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Estudio de la bioactividad de fracciones y proteínas lácteas para su aplicación como ingredientes funcionales: efecto de los tratamientos tecnológicos

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La presente Tesis Doctoral está constituida por un compendio de trabajos de investigación previamente publicados en diferentes revistas científicas internacionales. A continuación se enumeran las referencias bibliográficas de estos artículos:

1. Protective effect of bovine lactoferrin against *Cronobacter sakazakii* in human intestinal Caco-2/TC7 cells. Inés Abad, Andrea Sangüesa, María Ubieto, Juan José Carramiñana, María Dolores Pérez, Berta Buey, José Emilio Mesonero, Laura Grasa, y Lourdes Sánchez. *International Dairy Journal*, **2022**, 133, 105428. DOI: 10.1016/j.idairyj.2022.105428. FI: 3,1 - *Food Science & Technology* (JCR). Autora principal del trabajo de investigación.
2. Dairy by-products and lactoferrin exert antioxidant and antigenotoxic activity on intestinal and hepatic cells. Inés Abad, Julien Vignard, Catherine Bouchenot, Dimitra Graikini, Laura Grasa, María Dolores Pérez, Gladys Mirey, y Lourdes Sánchez. *Foods*, **2023**, 12(10), 2073. DOI: 10.3390/foods12102073. FI: 5,2 - *Food Science & Technology* (JCR). Autora principal del trabajo de investigación.
3. Development of encapsulation strategies and composite edible films to maintain lactoferrin bioactivity: A review. Inés Abad, Celia Conesa, y Lourdes Sánchez. *Materials*, **2021**, 14(23), 7358. DOI: 10.3390/ma14237358. FI: 3,748 - *Chemistry, Physical* (JCR). Autora principal del trabajo de investigación.
4. Effect of *in vitro* gastrointestinal digestion on the antibacterial activity of bioactive dairy formulas supplemented with lactoferrin against *Cronobacter sakazakii*. Inés Abad, Laura Serrano, Dimitra Graikini, María Dolores Pérez, Laura Grasa, y Lourdes Sánchez. *BioMetals*, **2022**, 36(3), 667-681. DOI: 10.1007/s10534-022-00459-5. FI: 3,5 - *Biochemistry & Molecular Biology* (JCR). Autora principal del trabajo de investigación.
5. Does lactoferrin, free, encapsulated or in dairy matrices, maintain its antibacterial activity after *in vitro* digestion? Inés Abad, Laura Pemán, María Dolores Pérez, Laura Grasa, y Lourdes Sánchez. *Journal of Functional Foods*, **2024**, 112, 105936. DOI: 10.1016/j.jff.2023.105936. FI: 5,6 - *Food Science & Technology* (JCR). Autora principal del trabajo de investigación.
6. Gastrointestinal digestion and technological treatments modify the antibacterial activity of lactoferrin supplemented dairy matrices against *Staphylococcus aureus*. Inés Abad, Aroa Bailac, María Dolores Pérez, Juan José Carramiñana, Miguel Calvo, y Lourdes Sánchez. *International Dairy Journal*, **2024**, 105899. DOI:

10.1016/j.idairyj.2024.105899. FI: 3,1 - *Food Science & Technology* (JCR). Autora principal del trabajo de investigación.

7. Lactoferrin modulates oxidative stress and inflammatory cytokines in a murine model of dysbiosis induced by clindamycin. Inés Abad, Andrea Bellés, Ana Rodríguez-Largo, Ignacio de Blas, Laura Grasa, y Lourdes Sánchez. Pendiente de enviar a la revista *International Journal of Biochemistry and Cell Biology*. FI: 4 - *Biochemistry & Molecular Biology* (JCR). Autora principal del trabajo de investigación.

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RESUMEN/SUMMARY

RESUMEN

La leche es una fuente de proteínas bioactivas, algunas con propiedades defensivas, lo que le otorga un potencial beneficioso para la salud. Entre ellas, encontramos las proteínas del lactosuero, como la lactoferrina, y las proteínas de la membrana del glóbulo graso. La industria láctea genera ciertos subproductos, como el lactosuero y la mazada, que pueden ser de gran interés por su composición y propiedades tecnológicas y funcionales.

Las bacterias patógenas, como *Cronobacter sakazakii*, *Listeria monocytogenes* o *Staphylococcus aureus* suponen una gran preocupación para la salud pública, ya que se han asociado con intoxicaciones de origen alimentario debidas a la ingesta de alimentos contaminados o al manejo o almacenaje inadecuados de alimentos. Entre los alimentos que pueden ser causa de estas intoxicaciones se encuentran los productos lácteos, constituyendo las fórmulas infantiles en polvo un alimento de especial preocupación por ser sus destinatarios una población de mayor riesgo.

El objetivo principal de este estudio ha consistido en la caracterización de la actividad antibacteriana de algunas fracciones y proteínas lácteas, evaluando el efecto de distintos tratamientos tecnológicos en su actividad. Este estudio se ha realizado *in vitro* utilizando la línea celular Caco-2/TC7, que se considera un modelo de epitelio intestinal humano de referencia en la mayoría de los ensayos farmacológicos y adecuado para testar los efectos biológicos de compuestos de actividad desconocida.

Para la obtención y caracterización de las fracciones se partió de leche cruda de vaca suministrada por la empresa Villacorona (El Burgo de Ebro, España). A partir de ella se obtuvo el lactosuero, la mazada y la fracción enriquecida en membrana del glóbulo graso que, junto con la lactoferrina bovina cedida por la compañía Tatua Nutritionals (Morrinsville, Nueva Zelanda), se emplearon para analizar el efecto antioxidante y antigenotóxico sobre la línea celular intestinal humana Caco-2 y la línea epitelial hepática

humana HepG2. Esta parte del estudio se realizó en el Centro de Investigación en Toxicología Alimentaria – Toxalim, de la Universidad de Toulouse - INRAE (Francia), durante una estancia de investigación. Estos resultados se muestran en el artículo 2 del presente compendio, en los que se observó que tanto la lactoferrina como el lactosuero y la mazada ejercen un poder antioxidante y de protección del ADN en las células Caco-2 y HepG2.

La determinación de la actividad antibacteriana de la lactoferrina bovina frente a *C. sakazakii*, *L. monocytogenes* y *S. aureus* se llevó a cabo tanto en fase exponencial como en fase estacionaria y a dos tiempos de incubación con la bacteria (4 y 24 h). Los resultados expuestos en los artículos 1, 5 y 6 muestran que la lactoferrina presenta actividad antibacteriana frente a *C. sakazakii* y *L. monocytogenes*, tanto en fase exponencial como estacionaria, y frente a *S. aureus* en fase estacionaria; siendo especialmente activa en la inhibición del crecimiento de *L. monocytogenes*.

Además, en el artículo 1 de la presente Tesis se analizó el efecto de la lactoferrina en la internalización de *C. sakazakii* por las células Caco-2, así como su efecto en el estrés oxidativo y en la expresión de los receptores tipo Toll causado por dicha bacteria en las células.

Partiendo de los subproductos lácteos obtenidos, lactosuero y mazada, y con la membrana del glóbulo graso y la lactoferrina como suplementos, se elaboraron seis fórmulas lácteas. Dos de ellas sin tratamiento tecnológico, otras dos con un tratamiento de homogeneización de la mazada, y las dos últimas sometidas a un tratamiento térmico de pasteurización. La determinación de la actividad antibacteriana de la lactoferrina bovina y de estas fórmulas lácteas frente a *C. sakazakii*, *L. monocytogenes* y *S. aureus* se llevó a cabo antes y después de una disgestión gastrointestinal *in vitro*. Los resultados expuestos en los artículos 4, 5 y 6 mostraron que los digeridos de lactoferrina generados tras la digestión poseen efecto antibacteriano frente a las tres bacterias estudiadas, siendo *L. monocytogenes* la más sensible. Además, los digeridos de las fórmulas lácteas también presentan actividad antibacteriana frente a estas bacterias, siendo los digeridos gástricos los más efectivos. En este estudio, los tratamientos tecnológicos de homogeneización y pasteurización mantuvieron e incluso mejoraron el efecto antibacteriano de las fórmulas lácteas y sus digeridos.

La encapsulación de compuestos bioactivos permite su protección y liberación controlada. Así, se llevó a cabo la encapsulación de la lactoferrina en microperlas de alginato, las cuales fueron sometidas a las condiciones de pH y a las enzimas específicas del proceso de digestión gastrointestinal. El artículo 3 del presente trabajo recoge una amplia información sobre la encapsulación de la lactoferrina, los métodos empleados en diferentes estudios y el efecto que producen en su actividad. Además, en el artículo 5 se muestra el análisis de la actividad antibacteriana frente a *L. monocytogenes* de la lactoferrina encapsulada en microperlas de alginato tras la digestión gastrointestinal *in vitro*. Los resultados obtenidos indicaron que la encapsulación de la lactoferrina con alginato permite la liberación controlada de la proteína en el intestino, aunque no asegura su actividad frente a la bacteria en dicha localización.

Por último, se evaluó el efecto de la lactoferrina bovina en el estrés oxidativo y la expresión de las citoquinas inflamatorias *in vivo* en un modelo murino de disbiosis intestinal causada por el antibiótico clindamicina. El artículo 7 recoge esta parte del presente estudio, y muestra cómo la lactoferrina protege frente a los efectos negativos de la clindamicina restableciendo los niveles normales de polimorfonucleares, principales células defensivas de epitelio intestinal, revirtiendo la oxidación proteica y disminuyendo la expresión de citoquinas proinflamatorias en el íleon de los ratones.

Así, los resultados obtenidos en esta tesis apoyan el interés en revalorizar y considerar al lactosuero y la mazada como fuentes de componentes bioactivos. Además, pueden ser utilizados en productos funcionales, protegiendo la integridad de proteínas como la lactoferrina en el proceso de digestión gastrointestinal.

SUMMARY

Milk is a source of bioactive proteins, some with defensive properties, which gives it a beneficial potentiall for health. These include whey proteins, such as lactoferrin, and fat globule membrane proteins. The dairy industry generates certain by-products, such as whey and buttermilk, which can be of great interest due to their composition and properties technological and functional.

Pathogenic bacteria like *Cronobacter sakazakii*, *Listeria monocytogenes* and *Staphylococcus aureus* are of great concern to public health, as they have been associated with poisoning of food origin, due to ingestion of contaminated food or improper handling or storage of food. Among the foods that can be the cause of these poisonings are dairy products, with powdered infant formulas being a food of special concern because their recipients are a highest risk population.

The main objective of this study has been to characterize the antibacterial activity of some milk fractions and proteins, evaluating the effect of different technological treatments on their activity. This study has been carried out *in vitro* using the Caco-2/TC7 cell line, which is consider a reference model of human intestinal epithelium in most pharmacological assays and suitable for testing the biological effects of compounds if unknown activity.

To obtain and characterize the fractions, raw cow's milk was supplied by the company Villacorona (El Burgo de Ebro, Spain). Whey, buttermilk and the fat globule membrane-enriched fraction were obtained from this milk, which, together tith bovine lactoferrin provided by the company Tatua Nutritional (Morrinsville, New Zealand), were used to analyze the antioxidant and antigenotoxic effect on the human epithelial intestinal cell line Caco-2 and the human hepatic cell line HepG2. This part of the study was carried out at the Food Toxicology Research Center – Toxalim, Univesity of Toulouse – INRAE

(France), during a research stay. These results are shown in article 2 of the present compendium, where it was observed that both lactoferrin and whey and buttermilk exert an antioxidant and DNA protection activity on Caco-2 and HepG2 cells.

The determination of the antibacterial activity of bovine lactoferrin against *C. sakazakii*, *L. monocytogenes* and *S. aureus* was carried out both in exponential and stationary phase and at two incubation times with the bacterium (4 and 24 h). The results presented in articles 1, 5 and 6 show that lactoferrin shows antibacterial activity against *C. sakazakii* and *L. monocytogenes*, both in exponential and stationary phase, and against *S. aureus* in stationay phase; being especially active in the inhibition of the growth of *L. monocytogenes*.

In addition, the effect of lactoferrin on the internalization of *C. sakazakii* into Caco-2 cells, as well as its effect on the oxidative stress and the imbalance in the expression of Toll-like receptors caused by this bacterium in the cells was analyzed in the article 1 of the present Thesis.

Based on the dairy by-products obtained, whey and buttermilk, and with fat globule membrane and lactoferrin as supplements, six dairy formulas were prepared. Two of them without technological treatment, another two with the homogenization of buttermilk, and the last two subjected to a pasteurization heat treatment. The determination of the antibacterial activity of bovine lactoferrin and of those dairy formulas against *C. sakazakii*, *L. monocytogenes* and *S. aureus* was carried out before and after *in vitro* gastrointestinal digestion. In the results presented in articles 4, 5 and 6, it was observed that the lactoferrin digests generated after digestion had antibacterial effect against the three bacteria, *L. monocytogenes* being the most sensitive to lactoferrin. In addition, digests of dairy formulas also showed antibacterial activity against these bacteria, with gastric digests being the most effective. In this study, the technological treatments of homogenization and pasteurization maintained and even improved the antibacterial effect of the dairy formulas and their digests.

Encapsulation of bioactive compounds allows their protection and controlled release. Thus, the encapsulation of lactoferrin in alginate microbeads was carried out, which were subjected to pH conditions and to the specific enzymes of the gastrointestinal digestion process. Article 3 of the present work contains extensive information on the encapsulation of lactoferrin, the methods used in different studies and their effect on its activity. In

addition, article 5 shows the analysis of the antibacterial activity against *L. monocytogenes* of lactoferrin encapsulated in alginate microbeads after *in vitro* gastrointestinal digestion. It was observed that the encapsulation of lactoferrin with alginate allows the controlled release of the protein in the intestine, although it does not ensure its activity against the bacteria in that location.

Finally, the effect of bovine lactoferrin on oxidative stress and the expression of inflammatory cytokines was evaluated *in vivo* in a murine model of intestinal dysbiosis caused by the antibiotic clindamycin. Article 7 reports this part of the present study, and shows how lactoferrin protects against the negative effects of clindamycin by restoring normal levels of polymorphonuclears, the main defensive cells of the intestinal epithelium, reversing protein oxidation and decreasing the expression of pro-inflammatory cytokines in the ileum of mice.

Therefore, the results obtained in this Thesis support the interest in revaluing and considering whey and buttermilk as sources of bioactive components. Furthermore, they can be used in functional products, protecting the integrity of proteins such as lactoferrin during the gastrointestinal digestion.

ABREVIATURAS

ABREVIATURAS

α-LA/ α-La: alfa-lactalbúmina (en inglés, *alpha-lactalbumin*)

β-LG/ β-Lg: beta-lactoglobulina (en inglés, *beta-lactoglobulin*)

4-HDA: 4-hidroxialqueno (del inglés, *4-hydroxyalkenals*)

A

ACN: alfa-caseína (en inglés, *alpha-casein*)

ADPH: adipofilina (del inglés, *adipophilin*)

ALG/ Alg: alginato (en inglés, *alginate*)

AMPs: péptidos antimicrobianos (del inglés, *antimicrobial peptides*)

ANOVA: análisis de varianza (del inglés, *analysis of variance*)

ANS: ácido 8-anilinonaftaleno-1-sulfónico (del inglés, *8-anilinonaphthalene-1-sulfonic acid*)

APCs: células presentadoras de antígenos (del inglés, *antigen-presenting cells*)

ATCC: Colección Americana de Cultivos Tipo (del inglés, *American Type Culture Collection*)

B

BCA: ácido bicinconínico (del inglés, *bicinchoninic acid*)

BCN: beta-caseína (en inglés, *beta-casein*)

BM: mazada (del inglés, *buttermilk*)

BNC: nanocelulosa bacteriana (del inglés, *bacterial nanocellulose*)

BSA: albúmina sérica bovina (del inglés, *bovine serum albumin*)

BTN: butirofilina (en inglés, *butirophilin*)

C

CECT: Colección Española de Cultivos Tipo (en inglés, *Spanish Type Culture Collection*)

ChS: sulfato de condroitina (del inglés, *chondroitin sulphate*)

Clin: clindamicina (en inglés *clindamycin*)

Ct: umbral de ciclos (del inglés, *threshold cycles*)

D

DMEM: medio de Eagle modificado de Dulbecco (del inglés, *Dulbecco's Modified Eagle's Medium*)

DNPH: 2,4-dinitrofenilhidrazina (del inglés, *2,4-dinitrophenylhydrazine*)

E

EDC: 1-etil-3- (3-dimetilaminopropil) clorhidrato de carbodiimida (del inglés, *1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride*)

EDTA: ácido etilendiaminotetraacétido **(**del inglés**,** *ethylenediaminetetraacetic acid*)

EE: eficiencia de encapsulación (en inglés, *encapsulation efficiency*)

EGF: factor de crecimiento epidérmico (del inglés, *epidermal growth factor*)

F

FBS: suero fetal bovino (del inglés, *fetal bovine serum*)

G

GA: goma arábiga (en inglés, *gum arabic*)

GD: digerido gástrico (del inglés, *gastric digest*)

GF: fracción gástrica (del inglés, *gastric fraction*)

GIT: gastrointestinal (en inglés, *gastrointestinal*)

GMP: glicomacropéptido (en inglés, *glicomacropeptide*)

H

HIPEs: emulsiones de elevada fase interna (del inglés, *high internal phase emulsions*)

HM: altamente metilado (del inglés, *highly methylathed*)

HTST: alta temperatura, tiempo corto (del inglés, *high temperature short time*)

I

IBD: enfermedades inflamatorias intestinales (del inglés *inflammatory bowel diseases*)

ID: digerido intestinal (del inglés, *intestinal digest*)

IF: fracción intestinal (del inglés, *intestinal fraction*)

IFN- α: interferón alfa (en inglés, *alpha interferon*)

IFN- β: interferón beta (en inglés, *beta interferon*)

Igs: inmunoglobulinas (en inglés, *immunoglobulins*)

IP: intraperitoneal (en inglés *intraperitoneal*)

K

KCN: kappa-caseína (en inglés, *kappa-casein*)

L

LBP: baja unión a proteínas (del inglés, *low binding protein*)

LC: capacidad de carga (del inglés, *loading capacity*)

LD: lactadherina (en inglés, *lactadherin*)

LF: lactoferrina (en inglés, *lactoferrin*)

LM: de baja metilación (del inglés, *low methylated*)

LP: lactoperoxidasa (en inglés, *lactoperoxidase*)

LPS: lipopolisacárido (en inglés, *lipopolysaccharide*)

LYS: lisozima (del inglés, *lysozyme*)

M

MDA: malondialdehído (en inglés, *malondialdehyde*)

MEM: medio esencial mínimo de Eagle (del inglés, *Eagle's Minimum Essential Medium*)

MFGM: membrana del glóbulo graso de la leche (del inglés, *milk fat globule membrane*)

MIC: concentración mínima inhibitoria (del inglés, *minimum inhibitory concentration*)

MRSA: *S. aureus* resistente a meticilina (del inglés, *methicillin-resistant S. aureus*)

MT: millones de toneladas (en inglés, *million tones*)

MTS: 3- (4, 5- dimetil tiazol- 2- yl)- 5- (3- carboxi metoxi fenil)- 2- (4-sulfenil)- 2H tetrazolio (del inglés, *3- (4, 5- dimethyl thiazol- 2- yl)- 5- (3- carboxy methoxy phenyl)- 2- (4-sulfophenyl)- 2H tetrazolium*)

MUC: mucina (en inglés, *mucin*)

N

NAC: N-acetilcisteína (en inglés, *N-acetylcysteine*)

NaCas: caseinato de sodio (en inglés, *sodium caseinate*)

NEAA: aminoácidos no esenciales (del inglés, *non-essential amino acids*)

NES: enzima melladora en *S. aureus* (del inglés, *nicking enzyme in S. aureus*)

nLF: lactoferrina nativa (del inglés, *native lactoferrin*)

NOD: dominio de unión a nucléotidos y oligomerización (del inglés *nucleotide-binding and oligomerization domain*)

P

PAMPs: patrones moleculares asociados a patógenos (del inglés, *pathogen-associated molecular patterns*)

PBS: solución salina tamponada con fosfato (del inglés, *phosphate-buffered saline*)

PC: fosfatidilcolina (del inglés, *phosphatidylcholine*)

PE: fosfatidiletanolamina (del inglés, *phosphatidylethanolamine*)

PFA: paraformaldehído (en inglés, *paraformaldehyde*)

pI: punto isoeléctrico (en inglés, *isoelectric point*)

PMN: polimorfonucleares (en inglés, *polymorphonuclear*)

PMSF: fluoruro de fenilmetilsulfonilo (del inglés, *phenylmethylsulfonyl fluoride*)

PPI: aislado de proteína de guisante (del inglés, *pea protein isolate*)

R

ROS: especies reactivas de oxigeno (del inglés, reactive oxygen species)

S

SD: digerido salivar (del inglés, *salivary digest*)

SDS: dodecilsulfato de sodio (del inglés, *sodium dodecyl sulphate*)

SDS-PAGE: electroforesis en gel de poliacrilamida y dodecilsulfato de sodio (del inglés, *sodium dodecyl sulphate polyacrylamide gel electrophoresis*)

SGS: solución gástrica simulada (en inglés, *simulated gastric solution*)

SIS: solución intestinal simulada (en inglés, *simulated intestinal solution*)

sLF: lactoferrina saturada en hierro (del inglés, *saturated lactoferrin*)

SM: esfingomielina (del inglés, *sphingomyelin*)

SPI: aislado de proteína de soja (del inglés, *soy protein isolate*)

SRM: monitoreo de reacciones seleccionadas (del inglés, *selected reaction monitoring*)

SSS: solución salivar simulada (en inglés, *simulated salivary solution*)

T

TA: ácido tánico (del inglés, *tannic acid*)

TAME: p-tolueno-sulfonil-l-arginina-metil-ester (del inglés, *p-toluene-sulfonyl-l-arginine-methyl-ester*)

TG: triglicéridos (en inglés, *triglycerides*)

TGA: transglutaminasa (en inglés, *transglutaminase*)

TGI: tracto gastrointestinal (en inglés, *gastrointestinal tract*)

TLRs: receptores tipo Toll (del inglés, *Toll-like receptors*)

TNBS: ácido trinitrobenceno-sulfónico (del inglés, *2,4,6-trinitrobencenesulfonic acid*)

TPS: almidón termopláctico (del inglés, *thermoplastic starch*)

TSA: agar de tripticasa de soja (del inglés, *trypticase soy agar*)

TSB: caldo de tripticasa de soja (del inglés, *trypticase soy broth*)

U

UHT: tratamiento a altas temperaturas (del inglés, *ultra-high-temperature*)

u.log: unidades logarítmicas (en inglés, *logarithmic units*)

W

WBM: mazada lavada (del inglés, *washed buttermilk*)

WPI: aislado de proteína de suero (del inglés, *whey protein isolate*)

X

XO: xantina oxidorreductasa (en inglés, *xanthine oxidoreductase*)

Y

YE: extracto de levadura (del inglés, *yeast extract*)

Z

ZO-1: zónula occludens-1 (en inglés, *zonula occludens-1*)

JUSTIFICACIÓN Y OBJETIVOS

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1. Introducción y justificación del tema

Los alimentos, además de tener una función nutritiva, contienen componentes con efectos beneficiosos para la salud. De entre ellos, las proteínas y péptidos, y en particular los procedentes de la leche, tienen un gran interés por las funciones biológicas identificadas para ellos. Estas funciones tienen una gran relevancia en el desarrollo del recién nacido, por lo que los componentes que las poseen pueden ser utilizados en fórmulas infantiles y también en productos para personas con necesidades especiales.

Entre los componentes bioactivos de la leche existe un grupo de factores protectores frente a agentes infecciosos. De hecho, los recién nacidos alimentados con lactancia natural tienen menor incidencia de infecciones gastrointestinales que los alimentados con leches de fórmula (Gopal y Gill, 2000). Aunque se han estudiado algunas proteínas lácteas con actividad antibacteriana como las inmunoglobulinas y la lactoferrina, todavía quedan aspectos por conocer de proteínas minoritarias con dicha actividad, como las que se encuentran en la membrana del glóbulo graso.

La lactoferrina es una glicoproteína fijadora de hierro, presente en la leche de numerosas especies de mamíferos, incluyendo el ser humano (García-Montoya et al., 2012). La lactoferrina posee numerosas propiedades (García-Montoya et al., 2012), entre las que destaca su actividad antibacteriana frente a patógenos Gram-positivos y Gram-negativos. Esta actividad puede ser bacteriostática, por su capacidad para quelar el hierro, o bactericida mediante su interacción con la pared bacteriana provocando su desestabilización (González-Chávez, Arévalo-Gallegos, y Rascón-Cruz, 2009). La concentración de lactoferrina es mayor en el calostro, siendo en la leche bovina definitiva de 0,1 g/L, mientras que en la leche humana es de 2 g/L (Sánchez, Calvo, y Brock, 1992). En los años 90, la lactoferrina bovina se comenzó a añadir como suplemento en fórmulas

infantiles, yogures y bebidas en Japón. Sin embargo, en Europa la lactoferrina no fue autorizada como ingrediente alimentario hasta 2012 (Comisión Europea, 2012).

La membrana del glóbulo graso de la leche tiene como función recubrir las microgotas lipídicas de la leche, impidiendo su coalescencia y la degradación de la grasa por lipasas (Dewettinck et al., 2008). Las proteínas presentes en esta membrana se han relacionado con interesantes propiedades biológicas y tecnológicas. Dependiendo de su localización, la forma de obtener estas proteínas es más o menos compleja y su presencia en el lactosuero y en la mazada o suero de mantequilla, más o menos abundante (Hvarregaard et al., 1996). Entre estas proteínas, se encuentra la lactadherina, que es una glicoproteína débilmente unida a la membrana del glóbulo graso, lo que permite su extracción fácilmente (Hvarregaard et al., 1996). Entre las funciones biológicas atribuidas a la lactadherina destaca su actividad neutralizante frente a los rotavirus, uno de los principales agentes causantes de diarreas en niños (Inagaki et al., 2010).

Por todo lo expuesto, es evidente que la leche es una interesante fuente de proteínas bioactivas. La industria láctea genera un gran volumen de subproductos que suponen un problema medioambiental y cuyo principal destino es la obtención de ingredientes tecnológicos o la alimentación animal. La obtención de componentes bioactivos de estos subproductos permitiría revalorizarlos. Así, el lactosuero obtenido en la fabricación de queso, contiene más del 50% de los sólidos de la leche, e incluye lactosa, proteínas del lactosuero, minerales y vitaminas (Pires et al., 2021). Además, el lactosuero procedente de la coagulación enzimática de la leche, contiene también el caseinmacropéptido. Este se produce cuando el enzima coagulante actúa sobre la caseína kappa, liberando la parte externa de dicha caseína, que presenta carácter hidrofílico, y que corresponde con el caseinmacropéptido (Corredig y Salvatore, 2016). Un elevado porcentaje de las moléculas del caseinmacropéptido están glicosiladas, por lo que genéricamente también recibe el nombre de glicomacropéptido. Parece que estos glicanos tienen relación con las funciones biológicas que se han propuesto para esta molécula, aunque todavía no han sido muy estudiadas. Además de su función como ingrediente alimentario, el lactosuero también presenta propiedades biológicas, como cierta actividad antioxidante (Zulueta et al., 2009), actividad inhibitoria de la adhesión de bacterias a epitelios (Halpin et al., 2010) y actividad antirrotavirus (Bojsen et al., 2007).

Otro subproducto de la industria láctea es el suero de mantequilla o mazada, que contiene lactosa, minerales y proteínas en la misma proporción que la leche desnatada, y un alto

contenido en fragmentos de membrana del glóbulo graso y fosfolípidos (Spitsberg, 2005). Actualmente, la mazada se destina a alimentación animal y como ingrediente en la industria alimentaria. La mazada y sus hidrolizados podrían constituir una fuente importante de antioxidantes naturales (Conway et al., 2014) e inhibidores de la adhesión de patógenos a las células (Spitsberg, 2005).

El sistema digestivo es la primera diana con la que interaccionan los alimentos y el intestino tiene también una proyección hacia otros sistemas, como el sistema nervioso central (eje intestino-cerebro). Además, el intestino es una barrera física y fisiológica de protección y señalización frente a microorganismos y moléculas potencialmente dañinas. La luz intestinal está colonizada por una comunidad microbiana denominada microbiota comensal, que parece influir tanto en el mantenimiento de la homeostasis intestinal y defensa frente a microorganismos patógenos, como en la génesis y/o prevalencia de patologías inflamatorias intestinales (Kamada et al., 2013). La microbiota intestinal activa vías de señalización en las células epiteliales e inmunitarias de la mucosa intestinal. Para ello, determinadas moléculas de los microorganismos y del daño celular interaccionan mediante receptores de reconocimiento de patrones con las células del hospedador. Entre éstos, se encuentran los receptores tipo Toll, capaces de reconocer los patrones moleculares asociados a patógenos e inducir una respuesta, activando vías de señalización intracelular, que estimulan la síntesis de citoquinas proinflamatorias o del interferón alfa o beta (Abreu, 2010). La alteración de la señalización mediada por estos receptores tipo Toll parece influir en las enfermedades inflamatorias intestinales crónicas (Wlodarska, Kostic, y Xavier, 2015; Ramanan y Cadwell, 2016). Además, se ha descrito que la microbiota intestinal contribuye al mantenimiento de la barrera epitelial y de las uniones intercelulares (Ramanan y Cadwell, 2016). Nuestro objetivo es estudiar cómo las fracciones lácteas bioactivas pueden influir en la defensa del intestino frente a ciertos agentes patógenos.

2. Hipótesis y objetivos

Como se ha demostrado en estudios previos, algunas de las proteínas del lactosuero y de la membrana del glóbulo graso de la leche tienen una actividad protectora frente a agentes patógenos, lo que tiene un gran interés para su utilización en productos funcionales. Sin embargo, todavía es necesario conocer más datos científicos de cómo pueden influir estos componentes bioactivos, y los tratamientos tecnológicos a los que se someten, en la respuesta de las células epiteliales del intestino frente a la acción de algunos agentes

patógenos. Este proyecto de tesis pretende obtener estos datos en modelos celulares *in vitro* y también *in vivo*, en un modelo murino afectado por un proceso de desequilibrio de la microbiota intestinal causado por antibióticos.

El objetivo principal de esta Tesis Doctoral ha sido la caracterización de la actividad antibacteriana de algunas fracciones y proteínas lácteas, evaluando el efecto de distintos tratamientos tecnológicos y de la digestión gastrointestinal *in vitro* en su actividad. Asimismo, se ha evaluado el efecto de dichas fracciones y proteínas lácteas en células humanas intestinales y hepáticas y en un modelo murino de disbiosis intestinal causada por antibióticos.

Para conseguir el objetivo principal se han llevado a cabo diferentes objetivos parciales:

1. Obtención y caracterización de las fracciones derivadas de la leche de vaca (lactosuero, mazada y fracción enriquecida en membrana del glóbulo graso).
2. Evaluación de la actividad antioxidante y antigenotóxica de la lactoferrina bovina y las fracciones lácteas en líneas celulares de origen intestinal y hepático.
3. Determinación de la actividad antibacteriana de la lactoferrina bovina y de fórmulas lácteas a base de lactosuero y mazada suplementadas con lactoferrina y membrana del glóbulo graso frente a *Cronobacter sakazakii*, *Listeria monocytogenes* y *Staphylococcus aureus*.
4. Evaluación del efecto de los tratamientos tecnológicos y de la digestión gastrointestinal *in vitro* en la actividad antibacteriana de la lactoferrina bovina y de las fórmulas lácteas a base de lactosuero y mazada suplementadas con lactoferrina y membrana del glóbulo graso frente a *Cronobacter sakazakii*, *Listeria monocytogenes* y *Staphylococcus aureus.*
5. Determinación de la actividad antibacteriana frente a *Listeria monocytogenes* de la lactoferrina encapsulada en microperlas de alginato tras la digestión gastrointestinal *in vitro*.
6. Evaluación del efecto de la lactoferrina bovina en el estrés oxidativo y la expresión de las citoquinas inflamatorias en un modelo murino de disbiosis intestinal causada por clindamicina.

REVISIÓN BIBLIOGRÁFICA

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1. Composición de la leche

La leche es fuente de proteínas bioactivas, algunas de ellas con propiedades defensivas, de gran valor para la protección del recién nacido. El objetivo natural de la leche es satisfacer las necesidades nutricionales y fisiológicas de los neonatos, garantizando así su crecimiento y desarrollo (Svanborg et al., 2015). En cualquier caso, la leche se caracteriza por ser un alimento muy completo, por lo que se considera un alimento básico en la dieta de niños y adultos, ampliando su función más allá de la subsistencia nutricional de los lactantes (Park y Nam, 2015).

El Codex Alimentarius (1999) define le leche como la secreción mamaria producida por animales de abasto en uno o más ordeños, sin ninguna adición ni extracción, destinada al consumo como leche líquida o a un procesamiento posterior. De manera similar, el Reglamento (CE) Nº 853/2004 determina que la leche cruda es la secreción producida por la glándula mamaria de animales de abasto que no haya sido sometida a ningún tratamiento equivalente a un calentamiento a temperatura de 40 °C o mayor (Comisión Europea, 2004).

Los principales componentes de la leche son: agua, proteínas, lípidos, carbohidratos, vitaminas y minerales. La composición de la leche varía entre especies (**Tabla 1**) e incluso en una misma especie en función de la raza, la etapa de lactación, el tipo de alimentación, el clima, etc. (Roy et al., 2020).

El agua es el componente principal de la leche, tratándose del medio en el que se disuelven o se dispersan sus componentes. En ella, los glóbulos grasos se encuentran en emulsión; la lactosa, algunas proteínas y los minerales en solución, y las caseínas en suspensión coloidal (Agudelo Gómez y Bedoya Mejía, 2005).

Tabla 1. Composición de la leche de diferentes especies en g por 100 g de leche. MST: Materia seca total. *Energía expresada en kJ/100 g de leche (Gantner et al., 2015).

Leche	MST	Grasa	Proteína	Lactosa	Cenizas	Energía*
Humana	10-13	2,1-4,0	0,9-1,9	6,3-7,0	0,2-0,3	270-209
Vaca	12-13	3,3-6,4	3,0-4,0	4,4-5,6	0,7-0,8	270-280
Búfala	16-17	5,3-15,0	2,7-4,7	3,2-4,9	0,8-0,9	420-480
Cabra	12-16	3,0-7,2	3,0-5,2	3,2-4,5	0,7-0,9	280-290
Oveja	18-20	4,9-9,0	4,5-7,0	4,1-5,9	0,8-1,0	410-440

1.1. Carbohidratos de la leche

El principal carbohidrato de la leche de los mamíferos es la lactosa, constituyendo alrededor del 99% de los carbohidratos totales. Se trata de un disacárido compuesto por D-glucosa y D-galactosa (**Figura 1**), cuya concentración es inversamente proporcional a las concentraciones de lípidos y proteínas, y varía en función de la especie. El contenido de lactosa en la leche bovina es de aproximadamente un 4,8%, y se distinguen dos isómeros diferentes: la α-lactosa y la β-lactosa. La leche contiene trazas de otros azúcares, incluyendo monosacáridos como la glucosa, fructosa, y galactosa, oligosacáridos neutros o ácidos, y grupos de glicanos unidos a proteínas y lípidos, como la glucosamina (Fox et al., 2015a).

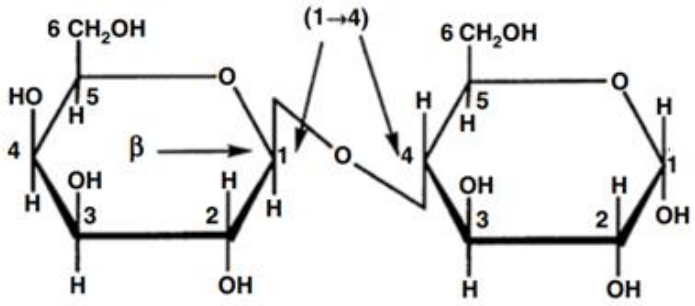

Figura 1. Estructura de la lactosa (Fox *et al.*, 2015a).

1.2. Lípidos de la leche

La grasa de la leche se presenta en forma de glóbulos emulsionados en la fase acuosa, con un diámetro que varía de 0,1 a 10 μm, siendo el tamaño medio de 4 μm. Cuando el tamaño del glóbulo graso es mayor, disminuye el contenido de ácidos grasos de cadena media, mientras que aumenta el número de ácidos grasos de cadena larga con respecto a los glóbulos grasos de menor tamaño (López et al., 2011). Los glóbulos grasos se sintetizan en la glándula mamaria y se liberan a la leche en forma de vesículas, aportando

triglicéridos, nutrientes y moléculas bioactivas al tracto gastrointestinal (TGI) del neonato (López, 2011). Los glóbulos grasos son digeridos por las enzimas lipolíticas presentes en el tracto digestivo, facilitando la lipólisis de los triglicéridos (TG) de la leche y la absorción de los productos de la digestión (Jensen, 1999). Los TG representan el 98% de los lípidos de la leche de rumiantes y humanos, confiriéndole a esta sus propiedades principales como el punto de fusión, la densidad y la hidrofobicidad. Además, los lípidos de la leche proporcionan unas propiedades nutricionales, texturales y organolépticas distintivas a la leche entera y a los productos lácteos como la mantequilla y el queso (MacGibbon y Taylor, 2006). La composición de los lípidos de la leche varía ligeramente entre especies como se muestra en la siguiente tabla (**Tabla 2**).

Tabla 2. Composición de los lípidos en la leche de diferentes especies en porcentaje respecto a la grasa total (Gantner et al., 2015).

Leche	Triglicéridos	Ácidos grasos libres	Fosfolípidos
Humana	97-98	0,7-1,5	0,5-1,5
Vaca	97-98	0,7-1,5	0,5-1,5
Yegüa	80-85	9,5	5-10
Búfala	97-98	0,7-1,5	0,5-1,5
Burra	80-85	9,5	5-10
Cabra	97-98	0,7-1,5	0,5-1,5
Oveja	97-98	0,7-1,5	0,5-1,5

1.2.1. Membrana del glóbulo graso

Los glóbulos grasos están rodeados por una membrana, denominada membrana del glóbulo graso (MFGM, del nombre en inglés *milk fat globule membrane*), que los mantiene en emulsión y los protege de la degradación enzimática y la coalescencia (Manoni et al., 2020). La MFGM posee una estructura en tres capas, cuyos componentes se originan en el retículo endoplásmico y en la membrana plasmática apical de las células epiteliales mamarias. Antes de la secreción, la capa interna de la MFGM, compuesta por proteínas y lípidos polares, cubre los lípidos centrales ricos en TG, acumulados en puntos focales de la membrana del retículo. Por otro lado, la porción de la MFGM que se origina en la membrana plasmática apical tiene una apariencia típica de membrana bicapa, con un material denso en electrones en la cara interna de la membrana (Heid y Keenan, 2005). Esta bicapa externa se compone de enzimas, proteínas y lípidos polares. La MFGM es

particularmente rica en proteínas y fosfolípidos con actividades específicas, lo que le aporta un gran potencial para aplicaciones funcionales y nutracéuticas (Barukčić, Lisak Jakopović, y Božanić, 2019). Estos precursores de los glóbulos, originados en el retículo, se liberan al citosol como gotas pequeñas, de en torno a 0,5 μm de diámetro. Estas gotas de microlípidos se fusionan entre sí, creciendo en volumen. En este proceso de fusión se ven involucrados el calcio y una fracción de proteína de alto peso molecular (Heid y Keenan, 2005).

Las proteínas de la MFGM representan el 25-70% de su masa, y poseen funciones de gran relevancia para la estimulación del crecimiento celular y la protección frente a patógenos intestinales (Riccio, 2004). Estas proteínas se describirán con detalle en la sección 1.3.3.

Los fosfolípidos son los lípidos mayoritarios en la MFGM, principalmente la fosfatidilcolina (PC), la fosfatidiletanolamina (PE) y la esfingomielina (SM), un esfingolípido altamente bioactivo (Douëllou, Montel, y Sergentet, 2017). Se sabe que los lípidos polares no se organizan de manera homogénea en la MFGM, sino que la PE, la fosfatidilserina y el fosfatidilinositol predominan en la capa interna de la MFGM, mientras que la PC y SM se concentran, principalmente, en la bicapa externa del glóbulo. En función del tamaño del glóbulo graso, la composición de la MFGM puede variar. A menor tamaño del glóbulo, menor contenido de PC y SM en la membrana. Esta diferencia podría deberse a que la secreción de pequeños glóbulos podría inducir un cambio en la curvatura de la bicapa, dando lugar a un proceso dinámico con una pérdida de ciertos componentes como la PC y la SM. Esta pérdida también podría deberse a un reordenamiento de la membrana plasmática apical tras la secreción, causando una pérdida de material de la MFGM. La formación de vesículas que se desprenden del glóbulo graso podría llevar a una pérdida de PC y SM (López et al., 2011).

La principal función de la MFGM es actuar como agente emulsionante natural, impidiendo la coalescencia de los glóbulos grasos en la leche y protegiendo a la grasa de la degradación enzimática de las lipasas (López, 2011). Además, se han propuesto para la MFGM numerosos efectos beneficiosos para la salud, como actividad anticancerígena, antimicrobiana, antiinflamatoria y anticolesterolémica (Manoni et al., 2020). En concreto, se ha observado que los oligosacáridos de la leche bovina y los glicoconjugados ligados a la MFGM inhiben la adhesión de los microorganismos vehiculados por los alimentos a las células y tejidos intestinales (Douëllou, Montel, y Sergentet, 2017). Asimismo, se ha

propuesto que las propiedades antiinflamatorias de la leche se pueden atribuir a la presencia de fosfolípidos y ácidos grasos saturados de cadena corta o media (Cichosz, Czeczot, y Bielecka, 2020). Sin embargo, la MFGM puede verse afectada por los tratamientos tecnológicos que se aplican a la leche, tales como el tratamiento térmico, la refrigeración, la liofilización y la homogeneización, haciendo que pierda su actividad o disminuyendo sus efectos beneficiosos (Singh, 2006).

En los últimos años, ha habido un gran interés por el uso de la MFGM como ingrediente adicional en las fórmulas infantiles (Ross et al., 2015), ya que se ha demostrado la capacidad de las glicoproteínas de la MFGM para inhibir la infección por rotavirus, reduciendo la incidencia de diarrea en niños (Da Silva, Colleran, e Ibrahim, 2021).

1.3. Proteínas de la leche

Las proteínas de la leche bovina se pueden clasificar en tres grupos: (1) caseínas, que representan un 80%, (2) proteínas del lactosuero, representando un 20% de las proteínas totales, y (3) proteínas de la membrana del glóbulo graso, en una proporción minoritaria (Hazlett, Schmidmeier, y O'Mahony, 2019).

1.3.1. Caseínas

La principal proteína láctea es la caseína. Esta proteína se encuentra exclusivamente en la leche, y se considera de alto valor biológico por su contenido en aminoácidos esenciales (Fox et al., 2015b). Además, es fuente de calcio, fósforo y vitamina B2 (riboflavina), contribuyendo significativamente a los requerimientos de vitamina A y B1 (tiamina) (Agudelo Gómez y Bedoya Mejía, 2005).

Las caseínas son fosfoproteínas, la mayoría hidrofóbicas, sintetizadas en la glándula mamaria y secretadas en forma de grandes agregados coloidales denominados micelas. Estas micelas están compuestas por un 92% de caseína y un 8% de sales, entre las que destaca el calcio (Ferrandini et al., 2006), comprendiendo el 65% del calcio total de la leche (Haug, Hostmark, y Harstad, 2007). Las micelas de caseína son muy estables, lo que las hace responsables de gran parte de las propiedades físicas de la leche (Ginger y Grigor, 1999); sin embargo, precipitan por acidificación, a un pH alrededor de 4,6 que es su punto isoeléctrico (pI), y por la acción de enzimas proteolíticas (cuajo animal o enzimas coagulantes vegetales o microbianas) (Dalgleish, 2011). El diámetro medio de las micelas es de 160 nm y se componen de cuatro tipos principales de caseínas: αs1-, αs2-, β- y κ-caseína (Lomholt, 1996).

A lo largo de los años se han propuesto distintos modelos que describen la estructura interna de las micelas de caseína. El más antiguo y citado es el modelo submicelar, propuesto por Morr en 1967, que defiende la idea de que las micelas están compuestas por submicelas de caseínas que se unen mediante enlaces hidrofóbicos de fosfato cálcico coloidal (De Kruif et al., 2012). Este modelo se basa en la hipótesis de que hay dos formas de unidades submicelares presentes en una micela de caseína: unas submicelas ricas en κ-caseína y unas submicelas pobres en κ-caseína. Las submicelas ricas en κ-caseína se distribuirían en la superficie, mientras que las pobres en κ-caseína se encontrarían en la estructura interna de la micela (**Figura 2A**) (Horne, 2006). Posteriormente, en 1992, Holt propuso el modelo de nanocluster, en el que describe la micela como un entramado flexible de caseínas α y β fuertemente hidratado y mineralizado. Esta red de caseínas contiene nanoclusters de fosfato cálcico coloidal, que actúan para estabilizar la estructura y constituyen el centro de crecimiento de la micela (**Figura 2B**). Este modelo presenta una distribución más escasa de κ-caseína en la superficie de la micela (Ferrandini et al., 2006).

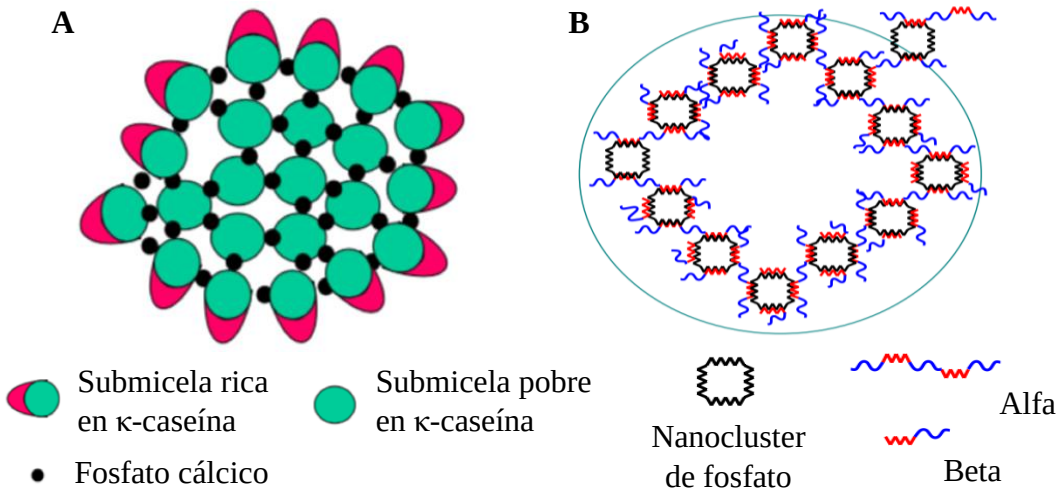

Figura 2. (**A**) Esquema del modelo submicelar de las micelas de caseína propuesto por Morr en 1967, con submicelas ricas y pobres en κ-caseína. (**B**) Esquema del modelo de nanocluster propuesto por Holt en 1992. [Adaptada de Horne (2006)].

La parte hidrofílica C-terminal de la κ-caseína se dispone hacia la superficie, permitiendo la regulación del tamaño micelar, evitando la agregación de submicelas y asegurando el mantenimiento de la suspensión de las micelas en la leche (Ferrandini et al., 2006). Esta propiedad estabilizante se destruye con la acción del cuajo en la coagulación enzimática, momento en el que la quimosina, principal enzima del cuajo, hidroliza la κ-caseína,

liberando su extremo C-terminal, denominado caseinmacropéptido (Corredig y Salvatore, 2016).

Los dos modelos de micela descritos conservan la característica de que el fosfato cálcico coloidal, que se encuentra dentro de la micela de caseína, desempeña un papel esencial en la estabilización de la micela en la leche (Phadungath, 2005).

La principal función biológica de las caseínas en la leche es transportar el calcio y el fósforo (Haug, Hostmark, y Harstad, 2007). Además, las caseínas se han identificado como la principal fuente de péptidos bioactivos tras su hidrólisis, tanto en digestiones *in vitro* como *in vivo*. Una gran mayoría de las proteínas de la leche se absorben en forma de péptidos y no como aminoácidos libres. Tras la acción de las enzimas proteolíticas, los péptidos sufren una digestión adicional mediante las peptidasas (Baró et al., 2001).

Se ha descrito que determinados péptidos derivados de las caseínas muestran un efecto protector en el organismo, potenciando el sistema inmune o ejerciendo una actividad antimicrobiana. Las casomorfinas, de entre 4 y 10 aminoácidos, derivan de la α- y β-caseína e influyen positivamente en el sistema digestivo, presentando un rol terapéutico en el tratamiento de desórdenes gástricos y diarrea. Por otro lado, se liberan péptidos como las casoxinas y lactoferroxinas, que presentan un efecto antagonista frente a la inhibición de la motilidad gástrica inducida por las casomorfinas. Otros péptidos de acción sobre el sistema gastrointestinal son los caseinmacropéptidos (Baró et al., 2001).

1.3.2. Proteínas del lactosuero

Las proteínas del lactosuero tienen un papel importante como aporte de aminoácidos, y pueden tener efectos biológicos y fisiológicos. Entre ellas, encontramos la β-lactoglobulina, la α-lactalbúmina, la albúmina sérica bovina, las inmunoglobulinas, la lactoperoxidasa y la lactoferrina (Madureira et al., 2007).

La β-lactoglobulina (β-LG) es una proteína globular perteneciente a la familia de las lipocalinas. Su estructura cuaternaria depende del pH del medio en el que se encuentra, y se presenta principalmente como un dímero estable al pH de la leche. Se sintetiza en la glándula mamaria y está constituida por 162 residuos de aminoácidos, siendo una fuente rica de cisteína, aminoácido esencial para la síntesis de otros péptidos y proteínas (Madureira et al., 2007). La β-LG es una de las principales proteínas del lactosuero de los rumiantes, representando aproximadamente el 50% de la proteína total del suero de la leche bovina (Tai, Chen, y Chen, 2016). Sin embargo, esta proteína no se encuentra

presente en la leche humana, por lo que es un alérgeno relevante en la infancia (Barbiroli, Iametti, y Bonomi, 2022). Esta proteína presenta una gran afinidad de unión por los ácidos grasos, fosfolípidos y compuestos aromáticos, y participa en el transporte de micronutrientes de baja polaridad y en la regulación del metabolismo del fósforo en la glándula mamaria (Madureira et al., 2007).

La α-lactalbúmina (α-LA) es la segunda proteína más abundante del lactosuero de los rumiantes, representando el 20% del total de sus proteínas. Está constituida por 123 aminoácidos y es rica en triptófano, lisina y cisteína, lo que favorece su papel fundamental en la síntesis de la lactosa en la leche (Kamau et al., 2010). La α-LA se sintetiza en la glándula mamaria y presenta una estructura globular que se estabiliza mediante cuatro puentes disulfuro (Madureira et al., 2007). Debido a su perfil proteico, los péptidos y aminoácidos liberados en la digestión presentan un interesante potencial que convierte a la α-LA en un buen suplemento para promover la salud gastrointestinal influyendo en la motilidad, y aportando péptidos con actividad antimicrobiana (Layman, Lönnerdal, y Fernstrom, 2018). Esta actividad antimicrobiana de los péptidos de la α-LA parece estar asociada con los puentes disulfuro que posee la proteína, ya que se demostró que la digestión de la α-LA con tripsina producía dos pentapéptidos, unidos por un puente disulfuro, que mostraron propiedades antimicrobianas frente a *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermis*, y *Candida albicans*, mientras que los péptidos individuales no mostraron dichas propiedades (Pellegrini et al., 1999). En el estudio realizado por Brück et al. (2003), la fórmula infantil suplementada con α-LA presentó efecto inhibidor sobre la diarrea inducida por *E. coli* en monos Rhesus lactantes.

La albúmina sérica bovina (BSA, del inglés *bovine serum albumin*) no se sintetiza en la glándula mamaria, sino que se encuentra en la leche tras una transferencia pasiva del torrente sanguíneo. Posee 17 puentes disulfuro intermoleculares y un grupo tiol. Esta proteína puede unir ácidos grasos libres y otros lípidos. Una función importante asociada a la BSA es la inhibición del crecimiento tumoral (Madureira et al., 2007).

Las inmunoglobulinas (Igs) forman una parte importante de la actividad inmunológica que posee la leche, y especialmente el calostro, en el que se encuentran en elevados niveles. Estas proteínas también están presentes en el resto de fluidos fisiológicos de los mamíferos, y se sintetizan en respuesta a la estimulación por antígenos específicos. Las Igs pertenecen a una familia de proteínas de alto peso molecular y se dividen en varias

clases: IgG, IgA, IgM, IgE e IgD. Las principales clases de Igs presentes en las secreciones mamarias son las IgG, IgA e IgM, siendo las IgG y las IgA las más abundantes en la leche bovina y humana, respectivamente. Todas las Igs monoméricas tienen la misma estructura molecular básica, y están compuestas por dos cadenas pesadas y dos cadenas ligeras idénticas, con una masa molecular total de aproximadamente 160 kDa (Hurley y Theil, 2011). Su principal función es transferir inmunidad pasiva al recién nacido, protegiéndolo de numerosas enfermedades (Madureira et al., 2007).

La lactoperoxidasa (LP) está presente en varias secreciones de los animales como las lágrimas, saliva y leche. En la leche, esta peroxidasa es una de las enzimas más abundantes, aunque representa solamente alrededor del 1% de las proteínas totales del lactosuero. La LP, en presencia de tiocianato y peróxido de hidrógeno, cataliza la formación de diferentes compuestos con capacidad antibacteriana frente a diversas bacterias Gram-positivas y Gram-negativas (Madureira et al., 2007). Debido a sus propiedades, en algunos países se emplea la activación del sistema LP para la conservación de la leche. Este sistema consiste en la adición de tiocianato de sodio y peróxido de hidrógeno, que reactivan la enzima LP presente en la leche para mantener la calidad de este producto, sin necesidad de refrigeración, hasta su procesamiento o pasteurización. El sistema LP permite reducir las pérdidas de leche por su alteración y aumentan su disponibilidad cuando no es posible su refrigeración por razones técnicas y/o económicas (FAO y OMS, 2005).

La lactoferrina (LF) es una proteína minoritaria del lactosuero perteneciente a la familia de las transferrinas, que participa en diversas funciones fisiológicas y protectoras mediante su actividad antioxidante, antitumoral, antiinflamatoria y antimicrobiana (Sánchez, Calvo, y Brock, 1992). La LF es una glicoproteína monomérica quelante de hierro con un peso molecular de 80 kDa. Está presente en el cuerpo humano como una proteína secretora, sintetizada por las células epiteliales glandulares, encontrándose en la leche, saliva, lágrimas, secreciones nasales e intestinales, jugo pancreático, líquido seminal, etc. (Adlerova, Bartoskova, y Faldyna, 2008). La concentración de LF en la leche varía según las especies y la etapa de lactación, siendo menor en la leche madura que en el calostro y en la leche bovina que en la humana (**Tabla 3**) (Wang et al., 2019).

Tabla 3. Concentración de LF en la leche en función de la especie y la etapa de lactación, expresada en g/L (Wang et al., 2019).

ESPECIE	ETAPA	
	Calostro	Leche madura
Humana	5	2-3
Bovina	0,80	0,03-0,49

La LF está compuesta por una cadena de 703 aminoácidos plegados en dos lóbulos globulares, también conocidos como regiones C- (carboxi) y N- (amino), unidos por una hélice α. Cada lóbulo se compone de dos dominios que albergan un sitio de unión de hierro (**Figura 3**) (Wang et al., 2019). Dependiendo del grado de saturación de hierro de la molécula de LF, se distinguen tres formas: apolactoferrina (libre de hierro), forma monoférrica (un ión férrico) y hololactoferrina (dos iones férricos) (Adlerova, Bartoskova, y Faldyna, 2008). La LF se secreta mayoritariamente como apolactoferrina que es la forma abierta y cuando une dos iones de hierro da lugar a la hololactoferrina que es la forma cerrada (Mayeur et al., 2016). Además, la LF posee en su superficie sitios de glicosilación, variables en número en función de la especie (Adlerova, Bartoskova, y Faldyna, 2008).

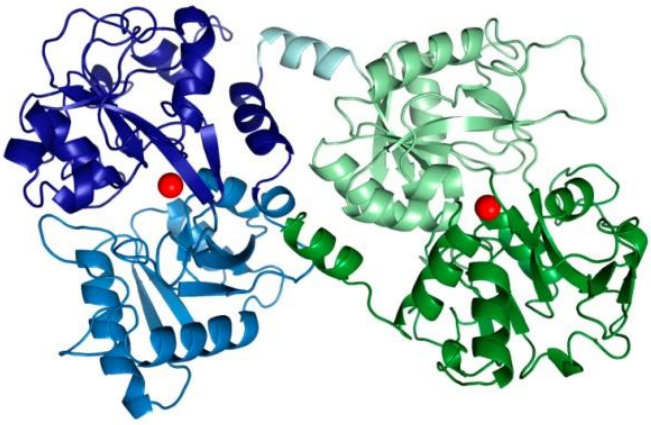

Figura 3. Representación de la estructura de la lactoferrina bovina. El lóbulo N-terminal está a la izquierda, formado por dos dominios N1 y N2. En el lado derecho se representa el lóbulo C-terminal, formado por los dominios C1 y C2. Las esferas rojas representan los átomos de hierro (Superti, 2020).

La LF es fundamental en la dieta del lactante, puesto que desempeña funciones tanto protectoras de infecciones como promotoras de la maduración del sistema inmunológico innato y adaptativo (Superti, 2020). La LF desempeña una actividad antimicrobiana frente

a un amplio espectro de microorganismos, como bacterias, hongos, levaduras y virus. La LF puede ejercer esta actividad mediante un efecto bacteriostático o bactericida. El primer mecanismo se lleva a cabo cuando la molécula de LF se encuentra libre de hierro, presentando una mayor capacidad para el secuestro de este ion del medio y privando al microorganismo de dicho nutriente (Superti, 2020). Sin embargo, cuando la molécula se encuentra en su forma saturada, la LF también puede ejercer su actividad antibacteriana, interaccionando directamente con la membrana de la bacteria. En el caso de las bacterias Gram-negativas, la LF se une y libera lipopolisacáridos (LPS) de la superficie de la membrana, desestabilizándola y alterando consecuentemente su metabolismo y causando un efecto bactericida (Wang et al., 2019). Además, tanto la LF bovina como la humana tienen el mismo sitio de unión para el LPS (Gruden y Poklar Ulrih, 2021). En el caso de las bacterias Gram-positivas, la LF se une a las moléculas aniónicas de la superficie celular, reduciendo la carga negativa y facilitando el efecto de algunos compuestos antibacterianos como los antibióticos (Wang et al., 2019). Otros mecanismos de acción antimicrobiana son la proteólisis de factores de virulencia, la inhibición de la adhesión microbiana a las células huésped mediante la unión con los glicosaminoglicanos de las membranas, así como la estimulación del crecimiento de la microbiota probiótica comensal del intestino (Superti, 2020). Este efecto antimicrobiano fue la primera actividad protectora identificada para la LF, y se ha demostrado ampliamente tanto *in vitro* como *in vivo*.

Además, la LF puede disminuir la producción de especies reactivas de oxígeno (ROS, del inglés *reactive oxygen species*) intracelulares, modular las citoquinas proinflamatorias o suprimir la senescencia de las células inducida por el peróxido de hidrógeno (Kruzel, Zimecki, y Actor, 2017; Park et al., 2017).

Basándose en las diversas funciones de la LF, en particular en las antimicrobianas, antioxidantes y antiinflamatorias, su introducción como un nuevo ingrediente alimentario se aprobó por la Comisión Europea en 2012 (Reglamento (CE) Nº 258/97). Este Reglamento especifica que la LF bovina, como proteína que se encuentra naturalmente en la leche de vaca, puede comercializarse como ingrediente alimentario, estableciendo unos límites para su uso en diferentes categorías de alimentos, como fórmulas infantiles y de continuación, bebidas a base de leche, etc. Para las fórmulas infantiles, el nivel máximo de uso propuesto es de 1 mg/mL, y para bebidas a base de leche, el nivel indicado está entre 200 y 330 mg/100 g de producto, dependiendo de si se presenta en forma líquida

o en polvo (Comisión Europea, 2012). En el estudio realizado por Superti (2020) se observó que la adición de LF a las fórmulas infantiles tenía importancia en la prevención de infecciones en recién nacidos, así como en la reducción de la mortalidad.

1.3.3. Proteínas de la MFGM

Las proteínas de la MFGM representan el 1-2% de la fracción proteica total de la leche. Estas proteínas se dividen en dos grupos: (1) las glicoproteínas, entre las que se encuentran la lactadherina (LD), la mucina (MUC), la butirofilina (BTN) y la xantina oxidorreductasa (XO); y (2) las proteínas no glicosiladas, siendo la adipofilina (ADPH) y la proteína de unión a ácidos grasos las más importantes (**Figura 4**). Estas proteínas varían durante la etapa de lactación, y desempeñan funciones importantes para la actividad celular, como la promoción del crecimiento celular y los mecanismos defensivos (Riccio, 2004). Los compuestos glicosilados de la MFGM, así como los productos obtenidos en su hidrólisis, han mostrado actividad antibacteriana y antiviral (Dewettinck et al., 2008).

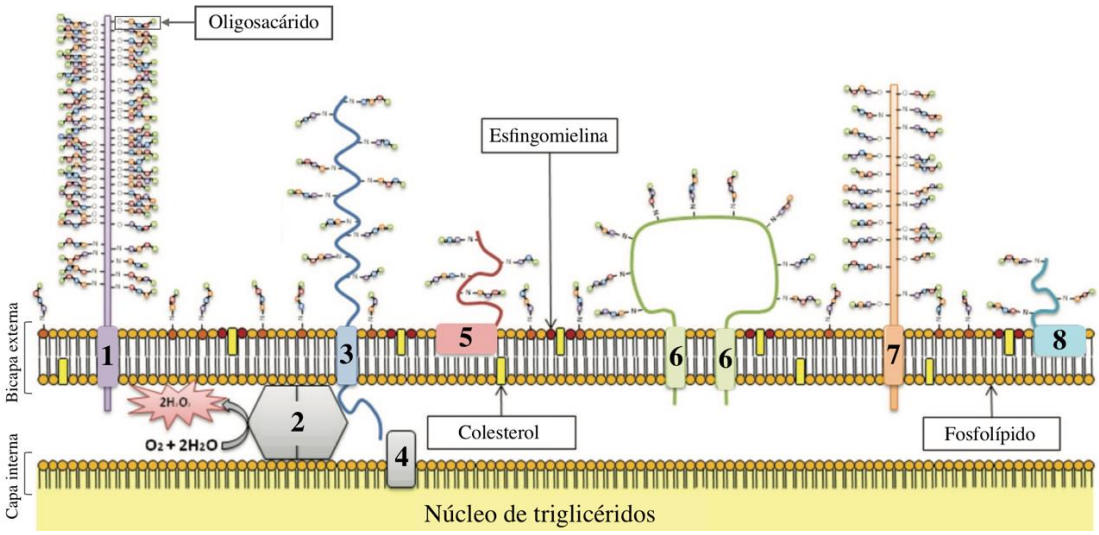

Figura 4. Estructura esquemática de la disposición de las proteínas y lípidos en la MFGM, adaptada de Douëllou, Montel, y Sergentet (2017). Las proteínas MUC1 (1), BTN (3), CD36 (6) y MUC15 (7) se encuentran en la bicapa lipídica externa. La XDH/XO (2) se sitúa entre la capa interna y la bicapa externa, y está estrechamente conectada con la proteína transmembranal BTN. La ADPH (4) se encuentra en la capa polar interna por su afinidad por los triglicéridos. Las proteínas LD (5) y PP3 (proteosa peptona 3) (8) están situadas en la zona exterior de la membrana. La mayor parte de los lípidos se encuentran en la membrana externa.

La LD es una glicoproteína multifuncional de 409 aminoácidos con dos variantes de glicosilación, PAS-6 y PAS-7, de 47 y 50 kDa, respectivamente (Ripollés et al., 2018). La LD bovina se encuentra formada por cuatro dominios: EGF1 y EGF2, donde EGF (del

inglés, *epidermal growth factor*) indica la homología con el dominio del factor de crecimiento epidérmico; y C1 y C2, representando los dominios carboxilo terminales, homólogos a los factores de coagulación V y VIII (**Figura 5**). Se ha demostrado que la LD puede actuar como un enlace entre dos superficies, ya que presenta afinidad por las integrinas gracias a la secuencia de adhesión celular Arg-Gly-Asp presente en el dominio N-terminal y permite una unión directa a los fosfolípidos por su extremo C-terminal (Andersen et al., 2000).

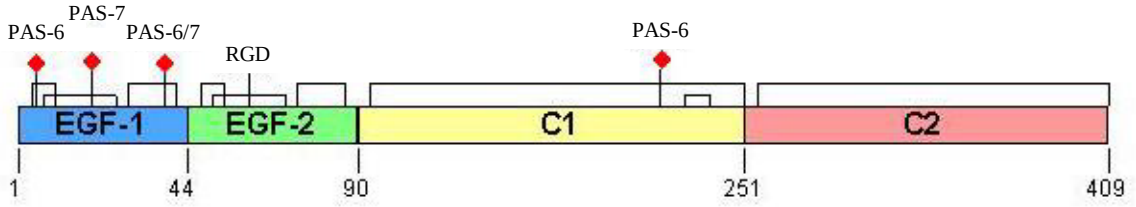

Figura 5. Representación de la estructura de la lactadherina bovina. Los sitios de glicosilación de PAS-6 y PAS-7 están marcados con rombos rojos. Los puentes disulfuro se muestran con líneas negras. RGD: secuencia Arg-Gly-Asp [adaptada de Hvarregaard et al., (1996)].

Gracias a su estructura multidominio, la LD participa en procesos biológicos fisiológicos y patológicos como la fagocitosis, angiogénesis, aterosclerosis, la coagulación de la sangre, etc. (Kamińska, Enguita, y Stępień, 2018). Además, se ha demostrado la capacidad de inhibición de la LD humana frente a la infección por rotavirus, protegiendo el TGI de los lactantes (Parrón et al., 2018).

Las MUC son glicoproteínas con un alto nivel de glicosilación. Las mucinas más destacables son la MUC1, mucina predominante en la leche bovina, la MUC15 y la MUCX, cuyos pesos moleculares varían entre 95 y 200 kDa. La MUC1 presenta una región N-terminal exoplásmica, un dominio transmembrana, y una corta cola entre la bicapa externa y la capa interna de la MFGM. Posee una región central muy glicosilada con repeticiones en tándem de serina-treonina-prolina, y una región C-terminal rica en cisteína (Bansil y Turner, 2006) (**Figura 6**). Por otro lado, la MUC15, al igual que la MUC1, presenta un extenso dominio orientado hacia el exterior de la MFGM y un dominio transmembrana; sin embargo, no presenta repeticiones en tándem (Keenan y Mather, 2006). La MUCX, de mayor peso molecular que las anteriores, posee carbohidratos con estructuras más complejas, facilitando su unión a un espectro más amplio de microorganismos patógenos (Liu, Erickson, y Henning, 2005).

Las MUC forman parte del sistema inmunitario, y contribuyen a la inhibición de ciertas bacterias patógenas, destacando así su capacidad antimicrobiana (Kutta et al., 2008). Las MUC podrían desempeñar un papel específico como inhibidores competitivos en la unión microorganismo-célula, uniéndose a los microorganismos e impidiendo su adherencia a la superficie de las células del huésped (Liu y Newburg, 2013).

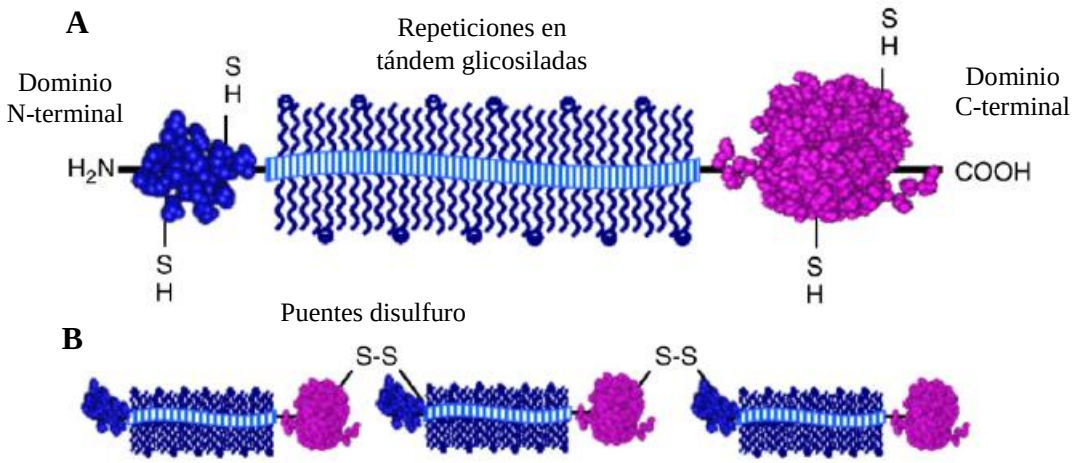

Figura 6. Representación de la estructura de (**A**) un monómero típico de mucina, organizado con los distintos dominios y (**B**) una molécula de mucina compuesta por varias subunidades unidas por puentes disulfuro [adaptada de Coles, Chang, y Zauscher, (2010)].

1.4. Minerales y vitaminas

La leche contiene minerales, entre los que encontramos, principalmente, el calcio, fósforo, sodio, cloruro, zinc, potasio y magnesio.

Además, la leche es rica en vitaminas, como la A, C, D, E, K, la riboflavina (B2), el ácido fólico (B9), la vitamina B12, los carotenos, la nicotinamida y la biotina (Agudelo Gómez y Bedoya Mejía, 2005).

Por otro lado, en la leche se distinguen dos grupos de enzimas; las hidrolasas, entre las que se encuentran las lipasas, proteasas y esterasas, y las enzimas oxido-reductasas, entre las que se identifican la catalasa y la lactoperoxidasa (Agudelo Gómez y Bedoya Mejía, 2005).

2. La industria láctea

2.1. Subproductos de la industria láctea

La industria láctea genera un gran volumen de subproductos cuyo destino es, principalmente, la obtención de ingredientes tecnológicos o la producción de suplementos para la alimentación animal. Además, en el caso de que dichos subproductos no se destinen a ningún uso en concreto, su eliminación supone un serio problema por ser fuente de contaminación. Teniendo en cuenta que la leche y sus derivados contienen proteínas y otros compuestos con propiedades biológicas (Svanborg et al., 2015), su aislamiento a partir de subproductos como el lactosuero y la mazada les aporta un valor extra y supone una fuente adicional de ingresos para la industria láctea.

El lactosuero se obtiene durante la fabricación del queso tras la precipitación de las caseínas por acidificación o por coagulación enzimática, o bien por separación de las caseínas mediante ultrafiltración por membrana (Pires et al., 2021). En función del procedimiento de obtención, se distingue entre el lactosuero ácido, que se obtiene mediante la precipitación de las caseínas utilizando ácidos orgánicos o fermentos lácticos que producen un descenso del pH por debajo de 4,6; y el lactosuero dulce, que se obtiene mediante la coagulación de las caseínas a pH 6 o al pH normal de la leche (6,6-6,8) con enzimas coagulantes como la quimosina (Mollea, Marmo, y Bosco, 2013). Independientemente de su proceso de obtención, el lactosuero es una fuente de proteínas de alto grado nutricional, aunque durante mucho tiempo se consideró un líquido de poco valor. Para la producción de 1 kg de queso se requieren 10 kg de leche, generándose así en este proceso 9 kg de lactosuero (Domingos et al., 2017). Actualmente, el lactosuero se utiliza para la alimentación animal en forma líquida y en polvo como ingrediente en la industria de alimentos. En 2016, se estimó la tasa de producción mundial de lactosuero en 200 millones de toneladas (MT), mostrando un aumento anual del 3% entre 1997 y 2017 (Domingos et al., 2018). Sólo en Europa, se produjeron 56 MT de lactosuero en 2022 (Eurostat, 2022). Cerca del 50% del lactosuero producido a nivel mundial es transformado en distintos productos para alimentación humana y animal. Aproximadamente el 50% de este volumen transformado se utiliza como lactosuero en forma líquida, el 30% en polvo, el 15% como lactosa y productos derivados, y el 5% como concentrado de proteína de lactosuero (Spalatelu, 2012).

El lactosuero contiene alrededor del 25% de las proteínas de la leche, el 8% de la grasa y cerca del 95% de la lactosa, lo que lo convierte en una importante fuente de nutrientes

(Torres et al., 2009). Los componentes proteicos del lactosuero incluyen, como se ha comentado anteriormente, la β-LG, la α-LA, la BSA, las Igs, la LF, la LP y el glicomacropéptido cuando el lactosuero se obtiene por coagulación enzimática (Kerasioti et al., 2014). Las proteínas del suero permanecen solubles después de la coagulación ácida o enzimática de la leche, lo que las distingue de las caseínas, que precipitan en estas condiciones (Farkye y Shah, 2014). Las proteínas del lactosuero tienen excelentes propiedades funcionales, como buena solubilidad, viscosidad, propiedades emulsionantes y gelificantes, y son las principales responsables del alto valor nutricional y tecnológico del lactosuero (Barukčić, Lisak Jakopović, y Božanić, 2019). Además, cuando estas proteínas son digeridas, dan lugar a aminoácidos esenciales y a péptidos bioactivos que presentan numerosas actividades fisiológicas. Por ello, los concentrados y aislados de lactosuero son ampliamente utilizados en la industria alimentaria (Mollea, Marmo, y Bosco, 2013).

A principios del siglo XXI, el interés en las proteínas y péptidos bioactivos del suero de la leche experimentó un aumento, al publicarse numerosos estudios que demostraban sus beneficios para la salud y proponían su uso como ingredientes en alimentos funcionales (Madureira et al., 2007; Solak y Akin, 2012).

La mazada o *buttermilk* (BM) es otro subproducto de la industria láctea, liberado en el proceso de fabricación de la mantequilla. Estimando el impacto ambiental mediante una metodología de evaluación del ciclo de vida, se ha observado que la mazada no influye en el impacto causado durante la fabricación de la mantequilla en un grado tan alto como lo hace el lactosuero en el proceso de producción del queso (Santos et al., 2022). Durante muchos años, se ha infravalorado la mazada, aunque algunos estudios realizados en las últimas décadas han demostrado su potencial, especialmente debido a las proteínas y lípidos presentes en la MFGM que contiene (Vanderghem et al., 2010). Durante el proceso de batido de la nata (emulsión de grasa en fase acuosa) en la elaboración de la mantequilla, una parte de los glóbulos grasos se rompen, lo que permite la liberación de la grasa envuelta por la MFGM. Este proceso produce una inversión de fases que da lugar a la mantequilla (emulsión de fase acuosa en grasa) con la liberación de la mazada como excedente de la fase acuosa. Esta mazada es similar a la leche desnatada en las proporciones de lactosa, minerales y proteínas; sin embargo, la mazada presenta mayor cantidad de grasa, siendo su concentración de fosfolípidos cinco veces mayor que la de la leche entera (Vanderghem et al., 2010). En función del tipo de nata a partir de la que

se elabora la mantequilla se distinguen tres tipos de mazada: dulce, ácida y de lactosuero. La mazada dulce se libera mediante el batido de la nata no sometida a fermentación láctica; mientras que la mazada ácida se produce en el batido de la nata fermentada por bacterias lácticas, presentando un pH menor de 5,4. La mazada de lactosuero se obtiene en el proceso de elaboración de mantequilla a partir de la nata obtenida de la grasa que queda en el lactosuero producido en la fabricación de queso (Sodini et al., 2006).

Considerando el gran volumen de lactosuero producido anualmente, la mazada derivada de la mantequilla elaborada con grasa de suero tiene un gran mercado potencial. Además, la mazada tiene propiedades emulsionantes debido a la presencia de caseínas y cantidades considerables de fosfolípidos, lo que la hace apta para su aplicación tecnológica en la industria alimentaria (Ali, 2019). La mazada es una buena fuente de potasio, fósforo, vitamina B12 y calcio, lo que le confiere buenas propiedades como ingrediente en alimentación humana (Conway et al., 2014).

Por último, el *butterserum* o suero de mantequilla es la mazada que queda retenida entre los granos de mantequilla. Es la fase líquida que se obtiene cuando la mantequilla se transforma por calentamiento y centrifugación en grasa láctea anhidra (Bourlieu et al., 2018).

El 71% de toda la leche entera disponible en la Unión Europea se utiliza para la fabricación de queso y mantequilla (Eurostat, 2022), lo que genera una gran producción colateral de lactosuero, mazada y *butterserum*. Numerosos estudios han mostrado resultados que indican una clara influencia del lactosuero y la mazada en la salud y el bienestar humanos gracias a las propiedades bioactivas de estas fracciones y, especialmente, de sus proteínas y lípidos (Brandelli, Daroit, y Corrêa, 2015; Egger y Ménard, 2017; Harouna et al., 2020; Buey et al., 2021).

2.2. Tratamientos tecnológicos de la leche

La leche destinada a su comercialización y consumo se procesa a partir de leche cruda obtenida de vacas sanas, sometida a diversas fases de transformación. Este proceso tiene como principal objetivo recoger y conservar la leche a lo largo de la cadena de suministro mediante las siguientes operaciones: ordeño mecánico, refrigeración, almacenamiento en frío, homogeneización, tratamiento térmico, envasado y almacenamiento. Cada uno de estos pasos de procesado induce cambios en la calidad intrínseca de la leche (Michalski y Januel, 2006).

2.2.1. Homogeneización

La homogeneización, que normalmente se emplea para reducir el tamaño de los glóbulos grasos presentes en la leche (Bourlieu et al., 2015), provoca los cambios más profundos en la estructura física de la leche y podría alterar sus propiedades (Michalski y Januel, 2006). Tras el proceso de homogeneización, el tamaño de los glóbulos grasos disminuye, liberando fragmentos y proteínas de la MFGM y provocando un aumento de la superficie de grasa láctea. La cantidad de MFGM nativa es insuficiente para cubrir las superficies de los nuevos glóbulos de grasa que se forman durante la homogeneización. Por ello, para cubrir este aumento de la superficie se produce una adsorción de componentes, favoreciendo la unión de las caseínas y las proteínas del lactosuero a la membrana (Cano-Ruiz y Richter, 1997) (**Figura 7**).

Las consecuencias de la homogeneización en la organización de la leche dependen del tipo de homogeneización, así como de si se ha sometido también a un tratamiento térmico. El principal efecto de la homogeneización sobre los componentes solubles de la leche es la disrupción de las micelas de caseína, que son adsorbidas en forma micelar o como fragmentos sobre los glóbulos grasos (Michalski y Januel, 2006).

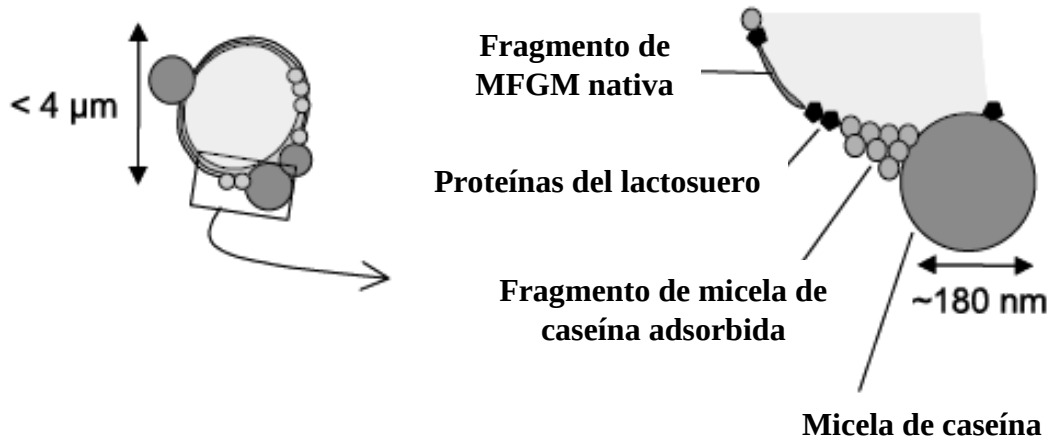

Figura 7. Reorganización de una gota lipídica tras la homogeneización [adaptada de Michalski y Januel, (2006)].

La XO y la BTN se encuentran unidas covalentemente a los ácidos grasos de la MFGM, lo que contribuye a la lipofilia de estas proteínas y a la conservación de su afinidad por el glóbulo graso a pesar del estrés físico causado por la homogeneización. Sin embargo, el contenido en fosfolípidos de la nueva membrana es menor al de la MFGM nativa (Michalski y Januel, 2006).

2.2.2. Tratamiento térmico

El tratamiento térmico de pasteurización sirve para eliminar cualquier microorganismo patógeno, garantizando la seguridad de los productos lácteos, y alargando su vida útil (Claeys et al., 2013). La pasteurización puede realizarse de dos maneras: por el proceso HTST (del inglés *high-temperature short-time*) o por el proceso UHT (del inglés *ultra-high temperature*). El proceso HTST consiste en calentar la leche a 72 ºC durante 15 s y enfriarla inmediatamente. El proceso UHT permite acortar la fase de calentamiento, aumentando la temperatura: 140-150 ºC durante 2 s (Qi et al., 2015).

El grado de desnaturalización de los componentes de la leche depende del tipo de tratamiento térmico y del tipo de proteína. Las caseínas, en especial la β-caseína, son más resistentes al calor que las proteínas del lactosuero, y en concreto, la β-LG es más sensible que la α-LA. Entre las vitaminas, la B1, B6, B12 y C y el ácido fólico son sensibles al calor. Además, en la leche desnatada, la unión inducida por el calor de las proteínas desnaturalizadas a las micelas de caseína provoca un aumento, dependiente del pH, del tamaño de las micelas y de las interacciones entre micelas (Michalski y Januel, 2006).

Durante el calentamiento de la leche aumenta la velocidad de las reacciones químicas y enzimáticas. El calor favorece la glicosilación no enzimática (también conocida como reacción de Maillard), que consiste en una reacción química entre un grupo carbonilo de la lactosa y un grupo amino libre de una proteína (Shimamura y Ukeda, 2012). Los productos derivados de la reacción de Maillard podrían modificar las funciones de las proteínas y péptidos de la leche, si bien es un tema que requiere más investigación.

3. Fórmulas lácteas infantiles

La leche materna es la mejor fuente de nutrición para el recién nacido, ya que contiene una gran cantidad de agentes bioactivos. Sin embargo, la lactancia materna no siempre es posible, por lo que es importante disponer de fórmulas infantiles de alta calidad. Estas se definen como un alimento adecuado para sustituir total o parcialmente a la leche humana, cubriendo los requerimientos nutricionales del lactante (Koletzko et al., 2005). La leche que se utiliza principalmente como base para la elaboración de fórmulas es la leche de vaca, aunque últimamente está aumentando la demanda de leche de cabra para este fin (Maathuis, Havenaar, y Bellmann, 2017). Sin embargo, la composición de la leche materna difiere cuantitativa y cualitativamente de la de vaca. Por esta razón, se toma como referencia el contenido promedio de nutrientes de la leche materna para elaborar las

fórmulas infantiles, ajustando su composición en macronutrientes, minerales, vitaminas y aporte energético (He et al., 2022). Además, la grasa de la leche de vaca se sustituye por mezclas de aceites vegetales para compensar el déficit de ácidos grasos insaturados y hacerla más similar a la grasa de la leche humana (Pan et al., 2022). Por otro lado, los glóbulos de grasa en la leche de vaca tienen un tamaño bastante uniforme, con un diámetro máximo de 4 μm (Bourlieu et al., 2015), mientras que la leche humana contiene glóbulos de grasa más pequeños, especialmente en las primeras etapas de la lactancia, con un tamaño máximo de 1,8 μm en el calostro (Rüegg y Blanc, 1981). Por este motivo, la homogeneización y la pasteurización se utilizan para tratar y estabilizar las fórmulas infantiles, reduciendo el tamaño de los glóbulos de la leche de vaca y haciéndolos más similares a los de la leche materna (Bourlieu et al., 2015). En estos procesos se forma una membrana en la que también participan las caseínas y las proteínas del suero, manteniendo así la estructura del glóbulo de grasa (Pan et al., 2022). Estos tratamientos tecnológicos también modifican el efecto de la digestión en los preparados lácteos, ya que se ha observado una mayor liberación de ácidos grasos y una mayor proteólisis de las caseínas en la leche procesada con tratamientos de homogeneización y pasteurización (Bourlieu et al., 2015).

El Reglamento (CE) Nº 2016/127 de la Comisión Europea recoge los requisitos específicos de composición de los preparados para lactantes y de continuación, así como la información sobre los alimentos destinados a niños de corta edad (Comisión Europea, 2016).

Existen diferentes procesos para elaborar una fórmula infantil. Se puede realizar una mezcla de componentes en polvo en condiciones higiénicas, o una reconstitución de los ingredientes en polvo, seguida de una aplicación de un tratamiento térmico y un secado por atomización (Watson, 2022). En cualquier caso, las fórmulas en polvo no son un producto estéril, y las condiciones inadecuadas de producción, almacenamiento y manipulación pueden ser un riesgo para la salud infantil (Vargas-Leguás et al., 2009). Por este motivo, los productos lácteos en polvo deben almacenarse en un lugar seco, a temperaturas inferiores a 20 °C y con una humedad relativa máxima del 70%. Además, se recomienda que la leche en polvo se consuma en un plazo máximo de un mes una vez abierto el envase y que, tras ser reconstituida, se administre inmediatamente o se mantenga en refrigeración durante un periodo no superior a 24 h (Vargas-Leguás et al., 2009).

En la última década, el uso de la MFGM en las fórmulas infantiles ha despertado un gran interés (Ross et al., 2015). Aunque la concentración de MFGM en la leche humana es muy baja, presenta un papel fundamental en el desarrollo del lactante. La presencia de MUC y LD en la MFGM añadida a las fórmulas infantiles tiene un papel protector debido a su capacidad para inhibir la infección por rotavirus, reduciendo así la incidencia de diarrea en bebés lactantes (Da Silva, Colleran, e Ibrahim, 2021).

Por otro lado, la suplementación de las fórmulas infantiles con LF ha aumentado en los últimos años, contribuyendo también a aumentar la capacidad protectora de las fórmulas frente a diversos agentes patógenos. Tal y como se ha comentado en el apartado 1.3.2., la Comisión Europea permitió la utilización de LF bovina como nuevo ingrediente alimentario en su Reglamento (CE) Nº 258/97 (Comisión Europea, 2012).

4. Bacterias causantes de enfermedades intestinales y sistema inmunitario

La composición del microbioma intestinal es dinámica y se ve influenciada por factores ambientales, incluyendo la dieta, las infecciones y la exposición a medicamentos y probióticos, así como por factores genéticos (Rangan y Hang, 2017).

Existe un gran número de funciones inmunomoduladoras de las bacterias intestinales que influyen en la acción de los patógenos y la respuesta del huésped a la infección. La inhibición del crecimiento de agentes patógenos puede ocurrir mediante la competencia por los recursos o por mecanismos microbicidas directos. Además, la infección por microorganismos patógenos se puede atenuar por la modulación específica de mecanismos de virulencia (Rangan y Hang, 2017).

Un gran número de enfermedades gastrointestinales son causadas por la contaminación bacteriana de los alimentos. Tras una infección, el agente microbiano coloniza el intestino y libera toxinas que dañan las células del huésped. La intoxicación alimentaria también puede deberse a la ingesta de toxinas presentes en los alimentos, que alteran las células del TGI (Foster, 2002).

La infección con agentes patógenos puede causar estrés oxidativo en las células (Ivanov, Bartosch, e Isaguliants, 2017). Por ello, existe un gran interés por encontrar sustancias naturales que eviten o disminuyan el estrés celular que se produce cuando las ROS superan ciertos niveles y los trastornos fisiológicos derivados de dicho estrés (Park et al., 2017). Uno de los principales focos del daño oxidativo es el epitelio intestinal, incluso en

un intestino no afectado por patógenos, ya que está sujeto a una exposición constante a ROS por su contenido luminal y microbiota (Gill, Tsung, y Billiar, 2010).

4.1. *Cronobacter sakazakii*

Durante las últimas décadas, *Cronobacter sakazakii* ha emergido como un patógeno oportunista causando gran preocupación en los organismos de salud pública, ya que se ha asociado al consumo de fórmulas infantiles y leche en polvo. Para evitar y reducir el riesgo de infección por este patógeno, se ha recomendado tener especial cuidado en el manejo y almacenamiento de dichas fórmulas (Drudy et al., 2006). Además, *C. sakazakii* también se ha aislado de diversos ambientes y de una gran variedad de alimentos, como el queso, pan, tofu, embutidos, carne picada, etc. Dado que *C. sakazakii* no forma parte de la microbiota intestinal animal ni humana, es probable que el agua y el suelo sean las principales fuentes de contaminación de los alimentos (Iversen y Forsythe, 2003).

El género *Cronobacter* pertenece a la familia Enterobacteriaceae y está compuesto por siete especies: *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. dublinensis*, *C. muytjensii*, *C. universalis* y *C. condimenti.*

C. sakazakii es un bacilo móvil Gram-negativo, no formador de esporas y anaerobio facultativo. Su tamaño es de aproximadamente 1-3 μm. Crece en un amplio intervalo de temperatura, entre 6 y 47 °C, por lo que es capaz de crecer en refrigeración, aunque su temperatura óptima es de 37 a 43 °C (Iversen y Forsythe, 2003). *C. sakazakii* también tiene una alta resistencia a las actividades de agua bajas, en el rango de 0,3 a 0,83. Estas propiedades le dan a este patógeno la capacidad de crecer en una gran variedad de alimentos (Lin y Beuchat, 2007).

C. sakazakii presenta dos tipos de colonias morfológicamente diferentes cuando se aísla en agar tripticasa de soja. Las colonias de tipo A, también llamadas tipo mate, incluyen colonias grandes y con bordes ondulados que presentan texturas gomosas al tocarlas con un asa. Las colonias de tipo B o brillante y liso, son de textura suave y con pequeñas cantidades de producción de pigmento. En torno a un 80% de las cepas producen un pigmento amarillo dependiente de la temperatura (Yan y Gurtler, 2014).

El mecanismo de infección de *C. sakazakii*, transmitido tras la ingesta de alimentos contaminados, se basa en el daño que produce esta bacteria en las células epiteliales, lo que le permite atravesar la barrera intestinal, llegando así al sistema circulatorio (Ribet y

Cossart, 2015). Este patógeno interactúa con las células del epitelio intestinal, posiblemente mediante las proteínas de su membrana externa (OmpA y OmpX), provocando inflamación intestinal y alteraciones en las microvellosidades (Kim et al., 2010). Se ha reportado que los glicanos, tanto oligosacáridos como glicoconjugados, presentes en la leche impiden la adhesión microbiana. De esta forma, disminuyen la capacidad del patógeno para identificar la célula y unirse a ella, impidiendo la infección (Newburg, Ruiz-Palacios, y Morrow, 2005). Se ha demostrado que los glicanos presentes en la LF o en las MUC interactúan con estructuras de la membrana de las bacterias destinadas al reconocimiento y unión a la célula eucariota, impidiendo así la infección (O'Riordan et al., 2014). Además, existen evidencias que respaldan que la adición de algunos compuestos antimicrobianos naturales, como la LF, pueden evitar el crecimiento de *C. sakazakii* en las fórmulas infantiles en polvo reconstituidas (Harouna et al., 2020).

C. sakazakii afecta principalmente al sistema intestinal y digestivo y, aunque causa enfermedades en todos los grupos de edad, sus efectos son más severos en niños y, especialmente, en bebés de bajo peso, causando enfermedades como meningitis, enterocolitis necrotizante y sepsis en bebés prematuros y nacidos a término (Henry y Fouladkhah, 2019). Se han registrado tasas de mortalidad del 40-80% debidas a estos procesos, y los supervivientes suelen padecer secuelas neurológicas severas (AECOSAN, 2015). Del año 1961 al 2008, se notificaron 120 casos en niños de hasta 3 años de edad en todo el mundo (FAO y OMS, 2008).

C. sakazakii es capaz de formar biopelículas o *biofilms*, que consisten en agregaciones de bacterias en una superficie o incrustadas en una matriz compuesta, principalmente, por polisacáridos, proteínas y ácidos nucleicos. La formación de estos *biofilms* mejora la resistencia de las células bacterianas al estrés ambiental y les confiere protección frente a medicamentos y desinfectantes. En la industria alimentaria, los *biofilms* son un grave problema, ya que son fuente de contaminación de las materias primas, se crean en las superficies en contacto con los alimentos y su eliminación es difícil (Du et al., 2012).

4.2. *Listeria monocytogenes*

Listeria monocytogenes es un pequeño bacilo Gram-positivo perteneciente a la familia Listeriaceae. Es un microorganismo móvil que no forma esporas. Es una bacteria anaerobia facultativa y mide en torno a 1-1,5 μm de longitud. Su temperatura óptima de crecimiento es de 37 °C, aunque puede sobrevivir en diferentes condiciones adversas, lo

que favorece su desarrollo en alimentos y superficies de equipos industriales (Shamloo et al., 2019).

El género *Listeria* está formado por 17 especies, siendo las más frecuentes *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri* y *L. grayi* (Leclercq et al., 2019).

La listeriosis es la enfermedad causada por el consumo de alimentos contaminados por *L. monocytogenes*. Esta bacteria es una de las principales bacterias causantes de enfermedades transmitidas por los alimentos en el mundo (Liu et al., 2005). Las infecciones causadas por *L. monocytogenes* en personas sanas suelen ser asintomáticas o pueden provocar una gastroenteritis leve con fiebre. Sin embargo, en determinados grupos de riesgo, estas infecciones pueden llegar a causar septicemina, meningitis, aborto espontáneo, parto prematuro e incluso la muerte (Sibanda y Buys, 2022). Desde el año 2015, la listeriosis es una enfermedad de declaración obligatoria en España. El periodo de incubación de esta bacteria suele ser de 1 ó 2 semanas, aunque puede llegar a 3 meses, lo que hace que en ocasiones resulte difícil identificar el alimento origen de la infección. En 2020 se confirmaron 191 casos de listeriosis en España (AESAN, 2022).

A lo largo de la cadena alimentaria, *L. monocytogenes* se enfrenta a diferentes barreras físicas y químicas que dificultan su crecimiento y supervivencia. En respuesta a la exposición a situaciones de estrés, los agentes patógenos desarrollan mecanismos que les permitan su supervivencia. Esta adaptación favorece la colonización y viabilidad de *L. monocytogenes* en diferentes condiciones de procesado y conservación de los alimentos (Sibanda y Buys, 2022). Uno de los principales mecanismos de adaptación de *L. monocytogenes* es la formación de *biofilms*, como se ha comentado anteriormente en el caso de *C. sakazakii*.

Se ha confirmado que *L. monocytogenes* es un agente causante de mastitis en vacas lecheras. Sin embargo, aunque la infección de la ubre por esta bacteria puede contaminar la leche, la acidificación producida durante la elaboración del queso es capaz de inhibir el crecimiento bacteriano, especialmente en los quesos blandos (Gérard et al., 2018). Por otro lado, en los quesos elaborados con leche pasteurizada, la probabilidad de infección es baja, aunque no se puede descartar, ya que la contaminación puede darse tras la pasteurización de la leche (Shamloo et al., 2019).

La transmisión de *L. monocytogenes* se asocia, principalmente, con alimentos listos para su consumo, refrigerados y con una vida útil prolongada por algún tratamiento de conservación. Estos alimentos son, entre otros, salchichas cocidas, patés, productos lácteos, pescados ahumados y verduras y frutas frescas (AESAN, 2022).

4.3. *Staphylococcus aureus*

Staphylococcus aureus es una bacteria Gram-positiva con un tamaño de entre 0,5 y 1 μm. Es un coco, anaerobio facultativo, catalasa positivo y coagulasa positivo (Pasachova, Ramirez, y Muñoz, 2019). Es una bacteria inmóvil que no forma esporas. Su temperatura de crecimiento puede variar entre 18 y 40 ºC.

S. aureus pertenece a la familia Staphylococcaceae, género *Staphylococcus*, compuesto por 52 especies. Esta bacteria causa, principalmente, infecciones nosocomiales o intrahospitalarias, que se producen debido a que el paciente ingresado presenta las defensas bajas, siendo más susceptibles los niños menores de 2 años, diabéticos, o pacientes con enfermedades pulmonares (Pasachova, Ramirez, y Muñoz, 2019). *S. aureus* puede ingresar al torrente sanguíneo, alcanzando el corazón y causando una endocarditis bacteriana, y también puede afectar al aparato gastrointestinal. Se trata de un patógeno relevante clínicamente, ya que presenta resistencia a algunos antibióticos, como es el caso de la penicilina (Foster, 2002).

La catalasa producida por *S. aureus* es una enzima que, al descomponer el peróxido de hidrógeno generado como mecanismo de defensa del huésped, permite la supervivencia intracelular de la bacteria. Por otro lado, la coagulasa es una proteína presente en la superficie de la bacteria que favorece la producción de fibrina y que, junto a las toxinas y otros factores de virulencia, permite que *S. aureus* provoque una amplia variedad de síndromes clínicos (Ondusko y Nolt, 2018).

Las enfermedades causadas por *S. aureus* se deben, principalmente, a dos tipos de determinantes de virulencia: las proteínas asociadas a la superficie celular y las toxinas extracelulares (Foster, 2002).

La intoxicación alimentaria por *S. aureus* puede ocurrir tras la ingesta de alimentos contaminados con enterotoxinas, a causa de un manejo o un almacenaje inadecuado. Las toxinas producidas por esta bacteria estimulan el nervio vago y el centro del vómito

localizado en el cerebro. Además, provocan un aumento del peristaltismo intestinal y una inhibición de la absorción de agua, causando diarrea (Foster, 2002).

Los alimentos principalmente implicados en este tipo de intoxicaciones son los productos cárnicos, de panadería y productos lácteos. A nivel nacional, *S. aureus*, junto con *L. monocytogenes*, es uno de los agentes principales responsable de los brotes relacionados con el consumo de leche cruda o derivados. *S. aureus* es fuente de contaminación ambiental y causante de mastitis en rumiantes. Las alertas alimentarias por toxinas estafilocócicas no son muy frecuentes en España; sin embargo, en el año 2022 se produjo una alerta asociada al consumo de queso contaminado con dichas toxinas (AESAN, 2022).

S. aureus, al igual que las bacterias mencionadas anteriormente, es capaz de formar *biofilms*, especialmente en ambientes húmedos como los que se encuentran en las plantas procesadoras de lácteos. La legislación de la Unión Europea establece límites microbiológicos para el control de enterotoxinas estafilocócicas en ciertas categorías de alimentos, garantizando las prácticas de higiene y la disminución del riesgo para la salud del consumidor (AESAN, 2022).

4.4. Sistema inmunitario intestinal

Las infecciones gastrointestinales causadas por microorganismos pueden causar inflamación intestinal, importante para la protección del huésped, ya que desencadena una serie de procesos dirigidos a la destrucción de los agentes patógenos. La inmunidad innata juega un papel clave en la inflamación al actuar como el principal sistema de defensa y responder rápidamente al ataque microbiano y al daño tisular (De Nardo, 2015). Entre los componentes del sistema inmunitario que inician la respuesta inmunitaria innata en el intestino se encuentran los receptores tipo Toll (TLRs, del inglés *Toll-like receptors*), que actúan reconociendo patrones moleculares asociados a patógenos (PAMP, del inglés *pathogen-associated molecular patterns*). Los TLRs son glicoproteínas integrales de membrana tipo I con una estructura trimodular. En los mamíferos se han identificado 13 TLRs diferentes, algunos de los cuales están especializados en el reconocimiento de bacterias, como el TLR1, TLR2, TLR4, TLR6 y TLR9 (Bellés et al., 2022). Los TLRs presentan un dominio N-terminal extracelular rico en leucina y un dominio C-terminal intracelular que se conoce como dominio del receptor Toll/IL-1. Este dominio es necesario para la interacción y reclutamiento de moléculas capaces de activar

la vía de señalización en cadena (Kumar, Kawai, y Akira, 2009). Cuando los TLR detectan los PAMP, experimentan un cambio conformacional que promueve la activación de las vías de señalización intracelular, desencadenando una respuesta intestinal y estimulando la síntesis de citoquinas proinflamatorias, como el interferón alfa y beta (IFN-α, IFN-β) (Layunta et al., 2021). En este contexto, el TLR2 reconoce una amplia variedad de PAMPs, como los lipopéptidos y peptidoglicanos en bacterias Gram-negativas, y el ácido lipoteicoico presente en la pared celular de bacterias Gram-positivas. Se ha demostrado que el TLR2 mejora la función de la barrera epitelial intestinal a través de diferentes mecanismos, incluida la organización de la proteína zonula occludens-1 (ZO-1) de las uniones estrechas (Burgueño y Abreu, 2020). El TLR4, localizado en la superficie de la célula como el TLR2, reconoce principalmente el LPS o endotoxina de la membrana externa de las bacterias Gram-negativas, teniendo un efecto protector en las lesiones intestinales. El TLR9, expresado en las vesículas intracelulares, reconoce el DNA genómico de bacterias internalizadas en las células (Burgueño y Abreu, 2020).

En condiciones fisiológicas, la activación de los TLR por agentes patógenos induce varias cascadas de señalización intracelular, lo que resulta en la producción de citoquinas, quimiocinas y factores de transcripción que desencadenan una respuesta inflamatoria. Sin embargo, la exposición constante a las bacterias de la microbiota suprime la activación del sistema inmunitario, protegiendo así a la mucosa de una inflamación innecesaria. Por ello, la composición de la microbiota debe mantenerse estable para asegurar la respuesta del sistema inmunitario intestinal en caso de infección. Si la composición de la microbiota cambia debido, por ejemplo, al efecto de los antibióticos, la comunidad bacteriana presentará distintos patrones moleculares a los TLR de las células intestinales. Este proceso se conoce como disbiosis (Bellés et al., 2022).

La LF puede contribuir al mantenimiento de la homeostasis intestinal, contrarrestando la disbiosis causada por los antibióticos. Ciertos péptidos de la LF, como la lactoferricina y la lactoferrampina, pueden emplearse como un tratamiento alternativo a los antibióticos, aprovechando su acción antimicrobiana selectiva y su efecto prebiótico. Además, se ha visto que, en función de la saturación en hierro, la LF muestra efectos divergentes en el crecimiento de las bacterias probióticas. Se ha demostrado que la LF libre de hierro inhibe o no afecta al crecimiento de diferentes cepas de lactobacilos y bifidobacterias. Sin embargo, la LF saturada en hierro al 66% inhibe el crecimiento de las bifidobacterias pero no de los lactobacilos; mientras que la LF saturada al 98% tiene un efecto contrario,

inhibiendo el crecimiento de los lactobacilos pero no de las bifidobacterias (Vega-Bautista et al., 2019). La capacidad de la LF bovina para modular positivamente la microbiota intestinal se ha demostrado en un modelo de ratones con colitis inducida por sulfato de dextrano sódico (Wang et al., 2021). En dicho estudio, se demostró que la LF bovina protegía la barrera intestinal y reducía la inflamación del colon. Además, disminuía los síntomas patológicos de la colitis y evitaba la pérdida de peso en los ratones enfermos. La LF también reguló la composición estructural y la función metabólica de los microorganismos intestinales.

5. Digestión de la leche y liberación de péptidos bioactivos

La digestión es el proceso de descomposición mecánica y enzimática de los alimentos en sustancias que, posteriormente, serán absorbidas al torrente sanguíneo. Los alimentos contienen grasas, carbohidratos y proteínas, que deben ser digeridos para poder ser absorbidos. Tras el proceso de digestión, estos macronutrientes se descomponen en moléculas que pueden atravesar el epitelio intestinal y alcanzar el torrente sanguíneo para su uso en diversas células del cuerpo (Patricia y Dhamoon, 2022).

La mayoría de las proteínas de la dieta, y en particular las proteínas de la leche, se consideran una fuente importante de péptidos bioactivos. Estos se encuentran inactivos en la proteína nativa y se liberan por acción de las enzimas digestivas durante la digestión gastrointestinal y también en la fermentación que la leche experimenta en la elaboración de algunos productos. La secreción gástrica altera la conformación del pepsinógeno, transformándolo en pepsina activa, que comienza a hidrolizar las proteínas en el estómago. Las proteasas pancreáticas se secretan junto con el bicarbonato pancreático, que neutraliza el ácido del estómago y aumenta el pH a un nivel óptimo para la actividad de las proteasas. Estas proteasas, como la tripsina y la quimotripsina, son responsables de la rotura de los polipéptidos, dando lugar a oligopéptidos y aminoácidos (Mohanty et al., 2016). Una vez liberados, los péptidos se dirigen a la luz intestinal, atraviesan el epitelio y alcanzan la circulación sistémica para interactuar con los receptores diana (Tidona et al., 2009).

Durante este proceso de digestión, una proteína puede hidrolizarse de diferente manera, en función de si se ha sometido previamente a algún tratamiento tecnológico o no. Se conoce que procesos como la pasteurización y la homogeneización desnaturalizan las

caseínas y las proteínas del lactosuero, de forma que son más fácilmente digeribles (Atallah et al., 2020).

En general, las proteínas de la leche son reconocidas como la principal fuente de péptidos biológicamente activos, liberados durante la digestión gastrointestinal. Estos péptidos bioactivos son fragmentos proteicos de bajo peso molecular, compuestos por aminoácidos unidos por enlaces covalentes. Pueden desencadenar efectos fisiológicos que influyen en las funciones corporales, siendo capaces de disminuir el riesgo de ciertas enfermedades e impactando positivamente en la salud. Entre estos efectos se distinguen las actividades antimicrobiana, antihipertensiva, antitrombótica, antiinflamatoria y antioxidante (Hernández-Ledesma et al., 2007; Sánchez y Vázquez, 2017). Estas actividades de las proteínas de la leche, así como las propiedades de sus hidrolizados se han revisado recientemente por Bielecka, Cichosz, y Czeczot (2022).

En las últimas décadas, ha surgido un gran interés por los péptidos antimicrobianos (AMPs, del inglés *antimicrobial peptides*) como alternativa a los antibióticos. Estos péptidos constan de menos de 100 aminoácidos y su composición incluye residuos de aminoácidos cargados positivamente, como lisina, arginina e histidina, y una gran cantidad de residuos hidrofóbicos (Browne et al., 2020). Los péptidos con actividad antimicrobiana interactúan específicamente con las membranas bacterianas, creando poros transmembrana, alterando el gradiente osmótico y, en consecuencia, la permeabilidad de la membrana (Hartmann et al., 2010). Se han identificado un gran número de péptidos bioactivos derivados de la leche con actividades interesantes. Específicamente, la hidrólisis de la LF da lugar a algunos AMPs, como la lactoferricina y la lactoferrampina.

La lactoferricina es un potente péptido antimicrobiano presente en el dominio N1 de la LF y que se genera por la acción de la pepsina gástrica. Se trata de un péptido de bajo peso molecular formado por una cadena peptídica de 25 aminoácidos con la secuencia FKCRRWQWRMKKLGAPSITCVRRAF, localizado entre los residuos 17-41 de la LF. La lactoferricina presenta una estructura tridiminensional cuasicíclica debido al enlace disulfuro presente entre sus dos residuos de cisteína (Wakabayashi et al., 1992). La lactoferricina ha mostrado efecto antibacteriano frente a una gran selección de bacterias, entre ellas *C. sakazakii* (Wakabayashi, Yamauchi, y Takase, 2008), *L. monocytogenes* (Wakabayashi et al., 1992) y *S. aureus* (Bellamy et al., 1992).

Por otro lado, la lactoferrampina es otro péptido de la LF, presente también en el dominio N1 de su estructura, entre los residuos 268-284. Este péptido presenta residuos cargados positivamente, así como un dominio hidrofóbico que contiene triptófano, residuo que interviene en la inserción en la membrana. Estas características de su estructura son rasgos característicos de los AMPs (Van der Kraan et al., 2004).

Además, se han atribuido varias bioactividades a péptidos derivados de otras proteínas de la leche, como las caseínas, la β-LG y la α-LA (Théolier et al., 2014). Por todo ello, los péptidos bioactivos son idóneos para su uso como fármacos o suplementos alimenticios.

La digestión de las proteínas de la leche es diferente según el tipo de proteína. Mientras que las proteínas del lactosuero se hidrolizan rápidamente, las caseínas llevan un proceso de digestión más lento y prolongado. Esto podría deberse a las condiciones ácidas del estómago, que producen la coagulación de las caseínas, ralentizando su paso al intestino. Sin embargo, las proteínas del lactosuero permanecen solubles siendo fácilmente digeridas y pasando rápidamente al intestino delgado (Mulet-Cabero et al., 2020).

Así, la LF es una proteína inestable en las condiciones del estómago y el intestino debido al bajo pH gástrico y la alta actividad de las proteasas pancreáticas, que provocan cambios conformacionales y la pérdida de actividad de la proteína (Raei et al., 2015, Braim et al., 2019). Teniendo en cuenta esta susceptibilidad, es importante lograr la estabilización de la LF, y otras proteínas del lactosuero, mediante el uso de algunas tecnologías, como la encapsulación o el *composite*, material creado con dos o más compuestos, evitando su degradación a lo largo del TGI (Balcão et al., 2013).

6. Encapsulación de compuestos bioactivos

La encapsulación permite proteger componentes bioactivos, como aceites esenciales (Bastos et al., 2020) o bacterias probióticas (Yucel Falco et al., 2019). Además, esta estrategia protege a algunas proteínas, como la LF, favoreciendo su biodisponibilidad, mejorando su estabilidad en el tracto digestivo y, consecuentemente, permitiendo que desarrollen sus funciones de manera localizada (Balcão et al., 2013).

En las últimas décadas se han desarrollado técnicas de encapsulación para preservar compuestos bioactivos, que se han incorporado en productos farmacéuticos, alimentarios y cosméticos (Castro-Rosas et al., 2017). La microencapsulación se define como un proceso en el que partículas o gotitas muy pequeñas se rodean de un revestimiento o se incrustan en matrices homogéneas o heterogéneas. Este proceso tiene como objetivo

preservar los compuestos bioactivos e impedir su inactivación, que puede ser causada por ciertas condiciones e interacciones con el medio donde esos compuestos deben ejercer su actividad (Gouin, 2004). Las nanopartículas portadoras permiten mejorar la biodisponibilidad y solubilidad de los compuestos bioactivos encapsulados, así como mejorar su estabilidad en el TGI y favorecer su capacidad para penetrar en tejidos y células (Mohammadian et al., 2020).

La selección de los materiales adecuados para encapsular compuestos es fundamental, pues deben presentar ciertas características que los hagan aptos para este fin. Es importante que sean químicamente compatibles y no reaccionen con la sustancia encapsulada. Además, deben presentar ciertas propiedades necesarias para este fin, como la resistencia, flexibilidad, impermeabilidad y estabilidad (Jyothi et al., 2012).

La encapsulación con biopolímeros ha ganado popularidad gracias a sus excepcionales características físicas como su biodegradabilidad, biocompatibilidad, baja toxicidad y gran capacidad para unir compuestos bioactivos hidrófobos (Il'ina et al., 2016).

6.1. Encapsulación de la lactoferrina

La encapsulación de la LF puede llevarse a cabo con proteínas lácteas, otras proteínas o polisacáridos como el alginato, la goma arábiga, la pectina, etc.

En el caso de la encapsulación con polisacáridos, la atracción electrostática proteína-polisacárido iónico requiere un pH específico, en el que las cargas de estas moléculas sean opuestas. La carga neta de una proteína en su pI es cero, siendo positiva por debajo del pI y negativa a un pH superior a su pI. Así, los polisacáridos catiónicos y aniónicos pueden formar complejos electrostáticos con proteínas dependiendo del pH. A pH fisiológico, la LF tiene carga positiva (pI 8,3-8,7); por tanto, su capacidad para formar complejos con varios polisacáridos aniónicos ha hecho posible la extensa investigación producida en esta área (Il'ina et al., 2016).

El tipo y la concentración de proteína y polisacárido, así como la temperatura, el pH y la composición iónica de la solución en la que tiene lugar la encapsulación, determinan las características funcionales del complejo proteína-polisacárido (Cheng et al., 2021). Además, la formación de coacervados complejos está influenciada por el peso molecular, la densidad de carga y la naturaleza química del polímero empleado (Gulão et al., 2014).

6.1.1. Encapsulación con alginato

El alginato (Alg) es un polisacárido aniónico natural, biodegradable y biocompatible. Deriva del ácido algínico y se encuentra en la pared celular de las algas pardas. Su estructura está formada por cadenas lineales compuestas por monosacáridos D-manurónico y L-gulurónico (Bastos et al., 2020).

El Alg debe su carácter polianiónico a los grupos carboxilo que aparecen a lo largo de su cadena. La distribución de monómeros en la cadena polimérica y la carga y volumen de grupos carboxilo dan al gel formado características de flexibilidad o rigidez dependiendo del contenido de gulurónico. Cuanto mayor es el número de bloques gulurónicos, más duro, rígido y quebradizo es el gel, mientras que, si predominan los grupos manurónicos, el gel es más blando y elástico (Avendaño-Romero, López-Malo, y Paolu, 2013).

Este polímero se presenta como alginato de sodio (Na-Alg) cuando se utiliza como aditivo alimentario, siendo de fácil disponibilidad y amplia aplicación en la industria. Las principales características del Na-Alg son su buena afinidad con el agua y su alta carga aniónica a valores de pH superiores a 2 (Wang et al., 2017). Por otro lado, el Alg combinado con calcio (Ca-Alg) forma un gel, empleado principalmente para la elaboración de perlas. Estas perlas de gel son útiles para encapsular una amplia variedad de agentes bioactivos, debido a su sencillez, biocompatibilidad, no toxicidad y bajo coste (Raei et al., 2015).

Cuando se alinean dos cadenas de gulurónicos, se crean sitios de coordinación. Estas cadenas tienen forma de bucles, creando cavidades con el tamaño adecuado para acomodar el ion de calcio. Tras la incorporación del calcio, el Alg sufre cambios conformacionales, produciendo un patrón de gelificación conocido como “caja de huevos”. Este patrón se basa en la dimerización de la cadena y, posteriormente, en una mayor agregación de los dímeros (Avendaño-Romero, López-Malo, y Paolu, 2013).

El Alg es capaz de formar, en función del pH del medio, complejos electrostáticos con la LF. Además, el Alg es una buena opción para la encapsulación de LF, ya que permite una liberación controlada en el intestino (Braim et al., 2019). Algunos autores han confirmado que la encapsulación de la LF con el Alg es un buen método para transportar la LF intacta al intestino (Raei et al., 2015). Además, Bokkhim et al. (2016) demostraron que las microperlas de LF-Alg protegen a la LF de la acción de la pepsina durante la digestión gástrica y permiten su liberación en el intestino.

6.1.2. Otros métodos de encapsulación

Además del alginato, la LF puede encapsularse empleando otros polisacáridos, como el condroitin sulfato (Dai et al., 2015), los galactomananos (Il'ina et al., 2011), la goma arábiga (Cheng et al., 2021) y la pectina (Raei et al., 2017), entre otros.

Por otro lado, las proteínas del lactosuero, como la β-LG y la α-LA (Darmawan et al., 2020) y las caseínas (Anema y de Kruif, 2012) pueden ejercer de vehículos naturales para las moléculas bioactivas como la LF. Gracias a sus propiedades estructurares y fisicoquímicas facilitan su funcionalidad en los sistemas de administración oral (Livney, 2010).

Estos variados métodos de encapsulación se analizarán más en profundidad en uno de los artículos que componen la presente Tesis Doctoral.

RESULTADOS Y DISCUSIÓN

RESULTADOS PUBLICADOS

Los resultados derivados de la presente Tesis Doctoral se han plasmado en los siguientes artículos publicados en revistas internacionales:

- Artículo 1: Protective effect of bovine lactoferrin against *Cronobacter sakazakii* in human intestinal Caco-2/TC7 cells.

- Artículo 2: Dairy by-products and lactoferrin exert antioxidant and antigenotoxic activity on intestinal and hepatic cells.

- Artículo 3: Development of encapsulation strategies and composite edible films to maintain lactoferrin bioactivity: a review.

- Artículo 4: Effect of *in vitro* gastrointestinal digestion on the antibacterial activity of bioactive dairy formulas supplemented with lactoferrin against *Cronobacter sakazakii.*

- Artículo 5: Does lactoferrin, free, encapsulated or in dairy matrices, maintain its antibacterial activity after *in vitro* digestion?

- Artículo 6: Gastrointestinal digestion and technological treatments modify the antibacterial activity of lactoferrin supplemented dairy matrices against *Staphylococcus aureus.*

- Artículo 7: Lactoferrin modulates oxidative stress and inflammatory cytokines in a murine model of dysbiosis induced by clindamycin.

A continuación, se reproducen dichos artículos con su contenido original y adaptados al formato de la presente Tesis Doctoral para facilitar su lectura.

ARTÍCULO 1

Protective effect of bovine lactoferrin against *Cronobacter sakazakii* in human intestinal Caco-2/TC7 cells

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Protective effect of bovine lactoferrin against *Cronobacter sakazakii* in human intestinal Caco-2/TC7 cells

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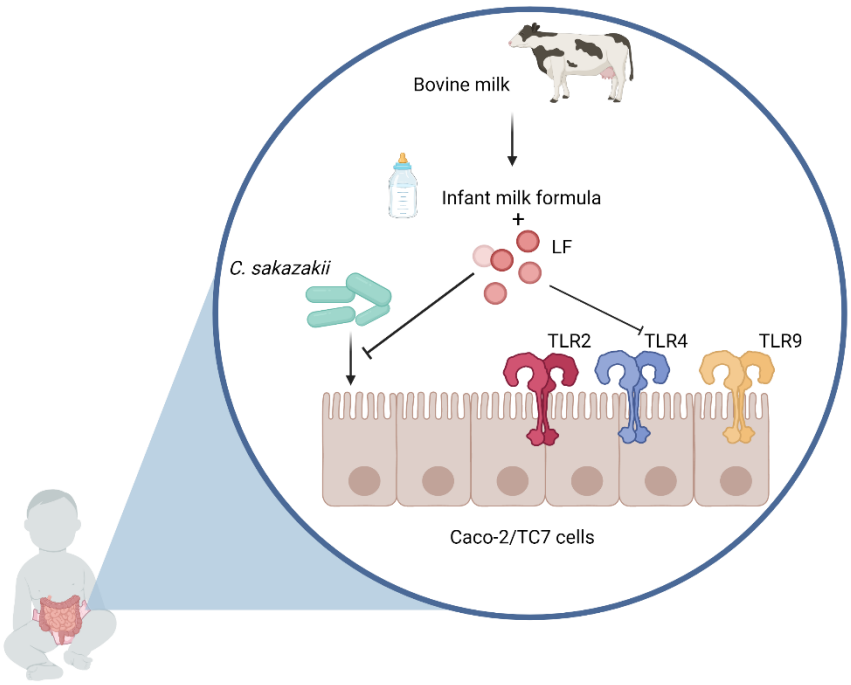
Abstract

Milk is a source of bioactive proteins with defensive properties of great value for protecting the newborn.The activity of bovine milk lactoferrin (LF) was investigated as an antibacterial agent in the internalisation of the emergent pathogen *Cronobacter sakazakii* into Caco-2/TC7 cells, a model of human intestinal epithelium. The effect of LF on oxidative stress and expression of Toll-like receptors (TLR) was also investigated. LF exerted a clear antibacterial activity against C. sakazakii, as well as an inhibitory effect on C. sakazakii adhesion and internalisation into Caco-2/TC7 cells. Incubation with C. sakazakii induced an increase in oxidative stress on the lipid fraction of Caco-2/TC7 cells, which was reversed by LF. Additionally, LF altered the expression of TLR, with a clear decrease in the expression levels of TLR4 at 24 h of incubation. These results suggest that LF has very interesting properties as a potential ingredient for functional foods.



1. Introduction

Milk from mammals is a source of bioactive proteins, some of them with defensive properties, which are of great value for protecting the newborn (Svanborg et al., 2015). The dairy industry generates a large volume of by-products that implicate an environmental problem. The current destination of those dairy by-products is mainly to obtain technological ingredients or to become supplements for animal feeding. The isolation of bioactive components from these by-products would add an extra value to them.

In 2016, the global generation rate of whey was estimated at 200 million tones (MT) per year, with an annual increase of 3% (Domingos et al., 2018). Whey obtained in cheese-manufacture accounts for 95% of the world's liquid whey; the remaining 5% derives from industrial isolation of caseins, which has decreased in the last few years. Whey contains more than 50% of milk solids, and includes lactose, whey proteins, minerals and vitamins. Its composition depends on the procedure of casein coagulation (De Wit, 1998). Whey proteins can be defined as the milk proteins that remain soluble after precipitation of caseins by acidification or enzymatic coagulation (Madureira et al., 2007).

The main whey proteins are β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulins, lactoferrin and lactoperoxidase, among others. Some of them are associated with antimicrobial and antiviral activities, immune system stimulation, anticarcinogenic activity and other metabolic functions (Madureira et al., 2007). Lactoferrin (LF), one of the milk defensive proteins, is present in many exocrine secretions of mammals, such as milk, tears and saliva (Siqueiros-Cendón et al., 2014). LF is a cationic glycoprotein from the transferrin family, with iron-binding capacity and numerous properties (García-Montoya et al., 2012), including antibacterial activity against Gram-positive and Gram-negative bacteria. LF is secreted in its open form without iron (apo-LF) and binds two ferric ions (Fe^{3+}) to give rise to its closed form (holo-LF) (Mayeur et al., 2016). The most recognised activity of LF is bacteriostatic when it does not have iron, because of its ability to sequester iron from the environment (Jenssen & Hancock, 2009). However, the iron-saturated LF can also be active by interacting with the bacterial membrane by destabilising it (González-Chávez, Arévalo-Gallegos, & Rascón-Cruz, 2009) and, consequently, altering the metabolism of bacteria.

LF is one of the most important elements for the defence against infections and the proper development and maturation of the intestinal mucosa (Aly, Ros, & Frontela, 2013). LF is also involved in some immunological processes, as it can inhibit inflammation or promote the innate and adaptive responses of the immune system (Actor, Hwang, & Kruzel, 2009). This activity of LF causes a decrease in reactive oxygen species (ROS) production and modulates the pro-inflammatory cytokines (Kruzel, Zimecki, & Actor, 2017).

During the last few decades, *Cronobacter sakazakii* has appeared as an emerging pathogen, causing great concern because it has been mainly associated with the consumption of infant formula and milk powder. Furthermore, this pathogen has also been isolated from various environments and food products (Iversen & Forsythe, 2003). *C. sakazakii* mainly affects the intestinal and digestive system, being especially dangerous for infants and neonates, to whom it can cause other severe symptoms (Iversen & Forsythe, 2003) and diseases like meningitis, necrotising enterocolitis or sepsis (Quintero et al., 2011).

C. sakazakii belongs to the Enterobacteriaceae family and is a Gram-negative motile bacillus, non-spore-forming and facultative anaerobic. It is capable of growing in a wide temperature range, from 6 to 47 ºC, its optimum temperature being 39 ºC (Iversen & Forsythe, 2003). *C. sakazakii* also has high resistance to low water activities, in the range from 0.3 to 0.83. All these properties give this pathogen the ability to grow in a great variety of foods (Lin & Beuchat, 2007).

The infection mechanism of *C. sakazakii*, after oral intake of contaminated foods, is based on the epithelial cell damage to cross the intestinal barrier, thus reaching the circulatory system (Ribet & Cossart, 2015). This pathogen interacts with intestinal epithelial cells, causing intestinal inflammation and villus alterations. *C. sakazakii* requires cellular structures to multiply and invade the intestine (Kim et al., 2010), stage that is considered critical to the disease pathogenesis (Giri et al., 2012). There are some evidences supporting that the addition of natural antimicrobials, such as LF, can avoid the growth of *C. sakazakii* in reconstituted powdered infant formula (Harouna et al., 2020). It has been reported that the glycans present in milk, similar to the carbohydrates of the cell surface, interfere with the adhesion of bacteria to cells by binding to pathogens (Newburg, Ruiz-Palacios, & Morrow, 2005). Therefore, if milk glycans can bind to receptors located on bacterial membrane, they could also bind to specific regions of the cellular surface avoiding bacterial adhesion (O'Riordan et al., 2014).

Infection with pathogenic agents can cause oxidative stress in the cells that are affected by them (Ivanov, Bartosch, & Isaguliants, 2017). Therefore, there is great interest to find natural substances that can avoid or decrease cellular stress, which is caused when ROS exceed certain levels, producing physiological disorders (Park et al., 2017). An important target for oxidative damage is the intestinal epithelium, as it is subjected to constant exposure to ROS due to luminal content and microbiota, even in the intestine not affected by pathogens (Gill, Tsung, & Billiar, 2010).

Innate immunity plays a key role in inflammation by acting as the primary host defence system to respond quickly to microbial attack and tissue damage (De Nardo, 2015). Among the components of immune system that initiate the innate immune response are Toll-like receptors (TLRs), which act recognising pathogen associated molecular patterns (PAMPs). When TLRs detect PAMPs, they undergo a conformational change that promotes the activation of intracellular signalling pathways, triggering an intestinal response, and stimulating the synthesis of pro-inflammatory cytokines or interferon alpha or beta (Layunta et al., 2021). In this context, TLR2 recognises a wide variety of PAMPs, such as lipopeptides, peptidoglycan and lipoteichoic acid, present in the cell wall of Gram-positive bacteria. TLR2 has been reported to improve the epithelial barrier function through different mechanisms, including the organisation of the tight junction zonula occludens-1 protein. TLR4 recognises cellular components of bacteria, such as the lipopolysaccharide (LPS) of the outer membrane of Gram-negative bacteria, having a protective effect in gut injuries, and TLR9 recognises intracellular bacteria (Burgueño & Abreu, 2020).

Therefore, the main aim of this study was to evaluate bovine LF as an antimicrobial agent against the emergent pathogen *C. sakazakii*, in the cellular line Caco-2/TC7 differentiated in enterocytes, used as a model of human intestinal epithelium. In addition, the activity of this protein on oxidative stress and expression of some intestinal Toll-like receptors in Caco-2/TC7 cells infected with *C. sakazakii* were analysed.

2. Material and methods

2.1. Preparation of lactoferrin

Bovine LF used for all assays was kindly donated by Tatua Nutritionals (Morrinsville, New Zealand). LF had an iron saturation below 10% and a purity higher than 90%, which was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-

PAGE), following the procedure described by Laemmli (1970), and showing a single band corresponding to a protein of about 80 kDa. Furthermore, a double immunodiffusion was performed to confirm that commercial LF was free of lactoperoxidase using specific rabbit polyclonal antibodies obtained previously in our laboratory, following the procedure described by Navarro et al. (2015). These procedures were approved by the Ethic Committee for Animal Experiments of the University of Zaragoza (Project Licence PI48/10). The care and use of animals were performed as stated in the Spanish Policy for Animal Protection RD 53/2013, which meets the EU Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

In addition, the presence of LPS in commercial LF was determined using the ToxinSensor™ Chromogenic LAL colorimetric kit (GenScript, NJ, USA) for the detection of bacterial endotoxins, according to manufacturer's instructions. In brief, the sample was incubated for 9 min at 37 °C with LAL reagent and the change in absorbance at 545 nm was measured. Kruzel et al. (2013) used the same kit and procedure to assess endotoxin levels present in human recombinant LF.

For the assays, a concentrated stock solution of LF was prepared in ultrapure water and sterilised using a low-binding protein filter of 0.22 μm. LF concentration was determined after filtration from its molecular extinction coefficient ($E^{1\%}_{280}$ =1.27 mL/cm · g). The concentration of LF solutions used in the different assays was adjusted considering the concentration of the stock solution.

2.2. Culture conditions of *C. sakazakii*

The bacterial strain used in this study was *C. sakazakii* CECT 858, supplied by the Spanish Type Culture Collection (CECT, Valencia, Spain), which corresponds with the strain ATCC 29544 of the American Type Culture Collection. This strain is of clinical origin from a child throat and is recommended as reference strain by international standards ISO 22964:2017 (ISO, 2017) and ISO 11133:2014/Amd 2:2020 (ISO, 2020). For the reference stock, the bacteria were fixed to porous rings and stored in cryovials at - 80 °C. To cultivate *C. sakazakii*, 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 aerobiosis 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.

2.3. Cell culture

This study was carried out in the human cell line Caco-2, clone TC7. Several authors have used this cell line to study intestinal epithelial physiology because it is an excellent model of human enterocyte-like cells (Latorre etal., 2014; Mesonero et al., 1994; Vašíček et al., 2020). It was maintained with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% (v/v) heat-inactivated foetal bovine serum, 1% (v/v) penicillin (10,000 units/mL)-streptomycin (10 mg/mL), 1% (v/v) non-essential amino acids (NEAA) (100 x) and 1% (v/v) L-glutamine (200 mM) (all reagents from Biological Industries, Kibbutz Beit Haemek, Israel). The culture flasks were maintained at 37 °C in a saturated humid atmosphere with 95% air and 5% CO_2. A solution of 0.25% (w/v) trypsin with 1 mM EDTA (ThermoFisher Scientific, Rockford, IL, USA) was used to enzymatically passage the cells, which were subcultured in 25 cm^2 cell culture flasks (Sigma Aldrich, St. Louis, MO, USA) at a density of 10^4 cells per cm^2. The experiments were carried out with cells cultured for 15 days to obtain a differentiation stage and morphology similar to functional enterocytes (Mesonero et al., 1994). The medium was changed every 48 h. For the assays with LF, the medium used was basic DMEM, supplemented with 1% (v/v) L-glutamine and 1% (v/v) NEAA, to avoid interactions with foetal bovine serum.

2.4. Bioactivity assays

2.4.1. Antibacterial activity of lactoferrin against C. sakazakii

This assay was conducted using *C. sakazakii* culture obtained from exponential and stationary phases, to check whether the effect of LF on bacterial viability depended on the growth phase.

A single colony of *C. sakazakii*, isolated as described above, was incubated in 10 mL TSB with 0.6% (w/v) YE for 8 h (exponential phase) or for 18-20 h (stationary phase) at 37 ºC in aerobiosis. Then, serial dilutions of the bacterial suspension were made with 1% (w/v) peptone water to reach a 4-5 log colony forming units (cfu)/mL suspension that was added to a 96-well microtitre plate (100 mL per well). Samples of native LF, at different concentrations (0.5, 1, 2, 5 and 10 mg/mL), were mixed with the bacterial suspension at a 1:1 ratio (v/v). All samples were assayed in duplicate in three independent experiments.

A control, only with bacterial suspension, was included in each experiment. The plate was maintained at 37 °C in aerobiosis and an aliquot of each sample was extracted at 4 and 24 h of incubation, subjected to decimal dilutions in peptone water and seeded on TSA plates. The colonies were counted after 24 h of incubation of the plates at 37 °C.

2.4.2. Effect of lactoferrin on Caco-2/TC7 cell viability

The effect of LF on viability of Caco-2/TC7 cells was evaluated before the inhibition of bacterial internalisation assays. First, 96- well plates were seeded with Caco-2/TC7 cells at a density of 5 $x10^3$ cells per well and maintained in culture for 15 days to achieve their differentiation into enterocytes. Cells were incubated with LF solutions dissolved in basic DMEM at different concentrations (0.5, 1, 2, 5 and 10 mg/mL), each in triplicate. In this assay, the effect of LF was tested incubating the cells with this protein at two different times (1 and 24 h) in three independent experiments.

Differentiated cells were washed with 200 mL of sterile phosphate-buffered saline (PBS). Afterwards, 200 mL per well of basic medium were added to the cells for them to get used to this simple medium and the plate was incubated for 2 h at 37 °C. Afterwards, the cells were washed and 50 mL of LF at different concentrations were added per well. A solution of 50 mM H_2O_2 was used as positive control for cytotoxicity and basic DMEM as negative control. The plate was incubated for 1 h at 37 °C.

In the second type of assay, the cells were previously incubated with different concentrations of LF at 37 °C for 24 h, to assess whether a longer incubation with LF had any effect on the cell response. Then, the assay was completed with the process detailed above.

The evaluation of the viability of the cells, after incubation with the samples, was carried out using the CellTiter 96 AQueous One Solution Cell Proliferation kit (Promega, Madison, WI, USA). The cells were washed and 200 mL per well of basic medium and 20 mL of 3- (4, 5- dimethyl thiazol- 2- yl)- 5- (3- carboxy methoxy phenyl)- 2- (4- sulfophenyl)- 2H tetrazolium (MTS) reagent were added. MTS allows the determination of the number of viable cells, as this reagent is bioreduced in the formazan dye by the activation of NADPH dependent dehydrogenase enzymes of metabolically active cells. The formazan is soluble in the culture medium, and after 2 h of incubation at 37 °C, the absorbance can be read at 492 nm on a Multiskan MS ELISA plate reader (Labsystem, Helsinki, Finland) and is directly proportional to the number of viable cells.

2.4.3. Inhibition of C. sakazakii internalisation by Caco-2/TC7 cells

The objective of this assay was to evaluate if LF was able to inhibit the internalisation of *C. sakazakii* into Caco-2/TC7 cells by incubating them with different concentrations of the protein before the contact with bacteria.

This assay was carried out in 24-well plates cultured with cells at a density of 3 $x10^4$ cells per well for 15 days. The effect of LF was tested in three independent experiments at two different times (1 and 24 h). The only difference between both assays was that in the second case, the cells were previously incubated with different concentrations of LF for 24 h, as it has been explained in the viability assay.

Differentiated cells were washed with 2 mL per well of sterile PBS and left for 2 h at 37 °C with basic DMEM. Afterwards, each well was washed with the same amount of PBS, and 150 mL of LF at concentrations of 0.5, 1, 2, 5 and 10 mg/mL diluted in the basic medium were added. Two wells were assigned as controls, which consisted in cells incubated only with basic medium without LF. The plate was incubated for 1 h at 37 °C. After incubation, cells were washed again with PBS, and 50 mL per well of a 4-5 log cfu/mL *C. sakazakii* suspension were added (also in control wells) to 150 mL per well of basic DMEM, and incubated for 4 h at 37 °C. Then, cells were washed and 200 mL per well of 150 mg/mL gentamicin were added, incubating them for 2 h at 37 °C to inactivate the bacteria that have remained outside the cells, which were eliminated with a final washing.

Afterwards, the plate was incubated with 200 mL per well of trypsin for 15 min at 37 °C to permeabilise and detach the cells. Finally, serial dilutions of the suspensions with peptone water were spread with Digralsky handle on TSA plates. The plates were incubated for 24 h at 37 °C and the colonies were counted.

2.4.4. Oxidative stress assays

The oxidative stress in proteins and lipids was evaluated in Caco-2/TC7 cells cultured with *C. sakazakii* and LF. To perform these assays, the cells were cultured in 6-well plates at a density of 1 $x10^5$ cells per well for 15 days and the same protocol described above in the inhibition of bacterial internalisation assay was followed to the step of incubation with bacteria. Two control wells were included, one without LF and bacteria, and one only with bacteria. The LF concentrations evaluated were 0.5 and 10 mg/mL assayed in duplicate in three independent experiments.

After 4 h incubation with bacteria, as described in the inhibition of bacterial internalisation assay, cells were washed twice with PBS and 200 mL per well of cold Tris-mannitol buffer were added. This buffer is composed of 200 mM Tris, 500 mM mannitol, 2% (v/v) sodium azide, 100 mM phenylmethylsulfonyl fluoride (PMSF), 1 pill of protease inhibitor and 25 mg/mL of benzamidine, pH 7.1 (all reagents from Sigma Aldrich).

Next, the cell monolayer was scraped over with a scraper brush and the cells were taken to a homogeniser. The homogenate was kept on ice and disrupted by sonication (15 x 1 s bursts, 60 W). Then, the homogenate was centrifuged at 3000 xg for 10 min at 4 °C and the supernatant, corresponding to the cell homogenate, was collected to analyse lipid and protein oxidation.

The protein content of cell homogenates was determined by the Bradford method and protein oxidation was analysed by the measurement of carbonyl levels (Latorre el at., 2014). For this purpose, the optimum concentration of protein was 0.5 mg/mL. The samples were analyzed in triplicate. Cell homogenates were incubated with 2,4-dinitrophenylhydrazine (DNPH), a carbonyl reagent, and protein carbonylation was measured at 375 nm. Results were calculated in nanomoles of carbonyl groups per mg of protein and expressed as the percentage respect to the control value (100%).

Lipid oxidation was assayed as described in Latorre et al. (2014). The oxidative stress in lipids was determined by measuring the concentration of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA), which reacted with N-methyl-2-phenyl-indole, resulting in a chromophore, whose absorbance was measured at 586 nm. Results were calculated in nanomoles of MDA + 4-HDA per mg of protein and expressed as the percentage respect to the control value (100%).

2.4.5. RNA extraction, reverse transcription and real-time PCR

TLR and LF receptor (also known as intelectin-1) expression was determined in Caco-2/TC7 cells cultured for 15 days in 24-well plates at a density of 3 $x10^4$ cells per well, following the same protocol of inhibition of *C. sakazakii* internalisation to the step of gentamicin addition. Cells were incubated (24 h or 1 h) with samples of LF at 0.5 and 10 mg/mL, with and without *C. sakazakii*. Four wells were included as controls without LF, two with bacteria and two only with cells. Additionally, two wells were assigned as

positive control, with 30 and 60 mg/mL of LPS from Escherichia coli (Sigma Aldrich). All samples were assayed in duplicate in, at least, two independent experiments.

mRNA was extracted from cells using the RNeasy mini kit (Qiagen, Hilden, Germany), following manufacturer's recommendations The complementary DNA (cDNA) was obtained by the action of reverse retrotranscriptase using the qScrit cDNA SuperMix kit (Quantabio, Beverly, MA, USA). Samples were taken to a thermocycler (Bio-Rad Laboratories, Madrid, Spain) and were incubated for 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C, maintaining the samples at - 20 °C until subsequent use. The cDNA obtained was amplified with the Real-time Polymerase Chain Reaction technique (qPCR) in qPCR Step One equipment (Applied Biosystems, Foster City, CA, USA), using SYBR Green Master Mix (Applied Biosystems) to determine the expression of TLR2, TLR4, TLR9 and LF receptor genes. GAPDH and HPRT1 were used as housekeeping genes. The specific primers are detailed in **Table 1**.

Table 1. Primer sequences used for qPCR analysis of the expression of housekeeping genes and TLR in Caco-2/TC7 cells. Fw: forward primer, Rv: reverse primer.

Gene	Primers (5' – 3')		Reference
GAPDH	Fw	CATGACCACAGTCCATGCCATCACT	Buey et al., 2021
	Rv	TGAGGTCCACCACCCTGTTGCTGTA	
HPRT1	Fw	CTGACCTGCTGGATTACA	Buey et al., 2021
	Rv	GCGACCTTGACCATCTTT	
TLR2	Fw	GAAAGCTCCCAGCAGGAACATC	Buey et al., 2021
	Rv	GAATGAAGTCCCGCTTATGAAGACA	
TLR4	Fw	TTGAGCAGGTCTAGGGTGATTGAAC	Buey et al., 2021
	Rv	ATGCGGGACACACACACTTTCAAATA	
TLR9	Fw	AGTCCTCGACCTGGCAGGAA	Buey et al., 2021
	Rv	GCGTTGGCGCTAAGGTTGA	
LFr	Fw	AATGGACCTGTTCTTCGT	Zheng et al., 2012
	Rv	TCTGGGTAGACTGCTTTG	

The results obtained for the threshold cycles (Ct) were statistically analysed by subtracting the mean of the Ct values corresponding to the housekeeping genes from the Ct for amplification of TLR genes ($\Delta Ct_{treatment} = Ct_{gene} - Ct_{housekeeping}$). The mean values of the negative controls were subtracted from the previously obtained value ($\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{control}$). Finally, the relative gene expression was calculated and expressed as fold difference ($2^{-\Delta\Delta Ct}$).

2.5. Statistical analysis

The results are presented as the mean ± standard deviation. Statistical analysis of the results was performed using the statistical software GraphPad Prism v8.0.2 (GraphPad Software, San Diego, CA, USA). The normality of data was checked with the Shapiro-Wilk test. To compare the means of three or more unpaired groups, an analysis of variance (ANOVA) was performed and Dunnet's test was used as a multiple comparison test. Data that did not follow a normal distribution were submitted to the nonparametric 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

LF is known as a multifunctional protein with several properties, including antibacterial activity among them (García-Montoya et al., 2012). It is also involved in some immunological processes, as it can inhibit inflammation or promote the innate and adaptive responses of the immune system (Actor, Hwang, & Kruzel, 2009). Different approaches have been carried out in this study to evaluate the bioactivity of LF against *C. sakazakii* in a model of human intestinal cells.

3.1. Antibacterial activity of lactoferrin against *C. sakazakii*

Based on the antibacterial activity of bovine LF, we have studied its effect against *C. sakazakii,* an emerging pathogen associated with some food products, such as powdered infant formula (Iversen & Forsythe, 2003). In particular, LF is being used as a supplement in infant milk products to mimic the composition of human milk and enhance child defences (Telang, 2018). Moreover, the presence of LF can be useful to prevent the contamination of infant formula with some pathogens, such as *C. sakazakii* (Harouna et al., 2020).

The high affinity of LF for bacterial LPS is well known and, consequently, bovine LF can be contaminated with this endotoxin, as it is isolated from milk that contains a wide variety of bacteria (Drago-Serrano et al., 2012). Therefore, although we were aware of the high quality of the LF used in our assays, the possible presence of LPS bound to LF was investigated. The level found was of 8 $x10^{-3}$ endotoxin units per mg protein, so the amount of LPS should be very low and, consequently, it did not affect the results of the assays.

In this study, the antibacterial activity of bovine LF against *C. sakazakii* was evaluated in different stages of bacterial growth. The results obtained in the exponential and in the stationary phases after 4 and 24 h of incubation with LF are shown in **Figure 1A** and **1B**.

As shown in **Figure 1**, LF exerted a clear antibacterial activity against *C. sakazakii*, which was directly proportional to LF concentration. The bacteria were more sensitive to LF in the exponential phase and after a shorter incubation time (4 h versus 24 h). When *C. sakazakii* was in the exponential phase (**Figure 1A**), treatment with LF at 1 mg/mL for 4 h caused a 1.5 logartihmic units (u.log) decrease respect to the control. Furthermore, when the concentration of LF increased to 2, 5 or 10 mg/mL, the antibacterial activity was more effective, reducing the bacterial counts by 4 u.log respect to the control. The antibacterial effect at longer incubation times (24 h) was only effective with statistically significant differences when the concentration of LF was, at least, of 5 mg/mL.

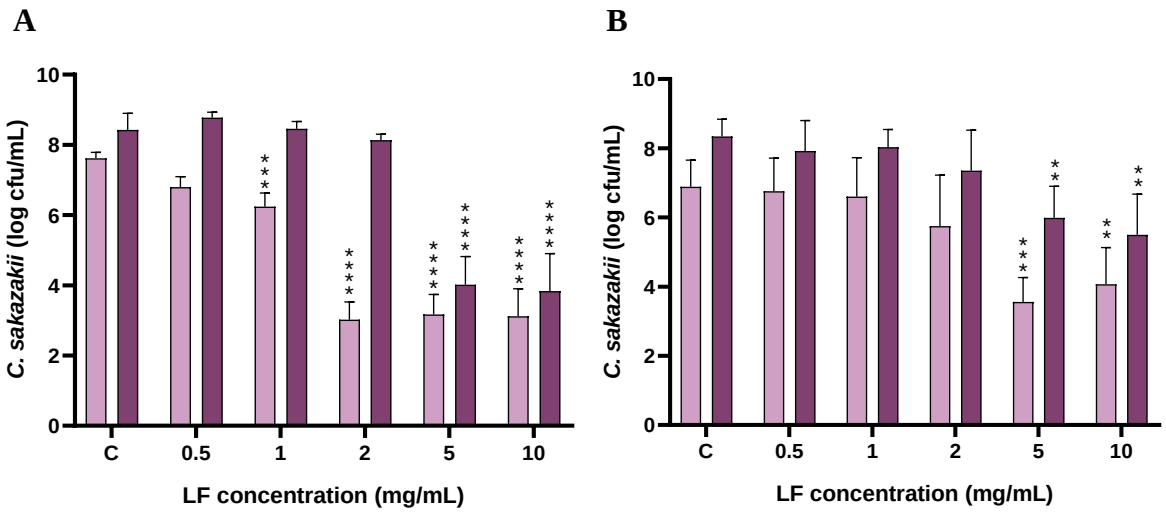


Figure 1. Antibacterial effect of bovine LF against *C. sakazakii* in two different growth stages after incubation for 4 h ( ) and 24 h ( ): (**A**) exponential phase after 8 h growth and (**B**) stationary phase after 18-20 h growth. C: control without LF. The values represent the mean ± standard deviation of two replications in three independent experiments (n = 6). Asterisks indicate significant differences respect to control (**p< 0.01, ***p< 0.001, ****p< 0.0001).

On the other hand, LF at 5 and 10 mg/mL reduced *C. sakazakii* growth in 3 and 2 u.log, respectively, when it was in the stationary phase after 4 h of incubation (**Figure 1B**). At 24 h of incubation, LF also reduced bacterial counts at the highest concentrations.

According to Embleton et al. (2013), the antibacterial activity of LF is attributed mainly to its ability to bind free iron, preventing its use by bacteria. LF also destabilises the cell

membrane of bacteria, adhering to the porins located on the surface of bacteria and causing the release of LPS, thus increasing bacterial fragility.

In our previous studies (Harouna et al., 2015, 2020), the activity of native and saturated bovine LF was evaluated against *C. sakazakii* only in the stationary phase. The results showed that native LF had a high inhibitory activity on *C. sakazakii* growth, while the iron-saturated LF did not have this effect. In addition, it was also shown that the presence of native LF in powdered infant formula was useful to minimise the growth of *C. sakazakii* when it had eventually contaminated the reconstituted formula. Our results complement these previous studies, analysing the even greater effect of native LF against *C. sakazakii* in the exponential phase of growth.

Alugupalli et al. (1995) determined the interaction between LF and *Actinobacillus actinomycetemcomitan*, a Gram-negative bacterium. They concluded that a specific interaction between LF and the bacterial outer membrane proteins occurred. They claimed that the binding of LF to bacteria was higher in the exponential phase than in the stationary phase of growth. On the other hand, in the study by Arnold et al. (1981), they also reported that the sensitivity of *Streptococcus mutans* to LF was higher in cultures at early exponential phase compared with cultures at early stationary phase, which were more resistant.

3.2. Effect of lactoferrin on Caco-2/TC7 cell viability

To carry out the different assays of antibacterial activity of LF in Caco-2/TC7 cells, it was necessary to evaluate previously the effect of LF on cell viability. This analysis allowed us to disregard the possible cytotoxic effect of LF on Caco-2/TC7 cells at the concentrations tested in the assays.

Some authors have reported that bovine or human LF promotes the proliferation of some cells, as a fibroblastic cell line (Azuma et al., 1989), bone forming cells, osteoblasts and cartilage cells (Cornish, 2004). In the study of Azuma et al. (1989), this stimulating effect of LF seems to be due to the iron carried by the molecule. However, in the study of Cornish (2004), the degree of iron saturation did not appear to influence the osteogenic behaviour. Furthermore, Hirotani et al. (2008) reported that human LF was able to reduce epithelial cell damage and tight junction opening in Caco-2 cells caused by bacterial LPS.

In addition, it has been reported that human recombinant LF is internalised by Caco-2 cells from the apical side, and subsequently localises in the nucleus (Ashida et al., 2004).

This internalisation from the apical side of Caco-2 suggests that it may be involved in iron uptake from the luminal fluid. Furthermore, the nuclear localisation of LF may indicate another function in the cells, such as modulation of intestinal function through gene regulation, as LF has been reported to bind to a specific DNA sequence (Ashida et al., 2004).

With our results, we can conclude that LF does not affect the viability of Caco-2/TC7 when incubated for a maximum time of 24 h (**Figure 2**), although a certain decrease in cell viability was observed at LF concentration of 10 mg/mL. In any case, this decrease did not present statistically significant differences respect to the control. These results agree with those of the study by Atef Yekta et al. (2010), which also confirmed that none of the bovine LF concentrations they assayed, up to 10 mg/mL, was cytotoxic to Caco- 2 cells.

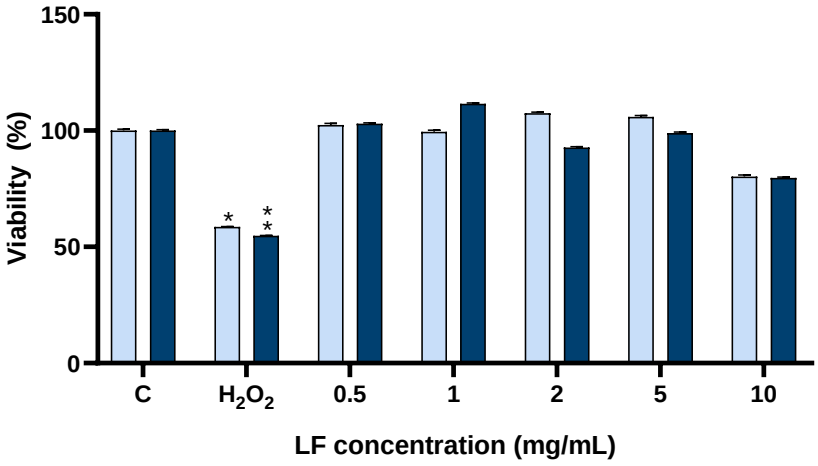


Figure 2. Viability of Caco-2/TC7 cells after 1 h (□) or 24 h (■) of incubation with bovine LF. C: Control of cells without treatment. H_2O_2: positive control of cytotoxicity consisting of cells treated with H_2O_2. The viability is expressed in percentage respect to the control that consisted of cells grown in complete medium. The values represent the mean ± standard deviation of two replications in three independent experiments (n=6). Asterisks indicate significant differences respect to control (*p< 0.05, **p< 0.01).

3.3. Effect of lactoferrin on inhibition of *C. sakazakii* internalisation by Caco-2/TC7 cells

The colonisation process of the intestine by a pathogen requires its adhesion to the host cells. After adhesion, the pathogen crosses the intestinal barrier and enters the bloodstream, causing the development of the disease (Ribet & Cossart, 2015). In the case of *C. sakazakii*, it has been reported that the outer membrane protein A (OmpA) plays an

important role in the invasion of intestinal cells by this bacterium. It seems that *C. sakazakii* interacts with the target cell through fibronectin-mediated binding. This could be a nonspecific first step, facilitating further specific interactions between bacterial ligands and host receptors (Mohan Nair & Venkitanarayanan, 2007).

In this study, we evaluated the capacity of bovine LF to inhibit *C. sakazakii* internalisation into Caco-2/TC7 cells. This cell line derives from human colon adenocarcinoma cells and, after differentiation, expresses morphological and functional characteristics of enterocytes (Sánchez et al., 1996). For this purpose, we evaluated two different incubation times of the cells with LF, 1 and 24 h, before the addition of bacterial suspension, to test if the cells had different responses depending on the time exposed to LF.

As shown in **Figure 3**, LF had an inhibitory effect on the internalisation of bacteria into Caco-2/TC7 cells. This effect was directly proportional to LF concentration when the previous incubation time of cells with the protein was 1 h. However, when the time of cell exposure to LF was increased up to 24 h, the protective effect disappeared at high concentrations of LF. A possible explanation for this effect would be that a long exposure to LF would increase the expression of LF receptors in the cells and, consequently, the binding of LF to them, thus reducing its activity against bacteria. To verify this hypothesis, the expression of LF receptor was also determined. The results indicated that only in the cells incubated with LPS, which was used as control, there was an increase in LF receptors although it was not significant; whereas the cells incubated with LF (0.5 and 10 mg/mL), with or without *C. sakazakii*, did not over-express LF receptors (results not shown). It has been reported that Caco-2 cells express a maximum level of LF receptors about 16 days of culture (Lönnerdal, Jiang, & Du, 2011). Therefore, it is possible that at the differentiation stage of our cells the level of LF receptors cannot be further increased by LF or *C. sakazakii*.

In relation to our results, Valenti et al. (1999) demonstrated that LF has the capacity to reduce significantly the invasion of intestinal Caco-2 cells by *Listeria monocytogenes*. Furthermore, both human and bovine LF have been shown to inhibit the adherence *of E. coli* to Caco-2 cells and the inhibition is dose dependent (Atef Yekta et al., 2010).

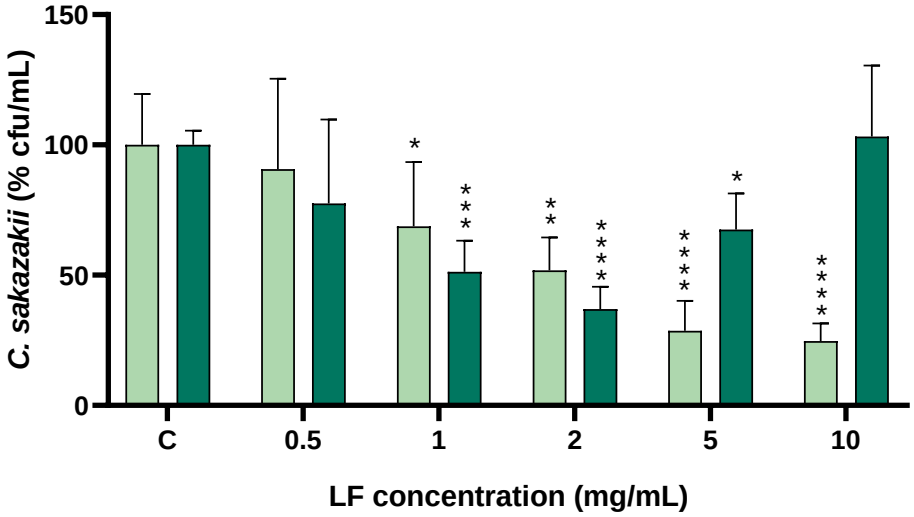


Figure 3. Inhibition of *C. sakazakii* internalization into Caco-2/TC7 cells caused by bovine LF at different concentrations at 4 h ( ) and 24 h ( ) of incubation. C: Control of cells without treatment. The growth is expressed in percentage of UFC/mL respect to the control. The values represent the mean ± standard deviation of two replications 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).

There have also been some previous studies reporting the effect of some compounds interfering in the internalisation of *C. sakazakii* into Caco-2 cells. Thus, Quintero et al. (2011) studied the inhibition of *C. sakazakii* adhesion to Caco-2 cells mediated by galactooligosaccharides of commercial origin. They observed that these oligosaccharides caused a 31% reduction in *C. sakazakii* adhesion, although the concentration used was very high (16 mg/mL), more than that usually added to supplement infant formula.

In a previous study, we observed that different dairy by-products, such as commercial skim milk, butterserum and buttermilk, and also raw buttermilk, inhibited the adhesion of *C. sakazakii* to Caco-2/TC7 cells at a level no higher than 22% (Ripollés et al., 2017). It was found that the specific activity of raw buttermilk (percentage of adhesion inhibition per mg of protein) was lower than that of commercial products. It is possible that the higher effect of commercial products was due to the heat treatment applied to them, as it can denature milk proteins, exposing their hydrophobic groups, which would interact with the surface molecules of bacteria, avoiding their binding to cells (Ripollés et al., 2017).

The inhibition of microorganism adhesion to host cells by LF has been previously studied. LF has been shown to have an inhibitory effect on the internalisation of viruses, such as human papillomavirus. This activity was more potent for bovine LF than for the human

counterpart (Drobni, Näslund, & Evander, 2004). The same authors suggested that LF had two mechanisms to prevent the adsorption of different viruses to target cells. It could bind directly to the virus (like for rotavirus, HIV or poliovirus) or it could link to heparin sulphate proteoglycans on the cell surface, interfering in both cases with the binding of the virus to host cells.

Furthermore, both apo-LF and holo-LF have been shown to have a significant capacity of inhibiting the adhesion and internalisation of *E. coli* to human epithelial cells (Longhi et al., 1993). These authors have shown that in their experimental conditions, LF bound both to the cell membrane and the bacterial outer membrane. Another study by Kawasaki et al. (2000), also confirmed that bovine LF had an adhesion-inhibiting activity against different *E. coli* strains. This capacity was tested *in vitro* on human epithelial cells (JTC-17) and *in vivo* on intestinal mucosa of mice. These authors confirmed that the inhibitory effect of LF was concentration dependent, which agrees with our results.

Finally, Quintero-Villegas, Wittke, and Hutkins (2014) performed assays evaluating the adherence of *C. sakazakii* to Hep-2 cell line. They observed that the adherence of this bacterium to those cells decreased in presence of LF. This effect was observed in a percentage of 80-99% of efficiency at a minimum LF concentration of 10 mg/mL incubated for 30 min, without additional significant effect with higher concentrations, similar to the results we have found in Caco-2/TC7 cells (**Figure 3**).

3.4. Effect of lactoferrin on oxidative stress induced by *C. sakazakii* in Caco-2/TC7 cells

Some studies have shown that the presence of pathogens, such as *Helicobacter pylori*, in the intestine can produce ROS enzymes and subsequently, oxidative stress (Ivanov, Bartosch, & Isaguliants, 2017; Naito & Yoshikawa, 2002). Furthermore, LPS, present in the outer membrane of Gram-negative bacteria, has been reported to increase the protein and lipid oxidation on cells (Latorre et al., 2014). For this reason, it is of great interest to find natural substances that can avoid or decrease cellular oxidative stress. In this study, the effect of LF was analysed on the oxidative stress possibly induced in Caco-2/ TC7 cells by a stressor as it can be a pathogen like *C. sakazakii*.

In our previous study of Buey et al. (2021), the effect of LF incubated for 24 h was evaluated on the basal oxidative stress of Caco-2/TC7 cells. When the cells were incubated with LF, it did not seem to modify by itself the lipid or protein oxidative status

of Caco-2/TC7 cells. Moreover, according to Zhao et al. (2019), LF decreases paracellular permeability and increases the activity of alkaline phosphatase and transepithelial electrical resistance, strengthening the barrier function of intestinal epithelium.

The incubation of Caco-2/TC7 cells with *C. sakazakii* caused a clear and significant increase in the oxidative stress in the lipid fraction (**Figure 4B**). The treatment of cells with LF allowed a decrease of this oxidative stress caused by the bacteria. However, this pathogen did not seem to alter the oxidative stress in proteins in the same way (**Figure 4A**), at least for the incubation time of 4 h. In any case, treatment of the infected cells with LF at low concentrations (0.5 mg/mL) reduced significantly the presence of carbonyls (**Figure 4A**). These results agree with those obtained in the study by Buey et al. (2021), in which LF showed a positive effect in reducing oxidative stress caused to the cells by LPS. However, in our study, the protective effect of LF against *C. sakazakii* was greater at lower concentration (0.5 mg/mL), while in the study by Buey et al. (2021), the concentration of 10 mg/mL was the one with greater effect reversing oxidation caused by LPS.

In the study by Liu et al. (2019), human recombinant LF showed an effect of down-regulation of some inflammatory markers and upregulation of cell proliferation when Caco-2 cells were treated with hydrogen peroxide to mimic epithelial damage during intestinal injury.

3.5. Effect of lactoferrin on TLR expression

Some studies show that oxidative stress and liberation of ROS are related with the activation of TLR pathways. In the study by Yoshino and Kashiwakura (2017), ROS induced by radiation promoted the expression of TLR2 and TLR4, while Latorre et al. (2014) showed that the activation of these receptors improved the oxidative status of intestinal epithelial cells.

Milk proteins and LF in particular, have been reported to be involved in processes of inhibition and promotion of cellular immune responses (Legrand et al., 2005). Puddu et al. (2011) demonstrated that the induction of IL-6 mediated by bovine LF was affected when TLR2 and TLR4 of human monocytes were blocked with antibodies, which indicates a critical role of LF in modulating host immune function.

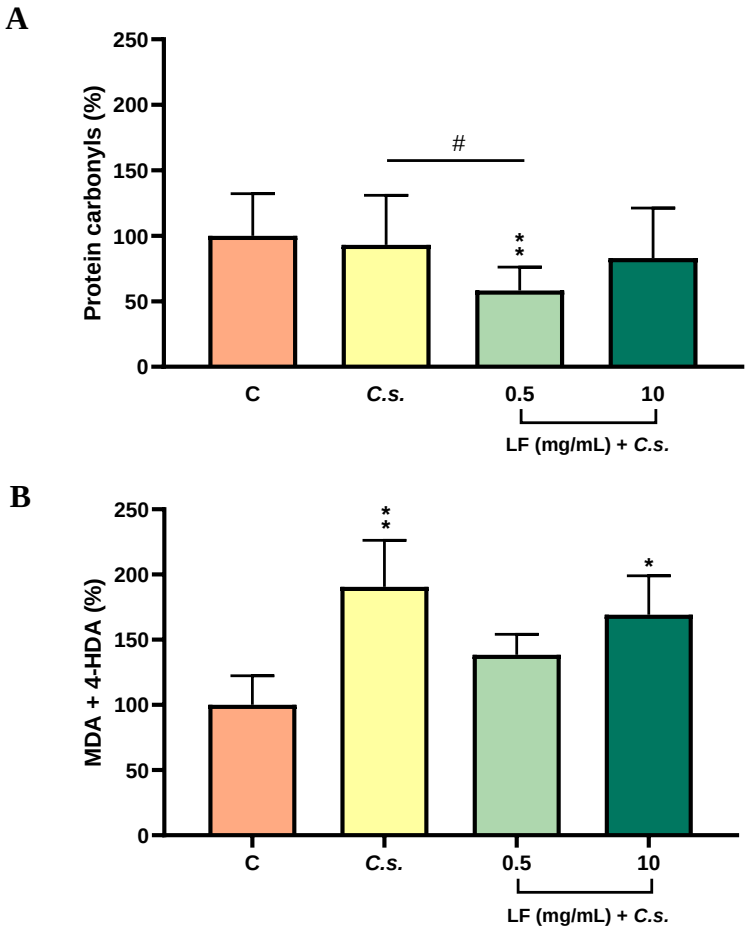


Figure 4. Effect of bovine LF on oxidative stress caused by *C. sakazakii* in Caco-2/TC7 cells. (**A**) Protein oxidation, analyzed by the measurement of carbonyl level. (**B**) Oxidative stress in lipids, determined by the concentration of MDA + 4-HDA. C: control of cells without LF or *C. sakazakii*; *C.s.*: cells treated with *C. sakazakii* without LF. The values represent the mean ± standard deviation of two replications in three independent experiments (n=6). Asterisks indicate significant differences respect to control (*p< 0.05, **p< 0.01). Hash indicate significant differentes respect to *C.s* (#p< 0.05).

In this study, the effect of LF on Caco-2/TC7 cells after incubation with *C. sakazakii* was analysed by determining the expression of TLR2, TLR4 and TLR9, using LPS as an internal and comparative control. We found that LF decreased the expression of TLR2 at short and long incubation times, being statistically significant respect to the control when LF was added at a concentration of 10 mg/mL after 1 h of incubation (**Figures 5A** and **6A**). This decrease can be explained by the fact that there was no active inflammatory process, since TLR2 is believed to have a role in ameliorating intestinal injury induced by chronic inflammatory processes (Burgueño & Abreu, 2020). However, in the presence of bacteria, differences were observed between 1 h and 24 h exposure to LF, with

significant differences observed only at short term. This effect could be due to the activation of compensation mechanisms in the 24 h incubation assay. The infection of the cells with *C. sakazakii* caused an increase in TLR2 expression, which was reversed by LF, causing a significant decrease at 10 mg/mL (**Figure 5A**).

With respect to TLR4 expression, the results obtained were different depending on the exposure times with LF, showing a marked decrease in the expression of TLR4 when the cells were incubated with LF, with or without bacteria, for 24 h (**Figure 6B**). These results agree with those obtained by Buey et al. (2021), in which LF significantly decreased the expression of this receptor after 24 h of exposure. However, this decrease was not observed when the cells were incubated with LF only for 1 h (**Figure 5B**). The results observed with LPS, used as a comparative control, showed a marked activation of TLR4 expression after 24 h of incubation, showing that a prolonged time of exposure to bacteria or to LPS endotoxin enhanced the cell response. In fact, TLR4 has been reported to recognise cellular components of bacteria, such as LPS, having a protective effect in gut injuries (Burgueño & Abreu, 2020).

In other cell types, the effect of LF reported on TLR4 expression is different from that found in our study. Thus, Figueroa-Lozano et al. (2018) showed that LF induced strong activation of TLR4, being the protein core, without attached glycans, the part of the molecule responsible for the stimulation of this receptor. In addition, Na et al. (2004) suggested that LPS was required for TLR4 signalling in macrophages and it was part of the immunomodulatory function and antimicrobial activity of LF. According to this, in the present study we found that LPS tended to increase the expression of TLR4 (**Figure 6B**). In any case, the mechanisms of LF influence on TLR4 expression can be very different depending on the cell type.

As far as TLR9 is concerned, the presence of *C. sakazakii* or LPS in the cells produced a significant decrease in the expression of this receptor (**Figure 5C**). However, when treating the cells with LF for 1 h prior the addition of bacteria, the expression of TLR9 began to increase, reversing their effect (**Figure 5C**).

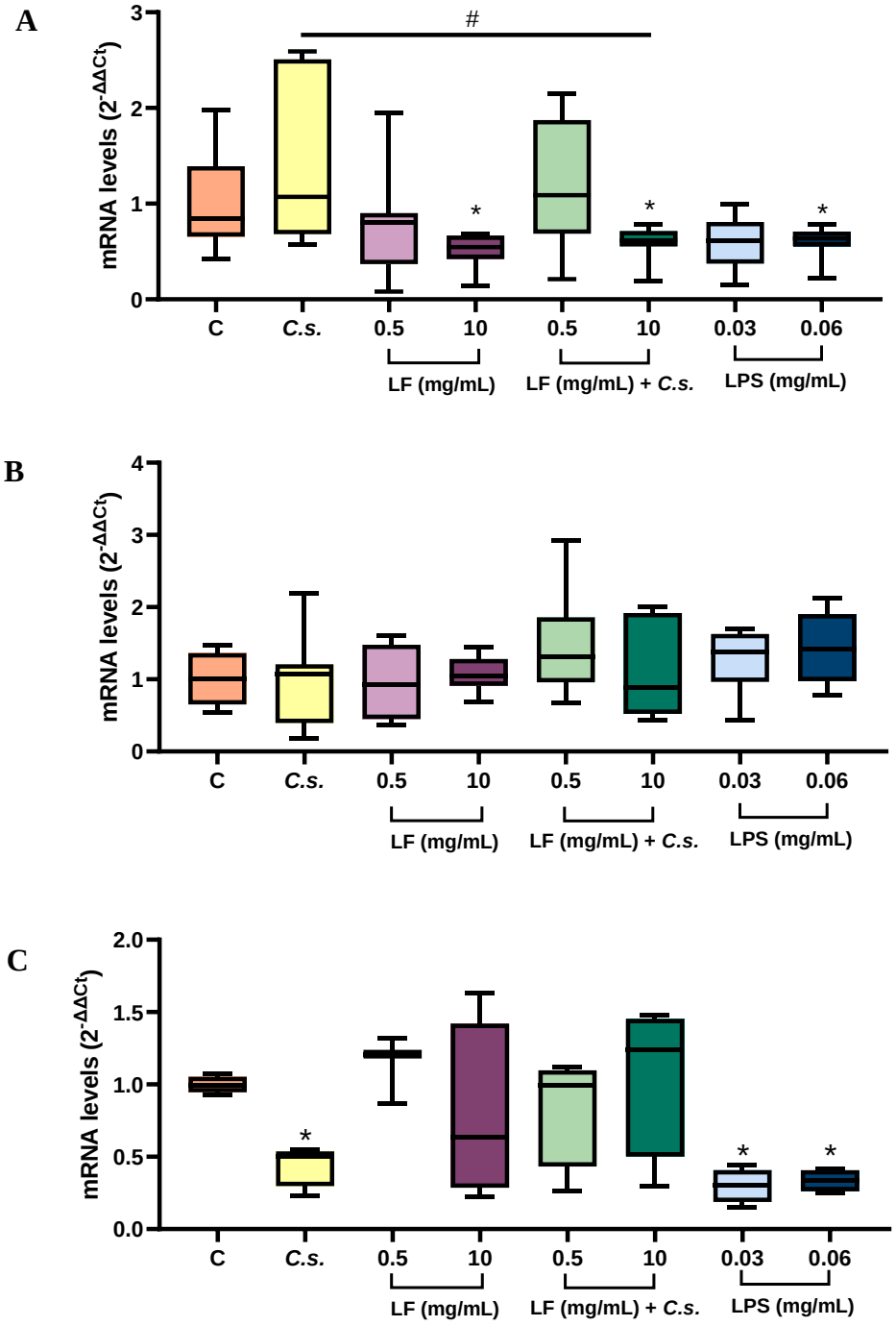


Figure 5. Effect of an incubation of 1 h with bovine LF at two concentrations (0.5 and 10 mg/mL) on the expression of (**A**) TLR2, (**B**) TLR4 and (**C**) TLR9 in Caco-2/TC7 cells. Incubation of LF with cells for 1 h. C: Control of cells without LF and without *C. sakazakii*; *C.s.*: cells with *C. sakazakii* without LF; LPS: cells with LPS without LF. The values represent the mean ± standard deviation of four experiments (n = 4). Asterisks indicate significant differences respect to control (*p< 0.05). Hash indicate significant differentes respect to *C.s* (#p< 0.05).

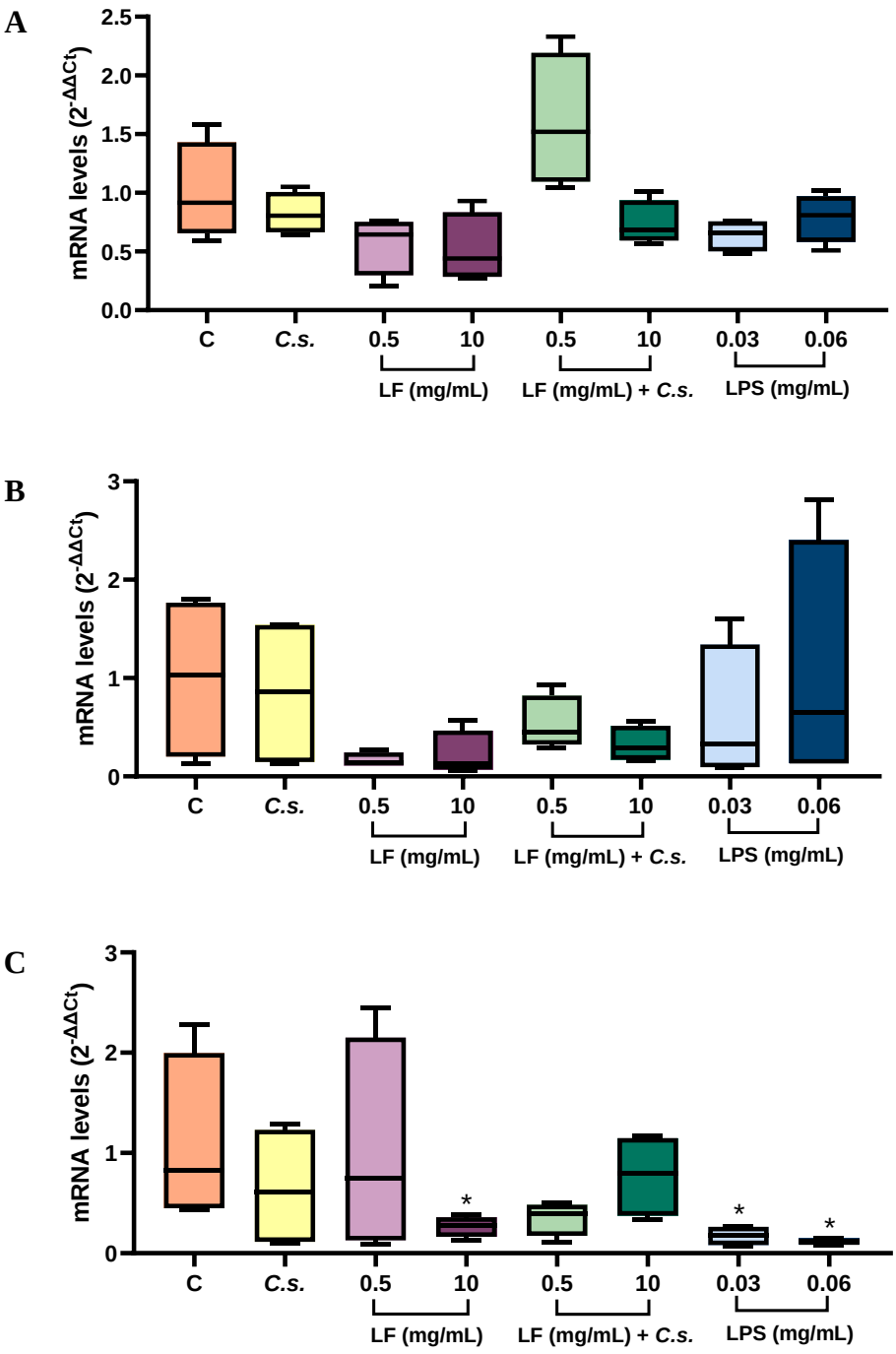


Figure 6. Effect of an incubation of 24 h with bovine LF at two concentrations (0.5 and 10 mg/mL) on the expression of (**A**) TLR2, (**B**) TLR4 and (**C**) TLR9 in Caco-2/TC7 cells. Incubation of LF with cells for 24 h. C: Control of cells without LF and without *C. sakazakii; C.s.*: cells with *C. sakazakii* without LF; LPS: cells with LPS without LF. The values represent the mean ± standard deviation of four experiments (n = 4). Asterisks indicate significant differences respect to control (*p< 0.05).

On the other hand, LF diminished significantly the expression of TLR9 in the absence of bacteria when Caco-2/TC7 cells were incubated with the highest protein concentration for 24 h (**Figure 6C**). This receptor plays an important role in one of the main pathways responsible for anti-inflammatory effects, being able to neutralise the inflammatory signals induced by other TLRs (Vijay, 2018). In the study by Buey et al. (2021), LF induced a slight increase in the expression of TLR9, which might help in the anti-inflammatory effects.

4. Conclusions

Milk is a source of bioactive proteins, LF among them, which is considered a multifunctional protein, with very interesting properties to be a potential ingredient for functional foods and in special for protecting the newborn.

The results derived from this study indicate that LF is a dairy protein with a potential protective effect against *C. sakazakii*. This pathogen has been mainly associated with the consumption of infant formula and dairy powders. It interacts with intestinal epithelial cells, causing intestinal inflammation and villus alterations. We have found that LF presents antibacterial activity in both exponential and stationary phases of bacterial growth.

Furthermore, LF inhibits the internalisation of the pathogen into Caco-2/TC7 cells, a model of human intestinal cells. This study has also shown the effect of LF reversing the oxidative stress caused in the lipid fraction of cells infected with *C. sakazakii*. Additionally, LF modifies the expression of TLR and in particular decreases the overexpression of TLR2 caused by the pathogen when the cells are incubated for 1 h with LF previously to the infection with *C. sakazakii*. This study suggests that LF has a great value and can be a potential ingredient for functional foods. However, further experiments should be performed to improve our knowledge about the influence of dairy proteins, and LF in particular, on the innate immune system.

Credit author statement

Inés Abad: Writing - original draft preparation, Methodology, Investigation, Data curation; Andrea Sangüesa: Methodology, Investigation, Software, Data curation; María Ubieto: Methodology, Investigation, Software, Data curation; Juan J. Carramiñana: Conceptualization, Methodology; María D. Pérez: Writing - review & editing, Reviewing and Editing; Berta Buey: Methodology; José E. Mesonero: Methodology, Writing -

review & editing, Reviewing and Editing; Laura Grasa: Methodology, Writing - review & editing, Reviewing and Editing; Lourdes Sánchez: Conceptualization, Supervision, Writing - review & editing, Reviewing and Editing.

Declaration of competing interest: None.

Data availability: Data will be made available on request.

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ARTÍCULO 2

Dairy by-products and lactoferrin exert antioxidant and antigenotoxic activity on intestinal and hepatic cells

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Dairy By-Products and Lactoferrin Exert Antioxidant and Antigenotoxic Activity on Intestinal and Hepatic Cells

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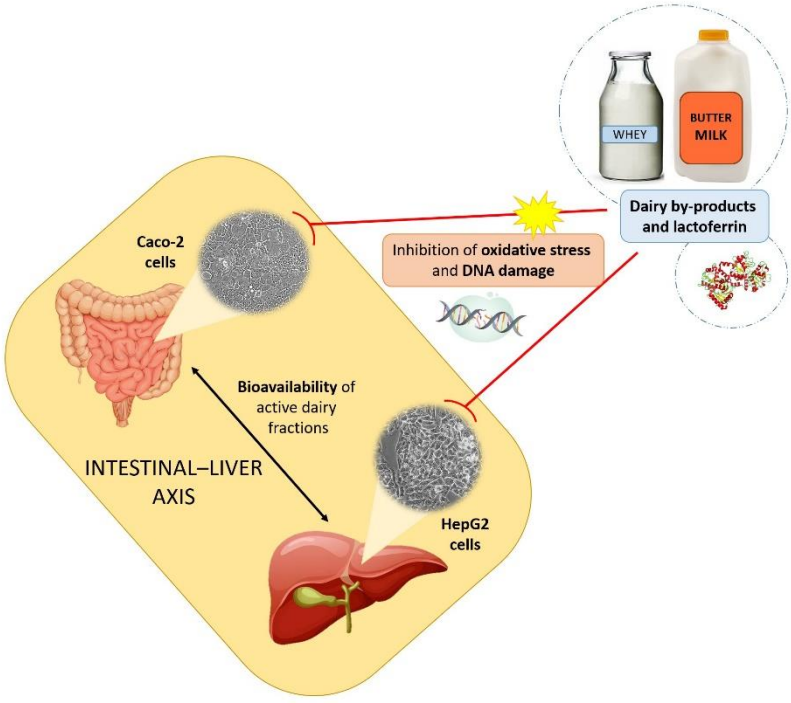
Abstract

The dairy industry generates a large volume of by-products containing bioactive compounds that may have added value. The aim of this study was to evaluate the antioxidant and antigenotoxic effects of milk-derived products, such as whey, buttermilk, and lactoferrin, in two human cell lines: Caco-2 as an intestinal barrier model and HepG2 as a hepatic cell line. First, the protective effect of dairy samples against the oxidative stress caused by menadione was analyzed. All these dairy fractions significantly reversed the oxidative stress, with the non-washed buttermilk fraction presenting the greatest antioxidant effect for Caco-2 cells and lactoferrin as the best antioxidant for HepG2 cells. At concentrations that did not impact cell viability, we found that the dairy sample with the highest antigenotoxic power against menadione, in both cell lines, was lactoferrin at the lowest concentration. Additionally, dairy by-products maintained their activity in a co-culture of Caco-2 and HepG2, mimicking the intestinal-liver axis. This result suggests that the compounds responsible for the antioxidant activity could cross the Caco-2 barrier and reach HepG2 cells on the basal side, exerting their function on them. In conclusion, our results show that dairy by-products have antioxidant and antigenotoxic activities, which would allow revaluing their use in food specialties.



1. Introduction

The dairy industry generates a large volume of by-products whose current destination is mainly to obtain technological ingredients or become supplements for animal feeding. Considering that milk and its by-products, such as whey and buttermilk, contain proteins and other compounds with biological properties (Svanborg et al., 2015), their isolation would give them extra value and an additional source of income for the dairy industry.

Milk proteins are recognized as the main source of biologically active peptides, some displaying beneficial activities, such as antihypertensive, antidiabetic, antioxidant, immunomodulatory, and mineral binding properties (Lin et al., 2018). In addition, milk anti-inflammatory properties can be attributed to the presence of phospholipids and short- or medium-chain saturated fatty acids (Cichosz, Czeczot, & Bielecka, 2020).

Whey is obtained during cheese or casein manufacturing, and it has long been considered of little value. Nowadays, whey is used for animal feed in liquid form and as an ingredient in the food industry in dried form. The global production rate of whey in 2016 was estimated at 200 million tones (MT) per year, showing an annual increase of 3% (Domingos et al., 2018). Only in Europe were 57 MT of whey produced per year (Eurostat, 2021).

Whey contains about 20% of milk proteins, about 8% of fat, and 70% of lactose, making it an important source of nutrients (Torres et al., 2009). The protein components of whey include β-lactoglobulin, bovine serum albumin, α-lactalbumin, immunoglobulins, lactoferrin, lactoperoxidase, other enzymes, and glycomacropeptide (Kerasioti et al., 2014). Whey proteins remain soluble after acid or enzymatic coagulation of milk, which distinguishes them from caseins that precipitate under these conditions (Farkye & Shah, 2014). Whey proteins have excellent functional properties, such as good solubility, viscosity, emulsifying, and gelling properties, and they are mainly responsible for the high nutritional and technological value of whey (Barukčić, Lisak Jakopović, & Božanić, 2019). Thus, whey concentrates and isolates are widely used in the food industry (Mollea, Marmo, & Bosco, 2013). A few years ago, there was an increased interest in some bioactive whey proteins and peptides, as they have health benefits and can be used as ingredients in functional foods (Madureira et al., 2007; Solak & Akin, 2012). The high content of proteins in whey is responsible for the high nutritional and technological value of this by-product (Barukčić, Lisak Jakopović, & Božanić, 2019). Among them,

lactoferrin (LF) is one of the main defensive proteins. LF is a cationic glycoprotein belonging to the transferrin family, with iron-binding capacity and numerous properties, such as antimicrobial activity and the ability to protect cells from oxidative stress (García-Montoya et al., 2012). LF may decrease the production of intracellular reactive oxygen species (ROS) or suppress the senescence of cells induced by hydrogen peroxide (Park et al., 2017; Kruzel, Zimecki, & Actor, 2017). In 2012, bovine LF was allowed as a novel food ingredient under Regulation (EC) Nº 258/97. This regulation specified that bovine LF, as a protein that occurs naturally in cow's milk, could be placed on the market as a novel food ingredient. In addition, this regulation establishes the limits for its use in different food categories, such as infant and follow-on formulas, beverages based on milk, etc. For infant formulas, the maximum use level proposed is 1 mg/mL, and for drink mixes based on milk, the indicated level is between 200 and 330 mg/100 g, depending on whether the product is in liquid or powder form (European Commission, 2012). The broad properties of LF on human health have been reviewed recently (Cao et al., 2022; Kowalczyk et al., 2022).

Buttermilk (BM) is another dairy by-product released by butter manufacturing. Estimating the environmental impact through a life cycle assessment methodology, it has been observed that BM does not influence the impact caused by the butter manufacturing process in as high a proportion as whey does in the cheese production process (Santos et al., 2022). For many years, BM has been undervalued, although some studies carried out in the last decade have shown its potential, especially due to the proteins and lipids present in the milk fat globule membrane (MFGM) (Vanderghem et al., 2010). MFGM is particularly rich in proteins and phospholipids and has great potential for functional and nutraceutical applications (Barukčić, Lisak Jakopović, & Božanić, 2019). During churning in butter making, milk fat globules are disrupted, allowing the release of the fat enveloped by the MFGM and the phase inversion with the release of BM as the aqueous phase. BM is similar to whey in lactose content but contains a higher amount of protein (Vanderghem et al., 2010). Furthermore, BM contains a high amount of fat, with its phospholipid concentration being seven times higher than that of whole milk (Libudzisz & Stepaniak, 2002). Considering the large volume of whey produced annually, BM derived from butter elaborated with whey fat has a huge potential market. In addition, BM has emulsifying properties due to the presence of caseins and considerable amounts

of phospholipids, making it suitable for technological application in the food industry (Ali, 2019).

Seventy-one percent of all the whole milk available in European Union dairy is used for the manufacture of cheese and butter (Eurostat, 2021), which generates a large collateral production of whey and buttermilk. Numerous studies have clearly presented the valuable influence of whey, LF, and BM on human health and well-being. These fractions and some of their proteins have antimicrobial, antioxidant, antihypertensive, antidiabetic, or immunomodulatory properties (Brandelli, Daroit, & Corrêa, 2015; Buey et al., 2021; Egger & Ménard, 2017; Harouna et al., 2020). The antioxidant, antimicrobial, and anticarcinogenic activities of bovine milk proteins, as well as the properties of their hydrolysates, have been reviewed recently (Bielecka, Cichosz, & Czeczot, 2022). With all this information gathered, it can be expected that the dairy fractions show some activity against reactive oxygen species and exert a protective role on DNA. The aim of this study was, therefore, to evaluate the antioxidant and antigenotoxic effects of milk-derived products, such as whey and BM, and compare them to LF, a milk protein with known antioxidant properties, in a hepatic and a colon carcinoma cell lines, simulating the conditions of the liver-intestine axis. In this study, we show evidence to consider milk fractions as potential antioxidants after oxidative stress caused by menadione. Consequently, this work intends to add value to the dairy by-products beyond their current applications as animal feed or complementary technological ingredients.

2. Materials and methods

2.1. Chemicals and preparation of milk samples

Calicheamicin (Pfizer, France) is a cytotoxic agent that causes double-strand DNA breaks, used as a positive control for viability assays and genotoxicity studies. Menadione (Sigma-Aldrich, St. Louis, MO, USA) was used to induce oxidative stress and N-acetylcystein (Sigma-Aldrich) as an antioxidant.

The dairy fractions used in this study for evaluating their bioactivity were obtained by different processes, as follows:

Raw bovine milk was supplied by the dairy company Villacorona (El Burgo de Ebro, Spain). The quality of milk was verified after reception by checking the pH, acidity, fat percentage, and alkaline phosphatase and lactoperoxidase activities, and it was processed as explained in a previous study (Abad et al., 2022a) at the Food Science and Technology

Pilot Plant of the University of Zaragoza, located in the Veterinary Faculty. The whey and BM obtained were lyophilized and kept at -20 °C for later use. After freeze-drying, the concentration of whey obtained was 0.068 g of dry matter per mL and that of BM was 0.029 g of dry matter/mL. 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 121.8 mg of protein per g of BM.

In the process of making BM, a step of cream washing was included to reduce the content of milk proteins, based on the method described by Le et al. (2009). Cream obtained from raw milk was washed twice with four volumes of phosphate-buffered saline (PBS), composed of 140 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4, and 1 mM ethylenediaminetetraacetic acid (EDTA), centrifuging between each wash at 3000× *g* for 15 min at 4 °C. Washed cream was stirred to obtain butter grains, and washed buttermilk (WBM) was obtained, lyophilized, and kept at -20 °C.

Bovine LF was kindly donated by Tatua Nutritionals (Morrinsville, New Zealand). This LF was used in our previous assays, in which iron saturation, purity, and content of LPS were determined (Abad et al., 2022b).

All samples were diluted in PBS to obtain a concentration of 40 mg/mL and filtered with 0.22 µm low-binding protein Millipore filters (Merck, Darmstad, Germany). The final concentrations of LF, whey, and BM used in the assays were 10, 5, 2, 1, and 0.5 mg/mL. All samples were tested in duplicate in each assay.

2.2. Cell culture

The cell lines used in this study were HepG2, a human liver cancer cell line, and Caco-2, a human colon carcinoma cell line, both obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HepG2 cells were maintained with Eagle's Minimum Essential Medium (MEM) (Thermo Fisher Scientific, Rockford, IL, USA), supplemented with 10% (*v*/*v*) fetal bovine serum (FBS) (Thermo Fisher Scientific), 1% (*v*/*v*) antibiotics (penicillin 10,000 units/mL-streptomycin 10 mg/mL, Thermo Fisher Scientific), and 1% (*v*/*v*) 200 mM L-glutamine (Thermo Fisher Scientific). Caco-2 cells were maintained with Dulbecco's Modified Eagle's Medium (DMEM) with glutamine (Thermo Fisher Scientific) supplemented with 20% (*v*/*v*) FBS and 1% (*v*/*v*) antibiotics. Caco-2 cells were differentiated for 17 days as previously described (Sánchez et al., 1996).

The cells were cultured at 37 °C in a 5% CO_2 humidified atmosphere. The medium was changed every 2-3 days for the maintenance of cells.

2.3. Bioactivity assays

2.3.1. Cell viability assay

The CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA) was performed to evaluate any cytotoxic effect of dairy samples on Caco-2 and HepG2 cell lines. For each cell line, 96-well plates were seeded at a density of 15,000 cells/well in the case of HepG2 and 30,000 cells/well in the case of Caco-2. After two days of growth of HepG2 and after differentiation of Caco-2, cells were treated with dairy samples (LF, whey, WBM, and non-WBM) at different concentrations (10, 5, 2, 1, and 0.5 mg/mL) or 5 µM calicheamicin as a positive control of cytotoxicity for 24 h (100 µL/well). The CellTiter-Glo® Luminescent Cell Viability Assay was performed according to the manufacturer's instructions. Luminescence was measured using the Infinite® 200 PRO instrument (Tecan, Männedorf, Switzerland). All samples were analyzed in duplicate in three independent viability assays.

2.3.2. Oxidative stress assay

The protective effect of dairy samples against oxidative stress was analyzed. In this case, 50 µM menadione was used as an oxidizing agent. To measure oxidative stress in living cells, CellRox® Green Reagent (Thermo Fisher Scientific) was used, which is a fluorogenic probe. The dye penetrates cells and is not fluorescent in its reduced state but exhibits green photostable fluorescence after oxidation by ROS.

The cells were seeded at a density of 300,000 cells/mL on coverslips inserted in 24-well plates. After two days of growth for HepG2 and after differentiation for Caco-2, cells were incubated for 1 h with the four dairy samples at different concentrations (5, 1, and 0.5 mg/mL) or with 1 mM N-acetylcysteine (NAC) as a control of antioxidant activity. After the incubation time, 50 µM menadione was added for 1 h and then CellRox® Green Reagent was added for 30 min. Cells were then washed and fixed with 4% paraformaldehyde (PFA). After three washes in PBS, including 30 nM DAPI to stain the cell nuclei in the last wash, coverslips were mounted and observed under a Nikon 50i fluorescence microscope (NIKON, Tokyo, Japan) equipped with a Luca S camera (**Figure S1A - S1C**). The green reagent signal intensity of each nucleus was quantified with an ImageJ macro of the image analysis software ImageJ 1.52p (National Institutes

of Health, Bethesda, MD, USA). The green reagent signal intensity of the whole cell population was averaged for each condition. Results were normalized to 1 for the untreated condition. For each experiment, 150-200 cells were counted, and three independent experiments were performed.

2.3.3. Genotoxicity assay

Genotoxicity was assessed by measuring the level of gamma-H2AX (the phosphorylated form of H2AX at serine 139 that is induced after activation of the DNA damage response, noted γ-H2AX) as described previously (Pons et al., 2019). Briefly, the same process as above was carried out, using NAC as an antigenotoxic control (**Figure S1D - S1F**). After fixation with PFA, cells were incubated for 1 h at room temperature with the γ-H2AX antibody (05-636, Sigma-Aldrich) in a dilution of 1/1000 (*v*/*v*). Subsequently, the cells were washed three times with PBS and incubated with the secondary antibodies (Alexa Fluor 546 goat anti-mouse, Thermo Fisher Scientific) diluted 1/800 (*v*/*v*) for 40 min at room temperature. Cells were washed three times with PBS, and 30 nM DAPI dye was added to the last wash to stain nuclei. Coverslips were mounted onto slides with PBS-glycerol (90%) containing 1 mg/mL paraphenylenediamine and observed under a Nikon 50i fluorescence microscope equipped with a Luca S camera, analyzing the intensity of fluorescence through ImageJ macro as described above. γ-H2AX signal intensity of each nucleus was automatically determined by an ImageJ macro. γ-H2AX signal intensity was averaged for each condition, and these results were normalized to 1 for the untreated condition. For each experiment, 150-200 cells were counted, and three independent experiments were performed.

2.3.4. Bioavailability assay

To assess bioavailability, a system of co-culture of Caco-2 and HepG2 cell lines was carried out using a 24 well plate with standard transwell inserts (Corning Incorporated, New York, NY, USA) in order to mimic the intestinal-liver axis. The Caco-2 cells were seeded and grown on transwell inserts (3000 cells/transwell). Once differentiated (**Figure S2**), Caco-2 cells were adapted to MEM HepG2 medium, and HepG2 cells were seeded on coverslips (180,000 cells/well), associating the two cell lines (**Figure S3**).

After two days of co-culture, Caco-2 cells were exposed to dairy samples (LF, whey, WBM, and non-WBM) at final concentrations of 5 and 1 mg/mL and NAC (final concentration of 1 mM) and incubated at 37 °C for 24 h. Subsequently, 50 µM menadione was added for 1 h to both Caco-2 and HepG2 cells. The CellRox Green Reagent was then

added to both cell lines at a final concentration of 5 µM for 30 min. Afterwards, the medium was collected from the upper and lower chambers and stored at -80 °C for further analysis. Immunofluorescence was performed as described previously (Gillois et al., 2021), with minor changes. Briefly, cells were washed and fixed with 4% PFA, permeabilized with 0.5% Triton X-100, and blocked with 0.1% PBS-NP40 with 3% bovine serum albumin (BSA) for 45 min. Cells were then incubated with γ-H2AX primary antibody at a 1/1000 dilution to measure genotoxicity in the two cell lines, as detailed in the previous section. For HepG2 cells grown on coverslips, the primary antibody was incubated for 1 h at room temperature. For Caco-2 cells grown in the transwell, the primary antibody was incubated overnight at 4 °C. The secondary antibody, labeled with Alexa 546, was incubated at room temperature at a 1/800 dilution for 40 min for HepG2 cells and for 1 h for Caco-2 cells. Subsequently, cells were stained with DAPI and rinsed, then coverslips with HepG2 cells or membranes cut out from the transwell inserts with Caco-2 cells were mounted and observed under the fluorescence microscope to quantify the oxidative stress and genotoxicity, as described above. For each experiment, 150-200 cells were counted, and three independent experiments were performed.

2.3.5. Proteomic analysis

To assess the possible passage of LF from the upper to the lower compartment of the transwell through the Caco-2 cell barrier, proteomic analyses were performed (Proteomics Platform of Servicios Científico Técnicos of CIBA, IACS-Universidad de Zaragoza). For controlled quantification of proteins, the Selected Reaction Monitoring (SRM) technique was used. The digestion of samples was carried out in solution, starting with a sample of approximately 40 µg of protein. For this, 10 µL of denaturing buffer (6 M urea, 100 mM TRIS buffer, pH 7.8) was added. Cysteines were then reduced by adding 1.5 µL of 200 mM dithiothreitol for 30 min at 37 °C and alkylated with 6 µL of 200 mM iodoacetamide for 30 min in darkness. Afterwards, the samples were diluted with 50 mM ammonium bicarbonate to reach a final concentration of less than 1 M urea. Digestion was carried out overnight with trypsin (Gold Trypsin, Promega) at 37 °C in a 1:20 ratio (enzyme/protein). The reaction was stopped the next day by adding concentrated formic acid (Sigma-Aldrich).

The development of the SRM method for the quantification of the proteins of interest was carried out using Skyline software (MacCoss Lab Software, Seattle, WA, USA, version

20.2.0.343). SRM analyses were performed in a hybrid triple quadrupole-linear ion trap mass spectrometer (6500QTRAP+, Sciex, Foster City, CA, USA) coupled to a nano/micro-HPLC (Eksigent LC425, Sciex).

2.4. Statistical analysis

The results are presented as the mean ± standard deviation. The statistical analysis of the results was performed using the statistical software GraphPad Prism v8.0.2 (GraphPad Software, San Diego, CA, USA). The normality of the data was checked with the Shapiro-Wilk test. To compare the means of three or more unpaired groups, an analysis of variance (ANOVA) was performed, and Dunnet's test was used as a multiple comparison test. Data that did not follow a normal distribution were submitted 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. Effect of dairy fractions on viability of Caco-2 and HepG2 cells

First, we carried out viability assays on Caco-2 and HepG2 cell lines in order to identify the concentrations that may have a cytotoxic effect. Calicheamicin, a cytotoxic agent that causes DNA double-strand breaks, was used as a positive control at 5 µM.

The results obtained showed that LF, whey, and non-WBM did not affect the Caco-2 cells viability when incubated for a maximum of 24 h. However, WBM induced some cell toxicity, although without going below 88% viability (**Figure 1A**).

In the case of HepG2 cells, a dose-dependent response was observed for whey and non-WBM, with a significant decrease (about 30%) in cell viability for the highest concentration (10 mg/mL) when compared to non-treated cells. Surprisingly, no significant decrease was observed for WBM, in contrast to Caco-2 cells. In addition, there was also no decrease in cell viability with LF at concentrations from 1 to 5 mg/mL (**Figure 1B**). Some authors reported that LF may promote the proliferation of some cells, such as bone-forming cells, osteoblasts, and cartilage cells (Cornish, 2004). It was thought that LF supported cell proliferation due to its ability to transport iron into cells. Moreover, LF has been shown to act as a growth factor activator. Actually, the effect of LF on intestinal epithelial cells has been reported to be more potent than that of epidermal growth factor (Habib et al., 2013).

Due to a decrease in viability observed with some samples, the highest concentration of 10 mg/mL was suppressed for the rest of the tests.

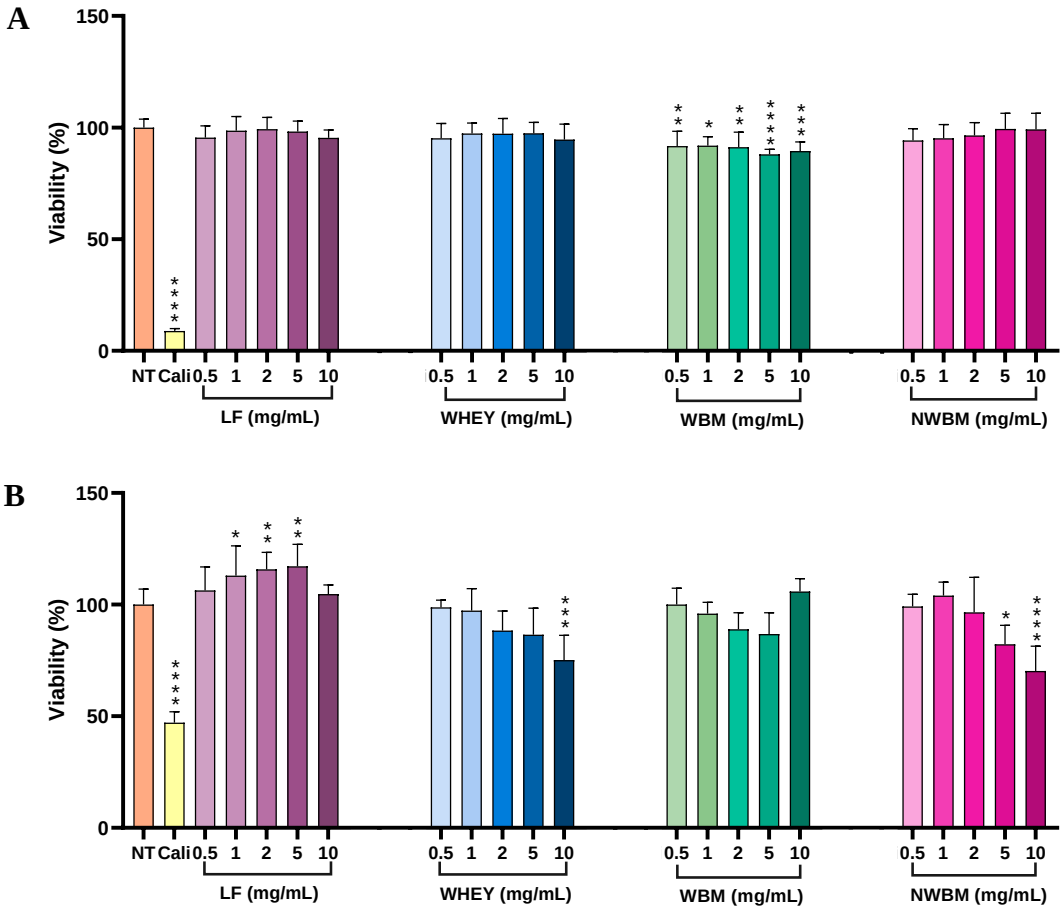


Figure 1. Viability of (**A**) Caco-2 and (**B**) HepG2 cells after 24 h of incubation with dairy fractions. NT: non-treated cells. Cali: positive control of cytotoxicity consisting of cells treated with 5 µM calicheamicin. LF: lactoferrin. WBM: washed buttermilk. NWBM: non-washed buttermilk. The viability is expressed in percentages with respect to NT. The values represent the mean ± standard deviation of two replicates in three independent experiments (n = 6). Asterisks indicate significant differences respect to NT (*p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001).

3.2. Effect of dairy fractions on oxidative stress caused by menadione on Caco-2 and HepG2 cells

Oxidative stress is the result of an imbalance between ROS production and the cells' ability to eliminate these reactive species. Organisms have endogenous antioxidant defense mechanisms to deal with oxidative stress (Kerasioti et al., 2014). It may also be important to provide an exogenous source of antioxidants to complement the endogenous activity. Thus, it was shown that a high dairy diet in mice resulted in a decrease in ROS and malondialdehyde (Zemel & Sun, 2008).

To evaluate the effect of the different dairy fractions on oxidative stress, cells were treated with menadione as an oxidizing agent. In this assay, the fluorescent signal given by the probe that detected oxidative stress was measured. After 1 h of treatment with menadione, the fluorescent signal was highly increased for both Caco-2 cells (**Figure 2A**) and HepG2 cells (**Figure 2B**). In contrast, pre-incubation with 1 mM NAC before the menadione treatment impeded the oxidative stress response, inducing a strong decrease in the level of oxidative stress (**Figure 2**). Interestingly, all the dairy products tested significantly reversed the oxidative stress caused by menadione on Caco-2 (**Figure 2A**) and HepG2 (**Figure 2B**) cells.

In Caco-2 cells, whey showed a dose-dependent protective effect (**Figure 2A**); furthermore, dairy fractions at a concentration of 1 mg/mL (whey, WBM, and non-WBM) showed a stronger antioxidant effect compared to LF, suggesting the presence of an antioxidant compound in these fractions. In HepG2 cells, this relationship with the amount of by-product added to the cells was not observed **(Figure 2B**), for which the whey concentration of 0.5 mg/mL decreased oxidative stress more than the 1 mg/mL concentration. However, it is noteworthy that the highest concentration of whey (5 mg/mL) showed the greatest effect in both cell lines. It is well known that some whey components, such as α-lactalbumin, have antioxidant activity, producing a reduction in oxidative stress due to their iron chelating-properties. In addition, whey contains sulphur-rich amino acids, which may also improve antioxidant defense (Kerasioti et al., 2014).

The highest concentration of non-WBM (5 mg/mL) did not reverse oxidative stress as properly as lower doses of non-WBM (0.5 and 1 mg/mL) in HepG2 cells (**Figure 2B**), when compared to Caco-2 cells (**Figure 2A**). This could be the result of cell cytotoxicity induced in HepG2 cells at this concentration, as shown previously (**Figure 1B**). In the case of the WBM fraction, all the concentrations tested had a protective effect on Caco-2 and HepG2 cells from the stress caused by menadione.

Overall, the dairy by-product with the highest antioxidant activity on Caco-2 cells was the non-WBM at a concentration of 1 mg/mL, decreasing the effect of menadione by 11-fold compared to the menadione control. This result suggests strong antioxidant activity in this dairy fraction. A similar effect was shown by WBM at the same concentration, with a 10.8-fold decrease in respect to menadione (**Figure 2A**). However, in HepG2 cells, the greatest antioxidant effect was shown with 1 mg/mL of LF (**Figure 2B**), which reduced menadione stress by 8.4-fold, almost as much as NAC (9-fold decrease over

menadione control). These results were satisfying since the accepted dose for the addition of LF to food is around 1 mg/mL (European Commission, 2012). On HepG2 cells, whey and non-WBM at 1 mg/mL reduced menadione stress by 5.9 and 6-fold, respectively.

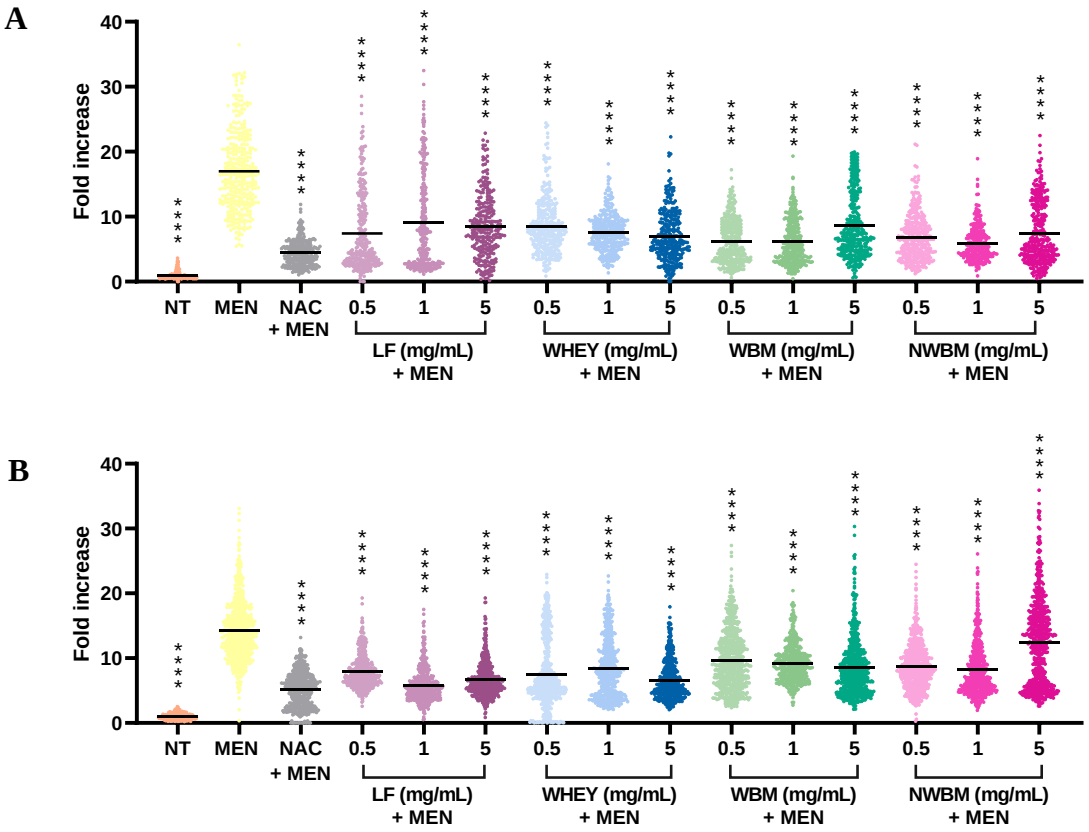


Figure 2. Effect of dairy fractions on oxidative stress caused by menadione on (**A**) Caco-2 and (**B**) HepG2 cells. NT: non-treated cells. MEN: positive control of oxidative stress, cells treated with 50 µM menadione for 1 h. NAC + MEN: cells treated for 1 h with 1 mM NAC as a control of antioxidant effect before menadione treatment. LF: lactoferrin. WBM: washed buttermilk. NWBM: non-washed buttermilk. The results were normalized to 1 for the untreated condition. The values represent the intensity of 150-200 cells counted in each experiment, on three independent experiments. The bars represent the means of all counts. Asterisks indicate significant differences respect to MEN (****p< 0.0001).

In the study by Buey et al. (2021), the effect of these dairy by-products on oxidative stress caused by the lipopolysaccharide (LPS) present in the membranes of Gram-negative bacteria was analyzed. They showed that both whey and LF had antioxidant power, reducing lipid oxidation and protein damage caused by LPS. In the same study, BM did not seem to reduce the oxidative stress caused by LPS, while in our study this dairy by-product did protect cells against the oxidative effect of menadione. Furthermore, Prakash et al. (2022) affirmed that protein hydrolysates from whey and BM contain antioxidant

agents and enhancers of phagocytic activity in macrophages. As in our experimental set-up, the dairy fractions were used without hydrolysis, so proteins may not enter the cells. However, proteins may pass through Caco-2 cells through the paracellular route or via transcytosis (Ballegaard & Bøgh, 2022). In addition, some specific transport mechanisms may be involved, such as intelectin 1, a LF-receptor, expressed in Caco-2 cells (Akiyama et al., 2013). It has also been shown that LF binds to the asialoglycoprotein receptor (ASGPR) in rat liver (McAbee, Bennatt, & Ling, 1998). Yet, small molecules such as amino acids (Bielecka, Cichosz, & Czeczot, 2022) or other small metabolites—to be identified—could explain the antioxidant effect observed here. Further studies are needed to characterize these compounds.

3.3. Effect of dairy fractions on genotoxicity caused by menadione on Caco-2 and HepG2 cells

Human cells are continuously exposed to physiological and external influences that can cause cytotoxic, oxidative, and genotoxic damage. When an imbalance occurs in the dynamics between ROS generation and antioxidant systems, oxidative damage to all cellular targets, including DNA, arises (Markkanen, 2017). HepG2 cells are capable of performing oxidative metabolism of nutrients, metabolites, and xenobiotics. Furthermore, the intestine is exposed to high levels of ingested oxidants, and Caco-2 cells show many functions similar to those of enterocytes (O'Brien et al., 2000).

Therefore, we have analyzed the protective effect of dairy by-products against DNA damage indirectly caused by menadione after the generation of reactive species that finally generate DNA damage. To evaluate the effect of the different dairy fractions on a genotoxic stress, we again used menadione treatment as a positive control. Under these conditions, after immunostaining, we quantified the fluorescent signal due to γ-H2AX, a classic biomarker of DNA damage. We observed a strong induction of DNA damage after the menadione treatment (**Figure 3**). NAC used as an antioxidant allowed the reversion of the γ-H2AX signal, indicating that DNA damage was caused indirectly through oxidative stress (**Figure 3**). Overall, the dairy products significantly reversed the genotoxic stress caused by menadione on Caco-2 (**Figure 3A**), with whey showing the same dose-dependent effect as observed in the antioxidant capacity analysis (**Figure 2A**).

The dairy sample with the highest antigenotoxic activity in Caco-2 cells was non-WBM at low concentrations, followed by LF at 0.5 mg/mL (below the maximum allowable dose as detailed in Regulation (EC) Nº 258/97, European Commission (2012)) and WBM at 1 mg/mL (**Figure 3A**), reducing the genotoxic effect of menadione by about 7.3-fold. For HepG2 cells, the greatest DNA protector was LF at 0.5 mg/mL (**Figure 3B**) with an effect inversely proportional to the concentration. The same dose-response relationship was shown by WBM. Altogether, our results suggest that some antioxidant compounds present in the tested dairy fractions may help prevent DNA lesions associated with oxidative stress.

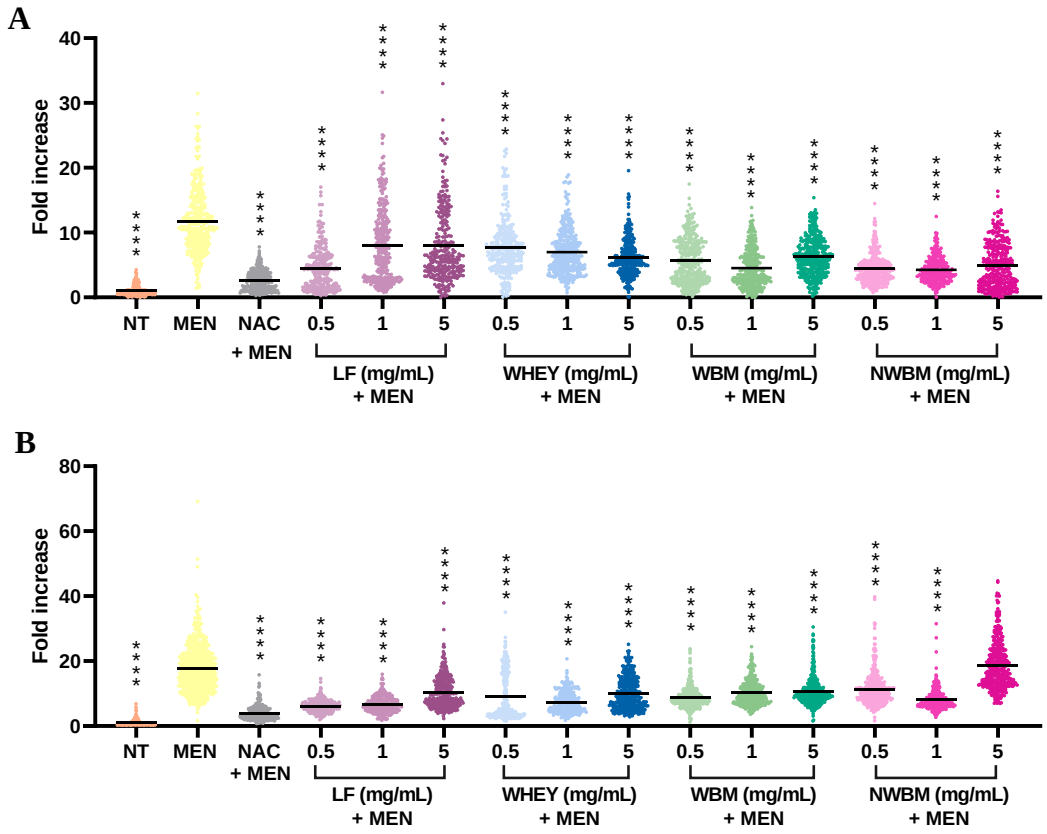


Figure 3. Effect of dairy fractions on genotoxicity caused by menadione on (**A**) Caco-2 and (**B**) HepG2 cells. NT: non-treated cells. MEN: positive control of genotoxicity, cells treated with 50 µM menadione for 1 h. NAC + MEN: cells treated with 1mM NAC as a control of antigenotoxic effect before treatment with 50 µM menadione for 1 h. LF: lactoferrin. WBM: washed buttermilk. NWBM: non-washed buttermilk. The results were normalized to 1 for the untreated condition. The values represent the intensity of 150-200 cells counted in each experiment, on three independent experiments. The bars represent the means of all counts. Asterisks indicate significant differences respect to MEN (****p< 0.0001).

Several studies on the antigenotoxic effect of fermented milk against mutagens have been condensed in an extensive review (Saikali et al., 2004). In this review, *in vivo* studies with rodents fed fermented milk showed a reduction in the incidence of carcinogen-induced colon cancer tumors and a decrease in DNA damage. In addition, the *in vitro* studies reviewed also demonstrated the antigenotoxic and antimutagenic effects of fermented milk (Saikali et al., 2004).

The results of Chiang et al. (2017) showed that hydrolyzed bovine colostrum whey presented oxidative damage inhibitory activities and an inhibitory effect on the decomposition of supercoiled DNA. In addition, in the study by Tian (2013), the antigenotoxic properties of LF were determined in HT29 cells, a model of human intestinal cells, after DNA had been damaged by fecal water, finding the greatest effect of LF at high concentrations of 10 and 20 mg/mL. Habib et al. (2013) also studied the antioxidant and DNA damage inhibitory activities of LF. They used camel milk LF and obtained results similar to those reported here. They analyzed the complete degradation of plasmid DNA treated with UV light, H_2O_2 and $FeSO_4$ and how DNA damage was reduced in the presence of LF, most likely through binding catalytic iron. Furthermore, Ogasawara et al. (2014) demonstrated that native LF and iron saturated LF clearly protected DNA from fragmentation caused by UV radiation in the presence of ROS. All these results indicated that LF could suppress ROS and protect DNA from damage.

In conclusion, we show here (**Figure 2** and **Figure 3**) that dairy by-products partially rescue the menadione effects (oxidative stress and subsequent genotoxic stress). The remaining oxidative stress could explain the remaining genotoxicity. It is possible that a treatment with a lower concentration of menadione would have allowed total protection against both oxidative and genotoxic stresses.

3.4. Bioavailability of dairy fractions in Caco-2/HepG2 co-culture

In order to study the bioavailability and metabolism of the different dairy compounds, a Caco-2/HepG2 co-culture intestinal cell monolayer model was used. This co-culture was chosen to model the intestinal-liver axis and study the transport and changes in the functional activity of the metabolites. This co-culture system has already been used previously (Yao et al., 2020). To understand the behavior of the dairy by-products across a barrier similar to the intestinal epithelium, we evaluated the transport and metabolism of dairy samples in differentiated Caco-2 cells and their exposure to HepG2 cells, in order

to analyze their bioavailability and their effects on oxidative stress and DNA damage caused by menadione (**Figure 4**). In addition, the proteomic analysis of the culture medium allowed us to confirm the passage of LF from the upper transwell to the lower compartment. The amount of LF detected was 63-fold higher in the upper chamber (in contact with the apical side of Caco-2 cells) than in the lower chamber (in contact with HepG2 cells).

Analyzing the bioavailability and effect of the samples, we noticed that menadione-induced oxidative stress was reverted by the different dairy fractions in Caco-2 cells (**Figure 4A**) but also in HepG2 cells (**Figure 4B**). This meant that at least part of the antioxidant compounds in the dairy products were bioavailable and protected HepG2 cells from menadione stress. The reversion patterns of the genotoxic stress in both cell lines led us to the same conclusion (**Figure 4C, 4D**). This confirms that menadione effects (oxidative stress and genotoxicity) are closely related, with cells displaying the highest level of oxidative stress being those displaying an increased DNA damage level. It is also observed that the protective effect of milk samples was effective against both stresses, strongly suggesting that the antioxidant effect impeded both oxidative and genotoxic stresses. This study also shows that these potential antioxidant compounds contained in the dairy products were not altered and maintained their activity when they crossed the Caco-2 barrier and reached HepG2 cells on the basal side, similar to that observed in the single cell model (see **Figure 2** for oxidative stress and **Figure 3** for genotoxic stress).

The single Caco-2 epithelial model lacks communication with other organs involved in the regulation of compound absorption, which may be a limitation for this model. However, the co-culture model with both Caco-2 and HepG2 cell lines could improve research on nutrient absorption (Scheers et al., 2014). Caco-2/HepG2 co-culture models have been used previously to analyze the intestinal transport of polyphenol extract (Yao et al., 2020) or for *in vitro* iron absorption studies (Scheers et al., 2014). In the study by Scheers et al. (2014), iron uptake on the apical surface of Caco-2 cells was higher in the co-culture model with HepG2 than in the Caco-2 cell monoculture. For the design of a successful combined liver and intestine model, some requirements must be considered.

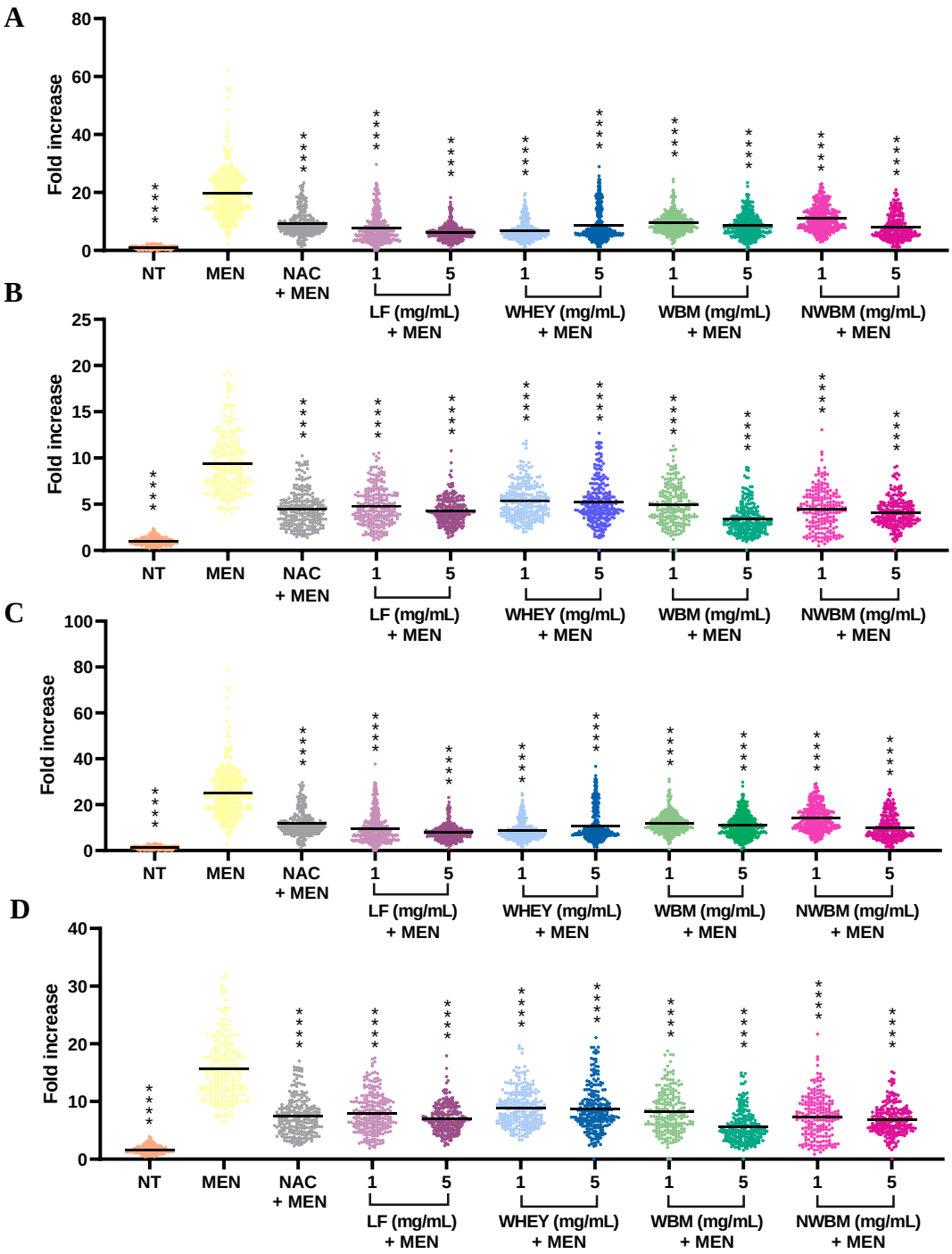


Figure 4. Effect of dairy fractions on oxidative stress (**A**, **B**) and genotoxicity (**C**, **D**) caused by menadione on Caco-2 cells (**A**, **C**) and HepG2 cells (**B**, **D**) co-cultured in standard transwell inserts. NT: non-treated cells. MEN: positive control of oxidative stress and genotoxicity, cells treated with 50 µM menadione. NAC + MEN: cells treated with 1 mM NAC as a control of antioxidant and antigenotoxic effects before treatment with 50 µM menadione. LF: lactoferrin. WBM: washed buttermilk. NWBM: non-washed buttermilk. The results were normalized to 1 for the untreated condition. The values represent the intensity of 150-200 cells counted in each experiment, on three independent experiments. The bars represent the means of all counts. Asterisks indicate significant differences respect to MEN (****$p < 0.0001$).

The chosen liver cell line should be easily accessible, have similar nutritional requirements to those of Caco-2 cells, and have reproducible secretion; these are prerequisites ensuring that the cells will not lose their phenotypes in culture, as occurs with primary liver cell lines (Scheers et al., 2014). For all these reasons, the chosen HepG2 cell line is a good choice for co-culture with Caco-2 cells.

In the present study, we have analyzed the antioxidant and antigenotoxic activity of dairy fractions and isolated proteins, although we have not considered what would occur in the case of a dynamic process within the gastrointestinal tract. However, we should point out that this study is the first part of a wider research, in which the effect of those samples after digestion will be investigated by analyzing their antioxidant and antigenotoxic potential in such conditions. Even though no digestion assay was performed, we observed antioxidant and antigenotoxic activities of all dairy fractions in the HepG2 cells when grown in co-culture with Caco-2 cells, suggesting that small compounds may play this role.

4. Conclusions

In conclusion, lactoferrin, whey, and buttermilk (washed and non-washed) display antioxidant and protective effects against DNA damage in Caco-2 and HepG2 cell lines. The co-culture between these two cell lines has made it possible to show the transfer of compounds that exert these protective functions on both cell lines.

Therefore, this study provides useful information to increase the commercial value of dairy by-products as multifunctional ingredients and their recognition as a natural source of antioxidants. It would be interesting to continue investigating the protective activity of milk compounds against genotoxicity as well as their bioavailability, since there are not many studies on this subject. Further characterization of the active compounds contained within these fractions will be key in the future.

Author Contributions

I.A.: Writing—Original draft, Methodology, Investigation, Software, Data curation, Formal Analysis; J.V.: Methodology, Investigation, Software, Writing—review and editing; C.B.: Methodology, Investigation; D.G.: Methodology, Investigation; L.G.: Methodology, Writing—review and editing; M.D.P.: Writing—review and editing; G.M.: Conceptualization, Resources, Supervision,Writing—review and editing; L.S.: Conceptualization, Resources, Supervision, Writing—review and editing, Funding

acquisition, Project administration. All authors have read and agreed to the published version of the manuscript.

Supplementary material

A.1. Oxidative stress and genotoxicity assays

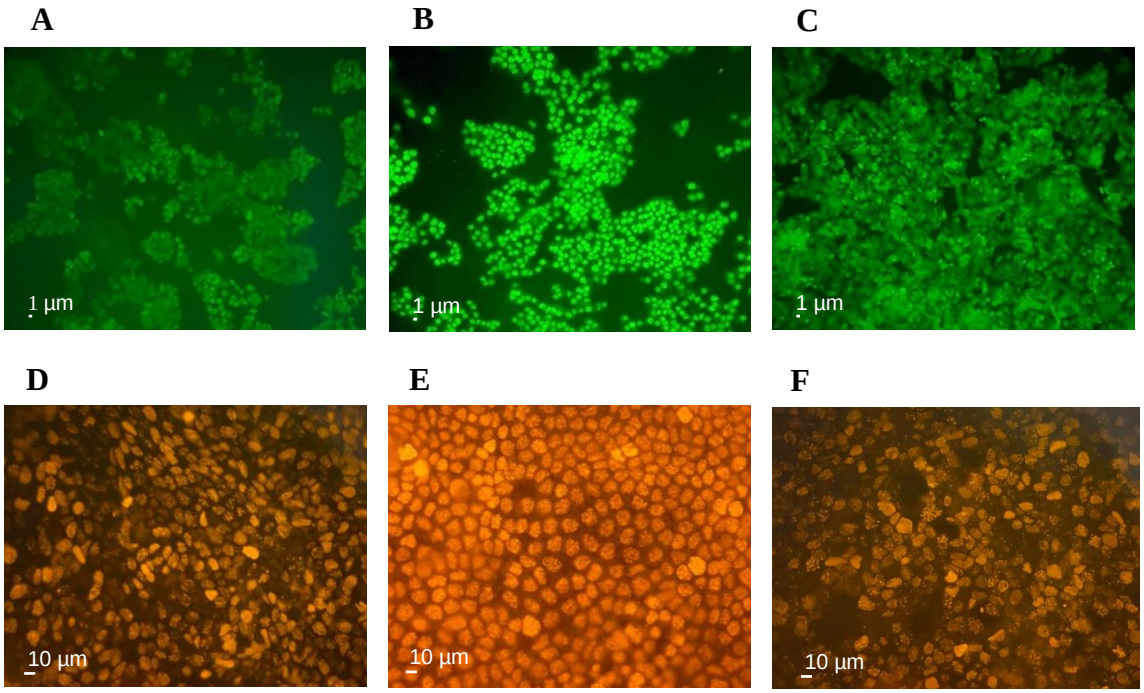


Figure S1. (**A, B, C**) Green fluorescence emitted by CellRox® Green Reagent (10x) (Bars, 1 µm) and (**D, E, F**) γ-H2AX signal (40x) (Bars, 10 µm) in (**A, D**) untreated cells, (**B, E**) cells treated with 50 µM menadione and (**C, F**) cells incubated with 1 mM NAC before treatment with 50 µM menadione.

A.2. Differentiation of Caco-2 cells

Differenciated Caco-2 cells were grown as monolayers for 17 days on transwell membranes. To ensure differentiation and the monolayer formation, cells were washed with PBS, fixed with 4% formaldehyde in PBS for 20 min, blocked and permeabilized in PBS containing 3% BSA and 0.3% Triton X-100 at room temperature for 30 min. Subsequently, monolayers were incubated with PBS containing 0.1% Tween-20, 2% BSA, rabbit anti-zonula occludens 1 (ZO-1) (1:25, v/v) antibodies (Rabbit Polyclonal Antibody (40-2300), ThermoFischer Scientific) and incubated overnight at 4°C. The incubation was followed by three washes in PBS and incubation for 1 h at room temperature in Alexa Fluor 488 labeled secondary antibody (1:500, v/v) and counterstained with DAPI. ZO-1 and DAPI signals were visualized with a Leica SP8

confocal microscope (Leica Microsystems, Wetzar, Germany) and software. Two independent experiments were conducted and independent fields examined.

Figure S2 shows the thigh junctions between cells marked with anti-ZO-1, and nuclei counterstained with DAPI. Cells were arranged in a monolayer, showing a polarized structure with nuclei at the basal side, and ZO-1 at the apical side.

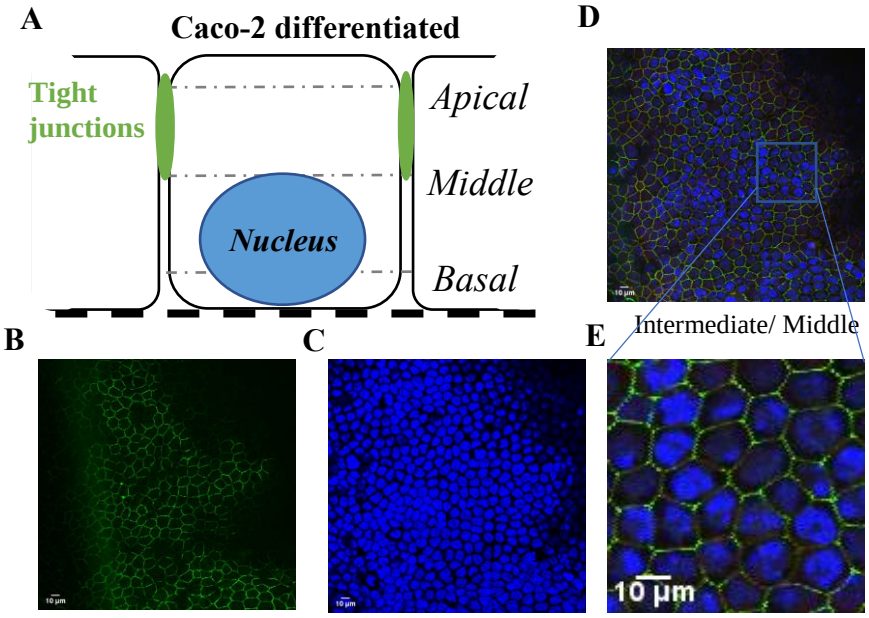


Figure S2. (**A**) Scheme of differentiated Caco-2 cells showing cellular structures (tight junction, nucleus) and confocal plans for the z-stack analysis. Cells were grown on transwell membranes (dotted line) for 21 days. (**B, C, D**) Confocal microscopic images (40x) of fixed and stained differentiated Caco-2. Cells were stained (**B**) with ZO-1 (green) for tight junction analysis and (**C**) with DAPI (blue) for nuclei staining. (**D**) Confocal analysis for the middle cellular plan shows an intermediate staining. (**E**) Close up of the confocal middle plan. Bars, 10 µm.

A.3. Bioavailability assay

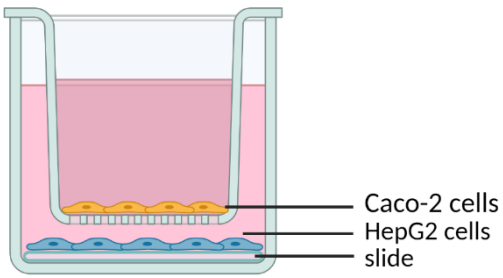


Figure S3. Bicameral chamber used for co-culture of cell lines, mimicking the intestinal-liver axis, with Caco-2 seeded on the standard transwell insert and HepG2 seeded on the coverslip at the bottom. Graphics elaborated with BioRender.com.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to reasons of privacy.

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ARTÍCULO 3

Development of encapsulation strategies and composite edible films to maintain lactoferrin bioactivity: A review

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Review

Development of Encapsulation Strategies and Composite Edible Films to Maintain Lactoferrin Bioactivity: A Review

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Abstract

Lactoferrin (LF) is a whey protein with various and valuable biological activities. For this reason, LF has been used as a supplement in formula milk and functional products. However, it must be considered that the properties of LF can be affected by technological treatments and gastrointestinal conditions. In this article, we have revised the literature published on the research done during the last decades on the development of various technologies, such as encapsulation or composite materials, to protect LF and avoid its degradation. Multiple compounds can be used to conduct this protective function, such as proteins, including those from milk, or polysaccharides, like alginate or chitosan. Furthermore, LF can be used as a component in complexes, nanoparticles, hydrogels and emulsions, to encapsulate, protect and deliver other bioactive compounds, such as essential oils or probiotics. Additionally, LF can be part of systems to deliver drugs or to apply certain therapies to target cells expressing LF receptors. These systems also allow improving the detection of gliomas and have also been used for treating some pathologies, such as different types of tumours. Finally, the application of LF in edible and active films can be effective against some contaminants and limit the increase of the natural microbiota present in meat, for example, becoming one of the most interesting research topics in food technology.

1. Introduction

Lactoferrin (LF) is an iron-binding glycoprotein present in the secretions of mammalian species. It was first isolated from milk, and afterwards, it was found in mucosal surfaces, specific granules of leukocytes and several secretory fluids including tears, saliva, seminal and nasal secretions (Farnaud & Evans, 2003). LF plays important roles, such as modulation of iron uptake and release by the intestinal mucosal cells in the suckling newborn; antimicrobial activity against bacteria, virus, fungus and parasites; antioxidant activity in several systems and regulation of immunity and cellular growth (Brock, 2002). When LF is intended to be used as an ingredient in some functional products, it is essential to know the effect that technological treatments exert on its activity (Franco et al., 2018).

LF is highly unstable under the conditions of the stomach and intestines due to the gastric low pH and the high protease activity present in both compartments that cause

conformational changes and loss of activity to this protein (Braim et al., 2019; Raei et al., 2015; Takeuchi et al., 2006). Taking this susceptibility into account, it is important to achieve LF stabilization by using some technologies, such as encapsulation or composite materials, to avoid its degradation along the gastrointestinal (GIT) tract (Balcão et al., 2013). Apart from the interest in encapsulating LF to preserve its beneficial biological activities, this protein is used as a component in complexes, nanoparticles, hydrogels and emulsions to encapsulate, protect and deliver other bioactive compounds (Liu et al., 2018a). In addition, LF is also being investigated to be part of systems to deliver drugs or to apply certain therapies to target cells expressing LF receptors on their surface (El-Fakharany, 2020).

Encapsulation techniques have been developed in the last decades to preserve bioactive compounds incorporated into pharmaceutics, food and cosmetic products (Castro-Rosas et al., 2017). Microencapsulation is defined as a process in which very small particles or droplets are surrounded by a coating or are embedded in homogeneous or heterogeneous matrices. This process is intended to preserve bioactive compounds from inactivation, which can be caused by certain conditions and interactions with the environment where those compounds are intended to exert their activity (Gouin, 2004). Nanocarrier systems have many advantages, such as improving the bioavailability and solubility of bioactive compounds, enhancing their residence time and stability in the GIT and giving them a better ability to enter and permeate tissues and cells (Mohammadian et al., 2020).

Nowadays, research in edible films is a relevant emerging area of study because the reduction of plastic waste is of high priority at the global level. Edible films and coatings avoid the desiccation of the food product, increasing its structure resistance and controlling gas exchange (Dhumal & Sarkar, 2018; Falguera et al., 2011). Furthermore, edible films and coatings can contain and deliver active ingredients that add extra properties to them, such as antioxidant and antimicrobial properties, by incorporating bioactive compounds like LF (Dhumal & Sarkar, 2018).

The development of nanotechnology has produced great changes in many areas, such as the way to administer some therapies or the design of particles that can be directed to specific targets. The present review covers the current emerging technologies that can be used to preserve the biological activity of LF in different systems. This report is not comprehensive, as the studies on LF are numerous and it is impossible to cover all of them; however, the articles revised here are representative of the research performed on

this field in recent years. We have focused on the mechanisms to encapsulate LF to preserve its integrity, the use of LF to decorate particles with the aim to improve the delivery of compounds to specific cells and finally, on the use of LF in active films for food preservation.

2. Materials and methods

2.1. Search Strategy

The three authors performed the electronic search from 1995 to September 2021, using Medline (PubMed) and ScienceDirect. The search strategy applied was a combination of medical subject headings (MeSH), terms and free text words, including the following keywords individually or combined: lactoferrin, nanocarriers, nanoparticles, microparticles, encapsulation, edible films, active films, active packaging, milk proteins, whey proteins, caseins, polysaccharides, alginate, chitosan, pectin, gums, chondroitin sulphate and galactomannans.

Review articles and the different research articles found were also sources of references to locate other articles. The procedure used for selecting the articles and type of articles finally included in the review was discussed by all the authors and finally, 153 articles were revised and included in the review. Furthermore, the evolution in the number of publications related to some terms used in the search was analysed over the course of twenty-five years, as is represented in **Figure 1**.

2.2. Selection of Studies

The review process was divided into three major sections to be revised initially by each of the three authors (I.A., C.C. and L.S.), and afterwards, all authors revised the three sections. The selection of the articles was first screened for relevance by reading the abstracts. Additionally, the relevant references of the selected articles were searched manually. The following exclusion and inclusion criteria were used to select the obtained articles:

2.2.1. Inclusion Criteria

1. The article includes a description of the procedure to make LF composites;
2. The article includes applications of LF composites related to medicine;
3. The article includes applications of LF composites related to foods;
4. Review articles that help to identify articles related to the topics for review.

2.2.2. Exclusion Criteria

1. Articles not providing detailed information on the preparation of LF composites;
2. Articles not providing detailed information on the applications of LF composites;
3. Articles published before the year 1999.

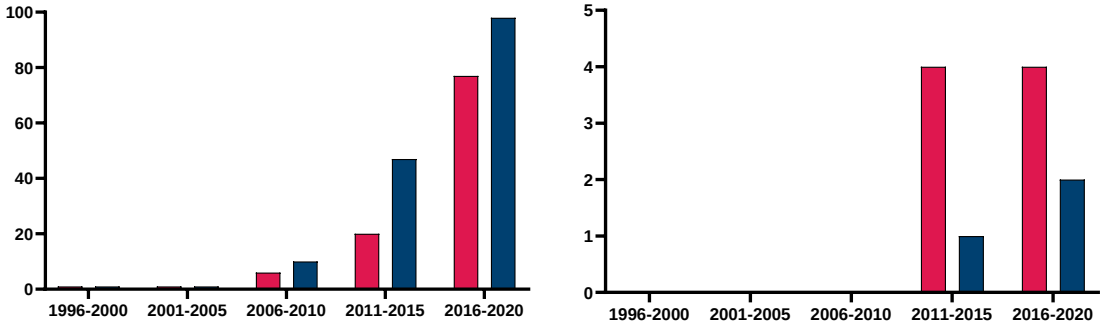

Figure 1. Graphs representing the publications found by combining the term lactoferrin with (**A)**: nanoparticles, microparticles or encapsulation, (**B**): edible films, active films or active packaging, in ScienceDirect (■) and PubMed (■) shown with years.

3. Results

3.1. Encapsulation of lactoferrin

The selection of appropriate materials for encapsulating bioactive compounds is essential. These materials should be chemically compatible, non-reactive with the encapsulated substance and are expected to show certain properties, such as strength, flexibility, impermeability and stability, which are required for their application (Bansode et al., 2010; Maresca et al., 2016).

There are various food biopolymers, including polysaccharides, lipids, proteins, and their conjugates used to develop a wide range of nanocarriers. These are designed to protect, entrap, encapsulate and control the delivery of bioactive compounds and nutraceuticals (Mohammadian et al., 2020). The systems based on lipid carriers can be classified as nanoemulsions, solid lipid nanoparticles, nanostructured lipid carriers, nanoliposomes, micelles and nanosuspensions (Shin, Kim, & Park, 2015). Polysaccharide-based nanocarriers also include polymer nanoparticles, polymeric micelles and inclusion complexes (Mohammadian et al., 2020). However, nanocarriers based on food proteins can be prepared with different structures, including nanoparticles, hollow nanoparticles, nanohydrogels, heat-induced nanofibrillar aggregates, electrospun nanofibers and tubular nanostructures (Fathi, Donsi, & McClements, 2018).

Food proteins normally used to construct nanocarriers can derive from animals and plants, such as whey, soy, egg, corn proteins, gelatin and bovine serum albumin (BSA), among others. The nanocarriers produced with those proteins are adequate for the delivery of both hydrophobic and hydrophilic bioactive ingredients and can be included in products for nutrition, drugs or can be directed to apply several therapies (Mohammadian et al., 2020).

Encapsulation can be conducted by chemical or mechanical procedures, as shown in **Figure 2**. Chemical encapsulation can be done by simple and complex coacervation, co-crystallization, interfacial polymerization, ionic gelation, entrapment in liposomes, molecular inclusion and ionic gelation plus electrostatic interactions. The mechanical procedures are spray-drying, freeze/cold-drying, extrusion and fluidized bed (Madene et al., 2006).

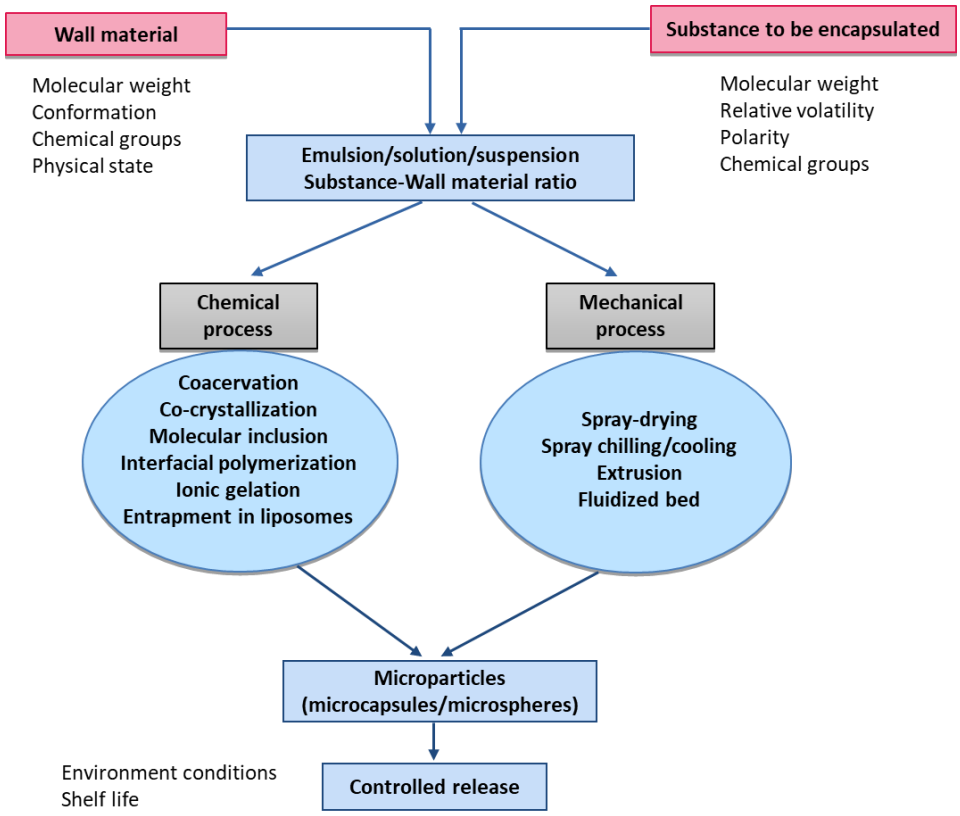

Figure 2. A schematic illustration of different processes of encapsulation used in food and flavour industries [adapted from Madene et al. (2006)].

The complex coacervates are generated by an encapsulation method that usually consists of three stages: emulsification, coacervation and cross-linking. The complex coacervation is a liquid–liquid phase separation that occurs when there is an interaction, mainly electrostatic, between biopolymers of opposite charges, the interaction being influenced

by pH. The polymer matrix, considered as the wall, mainly made up of positively charged proteins and negatively charged polymers surrounds the bioactive compound, considered as the core (Corrêa-Filho, Moldão-Martins, & Alves, 2019). The polymer-based coacervates have many applications as food and biomaterial ingredients or encapsulants in personal care and functional foods. Recently, there has been an increase in the interest to study heteroprotein complex coacervates, which are a special case of coacervates in which the dense phase is formed by at least two different proteins with opposite charges (Zheng et al., 2020).

The potential benefits of bioactive compounds, also known as nutraceuticals, are not optimally achieved because of their low and/or variable bioavailability (McClements, Li, & Xiao, 2015). This availability is a measure of the bioefficiency of bioactive compounds and is the result of processes that those components undergo within four gastrointestinal segments: the mouth, stomach, small intestine and large intestine (Dima et al., 2020).

The factors that mainly affect the bioavailability of encapsulated bioactive compounds are the composition of nanocarriers and loading capacity, size and surface. For example, nanocarriers based on starch and proteins can be hydrolysed by amylases and proteases, while other materials, such as resistant starch, pectin and alginate are resistant to enzymes and pH, and are attacked only by the colonic microbiota (Dima et al., 2020). However, the release of bioactive compounds from lipid-based nanocarriers is favoured by the action of lipase in the mouth, stomach and intestines, which breaks down triacylglycerides into free fatty acids and monoacylglycerides, contributing, with the bile salts, to the solubilisation of bioactives into mixed micelles (McClements, 2017). Furthermore, the bioavailability of many bioactive components depends on the food matrix that is coingested with them, in the case that those are contained in a food product or in some nutraceuticals.

3.1.1. Encapsulation of lactoferrin with milk proteins

Milk proteins have a rich diversity of physicochemical characteristics and biodegradable properties, which make them appealing for different food and pharmaceutical applications (Delboni & Da Silva, 2016). Proteins are natural vehicles for bioactive molecules, thanks to their structural and physicochemical properties that facilitate their functionality in delivery systems. For oral delivery applications, the biocompatibility of milk proteins is usually positive (Livney, 2010), although it is necessary to consider that, a small portion of the population is allergic to some milk proteins.

- Whey proteins

Generally, whey proteins and LF are electrostatically attracted to each other over a range of pH values due to their different isoelectric points (pI), and so they can be assembled in various interfacial structures, such as single, double and mixed layers in emulsions (Teo et al., 2016). In that sense, Anema and de Kruif (2014) assayed the mixture of LF with the anionic protein β-lactoglobulin (β-Lg) at a range of pH and mixing ratios. Complexation was monitored through turbidity and zeta potential measurements, and the formation of complex coacervates was observed. Complexes were formed in the pH region of 5-7.3, and at the maximum turbidity, the complexes were neutrally charged. Moreover, although the charge ratios of LF/β-Lg varied with pH, LF/β-Lg complexes were found to have a constant stoichiometry of 1:3 at all pHs due to charge neutralization. With the addition of NaCl, the complexation diminished and disappeared at a salt concentration of about 100 mM.

Chapeau et al. (2016) showed that electrostatic complexes formed between LF and β-Lg can be used to successfully encapsulate vitamin B9 at its recommended daily intake levels. They identified two types of B9/LF/β-Lg co-assembly: aggregates (at low and high protein concentrations) and heteroprotein coacervates (at intermediate protein concentrations), both exhibiting different kinetics and stability over time. The B9/LF/β-Lg coacervates showed high entrapment of B9 (from 6 to 16 mg B9/g protein), which means that a few milligrams of these coacervates would be enough to cover the recommended daily intake of this vitamin. The same research group also demonstrated the scale-up of this system, confirming the efficiency of this type of biocarrier for developing natural functional foods (Chapeau et al., 2017).

Whey protein isolate (WPI) is obtained from the main by-product of cheese production and mainly consists of β-Lg and α-lactalbumin (α-La). Irreversible denaturation and aggregation of whey proteins occur at heating temperatures higher than 70 °C, and the denatured proteins interact with each other, forming a gel network. WPI has a strong binding affinity for LF as it can cross-link to form a stable protein complex with enhanced elasticity and gel strength under various heating conditions, as reported by Li and Zhao (2018). These researchers showed that the addition of LF into WPI gels increased the gel strength at the two pHs tested of 5.8 and 6.7, being stronger and more uniform at pH 6.7 in the presence of high concentrations of LF (20% and 30%). Those findings revealed

both the practical and theoretical significance for the utilisation of LF in gelled protein products.

Theoretical models and numerical simulations were applied by Delboni and Da Silva (2016) to gain insight into understanding the fundamental mechanisms responsible for the process of milk protein complexation under different conditions. This knowledge can be essential to use milk proteins in nanoscale encapsulation systems for food and pharmaceutical applications. The interactions between β-Lg and LF and between α-La and LF were investigated by means of Monte Carlo simulations. The comparison between the free energies associated with the complexation of LF with β-Lg and α-La at different pH and ionic strengths revealed the weaker attraction between α-La and LF in contrast with the stronger attraction measured between β-Lg and LF. The driving force for the complexation being studied of β-Lg with LF is due to an association of electrostatic interactions, where protein charge plays an important role.

The influence of small ligands on the complex coacervation between β-Lg and LF was studied by Tavares et al. (2017). In this work, 8-anilinonaphthalene-1-sulfonic acid (ANS), a fluorescent probe, was used as a model of a small ligand. While ANS did not interact with β-Lg, it presented two binding sites for LF inducing its self-aggregation. Depending on its concentration, ANS modulated the shape of the β-Lg-LF macromolecular assembly. Coacervates were observed for ANS/LF molar ratio <25 against amorphous aggregates found for higher ANS/LF molar ratios.

In 2020, Darmawan et al. explored a novel in silico approach to study the possibility of employing whey protein components to encapsulate LF. The interaction of apo-LF (the most unstable form of the protein) with β-Lg and α-La was studied at temperature conditions that are used during the spray drying process (95 and 180 °C). This method is commonly employed in the food industry to encapsulate food ingredients by using hot air drying. It was found that the interaction of β-Lg and α-La with LF allowed maintaining the structure of two regions of the molecule corresponding to antibacterial peptides (lactoferricin and lactoferrampin) under the spray drying conditions. In this study, it was also demonstrated that apo-LF was most probably readily dispersible during subsequent rehydration due to its tendency to agglomerate under those temperatures. Moreover, they showed that the interactions between apo-LF, β-Lg and α-La were due to the acidic and basic amino acid residues of these proteins.

In the study by De Figueiredo Furtado et al. (2021), LF was proposed as a promising ingredient for incorporation into powder infant formulas, because it improved their properties compared with formulas without this protein. The interaction of LF with WPI or whey protein hydrolysates used in the formulas, as encapsulating components of the oil mixture added, resulted in more stable emulsions. The powders obtained from the emulsions containing LF were those with a lower stickiness and consequently, with higher yield, encapsulation efficiency and wettability.

- Caseins

Caseins represent the major protein fraction of milk, making up 80% of the total proteins. They precipitate at pH 4.6 and individual caseins, including αs1, αs2, β and κ-casein, are unique proteins with respect to their structure and function. They have open and flexible conformations and consist of hydrophilic and hydrophobic segments. Due to their highly hydrophobic nature, individual caseins are stabilised by the creation of a micellar structure, which is chemically heterogeneous and is composed of the four types of caseins and by amorphous calcium phosphate (Holt et al., 2013). The stability of the caseins and casein micelles to some treatments, such as heating, freezing, and drying, make them valuable in delivering food ingredients and bioactive compounds (Ranadheera et al., 2016).

Anema and de Kruif investigated the interaction of LF with the casein micelles, as found in bovine skimmed milk (Anema & de Kruif, 2011), and with the individual caseins (Anema & de Kruif, 2012a). The cationic LF (pI 8.3–8.7) was found to bind to the anionic caseins (pI range from 4.9 to 5.6) and to the casein micelles (pI 4.6) at intermediate pH values. The binding of LF to the individual caseins can be described as the formation of complex coacervates. Moreover, Anema and de Kruif (2012b) assayed the binding of LF to transglutaminase (TGA) cross-linked casein micelles. They found that the internal cross-linking of the casein micelles by TGA did not alter the binding mechanism of LF to them. The binding of LF to the untreated micelles swelled the micelles, which was avoided in the TGA-micelles. Additionally, after several hours in the presence of added LF, the untreated micelles started to disintegrate, and their size decreased (increasing transparency of the milk was observed). In contrast, the internal cross-linking of the casein micelles prevented the disintegration of the casein micelles.

In 2016, Anema and de Kruif studied complex formation between some bovine casein types (α-casein, ACN; β-casein, BCN, and κ-casein, KCN) and LF. They showed that the optimum coacervation occurred at the mixing ratio in which charge neutrality happened and the turbidity was maximum. The kinetics of complex formation for LF/BCN and LF/KCN were rapid and occurred through a nucleation and coalescence process. However, the kinetics of complex formation for LF/ACN were much slower. They evidenced that when salt is added the coacervation diminishes, leading eventually to a one-phase system. Salt decreases the entropical contribution, and consequently, the coacervation is not observed. For the LF/BCN or LF/KCN coacervates, the samples were completely transparent (turbidity of ~0) at an ionic strength of ~25 mM, whereas for the LF/ACN, a much lower ionic strength of only ~10 mM was required for the sample to be completely transparent. It is interesting to read the review article of Anema (2016), in which he summarises all the results that have been obtained in his research group studying the spontaneous self-association of LF with the casein micelles in milk and with individual isolated caseins.

The complexation behaviour between LF and sodium caseinate (NaCas) before and after heat treatment was also studied by Li and Zhao (2017). The results show that the denaturation and aggregation of LF can be inhibited by forming soluble LF-NaCas complexes. The complexes formed at a ratio of 2:1 had an average diameter of 194 nm and exhibited a higher capacity for lowering the air/water interfacial tension compared to complexes with a lower proportion of LF. NaCas played a role as a stabiliser to protect LF against heat-induced precipitation. Therefore, the presence of NaCas is important for maintaining the structure and functionalities of LF during the production of LF-enriched food products. However, the authors have not demonstrated the biological activity of LF while being part of the complexes formed.

3.1.2. Encapsulation of lactoferrin with other proteins

The ability of LF to form positively charged droplets at neutral pH can have many important practical implications. Recently, there has been an increasing interest in the application of whey proteins, and especially LF, as emulsifiers (Çelebioğlu, Lee, & Chronakis, 2020). LF has been used as an emulsifier in multilayer emulsions for lipophilic nutraceutical delivery of active compounds, such as curcumin and β-carotene (Acevedo-Fani, Soliva-Fortuny, & Martín-Belloso, 2017). LF can interact with other proteins or polysaccharides, providing good stability to oil-in-water emulsions (Liu et al., 2018b).

BSA with tannic acid (TA) has been used to encapsulate LF for oral administration by alternating layers of BSA and TA on porous microparticles of $CaCO_3$ previously prepared with LF (Kilic et al., 2017). The authors examined two approaches to load LF into $CaCO_3$ particles, as shown in **Figure 3**. The first one used co-precipitation in which $CaCO_3$ microparticles were directly formed in an LF solution. The second one used post-loading in which $CaCO_3$ microparticles were prepared in a water solution and then dispersed in an LF solution. The $CaCO_3$ was dissolved after covering the microparticles with the layers of BSA and TA. The post-loading approach showed lower LF degradation than that of co-precipitation and was chosen as the oral delivery system in a murine model. The microcapsule shells formed conferred LF high stability and protection in gastric conditions, while they were degraded under intestinal conditions, thus releasing LF. The animals dosed with encapsulated LF showed a 6.5 times higher concentration of LF in the intestine than the control group dosed with free LF. Moreover, LF was later detectable in the liver, demonstrating that this encapsulation system has great potential for oral delivery of bioactive molecules.

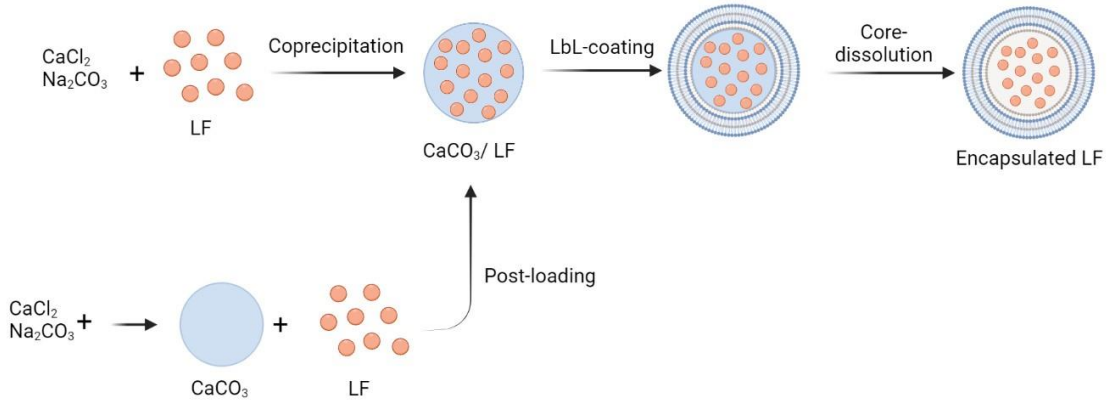

Figure 3. Scheme of LF encapsulation, co-precipitation of $CaCO_3$ and LF vs. post-loading of LF in porous $CaCO_3$ microparticles, both followed by Layer-by-Layer (LbL) deposition of bovine serum albumin and tannic acid and final dissolution of $CaCO_3$. (Redrawn and slightly changed from that contained in the article by Kilic et al. (2017), which is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/, accessed on 30 October 2021).

Pea protein isolate (PPI) has also been used to form complexes and coacervates with LF by electrostatic interactions under specific pH conditions (Adal et al., 2017). The maximum level of coacervate formation was observed at charge neutrality. The pH where

coacervation was most favourable was pH 5.4, and the soluble complexes were maximised at pH 7. The formation of heteroprotein complexes was studied by Small Angle X-ray Scattering (SAXS), confirming the formation of complexes of around 13 nm at pH 7 and around 80 nm at pH 5.4. A predominance of elliptical over spherical shapes for LF-PPI coacervates was found, and rare chain-like aggregations were observed, probably due to PPI-PPI complexes. These coacervates can be of interest in various food applications.

Zheng et al. (2020) investigated the conditions, thermodynamic mechanism, and morphological structure for the formation of soy protein isolate/lactoferrin (SPI/LF) complex coacervate. The stoichiometry of two optimal complex coacervates (SPI/LF = 1:3 at pH 6.25 and SPI/LF = 1:4 at pH 6.6) was identical to their mixing ratio. Moreover, the SPI/LF complex coacervation was exothermic and accompanied by significant entropy gain. This coacervation was established by electrostatic interactions and by hydrogen bonds. It was shown that the SPI/LF interaction improved the heat-stability of the LF heat-sensitive lobe. When the structure of the complexes was analysed, it was observed that the 1:3 SPI/LF complex exhibited distinct granules, whereas the 1:4 SPI/LF complex presented a uniform cross-linking structure. Analysis by atomic force microscopy showed the existence of sphere complexes with a diameter of 50-150 nm in the 1:3 SPI/LF complex and a chain-like structure with a length of 50-150 nm and a width of 20-80 nm in the 1:4 SPI/LF one. This different structure was hypothesised to be by SPI self-aggregation. It remains unanswered if LF maintains its properties in SPI/LF coacervates, although the increase observed in LF thermal resistance is very positive for the potential applications of those coacervates.

3.1.3. Encapsulation of lactoferrin with polysaccharides

Biopolymer nanoparticles have gained popularity thanks to their exceptional physical characteristics, such as biodegradability, biocompatibility, low toxicity and great ability to bind hydrophobic bioactive compounds (Dai et al., 2015; Il'ina et al., 2016). The protein-ionic polysaccharide electrostatic attraction requires a specific pH, in which the charges of these molecules are opposite. The net charge of a protein at its pI is zero, being positive below the pI, and negative at a pH higher than the pI. Thus, cationic and anionic polysaccharides can form electrostatic complexes with proteins depending on pH. At physiological pH, LF is positively charged (pI 8.3-8.7); therefore, its capacity to create

complexes with several anionic polysaccharides has made possible the extensive research produced in this area (Il'ina et al., 2016).

The type and concentration of protein and polysaccharide, as well as the temperature, pH and ionic composition of the solution, determine the functional characteristics of a protein-polysaccharide complex (Cheng et al., 2021). Additionally, the formation of complex coacervates is influenced by the molecular weight, charge density and chemical nature of the polymers used (Gulao et al., 2014).

- Alginate

Alginate (Alg) is a natural, biodegradable and biocompatible anionic polysaccharide obtained from brown algae (Bastos et al., 2020). It is an organic polymer derived from alginic acid and is located in the cell wall of the algae. Its structure consists of linear chains composed of monosaccharides D-annuronic and L- guluronic.

This polymer owes its polyanionic character to the carboxyl groups that appear along the chain. The distribution of monomers in the polymeric chain and the charge and volume of carboxyl groups give to the formed gel characteristics of flexibility or rigidity depending on the guluronic content. The higher the number of guluronic blocks, the harder and more rigid and brittle the gel is, while if the mannuronic groups predominate, the gel is softer and more elastic (Avendaño-Romero, López-Malo, & Paolu, 2013). This polymer is presented as sodium alginate (Na-Alg) when used as a food additive and is easily available and widely applied in the industry. The main characteristics of Na-Alg are its good affinity with water and its highly anionic charge at pH values higher than two (Wang et al., 2017).

On the other side, Alg combined with calcium (Ca-Alg) forms a gel that is a wellknown system used for the elaboration of gel beads. These beads are useful to encapsulate wide variety of bioactive agents, due to their simplicity, biocompatibility, non-toxicity and low cost (Raei et al., 2015).

When two chains of the guluronic blocks align, coordination sites are created. These chains are in the shape of loops, creating cavities with adequate size to accommodate the calcium ion. After the addition of calcium, the Alg undergoes conformational changes, producing the well-known "egg box" gelling pattern [Figure 1 in (Avendaño-Romero, López-Malo, & Paolu, 2013)]. This pattern is based on the dimerization of the chain and,

later on, further aggregation of the dimers (Avendaño-Romero, López-Malo, & Paolu, 2013).

Furthermore, Alg is a good option for the encapsulation of molecules since it has unique characteristics that allow a controlled release in the intestine (Braim et al., 2019). Additionally, Alg microbeads, reaching a mean diameter of 130 ± 47 μm, are one of the most studied encapsulation systems (Yucel Falco et al., 2019). Kanwar et al. (2012) and Braim et al. (2019) confirmed that the LF/Alg capsule can be coated with chitosan, another polycationic polymer, to improve the integrity of the capsule in GIT fluids.

Some studies have proven the efficacy of coating LF by Alg capsules to deliver this protein to the colon (Kanwar et al., 2012) and to impede the growth of Clostridiodes (Braim et al., 2019). Furthermore, the use of LF as a complement to Alg to encapsulate essential oil particles (Bastos et al., 2020) or probiotics (Yucel Falco et al., 2019) has also been achieved.

In the study by Raei et al. (2015), LF was encapsulated in Ca-Alg capsules, and it was proven that the effectiveness of these capsules increased with higher concentrations of Ca-Alg and with thermal treatment (61 °C for 10 min). However, the stability of the nanoparticles was greater when the polysaccharide concentration was lower (0.2%, w/w) and the thermal treatment was applied. These particles remained intact for 30 min at pH 2, conditions that can be considered equivalent to gastric digestion, which guarantees a controlled release during the first 30 min and a subsequent gradual release in acidic and neutral conditions. In this way, they confirmed that the encapsulation of LF with Ca-Alg is a technique that can be used for the targeted delivery of LF to the intestine.

Furthermore, Bokkhim et al. (2016) encapsulated three different forms of LF (apo-, native- and holo-) in micro-gel particles of Alg, using the aerosol technique. They produced the particles from a 2% (w/w) solution of LF/Alg (1:1), using 0.1 M $CaCl_2$ as the cross-linking solution. These authors demonstrated that a major concentration of $CaCl_2$ decreased the encapsulation efficiency. LF/Alg particles were maintained intact during simulated gastric digestion of 2 h, with 30% more protein compared to the non-encapsulated LF. The digestion of all forms of LF, pure or encapsulated, under intestinal fluids was rapid. Thus, these authors showed with this study that LF/Alg micro-gel particles can protect LF from the action of pepsin (the main enzyme of gastric digestion) and release it at the intestine.

Additionally, Wang et al. (2017) proved the efficacy of the LF/Na-Alg complex coacervates. This study showed that owing to the positive charge of LF at pHs lower than 8.5 and the negative charge of Na-Alg in the entire pH range studied (2-10), the electrostatic interaction between both molecules was excellent. This electrostatic interaction increased as the pH decreased from eight to five and remained constant at pH from five to four. Therefore, the authors concluded that the optimum pH for the formation of the LF/Na-Alg complex coacervates was 4.5. Additionally, they compared the degradation of the uncoated LF and that of the Alg capsule in the gastric stage. They found that 100% of the uncoated protein was degraded under gastric conditions. This percentage decreased to 70% when LF was protected with Na-Alg, keeping 30% of the protein intact at that stage of digestion. Alg provided a blockage of the sites of LF for the action of pepsin, reducing LF proteolysis. Several authors have defended that Alg can interact with pepsin, decreasing its catalytic mechanism during gastric digestion, while it does not have the same effect with trypsin, being degraded by this enzyme in the intestinal digestion (Bastos et al., 2020; Bokkhim et al., 2016; Wang et al., 2017).

The encapsulation of LF with Na-Alg allows the preservation of its properties, such as its antioxidant activity, increasing by 30% with respect to the uncoated LF under gastric conditions (pH 2). After gastric digestion, the antioxidant capacity of uncoated LF decreased by 12% due to the breakdown of peptides. However, the antioxidant capacity of LF/Na-Alg coacervates was not modified after this digestion stage (Wang et al., 2017).

- Chondroitin sulphate

Chondroitin sulphate (ChS) is a polyanionic mucopolysaccharide that is used as a food-grade material and is obtained from animal cartilage. ChS contains strongly acidic sulphate groups and weakly acidic carbonyl groups, so it has a high negative charge density and can form ionic complexes with positively charged molecules, such as LF. Furthermore, the ability of ChS as a potential carrier for drug delivery has been investigated, as it possesses numerous attractive properties, such as biosafety, biocompatibility and biodegradability (Dai et al., 2015). Additionally, ChS is considered a possible ligand targeting cancer cells, which overexpress CD44 receptors (Abdelaziz et al., 2020) and it is valued as a suitable polysaccharide for delivering active compounds to target cells (Dai et al., 2015).

In the same study by Abdelaziz et al. (2020), it was proposed to coat the surface of nanoparticles with ChS and LF through carbodiimide coupling or by electrostatic interactions, as a potential approach to attack cancer cells overexpressing LF receptors. Here, the use of LF and ChS as coating materials demonstrated their efficiency in tumour selection.

- Galactomannans

Galactomannans are industrial polysaccharides obtained from seeds of the Leguminosae family. They are storage polysaccharides formed by a skeleton of mannose with galactose branches. They are used to increase viscosity or film production. Galactomannans have applications in both food and clinical industries, due to their rheological properties (Albuquerque et al., 2017). In increasing the order of mannose:galactose ratio, we can distinguish different types of galactomannans: fenugreek gum (1:1), guar gum (2:1), tara gum (3:1) and locust bean gum (4:1) (Mathur & Mathur, 2005).

Nanoparticles containing LF have been formed from a soluble polyelectrolyte complex composed by LF/N-succinyl chitosan/galactomannan in a 1:3:2 ratio, after heat treatment below the protein heat denaturation temperature. The presence of galactomannan allows forming the particles, not only by electrostatic interaction but also by hydrophobic interactions and hydrogen bonds. Because of those interactions, LF is well integrated into these complexes, due to the hydrophobic interaction between the pyranose chains of chitosan, galactomannan and the hexose residues of the hydrocarbon component of LF (Il'ina et al., 2011). These biopolymer particles have a 250-400 nm size, regular spherical shape and good storage stability. The load of LF in the particles was 76-87% and they retained their characteristics after lyophilisation. Therefore, these particles can eventually be used for the targeted transport of biologically active substances (Il'ina et al., 2016).

The immobilization of LF in galactomannan films was proposed by Albuquerque et al. (2017) as an alternative to conventional edible solid dosages, such as capsules, particles, beads or tablets. This alternative can be effective for biotechnological applications in the pharmaceutical and the food industries, for individuals with dermal wounds or with difficulty in swallowing edible doses, for example.

The tara gum, like the rest of galactomannans, is a neutral polysaccharide; therefore, the insertion of charged groups into its structure allows its interaction with other polymers and proteins. The process of carboxymethylation consists of the insertion of negatively

charged carboxymethyl groups in the main polysaccharide, thus favouring the formation of complex coacervates (Santos et al., 2019). Taking the advantage of this property, Santos et al. (2021) performed the microencapsulation of vitamin D3 through complex coacervation in matrices formed by carboxymethyl tara gum and LF. To promote the electrostatic interaction between tara gum and LF, the system was shaken for 10 min at 300 rpm and then quickly placed in an ice bath to reduce the temperature. The addition of TGA to these structures catalysed the formation of covalent bonds between the proteins, increasing the stability of the particles formed. It was verified that a ratio of 1:3 (core:wall) was enough for correct encapsulation of the vitamin to ensure that it was protected by the polymeric matrix.

The complex coacervation of LF with biopolymers increases its stability during gastric digestion since the acidic pH of the stomach maintains both polymers with opposite charges supporting their interaction. However, intestinal pH (7) has a negative effect on coacervation since this pH modifies the positive charge of LF necessary for the interaction with tara gum (Santos et al., 2021). Therefore, these complexes improved the stability and facilitated the controlled release of the vitamin during GIT digestion.

- Gum arabic

Gum arabic (GA) is an anionic polyelectrolyte that mainly consists of three fractions of anionic branched arabinogalactans. These fractions are 80-90% arabinogalactan, 10-20% arabinogalactan-protein and 2-4% glycoprotein. The emulsifying properties of GA are mainly attributed to the arabinogalactan-protein (Cheng et al., 2021). Furthermore, due to its low viscosity and high solubility at high concentrations, and its emulsifying and encapsulating capacities, GA is extensively used in industry (Gulao et al., 2014).

The protein-polysaccharide electrostatic complexes can be used as stabilisers and emulsifiers in high internal phase emulsions (HIPEs). Some authors determined that the LF/GA complexes (1:1) can be used to form HIPEs over an ionic strength range of 0-300 mM NaCl at a pH range of 3-6. During the homogenisation applied for this process, GA is not as efficient as proteins at producing small droplets; but it is a better stabiliser once the droplets have been formed as reported by Cheng et al. (2021). These authors also showed that the antioxidant properties of LF allowed, in these HIPEs of LF/GA complexes, the encapsulation of nutraceutical compounds, such as curcumin, protecting them from chemical degradation.

- Pectin

Pectin is an anionic polysaccharide present in plants, mainly in fruits and young tissues. It is made up of galacturonic acid units with free carboxyl groups, which give it an acid tendency. In its native form, pectin is highly methylated. The degree of pectin methylation influences its ability to form gels. This polysaccharide is easily degradable under alkaline conditions and, especially, at high temperatures (Bengoechea et al., 2011). Pectin is a polysaccharide most used to produce multilayer encapsulation systems with whey proteins, and in particular, it has allowed the encapsulation of LF at pH between three and four with a size of around 700 nm and good characteristics to be used in pharmaceuticals and food products (Raei et al., 2017). In another study by Bengoechea et al. (2011), electrostatic complexes between high-methoxyl pectin and LF were formed at pH 7. These complexes were soluble at pH between 7 and 3.5 but underwent aggregation at lower pH. The properties of these pectin/LF complexes can be modulated through the pH, temperature or biopolymer concentration. Furthermore, it was observed that at pH 7 the stability of LF/pectin complexes to aggregation during heating was much better than that of LF alone.

Niu et al. (2019) also created LF/pectin complexes by mixing the two biopolymers in solution, leading to spontaneous colloid formation due to electrostatic interactions. They compared the potential of low (LM) and highly methylated (HM) pectin to interact with LF and determined the loading of LF in the capsules and their encapsulation efficiency. They concluded that LM pectin had a greater capacity to encapsulate LF, with an encapsulation efficiency of 40%, compared to 5% for HM pectin. Additionally, they verified the inhibitory effect on bacterial growth of the LF/pectin complexes at a concentration of 1 mg/mL, an effect that lasted at least 48 h. Therefore, they stated that LF encapsulation with pectin did not affect its bioactivity.

Moreover, in the study conducted by Niu et al. (2019), some LF/pectin complexes were coated with chitosan in order to increase their gastric stability and muco-adhesive properties. This coating improved the colloidal stability of the complex in simulated gastric fluid. Furthermore, in an HCl solution at pH 3, both systems, the uncoated and the chitosan-coated LF/pectin complexes, showed a gradual release of LF. After 2 h under these conditions, the uncoated complex showed a 30% release of LF, while the chitosan-coated complex only released 10%. However, under gastric simulated conditions (pH 3, no pepsin), the uncoated complex showed a negligible release of LF. This limitation of

LF release indicates that this protein would not be as exposed to digestive enzymes, such as pepsin. Therefore, these LF/pectin systems can be a good way to incorporate LF in foods or special products for oral administration, as they decrease the hydrolytic effect of pepsin on LF, protecting this bioactive molecule from gastric digestion up to at least 60 min for a gut-targeted delivery (Niu et al., 2019).

- Chitosan

Chitosan is a useful natural biopolymer, obtained by chemical or enzymatic deacetylation of chitin, a polysaccharide present in nature (insects, crustaceans and fungi) (Azmana et al., 2021). It has multiple reactive groups that can help in the transport of drugs due to its physicochemical properties, such as charge and hydrophobicity. Thus, due to these reactive groups, chitosan nanoparticles can be complemented with targeting vectors like LF (Lyalina et al., 2016). These authors showed that LF and succinyl-chitosan formed stable and high-yield nanoparticles and that the globular structure of LF allowed the formation of stable nanoparticles without chemical conjugation. These nanoparticles were designed to bind to immune cells, such as macrophages or phagocytic cells, in a few minutes and deliver LF by cross-presentation, the mechanism of antigen-presenting cells (APCs). Thus, the encapsulation of LF into chitosan can change the traffic of proteins inside APCs and modify the immune responses.

LF was found to form nanohydrogels by thermal gelation with the glycomacropeptide (GMP) showing high encapsulation efficiencies for curcumin and caffeine and a controlled release of those compounds (Bourbon, Cerqueira, & Vicente, 2016). Later, the same authors (Bourbon et al., 2016) studied the application of chitosan coating in LF/GMP nanohydrogels. This biopolymer did not affect the shape of nanohydrogels, maintaining their spherical shape and decreasing their aggregation in the solution. These authors evaluated the effect of chitosan-coat on release mechanisms of nanohydrogels at gastric digestion, using caffeine as the encapsulated compound. They concluded that the presence of chitosan improved the stability of LF/GMP nanohydrogels, keeping them intact until 60 min under acidic conditions, compared with the 15 min that the uncoated nanohydrogels remained intact. They continued their study and, in 2018, revealed that chitosan coating reduced the protein degradation from 86% to 76% for LF and from 71% to 53% for GMP. Furthermore, the chitosan increased the bioaccessibility of curcumin (bioactive compound encapsulated in the nanohydrogel) and protected its antioxidant

activity during the GIT digestion, losing only 30% of it compared to the 68% of activity loss for free curcumin (Bourbon et al., 2018).

Throughout this review, it has been shown that chitosan can be combined with other polysaccharides to favour the stability and integrity of the formed particles (Braim et al., 2019; Il'ina et al., 2011; Il'ina et al., 2016; Niu et al., 2019). As an example, chitosan together with pectin has been used to stabilise LF-loaded liposomes obtaining solid particles that are more resistant than liposomes to heat and to simulated intestinal digestion (Yao et al., 2015).

3.2. Nanocarriers coated with lactoferrin

The advances in the design of targeted drug delivery systems have been very relevant in the last few decades. These delivery systems must consider the surface properties and receptors of the target cell, the properties of the carrier and the suitable ligand to be utilised. Moreover, a targeted drug delivery system should be non-immunogenic, biocompatible, specific to the target site, and show chemical and physical stability, both *in vitro* and *in vivo* (Agwa & Sabra, 2020).

There are two strategies to design targeted delivery: passive and active. In the passive strategy, drugs are loaded into nanocarriers of a size around 200 nm, which can reach the tissues or organs by their enhanced permeability and retention. In the active strategy, drug-loaded nanocarriers are conjugated with a certain ligand, which can further bind to a specific receptor on the surface of target cells. This receptor is normally overexpressed in the affected cells by the disease and consequently, the drug accumulates on them (Agwa & Sabra, 2020).

Receptors for LF have been expressed in many cells, such as brain endothelial cells, liver cells, epithelial respiratory cells, cancer cells, etc. Therefore, LF can be used to cover nanocarriers loaded with drugs to increase their delivery to the cells via LF interaction with its receptors. Furthermore, the cationic nature of LF allows its binding to anionic cellular compounds, such as glycosaminoglycans (Agwa & Sabra, 2020).

In the last few years, inorganic nanoparticles have attracted considerable attention for drug delivery and imaging applications due to their characteristics. The positive features of these nanoparticles are their higher loading capacity, ease of functionalization with several types of ligands, biocompatibility and adaptable degradation rates. There are

several types of nanocarriers coated with LF that have been developed for delivery applications.

Thus, superparamagnetic iron oxide nanocarriers (Fe_3O_4-LF) with narrow size ranges were developed via surface functionalization with LF using the EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) coupling reaction aiming to target the receptors that are highly expressed on the surface of human fibroblasts (Gupta & Curtis, 2004).

Superparamagnetic iron oxide nanoparticles conjugated with LF (LF-SPIONs) (Xie et al., 2011) and LF-PEG-Fe_3O_4 (Qiao et al., 2012) were successfully fabricated via the EDC coupling reaction and were applied as a magnetic resonance imaging contrast agent for improving the detection of *in vivo* brain glioma.

Many other applications of superparamagnetic nanocarriers coated with LF have been applied in antitumour treatments (Fang et al., 2016; Sharifi et al., 2020; Song et al., 2017). LF has been covalently attached to silica nanoparticles by EDC coupling reaction and then PEGylated to extend the blood circulation time (Agwa & Sabra, 2020). The LF coated silica nanoparticles have been revealed as effective against breast cancer and as able to cross the brain–blood barrier (Ali et al., 2020; Song et al., 2017). There are many applications of nanoparticles and nanocarriers functionalised with LF to treat different pathologies, and they have been compiled in **Table 1**.

3.3. Edible and active film composites with lactoferrin

The processing of edible coatings and films is based on the use of various compounds with different properties, such as polysaccharides, proteins and hydrocolloids. A method to improve the properties of edible films is mixing polysaccharides and proteins, which is a promising area of research (Coughlan et al., 2004).

The development of new edible films has a great interest in the industry and for consumers, who are concerned about the need to reduce the use of plastic packaging for environmental reasons (Dinika et al., 2020). Several compounds have been used to develop edible films, trying to preserve the sensorial properties and safety of foods. On the one hand, the use of edible films containing antimicrobial agents has certain advantages over their direct application on the food surface, as they can be designed to control the speed of antimicrobial diffusion to the surface of food (Min & Krochta, 2005).

Table 1. Studies regarding the use of lactoferrin in different nanocarriers for therapeutic applications.

Application	Type of nanocarrier	Model of study	Reference
Cancer therapy			
Brain tumour	Magnetic nanocarriers	Rats	Fang et al., 2016
	Solid lipid nanoparticle	HBMECs, U87MG cells	Kuo & Cheng, 2016
Breast cancer	Polymeric nanoparticles	MDA-MB-231 cells, mice	Mahidhara et al., 2015
	Silk nanoparticles	MDA-MB-231, MCF-7 cells	Roy et al., 2016
	Protein nanocapsules	Mice	Kanwar et al., 2016
	Quantum dots-based ChS nanocapsules	MDA-MB-231, MCF-7 cells, mice	AbdElhamid et al., 2018
	Zein nanospheres	MCF-7, 4T1 cells, rats	El-Lakany et al., 2018
	Polymeric micelles	MDA-MB-231, MCF-7 cells	Sabra et al., 2018, 2019
	Silica nanoparticles	MCF-7 cells	Ali et al., 2020
	Betulinic acid nanoparticles	MDA-MB-231 cells	Halder et al., 2020
	Magnetic nanoparticles	4T1 cells	Sharifi et al., 2020
Bronchogenic carcinoma	Solid lipid nanoparticles	BEAS-2B cells, rats	Pandey, Gajbhiye, & Soni, 2015
Colon tumour imaging	Polymeric nanocarriers	Caco-2 cells, mice	Kanwar et al., 2012, 2015 Roy et al., 2015
	Protein nanoparticles	COLO-205 cells, rats	Ahmed et al., 2018
Glioblastoma	Polymeric nanoparticles	BCEC, C6 glioma cells, rats, mice	Miao et al., 2014
	Polymeric nanoparticles	U87MG, HBMEC, HA cells	Kuo & Chen, 2015
	Protein nanoparticles	U87MG, BCEC, HUVEC cells, mice	Mo et al., 2018
	Protein, FePt nanoparticles	U87MG, U-373 MG, MDCKII cells, rats	Pandey et al., 2019, 2020
	Gadolinium oxide nanoparticles	U87MG, MCF-7 cells, mice	Shen et al., 2020
Glioma	Magnetic nanoparticles	HEK 293, C6 glioma, ECV 304 cells, rats	Xie et al., 2011
	Magnetic nanogels	C6 glioma, ECV 304 rats	Jiang et al., 2013
	Protein nanoparticles	BCECs, C6 glioma cells, rats	Su et al., 2014
	Magnetic nanoparticles	C6 glioma cells	Tomitaka et al., 2015
	Magnetic nanoparticles	C6 glioma cells, rats	Zhou et al., 2015
	Polymeric nanoparticles	C6 glioma cells, rats	Li et al., 2018
	Magnetic nanocomposites	C6 glioma cells	Song et al., 2017
	Polymeric nanoparticles	BCECs, C6 glioma cells, mice	Xu et al., 2017
	Polymeric nanoparticles	L929, glioma 261 cells	Tammam, Azzazy, & Lamprecht, 2018
Hepatocellular carcinoma	PEGylated liposomes	HepG2, ECV304, BEL7402, NIH 3T3, SMMC7721 cells, mice	Wei et al., 2012, 2015a
	PEGylated liposomes	HepG2 cells	Wei et al., 2015b
	Protein nanoparticles, nanoemulsion	HepG2 cells, mice	Abdelmoneem et al., 2018, 2019

Table 1. *Cont.*

Laryngeal cancer	Betulinic acid nanoparticles	HEp-2 cells	Halder et al., 2020
Lung carcinoma	Inhalable nanocomposites	A549 cells, mice	Abd Elwakil et al., 2018
	Lipid nanocarriers	A549 cells, mice	Kabary et al., 2018
	Inhalable nanocomposites	A549 cells, mice	Abdelaziz et al., 2020
	Polymeric nanoparticles	A549 cells, mice	Rofeal et al., 2020
Pancreatic cancer	Polymeric nanoparticles	PANC-1 cells, mice	Etman et al., 2020 ; Etman, Abdallah, & Elnaggar, 2020
Retinoblastoma	Protein nanoparticles	Y79 cells	Ahmed, Ali, & Kondapi, 2014
Other pathologies			
Alzheimer	Protein nanoparticles	*In silico*	Akilo et al., 2018
	Polymeric nanoparticles	16HBE, SH-SY5Y cells, mice	Meng et al., 2018
	-	Review	Gopalan et al., 2020
	Polymeric nanoparticles	bEnd3 cells, mice, rats	Gothwal et al., 2019
Amyotrophic lateral esclerosis	Polymeric nanoparticles	Review	Mazibuko et al., 2015
Dental caries	Liposomes	Rats	Martínez-Gomis et al., 1999
Leishmaniosis	Polymeric nanoparticles	Mice	Halder et al., 2018
Infection by Clostridioides	Polymeric nanoparticles	Caco-2, Vero cells	Braim et al., 2019
Infection by Helicobacter pylori	Nanocrystals	Mice	Fulgione et al., 2016
HIV	Nanosuspensions	U-937 cells, rats	Kumar et al., 2017
	Protein nanoparticles	SupT1, HL2/3 cells	Senapathi et al., 2020
Infection by papilloma virus	Transferosomes	Hela cells	Hadidi, Saffari, & Faizi, 2018
Infection by Toxoplasma gondii	Protein nanocapsules	J7741 cells, mice	Anand et al., 2015
Infection by Plasmodium berghei	Polymeric nanoparticles	Mice	Anand et al., 2016
Osteoarthritis	Polymeric nanocarriers	Mice	Samarasinghe, Kanwar, & Kanwar, 2014
Parkinson	Protein nanoparticles	BCECs cells, rats	Huang et al., 2009, 2010
	Polymeric nanoparticles	bEnd.3 cells, mice	Hu et al., 2011
	Polymeric nanoparticles	SH-SY5Y, 16HBE cells, mice	Bi et al., 2016
	Polymer, solid lipid nanoparticles, liposomes, exosomes	Review	Kuo & Rajesh, 2018
	Polymeric nanoparticles	SH-SY5Y, 16HBE cells, rats	Tang et al., 2019
	Phosphorus nanosheets	SH-SY5Y, bEnd.3 cells, mice, rats	Xiong et al., 2020
Tendinitis	Polymeric	Tenocytes, rats	Choi et al., 2020

There are several techniques used to incorporate antimicrobial agents into packaging films. Some techniques are inappropriate for sensitive compounds, like those involving direct incorporation in the polymer matrix, whereas other strategies like film coating are much more adequate (Barbiroli, Farris, & Rollini, 2016).

Chitosan is a natural polymer that is biodegradable, biocompatible and presents antimicrobial properties. LF has been incorporated into chitosan films to improve barrier properties and to enhance the preservation of foods due to its antibacterial activity (Bourbon et al., 2011). In the study by Brown et al. (2008), a chitosan film containing LF alone was not found to be effective against *Listeria monocytogenes* and *E. coli* O157:H7, though when it was combined with lysozyme (LYS), the antibacterial activity of LYS was increased more than when it was combined with EDTA. A mixture of LF and LYS was also incorporated into cellulose-based packaging material and was proved to be effective against some meat contaminants, such as *Escherichia* and *Listeria*, also limiting the increase of natural microbiota present in veal meat (Barbiroli et al., 2012).

Cellulose films from bacterial origin containing LF have been proven to be non-toxic and have adequate technological characteristics to be used as bio-based meat product casings, showing a bactericidal activity against *E. coli* and *Staphylococcus aureus* (Padrao et al., 2016). The methodology used for binding LF to cellulose films has a great influence on its antibacterial activity. Thus, Padrao et al. (2020) compared the binding of LF to bacterial nanocellulose (BNC) by embedding the protein within the three-dimensional structure of BNC with its covalent binding to BNC nanofibrils. The concentration of LF in BNC obtained using the first method was twice that obtained with the second one, but LF only maintained a significant bactericidal activity against *E. coli* and *S. aureus* in the first type of BNC.

Starch is one of the most widely used materials within the bioplastic industry, due to its biodegradability, renewability, availability and low cost (Moreno, Atarés, & Chiralt, 2015). Starch does not have thermoplastic properties, though, with the aid of additives, it gels producing thermoplastic starch (TPS) that has been used for developing films. Thus, in the study by Moreno, Atarés and Chiralt (2015) LF and LYS were incorporated into the starch film, providing it with beneficial properties. Neither LF nor LYS was effective enough as antimicrobials when they were applied in the film separately, though the combination of both proteins weakly enhanced their antimicrobial activity against E. coli and coliform microbiota of pork-minced meat. The films containing a blend of LF and

LYS also reduced lard oxidation after long storage times (Moreno, Atarés, & Chiralt, 2015).

In the study by Tavassoli et al. (2021), multifunctional films were created by embedding different kinds of functional nanoparticles into a gelatin-based film prepared using a casting method. The nanoparticles were prepared by cross-linking cationic chitosan nanofibers, and afterwards, quercetin, LF, or both were introduced into these nanoparticles. The incorporation of the nanoparticles into the films decreased their mechanical strength and stiffness but increased their flexibility. The presence of LF, quercetin and chitosan in the gelatin-based films increased their antimicrobial and antioxidant activity. Furthermore, the incorporation of the nanoparticles into the films improved their degradation under simulated environmental conditions.

Bovine LF and its derived peptide lactoferricin B were individually immobilised on two different coatings. The coatings were functionalized with carboxyl groups deposited in the inner part of polyethylene microtubes by using a plasma deposition process fed with ethylene and acrylic acid vapours (Quintieti et al., 2013). The resulting functionalized tubes were tested for antimicrobial activity against three Pseudomonas strains responsible for casein hydrolysis and cheese pigmentation in Mozzarella. It demonstrated the antibacterial activity of immobilized lactoferricin B, with a significant reduction in the growth of the bacteria tested, though no activity was observed for the immobilised LF. Many studies have been conducted showing the incorporation of LF, alone or combined with other molecules, in active and edible films, which are compiled in **Table 2.**

4. Discussion

The current review has evaluated the encapsulation strategies to protect LF and its bioactivity. These techniques have been developed in recent years to preserve bioactive compounds incorporated into pharmaceutical, food and cosmetic products.

Nanoencapsulation is defined as a process in which very small particles are surrounded by a coating or embedded in homogeneous or heterogeneous matrices. This process can improve the bioavailability and solubility of bioactive compounds, enhance their residence time and stability in the GIT tract and give them a better ability to enter and permeate tissues and cells.

Table 2. Studies regarding the use of lactoferrin in different systems for applications in food technology.

System	Other molecules combined	Activity	Reference
Composite edible and active films			
Chitosan films	Glycomacropeptide	Water vapor, oxygen and carbon dioxide permeability decrease	Bourbon et al., 2011
	Lysozyme	Antimicrobial activity against *Listeria monocytogenes* and *E. coli* O157:H7	Brown, Wang, & Oh, 2008
Cellulose films	Lysozyme	Antimicrobial activity against *Escherichia, Listeria* and natural microbiota in veal meat	Barbirol et al., 2012
	-	Antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*	Padrao et al., 2016, 2020
Starch films	Lysozyme	Antimicrobial activity against *E.coli* and coliform microbiota of pork minced meat Lard oxidation reduction	Moreno, Atarés, & Chiralt, 2015
Gelatin-based films with chitosan nanoparticles	Quercetin	Antimicrobial activity, Antioxidant, Film degradation improvement	Tavassoli et al., 2021
Polyethylene microtubes	Lactoferricin B	Antimicrobial activity against *Pseudomonas* strains responsible for casein hydrolysis and cheese pigmentation in Mozzarella	Quintieri et al., 2013
LF nanoparticles			
	Cichoric acid	Antioxidant	Li et al., 2018
	Pectin and curcumin	Antioxidant	Yan et al., 2017
	Iron	Iron carrier	Martins et al., 2016
	-	Functional ingredients in commercial products	Bengoechea et al., 2011
Emulsions			
	Resveratrol	Emulsion stability and antioxidant activity	Acevedo-Fani et al., 2017
	WPI or whey protein hydrolysates with oil mixture	Ingredient in powder formula to mimic fat composition of human milk	De Figueiredo Furtado et al., 2021
	-	To deliver active compounds	Çelebioğlu et al., 2020 ; Liu et al., 2018
Coacervates			
Whey proteins	B9 vitamin	Non-specified	Anema & de Kruif, 2014
		For functional foods	Chapeau et al., 2016, 2017
		Food systems, bioactive encapsulation	Delboni & Da Silva, 2016; Tavares et al., 2017
Caseins		To deliver food ingredients and bioactive compounds	Anema & de Kruif, 2011, 2012a, 2012b, 2016

Table 2. *Cont.*

Pea protein isolate		Food applications	Adal et al., 2017
Soy protein isolate		Increase thermal stability of LF	Zheng et al., 2020
Gels			
Whey protein isolate		Improvement of gelled protein products	Li & Zhao, 2018
GMP	Curcumin and caffeine	Encapsulation to deliver compounds	Bourbon, Cerqueira, & Vicente, 2016
Polysaccharides		Food additive	Wang et al., 2017
		To deliver compounds	Raei et al., 2015
		To encapsulate essential oils	Bastos et al., 2020
		To encapsulate probiotics	Yucel Falco et al., 2019

During the last few years, many studies have detailed encapsulation by combining a protein, specifically LF, with milk proteins, other proteins or polysaccharides of different origins. Furthermore, numerous articles focus on nanocarriers coated with LF or edible and active film composites with LF.

Many studies have demonstrated the interaction of LF with proteins of different origins, including milk proteins (such as β-Lg, α-La and caseins), pea or soy proteins. The main interaction mode is by means of electrostatic interactions, based on the basic nature of LF. However, the biological activity of LF in these systems has not been demonstrated in many cases. The binding of LF to whey proteins, caseins or soy proteins increases LF thermal resistance, which shows great potential for different applications in industry.

LF can be useful for the delivery of different molecules. In that sense, it has been shown that LF, by forming coacervates with β-Lg, can act as a carrier for the release of vitamin B9. LF can also be used as an emulsifier for the release of other active compounds, such as curcumin or β-carotene and forms more stable emulsions in infant formulas.

Only one system based on proteins has been identified for the oral delivery of LF itself. BSA, with TA, has been used to encapsulate LF for oral administration by alternating layers of BSA and TA on porous microparticles of $CaCO_3$, previously prepared with LF. This system has been studied in an animal model showing promising results, as LF was identified in the liver, demonstrating that it can pass the GIT tract.

The formation of the protein-polysaccharide complex depends on various factors, such as pH, molecular weight and charge density. There are numerous polysaccharides that can be combined with LF: natural like Alg or chitosan, or industrial like galactomannans. In any case, all of them allow the encapsulation of LF or the combination with this protein to transport bioactive compounds such as vitamin D3, curcumin or nutraceutical compounds, protecting their bioactivity and integrity against chemical degradation.

Many studies have focused on the formation of these complexes by electrostatic interactions, taking advantage of the positive charge of LF and the opposite charge of the polysaccharides, such as Alg or ChS. This complex coacervation of LF with biopolymers increases its stability during gastric digestion since the acidic pH of the stomach maintains both polymers with opposite charges supporting their interaction. However, intestinal pH has a negative effect on coacervation, modifying the positive charge of LF necessary for the interaction with the polysaccharide. Therefore, these complexes improved the stability and facilitated the controlled release of the protein during GIT digestion. Furthermore, galactomannans allow, in addition to electrostatic interaction, the formation of films by hydrogen bonds or by hydrophobic interactions that favour a good integration of LF.

In addition to a controlled release in the gut, some polysaccharides allow delivery to target cells. While ChS combined with LF targets cancer cells, being effective for tumour selection, chitosan-LF nanoparticles bind immune cells and deliver LF by cross-presentation to macrophages or phagocytic cells. Several authors have used chitosan to coat LF-polysaccharide complexes, increasing the integrity of the capsule and its stability under gastric conditions.

The strategy of coating nanoparticles with LF has been developed intensively over the past few years. The receptors for LF are highly expressed on the surface of many cells; therefore, nanocarriers functionalised with LF have been used to treat tumours of several origins, such as mammary, hepatic, pancreatic and colonic, among others, with different results. However, there are still some issues to be investigated to know the interaction of LF-nanocarriers with the capillary endothelial cells in the blood-brain barrier and the kinetics of LF interaction with the surface of different types of cells. The use of LF nanocarriers to enhance the imaging analysis of some brain tumours is also an area of great interest for future development.

The food industry is currently investing in the development of edible and biodegradable films with properties directed to improve food preservation and to avoid the use of plastic packaging. Furthermore, the incorporation of bioactive molecules into the films has been encouraged over the past few years. These new films are able to maintain food quality and safety and, at the same time, allow reducing the use of chemical additives. In many studies, LF has been used in active films because of its antimicrobial properties. However, the results have indicated the existence of some problems in maintaining LF activity in the process of combining it with the base materials to build films or packaging. Some studies have proved that the combination of several active molecules with films, such as LF and LYS, gives better results. Nevertheless, the enrichment of films with bioactive molecules adds a high cost to the final product, a limitation that should be resolved to make those films applicable to food industry.

5. Conclusions

Lactoferrin is a molecule with many biological properties that must be preserved when it is used in products to improve health or is incorporated into active films designed to maintain the quality of food products. The encapsulation of this bioactive protein with other proteins or polysaccharides prevents its proteolysis during gastric digestion, allowing a targeted release of LF. This protein can also be used in the active targeted drug delivery, where drug-loaded nanocarriers are covered with LF, which can further bind to its receptors expressed on the surface of target cells. Future research on all these issues should be continued by evaluating the different LF activities, after being encapsulated or combined with certain composite materials, in *in vitro* assays and eventually, in humans.

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Conceptualization, L.S.; article reviewing, L.S., I.A. and C.C.; writing— original draft preparation, L.S., I.A. and C.C.; writing—review and editing, L.S., I.A. and C.C. All authors have read and agreed to the published version of the manuscript.

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ARTÍCULO 4

Effect of *in vitro* gastrointestinal digestion on the antibacterial activity of bioactive dairy formulas supplemented with lactoferrin against *Cronobacter sakazakii*

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Effect of in vitro gastrointestinal digestion on the antibacterial activity of bioactive dairy formulas supplemented with lactoferrin against *Cronobacter sakazakii*

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Abstract

Milk is a source of proteins with high nutritional value and relevant biological activities. Bioactive milk proteins, like lactoferrin, are important for newborn development and can also be used as ingredients in functional products to improve health. Lactoferrin is essential in infant's diet, since protects against infections and promotes immune system maturation. Bovine lactoferrin is used to supplement formula milk in order to strengthen baby's defences against some pathogenic bacteria. Thus, lactoferrin supplemented formula can be a barrier against emergent pathogens, such as *Cronobacter sakazakii*, which has caused great concern in the last few years. Milk proteins generate bioactive peptides in the digestion process, and it is known that industrial processing can modify their susceptibility to digestion. Treatments such as heating have been shown to denature whey proteins and make them more easily digestible. Therefore, the aim of this study was to analyze the effect of technological treatments and gastrointestinal digestion on the antibacterial activity against *C. sakazakii* of proteins present in dairy formulas supplemented with lactoferrin. Commercial bovine lactoferrin has been shown to have antibacterial activity against *C. sakazakii*, both in the native state and after static *in vitro* gastrointestinal digestion. In addition, the digests obtained from dairy formulas subjected to technological treatments, either homogenization or pasteurization, have higher antibacterial activity than non-treated formulas. The release of low molecular weight peptides during the *in vitro* gastric digestion is probably the cause that would explain the enhanced antibacterial activity of the digested dairy formulas.

1. Introduction

According to the Codex Alimentarius (1999), milk is defined as the normal mammary secretion obtained from dairy animals, without any addition or extraction, intended for consumption as liquid milk or for processing. Similarly, according to Regulation (EC) Nº 853/2004, milk is the secretion of the mammary glands of animals that has not been heated to a temperature above 40 °C or subjected to a treatment with equivalent effect (European Commission, 2004). In any case, milk intended for commercialisation must be subjected to pasteurization or sterilization to eliminate all pathogenic microorganisms.

Furthermore, milk can be subjected to other technological treatments, such as homogenization, which is usually applied to reduce milk fat globule size. In addition to ensuring dairy product safety, heat treatments also extend their shelf life (Claeys et al., 2013).

Milk is characterized by being a very complete food and it is considered a basic element in the diet. For this reason, there is a great demand for milk and dairy products. The main components of milk are protein, fat, lactose and minerals. The composition of milk varies among species and within the same species can also vary considerably depending on breed, stage of lactation, milking interval, type of feeding and climate (Roy et al., 2020). The major protein of most mammal milks is casein. This protein is only found in milk and it has a great biological value thanks to its content in essential amino acids (Fox et al., 2015). In addition to caseins, milk contains other proteins in the fraction called whey, which is obtained after acid or enzymatic precipitation of caseins, or by separation from casein by membrane filtration (Pires et al., 2021). The major whey proteins are β-lactoglobulin (β-LG), α-lactalbumin (α-LA), immunoglobulins, bovine serum albumin, lactoferrin (LF) and lactoperoxidase (Madureira et al., 2007).

Fat is present in milk as emulsified particles called fat globules, which are covered by a membrane known as the milk fat globule membrane (MFGM) and is mainly composed of proteins, phospholipids, cholesterol and glycerides (Manoni et al., 2020). The main proteins of MFGM are mucin, lactadherin, butyrophilin, xanthine oxidoreductase (XO) and adipophilin (Riccio, 2004). Several health promoting effects have been proposed for MFGM, such as anticarcinogenic, antimicrobial, anti-inflammatory and anticholesterolemic activities (Manoni et al., 2020). However, the MFGM can be affected by technological treatments applied to milk, such as heat treatment, refrigeration or freeze-drying, losing or minimizing its beneficial effects (Singh, 2006).

Most dietary proteins, and in particular milk proteins, produce bioactive peptides by the action of digestive enzymes during gastrointestinal digestion and milk fermentation. Once released, they are directed to the intestinal lumen, passing across the epithelium and reaching the systemic circulation to interact with target receptors (Tidona et al., 2009). These bioactive peptides can trigger physiological effects that influence body functions and consequently, impact positively on health. They exert functions such as antithrombotic, antihypertensive, antimicrobial and antiviral among others (Madureira et al., 2010).

In recent decades, a great interest in antimicrobial peptides has emerged, as an alternative to antibiotics. They consist of less than 100 amino acids and their composition includes positively charged residues, such as lysine, arginine and histidine, and a high amount of hydrophobic residues (Browne et al., 2020). Peptides with antimicrobial characteristics interact specifically with bacterial membranes, creating transmembrane pores and altering the osmotic gradient and consequently, membrane permeability (Hartmann et al., 2010). A great number of milk-derived bioactive peptides with interesting activities have been identified. Specifically, LF contains some antimicrobial peptides, such as lactoferricin and lactoferrampin. In addition, several bioactivities have been attributed to peptides from other milk proteins, such as caseins, β-LG or α-LA (Théolier et al., 2014).

Breast milk is the best source of nutrition for the newborn, as it contains a large number of bioactive agents. However, breast-feeding is not always possible, so it is important to have high-quality infant formula available. Milk mainly used as a base to make infant formulas is cow's milk, although the demand for goat's milk is increasing recently for this purpose. In addition, other ingredients are added to resemble mother's milk as much as possible (Maathuis et al., 2017). However, the composition of breast milk differs quantitatively and qualitatively from cow's milk. For this reason, the average nutrient content of breast milk is taken as a reference to elaborate infant formula (He et al., 2022). Furthermore, the fat of cow's milk is replaced by plant oil blends to compensate for the deficit of unsaturated fatty acids and make it more similar to human milk fat (Pan et al., 2022). In addition, fat globules in cow's milk are quite uniform in size, with a maximum of 4 μm (Bourlieu et al., 2015). However, human milk contains smaller fat globules, especially in the early stages of lactation, with a maximum size of 1.8 μm in colostrum (Ruegg and Blanc, 1981). For this reason, homogenization and pasteurization are used to treat and stabilize infant formulas, reducing the size of cow's milk fat globules (0.2-0.6 μm) and making them more similar to those of breast milk (Bourlieu et al., 2015). In these processes, a membrane composed of caseins and whey proteins is formed, thus maintaining milk fat globule structure (Pan et al., 2022). These technological treatments also modify the effect of digestion on dairy preparations, as it has been reported a higher release of fatty acids and a higher proteolysis of caseins in processed milk by homogenization or pasteurization (Bourlieu et al., 2015).

There are different processes to elaborate infant formula; some are based on mixing powder components in hygienic conditions and others on reconstituting powder

ingredients, applying heat treatment and spray drying (Watson, 2022). In any case, powder formulas are not a sterile product, and inadequate conditions of production, storage and handling can be a risk to infant health (Vargas-Leguas et al., 2009). For this reason, powdered milk products must be stored in a dry place, at temperatures below 20 °C and with a maximum relative humidity of 70%. In addition, it is recommended for powder milk to be consumed within a maximum period of one month once the container is opened, and to be administered immediately once reconstituted or keep in refrigeration no more than 24 h (Vargas-Leguas et al., 2009).

An emergent opportunistic pathogen that has caused great concern in the last few years has been *C. sakazakii*, as it has been associated to consumption of infant powder formula. To avoid and reduce the risk of infection by this pathogen, special care has been recommended in the handling and storage of powder formulas (Drudy et al., 2006). Although *C. sakazakii* causes illness in all age groups, its effects are most severe in children, and specially in low-weight babies, causing diseases like meningitis, necrotizing enterocolitis and sepsis in preterm and full-term infants (Henry & Fouladkhah, 2019).

In any case, milk and dairy products are a source of nutrients and defensive factors not only for the newborn, but also for children and adults (Park & Nam, 2015). Therefore, the main objective of this study was to evaluate the antibacterial activity against *C. sakazakii* of LF alone and in dairy formulas containing other milk bioactive proteins. The effect of technological treatments, such as homogenization or pasteurization, and of gastrointestinal digestion on the antibacterial activity of dairy formulas supplemented with LF have also been studied.

2. Material and methods

2.1. Obtaining dairy fractions

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. Previously to processing, milk pH, acidity, fat percentage, and alkaline phosphatase and lactoperoxidase activities were assessed. Milk was heated at 45-50 °C for 30 min in a cheese vat and skimmed in a skimming centrifuge model ARR-DES 125 (Suministros Quimicos Arroyo, Santander, Spain). Skim milk was subjected to a second centrifugation to obtain the maximum amount of cream.

Then, skim milk was heated at 35 °C in a 25 L cheese vat and 30% $CaCl_2$ was added into milk at dilution 1:8000 (v/v). Next, bovine rennet was added to milk at dilution 1:15,000 (v/v), evenly distributed and incubated for 1 h. After achieving casein coagulation, the curd was cut with lyres and whey was drained, filtered, lyophilized and kept at -20 °C for later use.

Cream containing 43% of fat, previously obtained from raw milk as described above, was used to obtain buttermilk. Cream was maintained at 4 °C overnight, and then it was 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. Buttermilk was filtered through cheese cloth and glass wool to remove the remaining butter grains, lyophilized and kept at -20 °C.

2.2. Preparation of samples and dairy formulas

Several dairy formulas were prepared to be subjected to static *in vitro* gastrointestinal (GIT) digestion and to subsequent evaluation of their antimicrobial activity against *C. sakazakii*. Commercial bovine LF used to supplement dairy formulas was donated by the company Tatua Nutritionals (Morrinsville, New Zealand).

Six dairy formulas were elaborated, based on whey or buttermilk and supplemented with LF and MFGM. Formula 1 (F1) was composed of whey (0.068 g/mL), LF (10 mg/mL) and MFGM obtained from the centrifugation at 40,000 g for 30 min at 4 °C of a volume (in 1:1 ratio with whey) of non-homogenized buttermilk. Formula 2 (F2) was made based on non-homogenized buttermilk, supplemented with LF (10 mg/ mL) and MFGM, obtained as in F1, from a volume of non-homogenized buttermilk (in 1:1 ratio with buttermilk). Formulas 3 and 4 (F3 and F4) were prepared with the same composition as F1 and F2, respectively, but buttermilk was previously subjected to one-phase homogenization at 250 bar. Formulas 5 and 6 (F5 and F6) were also prepared with the same composition as F1 and F2, respectively, but were subjected to a pasteurization process at 72 °C for 20 s.

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, where they were maintained for 20 s once they reached that temperature. After pasteurization, samples were cooled down immersing them into ice and afterwards, stored at -20 °C until use.

2.3. Culture of *Cronobacter sakazakii*

The bacterial strain used in this study was *C. sakazakii* CECT 858, supplied by the Spanish Type Culture Collection (CECT, Valencia, Spain), which corresponds with the strain ATCC 29544 of the American Type Culture Collection and is of clinical origin from a child throat. It is recommended as a reference strain by international standards ISO 22964:2017 and ISO 11133:2014/Amd 2:2020. For the reference stock, the bacteria were fixed to porous rings and stored in cryovials at -80 °C. To cultivate *C. sakazakii*, we followed the procedure described in our previous study (Abad et al., 2022).

2.4. *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). First, the simulated digestion solutions were prepared: salivary solution (SSS) at pH 7, gastric solution (SGS) at pH 3 and intestinal solution (SIS) at pH 7. These solutions were elaborated according to the concentrations of salts indicated in **Table 1** and sterilized with a 0.22 μm low binding protein (LBP) Millipore filter (Merck KGaA, Darmstadt, Germany).

After preparation of all solutions, a static *in vitro* digestion of dairy formulas was carried out. For this, an initial volume of 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. This first step of salivary digestion was incubated for 2 min at 37 °C under agitation. 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.

For 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 and 118 μL of milli-Q water were added. The final volume 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.

Table 1. Composition for a final volume of 200 mL of the simulated salt solutions for the different stages of *in vitro* GIT digestion (1.25x concentrations). SSS: simulated salivary solution; SGS: simulated gastric solution; SIS: simulated intestinal solution [adapted from Mackie & Rigby (2015)].

Salt	Stock concentration		Simulated solutions					
			SSS pH 7		SGS pH 3		SIS pH 7	
			Vol.	Conc. in SSS	Vol.	Conc. in SGS	Vol.	Conc. in SIS
	g/L	mol/L	mL	mM	mL	mM	mL	mM
KCl	37.7	0.5	7.55	15.1	3.45	6.9	3.4	6.8
KH_2PO_4	68	0.5	1.85	3.7	0.45	0.9	0.4	0.8
$NaHCO_3$	84	1	3.4	13.6	6.25	25	21.25	85
NaCl	117	2	-	-	5.9	47.2	4.8	38.4
$MgCl_2(H_2O)_6$	30.5	0.15	0.25	0.15	0.2	0.12	0.55	0.33
$(NH_4)_2CO_3$	48	0.5	0.03	0.06	0.25	0.5	-	-

To carry out 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 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 fractions obtained in the digestion were lyophilized and subsequently resuspended with the adequate volume of milli-Q water to obtain a final LF concentration of 5 mg/mL. The digests were filter sterilized using a 2 µm prefilter and a 0.45 µm LBP filter. Additionally, the original undigested formulas were also filtered for later use in the assays. In the case of F1 and F2, centrifugation at 3000 and 5000 g, respectively, for 5 min was required to eliminate some aggregates that made difficult the filtering. Formulas F3 to F6 were passed directly through 2 µm prefilter and 0.45 µm LBP filter. All the samples were stored at -20 °C until later use.

2.4.1. Trypsin activity assay

This analysis, described by Brodkorb et al. (2019), was carried out to assess the activity of the trypsin present in the pancreatin used in the intestinal phase of the *in vitro* digestion. For this assay, 10 mM p-toluene-sulfonyl-l-arginine-methyl-ester (TAME) (Sigma-

Aldrich) was used as trypsin substrate. The reaction of TAME in the presence of trypsin produces p-toluene-sulfonyl-l-arginine and methanol. The objective of the test was to measure the absorbance increase over time to determine the hydrolytic activity of trypsin per min. For this, a solution of trypsin at different concentrations: 10 μg/mL, 15 μg/mL and 20 μg/mL in 1 mM HCl and 46 mM Tris–HCl buffer with 11.5 mM CaCl2, pH 8.1, were prepared. First, 2.6 mL of Tris-HCl buffer was mixed with 300 μL of TAME and 100 μL of trypsin and then, continuous absorbance measurement was performed at 247 nm for 10 min. To measure the absorbance, the model 6505 UV/Vis spectrophotometer (Jenway, Stone, UK) was used. Subsequently, the same test was carried out with the pancreatin (1 mg/mL) used in the GIT digestion. The amount of pancreatin used in the digestion assays was based on the activity of the trypsin present in it.

2.4.2. Determination of the degree of hydrolysis after digestion

After digestion, the degree of hydrolysis of LF and the proteins in the dairy formulas was determined by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method (ThermoFisher Scientific, Rockford, IL, USA) based on the protocol by Spellman et al. (2003). TNBS was diluted to 0.1% (w/v) in distilled water. The standards were made with L-leucine (Sigma-Aldrich) at different concentrations, between 15 and 250 μM. In the case of the formulas, gastric and intestinal digests were adequately diluted to ensure that the values obtained fell within the L-leucine standard curve. The digests and standards were diluted in 1% (w/v) SDS and they were analyzed in duplicate. First, 0.25 mL of each sample was added to a tube with 2 mL of 0.2 M sodium phosphate buffer, pH 8.2. Then, 2 mL of TNBS reagent was added. Mix was incubated at 50 °C for 1 h in darkness. After incubation, and to stop the reaction, 4 mL of 0.1 N HCl was added to each tube and allowed to cool for 30 min. After this time, absorbance of the samples was measured at 340 nm.

2.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

The protein profiles of the formulas and their digests were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), using 4-20% polyacrylamide gels, which were stained with Coomassie Blue according to standard procedures. The samples to be characterized were diluted in a 1:1 (v/v) ratio with Laemmli buffer (Laemmli, 1970). The molecular weight marker used was the Page Ruler Prestained Protein Ladder (GE Healthcare, Buckinghamshire, UK).

2.6. Antibacterial activity assay against *Cronobacter sakazakii*

In this assay, the antibacterial activity of LF and dairy formulas, before and after different stages of digestion, against *C. sakazakii* was analyzed. First, an isolated colony of this bacterium, was cultured in 10 mL of TSB with 0.6% YE and incubated between 18 and 20 h at 37 °C. The bacterial suspension obtained in stationary phase of growth was diluted in 1% peptone water to an approximate concentration of 10^5 colony forming units (cfu)/mL.

In a sterile 96-well plate, 100 µL of the dairy samples were seeded with 100 µL of *C. sakazakii* suspension. As bacterial growth control, 100 µL of peptone water was added to 100 µL of the bacterial suspension. All samples were analyzed in duplicate in three independent experiments.

The plate was incubated at 37 °C for two incubation times, 4 and 24 h, at which antibacterial activity was assessed by taking 100 µL from each well and making several dilutions that were seeded on TSA plates. The plates were incubated for 24 h at 37 °C to subsequently count the colonies.

2.7. 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 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. Degree of protein hydrolysis after *in vitro* GIT digestion

The degree of hydrolysis of milk samples after digestion was determined by the method of Spellman et al. (2003). For this, the TNBS reagent was used, which reacts with the amino acids released in the digestion process. Standards were made with L-leucine solutions at concentrations between 0 and 250 µM (**Figure 1**) analyzing their absorbance

at 340 nm. The values of hydrolysis obtained for the digested samples were referred to those of L-leucine.

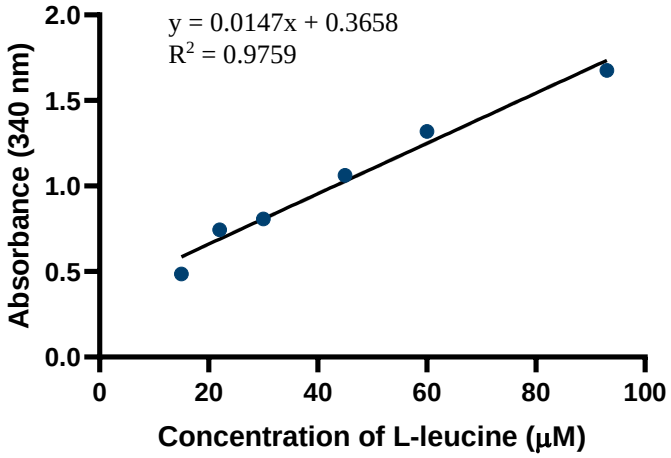

Figure 1. L-leucine standard curve to determine the degree of hydrolysis by TNBS method.

The results of the degree of hydrolysis of native LF and dairy formulas after the three stages of digestion are shown in **Table 2**. Each sample was analyzed in duplicate and an average absorbance value was obtained, which was taken to the standard curve for L-leucine (**Figure 1**) to determine the concentration of free amino acids present in each digest as L-leucine amino equivalents. The increase in absorbance at 340 nm is due to an increase in free amino acids release and a higher degree of hydrolysis.

The free amino acid concentration of the SD was considered basal, since the SSS did not contain any protease and consequently, hydrolysis should not have occurred. Comparing the concentration values of free amino acids of the GD with the basal values, an increase of between 1.2 and 1.7 times was observed, except for LF, which was 6 times higher. It is possible that the amount of pepsin present was not sufficient to hydrolyze the high protein content of dairy formulas. The concentration of free amino acids in the ID showed a higher increase than that of GD with respect to basal values, between 3 and 7 times higher. In the study by Corrochano et al. (2019), the TNBS method was also used to analyze the degree of hydrolysis of bovine whey proteins. Their results indicated that the degree of hydrolysis was greater for a pure protein, such as albumin, than for a whey protein isolate. This conclusion agrees with our study, in which the degree of hydrolysis of pure LF was higher than that of dairy formulas, probably due to a certain degree of protection exerted between proteins in the formulas.

Table 2. Degree of hydrolysis of LF and dairy formulas (F1-F6) from each stage of static *in vitro* GIT digestion expressed in L-leucine amino equivalents. SD: salivary digest, GD: gastric digest, ID: intestinal digest. Values represent the mean ± standard deviation of two replicates in one or two independent experiments (n ≥ 2).

Sample	L-leucine amino equivalent concentration (µM)		
	SD	GD	ID
LF	13.77 ± 3.51	83.02 ± 0.59	94.86 ± 0.23
F1	92.64 ± 1.77	194.70 ± 1.08	446.90 ± 0.53
F2	98.24 ± 0.24	120.29 ± 0.24	494.74 ± 1.90
F3	86.56 ± 1.90	144.78 ± 1.11	431.00 ± 1.90
F4	97.98 ± 0.12	198.08 ± 0.25	428.99 ± 0.94
F5	92.92 ± 2.35	152.87 ± 1.00	424.33 ± 0.17
F6	97.64 ± 0.36	161.05 ± 0.11	421.17 ± 0.75

3.2. Antibacterial activity against *Cronobacter sakazakii*

The first step before analyzing the antibacterial activity of the samples and their digests was to verify that the simulated solutions (SSS, SGS and SIS) used for the *in vitro* digestion process did not present antibacterial activity against *C. sakazakii*. It was shown that these solutions did not have a significant antibacterial effect against this bacterium (**Figure 2**) and, consequently, the activity of the dairy formula digests would be only due to the action of their components and in no case to the solutions used for digestion.

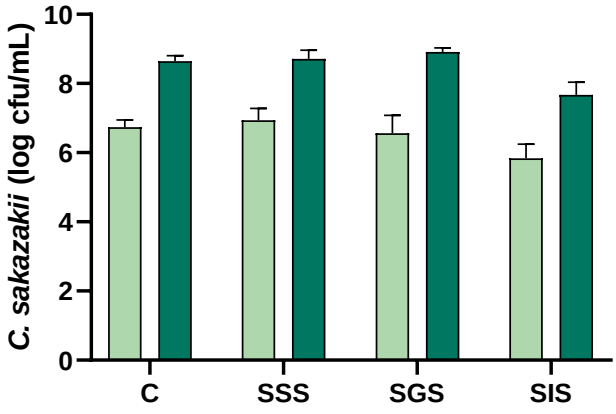

Figure 2. Antibacterial effect of simulated digestion solutions against *C. sakazakii* (□ 4 h; ■ 24 h). C: Control, SSS: simulated salivary solution, SGS: simulated gastric solution, SIS: simulated intestinal solution. Values represent the mean ± standard deviation of two replicates in three independent experiments (n=6).

In our previous studies, we have demonstrated that native LF had an antibacterial effect against *C. sakazakii* (Abad et al., 2022; Harouna et al., 2015). In the study by Harouna et al. (2015), it was shown that iron-saturated LF did not have antibacterial activity against this pathogen; therefore, the antibacterial effect of native LF was probably due to its ability to sequester iron from the medium. Furthermore, in the study by Abad et al. (2022) we showed that native LF was effective against *C. sakazakii* both in the exponential and in the stationary phase of growth.

The antibacterial effect of native LF and its digests, obtained after *in vitro* GIT digestion, was analyzed against *C. sakazakii,* in stationary phase of growth, at 4 and 24 h of incubation (**Figure 3**). The antibacterial activity of LF against the bacteria at 4 h of incubation was maintained after all stages of GIT digestion, which could be due to peptides generated in the gastric and intestinal digests, since LF did not appear as the whole molecule in SDS-PAGE. These results coincide with previous studies (Bellamy et al., 1992; Van der Kraan et al., 2004), in which some peptides derived from bovine LF, such as lactoferricin and lactoferrampin, showed antibacterial activity. However, although the antibacterial activity was maintained, it decreased as the digestion progressed. With the action of pepsin, the iron-binding centres of LF could be disrupted, resulting in free iron in the medium and fragments derived from LF unable to bind it (Hopp et al., 2022). Thus, as the digestion stages progress, there will be more iron available to favour the growth of *C. sakazakii*, reversing the possible antibacterial effect of bioactive peptides.

After 24 h of incubation, it was observed that bacteria recovered the ability to grow reaching a level similar to that of the control (**Figure 3**); therefore, the effect observed at 4 h was bacteriostatic and then, the bacteria probably developed some mechanisms to counteract the antibacterial activity of LF and their peptides. Digestion does not result in complete hydrolysis of all proteins, and consequently, although in this process peptides are generated and iron is released, it is possible that some LF-derived fragments maintain intact the iron-binding center (Hopp et al., 2022). Faced with the bacteriostatic effect caused by the lack of iron, the bacteria may develop defense mechanisms, including the production of siderophores. These siderophores could sequester iron from the medium and from LF fragments, forming stable complexes with it (Phair et al., 2022) and allowing the bacteria to recover.

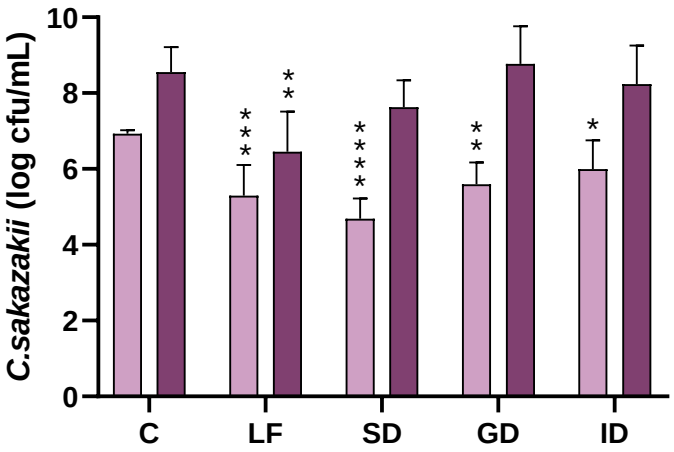

Figure 3. Antibacterial effect of LF and its digests against *C. sakazakii* (4 h; 24 h). C: Control, LF: LF at 5 mg/mL, SD: LF salivary digest, GD: LF gastric digest, ID: LF intestinal digest. Values represent the mean ± standard deviation of two replicates in three independent experiments (n=6). Asterisks indicate significant differences respect to control (*p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001).

Similarly, the antibacterial activity of the different dairy formulas (F1-F6) and their digests was also evaluated at 4 and 24 h. After digestion of these formulas, the extracts from each stage were analyzed by electrophoresis to determine their composition (**Figure 4**). It was observed that the salivary phase had no effect on the proteins, as it was expected since the SSS did not contain proteases. In the gastric digests, some changes were observed due to the proteolytic activity of pepsin, showing bands, corresponding to peptides of 10 kDa or smaller (lanes D, H and L in **Figure 4**). In the intestinal digest several bands were observed, corresponding to lipase (55 kDa) and several proteases, but the peptides that appeared in the gastric phase disappeared (lanes E, I and M in **Figure 4**). This may be due to the action of pancreatin proteases, such as trypsin, that continue the hydrolysis of those small peptides. The peptides obtained after intestinal digestion were probably so small that they were not retained in the electrophoresis gel. This electrophoretic pattern was very similar in all samples regardless of the dairy formula considered.

De Figueiredo Furtado et al. (2021) performed GIT digestion of some infant formulas based on whey protein isolate or whey protein hydrolysate, supplemented with LF, carbohydrates and fatty acids. In their study, α-LA was almost completely hydrolysed in the gastric phase. However, β-LG resisted the gastric phase of digestion and it was not proteolyzed until it was subjected to the intestinal phase, in which all remaining proteins

were hydrolyzed into small peptides. These results agree with those observed in our work when analyzing the formula digests by electrophoresis (**Figure 4**).

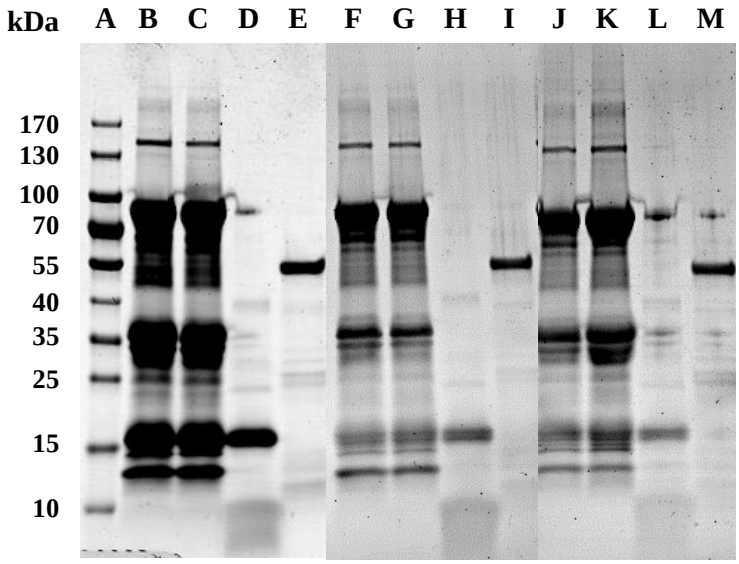

Figure 4. SDS polyacrylamide gel electrophoresis (4-20%) of digests of whey-based formulas (F1, F3 and F5) stained with Coomassie Blue. (A) Molecular weight marker, (B) F1, (C) F1 salivary digest, (D) F1 gastric digest, (E) F1 intestinal digest, (F) F3, (G) F3 salivary digest, (H) F3 gastric digest, (I) F3 intestinal digest, (J) F5, (K) F5 salivary digest, (L) F5 gastric digest, (M) F5 intestinal digest.

In the study by De Figueiredo Furtado et al. (2021), LF was resistant to the gastric phase and was hydrolyzed in the intestinal stage. In our case, LF was mainly hydrolyzed in the gastric phase, generating bioactive peptides in this stage. In the study by De Figueiredo Furtado et al. (2021), the iron saturation of LF was not specified. Therefore, the difference in the digestion stage, in which LF is hydrolyzed, could be due to the fact that their LF is more saturated than our LF, which has an iron saturation below 10%. It is well known that the structure of iron-saturated LF is more compact than that of the protein devoid of iron and, therefore, more resistant to hydrolysis (Sánchez, Calvo, & Brock, 1992). The different results of these two studies may also be explained by the difference in pH at the stages of digestion. In our study, the gastric stage was carried out at pH 3, which can cause that some bonds of LF molecule start to break, thus facilitating its hydrolysis. At that pH, some bonds that stabilize LF structure begin to break (Sánchez, Calvo, & Brock, 1992). However, it is possible that in the study by De Figueiredo Furtado et al. (2021), the gastric phase did not reach pH 3, and LF resisted more to enzymatic activity.

The results of the antibacterial activity of dairy formulas and their digests are shown in **Figure 5**. Mostly, formulas before digestion and the digests of the salivary phase did not show antibacterial activity, except for the SD of F4 and F6, both formulas based on buttermilk and subjected to a technological treatment. These digests did show an effect against *C. sakazakii* at 4 h of incubation (**Figures 5D, 5F**). However, the highest activity was presented by the GD, being higher in the formulas subjected to thermal treatment, F5 and F6 (**Figures 5E, 5F**). This antibacterial activity decreased after intestinal digestion, allowing greater bacterial growth, possibly due to the effect of proteolysis on the bioactive peptides.

LF at 5 mg/mL is shown as a positive control of the antibacterial effect. Some differences can be observed in LF activity between the different graphs (**Figures 5A-5F**), since all the tests were carried out independently, which could generate a small variation in its effect. It is important to point that LF used and the procedure followed for its preparation were the same in all assays. Although the bacteria came from the same stock and used always in the stationary phase, there could have been some variability between the experiments in the bacterial metabolic activity and susceptibility to LF. In any case, LF always showed great antibacterial activity, decreasing bacterial counts by 2-4 logarithmic units.

In addition, it is worth highlighting the difference between the effect of LF and the different formulas, the native protein always being the one that showed the greatest effect. According to the literature, this lower effect of LF when it is part of a dairy formula compared with LF alone in its native state could be due to the interaction with other proteins or components, like β-LG, albumin (Lampreave et al., 1990) or casein micelles (Anema, 2018), its effect being reduced because of its lower availability.

Comparing the activity of F1, based on whey, and F2, based on buttermilk (**Figures 5A, 5B**), a slightly greater antibacterial activity of F2 was observed after the gastric phase. This effect could be explained by the high content of MFGM, since F2 contains apart from that added to the formula, the MFGM naturally present in buttermilk. Additionally to bioactive proteins with antimicrobial properties present in MFGM (Parrón et al., 2018), other components such as glycosphingolipids and phospholipids are also being investigated, as they function as intracellular signalling molecules in a wide variety of biological processes (Fuller et al., 2013). For this reason, in recent years, the addition of MFGM, or fractions enriched in it, to infant formulas has been done in certain products.

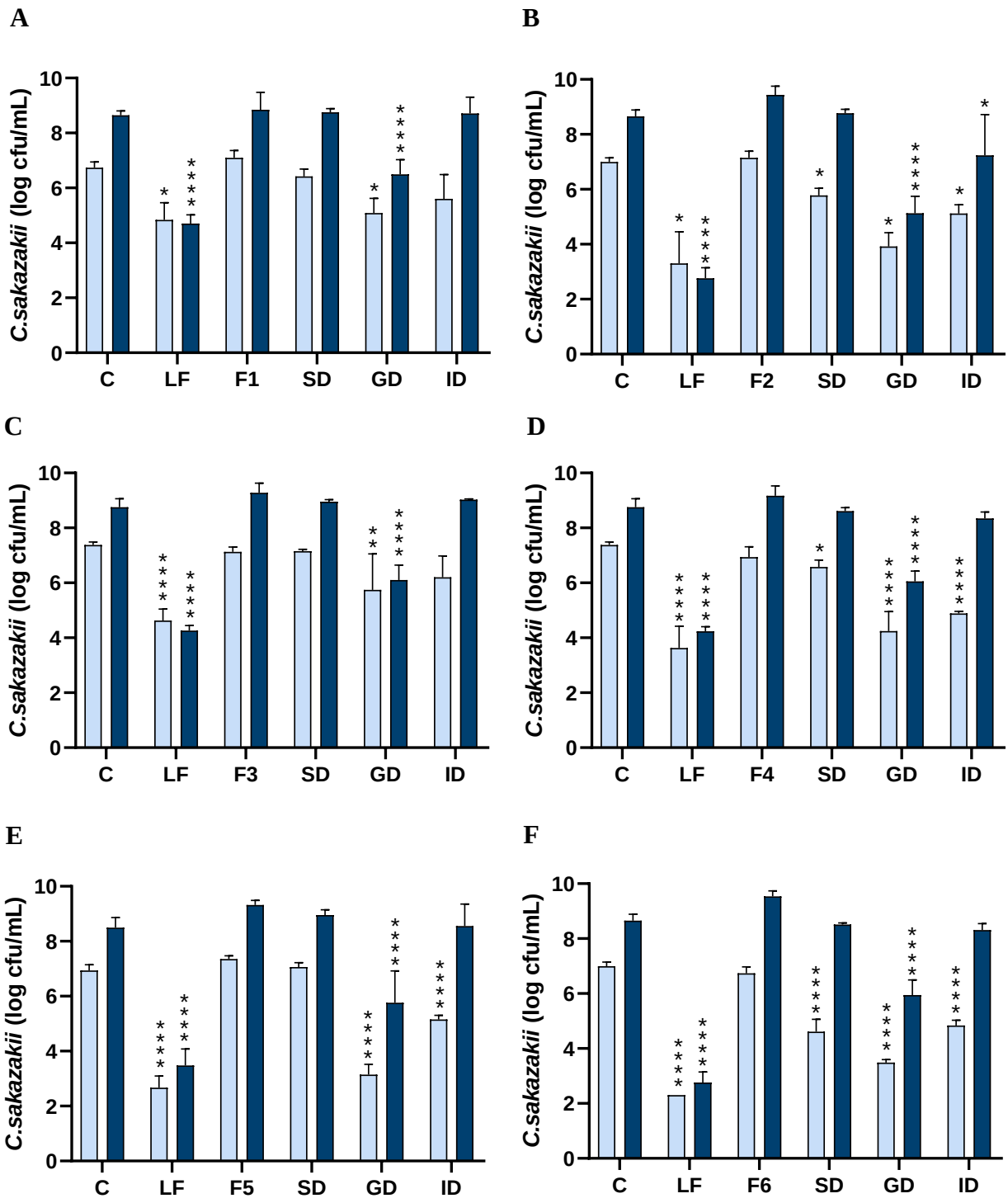

Figure 5. Antibacterial effect of (**A**) F1 and its digests, (**B**) F2 and its digests, (**C**) F3 and its digests, (**D**) F4 and its digests, (**E**) F5 and its digests, and (**F**) F6 and its digests against *C. sakazakii* (4 h; 24 h). C: Control, LF: LF at 5 mg/mL, SD: salivary digest, GD: gastric digest, ID: intestinal digest. Values represent the mean ± standard deviation of two replicates in, at least, two independent experiments (n ≥ 4). Asterisks indicate significant differences respect to control (*p< 0.05, **p< 0.01, ****p< 0.0001).

In the case of F3 and F4, the added MFGM was previously subjected to homogenization, which caused some small differences in the results (**Figures 5C, 5D**). As expected, it was observed that after the salivary phase there was no hydrolysis of the proteins and the SD of F3, based on whey, had no activity against the pathogen. However, F4 based on buttermilk, showed some antibacterial activity after the salivary phase, with little significant difference respect to the control. This could be because during homogenization, fat globules decrease their size and increase their surface, releasing the original MFGM proteins and favouring the binding of caseins to the membrane (Lee and Sherbon, 2002) and also of LF, as it has been reported that LF binds naturally to casein micelles (Anema, 2018). Therefore, the binding of LF to the homogenized MFGM could hinder its antibacterial ability. However, according to this argument, the MFGM proteins released in F4 could exert their activity.

On the other hand, GDs of F3 and F4 were effective at 4 and 24 h of incubation, with GD of F4 having greater antibacterial activity, producing greater decrease in the growth of *C. sakazakii*. The antibacterial activity of F3 and F4 decreased after the intestinal phase, the ID of F4 being the only one that maintained activity only at 4 h of incubation. Therefore, it can be concluded that the F4 digests, composed of homogenized buttermilk, had greater activity against *C. sakazakii* than the F3 digests based on whey.

Homogenization improves the digestibility of proteins (Tunick et al., 2016), facilitating the release of bioactive peptides. Furthermore, MFGM has a great amount of bioactive proteins, such as lactadherin, butyrophilin and mucin, with antibacterial effect (Lönnerdal, 2016) and it has been shown that the antibacterial activity of some MFGM hydrolysates may be due to the generation of biodefensive peptide sequences (Clare et al., 2008). In our study, F4 had more effect than F3, which could be due to the higher MFGM content of the former.

Formulas F5 and F6 were subjected to a thermal pasteurization treatment of 72 °C for 20 s. The SD of F5 did not show antibacterial activity, while the SD of F6 showed some effect, with significant differences respect to the control at 4 h of incubation (**Figures 5E, 5F**). The digests of F5 and F6 from the gastric phase showed antibacterial activity, with significant differences at 4 and 24 h, decreasing the growth of the pathogen by up to 4 log cfu/mL. The antibacterial effect of the ID of F5 and F6 was maintained at 4 h, although with less activity than the GD, and disappeared after 24 h of incubation.

The results obtained indicate that heat treatment has positive effects on the antibacterial activity of dairy formulas, possibly because it favours the release of active peptides. In the study by Halabi et al. (2020), it was observed that the application of thermal treatment to infant formulas made with whey increased the susceptibility of some proteins to pepsin hydrolysis. The resistance of α-LA and β-LG to hydrolysis was not modified by heating, while LF increased its susceptibility compared to the unheated form. The results obtained in that study also indicated that the kinetics of LF hydrolysis in intestinal digestion of heat-treated infant formulas were higher than those of untreated ones. The loss of activity of denatured and hydrolyzed LF could be compensated by the release of peptides with high antibacterial activity.

We can affirm from our results that dairy formulas showed antibacterial activity against *C. sakazakii* when were subjected to static *in vitro* digestion. In all the dairy formulas, the SD did not have antibacterial action, while the highest activity was observed in the digests of the gastric phase and it was maintained, to a greater or lesser extent, after the intestinal phase. There was greater antibacterial effect against the pathogen at 4 h of incubation than at 24 h, especially in the intestinal phase. In addition, it was observed that the formulas based on buttermilk (F2, F4 and F6) had greater antibacterial effect than those based on whey (F1, F3 and F5). This could be due to the effect of the proteins present in the MFGM, which were found in greater proportion in the formulas based on buttermilk. The XO, one of the main components of this membrane, generates H_2O_2 via an enzymatic reaction, which can cause bacterial death (Clare et al., 2008). Furthermore, it was observed that these biodefensive properties of XO were preserved after proteolysis (Clare et al., 2008). Likewise, the thermally treated formulas (F5 and F6) showed greater activity than those treated by homogenization (F3 and F4), and also than those with no treatment (F1 and F2), mainly in the gastric phase. It was also observed that heat treatment seemed to have a beneficial effect on formula F5, as it was the only formula based on whey with the highest activity against *C. sakazakii*.

In short, the GD presented the highest antibacterial activity against *C. sakazakii* in all formulas. This activity may be due to the release of bioactive peptides by the enzymatic proteolysis that occurs during digestion. After hydrolysis, proteins can become bioactive, increasing their inhibitory effect on pathogens (McEvoy et al., 2016).

To characterize the peptides generated after gastric digestion and to find out the molecular weight of those that were mainly responsible for the antibacterial action, the GD of one

formula was fractionated. Since the digestion of all the formulas followed the same procedure and generated a similar electrophoretic pattern of hydrolysates (as shown in **Figure 4**), the fractionation was performed only with one gastric digest. To make this selection, an untreated formula was preferred, since technological treatment could have generated protein–protein interactions and aggregates that would make more difficult the separation of peptides. Finally, F2 was chosen because it presented higher antibacterial activity. After fractionation, peptides with a size between 3 and 10 kDa and less than 3 kDa were differentiated (**Figure 6**).

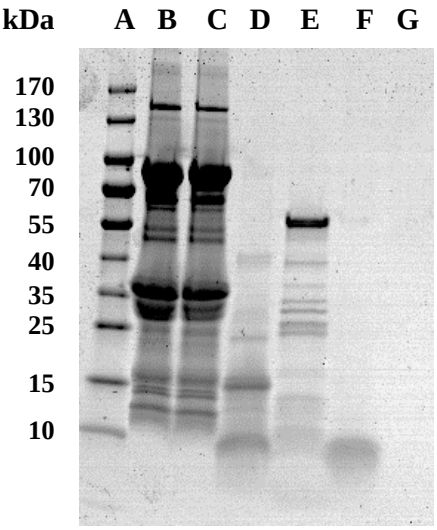

Figure 6. SDS polyacrylamide gel electrophoresis (4-20%) of digests of F2 stained with Coomassie Blue. (A) Molecular weight marker, (B) F2, (C) F2 salivary digest, (D) F2 gastric digest, (E) F2 intestinal digest, (F) 3-10 kDa fraction, (G) less than 3 kDa fraction.

In the lane corresponding to the fraction of peptides between 3 and 10 kDa (lane F), a band was observed at the lowest part of the gel, while in the fraction of peptides smaller than 3 kDa (lane G) no band was observed, probably because they are not retained in the gel due to their small size.

To complete the analysis, the antibacterial activity of the two fractions was evaluated (**Table 3**). It was observed that both had antibacterial effect and that the fraction between 3–10 kDa had greater activity than the fraction lower than 3 kDa. The 3-10 kDa fraction maintained its antibacterial activity at 24 h, while the fraction lower than 3 kDa significantly decreased its effect.

Table 3. Antibacterial activity of the fractions obtained from gastric digest of F2 by ultrafiltration. Values represent the mean ± standard deviation of *C. sakazakii* log cfu/mL from two replicates in two independent experiments (n=4) and are also expressed as a percentage respect to the control. Asterisks indicate significant differences respect to control (*p < 0.05, ****p < 0.0001).

Incubation of 4 h		**Incubation of 24 h**	
3-10 kDa peptides		**3-10 kDa peptides**	
Mean ± SD	4.09 ± 0.356 ****	**Mean ± SD**	5.34 ± 0.311 ****
Percentage	59.1	**Percentage**	65.1
<3 kDa peptides		**<3 kDa peptides**	
Mean ± SD	5.42 ± 0.03 ****	**Mean ± SD**	7.44 ± 0.355 *
Percentage	78.3	**Percentage**	90.7

The GIT digestion of food has great influence on the release of peptide sequences present in the proteins. The released peptides can carry out several biological functions, in the intestinal lumen and in the different organs of the body if they are absorbed and transferred to systemic circulation (Aspri et al., 2018). In the study by Aspri et al. (2018), fermented donkey milk showed activity against *Listeria monocytogenes* before digestion. After *in vitro* GIT digestion, its antibacterial activity against that pathogen increased. This enhanced antibacterial activity was also detected against *Staphylococcus aureus* and *Bacillus cereus*. The high level of LF and lysozyme contained in donkey milk could be, in part, responsible for the high antibacterial activity observed. Furthermore, these authors pointed that donkey milk fermented with lactic acid bacteria had different protective factors with antimicrobial activity, including the peptides released during the process of digestion. These factors can have an impact on intestinal health, especially beneficial for the immune defence system of children and elderly individuals.

In the study by Nielsen et al. (2018), 58 bioactive peptides were released during gastric digestion of proteins from human and bovine milk in premature infants. These peptides presented sequences closely related to known peptides with different activities: antioxidant, antithrombotic, antimicrobial, etc. The results of Nielsen et al. (2018) and those of the present study indicate the great interest in investigating bioactive peptides derived from milk proteins, as potential ingredients of functional foods.

4. Conclusions

Dairy by-products have an interesting potential as they contain bioactive compounds. Among these compounds, lactoferrin and the milk fat globule membrane have great value, and would allow the revaluation of those dairy by-products. However, it is necessary to know the effect of technological treatments when bioactive compounds are incorporated into products for consumption and also the effect of gastrointestinal digestion on their activity. The results obtained in this study show that LF maintains its antibacterial activity against *C. sakazakii* after being subjected to static *in vitro* digestion. In addition, it has been observed that dairy formulas containing bioactive proteins have antibacterial activity after being digested, especially after the gastric phase. Furthermore, it has been observed that pasteurization has positive effects on their antibacterial activity, possibly due to the partial denaturation of some proteins that facilitates the action of digestive proteases and the release of bioactive peptides.

In conclusion, this study provides useful information on dairy by-products, such as whey and buttermilk, and the effect of digestion and technological treatments on them; being potential ingredients for functional products directed to adults.

In the future, it would be necessary to carry out similar studies subjecting dairy formulas to digestion in different conditions, taking into account the variations that the digestion process may have in the infant's digestive tract, depending on age and intestinal maturation. This could extend our knowledge of how to use these by-products in the preparation of infant formulas and functional products.

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Author contributions

IA: Writing-original draft preparation, methodology, investigation, software, data curation; LS: methodology, investigation, software, data curation; DG: methodology, investigation; MDP: reviewing and editing; LG: methodology, reviewing and editing; LS: conceptualization, supervision, reviewing and editing.

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ARTÍCULO 5

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

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

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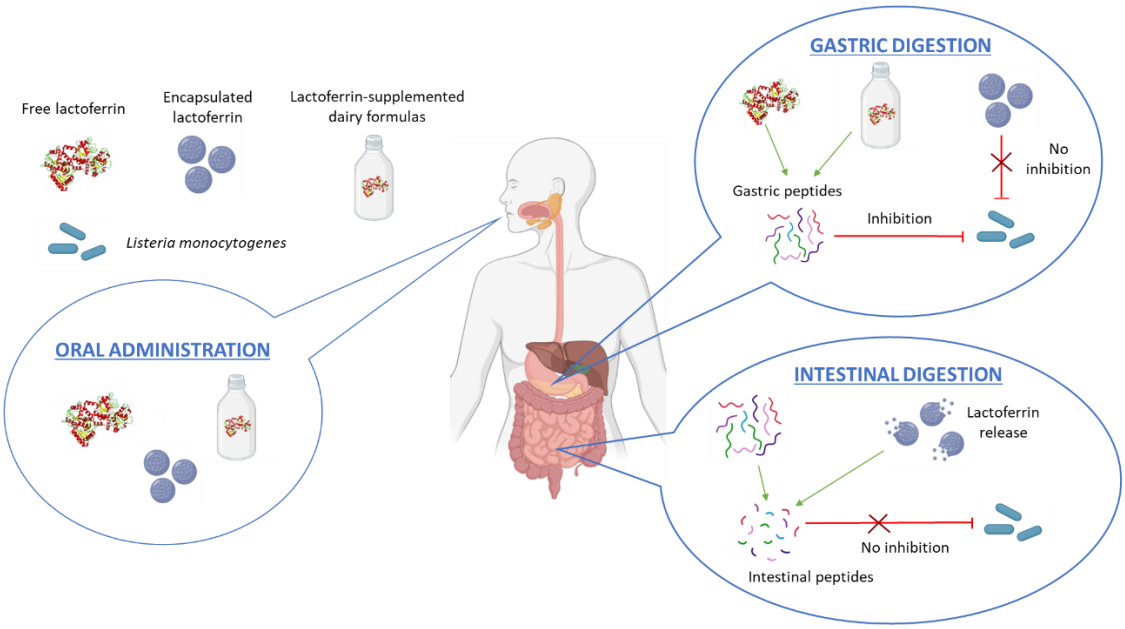
Abstract

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 (BM) 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 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 ALG 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 ALG/LF 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 ALG 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 ºC, 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 LF, LF encapsulated with ALG and LF 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., 2022a) 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 ºC 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 ºC. The composition of the dairy formulas is shown in more detail in **Table S1**.

Treatment of pasteurization at 72 ºC 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 ºC. 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 ºC.

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 ºC.

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).

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

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

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 ºC 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 ºC 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 ºC 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 LBP 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 ºC.

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 ºC. The final volume was adjusted to pH 3 and incubated for 2 h under agitation at 37 ºC. 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 ºC. The pH of the mixture was adjusted to 7 and incubated for 2 h under agitation at 37 ºC. 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 ($E^{1\%}_{280}$= 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 section 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 ºC. 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 ºC. 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 ºC. 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 ºC 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 ºC 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 ºC. 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 ºC 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 previous study (Abad et al., 2022a). 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 ºC, 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 ºC 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 ºC 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 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.

Figure 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 ºC 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.

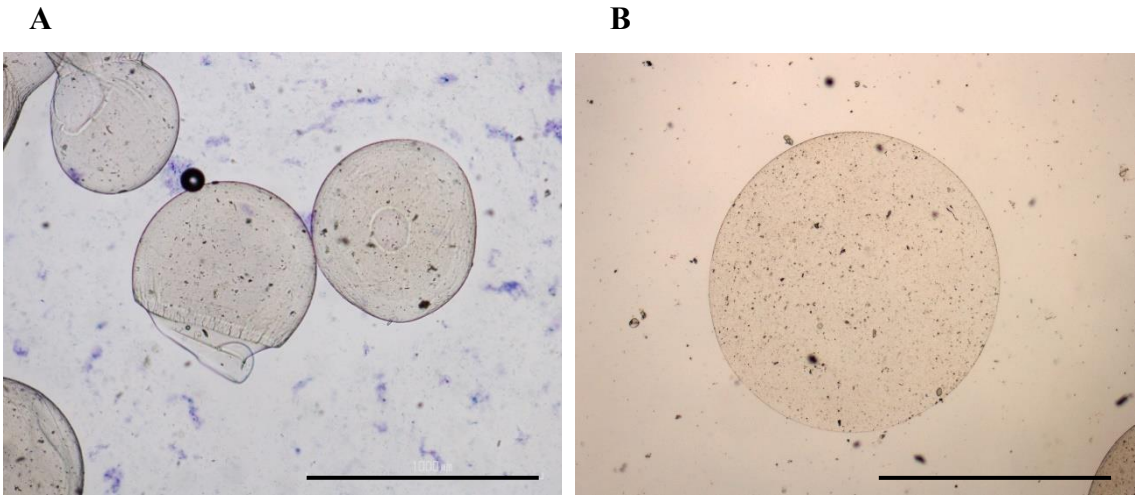


Figure 1. Microscope images of microbeads. The scale bar represents 1000 µm. (**A**) Freshly produced alginate 2% - LF 1% microbeads. (**B**) Alginate 2% - LF 1% freeze-dried microbead and rehydrated with PBS.

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 ALG matrix. As shown in **Figure 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.

Figure 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 40-50% of the milk protein captured by the ALG. It can, therefore, be concluded that the amount of LF released is pH dependent, as stated in our previous study (Abad, Conesa, & Sánchez, 2021).

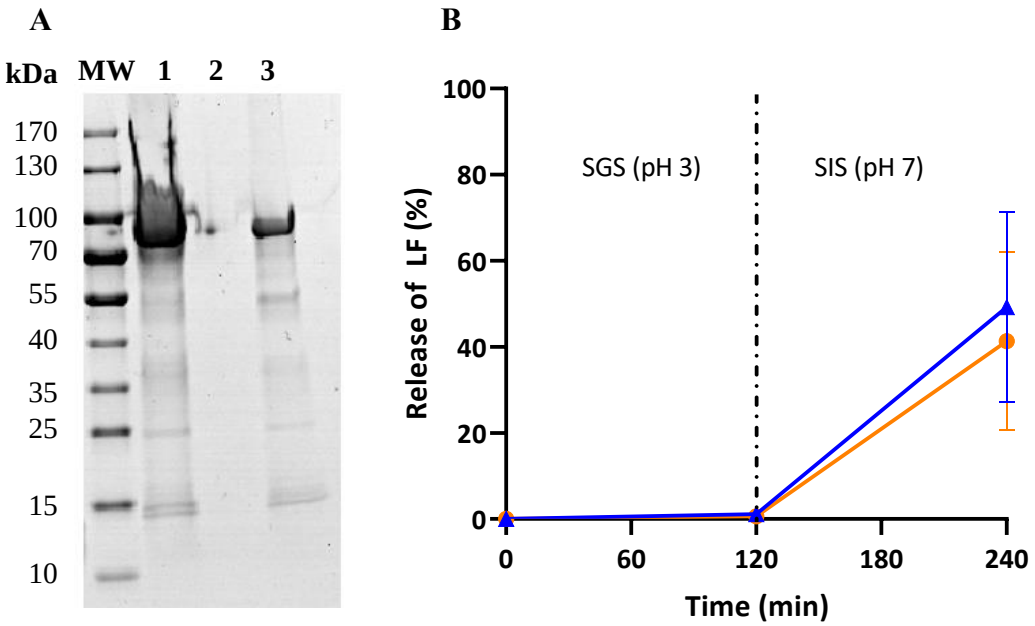


Figure 2. (**A**) SDS polyacrylamide gel electrophoresis (4-20%) of ALG/LF microbeads, stained with Coomassie Blue R. MW: molecular weight marker, 1: dissolved untreated microbeads, 2: supernatant of microbeads subjected to acidic pH, 3: supernatant of microbeads subjected to neutral pH. (**B**) Graphical 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).

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 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 (**Figure 3**), there were

differences in the results obtained by these two techniques. In **Figure 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.

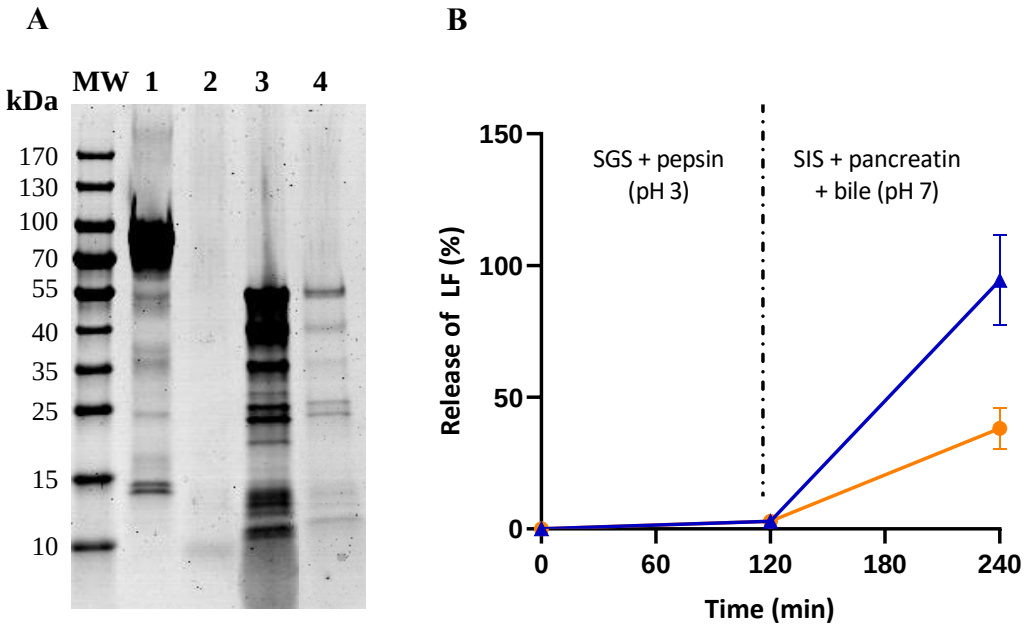


Figure 3. (**A**) SDS polyacrylamide gel electrophoresis (4-20%) of ALG/LF microbeads, stained with Coomassie Blue R. MW: molecular weight marker, 1: dissolved untreated microbeads, 2: supernatant of microbeads subjected to gastric digestion (pepsin and pH 3), 3: supernatant of microbeads subjected to 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).

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 (**Figure 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 (**Figure 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 (**Figure 4A**), and verified by the Western blotting performed (**Figure 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 ALG 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 (**Figure 2** and **Figure 3**), we can conclude that intestinal enzymes favours the liberation of LF from ALG 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.

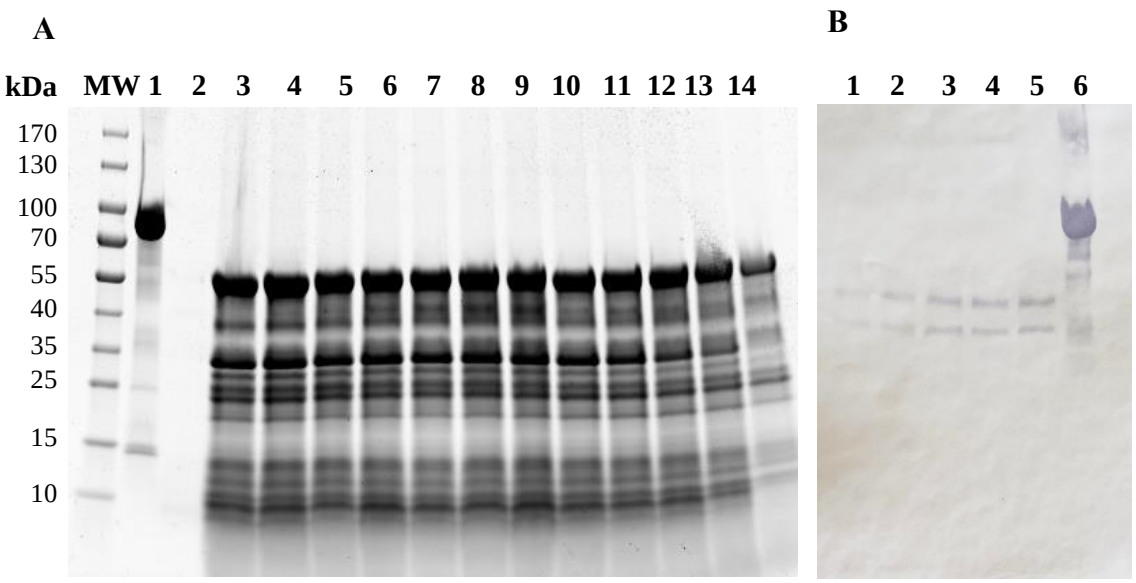


Figure 4. (**A**) SDS polyacrylamide gel electrophoresis (4-20%) of digested ALG/LF microbeads, stained with Coomassie Blue R. MW: molecular weight marker, 1: dissolved untreated microbeads, 2: GD of microbeads, 3-14: ID of microbeads for 1, 2, 4, 6, 8, 10, 12, 15, 30, 45 min, 1 h and 2 h, respectively.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

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. **Figure 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 colony forming units (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 (**Figure 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.

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 (**Figure 5A**) and in stationary phases (**Figure 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.

Figure 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.

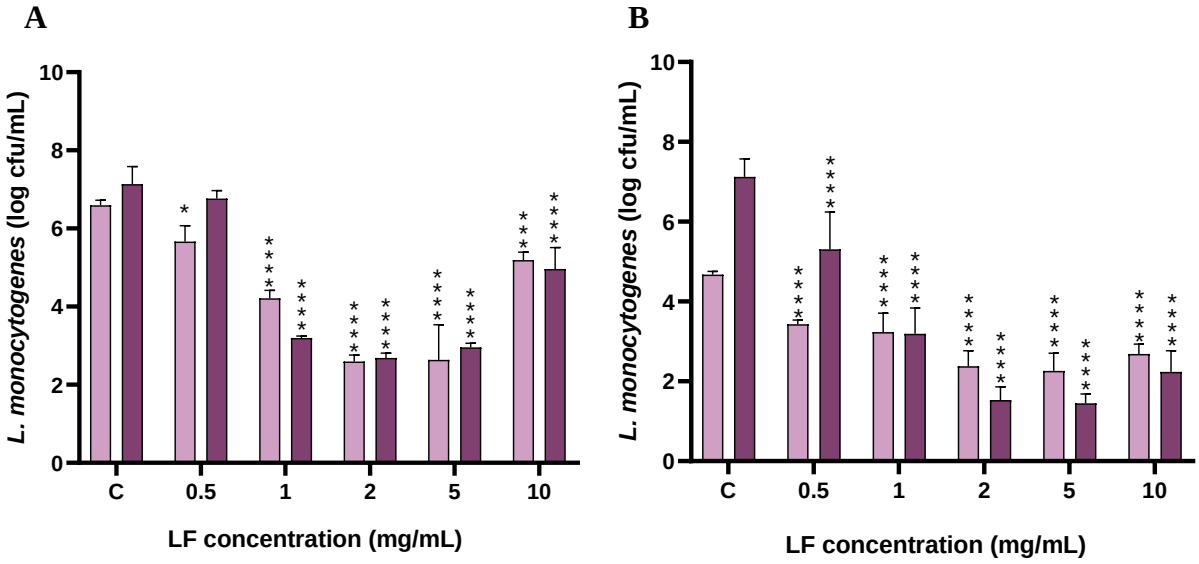


Figure 5. Antibacterial effect of LF at different concentrations against *L. monocytogenes* at (**A**) exponential phase and (**B**) stationary phase after an incubation of 4 h (▢) or 24 h (■). C: control. Values represent the mean ± standard deviation of two replicates in three independent experiments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

The results shown in **Figure 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 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., 2022a).

3.4.2. Antibacterial activity of digestion simulated solutions

Prior to the activity assays of the digests, we tested whether the 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 (**Figure 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.

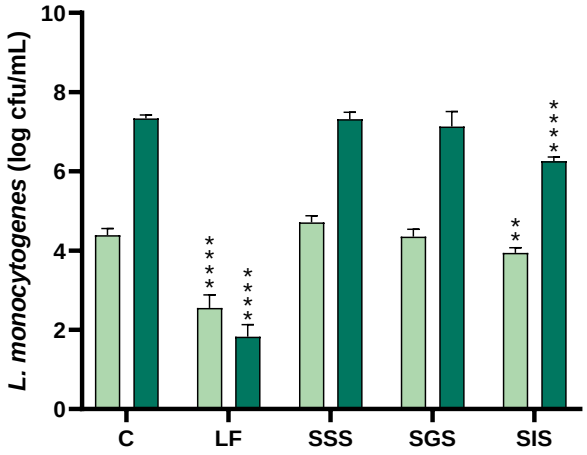


Figure 6. Antibacterial effect of simulated digestion solutions with digestive enzymes against *L. monocytogenes* (4 h; 24 h). C: control, LF: LF at 2.5 mg/mL, SSS: simulated salivary solution, SGS: simulated gastric solution, SIS: simulated intestinal solution. Values represent the mean ± standard deviation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

3.4.3. Antibacterial activity of lactoferrin and its digests

The results obtained after analyzing the antibacterial effect of LF digests are shown in **Figure 7**. Focusing on the electrophoresis (**Figure 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 (**Figure 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 Ripollés 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.

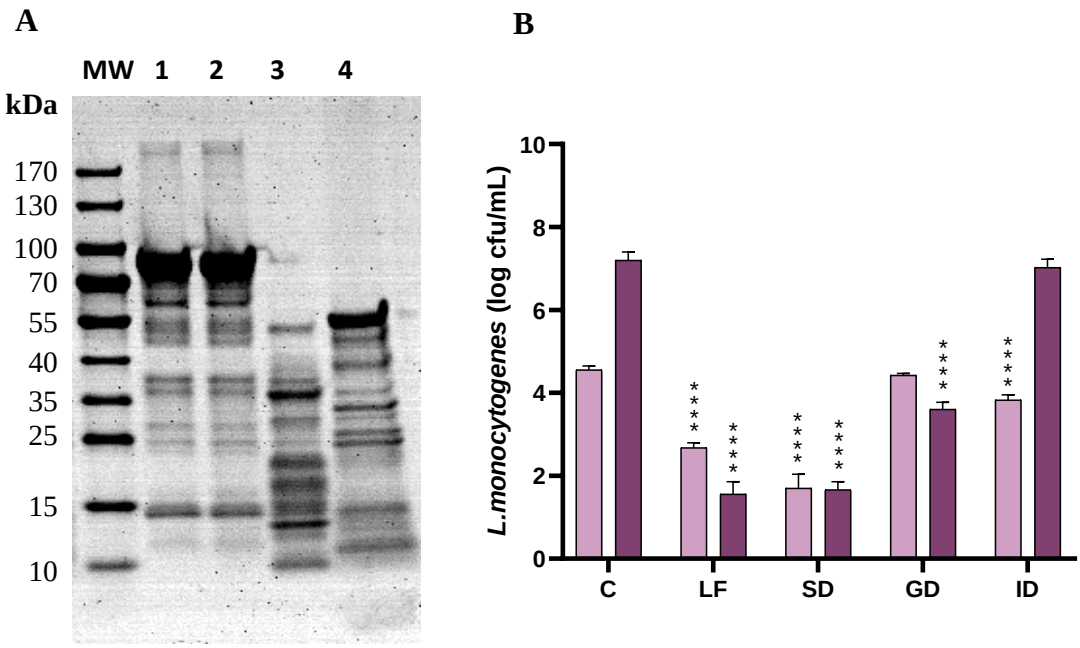


Figure 7. (**A**) SDS polyacrylamide gel electrophoresis (4-20%) of LF and its digests stained with Coomassie Blue R. MW: molecular weight marker, 1: native LF, 2: salivary digest of LF, 3: gastric digest of LF, 4: intestinal digest of LF. (**B**) Antibacterial effect of LF and its digests against *L. 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).

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* (**Figure 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 (**Figure 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 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.

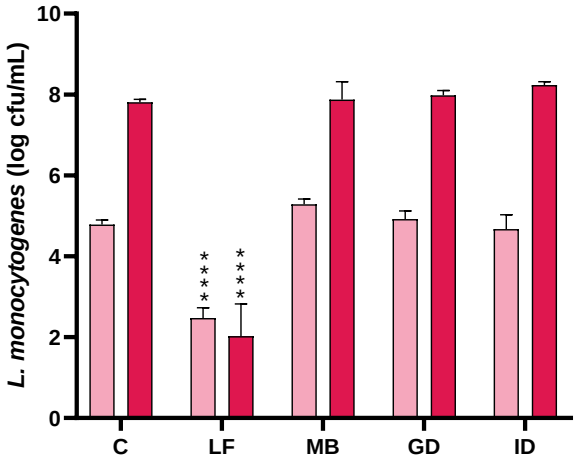


Figure 8. Antibacterial effect of ALG/LF microbeads and their digests against *L. monocytogenes* (□ 4 h; ■ 24 h). C: control, LF: LF at 2.5 mg/mL, MB: intact microbeads, GD: gastric digest of microbeads, ID: intestinal digest of microbeads. Values represent the mean ± standard deviation of, at least,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

The study by Raei et al. (2015) demonstrated that LF encapsulated in ALG 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, Moradian, & Rostami (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 (SD, GD and ID) 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.

Figure 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 (**Figure 7A**), in the GD of the formulas, some intact LF can be observed (**Figure 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 **Figure 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 (**Figure 9A**). Similarly results were obtained with F2, based on buttermilk. The antibacterial activity of the F2 digests (**Figure 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 (**Figure 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.

Figure 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 **Figure 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 ºC/20 s. As can be seen in **Figure 9E**, the SD of F5 did not show antibacterial activity, while that of F6 (**Figure 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.

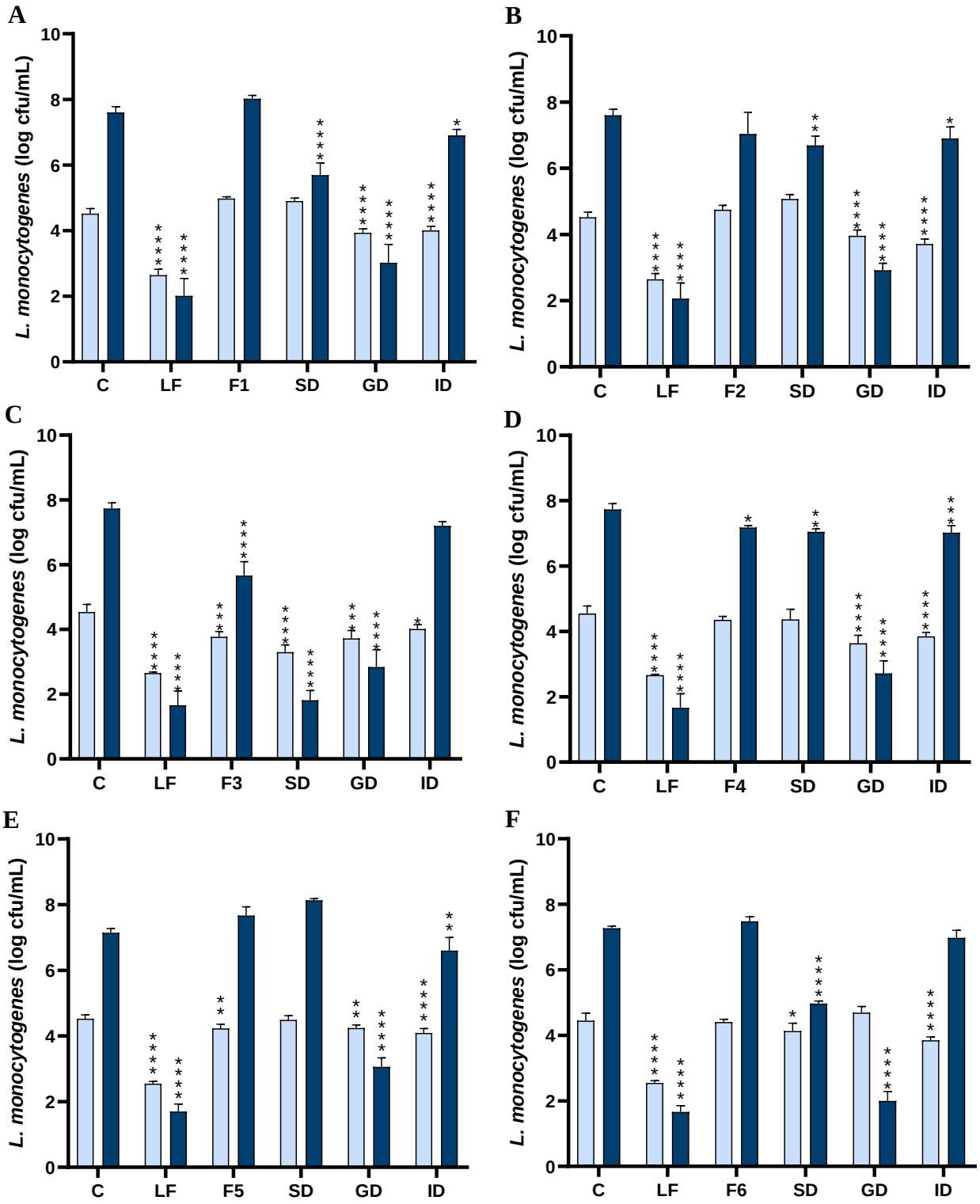


Figure 9. Antibacterial effect against *L. monocytogenes* of (**A**) F1 and its digests, (**B**) F2 and its digests, (**C**) F3 and its digests, (**D**) F4 and its digests, (**E**) F5 and its digests, and (**F**) F6 and its digests (□ 4 h; ■ 24 h). C: control, LF: LF at 2.5 mg/mL, SD: salivary digest, 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).

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 (**Figures 9A-9F**). 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 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 GD of the formulas (**Figure S2**), unlike the GD of free LF, in which the protein was completely digested (**Figure 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).

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

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 (**Figure 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 ALG 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 ALG 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.

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Supplementary material

1. Composition of lactoferrin-supplemented dairy formulas

Six dairy formulas, based on whey or buttermilk, were prepared to be subjected to static *in vitro* gastrointestinal digestion and to subsequent evaluation of their antibacterial activity against *L. monocytogenes*. Additionally, commercial bovine LF was used to supplement dairy formulas.

The six dairy formulas (F1-F6) were elaborated with a base of whey or buttermilk and supplemented with LF (10 mg/mL) and MFGM. The composition of each formula is detailed in **Table S1.**

2. Growth curve of *Listeria monocytogenes*

The growth curve of *L. monocytogenes* was obtained by colony counting and by determining the absorbance at 620 nm (**Figure S1**).

Table S1. Composition of the six dairy formulas (F1-F6) elaborated on a base of whey or buttermilk and subjected to homogenization or pasteurization. LF: lactoferrin. MFGM: milk fat globule membrane.

FORMULA	BASE	SUPPLEMENT	TREATMENT
FORMULA 1 (F1)	WHEY	LF (10 mg/mL) MFGM	-
FORMULA 2 (F2)	BUTTERMILK	LF (10 mg/mL) MFGM	-
FORMULA 3 (F3)	WHEY	LF (10 mg/mL) Homogenized MFGM	Homogenization of buttermilk
FORMULA 4 (F4)	BUTTERMILK (Homogenized)	LF (10 mg/mL) Homogenized MFGM	Homogenization of buttermilk
FORMULA 5 (F5)	WHEY	LF (10 mg/mL) MFGM	Pasteurization (72 ºC/ 20 s)
FORMULA 6 (F6)	BUTTERMILK	LF (10 mg/mL) MFGM	Pasteurization (72 ºC/ 20 s)

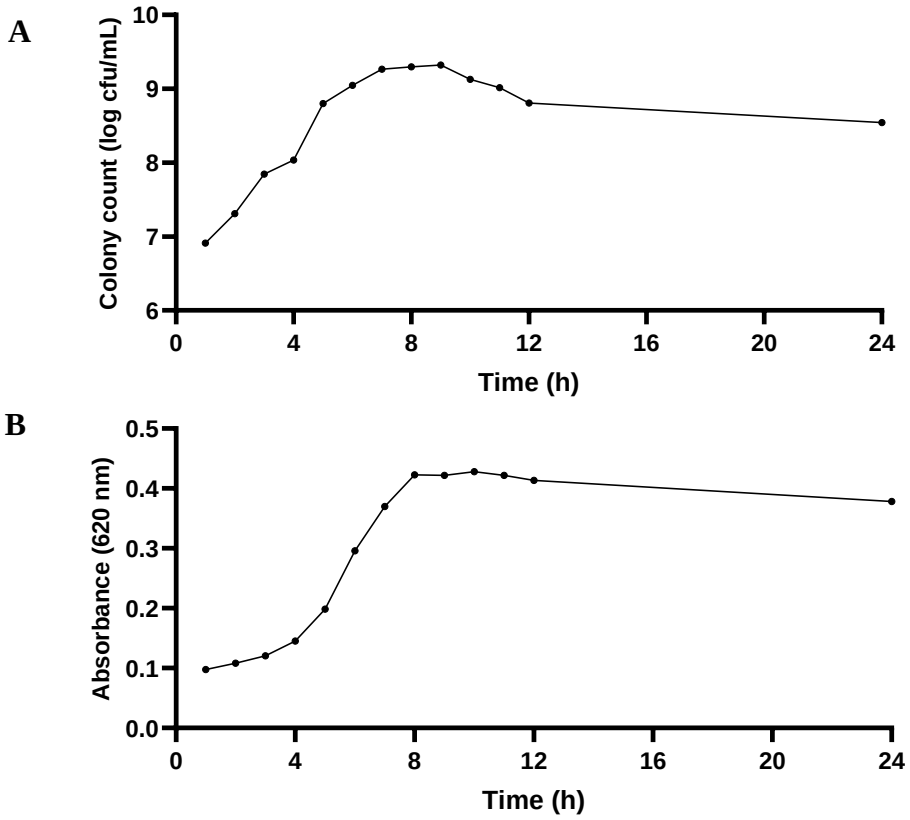

Figure S1. Growth curve of *L. monocytogenes* obtained by (**A**) the colony count in TSA plates incubated at 37 ºC for 24 h and by (**B**) the measurement of the absorbance at 620 nm.

3. Protein profile of lactoferrin-supplemented dairy formulas

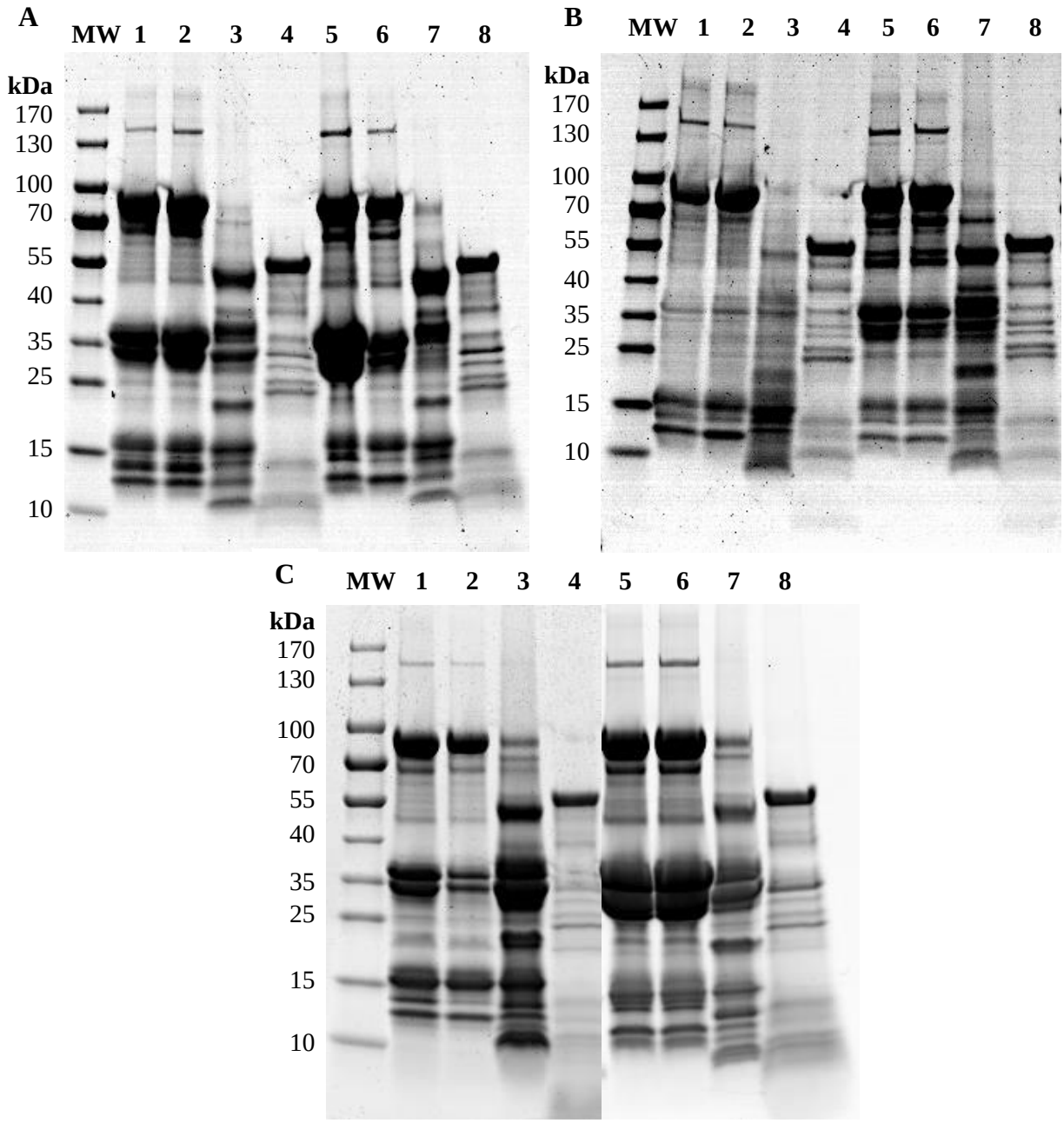

Figure S2. (**A**) SDS polyacrylamide gel electrophoresis (4-20%) of F1, F2 and its digests stained with Coomassie Blue R. MW: molecular weight marker, 1: F1, 2: salivary digest of F1, 3: gastric digest of F1, 4: intestinal digest of F1, 5: F2, 6: salivary digest of F2, 7: gastric digest of F2, 8: intestinal digest of F2. (**B**) SDS polyacrylamide gel electrophoresis (4-20%) of F3, F4 and its digests stained with Coomassie Blue R. MW: molecular weight marker, 1: F3, 2: salivary digest of F3, 3: gastric digest of F3, 4: intestinal digest of F3, 5: F4, 6: salivary digest of F4, 7: gastric digest of F4, 8: intestinal digest of F4. (**C**) SDS polyacrylamide gel electrophoresis (4-20%) of F5, F6 and its digests stained with Coomassie Blue R. MW: molecular weight marker, 1: F5, 2: salivary digest of F5, 3: gastric digest of F5, 4: intestinal digest of F5, 5: F6, 6: salivary digest of F6, 7: gastric digest of F6, 8: intestinal digest of F6.

4. Fractionation of peptides from gastric digest

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 (**Figure S3**).

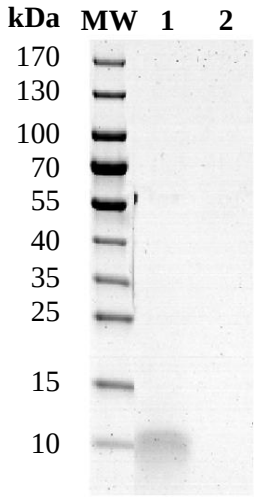

Figure S3. SDS polyacrylamide gel electrophoresis (4-20%) of fractions > 3 kDa (1) and <3 kDa (2) obtained from the fraction <30 kDa of the GD, stained with Coomassie Blue R. MW: molecular weight marker.

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.

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ARTÍCULO 6

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

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

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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.

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 et al., 2015).

On the one hand, 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 et al., 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, Lisak Jakopović, & Božanić, 2019) and some of these proteins ~~that~~ 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) Nº 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 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).

On the other hand, 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 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 et al., 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 et al., 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 to 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 ºC. *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 a 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 ºC 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 ºC 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 ºC. 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 ºC. 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) 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 ºC 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 ºC and 72 ºC, respectively. The samples were introduced into the first bath at 60 ºC and, upon reaching that temperature, they were transferred to the bath at 72 ºC. Once they reached 72 ºC, they were held for 20 s. After pasteurization, samples were immediately cooled down immersing them into ice and stored at -20 ºC until use.

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. 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

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 ºC. 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 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 ºC 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 x USP) to achieve 100 U/mL 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 ºC 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. 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 *Staphylococcus 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; ISO, 2023).

The bacteria were fixed to porous rings and stored in cryovials at -80 ºC 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 ºC 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 ºC to obtain isolated colonies for the assays.

A single colony of *S. aureus* was incubated at 37 ºC 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.

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 *Staphylococcus aureus*

Staphylococcus 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 concentrations (0.5, 1, 2, 5 and 10 mg/mL) was mixed with the bacterial suspension at 10^5 cfu/mL in a 1:1 ratio (v/v). Samples were incubated at 37 ºC 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 ºC, 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 *Staphylococcus aureus*

The same procedure detailed in the 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 ºC, 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 *Staphylococcus 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 **Figure 1**. The *S. aureus* culture was quite resistant to the effect of LF during the exponential phase (**Figure 1A**); while the stationary phase culture was slightly sensitive to this protein (**Figure 1B**).

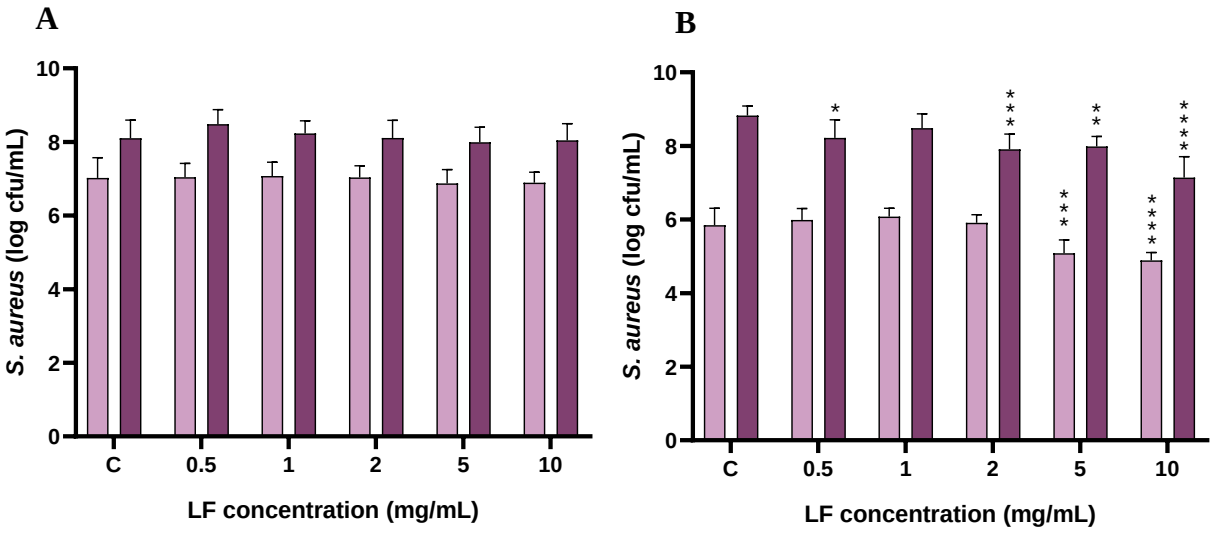

Figure 1. Antibacterial activity of bovine LF at different concentrations against *S. aureus* at (**A**) exponential phase and (**B**) stationary phase after an incubation of 4 h ( ) and 24 h ( ). C: control of bacteria with peptone water without LF. The values represent the mean ± standard deviation 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).

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 (**Figure 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) 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. 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.

In our study, the incubation of the bacteria with a concentration of 5 mg/mL 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 of LF was necessary to start inhibiting bacterial growth (**Figure 1B**). Kutila et al. (2003) obtained similar results to ours. In their study, LF at 1.67 and 2.67 mg/mL significantly slowed the growth of *S. aureus*; and LF at 2.67 mg/mL 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 (**Figure 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.

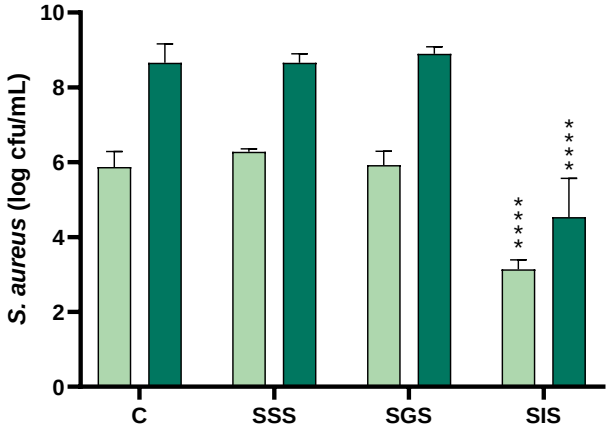

Figure 2. Antibacterial activity of simulated digestion solutions with digestive enzymes against *S. aureus* after an incubation of 4 h ( ) or 24 h ( ). C: control of bacteria with peptone water, SSS: simulated salivary solution, SGS: simulated gastric solution, SIS: simulated intestinal solution. The 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).

3.3. Antibacterial activity of lactoferrin and its digests against *Staphylococcus aureus*

The analysis of the antibacterial effect of LF and its digests is illustrated in **Figure 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 (**Figure 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.

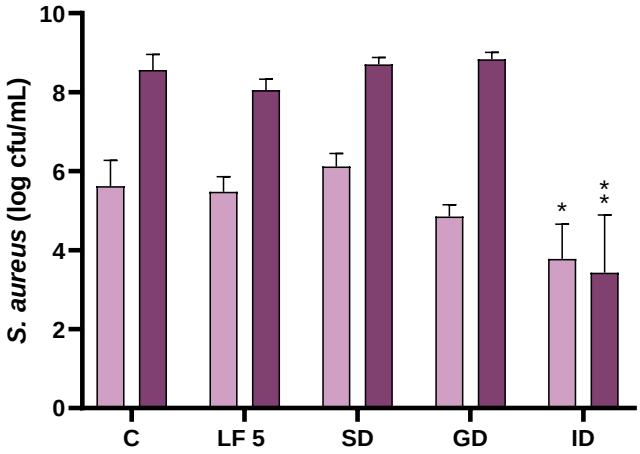

Figure 3. Antibacterial activity of LF and its digests against *S. aureus* after an incubation of 4 h ( ) or 24 h ( ). C: control of bacteria with peptone water without LF, LF 5: LF at 5 mg/mL, SD: salivary digest, GD: gastric digest, ID: intestinal digest. The values represent the mean ± standard deviation 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 *Staphylococcus 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 (**Figure 4**). Mostly, dairy formulas before digestion did not produce a decrease in the growth of *S. aureus*; however, their effect against this bacterium 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 (**Figure 2**). Possibly, the antimicrobial peptides (AMPs) generated in the digestion exerted some effect, reducing the number of bacteria up to 6 u.log (**Figure 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 (Yang et al., 2020). The MIC of peptide LCWAP was lower than the MIC of the entire protein (184.5 µg/mL). 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 et al., 2023), and an inhibitory effect on biofilm formation by *S. aureus* (Li et al., 2022).

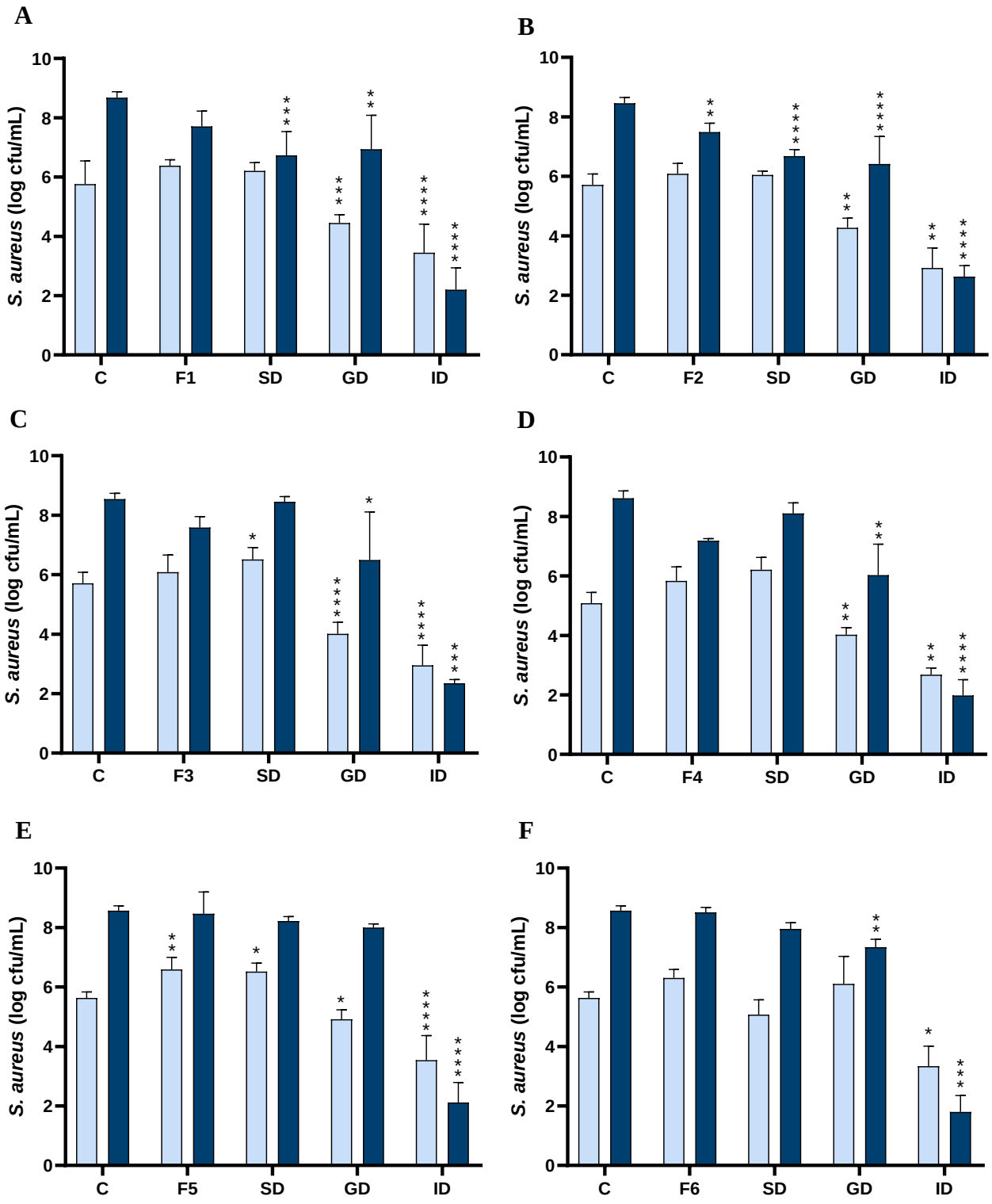

Figure 4. Antibacterial activity against *S. aureus* of (**A**) F1 and its digests, (**B**) F2 and its digests, (**C**) F3 and its digests, (**D**) F4 and its digests, (**E**) F5 and its digests and (**F**) F6 and its digests after an incubation of 4 h (□) or 24 h (■). C: control of bacteria with peptone 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).

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 (**Figure 4**).

Although more antibacterial effect was observed with the F2 (**Figure 4B**), based on buttermilk, than with the F1 (**Figure 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 et al., 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) Nº 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 (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.

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ARTÍCULO 7

Lactoferrin modulates oxidative stress and inflammatory cytokines in a murine model of dysbiosis induced by clindamycin

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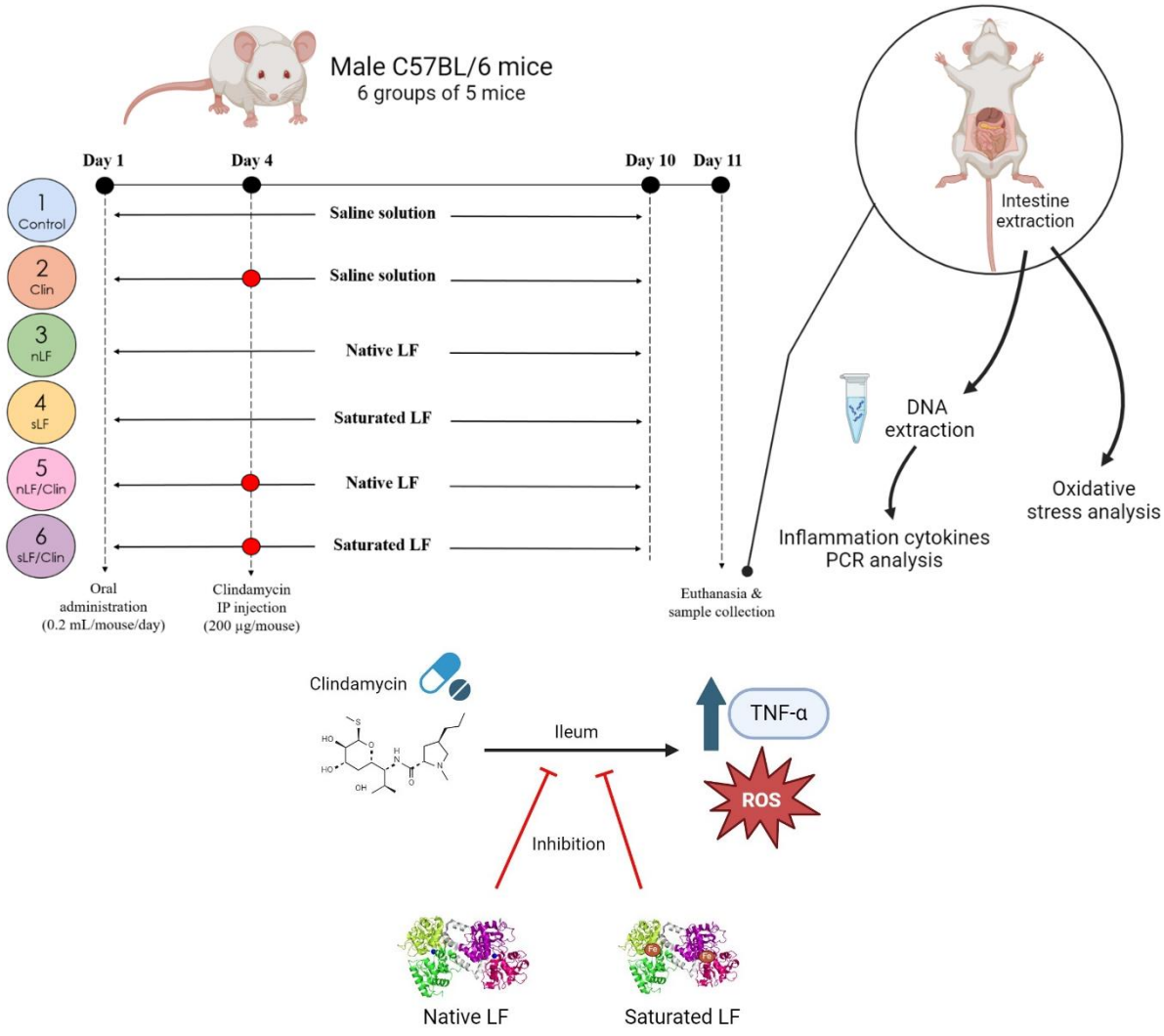
Abstract

Antibiotics, and specifically clindamycin, cause intestinal dysbiosis, reducing the microbiota with anti-inflammatory properties. Furthermore, clindamycin can induce alterations in the immune responses and oxidative stress. Lactoferrin, among other activities, participates in the maintenance of intestinal homeostasis and reduces dysbiosis induced by antibiotic treatment. The aim of this study was to analyze the effect of native and iron-saturated bovine LF in a murine model of dysbiosis induced by clindamycin. Six groups of male C57BL/6 mice were treated with saline (Control), clindamycin (Clin), native lactoferrin (nLF), iron-saturated lactoferrin (sLF), nLF + Clin or sLF + Clin. Oxidative stress caused in the intestinal cells of the ileum of animals subjected to different treatments was analyzed, focusing on lipid peroxidation and protein carbonyl content. The expression of inflammatory mediators was determined by qRT-PCR. Treatment with Clin did not modify lipid peroxidation, but significantly increased protein carbonyl levels, an effect that was reverted by orally administering sLF to mice. Furthermore, Clin significantly increased the expression of interleukin-6 and TNF-α. This effect was reverted by treatment with nLF and sLF, decreasing the expression to basal levels. In conclusion, this study indicates that lactoferrin can prevent some of the effects of Clin in intestinal cells and associated immune system.



1. Introduction

Antibiotics can lead to undesirable collateral effects, such as changes in intestinal microbiota, promoting dysbiosis and having an impact on the development of innate and adaptive immune responses against pathogens. Antibiotic-induced dysbiosis also increases the susceptibility to infections in later life (Shekhar & Petersen, 2020). Clindamycin is a lincosamide antibiotic whose action is based on slowing or stopping the growth of Gram-positive and anaerobic bacteria. This antibiotic is highly concentrated in the faeces, having a negative impact on the intestinal microbiota (Buffie et al., 2012). Clindamycin induces dysbiosis, reducing the presence of certain microorganisms with anti-inflammatory properties (Bellés et al., 2022).

Bovine lactoferrin (LF) is a cationic iron-binding glycoprotein present in exocrine secretions of mammals, such as milk, saliva, tears, etc. LF is secreted in its open form without iron (apo-LF), but it is able to bind two ferric ions to give rise to its closed form (holo-LF) (Adlerova, Bartoskova, & Faldyna, 2008). It has been shown that depending on LF iron saturation it can develop divergent effects on the growth of probiotic bacteria (Li et al., 2013; Fan et al., 2022). The increase of iron saturation in LF decreases its bacteriostatic capacity, with apo-LF being more functional (Orsi, 2004). However, holo-LF is not easily degraded and it is highly stable, especially when heated and stored for long time. For this reason, holo-LF is often used as an additional ingredient in some products (Fan et al., 2022). LF has numerous benefits at the intestinal level, such as favour iron absorption, increase the intestinal maturation or its barrier function, antibacterial and antiviral activity, or modulate the gut microbiota (Conesa et al., 2023). Furthermore, LF has been reported to contribute to the maintenance of intestinal homeostasis and to counter dysbiosis induced by antibiotic treatment (Bellés et al., 2022), and to inhibit the inflammatory response, promote natural killer cell death and rest reactive oxygen species (ROS) release at sites of inflammation (Cutone et al., 2020). The most widely adopted way for LF administration is orally. However, LF cannot pass intact through the stomach and is hydrolyzed during gastric digestion, releasing some bioactive peptides, such as lactoferricin and lactoferrampin. Therefore, although hydrolysis could affect the functional properties of LF, its degradation also could be beneficial (Wang et al., 2019). Furthermore, the susceptibility of LF to digestion depends on the matrix where it is found, being digested more easily when is in a liquid product than in a solid one. In addition, the

degree of glycosylation of LF also affects its stability in the gastrointestinal tract (Wang et al., 2019).

The inflammatory response in the gastrointestinal tract is highly regulated, ensuring a balance of pro-inflammatory and anti-inflammatory cytokines (Schenk & Mueller, 2008). Intestinal epithelial cells respond to external stimuli by expressing inflammatory factors and chemokines through the corresponding signalling pathways. Furthermore, these cells can recruit white blood cells to kill damaged cells or foreign pathogens. Therefore, intestinal development in the first period of life is crucial for the health of infants and children (Fan et al., 2022). LF plays an important role in modulating the immune system. It is considered an anti-inflammatory protein that helps prevent and treat inflammatory bowel diseases (IBD). Its immunomodulatory effect is based on inhibiting the synthesis of pro-inflammatory cytokines, such as TNF-α and interleukin-6 (IL-6), and promoting the production of anti-inflammatory cytokines, such as IL-4 and IL-10 (Conesa et al., 2023)

Some bactericidal antibiotics can induce a common oxidative damage, generating variable levels of deleterious ROS and causing damage to DNA, proteins and lipids, resulting in cell death (Guillouzo & Guguen-Guillouzo, 2020). Clindamycin, in particular, causes oxidative damage to DNA and lipids (Xiao et al., 2019). At low concentrations, ROS act as important mediators in almost all stages of the inflammatory process. However, overproduction of ROS can cause cellular damage and promote chronic inflammation (Chelombitko, 2018). On the other side, LF, as an anti-inflammatory agent, is also capable of capturing ROS, maintaining a physiological balance and reducing the inflammatory response (Conesa et al., 2023).

Therefore, the main objective of this study was to evaluate the effect of native and iron-saturated LF on oxidative stress and the expression of inflammatory mediators in the ileum from mice with dysbiosis induced by clindamycin.

2. Material and methods

2.1. Samples preparation

Native bovine LF (iron saturation below 10%) used for this study was kindly donated by Tatua Nutritionals (Morrinsville, New Zealand). Its purity of 90% was checked by SDS-PAGE, showing a single band at 80 kDa corresponding to the protein. For stock solution preparation, native LF (nLF) was dissolved in saline at a concentration of 200 mg/mL and

processed as detailed in our previous study (Bellés et al., 2022). The LPS level of this LF was determined (Abad et al., 2022) and it was considered minimal, so it did not influence the results.

To prepare saturated LF (sLF), the procedure described by Graikini et al. (2023) was followed. Briefly, an iron complex of 80 mM sodium nitrile acetate (NTA) and 20 mM $FeCl_3$ (FeNTA) and 10 mM $NaHCO_3$ (1:1, v:v) were added to LF (3 µL per mg of protein). The resulting sLF was considered 100% iron saturated.

Both, nLF and sLF, were adjusted to a concentration of 175 mg/mL for the animal experiments.

2.2. Animal model and treatments

The study was carried out with 30 male C57BL/6 mice of 8-12 weeks old (Janvier Labs, Le Genest-Saint-Isle, France). They were kept in a conventional laboratory animal facility at the University of Zaragoza at a range of temperature between 20-22 ºC, under a cycle of 12 h light/dark, with free access to water and animal chow. For the experiment, mice were randomly divided into 6 groups (n = 5 per group): control (receiving saline orally by gastric gavage for 10 days and representing the negative control group), clindamycin (Clin), native bovine lactoferrin (nLF), iron-saturated bovine lactoferrin (sLF), native bovine lactoferrin + clindamycin (nLF/Clin) and iron-saturated bovine lactoferrin + clindamycin (sLF/Clin) (**Figure 1**). All groups tested were treated equally, housed in the same room, kept in the same cages and maintained by the same personnel.

During the experiment, mice treated with Clin were fed for 10 days with saline and on day 4 received a single intraperitoneal (IP) injection of 200 µg of clindamycin (Normon Laboratories, Madrid, Spain) diluted in 0.2 mL of saline. Mice of the groups nLF and sLF were treated for 10 days orally by gastric gavage with 35 mg of nLF or sLF, diluted in 0.2 mL of saline. Mice from nLF/Clin and sLF/Clin groups were fed for 10 days with 35 mg nLF or sLF and on day 4 received an IP injection of 200 µg of Clin (**Figure 1**). The dose of 35 mg administered to the mice per day would be equivalent to about 140 mg/kg in humans (Nair & Jacob, 2016).

All procedures were conducted under Project Licence PI40/17 and approved by the in-house Ethics Committee for Animal Experiments of the University of Zaragoza. The care and use of animals were performed according to the Spanish Policy for Animal Protection

RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

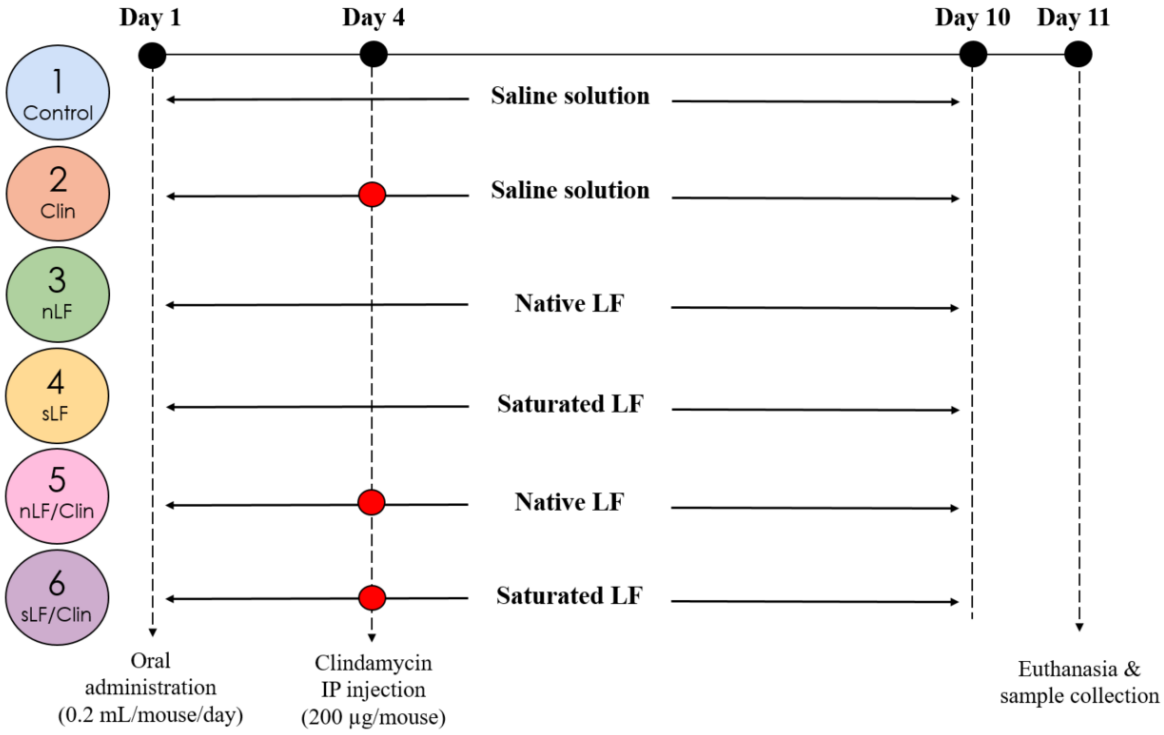


Figure 1. Division of the six groups of mice, 5 males per group. Control: oral administration of saline, Clin: oral administration of saline and intraperitoneal injection of clindamycin, nLF: oral administration of native lactoferrin, sLF: oral administration of iron-saturated lactoferrin, nLF/Clin: oral administration of native lactoferrin and intraperitoneal injection of clindamycin, sLF/Clin: oral administration of iron-saturated lactoferrin and intraperitoneal injection of clindamycin. During the first 10 days, all animals received an oral administration of saline for groups 1 and 2, native LF for groups 3 and 5, or saturated LF for groups 4 and 6. Additionally, groups treated with Clin (2, 5 and 6) received, on day 4, a single intraperitoneal injection of clindamycin. On day 11 of the experiment, the mice were humanely euthanised and organs were collected.

2.3. Microscopic assessment. Tissues histology

At postmortem, sections of the ileum, colon and cecum of each animal were collected for quantitative histopathological assessment of intestinal inflammation. Tissue sections were fixed in 10% neutral-buffered formalin for 48-72 h and routinely processed for paraffin embedding and hematoxylin-eosin staining. Total number of polymorphonuclear (PMN) cells in the intestinal lamina propria were quantified in 10 high-power fields (x400 magnification) by two pathologists blinded to the treatment groups.

2.4. Assessment of oxidative stress

Ileums were collected, washed with 0.9% NaCl and stored at -80 ºC until processed. Tissue samples were homogenized in cold 50 mM Tris buffer, pH 7.4, using Yellowline DI25 Basic Ultra-Turrax (IKA-Werke, Staufen Germany). The homogenate was centrifuged at 3000 g for 10 min at 4 ºC. The supernatant was collected for lipid peroxidation and protein carbonyl analysis.

The level of lipid peroxidation was determined by measuring the concentration of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA) as previously described (Gonzalo et al., 2011). In short, MDA + 4-HDA reacts with 1-methyl-2-phenylindole, giving rise a stable chromophore, whose absorbance can be measured spectrophotometrically at 586 nm. The samples were analyzed in duplicate. Lipid peroxidation was calculated in nmol MDA + 4-HDA per mg of protein. Protein concentration was determined by the Bradford method (Bio-Rad, Madrid, Spain). Results of lipid peroxidation were expressed as the percentage respect to the control value (100%).

The level of protein oxidation was analyzed by determining protein carbonyl content as previously described (Gonzalo et al., 2011). Tissue homogenates were incubated with 2,4-dinitrophenylhydrazine (DNPH), a carbonyl reagent, at 37 ºC for 1 h and protein carbonylation was measured at 375 nm. The samples were analyzed in triplicate. Results were calculated in nmol carbonyl groups per mg of protein and expressed as the percentage respect to the control value (100%).

2.5. RNA extraction and RT-PCR

Ileum samples were collected and preserved for 24 h in RNAlater solution (Ambion, Thermo Fisher Scientific, Madrid, Spain) and then, they were stored at -80 ºC until analysis. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). A fragment of each sample was taken and placed in a Precellys® tube (Precellys® Ceramic kit, Bertin Instruments, Montigny-le-Bretonneux, France) for homogenization. 600 µL of RLT lysis buffer supplemented with β-mercaptoethanol were added in a 100:1 (v/v) ratio. The samples were introduced into a Precellys®-24 homogenizer (Bertin Instruments) and processed with a program of 2 cycles of 20 s at 5000 rpm with a waiting time of 5 s. Subsequently, the samples were centrifuged at a maximum speed for 3 min and the supernatants with the cell lysate were carefully extracted. To each lysate, 600 µL of 70% ethanol were added, mixed by pipetting, and transferred to the extraction columns

of the RNeasy Mini Kit. The extraction of the RNA was performed according to the manufacturer instructions. The obtained RNA was stored at -80 ºC.

The complementary DNA (cDNA) was obtained using the qScript cDNA SuperMix kit (Quantabio, Beverly, MA, USA) and RT-PCR was performed as detailed in our previous study (Abad et al., 2022). This procedure was carried out to determine expression levels of IL-6, IL-10, IL-12 p35, IL-12 p40, TNF-α, and nucleotide-binding and oligomerization domain (NOD)-like receptors. The specific primers are detailed in **Table 1**.

The results obtained for the threshold cycles (Ct) were statistically analyzed by subtracting the mean of the Ct values corresponding to the housekeeping genes (GAPDH and HPRT1) from the Ct for amplification of studied genes ($\Delta Ct_{treatment} = Ct_{gene} - Ct_{housekeeping}$). The mean values of the negative controls were subtracted from the previously obtained value ($\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{control}$). Finally, the relative gene expression was calculated and expressed as fold change ($2^{\Delta\Delta Ct}$).

Table 1. Primer sequences used for RT-PCR analysis of housekeeping genes, interleukins, and NOD-like receptors. Fw: forward primer, Rv: reverse primer.

Gene	Primer (5' - 3')		Reference
GAPDH	**Fw**	CATGACCACAGTCCATGCCATCACT	Buey et al., 2021
	Rv	TGAGGTCCACCACCCTGTTGCTGTA	
HPRT1	**Fw**	CTGACCTGCTGGATTACA	Buey et al., 2021
	Rv	GCGACCTTGACCATCTTT	
IL-6	**Fw**	TCCTACCCCAATTTCCAATGC	Welter-Stahl et al., 2006
	Rv	TGAATTGGATGGTCTTGGTCCT	
IL-10	**Fw**	GGACAACATACTGCTAACCGAC	Wakabayashi et al., 2006
	Rv	AAAATCACTCTTCACCTGCTCC	
IL-12 p35	**Fw**	CATCGATGAGCTGATGCAGT	Wakabayashi et al., 2006
	Rv	CAGATAGCCCATCACCCTGT	
IL-12 p40	**Fw**	TGGAAGCACGGCAGCAGAATAAAT	Wakabayashi et al., 2006
	Rv	TGCGCTGGATTCGAACAAAGAACT	
TNF-α	**Fw**	AAATGGGCTTTCCGAATTCA	Korcheva et al., 2005
	Rv	CAGGGAAGAATCTGGAAAGGT	
NOD1	**Fw**	TCCCTTGCCTGTGAGCAGAAAGTA	Robertson et al., 2013
	Rv	GTGGGTATGTGCCATGCTTTGCTT	
NOD2	**Fw**	GGAGGAGCTTCCAGGAGTTT	Wakabayashi et al., 2006
	Rv	ACTCGTCCAAGCCATCAAAG	

2.6. Statistical analysis

The analysis was performed using the statistical software GraphPad Prism v8.0.2 (GraphPad Software, San Diego, CA, USA). The normality of the data was checked with the Saphiro-Wilk test. To compare the means of three or more unpaired groups, an

analysis of variance (ANOVA) was performed. Dunnet's post-hoc test was used as a multiple comparison test. Data that did not follow a normal distribution were submitted 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. Tissues histology

After checking the histology of the three tissues (colon, ileum and cecum), statistically differences were only observed in the ileum, so this tissue was the one chosen for the following experiments. Although there were no clear signs of tissue inflammation, differences were observed in the presence of PMN cells in the ileum of mice of different groups (**Figure 2**). In the control group (**Figure 2A**), the number of PMN cells was low; however, in the group of mice treated with clindamycin (**Figure 2B**), the presence of these cells was much more abundant, indicating an inflammation process. In the groups of mice treated with both native and saturated LF, the presence of PMN cells in the ileum was practically non-existent (**Figures 2C, 2D**). Finally, in the groups of mice treated with LF and clindamycin (**Figures 2E, 2F**), the presence of PMN cells in the ileum was very similar to that of the control group, and notably lower compared to the group treated only with the antibiotic.

3.2. Lipid and protein oxidation levels

Clin did not modify MDA + 4-HDA levels (**Figure 3A**), but increased significantly the protein carbonyl levels in ileum (**Figure 3B**). The oral administration of nLF increased slightly the carbonyl levels in ileum respect to the control (**Figure 3B**) and sLF also modified the oxidative stress in lipids and proteins (**Figure 3**). However, sLF reversed the oxidative stress of proteins caused by Clin to basal levels when administered to mice treated with the antibiotic.

In the study by Xiao et al. (2019), the authors analyzed the oxidative stress caused by different antibiotics *in vitro*, and concluded that clindamycin at 200 μg/mL caused oxidative lipid damage observed by the increase of MDA levels. These results do not coincide with ours, in which clindamycin did not affect oxidative stress in lipids (**Figure 3A**). This difference could be mainly because their study was carried out *in vitro* in different cell lines and ours was an *in vivo* experiment.

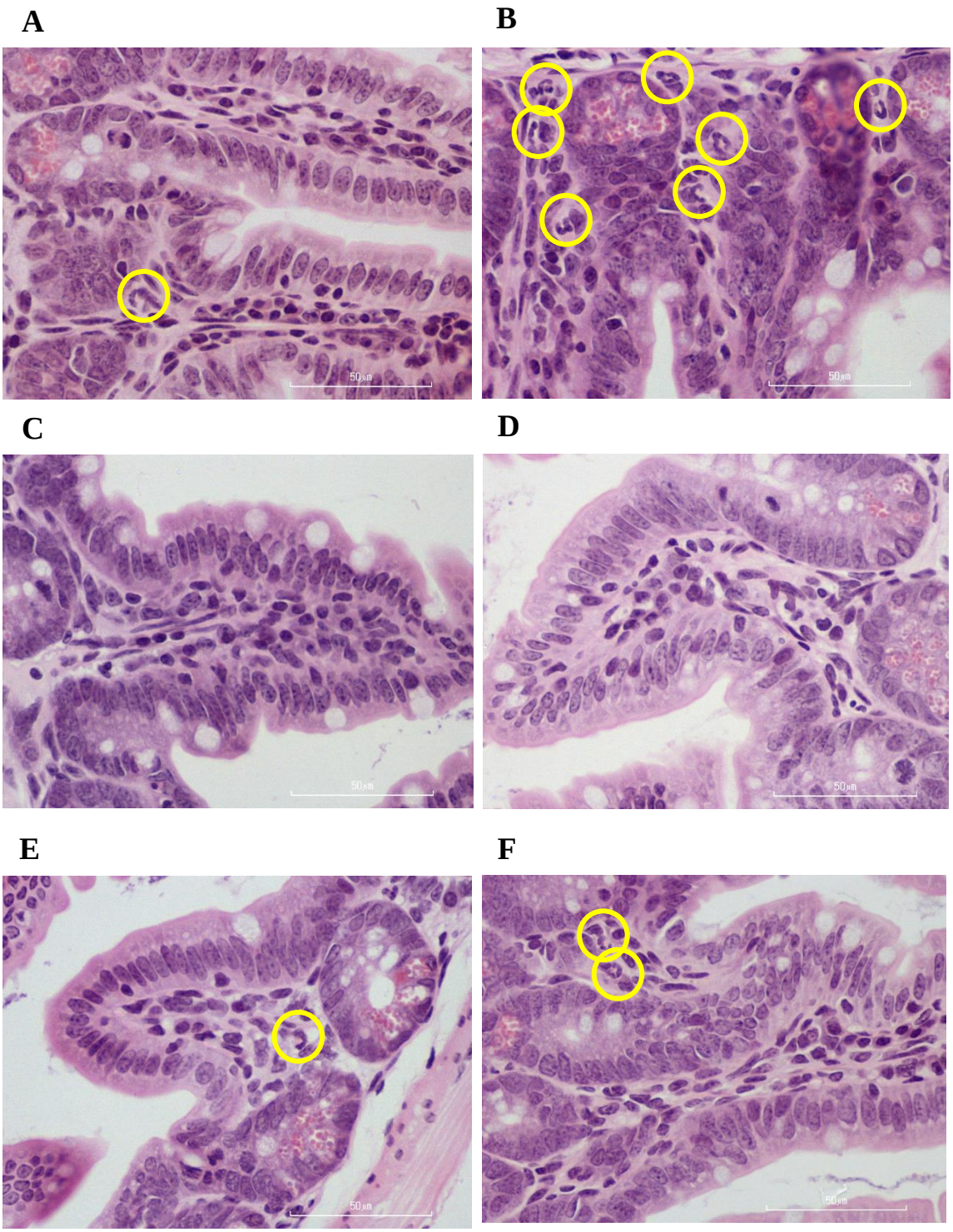


Figure 2. Polymorphonuclear cells in the ileum of mice of different groups. (**A**) Control group, (**B**) Clin group, (**C**) nLF group, (**D**) sLF group, (**E**) nLF/Clin group, (**F**) sLF/Clin group. Yellow circles indicate the PMN cells. Staining with hematoxylin and eosin.

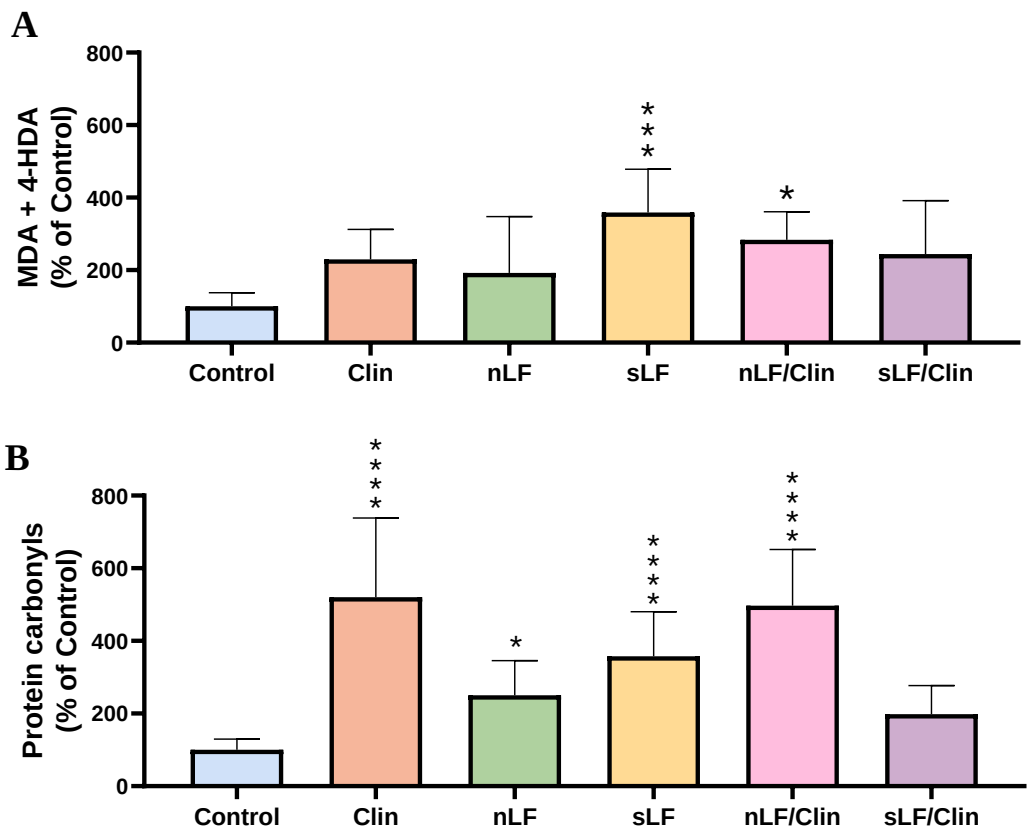


Figure 3. Effect of bovine LF on oxidative stress caused by clindamycin in mice ileum. (**A**) Levels of lipid peroxidation, determined by the concentration of MDA + 4-HDA. (**B**) Protein oxidation, analyzed by the measurement of carbonyls level. Clin: clindamycin; nLF: native LF; sLF: iron-saturated LF. The values represent the mean ± standard deviation of two replicates in five mice (n = 10). Asterisks indicate significant differences respect to control (*p< 0.05, ***p< 0.001, ****p< 0.0001).

Milk proteins, such as LF, have antioxidant effects, capturing ROS, chelating metals and modulating enzymes involved in ROS production (Bielecka, Cichosz, & Czeczot, 2022). LF binds iron, which increases its bioavailability and decreases its pro-oxidant effects. LF is able to control ROS levels by uptake of free iron, involved in ROS production (Cutone et al., 2020). Therefore, it can be expected that nLF will have greater effect than sLF, since it is more available to capture iron from the medium. However, in our study, sLF has shown a greater effect than native one, and this could be due to the fact that iron-saturated protein is more stable and resistant to digestion (Fan et al., 2022), which would allow it to reach the ileum and modulate in a positive way the effects of Clin. Although sLF alone had a negative effect of generating lipid and protein oxidation, when it acted in synergy with the antibiotic it had a positive effect, decreasing the oxidation. Therefore, although the mechanism of action of sLF is not known, it could be investigated whether

it would be effective to administer sLF preventively when an antibiotic is going to be administered, in particular, to reverse the negative effects of it.

It has been reported that milk proteins, like LF, generate bioactive peptides after digestion, whose activity is determined by their structure, molecular weight and sequence of amino acids (Bielecka, Cichosz, & Czeczot, 2022). Peptides with presence of tryptophan, histidine, tyrosine and proline in their sequence are the best antioxidants (Bielecka, Cichosz, & Czeczot, 2022). In fact, in a study by Hernández-Ledesma et al. (2007), they observed that after digestion of human milk, certain peptides containing tryptophan and tyrosine were generated, which could be responsible for its antioxidant activity. Furthermore, Guo et al. (2024) identified two peptides with antioxidant function in the intestinal digest of LF, QAYPNLCQLCK and NCPDKFCLFK, both with presence of tyrosine (Y) and proline (P). Thus, the LF administered orally to the mice could have been digested before reaching the ileum, giving rise to this type of peptides, responsible for the antioxidant effect observed in our study.

3.3. Inflammatory cytokine expression levels

In our results, Clin significantly increased the expression of some pro-inflammatory cytokines, such as IL-6 and TNF-α (**Figure 4**).

IL-6 is a single-chain protein, originally identified as a B-cell differentiation factor. This cytokine is produced in T cells, B cells, monocytes and fibroblasts, and is involved in the maturation of antibody-producing cells. IL-6 can bind to its receptor IL-6R whether it is soluble or located on the cell surface. The expression of IL-6 is increased in inflammatory diseases, and a blockage of this cytokine allows the control of symptoms and a slowdown in the progression of the disease (Cronstein, 2007). Excessive secretion of IL-6 and its dysregulation may play an important role in the pathogenesis of many diseases, including IBD (Suzuki, Yoshinaga, & Tanabe, 2011). Thus, it has been reported in mice that inactivation and blockage of IL-6 and its receptor decrease the incidence of colitis, indicating a key contribution of IL-6 in this process (Sander et al., 2008). In our results, the pro-inflammatory effect of Clin was reverted by oral treatment of nLF and sLF, decreasing the expression of IL-6 to control levels. However, nLF and sLF did not modify the expression levels of this cytokine by themselves (**Figure 4A**).

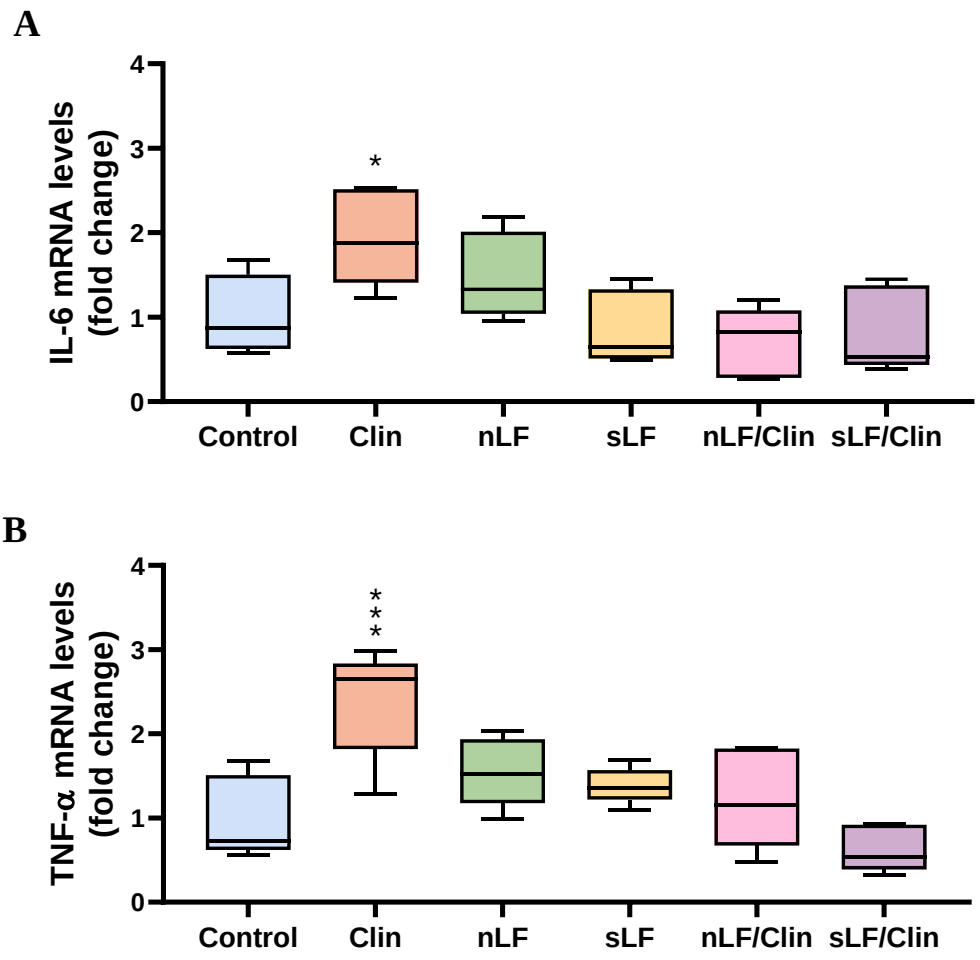


Figure 4. Effect of bovine LF on the expression levels of (**A**) IL-6 and (**B**) TNF-α in mouse ileum. Clin: clindamycin; nLF: native LF; sLF: iron-saturated LF. The horizontal line in the middle of each box represents the median, while the top and bottom borders of the boxes represent the 75 and 25 percentiles respectively, n = 5. Asterisks indicate significant differences respect to control (*p< 0.05, ***p< 0.001).

TNF-α is a key protein in the immune response, with multiple effects, from inflammation to apoptosis. TNF-α increases the production of other pro-inflammatory cytokines, such as IL-6, and promotes apoptosis by binding to its receptor (Victor & Gottlieb, 2002). It has been reported that some pro-inflammatory cytokines, such as TNF-α, decrease the expression of the tight junction structure and induce epithelial apoptosis (Suzuki, Yoshinaga, & Tanabe, 2011). In our study, there was a strong increase of TNF-α expression in the Clin group; while the administration of nLF and sLF improved the situation of the ileum cells, decreasing the Clin effect to basal levels. Furthermore, the single treatment with LF did not modify TNF-α expression (**Figure 4B**).

In the study by Fan et al. (2022), the authors analyzed the protective effect of LF with different iron saturations in a murine model of a lipopolysaccharide (LPS)-induced

intestinal inflammation. They concluded that LPS increased significantly the expression levels of IL-6 and TNF-α in the colon of mice. However, both apo- and holo-LF decreased significantly the levels of these inflammatory factors when administered orally to mice treated with LPS. These results are very similar to those obtained in the present study. Furthermore, it has been shown that doses of LF similar to those used in our study have effects on the intestinal immune system of mice (Bellés et al., 2022, Wakabayashi et al., 2006).

The main target of orally administrated LF is the intestinal immune system, since intact LF or its functional fragments, such as lactoferricin, are not transferred to the blood unless there is a lesion in the intestine (Wakabayashi et al., 2004). It has been observed that, in inflammatory bowel diseases, IL-6 levels are elevated (Suzuki, Yoshinaga, & Tanabe, 2011). In our case, the increase in IL-6 experimented by the Clin group could indicate an activation of the intestinal immune system, which is reversed by treating the mice with LF (**Figure 4A**). This process of activation caused in the ileum by clindamycin could be seen by the increase in the expression of inflammatory cytokines and the production of ROS, although it was not a clearly inflammation observed macroscopically or under the microscope at the histological analyze of the tissue (**Figure 2**).

Similar results have been obtained in previous studies with *in vitro* and *in vivo* models of LPS-induced inflammation, where LF modulated the immune response, downregulating pro-inflammatory cytokines, such as IL-6 and TNF-α, and enhancing the secretion of anti-inflammatory cytokines, like IL-10 and IL-4 (Legrand et al., 2005). Furthermore, oral administration of LF-derived lactoferricin decreased the inflammatory response in a murine model of intestinal dysfunction caused by *Escherichia coli*, suppressing the expression and release of IL-6 and TNF-α to basal levels (Haiwen et al., 2019).

On the other hand, Clin treatment did not alter the expression levels of other inflammatory cytokines analyzed, like IL-10, IL-12 p35, IL-12 p40, and NOD-like receptors (**Figure 5**).

IL-10 is one of the most important anti-inflammatory cytokines, besides IL-35 and TGF-β (Sabat et al., 2010). Neither Clin nor LF, in any of its iron saturation states, showed any effect on the expression of IL-10 (**Figure 5A**). IL-10 is a homodimer synthesized by several type of cells. Monocytes and macrophages secrete IL-10 after endogenous or exogenous activation, and this interleukin decreases the production of inflammatory mediators (Sabat et al., 2010).

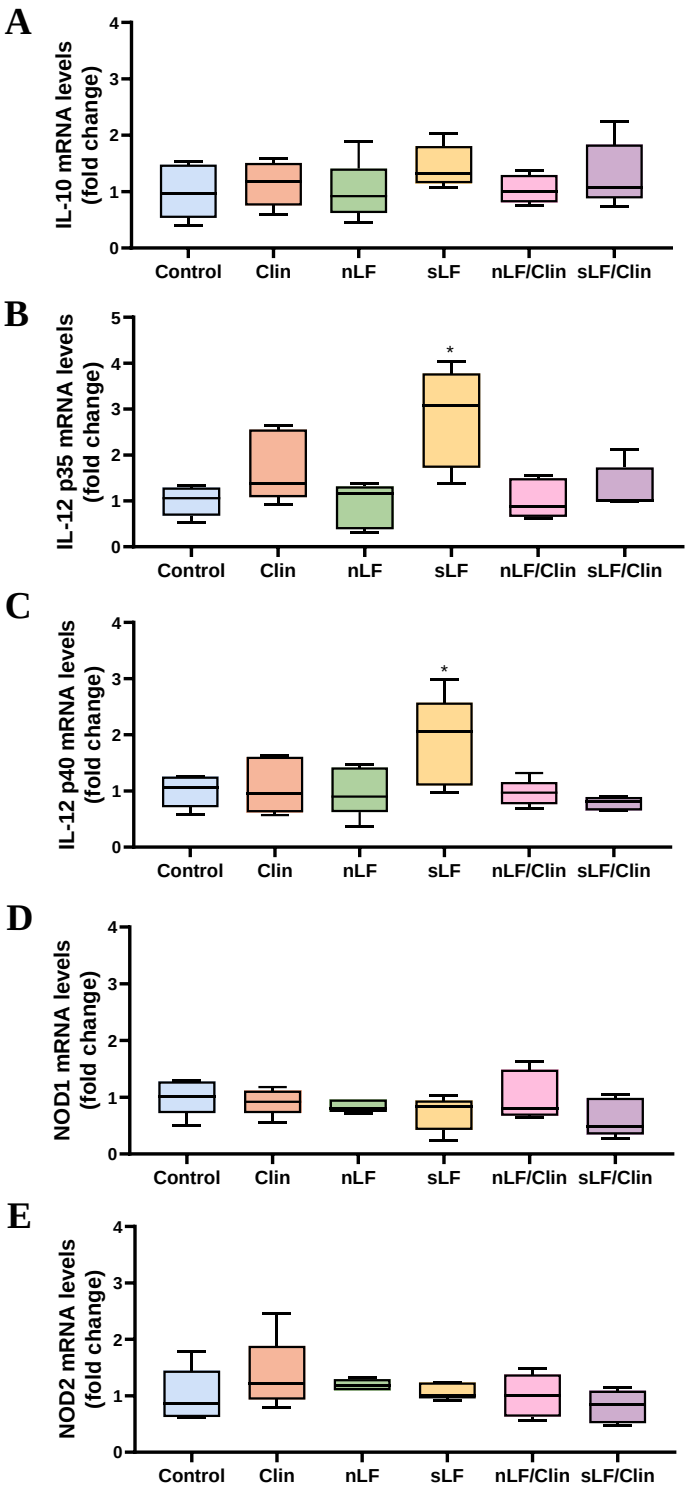


Figure 5. Effect of bovine LF on the expression of (**A**) IL-10, (**B**) IL-12 p35, (**C**) IL-12 p40, (**D**) NOD1 and (**E**) NOD2 in mice ileum. Clin: clindamycin; nLF: native LF; sLF: iron-saturated LF. The horizontal line in the middle of each box represents the median, while the top and bottom borders of the boxes represent the 75 and 25 percentiles respectively, n = 5. Asterisks indicate significant differences respect to control (*p< 0.05).

In the study by Madsen et al. (2020), IL-10 knockout mice developed chronic enterocolitis and showed significantly increased levels of bacteria in the colonic mucosa, indicating that this cytokine plays an important role in controlling the intestinal microbiota and regulating the intestinal inflammation process.

The expression levels of the two subunits of interleukin-12, IL-12 p35 and IL-12 p40, were significantly increased by sLF treatment (**Figures 5B, 5C**). Clindamycin did not show significant differences compared to the control group. This cytokine is a heterodimeric protein that has an important role as a mediator in the host's defense against infections and cancer (Del Vecchio et al., 2007). Wakabayashi et al. (2006) analyzed the effect of oral administered bovine LF (8.2% of iron saturation) in the gene expression in the small intestine of mice. That study showed that IL-12 p35 and IL-12 p40 were upregulated by nLF administration, while in our results, iron-saturated LF showed more effect than nLF.

In addition, Toll-like innate immune receptors (TLRs) and NOD-like receptors are also involved, with their signalling cascades, in the modulation of epithelial barrier repair and integrity (Parlato & Yeretssian, 2014). NOD-like receptors are strategically expressed in the intestine (Rubino et al., 2012). NOD1 and NOD2 play an important role in the activation of innate immune signalling after bacterial detection. These receptors distinguish between Gram-negative and Gram-positive bacteria by detecting specific peptidoglycan motifs. While NOD1 recognizes D-glutamyl-meso-diaminopimelic acid found in Gram-negative and certain Gram-positive bacteria, NOD2 detects the common bacterial muramyl-dipeptide (Parlato & Yeretssian, 2014). NOD1 is highly expressed in intestinal epithelial cells. This receptor has not been directly associated with intestinal inflammation, but is instead given a role in regulating host responses to normal intestinal microbiota and enteric pathogens in intestinal cells (Rubino et al., 2012). On the other hand, elevated levels of NOD2 have been observed in patients with IBD. Indeed, there is evidence that inflammation during IBD drives increased expression of NOD2 (Parlato & Yeretssian, 2014). However, the results obtained in our study did not show any significant modification in the expression of NOD1 and NOD2 (**Figure 5D, 5E**).

4. Conclusions

Although clindamycin did not cause clear inflammation of the small intestines in mice, it did activate an inflammatory process, recruiting polymorphonuclear cells in the ileum. This inflammation process seemed to improve with the ingestion of lactoferrin, both

native and iron-saturated, restoring the presence of these defensive cells in the ileum to basal levels. The results of this study, supported by previous studies, confirmed that oral administration of lactoferrin protects against the negative effects of clindamycin in oxidation and expression of inflammatory mediators in the ileum of mice. Lactoferrin reversed the increase in protein carbonyl content and reduced the expression of pro-inflammatory cytokines, such as IL-6 and TNF-α, mismatched by the action of clindamycin. Although these studies should be expanded and continued to better understand the mechanism of action of lactoferrin in the intestine, given the large number of beneficial properties for health that this protein presents, it could be an interesting ingredient for functional foods, being very useful as "protective" when taking antibiotics such as clindamycin.

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DISCUSIÓN GENERAL

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1. Actividad antibacteriana

1.1. Actividad antibacteriana de la lactoferrina

La presencia de LF puede favorecer la prevención de la contaminación por patógenos de las fórmulas lácteas infantiles (Harouna et al., 2020). Por ello, se ha estudiado el efecto antibacteriano de la LF frente a patógenos Gram-negativos como *C. sakazakii*, y Gram-positivos como *L. monocytogenes* y *S. aureus*.

En los estudios contenidos en esta Tesis Doctoral se ha analizado el efecto que presenta la LF frente a estas bacterias tanto en fase exponencial como en fase estacionaria de crecimiento. Los resultados expuestos en los **artículos 1, 5 y 6** se resumen en la **Tabla 4**, que permite una rápida comparación de las diferencias en la actividad de esta proteína en función de la bacteria y su etapa de crecimiento.

De acuerdo con el estudio realizado por Embleton et al. (2013), la actividad antibacteriana de la LF se le atribuye, principalmente, a su capacidad de captar hierro, privando al patógeno de este sustrato. Otros mecanismos para la acción antibacteriana de la LF son la liberación de LPS o la unión de la LF a las porinas (Gruden y Poklar Ulrih, 2021). Las porinas son proteínas transmembrana que generan canales para la difusión inespecífica de solutos a través de la membrana externa de las bacterias (Gruden y Poklar Ulrih, 2021). Sin embargo, tanto la liberación de LPS como la unión a porinas solo ocurre en bacterias Gram-negativas, por lo que el efecto de la LF varía mucho en función de la bacteria a la que se enfrenta, como se puede observar en la **Tabla 4**.

En los resultados obtenidos en la presente Tesis Doctoral, la LF fue más efectiva frente a *C. sakazakii* en fase exponencial que en fase estacionaria, disminuyendo el crecimiento de la bacteria en más de un 50% a partir de una concentración de 2 mg/mL. Sin embargo,

en el caso de *L. monocytogenes* el resultado fue contrario, siendo la LF más activa frente a la bacteria en la fase estacionaria de crecimiento, especialmente tras 24 h de incubación. No obstante, la LF mostró, de manera general, un mayor efecto antibacteriano frente a *L. monocytogenes* que frente a *C. sakazakii*, incluso en la fase exponencial, disminuyendo el crecimiento de la bacteria en un 50% a partir de una concentración de 1 mg/mL. Cabe destacar que este efecto comenzó a perderse a altas concentraciones de LF (10 mg/mL). Por otro lado, al analizar el efecto de la LF frente a *S. aureus* se detectó muy poca actividad antibacteriana, manteniéndose el nivel de bacteria independientemente del tiempo de incubación. La LF no mostró ningún efecto antibacteriano frente a *S. aureus* en fase exponencial, aunque sí disminuyó la bacteria en fase estacionaria en un 20%; resultados que coincidieron con los obtenidos por Bhimani, Vendrov y Furmanski (1999), que demostraron que *S. aureus* en fase exponencial era muy poco sensible a la LF humana y bovina.

Tabla 4. Actividad antibacteriana de la lactoferrina nativa a distintas concentraciones frente a *C. sakazakii*, *L. monocytogenes* y *S. aureus* en fase exponencial y fase estacionaria, expresando el crecimiento bacteriano en porcentaje con respecto al control. [LF]: concentración de lactoferrina en mg/mL, F. EXPON: fase exponencial, F. ESTAC: fase estacionaria. Se resaltan en azul las muestras que disminuyen el crecimiento bacteriano de manera significativa ($p \leq 0,05$).

[LF]	Fase de crecimiento	*C. sakazakii* ATCC 29544		*L. monocytogenes* ATCC 13932		*S. aureus* ATCC 25923	
		4 h	**24 h**	**4 h**	**24 h**	**4 h**	**24 h**
0,5	F. EXPON	89,18	104,15	**85,92**	94,83	100,26	104,71
	F. ESTAC	98,17	94,93	**73,44**	**74,49**	102,48	**93,10**
1	F. EXPON	**81,90**	100,40	**63,93**	**44,76**	100,80	101,65
	F. ESTAC	95,77	96,23	**69,22**	**44,76**	104,00	96,06
2	F. EXPON	**39,65**	96,54	**39,35**	**37,68**	100,23	100,11
	F. ESTAC	83,46	88,08	**50,84**	**24,02**	101,10	**89,59**
5	F. EXPON	**41,61**	**47,65**	**39,99**	**41,39**	97,92	98,65
	F. ESTAC	**51,72**	**71,82**	**48,45**	**23,84**	**86,94**	**90,48**
10	F. EXPON	**40,93**	**45,55**	**78,71**	**69,55**	98,14	99,32
	F. ESTAC	**59,00**	**65,87**	**57,46**	**31,41**	**83,66**	**80,84**

Por todo ello, y con los resultados obtenidos en los diferentes estudios, cabe destacar el alto poder antibacteriano que muestra la LF frente a la cepa CECT 935 (ATCC 13932) de *L. monocytogenes*, seguido por el efecto que presenta esta proteína láctea frente a *C.*

sakazakii, cepa CECT 858 (ATCC 29544). La LF mostró, de manera general, una acción antibacteriana dependiente de la dosis.

En estudios previos, la LF nativa mostró mayor efecto inhibitorio del crecimiento de *C. sakazakii* que la LF saturada con hierro (Harouna et al., 2015, 2020). En cualquier caso, estos estudios solo analizaron la actividad de la proteína en la fase estacionaria de la bacteria, y con los resultados detallados en el **artículo 1** podemos afirmar que la LF presenta un mayor efecto frente a *C. sakazakii* en fase exponencial. Este mismo efecto se observó en el estudio realizado por Afugupalli et al. (1995), en el que se concluyó que la interacción entre la LF y la membrana externa de *Actinobacillus actinomycetemcomitan*, bacteria Gram-negativa, fue mayor en la fase exponencial que en la fase estacionaria de crecimiento.

Los resultados obtenidos frente a *L. monocytogenes* fueron similares a los obtenidos en el estudio realizado por Conesa et al. (2010), en el que se evaluó el efecto antibacteriano de la LF bovina frente a esta bacteria Gram-positiva, midiendo la absorbancia del cultivo a 620 nm. En este estudio la LF también mostró mayor efecto frente a *L. monocytogenes* en fase estacionaria que en fase exponencial, siendo la concentración mínima inhibitoria (MIC, del inglés *minimum inhibitory concentration*) y la concentración mínima bactericida de la LF de 2 y 5 mg/mL, respectivamente. Estos resultados también coinciden con los obtenidos en el estudio realizado por Tidona et al. (2011), en el que analizaron el efecto de la leche de burra, rica en LF, frente a *L. monocytogenes* a diferentes tiempos de crecimiento, demostrando un efecto antibacteriano de la LF tanto en fase exponencial como estacionaria. Sin embargo, la reducción de colonias fue mayor tras 8 h de crecimiento, momento en el que la bacteria entraba en la fase estacionaria. Además, la LF humana también ha demostrado efecto antibacteriano frente a *L. monocytogenes* en fase estacionaria a concentraciones de 1 y 2 mg/mL (Lee et al., 2005).

El efecto antibacteriano de la LF observado en nuestro estudio frente a *S. aureus* fue bastante bajo, lo que pudo deberse a varios motivos. La cepa empleada fue la CECT 435, que corresponde con la ATCC 25923. Esta cepa es de origen clínico, y muestra características de cepa estándar para las pruebas de laboratorio (Cebrián et al., 2010). Algunos estudios previos que emplearon esta misma cepa, mostraron un mayor efecto inhibitorio por las LF empleadas. Sin embargo, en dichos estudios las condiciones empleadas diferían de las nuestras y, en algunos casos, el origen de la LF también era diferente. Así, en el estudio de Bai et al. (2010) emplearon LF bovina recombinante,

analizando el efecto de varios segmentos de esta LF, expresados en *Pichia pastoris*, frente a la misma cepa *de S. aureus* (ATCC 25923). Los resultados mostraron que ciertas regiones de la LF eran esenciales para ejercer el efecto antibacteriano. La proteína que incluía la región entre lóbulos y el lóbulo N mostró mayor efecto frente a *S. aureus* en fase exponencial que el lóbulo N solo o la proteína recombinante entera. El estudio realizado por Kai et al. (2002) en un cultivo primario de células secretoras de glándula mamaria bovina, mostró que la LF bovina inducía la eliminación fagocítica de *S. aureus* (ATCC 25923) mediada por el complemento. Esto indicaba que la LF era capaz de activar sinérgicamente tanto la vía alternativa de la cascada del complemento bovino como la fagocitosis. Sin embargo, esta inhibición del crecimiento de *S. aureus* se analizó en la glándula mamaria bovina durante periodos de no lactancia, mientras que nuestro estudio no aborda el entorno con las células secretoras de la glándula mamaria bovina. Además, en el estudio de Padrão et al. (2016), en el que se evaluó la función de un compuesto de celulosa bacteriana y LF como envase comestible con actividad antimicrobiana, la LF mostró efecto frente a *S. aureus* en fase exponencial. Todas las concentraciones de LF probadas en ese estudio (0,25; 0,5; 1; 2,5; 5 y 10 mg/mL) redujeron la tasa de crecimiento específico de *S. aureus*, aunque este efecto no fue dosis-dependiente, ya que la actividad antibacteriana fue muy similar en concentraciones entre 0,5 y 10 mg/mL. Sin embargo, en dicho estudio no se especificó la cepa de *S. aureus* utilizada, por lo que se podría haber utilizado una cepa más sensible a la acción de la LF.

Es conocido que *S. aureus* es capaz de producir sideróforos (Perry et al., 2019), responsables de la absorción del hierro presente en el medio y unido a la LF, evitando el efecto de esta proteína y manteniendo el crecimiento de la bacteria (Hussan et al., 2022). Cuando *S. aureus* y LF coexisten en un ambiente que no tiene suficiente hierro, como nuestro medio de cultivo, se produce una competencia entre las bacterias y la proteína para capturar el hierro del medio. Se ha demostrado que cuando *S. aureus* está en la fase exponencial de crecimiento, produce sideróforos que pueden captar hierro unido a transferrina, una proteína fijadora de hierro muy similar a la LF (Lindsay, Riley & Mee, 1995).

En fase estacionaria, Kutila et al. (2003) obtuvieron resultados similares a los nuestros. En su estudio, la LF bovina a concentraciones de 1,67 y 2,67 mg/mL disminuyó significativamente el crecimiento de *S. aureus*, y la LF a 2,67 disminuyó el crecimiento máximo de *S. aureus* tras 20 h de incubación.

Por otro lado, estudios como el de Redwan et al. (2016) demostraron el efecto antibacteriano de la LF de camella y humana frente a un aislado clínico de *S. aureus* resistente a meticilina (MRSA, del inglés *methicillin-resistant S. aureus*).

1.2. Actividad antibacteriana de los digeridos de la lactoferrina

Anteriormente, algunos estudios han analizado la actividad antibacteriana de los hidrolizados de la LF, generados por la acción de la pepsina, quimosina o del cuajo microbiano, frente a *C. sakazakii* (Wakabayashi, Yamauchi, y Takase, 2008), *L. monocytogenes* (Ripollés et al., 2015) o *S. aureus* (Dionysius y Milne, 1997).

En la **Tabla 5** se reflejan los resultados obtenidos en los ensayos antibacterianos de la LF y sus digeridos, productos de la digestión GIT *in vitro*, frente a las tres bacterias analizadas en los **artículos 4, 5 y 6**. La mayor efectividad de los hidrolizados tras 4 h de incubación se obtuvo frente a la bacteria Gram-negativa, *C. sakazakii*, disminuyendo su concentración de manera significativa con todos los digeridos. Sin embargo, aunque el efecto antibacteriano se mantuvo activo tras todas las etapas de la digestión, se apreció una disminución del efecto en cada etapa, siendo el SD el que mayor efecto mostró. Tras la acción de la pepsina en la etapa gástrica, los centros de unión al hierro de la LF pueden verse afectados, liberando al medio hierro libre y fragmentos de LF incapaces de captarlo (Hopp et al., 2022). De esta manera, conforme el proceso de digestión avance en el tiempo, habrá más hierro disponible para la bacteria, permitiendo así un mayor crecimiento de *C. sakazakii*, y revirtiendo el efecto de los péptidos con actividad antimicrobiana.

Tabla 5. Actividad antibacteriana de la lactoferrina nativa y sus digeridos frente a *C. sakazakii*, *L. monocytogenes* y *S. aureus* en fase estacionaria, expresando el crecimiento de la bacteria en porcentaje con respecto al control. LF: lactoferrina sin digerir, SD: digerido salivar, GD: digerido gástrico, ID: digerido intestinal. *La concentración de LF empleada fue de 5 mg/mL para *C. sakazakii* y *S. aureus* y de 2,5 mg/mL para *L. monocytogenes*. Se resaltan en azul las muestras que disminuyen el crecimiento bacteriano de manera significativa ($p \leq 0,05$).

MUESTRA	*C. sakazakii* ATCC 29544		*L. monocytogenes* ATCC 13932		*S. aureus* ATCC 25923	
	4 h	**24 h**	**4 h**	**24 h**	**4 h**	**24 h**
LF*	**76,46**	**75,44**	**58,79**	**21,68**	97,56	94,02
SD	**67,68**	89,19	**37,29**	**23,07**	108,93	101,59
GD	**80,76**	102,63	97,19	**50,04**	86,30	103,17
ID	**86,55**	96,34	**84,12**	97,59	**67,24**	**40,06**

En el estudio realizado por Wakabayashi, Yamauchi, y Takase (2008) analizaron el efecto de la LF, de su hidrolizado y del péptido lactoferricina frente a diferentes cepas de *C. sakazakii*, entre las que se incluyó la ATCC 29544. Demostraron que tanto la LF intacta, como el hidrolizado y la lactoferricina disminuían el crecimiento de la bacteria tras 17 h de incubación a 37 ºC. De estas tres muestras, la lactoferricina fue la que mostró una MIC menor, siendo por tanto la más efectiva frente a la bacteria. El hidrolizado con pepsina, que sería similar en composición a nuestro GD, mostró mayor efecto que la LF intacta, lo que difiere ligeramente de nuestros resultados, ya que tanto a las 4 como a las 24 h de incubación el efecto de la LF fue mayor que el del GD.

Además, en nuestro estudio la bacteria se recuperó tras 24 h de incubación, llegando a un nivel de crecimiento muy similar al del control. Por tanto, se podría afirmar que el efecto observado tras las 4 h de incubación fue bacteriostático y no bactericida, permitiendo la posterior recuperación de la bacteria.

Por otra parte, es importante señalar que la digestión no resulta en una hidrólisis completa de las proteínas, sobre todo si el medio en el que se encuentran es complejo. Por ello, aunque en el proceso de digestión se generan péptidos y se libera hierro, es posible que algunos fragmentos de la LF mantengan su centro de unión al hierro activo (Hopp et al., 2022). Ante el efecto bacteriostático causado a las 4 h por la falta de hierro en la bacteria, esta puede desarrollar mecanismos de defensa, como la producción de sideróforos comentada anteriormente. Estos sideróforos pueden secuestrar el hierro libre del medio o el que se encuentra en los fragmentos de LF, formando complejos estables con él (Phair et al., 2022) y permitiendo la recuperación de la bacteria. En el estudio realizado por Singh et al. (2017) se demostró que ciertas cepas de *C. sakazakii* eran capaces de producir sideróforos como mecanismo de defensa, facilitando la captación del hierro presente en el medio y favoreciendo su crecimiento.

En el estudio de Ripollés et al. (2015) se describe que los péptidos de la LF generados por la pepsina mostraron efecto frente a *L. monocytogenes* tras 24 h de incubación, lo que coincide con lo obtenido en nuestro estudio con el GD de la LF, que disminuye el crecimiento de *L. monocytogenes* en un 50% tras 24 h. En dicho estudio también se observó actividad antibacteriana de los hidrolizados de LF obtenidos tanto con quimosina recombinante como con cuajo bovino, aunque menor que la obtenida con pepsina.

En el estudio realizado por Wakabayashi et al. (1992) aislaron la lactoferricina, péptido de la LF generado por acción de la pepsina, mediante una hidrólisis a 37 ºC durante 4 h. El péptido fue purificado y mostró efectividad frente a *L. monocytogenes* tras una incubación de 16-20 h, disminuyendo su crecimiento a niveles similares a los de ciertos antibióticos. Si bien es cierto que la cepa ATCC 13932 empleada en nuestro estudio no se analizó en el trabajo citado, se emplearon cepas clínicas humanas muy similares. El GD obtenido en el presente trabajo, que debería contener la lactoferricina como péptido generado por la acción de la pepsina, no mostró una fuerte actividad tras 4 h de incubación con la bacteria, aunque sí lo hizo tras 24 h (**Tabla 5**), lo que coincidiría con los resultados aportados por Wakabayashi et al. (1992).

Por otro lado, Flores-Villaseñor et al. (2010) analizaron el efecto antibacteriano de péptidos sintéticos de la LF frente a *E. coli*, *S. aureus* ATCC 25923 y MRSA. En ese estudio, tanto la lactoferricina como la lactoferrampina sintéticas inhibieron el crecimiento de *S. aureus* en más del 85%. Estos resultados no coinciden con los obtenidos en nuestro estudio ya que el GD de la LF, en el que se detectó la secuencia LSKAQEKFGKNKSRSFQL correspondiente a una isoforma de la lactoferrampina, no mostró actividad frente a *S. aureus*. Sin embargo, es importante indicar la diferencia entre emplear péptidos sintéticos y purificados (Flores-Villaseñor et al., 2010) y un digerido gástrico de LF con una composición más compleja, como en nuestro estudio.

Bellamy et al. (1992) analizaron la actividad antibacteriana de la lactoferricina frente a un gran número de bacterias, tanto Gram-negativas como Gram-positivas en fase exponencial de crecimiento. Entre otras, se analizó la cepa de *S. aureus* JCM-2413 (correspondiente con *S. aureus* ATCC 25923) y se observó que, de todas las cepas de *S. aureus* analizadas, fue la que presentó una MIC de lactoferricina mayor. En cualquier caso, la lactoferricina mostró actividad frente a esta bacteria en fase exponencial tras 16-20 h de incubación. Estos resultados difirieron bastante de los nuestros, ya que el GD no inhibió el crecimiento de la bacteria tras 24 h de incubación, aunque nuestra bacteria se analizó en fase estacionaria.

Sin embargo, al comparar nuestros resultados con los obtenidos por Quintieri et al. (2020), podemos encontrar ciertas similitudes. En el citado estudio, analizaron el efecto de la LF y su hidrolizado con pepsina frente a diferentes especies y cepas del género *Staphylococcus*. De todas las analizadas, *S. aureus* fue la más resistente al efecto del hidrolizado de LF, presentando una MIC mayor de 20 mg/mL. Esto podría explicar que

nuestro GD no mostrara efecto frente a esta bacteria, pues la concentración empleada fue de 5 mg/mL. Asimismo, en este estudio también detectaron en el hidrolizado de LF la secuencia LSKAQEKFGKNKSRSFQL de la lactoferrampina (Quintieri et al., 2020).

En general, *L. monocytogenes* fue la bacteria más susceptible al efecto tanto de la LF (**Tabla 4**) como de los digeridos de la LF (**Tabla 5**), mostrando una menor variación de la actividad antibacteriana entre los dos tiempos de incubación, y reduciendo el crecimiento de la bacteria de manera estadísticamente significativa con prácticamente todas las muestras y concentraciones analizadas.

En definitiva, la acción antibacteriana de la LF y sus digeridos fluctúa bastante al comparar los resultados obtenidos en los distintos estudios realizados, en función de la cepa bacteriana empleada, del origen de la LF y de su saturación en hierro, de la formación de sideróforos, de la fase de crecimiento de la bacteria, etc.

1.3. Actividad antibacteriana de las fórmulas lácteas y sus digeridos

Las fórmulas lácteas infantiles se emplean para sustituir total o parcialmente a la leche humana, cubriendo las necesidades del recién nacido durante la época de lactancia (Koletzko et al., 2005). En cualquier caso, la leche se caracteriza por tener una composición muy completa, por lo que se considera un alimento básico en la dieta tanto de niños como de adultos, ampliando su función más allá de la subsistencia nutricional de los lactantes (Park y Nam, 2015). Además, la elaboración de productos o fórmulas con base láctea como las que se han preparado en el presente estudio, contribuyen al aprovechamiento y revalorización de subproductos de la industria láctea como el lactosuero o la mazada, y a la innovación en el desarrollo de alimentos con propiedades bioactivas interesantes.

En la elaboración de las fórmulas y productos lácteos se emplean tratamientos tecnológicos como la homogeneización para su estabilización, reduciendo el tamaño de los glóbulos grasos, y la pasteurización para su higienización y alargamiento de su vida útil (Bourlieu et al., 2015).

Sin embargo, en el caso de las leches de fórmula infantiles en polvo, una vez abiertas son productos no estériles que pueden ser contaminados por patógenos, como *C. sakazakii* y *S. aureus*, a causa de un mal manejo o por un almacenamiento inapropiado (Wang et al., 2012). Por esta razón, algunos casos clínicos de enfermedades transmitidas por los alimentos en niños están relacionadas con el consumo de preparados para lactantes

contaminados con patógenos, especialmente *C. sakazakii*, *Salmonella enterica* y *S. aureus* (Cho et al., 2019). Por lo tanto, el uso de compuestos antibacterianos naturales en las fórmulas lácteas, como la LF, no sólo es conveniente por su interesante papel en el desarrollo de la microbiota intestinal del lactante (Conesa et al., 2023), sino también como estrategia de tecnología de barrera (*hurdle technology* en inglés) para controlar la proliferación de contaminantes bacterianos.

La tecnología de barrera múltiple emplea diferentes medidas de conservación, que se aplican estratégicamente para controlar eficazmente el crecimiento de los microorganismos alterantes y patógenos en los alimentos (Yuan, 2003). Se trata de un área importante de la microbiología alimentaria que describe las posibilidades de un enfoque de conservación múltiple que actúa sinérgicamente para inhibir los microorganismos en los alimentos (Duffy, Chen, y Schaffne, 2003).

Así, el efecto antibacteriano de las seis fórmulas suplementadas con LF y de sus digeridos obtenidos tras el proceso de digestión GIT *in vitro* frente a las tres bacterias analizadas a lo largo del presente estudio se refleja en la **Tabla 6**.

En su mayoría, las fórmulas lácteas sin digerir y los SD no mostraron gran actividad frente a las bacterias, sino que la mayor actividad antibacteriana registrada la presentaron los GD, con alguna excepción en el estudio realizado con *S. aureus*, que se mostró más susceptible ante los ID.

Según la bibliografía, el bajo efecto de la LF cuando forma parte de una fórmula láctea en comparación con la potente actividad de la LF libre podría deberse a la interacción de la LF con otras proteínas de la leche como la β-LG, la albúmina (Lampreave et al., 1990) y las micelas de caseína (Anema, 2018), viéndose reducida su actividad por su baja disponibilidad. Además, esta interacción de la LF con el resto de proteínas de las fórmulas la protege hasta cierto punto de la digestión, apareciendo parte de la proteína intacta en los GD de las fórmulas. El mismo efecto protector se detalló en el estudio de Kuwata et al. (1998), en el que cuando se administró leche enriquecida con LF a ratones, aparecieron fragmentos activos de la LF en las heces, lo que sugirió que esta proteína se había mantenido íntegra en su mayor parte en su paso por el TGI.

Tabla 6. Actividad antibacteriana frente a *C. sakazakii*, *L. monocytogenes* y *S. aureus* de las fórmulas lácteas (**F1**: lactosuero, LF y MFGM, **F2**: mazada, LF y MFGM, **F3**: lactosuero, LF y MFGM homogeneizada, **F4**: mazada homogeneizada, LF y MFGM homogeneizada, **F5**: lactosuero, LF y MFGM, con tratamiento térmico, **F6**: mazada, LF y MFGM, con tratamiento térmico), y sus respectivos digeridos, expresando el crecimiento en porcentaje con respecto al control. F: fórmula sin digerir, SD: digerido salivar, GD: digerido gástrico, ID: digerido intestinal. Se resaltan en azul las muestras que disminuyen el crecimiento bacteriano de manera significativa ($p \leq 0,05$).

MUESTRA		*C. sakazakii* ATCC 29544		*L. monocytogenes* ATCC 13932		*S. aureus* ATCC 25923	
		4 h	**24 h**	**4 h**	**24 h**	**4 h**	**24 h**
F1	**F**	105,27	102,.7	110,07	104,87	96,07	88,82
	SD	95,28	101,27	108,49	**74,43**	107,67	**77,59**
	GD	**75,50**	**75,13**	**87,02**	**39,47**	**77,16**	**80,00**
	ID	83,22	100,77	**88,61**	**90,25**	**59,86**	**25,40**
F2	**F**	102,17	109,10	104,96	92,56	106,65	**88,53**
	SD	**82,63**	101,35	112,11	**88,00**	105,97	**78,98**
	GD	**56,03**	**59,31**	**87,63**	**38,38**	**74,83**	**75,88**
	ID	**73,20**	**83,68**	**82,16**	**90,80**	**51,16**	**31,06**
F3	**F**	96,58	106,04	**83,19**	**73,26**	106,58	88,79
	SD	96,97	102,25	**72,70**	**23,43**	114,05	98,97
	GD	**77,82**	**69,73**	**82,13**	**36,68**	**70,21**	**76,04**
	ID	84,06	103,17	**88,52**	93,13	**51,71**	**27,53**
F4	**F**	93,97	104,77	95,81	**92,89**	114,68	83,41
	SD	**89,14**	98,35	96,04	**91,18**	122,13	94,07
	GD	**53,12**	**69,20**	**80,00**	**35,13**	**79,19**	**69,99**
	ID	**66,27**	95,38	**84,57**	**90,87**	**52,65**	**22,99**
F5	**F**	106,02	109,76	**93,48**	107,38	116,94	98,77
	SD	101,95	105,40	99,28	113,87	115,67	95,92
	GD	**45,35**	**67,88**	**93,85**	**42,88**	**87,30**	93,38
	ID	**74,34**	100,73	**90,45**	**92,42**	**62,77**	**24,72**
F6	**F**	96,33	110,23	99,01	102,84	111,88	99,30
	SD	**66,00**	98,42	**92,83**	**68,30**	90,03	92,83
	GD	**49,89**	**68,69**	105,54	**27,50**	108,32	**85,64**
	ID	**69,13**	96,03	**86,52**	95,96	**59,25**	**21,02**

Sin embargo, la mayor actividad presentada por los digeridos de las fórmulas podría deberse a la liberación de péptidos durante las etapas de digestión. Tras la hidrólisis, las proteínas pueden aumentar su efecto inhibitorio frente a las bacterias patógenas (McEvoy et al., 2016). Además de la lactoferricina (Bellamy et al., 1992) y la lactoferrampina (Van der Kraan et al., 2004) procedentes de la LF, otras proteínas de la leche, como las caseínas, la β-LG o la α-LA, también dan lugar a diversos péptidos bioactivos con actividad antibacteriana (Théolier et al., 2014). En el presente estudio, en los GD de las fórmulas sin haber sido sometidas a tratamientos se detectaron secuencias peptídicas derivadas de

las caseínas, como el péptido LRLKKKYKVPQL procedente de la α-s1-caseína y el AMKPWIQPKTKKVIPYVR derivado de la α-s2-caseína, ambos con actividad antibacteriana documentada (McCann et al., 2006).

En el estudio realizado por Aspri et al. (2018), la leche de burra fermentada, que presenta un alto nivel de LF y lisozima, mostró cierta actividad frente a *L. monocytogenes* antes de la digestión. Sin embargo, tras una digestión GIT *in vitro*, su poder antibacteriano frente a este patógeno aumentó gracias a los péptidos de las caseínas, la β-LG, la α-LA y la lisozima liberados. Este incremento del efecto antibacteriano potenciado por el proceso de digestión también favoreció la inhibición de *S. aureus* y *Bacillus cereus*. Resultados muy similares se obtuvieron en el estudio realizado por Tidona et al. (2011), en el que los digeridos gástricos de leche de burra, obtenidos a pHs de 2 y 4, presentaron mayor efecto frente a *L. monocytogenes* que la leche sin digerir.

Algunos estudios han afirmado que los péptidos derivados de la leche presentan efecto antibacteriano frente a *S. aureus*. Folliero et al. (2022) analizaron la actividad de los AMPs derivados de la caseína del kashk, un producto lácteo iraní similar al suero de la leche, contra *S. aureus* en la cicatrización de heridas. Concluyeron que la fracción peptídica obtenida era eficaz, reduciendo la tasa de colonización cutánea de *S. aureus* con un efecto dosis-dependiente.

Además, recientemente se ha evaluado el potencial de diferentes AMPs para inhibir patógenos en distintas matrices alimentarias. Un nuevo AMP (LCWAP) derivado de una proteína ácida del pez *Larimichthys crocea* presentó actividad antibacteriana contra *S. aureus*, con una MIC de 15,6 µg/mL, inferior a la de la proteína completa (184,5 µg/mL). Esto demostró que el LCWAP tenía un fuerte efecto inhibidor del crecimiento de *S. aureus*, y este efecto era dependiente de la dosis. Además, el LCWAP redujo la formación de *biofilms* formados por *S. aureus* de forma directamente proporcional a la concentración del péptido (Yang et al., 2020).

Otro AMP, denominado BCp12, aislado de la caseína de leche de búfala hidrolizada, también mostró actividad antibacteriana, dañando la pared de *S. aureus* al provocar la aparición de poros en ella (Shi et al., 2023), e inhibiendo la formación de *biofilms* (Li et al., 2022).

Por tanto, si bien es cierto que la LF, en general, pierde efecto tras la digestión (**Tabla 5**), las fórmulas lácteas aumentan su actividad antibacteriana tras las etapas digestivas (**Tabla 6**), probablemente debido a los AMPs generados a partir de las proteínas de la leche.

El mayor efecto que mostraron algunas fórmulas con base de mazada con respecto a las de base de lactosuero podría deberse al alto contenido en MFGM, ya que además de la membrana añadida como suplemento a las fórmulas, contenían la membrana naturalmente presente en la mazada. Esta MFGM contiene proteínas bioactivas con propiedades antimicrobianas (Parrón et al., 2018). Además, la MFGM contiene otros componentes, como los glicoesfingolípidos y los fosfolípidos, que actúan como moléculas de señalización intracelular en una amplia variedad de procesos biológicos (Fuller et al., 2013). Por todo ello, la MFGM aislada o fracciones enriquecidas en MFGM se han empleado en los últimos años como ingredientes en algunas fórmulas infantiles (De Almagro García et al., 2017).

En el estudio *in vivo* realizado por Sprong et al. (2012), en el que investigaron el efecto antiinfeccioso de la MFGM en ratas, se observó que las ratas alimentadas con mazada en polvo (rica en MFGM) aumentaron su resistencia a la infección por *L. monocytogenes*. Esto podría deberse, principalmente, a la presencia de productos antibacterianos derivados de la hidrólisis tras la digestión de los lípidos polares (fosfolípidos y esfingolípidos) y de proteínas de la MFGM (XO, MUC o LD) (Huërou-Luron, Lemaire, y Blat, 2019). Así, la XO genera, mediante una reacción enzimática, H_2O_2, causando la muerte bacteriana. Además, se ha demostrado que estas propiedades biodefensivas de la XO se conservan tras la proteólisis (Clare et al., 2008), lo que permitiría su acción tras la digestión GIT.

2. Efecto de los tratamientos tecnológicos

2.1. Homogeneización de la mazada

El tratamiento de homogeneización causa una disminución del tamaño de los glóbulos grasos de la leche, liberando y exponiendo ciertas proteínas bioactivas de la MFGM que presentan actividad antibacteriana, como la LD, la BTN o la MUC, mejorando su digestibilidad (Tunick et al., 2016) y permitiendo así la liberación de secuencias peptídicas bioactivas (Clare et al., 2008).

En la leche desnatada, la adición de LF provoca una disminución de la turbidez, debido a la unión de la LF a las micelas de caseína. Este efecto varía con el tiempo, el pH y la

temperatura (Anema y De Kruif, 2011). Sin embargo, la homogeneización modifica o altera estas interacciones y puede dejar libre la LF, ya que este tratamiento provoca la adsorción de las caseínas a la MFGM (Lee y Sherbon, 2002), impidiendo la unión LF-caseína. Este efecto podría justificar la mayor actividad antibacteriana de las fórmulas sin digerir homogeneizadas, en las que la LF puede ejercer su actividad, en comparación con las que no han llevado este tratamiento. Además, la liberación de la LF de las caseínas favorecería su digestión y aumentaría la liberación de péptidos bioactivos (Tunick et al., 2016).

Por otro lado, las proteínas liberadas de la MFGM homogeneizada, como la MUC o la LD, podrían ejercer su actividad antibacteriana (Liu y Newburg, 2013), incrementando así las fórmulas su efecto.

2.2. Pasteurización de las fórmulas lácteas

El tratamiento térmico es comúnmente empleado para la conservación de los alimentos y la prolongación de su vida útil (Bourlieu et al., 2015). Sin embargo, este tratamiento puede afectar al valor nutricional y las propiedades sensoriales del producto, alterando principalmente la estructura y función biológica de las proteínas (Michalski y Januel, 2006). De esta manera, dependiendo de la intensidad del tratamiento térmico aplicado, se puede ver afectada la integridad de la LF y las proteínas lácteas (Franco et al., 2018). Sin embargo, en el estudio realizado por Conesa et al. (2010) se demostró que la pasteurización, comúnmente empleada como tratamiento para conservar la leche, no afectaba a la actividad antibacteriana de la LF bovina frente a *E. coli* O157:H7, *S. enterica serovar Enteriditis* y *L. monocytogenes*. De hecho, en el estudio realizado por Wakabayashi, Yamauchi, y Takase (2008), el calentamiento de la LF aumentó su efecto antibacteriano, cuando se encontraba a altas concentraciones, sobre *C. sakazakii* tras 17 h de incubación. Estos resultados coincidieron con los obtenidos en la presente Tesis Doctoral, en los que el tratamiento térmico de las fórmulas lácteas favoreció el efecto antibacteriano de sus digeridos, sobre todo frente a *C. sakazakii*.

Los resultados obtenidos en nuestro estudio indican que, en general, el tratamiento térmico tiene efectos positivos sobre la actividad antibacteriana de las fórmulas lácteas, posiblemente porque favorece la liberación de péptidos activos. Sin embargo, las fórmulas sometidas a tratamiento térmico sufrieron una pérdida de actividad frente a *S. aureus*, especialmente los GD.

Los tratamientos térmicos, como el HTST o el UHT, pueden causar alteraciones en los componentes de la leche o fracciones lácteas, aumentando la agregación de grasas y proteínas debido a la ruptura de la MFGM (Tunick et al., 2016). En el caso de la fórmula con base de lactosuero tratada térmicamente (F5), la MFGM podría estar interaccionando con las proteínas del lactosuero (Lee y Sherbon, 2002), disminuyendo así su disponibilidad y, por tanto, su efecto antibacteriano. Además, esto podría explicar la pérdida de actividad en los GD de F5 y F6 frente a *S. aureus*, al estar las proteínas agregadas, impidiendo así su correcta digestión y liberación de péptidos bioactivos.

En el estudio de Halabi et al. (2020), la aplicación de diferentes tratamientos de pasteurización a las fórmulas infantiles elaboradas con lactosuero aumentó la susceptibilidad de algunas proteínas a la hidrólisis por pepsina. La resistencia de la α-LA y la β-LG a la hidrólisis no fue modificada por el calentamiento, mientras que la LF aumentó su susceptibilidad en comparación con la forma no calentada. Los resultados obtenidos en ese estudio también indicaron que la cinética de la hidrólisis de la LF en la digestión intestinal de las fórmulas infantiles tratadas térmicamente era superior a la de las no tratadas. La pérdida de actividad de la LF desnaturalizada e hidrolizada podría compensarse con la liberación de péptidos con elevada actividad antibacteriana.

Con nuestros resultados podríamos concluir que, el tratamiento térmico de las fórmulas lácteas fue el más eficiente para disminuir el crecimiento de *C. sakazakii*, mientras que la homogeneización fue el tratamiento más favorable para la actividad de las fórmulas frente a *L. monocytogenes*. En la actividad frente a *S. aureus* no se observaron grandes diferencias entre la composición o tratamiento de las fórmulas.

2.3. Encapsulación de la lactoferrina

Como hemos visto anteriormente, en el **artículo 3**, se han evaluado las diferentes estrategias de encapsulación que permiten proteger la LF y su bioactividad. Además, en el estudio detallado en el **artículo 5** se llevó a cabo la encapsulación de LF en Alg para comprobar la biodisponibilidad de la LF sometida, en un ensayo *in vitro*. a las condiciones similares a las que experimentaría a lo largo del TGI.

La encapsulación con Alg es muy interesante debido a su resistencia a ciertas enzimas y pHs (Dima et al., 2020), lo que permite una liberación controlada de su contenido en el intestino (Braim et al., 2019).

En nuestro estudio, tras someter a las microperlas de Alg/LF a un pH de 3, se liberó aproximadamente el 1% de la LF encapsulada. Sin embargo, al simular el pH del intestino (pH 7), se observó un aumento significativo de la LF liberada, entre el 40-50% de la proteína capturada por el Alg. Por lo tanto, se puede concluir que la encapsulación y liberación de LF por el Alg es dependiente del pH.

Además, se comprobó el efecto de las enzimas digestivas sobre las cápsulas preparadas. Tras la digestión gástrica con pepsina a pH 3, sólo se liberó el 3% de la LF contenida en las microperlas. En cambio, tras la digestión intestinal con pancreatina y bilis a pH 7, se observó un aumento significativo de la cantidad de LF liberada, alcanzando prácticamente el 100%. Así pues, comparando la digestión de las microperlas de Alg/LF con y sin enzimas, pudimos concluir que las enzimas intestinales favorecieron la liberación de LF al medio.

Puesto que las microperlas de Alg/LF soportaron las condiciones gástricas, pero no las intestinales, se realizó una digestión simulada *in vitro* para comprobar el efecto antibacteriano de la fracción liberada de las cápsulas en cada etapa. Si bien se esperaba que las microperlas no digeridas y su GD no mostraran actividad porque la LF se encontraba encapsulada, habría sido interesante obtener cierto efecto antibacteriano con el ID de las microperlas, en el que sí se liberó la LF. Sin embargo, la digestión de la LF liberada con las enzimas intestinales fue tan rápida que el efecto no se llegó a producir. En cualquier caso, se sabe que la LF se digiere completamente *in vivo* en la fase gástrica, lo que dificulta su llegada al intestino (Furlund et al., 2013), por lo que esta encapsulación con Alg sí permitiría facilitar la llegada de LF, o al menos de una parte, al intestino.

Análogamente, en el estudio *in vitro* de Raei et al. (2015) se demostró que la LF encapsulada en Alg atravesaba la fase gástrica en su forma intacta, al menos durante los 30 primeros minutos, y se liberaba en la parte superior del intestino. Sin embargo, no analizaron ni la estabilidad de la LF ni su actividad una vez liberada.

Recientemente, en 2023, Hedyeloo, Moradian, & Rostami (2023) analizaron el efecto antibacteriano de la LF libre y encapsulada en quitosano frente a *E. coli*. No encontraron diferencias significativas entre el efecto antibacteriano de la LF libre y encapsulada. Sin embargo, aunque sí evaluaron el efecto de la digestión en las cápsulas, no se analizó el efecto antibacteriano de los digeridos.

3. Efecto de la lactoferrina en modelos *in vitro* e *in vivo*

3.1. Efecto de la lactoferrina en células intestinales y hepáticas

A lo largo de esta Tesis Doctoral se han estudiado diversas propiedades biológicas que posee la LF. Tal y como se ha tratado en el apartado 1 de esta Discusión general, la LF presenta efecto antibacteriano frente a patógenos como *C. sakazakii*, *L. monocytogenes* o *S. aureus*. Además, permite la liberación de péptidos bioactivos tras su hidrólisis. Sin embargo, se le pueden atribuir más actividades, que se han evaluado *in vitro* en la línea celular de carcinoma de colon humano Caco-2/TC7 diferenciada en enterocitos. Los resultados presentados en el **artículo 1** indican que la LF no influyó en la viabilidad celular, pudiendo afirmar la ausencia de citotoxicidad por parte de la proteína en las células epiteliales Caco-2/TC7 diferenciadas, resultados que coinciden con los del estudio realizado por Atef Yekta et al. (2010). En el **artículo 2** también se corroboró la ausencia de citotoxicidad de la LF en las células Caco-2 y en la línea celular hepática HepG2, y se comprobó que tanto el lactosuero como la mazada no presentaban efecto citotóxico sobre dichas líneas celulares.

En el estudio realizado por Ashida et al. (2004), se demostró que la LF recombinante humana se internalizaba en las células Caco-2 desde el lado apical y posteriormente, se localizaba en el núcleo. Esta localización nuclear podría indicar otra función de la LF en las células, como la modulación de la función intestinal a través de la regulación génica, ya que se observó que la LF se unía a una secuencia específica de ADN (Ashida et al., 2004). Posteriormente, Hirotani et al. (2008) informaron de que la LF humana era capaz de reducir el daño celular epitelial y evitar la apertura de las uniones estrechas causada por el LPS bacteriano en las células Caco-2.

Además, en el presente estudio, la LF presentó un efecto inhibidor sobre la internalización de *C. sakazakii* en las células Caco-2. En relación con estos resultados, Valenti et al. (1999) demostraron que la LF tiene la capacidad de reducir significativamente la invasión de las células Caco-2 por *L. monocytogenes*. Además, se ha demostrado que tanto la LF humana como la bovina inhiben la adherencia de *E. coli* a las células Caco-2 y que la inhibición depende de la dosis (Atef Yekta et al., 2010). Asimismo, Longhi et al. (1993) demostraron que tanto la apo-LF como la holo-LF humana tienen una capacidad significativa de inhibir la adhesión e internalización de esta bacteria a las células HeLa, de carcinoma uterino humano. La LF también ha mostrado un efecto inhibidor en la internalización a las células de ciertos virus, como el virus del papiloma humano. Esta

actividad parece ser más potente en el caso de la LF bovina que humana (Drobni, Näslund, y Evander, 2004).

En nuestro estudio, el tratamiento de las células Caco-2 con LF permitió disminuir el estrés causado por *C. sakazakii*. El LPS presente en la membrana externa de esta bacteria ha demostrado aumentar la oxidación de proteínas y lípidos en las células (Latorre et al., 2014). Estos resultados coincidieron con los obtenidos en el estudio realizado por Buey et al. (2021), en el que la LF mostró un efecto positivo en la reducción del estrés oxidativo causado a las células Caco-2/TC7 por el LPS.

Además, tanto la LF como el lactosuero y la mazada demostraron revertir el efecto oxidante generado por la menadiona en las células Caco-2 y HepG2. En este caso, se sabe que algunos componentes del lactosuero, como la α-LA, tienen actividad antioxidante, favoreciendo la reducción del estrés oxidativo debido a sus propiedades quelantes del hierro. Además, el lactosuero contiene aminoácidos ricos en azufre, que también pueden mejorar la defensa antioxidante (Kerasioti et al., 2014). En cuanto a la mazada, contiene proteínas que generan numerosos péptidos con potencial antioxidante (Conway, Gauthier, y Pouliot, 2013).

En el estudio realizado por Buey et al. (2021), tanto la LF como el lactosuero mostraron efecto antioxidante, reduciendo la oxidación lipídica y el daño a las proteínas causado por el LPS. Sin embargo, la mazada no redujo el estrés oxidativo causado por el LPS, a diferencia de lo observado en nuestro estudio, en el que este subproducto lácteo sí protegió a las células Caco-2 y HepG2 frente al efecto oxidante de la menadiona.

Las células humanas están continuamente expuestas a factores fisiológicos y externos que pueden causar daños citotóxicos, oxidativos y genotóxicos. Cuando se produce un desequilibrio en la dinámica entre la generación de ROS y los sistemas antioxidantes, surge el daño oxidativo que puede dirigirse a diversos objetivos celulares, incluido el ADN (Markkanen, 2017).

En nuestro estudio, tanto la LF como el lactosuero y la mazada disminuyeron significativamente el daño ocasionado en el ADN por acción de la menadiona. En el estudio realizado por Tian (2013), se evidenciaron las propiedades antigenotóxicas de la LF en células HT29, un modelo de células intestinales humanas, tras la alteración y el daño producido en el ADN por genotoxinas fecales. Habib et al. (2013) también estudiaron las actividades antioxidante e inhibidora del daño en el ADN de la LF

proveniente de leche de camella. Por su parte, Ogasawara et al. (2014) demostraron que la LF tanto nativa como saturada en hierro protegía claramente al ADN de la fragmentación causada por la radiación UV en presencia de ROS. Además, en un estudio realizado por Chiang et al., (2017), el suero hidrolizado de calostro bovino también presentó actividades inhibidoras del daño oxidativo y un efecto inhibidor sobre la descomposición del ADN superenrollado. Efectos similares de protección del ADN se observaron en diversos estudios recogidos en la revisión realizada por Saikali et al. (2004). En ella se resalta la actividad antigenotóxica y antimutagénica *in vitro* de la leche fermentada y la capacidad *in vivo* para reducir la incidencia de cáncer de colon inducido por carcinógenos y disminuir los daños en el ADN.

Algunos estudios muestran que tanto el estrés oxidativo como la liberación de ROS están relacionados con la activación de las vías TLR. En el estudio de Yoshino y Kashiwakura (2017), las ROS inducidas por la radiación promovieron la expresión de TLR2 y TLR4, mientras que Latorre et al. (2014) demostraron que la activación de estos receptores mejoraba el estado oxidativo de las células epiteliales intestinales Caco-2.

Las proteínas de la leche, y en particular la LF, están implicadas en estos procesos de inhibición y promoción de respuestas inmunitarias celulares (Legrand et al., 2005). En el presente estudio, la LF modificó la expresión de los TLR y, en particular, disminuyó la sobreexpresión de TLR2 causada por *C. sakazakii*.

3.2. Efecto de la lactoferrina en un modelo murino de disbiosis

Para completar los estudios realizados *in vitro* con la LF, se realizaron algunos ensayos *in vivo*, que permitieron evaluar el efecto de la LF, tanto nativa (nLF) como saturada con hierro (sLF), sobre el estrés oxidativo y la expresión de mediadores inflamatorios en el íleon de ratones con disbiosis inducida por clindamicina.

A partir del mismo experimento en el que se observaron los efectos de la LF sobre el íleon, expuestos en el **artículo 7**, también se analizó el efecto de la LF sobre la microbiota intestinal y la expresión de TLR en el colon de estos ratones (Bellés et al., 2022). En este estudio previo, se concluyó que la clindamicina provocaba modificaciones en la composición de la microbiota intestinal, reduciendo las bacterias con propiedades antiinflamatorias e induciendo alteraciones en la expresión de los receptores TLR, como TLR2, TLR8 y TLR9 (Bellés et al., 2022).

Según el estudio de Cutone et al. (2020), la LF es capaz de controlar los niveles de ROS mediante la captación de hierro libre. Sin embargo, en nuestro estudio la sLF administrada oralmente mostró un mayor efecto antioxidante que la nLF, disminuyendo el nivel de carbonilos desajustado por la clindamicina hasta prácticamente niveles basales; esto podría deberse a la mayor estabilidad en el TGI de la sLF (Fan et al., 2022).

Por otro lado, en los resultados obtenidos en el presente estudio, se observó que el pretratamiento con LF de los ratones a los que se les indujo una disbiosis por el tratamiento con clindamicina, permitió mantener los niveles basales de IL-6 y TNF-α en el íleon, reduciendo significativamente los efectos de la clindamicina sobre la expresión de estos mediadores inflamatorios.

En un estudio realizado en ratones, el tratamiento preventivo con LF bovina permitió reestablecer los niveles de IL-6 y TNF-α alterados por el LPS. La LF indujo por sí misma un nivel relativamente elevado de IL-6; sin embargo, el uso de la LF como un tratamiento previo a la inyección del LPS provocó una disminución significativa de los niveles de esta interleuquina (Machnicki, Zimecki, y Zagulski, 1993). De manera similar, en el estudio de Fan et al. (2022), se analizó el efecto protector de la LF con diferentes saturaciones de hierro en un modelo murino de inflamación intestinal inducida por LPS. La administración oral de apo- y holo-LF disminuyó notablemente los niveles de IL-6 y TNF-α aumentados por el LPS en el colon de los ratones. Estos resultados, aunque en distinta sección del intestino, son muy similares a los obtenidos en el presente estudio.

Otros estudios anteriores realizados en modelos *in vitro* e *in vivo* de inflamación inducida por LPS, también mostraron una modulación de la respuesta inmunitaria por parte de la LF, regulando a la baja las citoquinas proinflamatorias, como la IL-6 y el TNF-α, y potenciando la secreción de citoquinas antiinflamatorias, como la IL-10 y la IL-4 (Legrand et al., 2005). Además, este poder modulador también se le ha atribuido a los péptidos de la LF, pues la administración oral de lactoferricina suprimió la respuesta inflamatoria en un modelo murino de disfunción intestinal causada por *E. coli*, reduciendo la expresión y liberación de IL-6 y TNF-α a niveles basales (Haiwen et al., 2019).

Por ello, los resultados de este estudio, respaldados por otros anteriores, nos permiten afirmar que la leche es una fuente de proteínas bioactivas, entre ellas la LF, proteína multifuncional con propiedades muy interesantes como potencial ingrediente para alimentos funcionales y en especial, para la protección del recién nacido. Además, los

subproductos lácteos como el lactosuero y la mazada presentan propiedades beneficiosas para la salud, lo que permitiría aumentar su valor comercial como ingredientes multifuncionales y reconocer su potencial como fuente natural de compuestos bioactivos.

De manera adicional, se ha observado que los tratamientos tecnológicos como la homogeneización y la pasteurización no solo no perjudican las propiedades de las fórmulas lácteas, sino que potencian en algunos casos su actividad antibacteriana.

Así, podemos afirmar que, dado el gran número de propiedades beneficiosas para la salud que presentan la LF y los subproductos lácteos, podrían ser ingredientes interesantes para alimentos funcionales, siendo muy útiles como agentes antibacterianos, antioxidantes, antigenotóxicos y "protectores" ante el efecto de los antibióticos.

En cualquier caso, hay que seguir investigando en el efecto de la leche y sus componentes para mejorar los conocimientos sobre la influencia de las proteínas lácteas, y de la LF en particular, en el sistema inmunitario y, especialmente, cuando se encuentran en un entorno complejo como el TGI humano.

Además, sería necesario realizar una mayor caracterización de los compuestos activos contenidos en las fracciones lácteas, así como someter a las fórmulas lácteas empleadas a una digestión en diferentes condiciones, teniendo en cuenta las variaciones que el proceso de digestión puede tener en el sistema digestivo del lactante.

En nuestro estudio, la suplementación de LF en matrices lácteas ha mostrado ser más eficaz que la encapsulación en alginato para mantener la actividad de la LF. Sin embargo, sería interesante continuar con estos estudios para encontrar diferentes formas de preservar la actividad de la LF y enfocar el efecto antibacteriano de la LF en la sección intestinal del TGI, en el que las bacterias y la microbiota suponen un papel tan importante no solo a este nivel sino también para la salud integral del organismo.

CONCLUSIONES/ CONCLUSIONS

CONCLUSIONES

Los resultados obtenidos en la presente Tesis Doctoral nos han permitido obtener las siguientes conclusiones:

Primera: La lactoferrina bovina presenta actividad antibacteriana frente a *Cronobacter sakazakii* y *Listeria monocytogenes* tanto en fase exponencial como estacionaria, y frente a *Staphylococcus aureus* en fase estacionaria; siendo especialmente activa en la inhibición del crecimiento de *Listeria monocytogenes*.

Segunda: La lactoferrina inhibe la internalización de *Cronobacter sakazakii* en las células epiteliales intestinales Caco-2/TC7 y revierte el estrés lipídico y la alteración en la expresión de los receptores tipo Toll causados por la bacteria.

Tercera: La lactoferrina y los subproductos lácteos lactosuero y mazada ejercen un poder antioxidante y de protección del ADN en las células intestinales Caco-2 y hepáticas HepG2.

Cuarta: Los digeridos de lactoferrina generados tras la digestión gastrointestinal *in vitro* muestran efecto antibacteriano frente a *Cronobacter sakazakii*, *Listeria monocytogenes* y *Staphylococcus aureus*, siendo *Listeria monocytogenes* la bacteria más sensible.

Quinta: Los digeridos de las fórmulas lácteas elaboradas con base de lactosuero o mazada y suplementadas con membrana del glóbulo graso y lactoferrina presentan actividad antibacteriana frente a *Cronobacter sakazakii*, *Listeria monocytogenes* y *Staphylococcus aureus*, siendo los digeridos gástricos los más efectivos.

Sexta: Los tratamientos tecnológicos, como la homogeneización y la pasteurización, mantienen o incluso mejoran el efecto antibacteriano de las fórmulas lácteas y sus digeridos.

Séptima: La lactoferrina resiste mejor la digestión gastrointestinal *in vitro* cuando se encuentra como suplemento en las fórmulas lácteas con base de lactosuero o mazada.

Octava: La encapsulación de la lactoferrina con alginato permite la liberación controlada de la proteína en el intestino, aunque no asegura su actividad antibacteriana en dicha localización.

Novena: La lactoferrina, tanto nativa como saturada con hierro, restablece los niveles normales de los polimorfonucleares en el íleon, revierte la oxidación proteica y disminuye la expresión de citoquinas proinflamatorias en un modelo murino de disbiosis intestinal causada por la clindamicina.

CONCLUSIONS

The results obtained in this Doctoral Thesis have allowed us to obtain the following conclusions:

First: Bovine lactoferrin shows antibacterial activity against *Cronobacter sakazakii* and *Listeria monocytogenes* in exponential and stationary phase, and against *Staphylococcus aureus* in stationary phase; being especially active in inhibiting the growth of *Listeria monocytogenes*.

Second: Lactoferrin inhibits the internalization of *Cronobacter sakazakii* in Caco-2/TC7 intestinal epithelial cells and reverses the lipid stress and altered Toll-like receptor expression caused by the bacterium.

Third: Lactoferrin and the dairy by-products whey and buttermilk exert antioxidant and DNA-protective power on intestinal Caco-2 and hepatic HepG2 cells.

Fourth: Lactoferrin digests generated after *in vitro* gastrointestinal digestion show antibacterial effect against *Cronobacter sakazakii*, *Listeria monocytogenes* and *Staphylococcus aureus*, *Listeria monocytogenes* being the most sensitive bacterium.

Fifth: Digests of dairy formulas elaborated with whey or buttermilk and supplemented with fat globule membrane and lactoferrin present antibacterial activity against *Cronobacter sakazakii*, *Listeria monocytogenes* and *Staphylococcus aureus*, being the gastric digests the most effective.

Sixth: Technological tratments, such as homogenization and pasteurization, maintain or even improve the antibacterial effect of dairy formulas and their digests.

Seventh: Lactoferrin resists better *in vitro* gastrointestinal digestion when it is found as a supplement in whey or buttermilk-based formulas.

Eighth: Encapsulation of lactoferrin with alginate allows controlled release of the protein in the intestine, although it does not ensure its antibacterial activity in that location.

Ninth: Lactoferrin, both native and iron-saturated, restores normal levels of polymorphonuclears in the ileum, reverses protein oxidation and decreases the expression of pro-inflammatory cytokines in a murine model of intestinal dysbiosis caused by clindamycin.

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