Keywords:
Homogenization
Pasteurization
Dairy formulas
Alginate microbeads
Gastrointestinal tract
Digestive enzymes

ABSTRACT

Milk is a source of active compounds with defensive properties, such as lactoferrin and milk fat globule membrane proteins. These proteins generate bioactive peptides in the gastrointestinal tract and it is known that industrial processing can modify their susceptibility to digestion. However, does lactoferrin maintain its antibacterial activity after passing through the gastrointestinal tract? The aim of this study was to evaluate the effect of technological treatments, encapsulation and in vitro digestion on lactoferrin antibacterial activity against *Listeria monocytogenes*. The results showed that the gastric digest of free lactoferrin presented greater effect against *L. monocytogenes* than the intestinal digest; although less than undigested lactoferrin. Alginate-lactoferrin microbeads allowed the release of lactoferrin into the intestine, protecting it from pepsin, although it was not sufficient to maintain its antibacterial activity to the intestine. However, homogenization and pasteurization favoured the antibacterial activity of dairy formulas supplemented with lactoferrin and of their digests.

1. Introduction

Milk is one of the most complete food and, for this reason, it has an important role beyond infant feeding (Park & Nam, 2015). Milk proteins consist of caseins and whey proteins, and among this group the most relevant in bovine milk are β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA), immunoglobulins, lactoferrin (LF) and lactoperoxidase (Madureira et al., 2007). LF is a minor whey protein belonging to the transferrin family. It is an iron-binding glycoprotein with a molecular weight of approximately 80 kDa. It is produced in the epithelial cells of the mammary gland and secreted into milk. LF has an important defensive role, including antibacterial activity, anti-inflammatory effect and promotion of the immune system (García-Montoya et al., 2012).

Whey is a by-product derived from the cheese industry and it is a good source of proteins of high nutritional value. It contains soluble proteins that represent 20 % of the total milk proteins. When these proteins are partially digested, they are a source of bioactive peptides with numerous physiological activities (Mollea, Marmo, & Bosco, 2013).

On the other hand, buttermilk is the aqueous fraction resulting from cream churning to obtain butter, being the main by-product of the butter industry. It contains soluble components, such as lactose, proteins and minerals. In addition, it also consists of milk fat globule membrane (MFGM) fragments, rich in phospholipids and glycoproteins. The MFGM fragments are released from the fat globules when they agglomerate and are partially broken during the churning process (Vanderghem et al., 2010).

In recent years, the use of MFGM as an ingredient in infant formulas has attracted a great deal of interest (Ross et al., 2015). In addition, the ability of the main glycoproteins of MFGM mucin and lactadherin to inhibit rotavirus infection has been demonstrated, thus reducing the incidence of diarrhea in infants (Da Silva, Colleran, & Ibrahim, 2021). Furthermore, supplementation of infant formulas with LF has also increased in recent years because of its benefits for infants. These include protection against infections, immune and intestinal system development, brain and bone development, and improved iron absorption (Li et al., 2022). In 2012, the use of bovine LF was approved by the European Union as a novel food ingredient under Regulation (EC) No 258/97 (European Commission, 2012). This regulation specified that bovine LF could be placed on the market as a food ingredient, establishing the limits for its use in different food categories.

Milk proteins are considered one of the most important sources of

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https://doi.org/10.1016/j.jff.2023.105936
Received 19 July 2023; Received in revised form 24 November 2023; Accepted 4 December 2023
Available online 7 January 2024

bioactive peptides with different activities, which are mainly released during gastrointestinal digestion. Pepsin, together with other digestive enzymes, such as trypsin and chymotrypsin, are responsible for the hydrolysis of milk proteins (Mohanty et al., 2016). Milk-derived antimicrobial peptides act through diverse mechanisms, one of them is their interaction with the target bacterial membrane, with the consequent increase in cell permeability. Some antimicrobial peptides are derived from LF, including lactoferricin and lactoferrampin (Nielsen et al., 2017). However, these peptides are also ephemeral in the gastrointestinal tract. The digestion rate for LF and its peptides is influenced by the medium in which they are found. It has been shown that, in mice, LF administered alone is completely digested within 2 h (Fan et al., 2019), while when administered in a dairy base, some fragments of LF may appear in the mouse faeces (Kuwata et al., 1998). This difference is probably due to the interaction of LF with the rest of the dairy components, which could favour the protection of the protein and would reduce its degree of digestion in the gastrointestinal tract.

The digestion of milk proteins and the release of peptides from them can be controlled along the gastrointestinal tract thanks to encapsulation. This strategy can improve the bioavailability of LF, enhancing its stability in the gastrointestinal tract (Abad, Conesa, & Sánchez, 2021).

Alginate, a natural biodegradable polysaccharide, can form electrostatic complexes with LF depending on the pH of the media. It is a good option for the encapsulation of LF since alginate allows a controlled delivery of LF into the intestine, as it has been reported by several studies (Abad, Conesa, & Sánchez, 2021). Bokkhim et al. (2016) demonstrated that LF-alginate microbeads protected LF from the action of pepsin during the gastric digestion and allowed its release at the intestine. Furthermore, some authors have confirmed that the encapsulation of LF with alginate is a way to deliver intact LF to the intestine (Raei et al., 2015).

Listeria monocytogenes is a small Gram-positive bacillus belonging to the Listeriaceae family. It is a motile, non-spore-forming, facultative anaerobic, ubiquitous microorganism. Its size is approximately 1–1.5 μm in length. The optimal growth temperature is 37 °C, but it can survive in different conditions, which favours its growth and survival in food and surfaces of industrial equipment (Shamloo et al., 2019). Listeriosis is the disease caused by the consumption of foods contaminated with *L. monocytogenes*. It is one of the major causative agents of foodborne disease worldwide, resulting in high hospitalization and mortality rates (Liu et al., 2005). Furthermore, *L. monocytogenes* is able to colonize and survive in different food processing and preservation conditions, which is of great concern (Sibanda & Buys, 2022).

Therefore, the aim of this study was to analyze the effect of in vitro digestion on the antibacterial activity of lactoferrin in different states against *L. monocytogenes*. For this purpose, free lactoferrin, lactoferrin encapsulated with alginate and lactoferrin included in dairy formulas subjected to different technological treatments were analyzed.

2. Materials and methods

2.1. Preparation of samples and dairy formulas

Native bovine LF with an iron-saturation level below 10 % and a purity higher than 90 % (Abad et al., 2022) was kindly donated by the company Tatua Nutritionals (Morrinsville, New Zealand).

Six dairy formulas (F1-F6) were elaborated with a base of whey or buttermilk and supplemented with the commercial LF (10 mg/mL) and MFGM. To obtain the dairy by-products, raw bovine milk was provided by the dairy company Villacorona (El Burgo de Ebro, Spain), and processed at the Food Science and Technology Pilot Plant of the University of Zaragoza, located at the Veterinary Faculty, as detailed in our previous study (Abad et al., 2022b). A part of buttermilk was subjected to one-phase homogenization process at 250 bars. Whey and buttermilk were immediately frozen after being obtained and kept at −20 °C for later use. In addition, by performing a bicinchoninic acid test, the amount of protein present in each of these samples was analyzed, obtaining 153.8 mg of protein per g of whey and 136.7 mg of protein per g of BM. To obtain the MFGM, a volume (in 1:1 ratio with the base of the formula) of buttermilk (homogenized or not) was centrifuged at 40,000 g for 30 min at 4 °C. The composition of the dairy formulas is shown in more detail in Table S1.

Treatment of pasteurization at 72 °C for 20 s was applied to some dairy formulas as previously described (Abad et al., 2022b).

2.2. Lactoferrin encapsulated in alginate microbeads

LF encapsulation was performed following a procedure based on the studies of Bokkhim et al. (2016) and Braim et al. (2019), with some modifications.

Alginate (ALG) was prepared at 2 % (w/v) in milli-Q water at 40 °C. When complete dissolution was achieved, it was sonicated in an ultrasonic bath (Ultrasons, J.P. Selecta, Barcelona, Spain) for 10–15 min to facilitate air removal. Additionally, LF was prepared at a concentration of 10 mg/mL (1 % w/v), as in the dairy formulas. To favour electrostatic interaction between ALG and LF, LF was dissolved in 10 mM NaH_2PO_4 buffer, pH 5. LF was mixed with the ALG in a 1:1 ratio (v:v), and left to stand overnight at room temperature to allow the elimination of air bubbles.

The encapsulation of LF was performed in a B-395 Pro Encapsulator (BÜCHI Labortechnik AG, Flawil, Switzerland). The ALG-LF microbeads obtained were left in agitation for 30 min in a 100 mM $CaCl_2$ pH 5 solution, to favour their formation and stability. After this time, microbeads were washed twice with milli-Q water to remove excess $CaCl_2$, and kept at 4 °C.

The size of microbeads was measured using a Mastersizer 3000E (Malvern Instruments, Malvern, UK). This method is based on laser diffraction and the results are expressed as a volume-weighted average, D (4, 3). For this measurement, the refractive and absorption indices used were 1.333 and 0, respectively. Once the mean size of microbeads was measured, they were freeze-dried for storage at −20 °C.

ALG-LF microbeads were observed under the Eclipse 50i microscope equipped with the Digital Sight DS-2Mv camera (Nikon, Melville, NY, USA), comparing their morphology before lyophilization and after rehydration with a phosphate buffered saline (PBS).

Loading capacity (LC) and encapsulation efficiency (EE) were calculated using the following equations described in Zatorska-Płachta et al. (2021).

$$\mathrm{LC}\,(\%) = \frac{\text{Mass of encapsulated lactoferrin}}{\text{Mass of alginate}} \times 100$$

$$\mathrm{EE}\,(\%) = \frac{\text{Mass of encapsulated lactoferrin}}{\text{Total mass of lactoferrin}} \times 100$$

To determine the amount of encapsulated LF, 15 mg of microbeads were dispersed in 1 mL of 0.1 M sodium citrate, pH 8.4. They were shaken at 37 °C until completely dissolved. The supernatant was analyzed by spectrophotometry to measure the absorbance at 280 nm and with the LF molecular extinction coefficient ($E^{1\%}_{280} = 1.27$ mL/cm · g), the amount of encapsulated LF was determined.

The stability of the microbeads preserved at 4 °C was evaluated at different pHs and times. A phosphate buffer was prepared and adjusted to the desired pHs (3, 5, 7 and 8). The microbeads were left in those buffers at the different pHs at 4 °C at rest. After a period of time (1, 2, 4, 24, 48 or 72 h), the absorbance of the supernatant was measured at 280 nm to check if there had been LF release and to quantify it outside the microbeads, in case it has been discharged.

2.3. In vitro gastrointestinal digestion

The in vitro gastrointestinal digestion process followed the guidelines of the InfoGest Consensus Method detailed by Mackie and Rigby

(2015) and Brodkorb et al. (2019). Three simulated digestion solutions (simulated salivary solution, SSS; simulated gastric solution, SGS; and simulated intestinal solution, SIS) were prepared according to the salt concentrations detailed in our previous study (Abad et al., 2022b). These solutions were sterilized by using a 0.22 μm low binding protein Millipore filter (Merck KGaA, Darmstadt, Germany).

Commercial bovine LF and the six dairy formulas were subjected to a static in vitro gastrointestinal digestion consisting of three stages: salivary, gastric and intestinal phases and obtaining three digests: salivary digest (SD), gastric digest (GD) and intestinal digest (ID), as it has been described previously (Abad et al., 2022b). The digestion stages were consecutive, so that the digest obtained in the gastric phase had previously gone through the salivary stage and the ID had gone previously through salivary and gastric digestion.

All digests were lyophilized using Heto PowerDry DW8 equipment (Thermo Fisher Scientific, Rockford, IL, USA) and resuspended in a volume of milli-Q water necessary to obtain a final LF concentration of 5 mg/mL. To achieve sterility of the samples, they were filtered through a 0.45 μm Millipore low binding protein filter. The resulting samples were stored at −20 °C.

2.4. In vitro release of encapsulated lactoferrin

2.4.1. pH-change release

The ALG-LF microbeads were subjected to a pH variation process, similar to that occurring in the gastrointestinal phases, to know the protective capacity of microbeads and the percentage of encapsulated protein release. For this purpose, the protocol described by Braim et al. (2019) was followed with some adaptations. The simulated digestion solutions used were the same as those previously used for the digestion of dairy samples.

For treatment, 75 mg of the lyophilized microbeads were resuspended in 5 mL of SGS tempered at 37 °C. The final volume was adjusted to pH 3 and incubated for 2 h under agitation at 37 °C. After this time, the mixture was centrifuged at 4000 g for 5 min to achieve rapid sedimentation of the microbeads. The supernatant obtained was referred to as the gastric pH fraction (GF).

The precipitate with the microbeads obtained in the gastric phase was resuspended in 5 mL of SIS tempered at 37 °C. The pH of the mixture was adjusted to 7 and incubated for 2 h under agitation at 37 °C. In the same way as in the gastric stage, the sample was centrifuged and the supernantant corresponding to the fraction obtained at intestinal pH (IF) was collected.

The analysis of LF release from the microbeads in those phases was carried out by two methods. First, the concentration of LF released from the ALG-LF microbeads at pH 3 and 7 was analyzed spectrophotometrically by measuring the absorbance at 280 nm of the fractions. The quantity of LF was calculated according to its extinction coefficient ($E_{280}^{1\%} = 1.27$ mL/cm · g). Subsequently, and to know the percentage of LF release, the following formula proposed by Braim et al. (2019) was applied:

Protein release % = Mt/Mt_0 * 100, where Mt is the amount of protein at time t and Mt_0 is the amount of protein in untreated microbeads at time t = 0.

On the other hand, the amount of protein present in GF and IF was analyzed by bicinchoninic acid (BCA) assay using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific). The protein concentration thus obtained was compared with the initial amount present in the untreated mcirobeads, which were dissolved in 0.1 M sodium citrate solution, allowing the release of all encapsulated LF.

2.4.2. Enzyme-mediated digestion and release

The ALG-LF microbeads were subjected to an in vitro gastrointestinal digestion process, to analyze the capacity of microbeads to protect LF and the percentage of encapsulated protein release in conditions similar to those of the gastrointestinal tract. For these assays, the protocol described by Bokkhim et al. (2016) was followed with some adaptations. The simulated digestion solutions and the enzymes used were the same and in the same concentrations as those previously mentioned in point 2.3.

For this process, 75 mg of lyophilized microbeads were resuspended in 5 mL of SGS supplemented with pepsin (2000 U/mL) according to the InfoGest Consensus Method. The mixture was adjusted to pH 3 and incubated for 2 h under agitation at 37 °C. After this time, the mixture was centrifuged at 4000 g for 5 min and the supernatant obtained was referred to as the gastric digest (GD).

The microbeads that remained intact after the gastric phase were resuspended in 5 mL of SIS with intestinal enzymes (100 U/mL pancreatin and 10 mM bile), adjusted to pH 7 and incubated for 2 h under agitation at 37 °C. Some aliquots were taken from the supernatant at different times (1, 2, 4, 6, 8, 10, 12, 15, 30, 45 and 60 min). The final mixture was centrifuged and the supernantant corresponding to the intestinal digest (ID) was kept for analysis. The precipitate obtained after intestinal digestion was disolved with 0.1 M sodium citrate, in the same way as undigested microbeads, to check if there was LF left at the end of the digestion process.

The amount of LF released from the microbeads after each phase of digestion was calculated spectrophotometrically by measuring the absorbance at 280 nm and by the BCA technique. The presence of LF in the digests was checked by SDS-PAGE. In addition, a Western blotting assay was performed to identify if the bands observed in the digests corresponded to peptide fragments of LF, by following the method of Franco et al. (2010), using rabbit polyclonal anti-LF antibodies (1/100) and goat anti-rabbit IgG antibodies labeled with peroxidase (1/1000).

2.5. Culture of Listeria monocytogenes

The bacterial strain used in this study was *L. monocytogenes* CECT 935, supplied by the Spanish Type Culture Collection (CECT, Valencia, Spain), which corresponds with the strain ATCC 13932 of the American Type Culture Collection and is of clinical origin from the spinal fluid of a child with meningitis.

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

2.6. Listeria monocytogenes growth curve

To determine the evolution of the bacterial population, the growth curve of *L. monocytogenes* was performed. For this purpose, an isolated colony was cultured in TSB enriched with 0.6 % (w/v) YE and incubated at 37 °C. An aliquot was taken every hour during 12 h, and another one at 24 h, to measure the absorbance of the culture at 620 nm in a Multiskan MS ELISA plate reader (Labsystem, Helsinki, Finland).

In parallel, 50 μL of the bacterial suspension were taken and serial dilutions were performed in 1 % (w/v) peptone water for subsequent seeding on TSA plates. The plates were incubated for 24 h at 37 °C and colony counts were performed.

2.7. Antibacterial activity against Listeria monocytogenes

The antibacterial activity against *L. monocytogenes* of free LF, encapsulated LF and LF as a supplement in dairy formulas, and their respective digests, was analyzed. LF was evaluated at different concentrations (0.5, 1, 2, 5 and 10 mg/mL) against bacteria in exponential and stationary phase of growth, following the procedure detailed in our

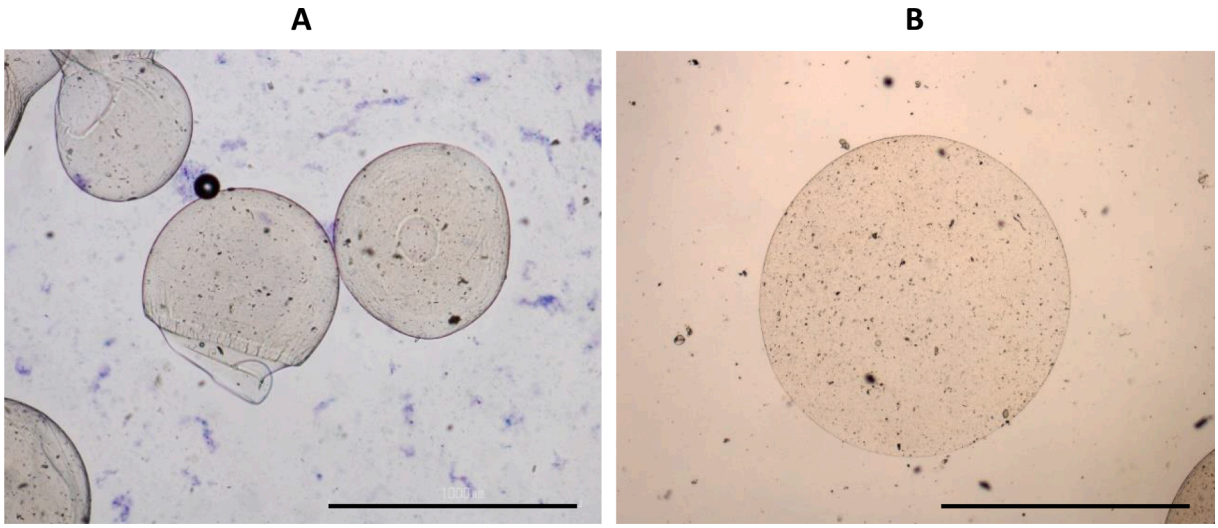

Fig. 1. Microscope images of microbeads. The scale bar represents 1000 μm. (**A**) Freshly produced alginate 2 % - LF 1 % microbeads. (**B**) Alginate 2 % - LF 1 % freeze-dried microbead and rehydrated with PBS.

previous study (Abad et al., 2022). The antibacterial activity of encapsulated LF, dairy formulas and digests was evaluated against *L. monocytogenes* only at stationary phase in the same way as in prior studies (Abad et al., 2022b).

All samples were evaluated in duplicate in three independent experiments. Two different incubation times, 4 and 24 h at 37 °C, were tested on the same plate. After the incubation time, 100 μL were taken from each well and serial dilutions were made for seeding in TSA plates. These plates were incubated for 24 h at 37 °C and colony counting was performed.

2.8. Fractionation of peptides from gastric digest

After the analysis of the antibacterial activity, a fractionation of the GD of LF and the GDs of F1 and F2, formulas that had not been subjected to any treatment, was performed with the aim of identifying the peptides responsible for the antibacterial effect. Using an Amicon ultrafiltration filter of 30 kDa (Amicon INC., Beverly, MA, USA), proteins of molecular weight greater and less than 30 kDa were separated. The volume corresponding to the fraction smaller than 30 KDa was processed with a dialysis membrane of 0.5–1 kDa (Spectrum Laboratories INC., CA, USA) to reduce the content of salts present in the SGS. After dialysis, the sample was subjected to a vacuum centrifugation with a SpeedVac (Genevac Ltd., Ipswich, UK) at 30 °C for 3 h to concentrate it.

An aliquot of the dialyzed and concentrated samples was analysed in the Proteomics Core Research Facility of Servicios Científico Técnicos del CIBA (IACS-Universidad de Zaragoza). Such analysis was carried out by protein identification on a hybrid triple quadrupole/linear ion trap mass spectrometer (6500QTRAP+, Sciex, Foster City, CA, USA) coupled to a nano/micro-HPLC (Eksigent LC425, Sciex). Peptide separation was

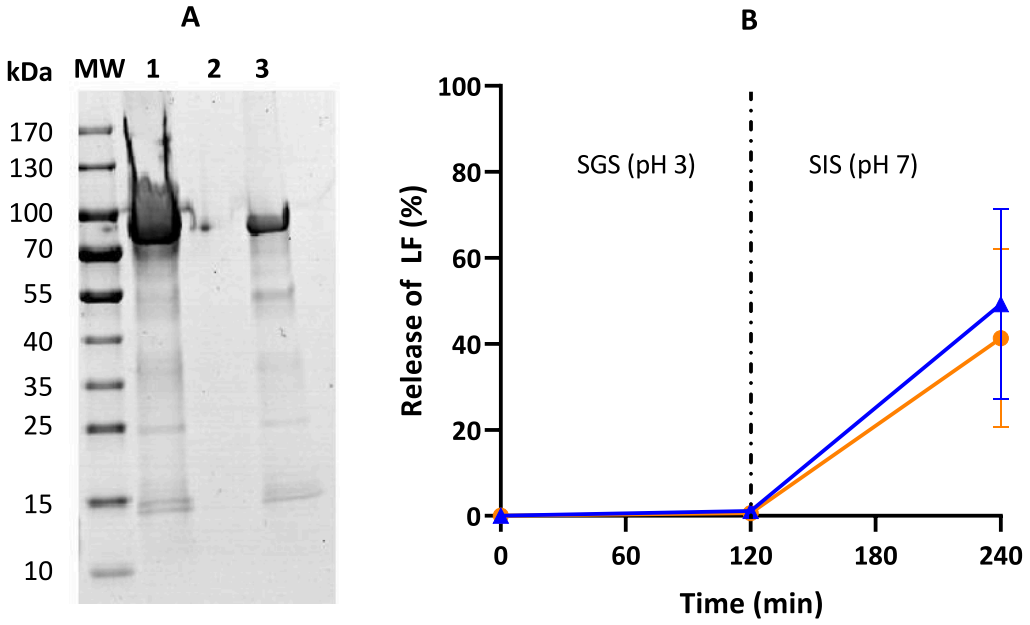

Fig. 2. (**A**) SDS polyacrylamide gel electrophoresis (4–20 %) of alginate-LF microbeads, stained with Coomassie Blue R. MW: molecular weight marker, 1: dissolved untreated microbeads, 2: supernatant of microbeads subjected to acidic pH, 3: supernatant of microbeads subjected to neutral pH. (**B**) Graphical representation of the percentage of in vitro pH-change release of LF from microbeads. Results obtained spectrophotometrically by absorbance at 280 nm (—) and by BCA (—). Values represent the mean of the eight batches (n = 8).

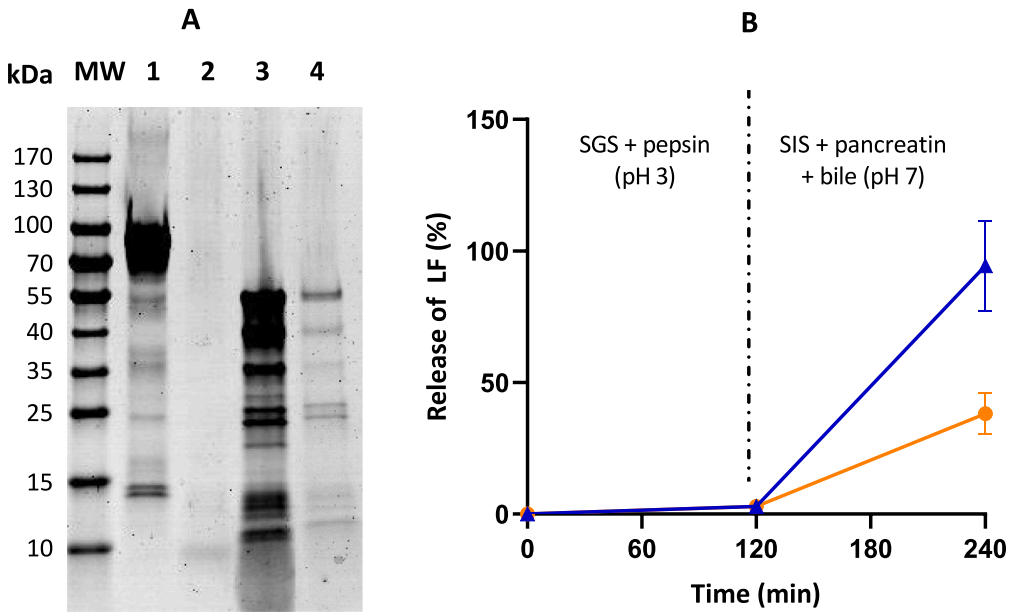

Fig. 3. (**A**) SDS polyacrylamide gel electrophoresis (4–20 %) of alginate-LF microbeads, stained with Coomassie Blue R. MW: molecular weight marker, 1: dissolved untreated microbeads, 2: supernatant of microbeads subjected to gastric digestion (pepsin and pH 3), 3: supernatant of microbeads subjected to intestinal digestion (pancreatin, bile and pH 7), 4: diluted microbeads after digestion process. (**B**) Graphical representation of the percentage of in vitro enzyme-mediate release of LF from microbeads. Results obtained spectrophotometrically by absorbance at 280 nm (—) and by BCA (—). Values represent the mean of four batches (n = 4).

performed using a C18 column (Luna® 0.3 mm id, 150 mm, 3 μm particle size, Phenomenex, CA, USA), at 5 μL/min. The search engine used was MASCOT (MatrixScience, UK) with public protein sequence databases (Swissprot, NCBI, etc.) according to the taxonomy of the bioactive peptides of interest.

Parallel to this analysis, the fraction smaller than 30 kDa was reprocessed using a 3 kDa ultrafiltration device. Two fractions were obtained and analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), using 4–20 % polyacrylamide gels (Bio-Rad Laboratories, Hercules, USA), which were stained with Coomassie Blue according to standard procedures.

2.9. Statistical analysis

In this study, results are presented as the mean ± standard deviation. Statistical analysis of results 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 Saphiro-Wilk test. For data that followed a normal distribution, analysis of variance (ANOVA) was used to compare the means of three of 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 and are indicated with asterisks (*) in the graphs.

3. Results and discussion

3.1. Encapsulation of lactoferrin in microbeads

Eight batches of microbeads were produced, each from a volume of 10 mL of ALG-LF mixture. Subsequently, the size of fresh microbeads was determined, using the Mastersizer 3000E, and a mean D (4, 3) of 710 ± 13.5 μm was obtained.

Fig. 1 shows the images taken with the microscope of freshly and freeze-dried microbeads rehydrated with PBS. It is observed that the spherical shape of microbeads is recovered after freeze-drying and subsequent rehydration, ensuring freeze-drying as a correct method of maintenance and preservation of ALG-LF microbeads.

The LC value obtained showed that the ALG-LF microbeads could absorb up to 13.4 % of LF respect to the biopolymer weight during the encapsulation process. In addition, the EE of the LF in the microbeads was 26.8 %.

The stability of the microbeads preserved at 4 °C was evaluated at different pHs (3, 5, 7 and 8) and times. At pHs 3, 5 and 7, LF was not released at any of the evaluated times (up to 72 h). However, at pH 8, LF began to be released after 4 h, so the preservation of these microbeads at basic pH is not recommended.

3.2. In vitro release of encapsulated lactoferrin

3.2.1. pH-change release

The microbeads produced were subjected to the pH conditions of in vitro gastrointestinal digestion, without enzymes, analyzing the release of the LF from the alginate matrix. As shown in Fig. 2A, in the GF, the band corresponding to LF was not observed; however, the IF did show the release of LF from microbeads, with the LF band at 80 kDa reappearing.

Fig. 2B shows the percentage of LF release in the two stages of treatment. The release was determined by measuring the absorbance of the samples at 280 nm and by performing a BCA test to quantify the protein present in the different samples. Both methods provided very similar results. After treatment with SGS at pH 3 during 2 h, about 1 % of the LF present in the microbeads was released. In contrast, a significant increase in LF released after treatment with SIS (pH 7) was observed, between 40 and 50 % of the milk protein captured by the alginate. It can, therefore, be concluded that the amount of LF released is pH dependent, as stated in our previous study (Abad, Conesa, & Sánchez, 2021).

The results obtained suggest that ALG-LF microbeads are suitable for encapsulation and controlled release of protein depending on pH. In the study by Braim et al. (2019), similar results were observed over time, releasing 100 % of LF from microbeads at 4 h of processing. In their case, the percentage of LF released at the gastric pH was higher, 60 % before 60 min. This difference with our results could be due to the different pHs used (1.2 in their case and 3 in our case) or to the solutions used. In our study, the protocol used for digestion followed the guidelines of the

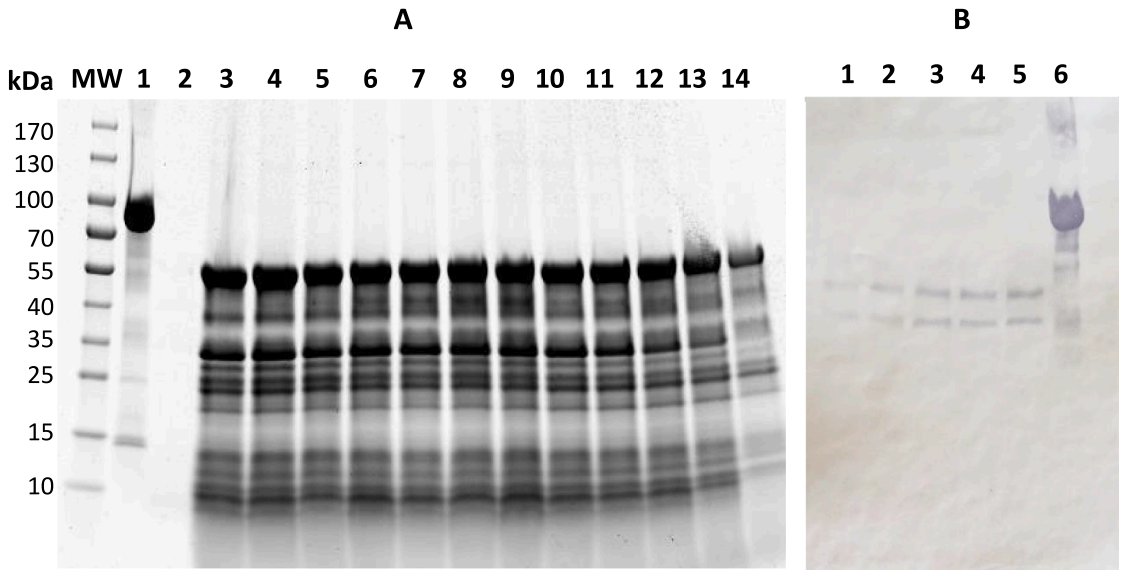

Fig. 4. (**A**) SDS polyacrylamide gel electrophoresis (4–20 %) of digested alginate-LF microbeads, stained with Coomassie Blue R. MW: molecular weight marker, 1: dissolved untreated microbeads, 2: GD of microbeads, 3–14: ID of microbeads for 1, 2, 4, 6, 8, 10, 12, 15, 30, 45 min, 1 h and 2 h, respectively. (**B**) Western blotting of LF. 1–5: ID of digested microbeads for 2, 4, 6, 8 and 10 min, respectively, 6: dissolved untreated microbeads.

InfoGest Consensus Method.

3.2.2. *Enzyme-mediated digestion and release*

The percentage of LF released from microbeads subjected to in vitro digestion with enzymes was also determined, as in section 3.2.1, by two methods, spectrophotometrically and by BCA technique. In this case (Fig. 3), there were differences in the results obtained by these two techniques. In Fig. 3A, it can be observed that LF (the 80 kDa band in lane 1) is practically not released from the ALG-LF microbeads to the supernatant after gastric digestion (lane 2). In GD, the main band does not appear and only small peptides around 10 kDa are observed, product of the pepsin digestion of the little amount of LF released from the microbeads in this step. However, we can affirm that LF is released from the microbeads in its totality in the intestinal phase since, although the 80 kDa band is not appreciated, a clear appearance of the peptides of smaller size are observed (10 kDa band in lane 3) due to the activity exerted by the intestinal enzymes. In addition, in the microbeads dissolved after digestion (lane 4), the LF band was not observed either, supporting the idea that all LF had been completely discharged.

After gastric digestion with pepsin at pH 3, only 3 % of the LF present in the microbeads was released. In contrast, after the intestinal digestion with pancreatin and bile at pH 7, a significant increase in the amount of released LF was observed (Fig. 3B). Although in the data obtained after gastric digestion there was unanimity in the value of LF released, the percentage of this protein release after intestinal digestion of the microbeads varied according to the technique used for its determination. The release percentage obtained by spectrophotometry reached almost 100 % of LF, while the determination by BCA reflected a lower release, around 40 % of total protein.

In this case and comparing the results with those obtained in the electrophoresis (Fig. 3A), it could be expected that the correct release percentage was the one obtained by the absorbance at 280 nm, wavelength at which the aminoacids are detected, allowing the detection of both the protein and the peptides generated. However, it is possible that the BCA technique does not correctly quantify all the peptides or amino acids generated after LF digestion, giving a lower percentage. In fact, the peptides containing tryptophan or tyrosine react with the BCA reagent, developing colour. However, free aminoacids cause a difference in the absorbance detected with this technique (Wiechelman, Braun, & Fitzpatrick, 1988).

When analyzing the fractions obtained at different incubation times in the intestinal phase, we could observe that LF hydrolysis was fast, being released from the ALG-LF microbeads and digested by the enzymes. It has been reported previously that the hydrolysis of LF by trypsin, one of the intestinal enzymes, generates two fragments of around 30 and 50 kDa (Mata et al., 1994). These fragments coincide with the molecular weight of those clearly present in our fractions (Fig. 4A), and verified by the Western blotting performed (Fig. 4B), confirming that these fragments belong to LF as they reacted with LF antibodies.

In addition, other encapsulation systems, such as liposomes, have been proven previously to decrease LF hydrolysis under intestinal conditions (Wang et al., 2019). In any case, and although liposomes protected LF to a greater extent than alginate microbeads, further studies should be carried out to design better encapsulation systems that permit LF to be active in the intestine.

Comparing the digestion of ALG-LF microbeads with and without enzymes (Figs. 2 and 3), we can conclude that intestinal enzymes favours the liberation of LF from alginate to the medium, since the percentage of release determined by the absorbance of the intestinal digests increases from 49 % without enzymes to 94 % with the action of pancreatin and bile.

3.3. *Growth curve of Listeria monocytogenes*

The growth curve of *L. monocytogenes* was obtained by colony counting and by determining the absorbance at 620 nm. Fig. S1A shows the results obtained after counting the *L. monocytogenes* colonies. The bacterial culture showed exponential growth up to 9 h, increasing the count rapidly from 10^7 to 10^9 cfu/mL. After 12 h of incubation, the count decreased by approximately 1 log unit (u.log) and remained in stationary phase until 24 h.

In the curve obtained by measuring the absorbance (Fig. S1B), it can be observed that, during the first 4 h, the growth was very slow, which could correspond to the latency phase. Subsequently, the bacterium enters the logarithmic or exponential phase, which is represented by a maximum of absorbance at 8 h of incubation and then, falls into a stationary phase until the end of the measurements.

These results confirm the possibility of determining the growth curve for *Listeria* quickly and economically, by making the measurement of absorbance instead of growing the bacteria on agar and counting the colonies.

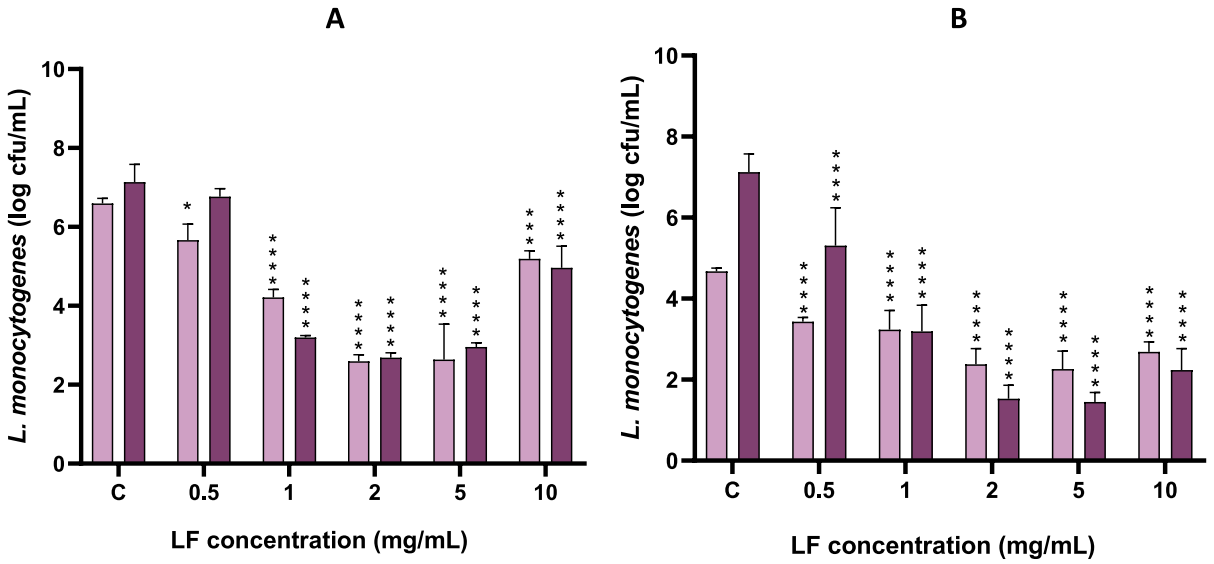

Fig. 5. Antibacterial effect of LF at different concentrations against *L. monocytogenes* at (**A**) exponential phase and (**B**) stationary phase after an incubation of 4 h (□) or 24 h (■). C: control. Values represent the mean ± standard deviation of two replicates in three independent experiments (n = 6). Asterisks indicate significant differences respect to control (*p < 0.05, ***p < 0.001, ****p < 0.0001).

3.4. Antibacterial activity against Listeria monocytogenes

3.4.1. Antibacterial activity of native lactoferrin

In this study, different assays were performed to evaluate the antibacterial activity of native bovine LF against *L. monocytogenes* in exponential (Fig. 5A) and in stationary phases (Fig. 5B). Two incubation times, 4 h and 24 h, and different concentrations of LF (0.5, 1, 2, 5 and 10 mg/mL) were tested. All samples were analyzed in duplicate in three independent assays for each growth phase.

Fig. 5A shows the results obtained when evaluating the antibacterial activity of LF against *L. monocytogenes* at exponential phase. It was observed that the sample with LF at 0.5 mg/mL had a slight activity after 4 h of incubation. However, the rest of LF concentrations showed higher activity, the concentrations of 2 and 5 mg/mL highlighting among others. In both cases, there was a reduction with respect to the control of 4 u.log at 4 and 24 h of incubation.

The results shown in Fig. 5B, concerning to the activity of LF against *L. monocytogenes* in stationary phase, reflect that the antibacterial effect presented by LF at 4 or 24 h of incubation was statistically significant for all concentrations. In both incubation times, the antibacterial effect increased with increasing LF concentration, with the exception of the highest one, 10 mg/mL. The LF concentrations that had the greatest effect were 2 and 5 mg/mL, as in the exponential phase, decreasing in this case the *L. monocytogenes* counts by up to 6 u.log at 24 h. These results are similar to those obtained in the study by Conesa et al. (2010), where the antibacterial activity of bovine LF against *L. monocytogenes* was evaluated by measuring the absorbance of the culture at 620 nm. In that study, the minimum inhibitory concentration and the minimum bactericidal concentration of LF were 2 and 5 mg/mL, respectively. Furthermore, these results show, in general terms, a greater effect of LF against *L. monocytogenes* in the stationary phase compared to the exponential phase. This is also in agreement with the study by Tidona et al. (2011), in which they analyzed the effect of donkey's milk, rich in LF, against *L. monocytogenes* over time. Their results showed an antibacterial effect in both exponential and stationary phases, with the greatest reduction after 8 h of growth, time in which the bacteria entered the stationary phase.

The antibacterial effect of LF is due, on the one hand, to its ability to bind free iron and prevent bacteria from obtaining this element necessary for their survival. On the other hand, it is also due to the direct

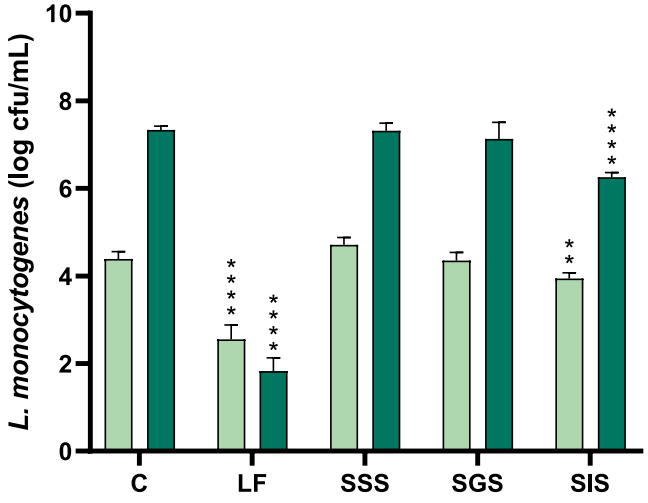

Fig. 6. Antibacterial effect of simulated digestion solutions with digestive enzymes against *L. monocytogenes* (□ 4 h; ■ 24 h). C: control, LF: LF at 2.5 mg/mL, SSS: simulated salivary solution, SGS: simulated gastric solution, SIS: simulated intestinal solution. Values represent the mean ± standard deviation of two replicates in three independent experiments (n = 6). Asterisks indicate significant differences respect to control (**p < 0.01, ****p < 0.0001).

interaction of LF with lipopolysaccharide (component of the outer cell membrane) of Gram-negative bacteria, or lipoteichoic acid (component of the cell wall) of Gram-positive bacteria (Berlutti et al., 2011; Embleton et al., 2013). Therefore, and since the interaction with Gram-negative and Gram-positive bacteria is different, the effect of LF may vary depending on the bacterium. In the case of *C. sakazakii*, a Gram-negative bacterium, the effect of LF against bacterial growth was clearly greater at the exponential phase, although LF at 5 mg/mL also showed activity reducing bacterial growth at stationary phase, decreasing the colonies in around 2 u.log with respect to the control at 4 and 24 h (Abad et al., 2022).

3.4.2. Antibacterial activity of digestion simulated solutions

Prior to the activity assays of the digests, we tested whether the

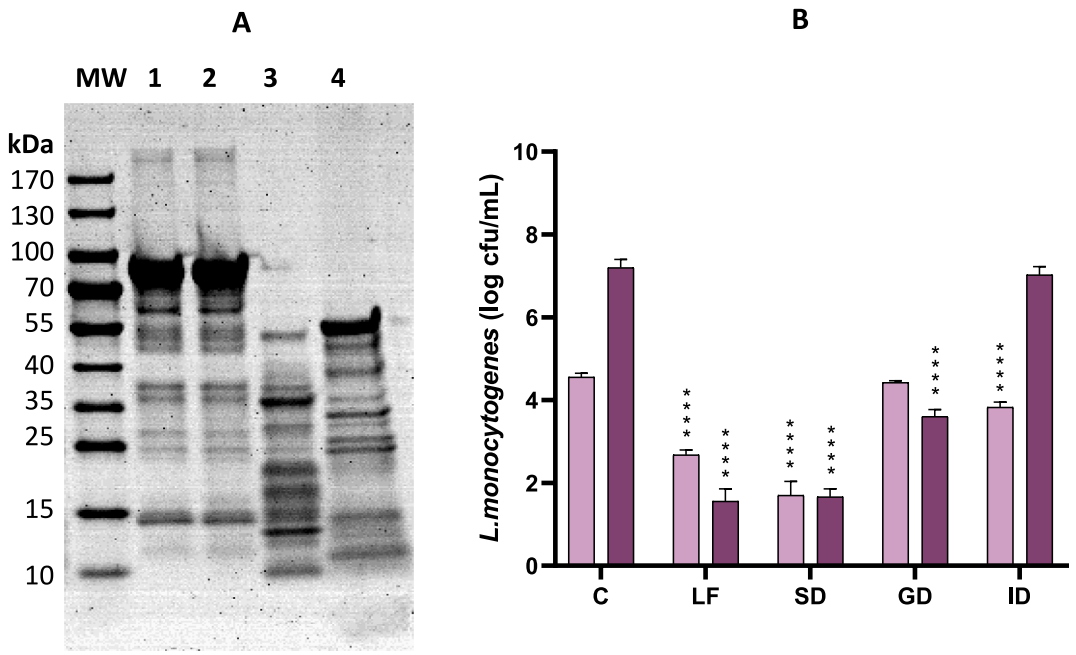

Fig. 7. (**A**) SDS polyacrylamide gel electrophoresis (4–20 %) of LF and its digests stained with Coomassie Blue R. MW: molecular weight marker, 1: native LF, 2: salivary digest of LF, 3: gastric digest of LF, 4: intestinal digest of LF. (**B**) Antibacterial effect of LF and its digests against *L. monocytogenes* (□ 4 h; ■ 24 h). C: control, LF: LF at 2.5 mg/mL, SD: salivary digest, GD: gastric digest, ID: intestinal digest. 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).

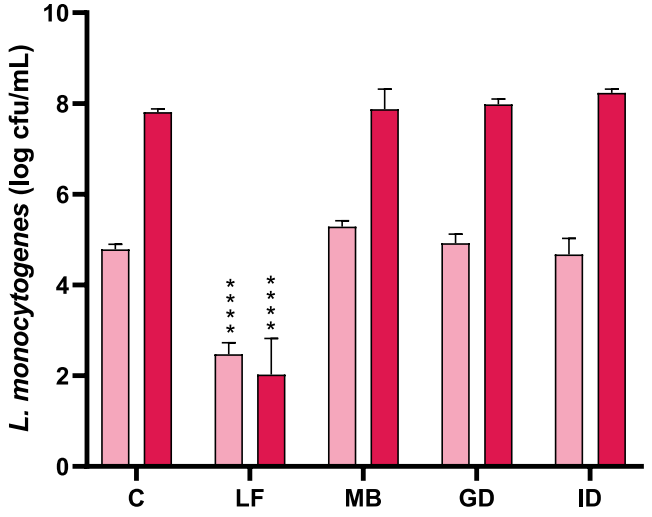

Fig. 8. Antibacterial effect of alginate-LF microbeads and their digests against *L. monocytogenes* (□ 4 h; ■24 h). C: control, LF: LF at 2.5 mg/mL, MB: intact microbeads, GD: gastric digest of microbeads, ID: intestinal digest of microbeads. Values represent the mean ± standard deviation of, at least, two replicates in two independent experiments (n ≥ 4). Asterisks indicate significant differences respect to control (****p < 0.0001).

simulated solutions and enzymes used in each phase of digestion had any effect on the growth of *L. monocytogenes*. Both SSS and SGS with pepsin showed no significant antibacterial effect against this bacterium, while SIS, supplemented with pancreatin and bile, did produce a small significant decrease in the bacterial count (Fig. 6). In the study by Akritidou et al. (2022), it is observed that pancreatin does not affect the growth of *L. monocytogenes* but bile acids can significantly reduce its growth.

3.4.3. Antibacterial activity of lactoferrin and its digests

The results obtained after analyzing the antibacterial effect of LF digests are shown in Fig. 7. Focusing on the electrophoresis (Fig. 7A), it can be observed that in the SD the band corresponding to LF (around 80 kDa) is maintained, in contrast to the GD and ID, where no intact protein residue is appreciated. However, in these two digests, a band of peptides of around 10 kDa can be distinguished in the GD and below 10 kDa in the ID. The digest obtained from the salivary phase is the one that showed the greatest antibacterial effect at 4 and 24 h of incubation (Fig. 7B). The low antibacterial activity observed for GD and ID could be due to the peptides generated, which are especially very evident in the GD. In the study carried out by Ripolles et al. (2015), it was found that native LF presented more activity against *L. monocytogenes* than the peptides derived from the pepsin-hydrolyzed protein, coinciding with our results.

In an experiment carried out in mice, it was observed that the majority of LF peptides are generated in the first hour of digestion. Two hours after ingestion, there were still peptides in the stomach, but no peptides were detected in the intestine of mice (Fan et al., 2019). This could justify the loss of activity in the digests, since LF is quickly digested.

3.4.4. Antibacterial activity of ALG-LF microbeads and its digests

Both the undigested microbeads and their gastric and intestinal digests did not show significant differences compared to the control in their effect against *L. monocytogenes* (Fig. 8). Although undigested microbeads and its GD were expected to have no activity because LF was encapsulated and cannot exert its action, it would have been interesting to obtain antibacterial effect with the ID. However, it has been observed that LF released from the microbeads in the intestine was quickly hydrolyzed (Fig. 4A), without giving time for the intact LF to exert its effect. It has been proven that, in vivo, LF is completely digested in the gastric phase, making it difficult to reach the absorption sites at the intestine (Furlund et al., 2013); therefore, the objective of LF encapsulation would have been to facilitate its arrival or, at least, part of the LF to the intestine. Furthermore, there are many other variants of the process in vivo that are not included in the in vitro process; for example, the presence of intestinal mucus, that could influence to a greater or

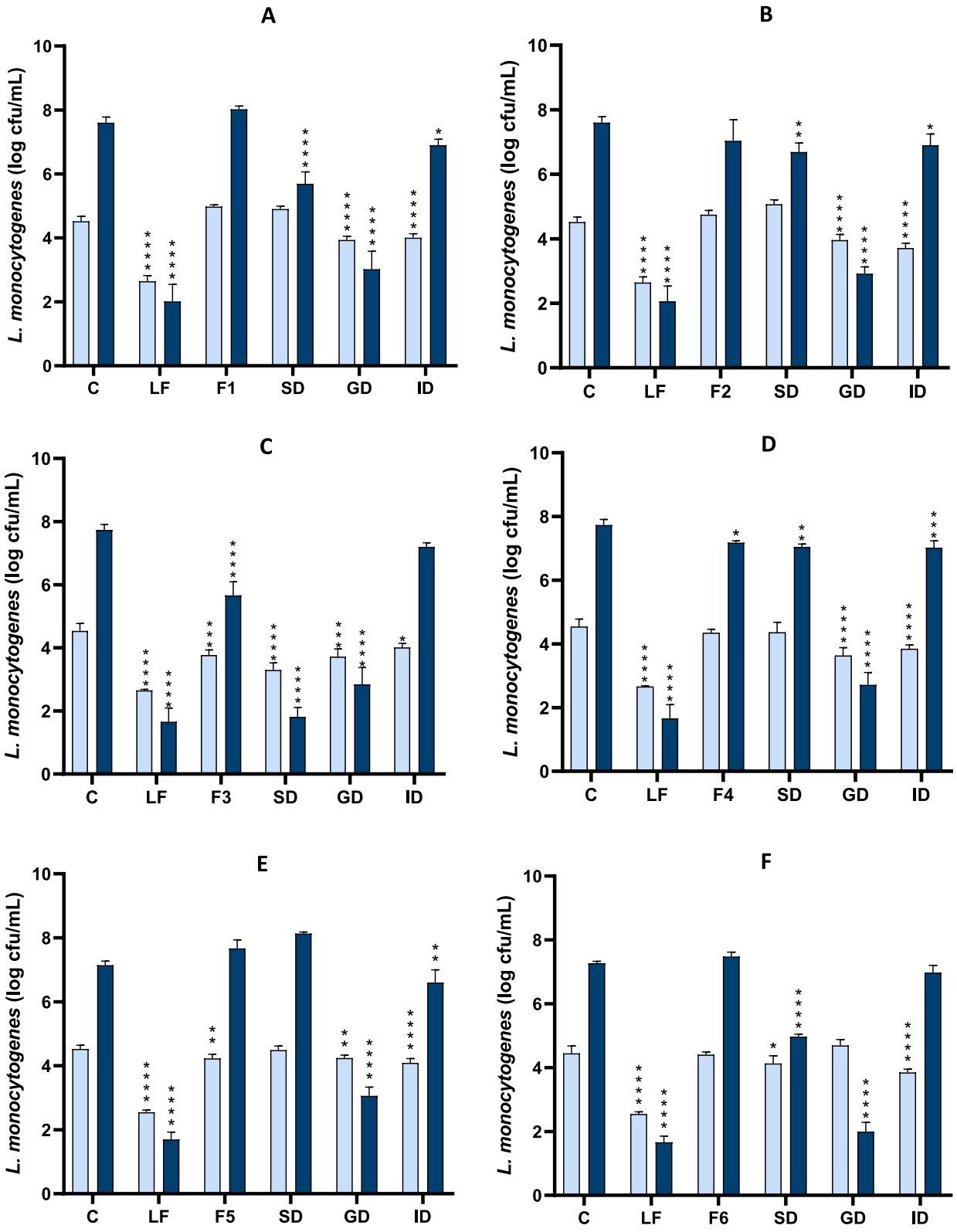

Fig. 9. Antibacterial effect against *L. monocytogenes* of (**A**) F1 and its digests, (**B**) F2 and its digests, (**C**) F3 and its digests, (**D**) F4 and its digests, (**E**) F5 and its digests, and (**F**) F6 and its digests (□ 4 h; ■ 24 h). C: control, LF: LF at 2.5 mg/mL, SD: salivary digest, GD: gastric digest, ID: intestinal digest. Values represent the mean ± standard deviation of two replicates in three independent experiments (n = 6). Asterisks indicate significant differences respect to control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

lesser extent the release of LF from the microbeads. In our case, the ALG-LF microbeads have been shown to be effective in overcoming the gastric stage, keeping the LF intact, but the ALG-LF system might not be optimal to preserve the antibacterial effect of the LF upon its arrival to the adult human intestine.

The study by Raei et al. (2015) demonstrated that LF encapsulated in alginate passed through the stomach in its intact form, and that it was released in the upper intestine. However, they did not analyse either the stability of LF once released or its activity in the intestine.

Recently in 2023, Hedyeloo et al. (2023) analyzed the antibacterial effect of free LF and LF encapsulated in chitosan against *E. coli*. In that study, they found that there were no significant differences between the antibacterial effect of free and encapsulated LF. However, although they evaluated the effect of digestion on the capsules, the antibacterial effect of the digests was not analyzed.

3.4.5. Antibacterial activity of lactoferrin-supplemented dairy formulas and their digests

The antibacterial activity of the dairy formulas (F1-F6) and their digests (DS, DG and DI) against *L. monocytogenes* in stationary growth phase was evaluated. Bovine LF was also included in these assays at a final concentration of 2.5 mg/mL, the same concentration as in the formulas, to have a positive control of antibacterial activity. All samples were tested in duplicate in three independent experiments.

Fig. S2 shows how the digestion process decreases the amount of protein in dairy formulas as the stages progress, finally observing the complete loss of LF (80 kDa), caseins (around 35 kDa), and α-LA and β-LG (around 15 kDa). Unlike the digestion of LF alone, in which after the gastric stage all the protein was digested (Fig. 7A), in the GD of the formulas, some intact LF can be observed (Fig. S2), showing that when ingested together with other dairy components, its degradation decreases. Furthermore, the bands of 50 and 30 kDa, and a band around 10 kDa, corresponding to the peptides generated by the digestion process, appears in the gastric phase. This 10 kDa band is observed very slightly in the ID, where the digestive enzymes continue to exert their activity reducing the size of the peptides, represented in the band that appears below 10 kDa.

The results of the antibacterial activity of the six dairy formulas and their digests against *L. monocytogenes* after 4 and 24 h of incubation are shown in Fig. 9.

The ID of F1, the dairy formula based on whey, showed greater effect than the SD after incubation for 4 h, and the inverse effect was obtained when incubated for 24 h. However, in this formula, the highest activity was observed with the GD, fraction in which part of the intact LF coexists with the peptides generated, decreasing bacterial growth by 1 u.log at 4 h and 5 u.log at 24 h (Fig. 9A). Similarly results were obtained with F2, based on buttermilk. The antibacterial activity of the F2 digests (Fig. 9B) could be explained by its high content of MFGM, since in addition to that supplemented to the formula, it also contains the MFGM naturally present in buttermilk. In the in vitro study conducted by Sprong et al. (2012), it was observed that rats fed with buttermilk powder (rich in MFGM) had increased resistance to *L. monocytogenes* infection. This may be mainly due to the presence of products with antibacterial activity derived from the hydrolysis after digestion of polar lipids (phospholipids and sphingolipids) and MFGM proteins (xannthine osidase, mucin and lactadherin) (Huërou-Luron, Lemaire, & Blat, 2019).

In the case of F3 (Fig. 9C), both the undigested formula and it SD had greater antibacterial effect on *L. monocytogenes* than the rest of the formulas. However, the ID showed less activity. Bacterial growth at 24 h decreased by approximately 2 u.log with respect to the control in the case of the undigested formula. In addition, antibacterial activity increased considerably with the SD and GD, reducing the bacterial count in 6 and 5 u.log at 24 h, respectively. This could be due to homogenization, because the milk fat globules have decreased in size and the MFGM bioactive proteins with antibacterial activity, such as lactadherin or mucin, may have become more available (Tunick et al., 2016). On the other hand, with respect to the GD, the results were very similar to those of the formula without treatment (F1), this digest being potentially inhibitory of bacterial growth. Therefore, it can be concluded that F3 had more effect against *L. monocytogenes* than F1, homogenization being the treatment more favourable in this case.

Fig. 9D shows the results obtained after analysis of the antibacterial activity of F4 and its digests. In this case, the undigested formula showed a slight antibacterial effect after 24 h of incubation. SD had no effect after 4 h, although it did significantly decrease the amount of bacteria after 24 h. Bacterial growth decreased during the first 4 h of incubation by approximately 1 u.log relative to the control with the GD and ID treatment. At 24 h, the antibacterial activity of the GD was more accentuated, decreasing 5 u.log of *L. monocytogenes* with respecto to the control. However, after this time, the ID was not able to reduce the growth of the bacteria. It should be noted that, possibly, in the ID of the formulas, the peptides with potential bioactivity have been completely digested and the observed effect can be due to the SIS that includes pancreatin and bile, as shown in Fig. 6. When comparing the antibacterial effect of F3 and F4, both containing homogenized MFGM but with different milk base (whey for F3 and buttermilk for F4), we did not find similar results. The undigested F4 and its SD were not as effective as in the case of F3. However, the GD of both F3 and F4 did show similar effect against *L. monocytogenes*.

Formulas F5 and F6 were subjected to a pasteurization treatment of 72 °C/20 s. As can be seen in Fig. 9E, the SD of F5 did not show antibacterial activity, while that of F6 (Fig. 9F) did show significant differences with respect to the control, at 4 and 24 h of incubation. Digests from the gastric phase showed antibacterial activity with significant differences at 24 h in both dairy formulas, and at 4 h only in F5. The antibacterial effect of the ID of the two formulas, possibly caused by digestive enzymes, was maintained at 4 h and disappeared at 24 h of incubation in F6. Heat treatment could have a beneficial effect on F6 compared with F5, possibly because it causes an alteration of the MFGM, favouring its interaction with whey proteins (Lee & Sherbon, 2002) in F5, decreasing its bioavailability to act against the bacteria.

Heat treatments are commonly used to preserve foods. However, they can affect their nutritional value and sensory properties, and especially, they can alter the structure and biological function of proteins. Thus, heat treatment, depending on its intensity, can affect integrity of LF present in milk, with a possible impact on its biological properties (Franco et al., 2018). Despite this, in the study by Conesa et al. (2010) it was shown that, pasteurization commonly used in milk treatments did not affect the antibacterial activity of bovine LF against *Escherichia coli* O157:H7, *Salmonella enterica serovar Enteriditis* and *L. monocytogenes*.

The same was observed in our previous study, in which heat treatment showed positive effects on the antibacterial activity of dairy formulas against *C. sakazakii* (Abad et al., 2022b). The results of these studies coincide with those presented in this work, since a positive effect of pasteurization on the antibacterial activity of F6 compared to F2 and F4 was observed, especially in the SD and GD at 24 h, where bioactive peptides responsible for this effect could have been released.

In dairy formulas, the added LF is possibly interacting with the rest of the components, which could decrease its availability and activity, explaining the low effect of undigested formulas compared to free native LF (Fig. 9A-F). In skim milk, the addition of LF causes a decrease in turbidity, due to the binding of LF to casein micelles. This efect varies with time, pH and temperature (Anema & De Kruif, 2011). However, homogenization modifies or alters these interactions and may leave the LF free, since this treatment causes the adsorption of caseins to the MFGM (Lee & Sherbon, 2002), preventing LF-casein binding. This effect could justify the higher activity in undigested F3 and its SD compared to F1 and F5, which have the same composition but have not undergone this treatment and, as a consequence, LF is less available. The antibacterial effect of SD of F3 could also be due to the high salt content of the solution used. In particular, it has been observed that bicarbonate

Table 1
Evaluation of the antimicrobial activity of LF, dairy formulas (F1-F6) and their respective digests against *L. monocytogenes*, expressing growth as a percentage with respect to the control (100%). LF: LF at 2.5 mg/mL, SD: salivary digest, GD: gastric digest, ID: intestinal digest.

	4 h				24 h			
	LF	**SD**	**GD**	**ID**	**LF**	**SD**	**GD**	**ID**
Native LF	58.79	37.29	97.19	84.12	21.68	23.07	50.04	97.59
	Formula	**SD**	**GD**	**ID**	**Formula**	**SD**	**GD**	**ID**
F1	110.07	108.49	87.02	88.61	104.87	74.43	39.47	90.25
F2	104.96	112.11	87.63	82.16	92.56	88.00	38.38	90.80
F3	83.19	72.70	82.13	88.52	73.26	23.43	36.68	93.13
F4	95.81	96.04	80.00	84.57	92.89	91.18	35.13	90.87
F5	93.48	99.28	93.85	90.45	107.38	113.87	42.88	92.42
F6	99.01	92.83	105.54	86.52	102.84	68.30	27.50	95.96

favours the binding of free iron to LF, which could explain the bacteriostatic effect of this digest (Sánchez, Calvo, & Brock, 1992). On the other hand, and as it has been previously mentioned, it has been observed that the interaction of LF with the proteins of the formulas protects it from digestion to a certain extent, with part of the protein appearing intact in the DG of the formulas (Fig. S2), unlike the GD of free LF, in which the protein was completely digested (Fig. 7). The same protective effect was detailed in the study by Kuwata et al. (1998), in which when milk enriched with LF was administered to mice, active fragments appeared in the faeces, suggesting that LF had survived to the transit through the gastrointestinal tract.

Gastric digests were the ones that presented the greatest antibacterial activity, reducing the growth of the bacteria by up to 30 % with respect to the control at 24 h. This significant antibacterial activity may be due to intact LF and the peptides generated during the digestion process, such as lactoferricin (Bellamy et al., 1992) and lactoferrampin (Van der Kraan et al., 2004). Very similar results were obtained in the study by Tidona et al. (2011), in which donkey milk digests obtained at pH 2 and 4, corresponding to the gastric phase, had a greater effect against *L. monocytogenes* than undigested milk. The high LF and lysozyme content of donkey milk could be responsible for this antibacterial activity.

As a summary, Table 1 shows the percentages of *L. monocytogenes* growth for the different samples with respect to the control, in the antibacterial activity assays of LF and dairy formulas after the stages of in vitro gastrointestinal digestion. It can be observed that LF was losing antibacterial activity throughout the digestion process. This is due to the proteolysis of LF that occurs during the gastric phase, and to the even greater hydrolysis performed by proteases (trypsin and chymotrypsin) present in pancreatin (Goulding et al., 2021).

3.5. Fractionation of peptides from gastric digest

In the proteomic analysis performed on the fraction smaller than 30 kDa of the GD of LF, a peptide with sequence LSKAQEKFGKNKSRSFQL, corresponding to an isoform of lactoferrampin, was detected. This peptide stands out for its antimicrobial activity also described by Quintieri et al. (2020). The identification of this peptide in the GDs of F1 and F2 was not possible due to the complexity of the sample. However, sequences derived from caseins were identified, such as the peptide LRLKKKYKVPQL from α-s1-casein and AMKPWIQPKTKKVIPYVR from α-s2-casein, both with recorded antibacterial activity (McCann et al., 2006).

The fraction smaller than 30 kDa from the GD was fractionated to separate peptides larger and smaller than 3 kDa. These two new fractions were subjected to electrophoresis (Fig. S3). When analyzing the fraction with peptides larger than 3 kDa, a band around 10 kDa was observed, corresponding to the peptides generated at this stage of digestion. The possible peptides smaller than 3 kDa, having such a small size, could not be observed in the electrophoresis gel.

4. Conclusions

Lactoferrin is a milk protein that shows antibacterial effect against *L. monocytogenes*, especially at concentrations of 2 and 5 mg/mL, both in the exponential phase and in the stationary phase of growth, being greater in the latter. It has been demonstrated that free LF is more active in its native form, losing part of its effect when digested in the gastrointestinal tract. The encapsulation of LF in alginate microbeads would allow its delivery in the intestine, protecting it from the action of pepsin in the stomach. However, this encapsulation system would not be the most appropriate to preserve the antibacterial activity of LF, since when it is released in the intestine is quickly digested.

In addition, LF can be added to dairy formulas favouring, together with other proteins, its antibacterial effect after gastrointestinal digestion, which causes the release of bioactive peptides. Technological treatments, such as homogenization and pasteurization, and in vitro digestion do not negatively alter the antibacterial activity of dairy formulas against *L. monocytogenes*, maintaining or enhancing it. Some digests of dairy formulas supplemented with LF present greater antibacterial effect than LF digests, which suggests that using LF as a supplement in dairy matrices is more effective than administering free LF. These dairy matrices exert an “encapsulation” effect, protecting LF in the gastrointestinal tract and providing other bioactive proteins and peptides. Therefore, LF supplementation in dairy matrices is more effective than alginate encapsulation in maintaining LF activity. In any case, it would be interesting to continue with these studies to find different ways to preserve LF activity and to focus the antibacterial effect of LF against *L. monocytogenes* in the intestinal section. Otherwise, it is important to know that biological activities of LF are not as potent as suggested, especially when it is found in a complex environment such as the human gastrointestinal tract.

Ethics statement

No animal or human experimentation has been conducted in this manuscript.

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) and of the Proteomics Core Research Facility of Servicios Científico-Técnicos

del CIBA (Zaragoza, Spain).

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-R), the European Social Found (ESF) and the Aragon Regional Government (A20_23R).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2023.105936.

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