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Entitled :

Extraction process, Characterization and biological activity evaluation (*in vitro* and *in vivo* study) of lactoferrin from bovine colostrum co-product

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AFNANE

SUMMARY

Bibliographic synthesis

Chapter II: Properties and applications of lactoferrin

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Abbreviations List

ACE2 : Angiotensin-converting enzyme 2

AKI : Acute kidney injury

ATPase : Proton pumping

bLF : Bovine lactoferrin

CFDA : Chinese Food and Drug Administration

cGMP : Degradation factor

cmDNA : Circulating mucoralssdeoxyrebo nucleic acid

ColI-1 : Raised collagen -1

CRP2 : Protein from allbomin family

CXCR4 : Motif cytokine receptor 4

DMN : Dimethylnitrosamine

DMT1 : Divalent metal ion receptor

DNA : Deoxyrebonucleicacid

ERK2 : Extracellular signal-regulator kinase 2

GSH : Gluthion

H2 : Hydrogene

HBE : Humain bronchical epithelial cell line

hCoV-NL63 : Human coronaviruses

HIV : Human immunodeficiency virus

HR : Heart rate

HS : Heparan sulphate receptors

HSPGs : Host cell surface

IGF-1 : Insulin- like growth factor-1

IgM : Immunoglobulin M

IV :	Intra vineuse
LBP :	LPS-binding protein
LD50 :	50% lethal dose
LER :	Negatively regulates telomerase activity
LF :	Lactoferrin
LPR :	Liver peyer receptor
LPS :	Deoxyrebonucleic acid
LRP1, 2 :	Lipoprotein related protein
MAP :	Mean arterial blood pressure
mRNA :	Messenger ribonucleicacid
NK :	Natural killer
NOAEL :	No observable adverse effect level
PDC :	Potassium dichromate
PI3K :	Phosphoinositide 3-kinase
PLGA NPs:	Polylactic-coglycolic acid nanoparticules
RES :	Reticuloendothelial system
RhLF :	Humain receptor lactoferrin
RTPCR :	Reverse transcriptase polymerase chain reaction
SARS-CoV :	Pseudotyped
SI6LFR :	Receptor of lactoferrin on enterocytes
SI-LFR :	Specific receptors
SOD :	Superoxide dismutase
TFR1, 2 :	Transferrin binding receptor
Th2 :	T helper2
TLR4 :	Tool-like receptor

TNF : Tumor necrosis factor

Abstract: Extraction process, characterization and biological activity evaluation (*in vitro* and *in vivo* studies) of lactoferrin from bovine colostrum coproduct.

Lactoferrin (LF) is a transferrin family glycoprotein with many biological and functional properties. It has a large range of uses especially in pharmaceutic and cosmetic industry.

The purpose of this research is to develop a process prototype of LF separation from bovine colostrum, and to evaluate its productivity in terms of yield and purity using new technological methods. Correspondingly, this research aimed also to evaluate the colostrum and its derivatives (LF and casein) activity as an antiulcer (*in vivo*) and as an antibacterial (*in vitro*).

Initially, LF is extracted from whole colostrum by sulfate ammonium adhesion followed by adsorption on a solid support, then elution by an acid solution. The product extracted is purified, dia-filtered and then lyophilized.

The characterization analysis of the obtained LF was carried out by electrophoresis (SDS-Page) and spectrophotometric (FTIR) methods, following to the quantification of proteins amount using Bradford's assay.

LF extracted has been tested *in vitro* in terms of its anti-bacterial activity using several clinical strains by disk method.

During work, we have extract casein and conserve colostrum to investigate their biological activity in parallel with the extracted LF. In this context, a complementary *in vivo* study intended to evaluate their anti-ulcer activity in adult female rats, following an ulcer induction by oral dose of ethanol.

Our result shows the quantitative and qualitative efficiency of our developed prototype of LF extraction process. The *in vitro* study results show no anti-bacterial effect of LF at the selected dose.

On the other hand, results show that the extracted LF and casein as well as the whole colostrum have a great gastro-protective activity, proven by their ability to inhibit the ulcer, to normalize the acidity of gastric juice, and to increase the percentage of healing. All the three pre-treatments demonstrate an improvement in hematological parameters Hb, HCT. etc.

Lastly, Macro and microscopic observations prove the anti-ulcer effect of LF, casein and especially colostrum by protecting the mucus gastric against ethanol-induced ulcer.

Other complementary studies are necessary to evaluate the hepatic and nephro-protection of LF by measuring oxidative stress parameters. etc.

Key words: Lactoferrin; Colostrum; Extraction process; Electrophoresis; Ulcer.

Résumé : Procédé d'extraction, caractérisation et évaluation de l'activité biologique (études *in vitro* et *in vivo*) de la lactoferrine à partir du coproduit du colostrum bovin.

La lactoferrine (LF) est une glycoprotéine de la famille des transferrines aux nombreuses propriétés biologiques et fonctionnelles. Il a une large gamme d'utilisations, en particulier dans l'industrie pharmaceutique et cosmétique.

L'objectif de cette recherche est de développer un prototype de procédé de séparation LF du colostrum bovin, et d'évaluer sa productivité en termes de rendement et de pureté à l'aide de nouvelles méthodes technologiques. Parallèlement, cette recherche visait également à évaluer l'activité du colostrum et de ses dérivés (LF et caséine) comme antiulcéreux (*in vivo*) et comme antibactérien (*in vitro*).

Dans un premier temps, la LF est extraite du colostrum entier par adhésion au sulfate d'ammonium suivie d'une adsorption sur un support solide, puis d'une élution par une solution acide. Le produit extrait est purifié, diafiltré puis lyophilisé.

L'analyse de caractérisation de la LF obtenue a été réalisée par des méthodes d'électrophorèse (SDS-Page) et de spectrophotométrie (FTIR), suite à la quantification de la quantité de protéines.La FL extraite a été testée *in vitro* en termes de sensibilité antibactérienne en utilisant plusieurs souches cliniques par la méthode des disques.

Au cours des travaux, nous avons extrait la caséine et conservé le colostrum pour étudier leur activité biologique en parallèle avec la LF extraite. Dans ce contexte, une étude complémentaire *in vivo* visait à évaluer leur activité anti-ulcéreuse chez des rats femelles adultes, suite à une induction d'ulcère par une dose orale d'éthanol.

Notre résultat montre l'efficacité quantitative et qualitative de notre prototype développé du procédé d'extraction de LF. Les résultats de l'étude *in vitro* ne montrent aucun effet antibactérien de la LF à la dose choisie.

D'autre part, les résultats montrent que la LF et la caséine extraites ainsi que le colostrum entier ont une grande activité gastro-protectrice, prouvée par leur capacité à inhiber l'ulcère, à normaliser l'acidité du jus gastrique et à augmenter le pourcentage de guérison. Les trois prétraitements démontrent une amélioration des paramètres hématologiques Hb, HCT. etc.

Enfin, des observations macro et microscopiques prouvent l'effet anti-ulcéreux de la LF, de la caséine et surtout du colostrum en protégeant le mucus gastrique contre l'ulcère induit par l'éthanol.

D'autres études complémentaires sont nécessaires pour évaluer la protection hépatique et néphro-protectrice de la LF par la mesure des paramètres de stress oxydatif. etc.

Mots clés : Lactoferrine ; Colostrum; Processus d'extraction ; Electrophorèse ; Ulcère.

الخلاصة: عملية استخلاص وتوصيف وتقييم النشاط البيولوجي (دراسات في المختبر وفي الجسم الحي) لاكتوفيرين من منتج اللبأ البقري المشترك.

اللاكتوفيرين (LF) هو بروتين سكري لعائلة الترانسفيرين وله العديد من الخصائص البيولوجية والوظيفية. لها مجموعة كبيرة من الاستخدامات خاصة في صناعة الأدوية ومستحضرات التجميل.

الغرض من هذا البحث هو تطوير نموذج أولي لعملية فصل LF من اللبأ البقري، وتقييم إنتاجيته من حيث الإنتاجية والنقاء باستخدام الأساليب التكنولوجية الجديدة. في المقابل، يهدف هذا البحث أيضًا إلى تقييم نشاط اللبأ ومشتقاته (LF والكازين (كمضاد للقرحة (في الجسم الحي) وكمضاد للبكتيريا (في المختبر).

في البداية، يتم استخلاص LF من اللبأ الكامل عن طريق التصاق كبريتات الأمونيوم متبوعًا بالامتزاز على دعامة صلبة، ثم الشطف بمحلول حمضي. يتم تنقية المنتج المستخلص، وتصفيته بالقطر، ثم تجفيفه بالتجميد.

تم إجراء تحليل توصيفي LF الذي تم الحصول عليه عن طريق électrophorèse (SDS-Page) وطرق القياس الطيفي (FTIR) ، بعد القياس الكمي للبروتينات.

تم اختبار LF المستخلص في المختبر من حيث حساسيته المضادة للبكتيريا باستخدام العديد من السلالات السريرية بطريقة القرص.

أثناء العمل، تحصلنا على مستخلصات الكازين كما تم حفظ اللبأ للتحقق من نشاطهما البيولوجي بالتوازي مع LF المستخرج. في هذا السياق، تهدف دراسة تكميلية في الجسم الحي إلى تقييم نشاطهم المضاد للقرحة في إناث الجرذان البالغة، بعد تحريض القرحة بجرعة فموية من الإيثانول.

تظهر النتائج الكفاءة الكمية والنوعية لنموذجنا الأولي المطور لعملية استخراج LF. تظهر نتائج الدراسة في المختبر عدم وجود تأثير مضاد للبكتيريا من LF في الجرعة المحددة.

من ناحية أخرى، أظهرت النتائج أنLF و الكازين المستخرجين بالإضافة إلى اللبأ الكامل لهم فعالية كبيرة في حماية الجهاز الهضمي ، والتي أثبتت قدرتها على تثبيط القرحة ، وتعديل حموضة العصارة المعدية ، وزيادة نسبة معالجة المعدة.. تظهر جميع المعالجات الثلاثة المسبقة تحسنًا في معايير الدم Hb و HCT إلخ.

أخيرًا، تثبت الملاحظات العينية والميكروسكوبية التأثير المضاد للقرحة لـ LF والكازين وخاصة اللبأ من خلال حماية الجدار المعدي من القرحة التي سببها الإيثانول.

تعتبر الدراسات التكميلية الأخرى ضرورية لتقييم الحماية الكبدية والكلوية للـ LF عن طريق قياس معاملات الإجهاد التأكسدي. إلخ.

الكلمات الأساسية: لاكتوفيرين؛ اللبأ. عملية الاستخراج، électrophorèse، القرحة.

Bibliographic synthesis

General introduction

Colostrum of bovine origin has been known in popular medicine for many years and has been used in the treatment of infectious diseases in humans and domestic animals. In general, colostrum is a mixture of milk secretions and constituents blood serum that accumulates in the udder before parturition and can be harvested just before or after parturition (Foley 1978).

After calving, cows generally produce more colostrum than needed to meet the requirements of the calf. Exploitation of the excess colostrum was hampered because of its low stability at room temperature and the need for efficient refrigeration facilities. Initial efforts by veterinarians were stimulated by advances in immunological research (Stokes and Bourne 1989) and attention was focussed on the specificity of the main protein classes present in bovine colostrum.

Among these bioactive compounds, lactoferrin (LF) and immunoglobulin G are two important bioactive compounds in research interest which contribute to the preservation of milk itself as they possess various microbial infections and cancer-fighting properties (Legrand et. al, 2008).

The name LF is derived from its past classification as a major iron-binding protein in milk. LF, also referred to as lactotransferrin, subsequently it has also been shown to be a major iron-binding protein of other exocrine secretions such as bile, pancreatic juice and small intestinal secretions, and has been localized in a host of other tissues, both in man and in other mammals (Johanson et al. 1960).

That its functions and properties have been gradually characterized. It plays important roles in the regulation of iron absorption, and the modulation of immune responses, and has anti-microbial, anti-viral, antioxidant, anti-cancer, and anti-inflammatory activities (Brock 2012;Rastogi et al. 2016).

The first objective of this paper is the development of LF separation process from bovine colostrum in its pure and no toxic state. The second objective is to investigate the biological activity of LF, casein and the whole colostrum.

This Master's thesis consists of a general introduction, bibliographic synthesis, experimental studies, general conclusion and a list of bibliographical references.

In this paper, the bibliographic synthesis part includes 3 chapters: Characteristics of

LF (structure, physiochemical properties, biosynthesis, pharmacokinetics...), Properties and applications of LF (nutritional, functional, biological properties..), LF and gastric ulcer (relationship between alcohol and gastric ulcer, gastro-protective effect of LF …).

The experimental part also includes 3 chapters: Material and method, result, discussion.

In this context, three investigations were conducted in parallel:

The first part aimed to extract LF from whole colostrum at laboratory scale, which focused on:

- ✓ Development of a prototype process for the separation and purification of LF.
- ✓ Extraction of LF by adhesion to ammonium sulphate followed by adsorption on a solid support then elution by an acid solution.
- ✓ Purification of LF by diafiltration and ultrafiltration.
- ✓ Study of the capacity of this process to separate LF and casein from colostrum (yield / purity).
- ✓ Characteristic analysis of LF obtained via SDS-Page and FTIR technics.

The second part is an experimental in vitro aimed to evaluate the antibacterial activity of the extracted LF by testing several bacterial strains.

The third part is an experimental in vivo study focuses on evaluating the protective effect of LF, casein and the whole colostrum against ethanol-induced ulcer. We were interested in

Doing:

- ✓ Measuring the pH and total acidity in gastric juice.
- ✓ Evaluating the hematological parameters.
- ✓ Macro and microscopic observation of the stomach's sections.

We end this work with a general conclusion which summarizes the main results of this work.

I. Characteristics of lactoferrin

1. Structure

Human LF's molecular structure and amino acid sequence were found in 1984. LF was then identified as a transferrin family member since it shares 60% of its sequence with serum transferrin (Metz-Boutigue et al. 1984).

LF is a glycoprotein with a molecular weight of around 80 kDa and a strong affinity for iron. LF has been shown to contain three distinct isoforms. LF-α has no ribonuclease activity, but it is the iron binding form. LF-β and LF-γ, on the other hand, have ribonuclease activity but can't bind iron (Furmanski et al. 1989).

LF is made up of a single polypeptide chain that is folded into two globular lobes and has 703 amino acids. A -helix connects these lobes, also known as the C (carboxy) and N (amino) terminal regions. Each lobe is divided into two domains: C_1, C_2, N_1, and N_2. On each lobe, the domains generate one iron binding site (Fig 01).

LF molecules have different amounts of possible glycosylation sites, primarily on the surface, depending on the species and protein. Mannose is the most prevalent saccharide; hexoses make up around 3%, while hexosamines make up 1%. The rate of resistance to proteases or very low pH is determined by the degree of glycosylation.

LF's capacity to bind iron is two times greater than transferrin, which can act as a donor of Fe^{3+} ions for LF in some situations. One LF molecule can bind two ferric ions. LF always binds one carbonate ion at the same time as each ferric ion. Despite the fact that this connection is extremely strong and can withstand pH values as low as 4, its saturation does not surpass 10% in total.

LF is classified as apo-LF (iron-free), mono-ferric (one ferric ion), or holo-LF (binds two Fe^{3+} ions) depending on its iron saturation. Holo-LF and apo-LF have distinct tertiary structures (Jameson et al. 1998).

Histidine, twice tyrosine, and aspartic acid are the most significant amino acid residues for iron binding, whereas an arginine chain is responsible for carbonate ion binding .

Apart from iron, LF may bind a wide range of other chemicals and substances, including lipopolysaccharides, heparin, glycosaminoglycans, DNA, and metal ions such as $A^{l3+,}$ Ga^{3+},

Mn^{3+}, Co^{3+}, Cu^{+2}, Zn^{+2}, and others. Its affinity for these other ions, on the other hand, is substantially lower.

LF may bind a range of different anions, including oxalates, carboxylates, and others, in addition to CO_3^{2-}. LF can influence the metabolism and distribution of numerous chemicals in this way (Baker 1994) .

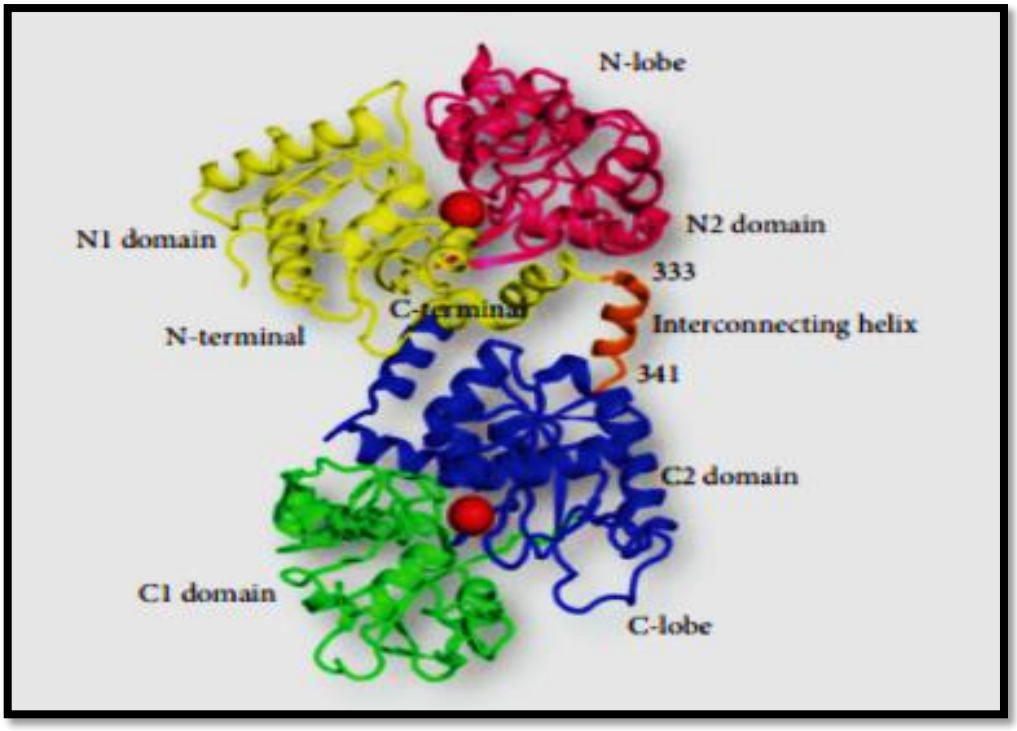

Figure01: Schematic diagram of the bovine lactoferrin molecule (PDB code: 1BLF) (Sharma et al. 2013)**.**

The N1 and N2 domains are colored in yellow and pink, respectively, while the C1 and C2 domains are colored in green and blue, respectively. The interconnecting helix between the lobes is colored in orange. The two iron atoms are shown as red spheres.

2. Physicochemical characteristics

Cationic iron-binding glycoprotein that was initially found in human milk and has a molecular weight of around 80 kDa. The physicochemical characteristics of three types of bovine LF, apo-, native-, and holo-, with 0.9 percent, 12.9 percent, and 99.7% iron content, respectively, were studied. The shape of LF was shown to alter the color, surface tension, thermal characteristics, particle charge, and rheological action of LF. More detail and essential physicochemical characteristics of LF as shown in (Table 01).

Table 01: Physicochemical characteristics of lactoferrin (Ye and Singh 2006; Bokkhim et al. 2013; PubChem.).

PHysics characteristic	**Synonyme**	LF 151186-19-5 LF (Bovine) J-008279 Lactotransferrin	**Other identification**	CAS : 339615-76-8 EC : 604-775-1
	Molecular formula	$C_{141}H_{226}N_{46}O_{29}S_3$	**Molecular weight**	80 kDa
	Size	Macromolecular 2lobs : C, N 4 sub-lobs :C_1 C_2,N_1 N_2	**Forms** (depending structure, dimention and Saturation iron)	**B** sheets 24% **α** helice 41% Apo-LF 0.9% Mono-LF 12.9% Holo-LF 99.9%
	Color	Salmon-pink (In natural state with saturation 15-20%)	**Concentration**	about 0.2 to 1 mg/mL from Bovine Colostrum Varied depending species
Chemics characteristic	**Solubility**	Distilled water (10mg/mL)	**Apparence viscosity**	Apo and Mono-LF : 1.4 mPas/S Holo-LF: 2.38mPas/S
	Surface tension (Depending forms)	Apo-LF: 53.1 Native-LF:56.5 Holo-LF:59.3	**Melting and Boiling points**	Not applicable
	Stability	Associated to degree of glycolisation	**Denaturation** (DSC analysis)	Apo-LF : 1 peak (71°C) Holo-LF : 2 peaks (91°C)
	Ability	Iron binding very high (Kd~10^{-20})	**pH**	Apo-LF: 5.7 Mono-LF: 5.4 Holo-LF: 6.2
	Isoelectric point	8-9	**Ionization**	Cation Strongly Binds polyanion

3. Biosynthesis

Bovine LF's mRNA code for a 708-amino-acid protein with a 19-amino-acid signal peptide immediately before a sequence that is similar to the human LF sequence. For bovine LF, the N-terminal has been reported (Goodman and Schanbacher 1991).

Transfer of LF to its storage granules acidification pathways appear to be important. The medial and trans cisternae are involved a component of the Golgi apparatus (Olsson et al. 1988). LF production regulation appears to be tissue specific and hormone dependent (Levay and Viljoen 1995).

LF is a protein found in milk that is produced by the epithelial cells of the mammary gland. The epithelial cells of the lacrimal gland, salivary gland, biliary tract. And pancreas synthesizes LF, which is found in tears, saliva, bile, and pancreatic fluid (Saito and Nakanuma 1992;Vorland 1999).

LF in plasma is mostly produced by neutrophils. LF is produced by neutrophils during the transition from promyelocyte to myelocyte and is retained in secondary granules (Iyer and Lönnerdal 1993). It is secreted when neutrophils are stimulated (Fig 2).

LF can be phagocytized by macrophages, monocytes, and other reticuloendothelial system cells by receptor-mediated endocytosis (Ismail and Brock 1993). Which involves Kupffer cells, liver endothelial cells, and hepatocytes, is an alternate method of LF elimination (Hu et al. 1993).

LF has been demonstrated to be exceptionally resistant to trypsin and chymotrypsin proteolytic destruction in *vitro*, indicating that it is at least partially resistant to digestion in the gut (Iyer and Lönnerdal 1993). Regardless of whether the infants are breastfed or formula-fed, complete LF and big pieces may be identified in substantial quantities in the feces of newborns in studies (Vorland 1999).

LF was discovered at quantities of 1 and 7 mg/mL in human natural milk and colostrum, respectively, and at 0.1 mg/mL (Mathur et al. 1990), 0.5 mg/mL in pooled pulmonary secretions, and more than 14 mg/mL in infected parotid fluid in bovine milk mid-lactation (Tabak et al. 1978).

LF is also released by polymorphonuclear leukocytes in response to cytokine stimulation. Gram-negative bacterial infection and stimulation LF levels in plasma are increasing. It can reach 0.2 mg/mL with acute sepsis. Local concentrations in inflammatory areas are most probable. Will be in the milligram rangein milliliters (LaForce and Boose 1987).

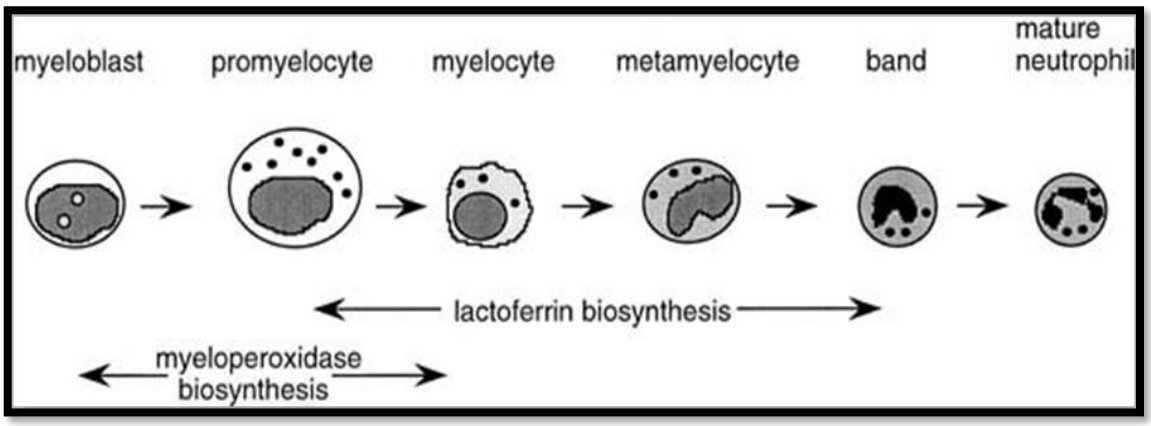

Figure 02: Lactoferrin biosynthesis (Saxon 2001).

4. Pharmacokinetic

4.1.Absorption

Because of its protein composition, bLF has an absolute oral bioavailability of roughly 1% after an oral dosage (Fischer et al. 2007). In addition to being hydrolyzed by pepsin, the stomach's low pH alters the ionization of the amino acids that retain LF structure, causing alterations in its secondary and tertiary structure (Yao 2015).The proteases in the small intestine then complete the breakdown of LF (trypsin, chymotrypsin, amino and carboxyl peptidase).

Other investigations reported that trypsin digested LF into fragments of varying sizes, with the bigger pieces (>30 kDa) demonstrating proteolysis resistance. LF, on the other hand, was discovered to be resistant to enzymes in the small intestine of adult rats (Yao 2015).

Furthermore, if the stomach acidity is insufficient, holo-LF can survive pepsin digestion . 10–20 minutes after intragastric intubation, intact LF molecules entered peripheral circulation from the colon and were found in several organs (Fischer et al. 2007). After bLF withstands intestinal enzymatic breakdown, it binds to LPR receptors on M cells in peyer's patches and is absorbed in an immunoreactive state into blood and tissues. Takeuchi and colleagues revealed that bLF injected intra-duodenal enters the body via the intestinal lymphatic system and travels throughout the body via the thoracic lymph fluid (Takeuchi et al. 2006).

Overall, gastric-mediated LF degradation is debatable. Troost and al discovered that LF is systemically absorbed via the gastrointestinal tract (more than 60%) in its intact form after 30 minutes in a clinical investigation. The pH range required for LF absorption (4.0–7.0) was greater than the pH required for pepsin digestion (1.5–2.0) (Troost et al. 2001). However, the

outcomes of another clinical investigation were inconclusive (Troost, Saris, and Brummer 2002).

The process of LF degradation, as well as the products of LF degradation, are yet unknown.

4.2.Half-life of LF

After intravenous administration, LF has a short half-life in the body, estimated to be around 10 minutes. Recombinant human LF, according to Peen and al. has a half-life of 12.6 minutes and is fully gone after 7 hours (Levay and Viljoen 1995). Once injection has been transported via the lymphatic system, it is quickly absorbed by the liver and discharged into the intestine wall. The gallbladder LF is quickly removed from circulation in the liver. Internalization of LRP-mediated proteins by hepatocytes (Kanwar J. R., Samarasinghe, and Sehgal 2012).

Intact and fragments of LF from the mother were discovered in the urine of breast-fed newborns, suggesting that the kidneys are responsible for some of the LF excretion

Another route for phagocytes to remove LF from the circulation and interstitial space is by receptor-mediated endocytosis. Ferritin transports the iron carried by the LF With such a limited half-life, it is hypothesized that LF transported inside lymphocytes will live longer and perform its job in the body (Kanwa J. R. r, Samarasinghe, and Sehgal 2012).

4.3.Cellular and tissue distribution

In the human body, lactotransferrin is present in two main tanks:

4.3.1. An extravascular pool

Mucous membranes and secretory surfaces vary. LF is a protein produced by epithelial cells in milk. Researchers discovered the existence of LF in the milk of every species investigated, save rats, dogs, and rabbits, in 1971, after screening several kinds of animals. They also discovered that the content of LF is greater in humans (Masson and Heremans 1971).

Other secretory fluids Lactotransferrin is produced by exocrine glands in the respiratory, reproductive, and digestive systems, according to reports.

LF has been detected in bronchial secretions epithelial cells of the mammary glands' alveolar ducts and the genital tract in both men and women, notably in the endometrium.

Finally, the salivary glands, stomach, pancreas, and duodenal epithelial cells express LF (Mason and Taylor 1978).

4.3.2. A pool circulating

It is present in the granules of neutrophil leukocytes as well as the plasma blood. LF is a protein produced by leukocytes and found in the blood. LF's plasma concentration is relatively low (1 to 2 g/mL) due to its effective hepatic clearance mechanism. However, depending on the research conducted and, in particular, the plasma preparation processes and test methodologies utilized, this rate can vary up to 1.6 mg/mL. This rate seems increase in patients with chronic myeloid leukaemias, and also seems be under hormonal control since it varies during pregnancy and menopause.

Corn lactotransferrin is mainly considered as a marker of inflammation, its concentration being greatly increased during inflammatory episodes. So, the correlation between the blood level of LF and the density of circulating neutrophils suggests a leukocyte origin of plasma LF (Azzouzi 2021) .

Concerning myeloid cells, the LF of these leukocytes is combined with the granules secondary specific and minority to other granules, probably granules tertiary. LF expression in neutrophils is also altered and affected during leukaemias characterized by the absence of secondary granules, due to myeloid cell maturation defects.

Also, the subcellular localization of LF in the mid- and trans-Golgi as well as in the endoplasmic reticulum, shows that LF undergoes maturation in through the secretory route. LF synthesized by neutrophils and stored in their granules specific, is currently often used as a marker of degranulation of neutrophils during different inflammatory processes (Azzouzi 2021).

4.4.Metabolism

LF is generated in neutrophils and kept in specific granules and perhaps tertiary granules in an iron-depleted condition (Iyer and Lönnerdal 1993). The steroid-thyroid receptor superfamily appears to function together to control LF gene expression. This backs up the theory that LF levels are influenced by hormones.

A more in-depth explanation is perhaps beyond the scope of this article. LF is not generated as a bigger precursor, unlike myeloperoxidase and several other granular products, and it was

discovered to be unphosphorylated. LF is transported to its storage granules via the Golgi apparatus's medial and transcisternae, which are acidification-dependent (Olsson et al. 1988). As a result, it appears to be processed similarly to proteins intended for secretion.

The neutrophil LF in these granules can travel one of two ways: it can be released into the surrounding tissues or blood, or it can fuse with phagosomes. Degranulation factors, which appear to be reliant on the activation of guanylate cyclase, cGMP, and protein kinase C, are required for secretion of polymorphonuclear cells into the circulation (calcium dependent). This happens in both aerobic and anaerobic environments, is unaffected by hydrogen sulphide, and is triggered by interleukin-8 and surface-bound IgG (Levay and Viljoen 1995).

LF release from neutrophils is frequently increased in iron excess, inflammation, infectious illnesses, and tumor formation, suggesting a multifactorial stimulatory mechanism(Kolb 1989). Upon release LF binds metal ions, of which iron has been the most intensively studied.

The precise relationship of serum apo- to holo-LF has not as yet been determined, because such determinations pose certain experimental difficulties. LF removal from circulation appears to occur in one of two ways ferritin; (Olofsson, Olsson, and Venge 1977) .

In experiments conducted with rats, the half-life of injected holo-LF was prolonged threefold by blocking the RES.There is still some debate over which cells are involved in this method of LF elimination (Ismail and Brock 1993).

The direct absorption of LF by the liver via an iron saturation-independent, clathrin-dependent, calcium-dependent mechanism of endocytosis is an alternate method of LF elimination (Hu et al. 1993).

Hepatocytes, as well as Kupffer and liver endothelial cells, appear to be implicated. LF has been demonstrated to limit transferrin absorption by rat hepatocytes. Therefore, the binding sites might be the same as for transferrin. According to Bennet and Kocinski (Hu et al. 1993). LF like transferrin, is not yet known to be recycled (Birgens et al. 1988). to completely comprehend LF metabolism in adults, more study is required

4.5.Elimination

LF may be removed from the body in two ways: by receptor-mediated endocytosis of phagocytic cells (macrophages, monocytes, and other RES cells) with subsequent iron transfer to ferritin, or through direct absorption by the liver. LF is removed through endocytosis, which

is accomplished by Kupffer cells, liver endothelial cells, and hepatocytes (Levay and Viljoen 1995).

LF and its fragments, mostly of maternal origin, have been identified in the urine of breast-fed newborns, suggesting that kidneys are involved in the removal of LF from circulation (Hutchens et al. 1991).

LF and LF fragments were identified in the urine of babies, suggesting that the kidneys play a role in LF clearance from the circulation. It's worth noting that the LF present in breast-fed babies comes mostly from their mothers. LF fragments with low molecular weights have also been found in stools (Hutchens et al. 1991). However, because there is still a lot of debate over fecal and urine LF removal, further research is needed.

4.6.Toxicity

According to the findings of (Dai et al. 2018), there were no observable side effects and no subchronic toxicity after giving 300 times / 90 days, the approved amount of Rh LF to the animals' bodies. The toxicological parameters of NOAEL were 10. 00 g/(kgd). Rh LF posed no concern to food safety.

The acute oral LD50 of recombinant Rh LF in mice was larger than 20000 mg/kg, indicating that RhLF had no harmful impact Rh LF was nonpoisonous according to the acute toxic dosage graduation criterion .

5. LF and iron binding

5.1.Binding sites

The LF has the ability to bind two Fe^{3+} ions reversibly with a synergistic bicarbonate (CO_3^2) anion (Groves 1960). LF therefore binds iron preferentially in the ferric form, but can also bind other metals *in vitro*. Stable complexes have been observed with Al^{3+}, Cr^{3+}, VO^{2+}, Mn^{3+}, Co^{3+}, Cu^{2+} and Zn^{2+} ions. Iron binding by LF requires the prior presence of a CO_3^{-2} anion. On the other hand, LF can bind *in vitro* other anions which are mostly carboxylate ions such as oxalate, malonate, glycolate, glycinate and nitriloacetate (Baker 1994).

The configuration of the iron-binding site located in the interdomain cavity is identical for each lobe of the protein. Each Fe^{3+} atom is linked to the hydroxyl group (OH) of two Tyr residues, to the carboxyl group (COOH) of an Asp residue and to an imidazole group of a His residue in coordination with a CO_3^{-2} anion. More specifically, the amino acids involved in iron

binding are Asp_{60}, Tyr_{92}, $TyrY_{192}$ and His_{253} for the N lobe and Asp_{395}, Tyr_{433}, Tyr_{526} and His_{595} for the C lobe (Moore et al. 1997). These different ligands form a coordination envelope around the Fe^{3+} ion, an envelope which allows the reversible binding of the latter (Fig 3).

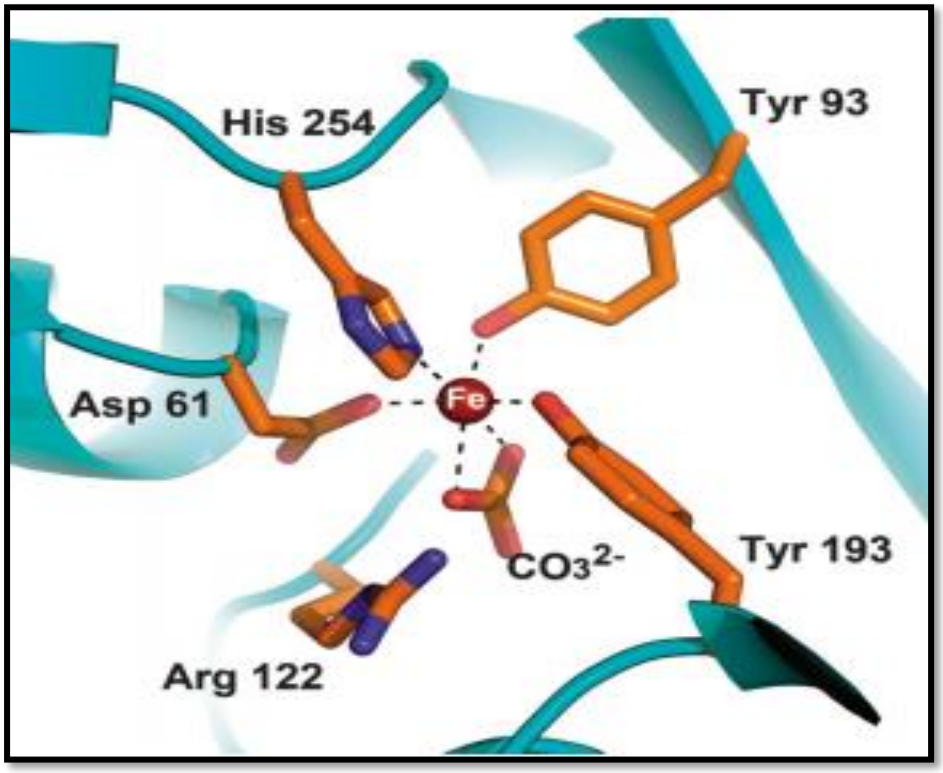

Figure 03: The canonical iron-binding site for lactoferrin (Baker H. M.and Baker 2012)**.**

The present figure showed the N-lobe site of human LF, this site includes 2 tyrosine side chains (Tyr 93 and Tyr 193), 1 aspartic acid (Asp 61), 1 histidine (His 254), plus a bidentate-bound carbonate ion that forms a bridge between the metal ion and an arginine residue (Arg 122) at the N-terminus of an a-helix on domain N2. A structural perspective on LF function

At the stoichiometry of the iron bond, the three negative charges of the nucleophilic groups of the side chains of the amino acids neutralize the three positive charges of the metal ion $Fe^{3+,}$ while the anion CO_3^{-2} can form two bonds with the latter (Brisson 2006) .

The anion CO_3^{-2} occupies an anionic pocket located on the inner surface of domain 2. This pocket is formed of the terminal amine (NH) of residues $Alan_{123}$ and Gly_{124} for the N lobe and Trp_{467} and Asp_{468} for the C lobe of a helix-a (helix 5). It is also formed from the side chain of an arginine residue (Arg_{121} for the N lobe and Arg_{463} for the C lobe) and the OH group of a threonine residue (Thr_{117} for the N lobe and Thr_{459} for lobe C) (Moore et al. 1997).These groups

form hydrogen bridges with the oxygen atoms of the CO_3^{-2} anion which make it possible to fix the anion CO_3^{-2}on the inner surface of domain 2 of each lobe protein (Fig 4).

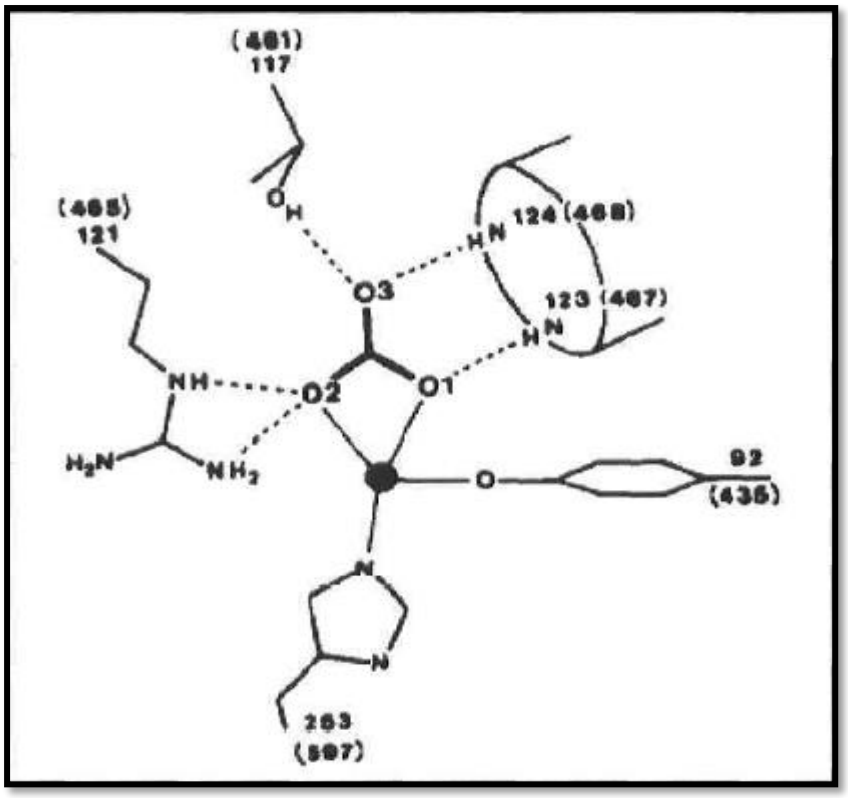

Figure 04: Diagram of the bicarbonate ion binding site of human lactoferrin on domain 2 of the N lobe (Shongwe et al. 1992)**.**

Moreover, the participation of the CO_3^{-2} anion in the coordination sphere of the Fe^{3+} ion is very important since it makes it possible to control the release of iron according to the ph. Thus, the protonation of CO_3^{-2} would be the first step in the release of iron from the interdomain cavity of each lobe of the protein (Abdallah and El Hage Chahine 2000).

LF begins to release its two Fe^{3+} ions almost simultaneously from approximately pH 4.0 and its desaturation is complete at pH 2.5. Iron retention as a function of pH is lower for serum transferrins which release their Fe^{3+} ions from pH 6.0 (Baker H. M.and Baker 2004).

Moreover, the release of the two Fe^{3+} ions by the serum transferrins does not take place simultaneously as for FL. The N lobe releases its Fe^{3+} ions from pH 6.0 compared to the C lobe which releases them when the pH is between 5.0 and 4.0. This difference in iron retention between LF and serum transferrins would be attributable to a pair of Lys residues, located on either side of the interdomain cavity of the N lobe. These two Lys residues are linked together by a hydrogen bridge. When the pH decreases, the two Lys protonate and become exhausted.

This repulsion leads to the opening of the interdomain cavity and the release of iron. The two Lys residues of the N lobe of LF (Lys_{210} and Lys_{301}) are, for their part, stabilized by the formation of an ion bridge between Lys_{301} and the side chain of a residue (Glu $_{216}$) (Edward N. Baker, Baker, and Kidd 2002).

The release of the Fe^{3+} ion therefore leads to the opening of the interdomain cavity of each lobe of the protein. The opening and closing of the interdomain cavity in the N-lobe requires a large conformational change that results in a rigid 54° rotation of one domain relative to the other. Two P-sheets located behind the iron binding site and which connect the two domains together form a hinge which allows rotational movement (Gerstein et al. 1993).

5.2.LF and iron metabolism

That LF is involved in iron transport. This might be because LF plasma concentrations are quite low under typical circumstances. When there is inflammation, however, the amount of LF rises. Because iron exchange from transferrin is simpler in this environment due to the lower pH, LF may have a role in local iron buildup at inflammatory sites (Jeremy H. Brock 2002).

LF has long been suspected of causing hypoferremia by binding free iron and transporting it back to macrophages (Van Snick, Masson, and Heremans 1974) LF has shown extraordinary resistance to trypsin and trypsin-like enzymes during proteolytic breakdown.

The degree of iron saturation determines the level of resistance (Lönnerdal and Iyer 1995) increase in LF in their bile, which might be explained by the mobilization of iron stored in the liver. Iron-treated rabbits, on the other hand, demonstrated suppression of LF secretion in the bile, even at modest dosages.

LF may thus serve as a regulator when large amounts of iron are liberated from its storage sites (Van Vugt et al. 1975). LF from the duodenal secretion was discovered to have a similar association with iron metabolism in humans (de Vet and van Gool 1974).

LF from human milk appears to alter newborns' intestinal iron absorption; however this is dependent on the organism's iron requirements. LF binding is mediated by specific receptors (SI-LFR) found on enterocytes. LF is destroyed 90% of the time after binding to the en-terocyte, releasing Fe^{3+} ions. The remaining 10% is carried through the cell membrane intact. A paucity of intracellular iron may cause an increase in the expression of particular receptors on the surface of enterocytes, resulting in enhanced LF-bound iron absorption (Suzuki Y. A., Lopez, and Lönnerdal 2005). Breastfed newborns are shown to have superior iron accessibility than

formula-fed infants (Fairweather-Tait et al. 1987). Despite this, several studies have found that LF has little effect on iron absorption in the intestines. Indeed, there might be a suppressive impact because increased iron absorption has been documented in newborns fed LF-free human milk; the effect of LF on absorption is detailed (Davidsson et al. 1994).

LF's capacity to bind Fe^{3+} ions has a considerable impact on many of its other biological features, even though it does not play the most crucial function in iron metabolism.

6. Receptors

LF's biological characteristics are mediated by particular receptors on target cells' surfaces. Mucosal epithelial cells, hepatocytes, monocytes, macrophages, polymorphonuclear leukocytes, lymphocytes, trombocytes, fibroblasts, and bacteria like Staphylococcus aureus and Pseudomonas hydrophila all have these receptors (Suzuk Y. A.i, Lopez, and Lönnerdal 2005). Some cells also include "main receptors," which allow them to bind not just LF, but also transferrin or LFs from different species. Nuclear receptors bind leukocyte cmDNA in addition to "conventional" receptors (Kanyshkova, Buneva, and Nevinsky 2001) .

LF interacts with sulfated proteoglycans on the cell surface first, and then binds to membrane receptors specifically to activate the ERK1/2 and PI3K/Akt pathways in cells. Low density lipoprotein related proteins (LRP1/CD91, LRP2/Megalin), transferrin binding receptors (TFR1, TFR2), ferritin and ferroportin, which are necessary for iron transfer, are all LF membrane internalization receptors. Due to their high metabolic rate, cancer cells often have increased receptor expression on their surfaces.

LF can also enter cells through a charge-based interaction, in which its positive charge allows it to bind with negatively charged glycosaminoglycans on the cell surface (Gupta et al. 2015; J. Kanwar et al. 2015).

Compared to negatively charged PLGA NPs, Meng et al. created LF-conjugated N-trimethylated chitosan NPs that showed much greater cellular absorption in 16HBE and SH-SY5Y cells (Meng et al. 2018). Because of electrostatic interactions between negatively charged cell membranes and positively charged NPs, cationic NPs were easily attracted to cells.

Intelectin 1 (Omentin-1) is another LF receptor that is present in intestinal epithelia and is responsible for LF absorption (Akiyama et al. 2013).Furthermore, intestinal cells produce iron absorption receptors, such as divalent metal ion receptor (DMT1) receptors, which improve Fe-LF uptake in the intestine (Kanwar J. R. et al. 2016) .

LF also binds to Toll-like receptor 4 (TLR4), which is responsible for LF-mediated NF-kB pathway activation and C-X-C-motif cytokine receptor 4 (CXCR4), which enhances LF-mediated Akt signaling stimulation (Takayama et al. 2017).

II. Properties and applications of lactoferrin

1. Properties

Lactoferrin has extensive antibacterial action against bacteria, fungi, yeasts, and viruses, as well as anti-inflammatory and anticancer properties. Lactoferrin also boosts the immunological response by promoting cell–cell contact and activation of polymorphonuclear leukocytes and natural killer (NK) cells. LPS stands for lipopolysaccharide (Cui et al. 2022).

Due to its particular structure, its basic surface and its ability to bind iron, LF has a multitude of nutritional, functional and biological properties as illustrated in (Fig 5).

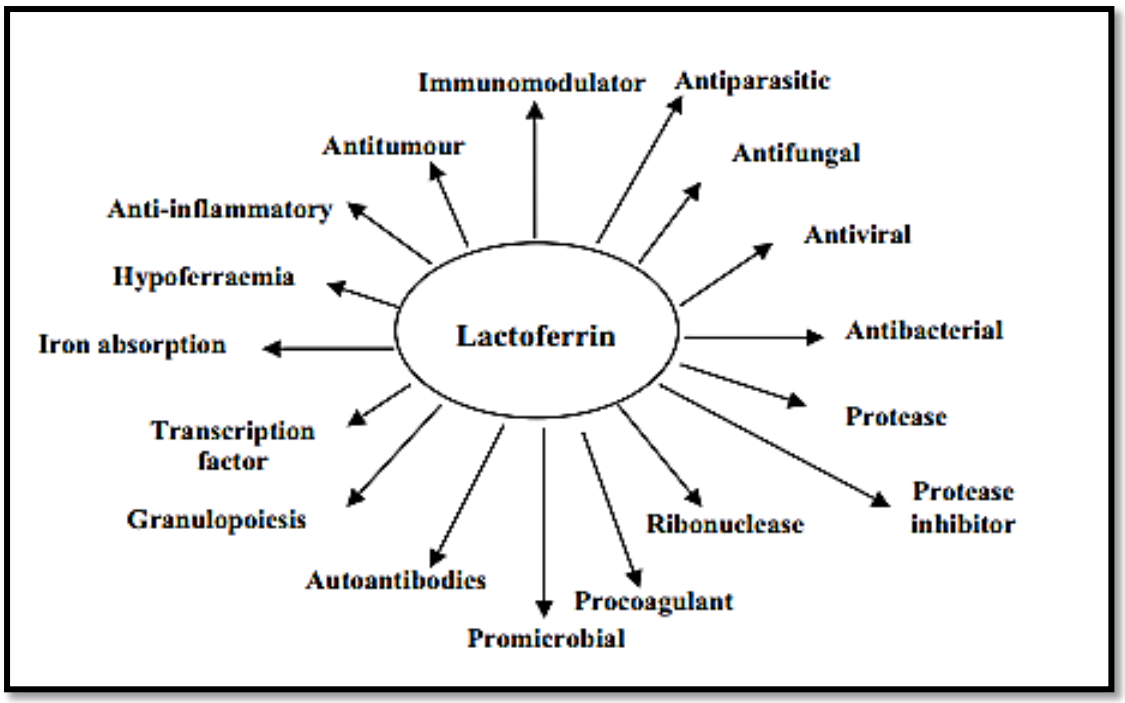

Figure 05: Properties of lactoferrin (Farnaud and Evans 2003)**.**

1.1.Nutritional properties

Like most whey proteins, LF has a sequence whose profile is rich in essential amino acids. . Although its biological value has not been determined, LF is known to be relatively resistant to enzymes related to digestion. The effect of gastric digestion by acid pepsin hydrolysis has been studied *in vivo* and the results demonstrate that a substantial part of LF (>60%) survives gastric transit (Troost et al. 2001).

In addition, *in vitro* tests revealed that LF only undergoes limited hydrolysis by intestinal enzymes (trypsin and chymotrypsin), releasing mainly high molecular weight fragments .

The digestibility of LF also depends on its level of iron saturation. Due to its more compact structure, holo-LF has greater resistance to proteolysis than apo-LF. LF glycovariant A is also 10 times more resistant to tryptic hydrolysis than glycovariant B due to the presence of an additional carbohydrate chain at position Asn28i which blocks a cleavage site of this enzyme (Van Veen et al. 2004) .

From a nutritional point of view, bovine LF is mainly used in infant formula to mimic the actual protein composition of human milk.

Indeed, human milk is rich in LF. The latter represents 10% to 20% of the total proteins of human milk , whereas it represents only 2% of the proteins of bovine milk (Cayot, and Lorient 1998). Additionally, the high concentration of LF and the high bioavailability of iron from human milk suggest that this protein is involved in iron absorption metabolism, especially since a specific receptor has also been identified on the surface of the intestine in humans (Gíslason et al. 1993). On the other hand, the existence of an iron transport mechanism by LF still remains a subject of debate. Finally, (Hernell and Lönnerdal 2002) demonstrated that formula milk with bovine LF supplements were added, and had no influence on iron absorption in infants.

Functional properties are desired characteristics that contribute to the non-nutritional qualities of food products. The functional properties of LF have been little studied, probably due to its low concentration in milk and the still high cost of LF isolates. On the other hand, increasing the use of LF as a bioactive ingredient in food matrices requires a better understanding of its functional properties.

The main functional property of LF to have been studied is its antioxidant power. The antioxidant mechanism of LF is mainly linked to its ability to chelate heavy metals (Fe^{3+}, $Cu2^{+}$), which catalyze acid oxidation reactions unsaturated fats, responsible for oxidative rancidity in food products. The antioxidant effect of LF has been studied in different model systems such as dry soybean fat extracts, oil-based emulsions rich in unsaturated fatty acids and liposomes (S.W. Huang et al. 1999).

The antioxidant capacities of LF have also been evaluated in a variety of food matrices such as milk drinks, mayonnaises and infant formulas (Nielsen et al. 2004). As with most globular proteins, LF exhibits good emulsifying properties. Ye and Singh 2006 investigated the ability of LF to adsorb at the interface of an oil-in-water emulsion to stabilize it. LF can form fine emulsions and is stable over a pH range of 3 to 7.

A distinguishing feature of these emulsions is that the LF-stabilized oil droplets possess a charge of positive surface at pH below the pi of the protein. The gelling properties of LF have also been studied and the mechanical properties of the gels formed depend on the gel formation temperature, the ionic strength and the presence of 3-LG (Paulsson and Elofsson 1994).

1.2.Functional properties

1.2.1. Cardiological and neurological effects

Hypotension is a physiologic state of low blood pressure caused by a variety of factors ranging from dehydration to major medical conditions. The purpose of this study was to see if LF, a natural immunomodulator, might help rats with lipopolysaccharide (LPS-induced) hypotension. LF has previously been shown to play a function in immune response mediation, including the regulation of inflammatory cytokine production during acute inflammation (Doursout et al. 2013).

Rats were given LF by gavage 1 or 18 hours before receiving LPS injections. After 6 hours of LPS injection, the heart rate (HR) and mean arterial blood pressure (MAP) were continually measured. TNF-, IL-6, and TGF- production were measured in the serum at the same time as hemodynamic measures. The proximal duodenum was histopathologically examined at the end of the study. LPS-induced hypotension was prevented in rats by giving them LF 1 hour before they were given LPS. These studies indicate a potential for clinical utility of LF to control hypotension (Doursout et al. 2013).

The effects of bovine milk-derived bLF on distress behaviors induced by maternal separation in 5- to 18-day-old rat pups were investigated in this study. 30 minutes before the behavioral test, the rat pups were given either BSA (100 mg/kg, i.p.; control) or bLF (100 mg/kg, i.p.). Body motions or ultrasonic vocalizations were used to evaluate distress activity (USVs). bLF substantially decreased body movements after 5 minutes of mother separation, especially in 10-day-old pups (Takeuchi et al. 2003).

According to the findings, milk-derived bLF reduces mother separation-induced pain through an opioid-mediated mechanism. Furthermore, bLF may activate NOS, and higher amounts of nitric oxide may induce opioid system alterations (Takeuchi et al. 2003).

1.2.2. Hepatic and renal effects

Liver illnesses, which can be caused by chemical toxicity, viral hepatitis infection, or autoimmune disorders, are a serious health concern because they can progress to cirrhosis and

fibrosis. LF is a siderophilic protein with two iron-binding sites that has been shown to have a wide range of biological activities. The researchers used dimethylnitrosamine (DMN) to cause hepatotoxicity in rats in order to test the hepatoprotective benefits of LF against hepatic damage (Tung et al. 2014).

The findings revealed that DMN-induced hepatic pathological damage reduced body weight and liver index, raised collagen -1(I) (ColI-1) and -smooth muscle actin mRNA and protein levels, and elevated hydroxyproline content. When compared to the DMN-treated group, however, LF administration raised body weight and liver index, lowered ColI-1 and -smooth muscle actin mRNA and protein levels, and suppressed hydroxyproline content.

Low-dose LF (100 mg/kg of body weight) or high-dose LF (300 mg/kg of body weight) significantly reduced the occurrences of liver lesions caused by DMN, according to liver histology. These findings imply that LF provides significant hepatoprotection in rats against DMN-induced liver damage, and that the hepatoprotective effects of LF may be linked to collagen inhibition and stellate cell activation (Tung et al. 2014).

The study of (Hegazy et al. 2016) discovered that oxidative stress and inflammation play a significant role in potassium dichromate (PDC)-induced acute kidney injury (AKI). Furthermore, the study revealed for the first time the role of Interleukin-18 IL-18, which may be one of the most important mediators of PDC-induced renal tissue and tubular injury. In the present model, activation of the T helper2 (Th2) response and IL-4-mediated inflammatory response is suggested as a mechanism of action for IL-18.

Furthermore, the study found that insulin- like growth factor-1 (IGF-1), which is known to have a role in pathogenic renal tissue hypertrophy, is involved in PDC-AKI. It also shows that phosphorylated form box protein 01 levels are upregulated, suggesting that it is a key activator of tubular epithelial hyperplasia and apoptosis in PDC-AKI.

Furthermore, the same findings revealed that pretreatment of rats with LF (200 and 300 mg/kg, p.o.) resulted in significant protection against PDC-induced acute nephrotoxicity, as demonstrated by biochemical data and confirmed by histological and immunohistochemical studies. The findings showed that LF's protective qualities may be due to its antioxidant, anti-inflammatory, and antiproliferative capabilities, as well as the suppression of IL-18 and IGF-1 (Hegazy et al. 2016).

1.3.Biological activities

LF is also attributed to a multitude of biological activities, which are properties capable of intervening in various physiological functions in humans. highlight the presence of 05 biological activities: antibacterial, antifungal and antiparasitic, antiviral, antitumor and immunomodulation (Gifford, Hunter, and Vogel 2005).

1.3.1. Antibacterial activity

LF's antibacterial effectiveness against Gram-positive and Gram-negative bacteria, as well as certain acid–alcohol-resistant bacteria, has been well proven *in vitro* and *in vivo*. The bacteriostatic action of LF is owing to its capacity to take up the Fe^{3+} ion, restricting bacteria's utilization of this nutrient at the infection site and suppressing their growth as well as the production of their virulence factors (Me 2005).

The direct contact of LF with bacterial surfaces has been linked to its bactericidal activity (Fig 6). It was discovered in 1988 that LF destroys Gram-negative bacteria's exterior membrane by interacting with lipopolysaccharide (LPS) (Ellison, Giehl, and LaForce 1988). The positively charged N-terminus of LF inhibits LPS from interacting with bacterial cations (Ca^{2+} and Mg^{+2}) resulting in LPS release from the cell wall, increased membrane permeability, and bacterial harm (Coughlin, Tonsager, and McGroarty 1983). Natural antibacterials such as lysozyme, which is released from the mucosa at high quantities along with LF, are also potentiated by the combination of LF and LPS (Ellison and Giehl 1991).

The mechanism of action of LF against Gram-positive bacteria is based on its net positive charge binding to anionic molecules on the bacterial surface, such as lipoteichoic acid, which results in a reduction of negative charge on the cell wall, favouring contact between lysozyme and the underlying peptidoglycan over which it exerts an enzymatic effect (Leitch and Willcox 1999).

The capacity of LF to inhibit the attachment of specific bacteria to the host cell has been demonstrated *in vitro* and *in vivo* experiments. The processes that prevent attachment are unknown, although they have been discovered.

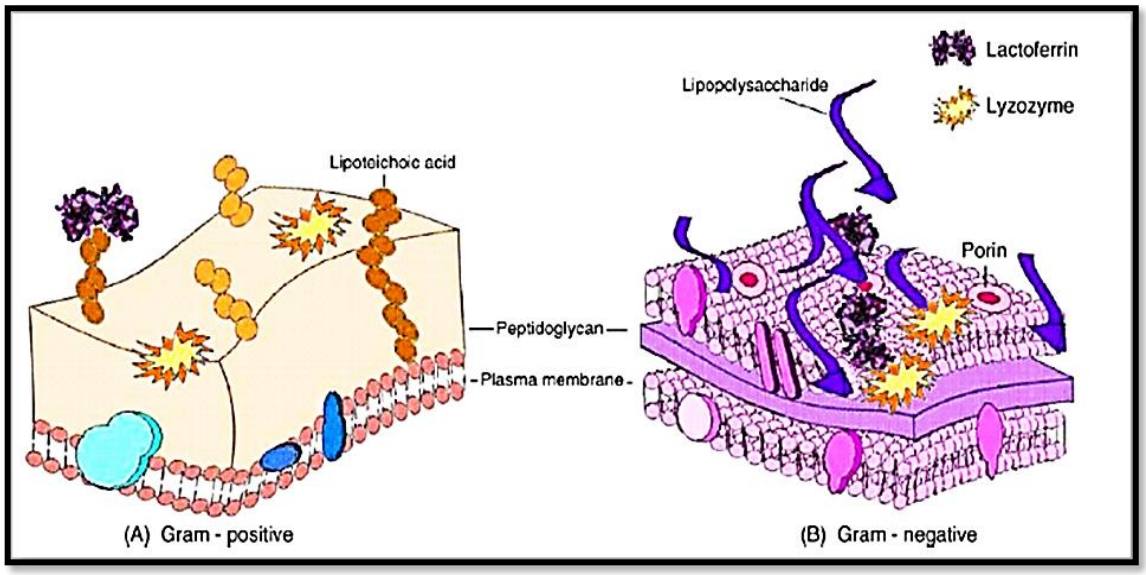

Figure 06: Mechanism of antibacterial action of lactoferrin (González-Chávez, Arévalo-Gallegos, and Rascón-Cruz 2009)**.**

(A) Gram-positive bacteria: LF is bound to negatively charged molecules of the cell membrane such as lipoteichoic acid, neutralizing wall charge and allowing the action of other antibacterial compounds such as lysozyme. (B) Gram-negative bacteria: LF can bind to lipid A of lipopolysaccharide, causing liberation of this lipid with consequent damage to the cell membrane. Which may suggest that LF's oligomannose glycans bind bacterial adhesins, preventing their interaction with host cell receptors.

1.3.2. Antiviral activity

Antiviral activity of LF has been found against a wide spectrum of RNA and DNA viruses that infect people and animals (Strate et al. 2001). At quantities 10 times lower than those seen in human milk, LF inhibits human respiratory syncytial virus. Non-enveloped viruses, such as adenoviruses and enteroviruses, are also susceptible to LF (Seganti et al. 2004). The human immunodeficiency virus (HIV) continues to be a serious medical issue, as existing treatments for the illness it produces are ineffective. *In vitro* studies demonstrate that LF has a significant anti-HIV activity among human plasma and milk proteins.

This impact is caused by viral replication inhibition in the host cell (Viani et al. 1999). The antiviral mechanisms of LF are yet to be fully understood. Certain viruses, including as poliovirus type 1 (which causes poliomyelitis in humans, herpes simplex virus types I and II and cytomegalovirus (Beljaars et al. 2004), can be prevented from entering the host cell by LF. Hepatitis C virus (HCV) and rotavirus are examples of other viruses (Ikeda et al. 2000).

LF suppresses viral replication in the host cell rather than inhibiting viral entrance. Several methods of action for LF's antiviral activities have been hypothesized (Fig 7). One of the most frequently held beliefs is that LF binds to and inhibits glycosaminoglycan viral receptors, particularly heparan sulphate receptors (HS). The binding of LF and HS inhibits the virus from making contact with the host cell and so prevents infection (Strate et al. 2001).

The antiviral action of LF has also been reported in viruses that infect animals, such as the Friend virus complex , which causes erythroleukemia in rats, and the feline calicivirus (Addie et al. 2003).

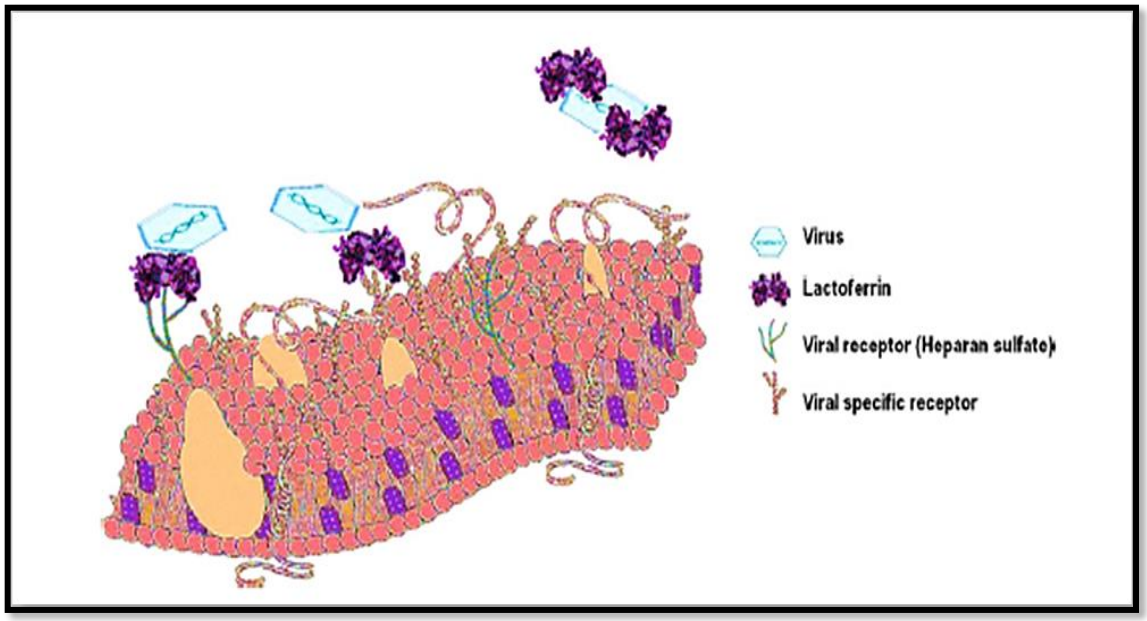

Figure 07: Mechanism of antiviral action of lactoferrin (González-Chávez, Arévalo-Gallegos, and Rascón-Cruz 2009)**.**

LF can be linked to the viral particle and to glycosaminoglycans, specific viral receptors or heparan sulphate to prevent internalisation of the virus into the host cell.

1.3.3. Lactoferrin as a potential preventative and adjunct treatment for COVID-19

In murine coronavirus (de Haan et al. 2005), as well as human coronaviruses hCoV-NL63 (Milewska et al. 2014) and pseudotyped SARS-CoV (Lang et al. 2011), LF has been discovered to block viral entry by binding to host cell surface HSPGs. There is no research on the impact of LF on SARS-CoV-2 and its entrance into host cells that have been published yet. Nonetheless, given the currently accepted 'viral surfing' model for the role of cell surface

HSPGs (Burckhardt and Greber 2009), in which invading virion particles surf' from low-affinity HSPG anchoring sites to high-affinity entry receptors during an invasion, as well as the homology of SARS-CoV and SARS-CoV-2 spike protein structures, as well as both viruses relying on the same angiotensin-converting enzyme 2 (ACE2) receptor in viral infections in particular, the amplitude of the immune response and inflammation may have a role in illness severity, and this is especially true for COVID-19 (Hoffmann et al. 2020). We believe it is reasonable to propose a similar mechanism in which HSPGs act as SARS-CoV-2 attachment sites, allowing the virus to concentrate on the cell surface and promote particular entrance receptors like ACE2. As with SARS-CoV, it is probable that LF can suppress SARS-CoV-2 invasion at micromolar concentrations and in a dose-dependent manner (Fig 8).

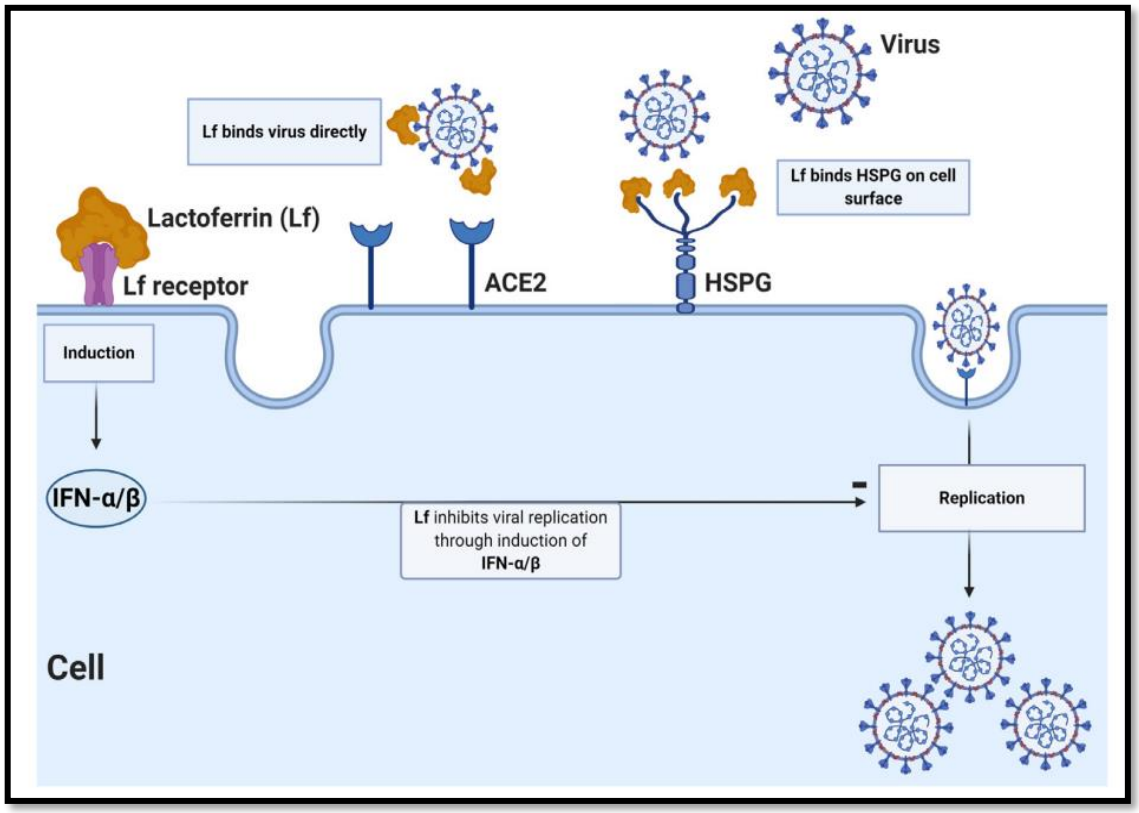

Figure 08: Potential antiviral mechanisms of lactoferrin (Chang, Ng, and Sun 2020)**.**

(1) direct binding of virus by LF; (2) LF binding heparan sulphate proteoglycans (HSPGs) on the host cell surface, reducing viral surfing and subsequent viral entry; and (3) LF inhibition of viral replication via induction of intracellular cell signals. ACE2, angiotensin-converting enzyme 2; IFN, interferon.

75 symptomatic SARS-CoV-2-positive patients recovered completely in 4–5 days after receiving a supplement containing 32 mg of LF administered four to six times per day for 10

days with zinc 10 mg two to three times daily, and the same treatment at a lower dose appeared to prevent the disease in healthy contacts (Serrano et al. 2020).

Because another important element of LF bioactivity is its immunomodulatory and anti-inflammatory actions, in the case of viral infections, the size of the immune response and inflammation may play a role in disease severity, which is especially true for COVID-19. According to current understanding, COVID-19 mortality is caused by a cytokine storm syndrome in a subset of patients, which causes hyperinflammation, severe respiratory distress, and death (Mehta et al. 2020).

Increases in cytokines and acute-phase reactants such interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF), and ferritin are characteristic of a cytokine profile in severe COVID-19 patients. In experimental conditions imitating sepsis, LF has been shown to lower IL-6 and TNF (Zimecki, Miedzybrodzki, and Szymaniec 1998) as well as downregulate ferritin (Rosa et al. 2017). If the concept that LF can moderate an overactive immunological and inflammatory response to viral infection is valid, LF could be a potential supplemental therapy for COVID-19 instances that are more severe anticovid properties.

1.3.4. Immunomodulatory and anti-inflammatory activity

The innate and acquired immune systems are both modulated by LF (Legrand et al. 2006). The fact that persons with congenital or acquired LF deficiency experience repeated infections demonstrates its link with the immune system. Wakabayashi et al. 2006 used the quantitative reverse transcriptase polymerase chain reaction (RTPCR) method to assay the expression of 20 immune-related genes (antimicrobial proteins, pattern recognition receptors, and lymphocyte movement related proteins) in the small intestine of mice given bLF (2.5 g/kg) in 2006, and found that LF can modulate the expression of those genes both specifically and non-specifically.

The positive charge of LF allows it to attach to negatively charged molecules on the surface of immune system cells (Baker E. N. and Baker 2005), and it has been proposed that this connection might activate signalling pathways that lead to cellular responses including activation, differentiation, and proliferation. The transfer of LF into the nucleus, where it may bind DNA, has been shown (Legrand et al. 2004; Baker E. N. and Baker 2005) and activate several signalling pathways (González-Chávez, Arévalo-Gallegos, and Rascón-Cruz 2009).

LF may increase skin immunity and reduce allergy reactions in addition to generating systemic immunity. It stimulates the immune system to respond to skin allergens, resulting in

dose-dependent inhibition of Langerhans cell migration and dendritic cell accumulation in lymph nodes (Conneely 2001). LF is released into the medium, where it inhibits pro-inflammatory cytokines such as interferon-gamma, tumor necrosis factor-alpha, and interleukin (IL)-1, IL-2, and IL-6 (Crouch, Slater, and Fletcher 1992), resulting in anti-inflammatory effects (González-Chávez, Arévalo-Gallegos, and Rascón-Cruz 2009).

LF raises the number of natural killer (NK) cells in the blood (Shimizu et al. 1996), increases polymorphonuclear cell recruitment in the blood (Kurose et al. 1994), stimulates phagocytosis (Szuster-Ciesielska, Kamińska, and Kandefer-Szerszeń 1995), and can modify the myelopoietic process (Broxmeyer et al. 1987).

By interacting with LPS and a variety of receptors on the surface of epithelial and immunological cells, the LF regulates the production of cytokines and the recruitment of immune cells to the site of inflammation, protecting against the septic attack (Ando et al. 2010).

Numerous animal studies have shown that an oral dosage of bLF protects effectively against lethal LPS doses. The protection's mechanism of action is depicted in Fig 09.

By reducing the pro-inflammatory environment, the LF protects against a variety of diseases, including rheumatoid arthritis, chronic gastrointestinal inflammation, neurodegenerative diseases, and skin allergies. Several studies using experimental animal models of inflammation in various tissues show that taking bLF orally reduces inflammation. In the vast majority of cases, this protection is also related to a decrease in TNF- (tumor necrosis factor) and IL-1 production, as well as an increase in IL-10 production. This alteration in the inflammatory process is accompanied by a decrease in the recruitment of immune cells, particularly leucocytes.

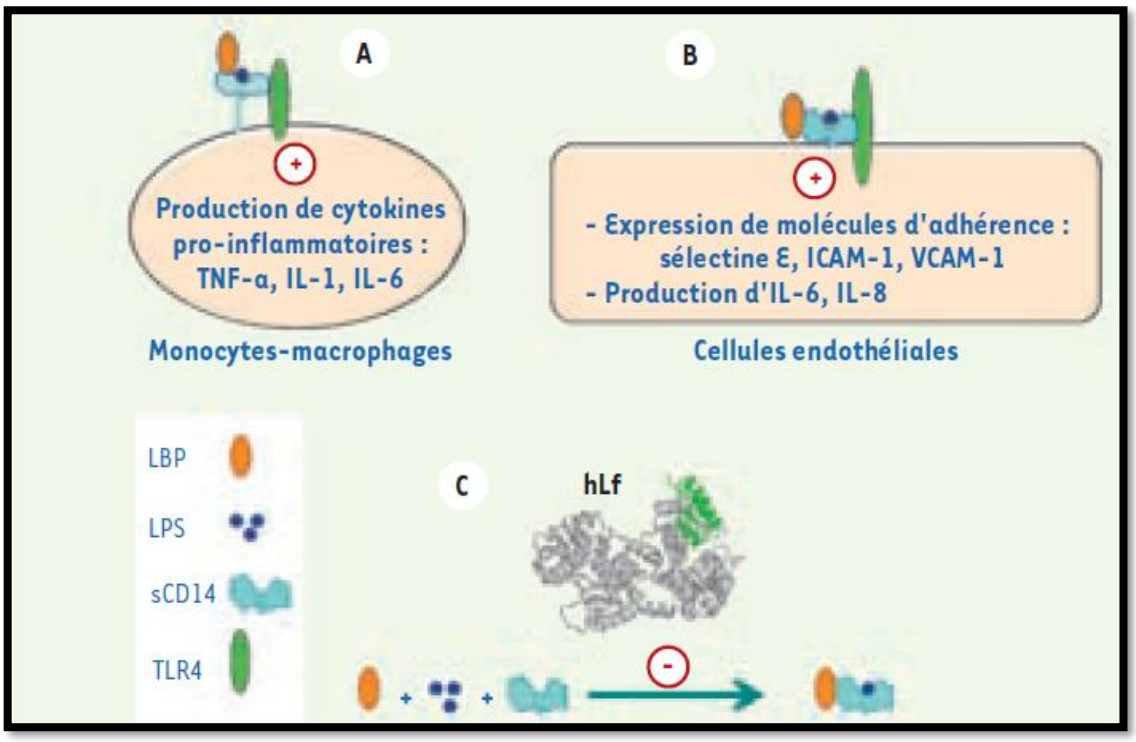

Figure 09: Neutralization of lipopolysaccharide by lactoferrin protects against septic shock (Pierce, Legrand, and Mazurier 2009)**.**

Production de cytokines pro-inflammatoires : Production of pro-inflammatory cytokines ; Expression de molécules d'adhérence : Expression of adhesion molecules ; Cellules endotéliales : Endothelial cells.

The LPS released into the bloodstream binds to the LBP (LPS-binding protein) and the membrane receptor CD14 found on monocytes and macrophages.

- ✓ A: The activation of these cells will necessitate the interaction of the tertiary complex with the TLR4 receptor (cell-signalling Toll-like receptor 4).
- ✓ B: The presentation of the LPSLBP-CD14 soluble complex to TLR4 will activate CD14-depleted endothélial cells. CD14 soluble is produced either by activated cells or by enzymatic cleavage of the CD14 membrane. The stimulation of monocytes/macrophages and endothelial cells results in the generation of pro-inflammatory cytokines. These molécules have a beneficial effect when produced in controlled quantities, but when produced in large quantities, they cause tissulary changes that can lead to a septic clot. The impact of these cytokines on endothelial cells also leads to an increase in the production of adhesion molecules, whose ligands are carried by neutrophils and monocytes. The adhesion of neutrophils,

followed by their dissemination in the tissues, plays a major role in the inflammatory process.

- ✓ C: By pinning the LPS, the LF prevents the development of various complexes and opposes the LPS's deleterious effects, providing protection against the septic shock (Ando et al. 2010) .

1.3.5. Antiparasitic activity

The majority of research on LF's antiparasitic efficacy has been done *in vitro*, with molecular associations being tested in the presence or absence of Fe^{3+}. Peptides derived from the whole molecule have also been proven to have this action.

A protozoan infection causes intestinal amoebiasis, which is one of the primary causes of diarrhea in children under the age of five and the world's fourth greatest cause of mortality.

Entamoe bahistolytica, which employs complicated ways to penetrate the intestinal mucosa and induce amoebic colitis (González-Chávez, Arévalo-Gallegos, and Rascón-Cruz 2009), causes the illness. Apo-LF is the milk protein that has the most amoebic activity against E. histolytica *in vitro*, since it may attach to the lipids on the trophozoite's membrane, producing membrane breakdown and parasite damage (Leon-Sicairos et al. 2006).

In vitro investigations have revealed that hLF may bind the intracellular parasite Toxoplasma gondii, which causes toxoplasmosis in people and animals. LF, on the other hand, is powerless to stop the parasite from infecting the host. Its method of action in this scenario is to stop T. gondii from growing into host cells (Dzitko et al. 2007).

The action of LF on the haemoparasites Babesiacaballiand Babesiaequi is dependent on whether it is linked to Fe^{3+} or not (Botteon et al. 2002). Babesiacaballi was shown to be strongly repressed by apo-LF but not by the other kinds of LF; none of the LF types had an inhibitory impact on B equinone (Ikadai et al. 2005).

2. Applications

2.1. Infant Formula Following

Aside from its inherent iron-binding capacity, LF can have a variety of positive benefits (Vogel 2012). LF has been employed in the pharmaceutical, food, and feed additive industries because of its antimicrobial, anticancer, antiviral, and immunomodulating properties. Bovine LF is a nutraceutical that does not appear to be harmful to one's health (Hernell and Lönnerdal 2002). LF has been linked to changes in glucose metabolism (Mayeur et al. 2016). In individuals

with type 2 diabetes and impaired glucose metabolism, José Maria Moreno-Navarrete et al. 2008 discovered a negative link between blood LF and fasting glucose concentrations but a positive correlation between blood LF and insulin sensitivity (Guo et al. 2017). It also modulates intestinal glucose absorption, suggesting that it might be effective in restoring glucose transport, particularly in inflammatory situations (Talukder et al. 2014).

In overweight adults, blood LF concentration has been shown to correlate with BMI and waist-to-hip ratio (José María Moreno-Navarrete et al. 2013). In humans, oral administration of enteric-coated LF for 8 weeks decreased overall adiposity, especially visceral fat formation, according to Ono et al. (Ono et al. 2010) . LF may be utilized as an adjuvant treatment for intestinal illnesses, according to recent research. Ingestion of LF as previously stated, may have beneficial effects on gastrointestinal illnesses by altering intestinal flora and avoiding diarrhea (Ochoa et al. 2012). In infants infected with rotavirus, clinical investigations have shown that LF lowers Giardia lamblia colonization and reduces vomiting and diarrhea (King et al. 2007 ; Ochoa et al. 2008).

In immunocompromised mice, LF has been shown to restore the humoral immune response (Artym et al. 2003). It can be utilized as an adjuvant treatment in immunocompromised individuals to restore T and B cells because of its immunomodulatory properties (Yen et al. 2011). Because LF prevents early cervical maturation and lowers the generation of proinflammatory factors, it may help to prevent preterm delivery in humans.Furthermore, LF aids in the development of the brain and intellect. During prenatal and neonatal stress, bovine LF has been found to protect the brain from neuronal death and decrease inflammation (Ochoa and Sizonenko 2017).

Future applications of LF might include therapeutic treatment of disorders affecting specific tissues and organs. LF might, for example, be used as an organ-targeting ligand for medication delivery. Many LF-drug carrier models have been developed since R. Huang et al. 2008 initially employed LF as a brain-targeting ligand. Future applications of LF are anticipated to include targeted therapy for liver and lung illnesses.

2.2.Food additives

LF may already be created in cows, goats, and rice because to the fast development of transgenic technology (Huang R.et al. 2008). Bovine LF has been used as a health-promoting addition in commercial food items in Japan for decades (Tomita et al. 2009), but the European Food Safety Authority just authorized it as an ingredient in food products (EFSA Panel 2012).

LF is also used as a food ingredient in China, according to the Chinese Food and Drug Administration (CFDA) (Wang et al. 2017). The processes behind LF synthesis, regulation, and action need to be better understood as the intake of milk grows, and this will aid knowledge of the critical roles of LF in metabolism.

2.3.Adjuvant therapy of metabolic diseases

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III. Lactoferrin and gastric ulcer

1. Gastric ulcer

One of the most common peptic ulcer illnesses is gastric ulcer (Sharifi-Rad et al. 2018; Serafim et al. 2020). Aside from the discomfort and issues that come with a gastrointestinal ulcer, it also causes bleeding and can result in mortality, albeit in small numbers on a worldwide scale (Dadfar and Edna 2020; Zhang et al. 2020). It's the unavoidable result of the stomach's damaging elements outnumbering its defense ones. Antioxidant enzymes, nitric oxide, mucin formation, surface phospholipids, prostaglandins, and growth factors are among the destructive factors, whereas antioxidant enzymes, nitric oxide, mucin production, surface phospholipids, prostaglandins, and growth factors are among the defensive factors (Ugwah et al. 2019).

Proton pump inhibitors, H_2-receptor blockers, and other contemporary gastric ulcer medicines may have a variety of adverse effects in addition to the risk of relapse and tolerance (Kuna et al. 2019). As a result, scientific validation of natural medicines that protect against stomach ulcers might provide an effective way to prevent the negative effects of traditional medications (Sharifi-Rad et al. 2018).

A gastric ulcer is a deep necrotic lesion that affects the entire mucosal depth as well as the muscularis mucosae. It arises as a result of a significant mismatch between aggressive elements and mucosal defense systems.

Ulcer formation is thought to be influenced by a variety of variables (genetic, neurological, hormonal, humoral, and iatrogenic) (Tarnawski et al. 1991).

2. Relationship between alcohol and gastric ulcer

The use of alcohol has long been linked to the development of peptic ulcers. When alcohol enters the stomach or duodenum, it can modify the mucosa (line) and cause inflammation, ulceration, or mucosal erosion (Harger 1958 ; Marotta and Floch 1991).

Alcohol inhibits the activity of gastroesophageal sphincters, promotes gastric secretion, and delays gastric emptying. Discovered that a large portion of alcohol eaten in tiny amounts is digested in the stomach. It has a negative impact by producing microvascular endothelial damage, which has been recognized as a key early event in the development of acute alcohol-induced stomach injury (Andrzej Tarnawski et al. 1988;Parodi et al. 1991).

3. Relationship between LF and gastric ulcer

Ethanol, or ethyl alcohol, comes from the fermentation of plants by yeasts (World Health Organization 2019). It is miscible with water, forming a mixture when heat is released and the liquid contracts: 1 volum to bethanol and 1 volum to water equal 1.92 volum. Mixture most common solvents are miscible with ethanol (Fiches toxicologiques).

Alcohol's effects are largely determined by its blood concentration and, as a result, the rate at which it is absorbed and metabolized in the body.

Many behavioral and environmental factors (time of day, amount of alcohol consumed, presence of food in the stomach, type of drink, chronic consumption, type of diet, age, association with smoking, etc.) but also genetic factors influence blood alcohol concentration (number of enzymes expressed allowing the metabolism of ethanol). As a result, depending on these various parameters, the speed of alcohol elimination can vary by a factor of three (Blanc 2015).

LF is a multifunctional protein generated from milk that has a variety of biological functions. Because it is safe for human consumption and is currently produced using low-cost, well-established large-scale procedures, the LF scientific community has committed itself to dissecting its mechanisms of action in order to make it more rational and efficient for diverse purposes. Proton pumping ATPases have been identified as molecular targets of LF in several cellular models connected to varied actions of this natural protein, according to research Information on this subject has not been systematically analyzed before, hence herein we review the current state of art on the effect of LF on proton pumping ATPases.

Though structurally different, we propose that LF holds a proton pump inhibitor (PPI)-like activity based on the functional resemblance with the classical inhibitors of the stomach H+/K+-ATPase. The downstream events and effects of LF's PPI-like behavior are also examined, as well as the impact on the creation of better LF applications (Cátia Santos-Pereira, María T. Andrés, et al. 2022).

4. Gastro-protective effect of LF

This study looked at the effects of lactoferrin extracted from cheese whey and marjoram tea on indomethacin-induced stomach ulcers in rats. The mice were given a 21-day pre-

treatment with lactoferrin, marjoram tea, or a combination of the two before being given stomach ulcers (via a single oral injection of 30 mg/kg indomethacin). LF, marjoram tea, and garlic were discovered to have gastroprotective effects.

LF, marjoram tea, and their combination had a gastro-protective effect against indomethacin-induced stomach ulcers in rats, according to the findings. After indomethacin treatment, LF, marjoram tea, and their combination reduced either the increase in ferritin, TNF-, lipid peroxidation, and nitric oxide or the decrease in GSH and SOD. LF, marjoram tea, and their combination also improved the pathological results of stomach tissue, highlighting the advantages of LF alone or in combination with marjoram tea in this regard (Ahmed M. Abdel-Salam1, Ahmed H. Zaghloul 1, Rasha S. Mohamed 2021).

Experimental study

I. Material and Methods

1. Chemicals

The chemicals utilized in the tests were in analytical grade and purchased from Sigma–Aldrich and Roche (see annex 01), they are delivered from Natural and Health Sciences Faculty, Mentouri University and from Research Centre on Pharmaceutical Sciences, Constantine, Algeria.

2. Bovine LF extraction and purification

Extraction and purification procedure was performed at the Research Centre on Pharmaceutical Sciences, Constantine, Algeria.

2.1.Sample Collection

3.3 L of colostrum samples were obtained immediately after postpartum of five bovines (mean age 30 ±6 months; first calving; feeding hay, fodder and herb). Sample Collection was done at a farm in El-Hamma, Constantine Algeria (in March, 2022). Samples were frozen at -20°C.

2.2.Extraction and purification process

Extraction process was achieved as described by (Devesh Parkar, Rahul N Jadhav, DR. Mukesh Ramesh Pimpliskar 2016; Ebrahim et al. 2019), with important modifications done by our own team. It was attained as elaborate in the synoptic diagram in Fig 10.

Fat layer (topmost) obtained was separated using a spatula and rejected after subjecting the sample to centrifugation. The pH of homogenized supernatant with equal volume of distilled water is justified to 4.6 and centrifuged again to separate the casein.

The rest of solution was added to ammonium sulphate 45% (v/v) after justifying the pH to 6. After 1h, pH of resulted solution was modified to reach 4 then 8 before adding ammonium sulphate 80% (v/v) and incubated for 16 h to separate the precipitate LF.

The separated LF was put into purification and desalting procedures following two methods. The first is made by adsorption on a solid using a column of silica gel. Then the second is carried out by diafiltration after washing with sterile milli-Q water to removal of salt as well as all the low molecular weight (15 kDa and below) proteins present in the sample (Peyrouset. A.G and Spring.F .P 1982; Frankinet. J 1988).

The final desalted sample of LF was further lyophilized by sublimation using a lyophilizer instrument (Cryotec ALPHA 1-2 LDplus / 101521,2006, see Annex 1).

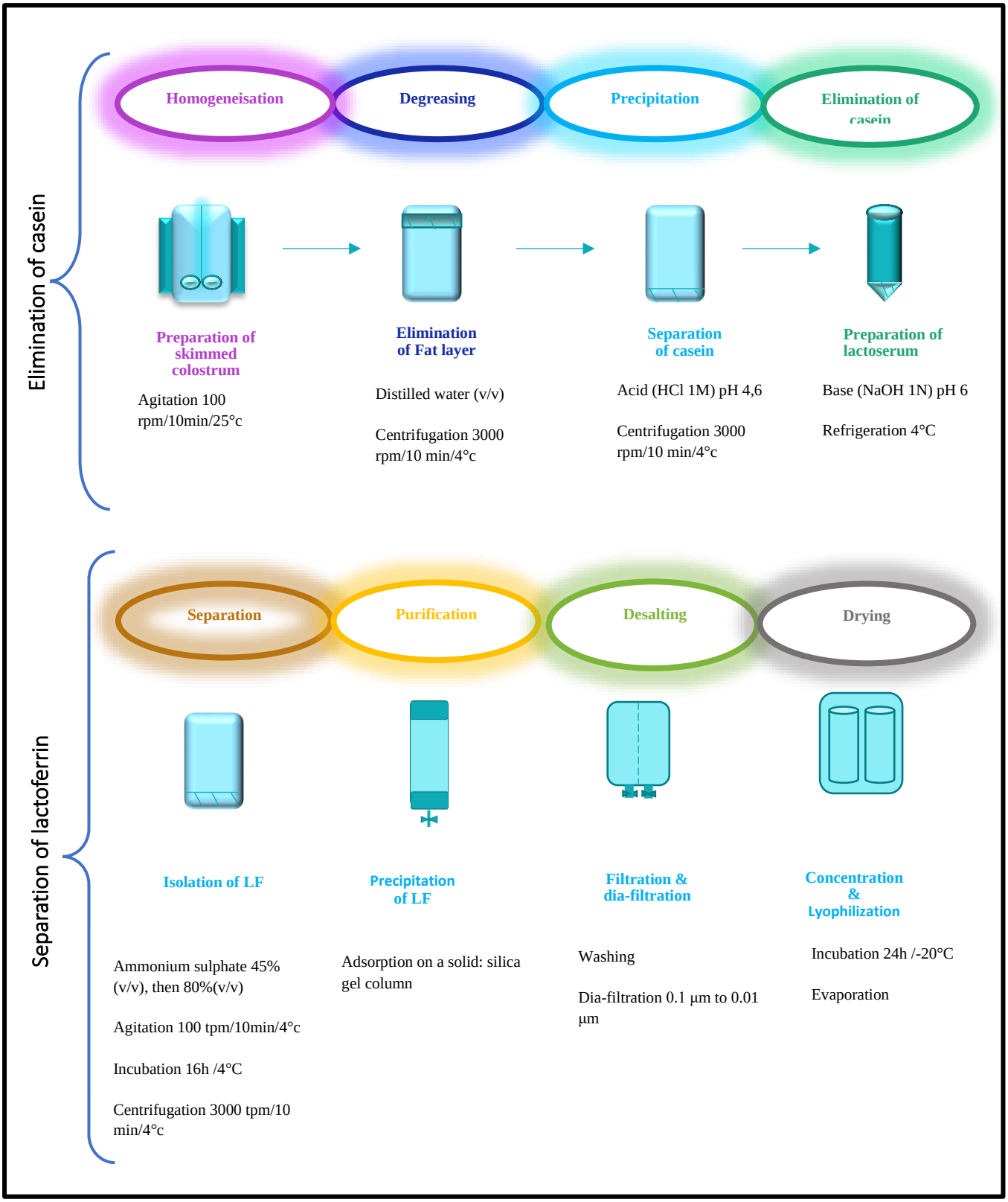

Figure 10: Synoptic diagram of bovine lactoferrin Extraction and purification.

3. Characterization of the extracted LF

3.1. Protien quantification by Bradford's assay

Bradford assays are Coomassie Dye-Binding assays for fast and simple protein quantification. The assay is performed at room temperature and no special equipment is required. Standard and unknown samples are added to preformulated Coomassie blue G-250 assay reagent and the resultant blue color is measured at 595 nm following a short room temperature incubation.

Bradford protein assays are compatible with most salts, solvents, buffers, thiols, reducing substances, and metal chelating agents encountered in protein samples.

The determination of proteins in the solution of bird droppings was carried by mixing 1 mL of protein solution with 4 mL of Bradford solution which prepared as mention by (Bradford 1976).

3.2. Determination of molecular weight by SDS – PAGE

Commercially available SDS-PAGE Kit was used for the analysis purpose. After electrophoresis, the gel was subjected to the 0.25% Coomassie Brilliant Blue R - 250 Staining Solution overnight at 4°C.

Next day, the stained gel was subjected to the destaining solution containing methanol, deionised water and glacial acetic acid. LF molecular weight was calculated using Rf values of Standard protein markers and extracted lactoferrin obtained by Gelanalyzer software (See Annex 1).

3.3. FTIR analysis

The FTIR Fourier-transform infrared spectroscopy analysis method uses infrared light to scan test samples and observe chemical properties.

The extracted LF was prepared by forming a uniform dispersion matrix with potassium bromide (KBr). 10 mg of a freeze-dried sample was mixed with 500 mg of KBr and ground to a fine powder using a mortar and pestle. In order to determine the structural characteristics of LF, spectroscopy measurement of the extracted sample through a spectrometer (Iraffinity-1S / Shimadzu) at room temperature at 500-4500 cm^{-1} wave length (see Annex 1). Graves were schematized using Origine® software.

4. In *vitro* experimental study

All tests were performed following standard laboratory procedures at the Research Center on Pharmaceutical Sciences, Constantine, Algeria.

4.1. Antibacterial activity

Antibacterial activity test was determined by the disk diffusion method using the (Bauer et al. 1966) method. The bacterial strains used for the test are: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* ATCC 25953, Salmonella typhi ATCC 14028 and *staphylococcus aureus* ATCC 43300. The 6 mm diameter discs were prepared with Whatman N° 3filter paper.

2 mg of LF was dissolved in 1 mL of sterile distilled water to get a concentration 2000 µg/mL. Then the following concentrations were prepared and applied for the antibacterial assay: 1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL in addition to 2000 µg/mL. 10µL was applied to each disc (Hoek et al. 1997).

The inoculum was prepared with the fresh cultures of bacterial strains which were cultivated on the nutrient agar for 18 h at 37°C, and it was standardized at 10^8 CFU/mL.

Then, Muller-Hinton agar was inoculated with the inoculum and the discs were placed on the surface of the inoculated agar plates and gently pressed to adhere to the agar surface. The plates were incubated for 24 h at 37°C. After incubation, the diameter of the zone of inhibition was measured. The test was repeated three times for each bacterial strain.

5. *In vivo* experimental study

All tests were performed following standard laboratory procedures at the Pharmaceutical Science Research Center, Constantine, Algeria.

5.1.Animals

Young adult female Wistar Albino rats (nulliparous and non-pregnant, n = 15, mean age = 85± 5 days) weighing 162 ± 26 g were used in this study. The rats were obtained from the Department of Biology, Mentouri University, Constantine, Algeria (in March, 2022).

They were housed in cages, kept in an air-conditioned room between 22 and 26 ° C and fed standard rat granules with free access to food and water at will. The rats were acclimatized to the laboratory environment for two weeks before the start of the study. The European Community Directive (86/609 / EEC) and the national rules on animal care have been followed.

5.2.Grouping and dosing animals

Rates were randomly divided into five groups each consisting of three rats. They were kept without food during the treatment period, which was three days, but had free access to water. They were kept in specially constructed cages to prevent coprophagia during the experiment. All animals were treated daily by gavage as following:

Group I and group II were served as vehicle treated control group and negative treated control group, respectively. Animals received 10 mg / kg / b.w of sodium chloride 0.9 %.

Group III, Group IV and Group V were served as treated groups and were received individually LF at 400 mg/kg b.w, CS at 400 mg/kg b.w and Col at 1 mL/ 200g / b.w. LF and CS were dispersed in sodium chloride 0.9 %, that suspension was sonicated for 10 min before the treatment.

5.3.Anti-ulcer activity test

The ulcer was induced by administering ethanol following the method referred to Dashputre and Naikwade, with a slight modification (Dashputre and Naikwade 2011).

The gastric ulcers were induced in rats by oral administration of ethanol at 1 mL/ 200g / b.w (90%), after 60 minutes of treatments of the negative treated control group and the others treated groups (i,e: group II,III,IV and V). while the first group was not exposed to gastric ulcer.

The animal's behaviours and any toxic effect within this 1 hour were closely observed and mentioned.

After 1 hour of drug treatment, animals were anesthetized and had undergone dissection. Stomach cardiac and pyloric ends were cut out. The gastric contents were drained into glass tubes for the determination of pH and total acidity (See Annex 2).

5.4.Determination of pH

An aliquot of 1 mL of each gastric juice was diluted with 1 mL of distilled water. Then the pH of the result solution was measured using pH meter (Bante PHS-3bW).

5.5.Determination of total acidity

The last result solution was taken into a 50 mL conical flask and two drops of phenolphthalein indicator was added and titrated with 0.01N NaOH until a permanent pink

color was observed. The volume of 0.01N NaOH consumed was noted. The total acidity was expressed as mEq/L and calculated by the following formula (Dashputre and Naikwade 2011):

$$Acidity = \frac{\text{V NaOH x Nx 100 mEq/L}}{0.1}$$

where V is volume and N is normality.

5.6.Macroscopic evaluation and ulcer scoring

The stomachs were opened along the greater curvature and rinsed with physiologic water to remove gastric contents and blood clots. Then they were examined by an optical magnifier 10× (Leica, see Annex 1) to evaluate and score the ulcers using the following notation (Abebaw, Mishra, and Gelayee 2017):

Normal colored stomach (0), Red coloration (0.5), Spot ulcer (1), Hemorrhagic streak (1.5), Deep ulcers (2), Perforation (3).

5.7.Ulcer index

The total mucosal area and total ulcerated area were measured using ImageJ® software. The ulcer index was then calculated as the following equation:

Ulcer index = 10/x

where x is the total mucosal area/ulcerated area.

5.8.Percentage of healing and ulcer inhibition

Percentage healing and inhibition of ulceration were calculated as below (Abebaw, Mishra, and Gelayee 2017):

$$\%\ Inhibition\ of\ ulceration = \frac{\text{Ulcer index control group} - \text{Ulcer index test group}}{\text{Ulcer index control group}} \text{x100}$$

$$\%\ Healing = \frac{\text{Total mucosal area} - \text{Ulcerated mucosal area}}{\text{Total mucosal area}} \text{x100}$$

5.9. Hematological analysis

The blood samples from animals (both treated and vehicle control groups) were collected in EDTA containing tubes for hematological study. CBC parameters as total red blood cells (RBC), hemoglobin (HB), hematocrite (HCT), platelet count (PLT) and white blood cells (WBC) count were determined with humalyzer.

6. Histopathological study

Stomachs were processed distinctly for histological interpretations. The material was preserved in the fixative (10 % formalin) for 48 hours, dried by serial ethanol cycles (70 % to absolute) and placed in paraffin.

The fragments were cut in 5 µm thinness which colored with Harris hematoxylin and eosin, and then observed using light microscopy (Leica DM 1000, Germany, see Annex 3), at the laboratory of anatomical and pathological cytology, EP0H El Modjahid Hafid Boudjamaa El Bir. Constantine. Algeria.

7. Statistical Analysis

All studies were performed in each test in triplicate. The data can be obtained on average ± standard deviation of the mean (SDM). The analyses were performed by the SPSS 18(Statistical Package for the Social Sciences) software and evaluated by a T-test. The $p < 0.01$ and $p < 0.05$ values were considered significant.

II. Results

1. Extraction yield

Extraction of LF from the Whole colostrum by ammonium sulfate adhesion, followed by adsorption on solid support and then elution by an acid solution, by realizing the extraction prototype developed by our research team, gaves us a very effective yield of LF and casien as shown in the following table (2).

Table 02: Extraction yield of fats, casiens and lactoferrin from the whole bovine colostrum.

Fats	Casein	Skimmed LF	Lyophilized LF
3,65%	15,54%	1,67%	1,33%

2. Characterization of the extracted LF

2.1.Protien quantification by Bradford's assay

On carrying out Bradford's Assay, it is notice according to our calibration range a variation of color according to the concentration of proteins extracted in each tube (the higher the concentration of proteins, the more the blue color intensifies). Following absorbance values at 595 nm were obtained using a spectrophotometer. Absorbance values are represented in the graph ulestrated in Fig 11:

The protein level in our extracted LF was calculated according to the calibration range. The protein content is estimated at 49.83 mg Eq BSA/mg of LF.

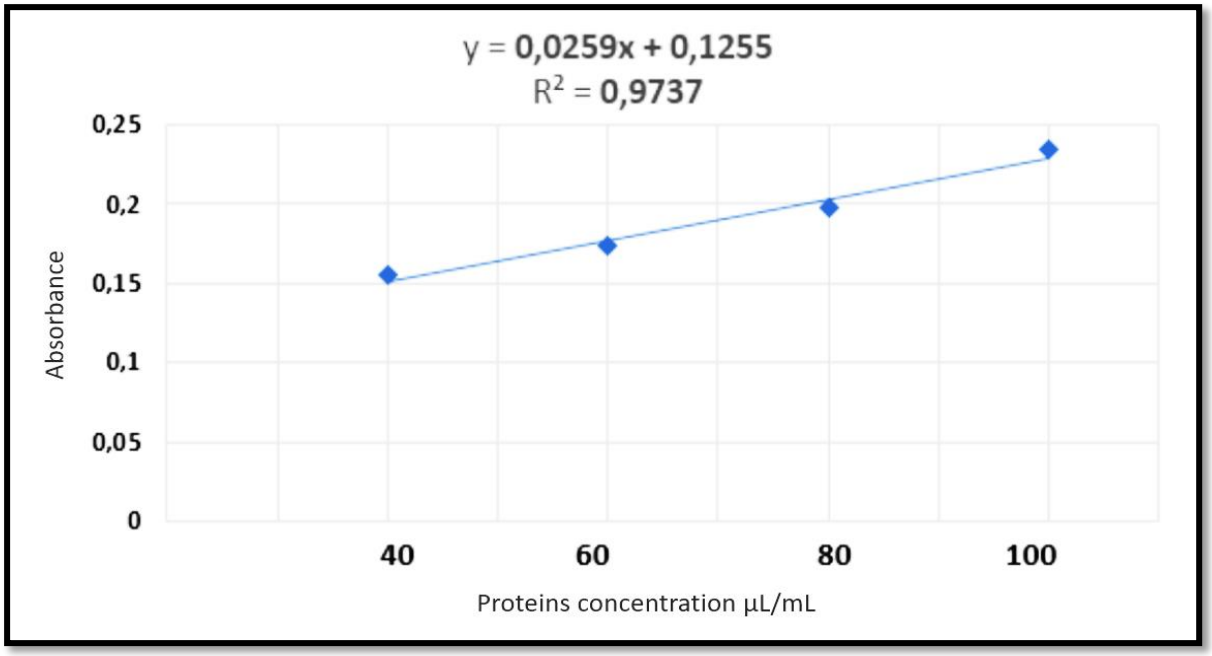

Figure 11: Calibration curve.

2.2.Determination of molecular weight by SDS – PAGE

On performing SDS-PAGE of the extracted and purified LF, following bands were obtained which can be seen in the following gel image (1). This image shows a single band mid 70 and 80 kDa.

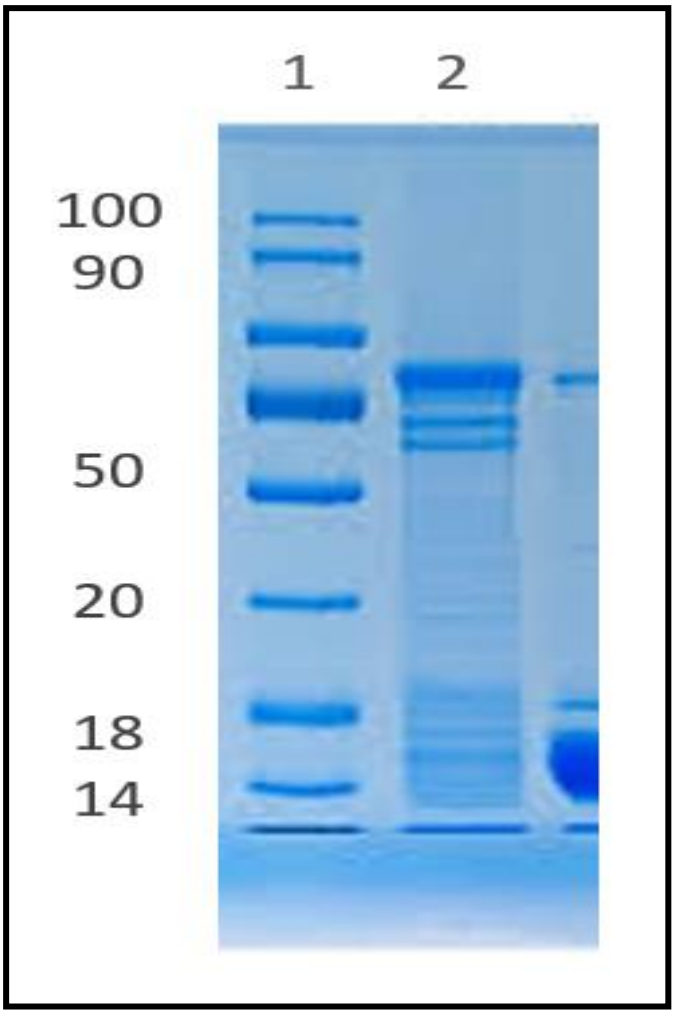

Image 01: Representative SDS-Page Coomassie gel of protein bands.

1 : Marker; 2: Lactoferrin.

2.3.FTIR analysis

The FTIR spectrum (Fig 12) of LF extracted from bovine colostrum showed major peaks in the amide region. The characteristic vibrations of the amide bonds which link the amino acids are: 1635.64, 1516.05, 1288.45, 3275.13 cm^{-1}.

The spectrum also shows a bond at 694.36 cm^{-1} and 2958.00 cm^{-1}. Other bonds appear at 1388 cm^{-1} and 1469.76 cm^{-1}. As well as between 1000 cm^{-1} and 1250 cm^{-1}.

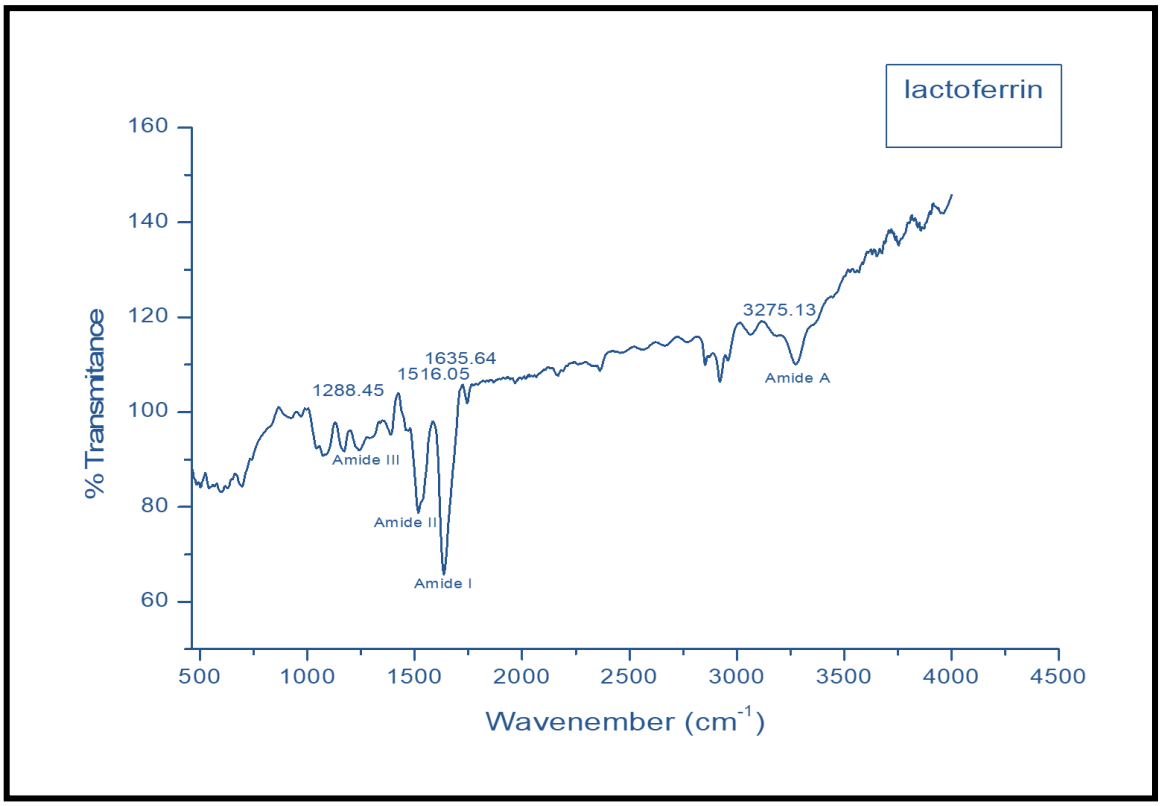

Figure 12: Fourier transform infrared spectroscopy (FTIR) spectra of lactoferrin.

3. *In vitro* study

3.1.Antibacterial activity

Our extracted LF showed no observed antibacterial activity at the selected concentration (2 mg/mL), using the disk diffusion method against *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* ATCC 25953, Salmonella typhi ATCC 14028 and *staphylococcus aureus* ATCC 43300 in comparison with the standard. Results are presented in Fig 13.

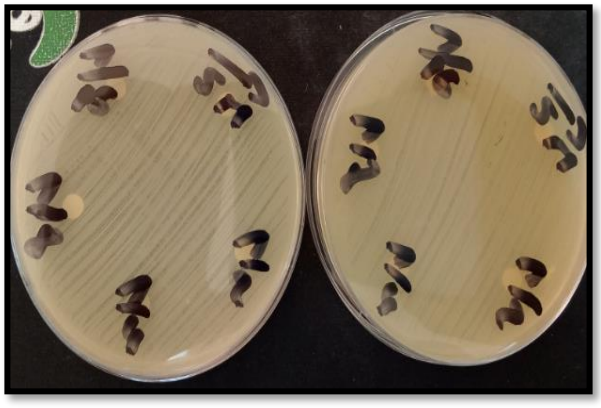

Image 02 : ***Salmonella typhi ATCC 14028***

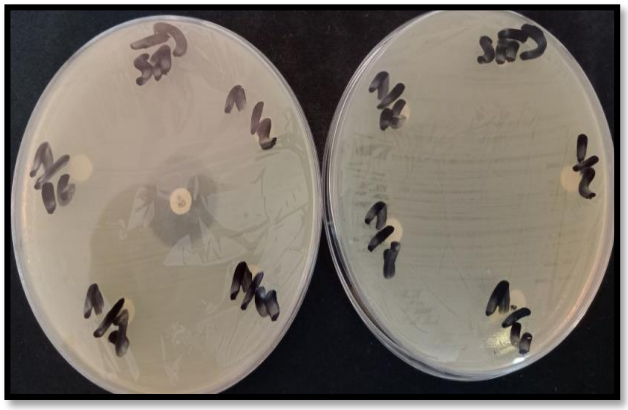

Image 03 : ***Escherichia coli ATCC 25922***

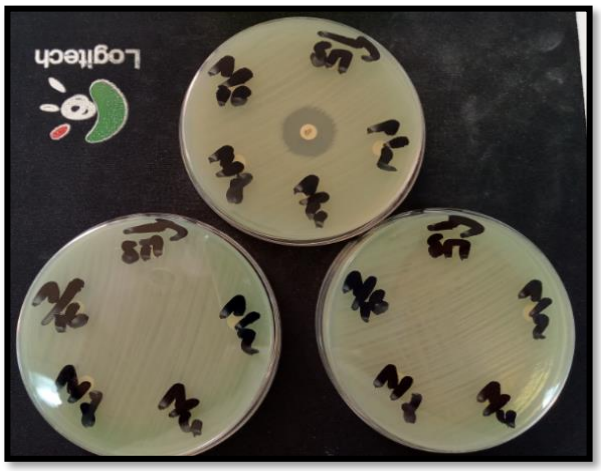

Image 04 : ***Pseudomonas aeruginosa ATCC 25953***

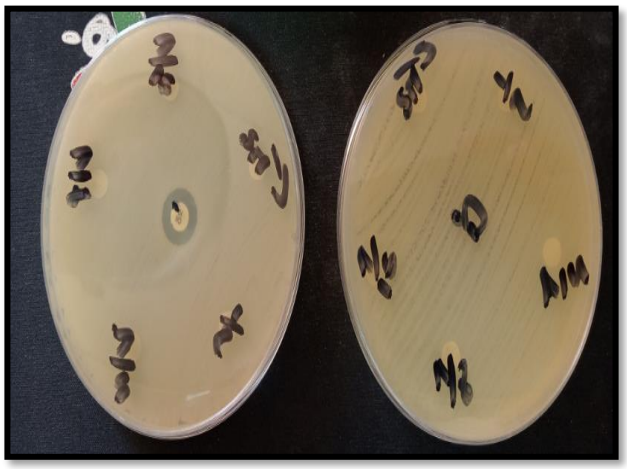

Image 05 : Klebsiella pneumoniae

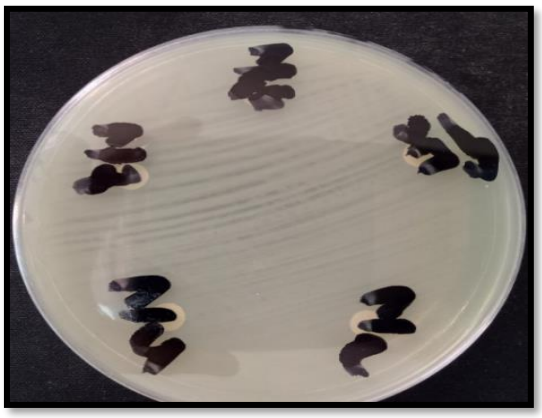

Image 06 : ***Staphylococcus aureus ATCC 43300***

Figure 13: Antibacterial activity evaluation of lactoferrin against *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* ATCC 25953, Salmonella typhi ATCC 14028 and *staphylococcus aureus* ATCC 43300 (ATCC: American Type Culture Collection/ DGRSDT**).**

4. *In vivo* studies: Evaluation of the gastroprotective effects of Lactoferrine, Casien and the whole Colostrum

4.1.Animal behaviours

The toxicological and behavioural symptoms of rats in each group after one hour of ulcer induction by oral administration of ethanol are represented in table (3). Rats in vehicle treated control were healthy and in normal physiological characteristics. While animals of the negative treated control worsened thier condition and suffered of sever pains and cramps and. Rats treated by LF and casein (CS) presented some symptoms as a decreased breathing, and fewer paining, cramping, itching, and diarrhea. Animals pretreated by colostrum had no change in their comportments as detailed in table (3).

4.2.Macroscopic evaluation and ulcer scoring

After dissection and gastrectomy, stomachs were examined by an optical magnifier to evaluate and score the ulcers. The microscopic observations are illustrated in Fig 14 and the scoring ulcer is represented in table (4).

Rats given casein, colostrum, and lactoferrin separately, had essentially identical symptoms, including redness of the stomach, ulcer patches, and hemorrhagic bands (image 9, 10 and 11), when compared to vehicle treated control (image 7). Animals of the negative treated control showed a severe symptom with a higher presence of deep ulcer and hemorrhagia (image 8).

The stomach area is less damage in the lactoferrin, casein, and colostrum groups compared to the negative treated control group.

Table 03: The toxicological and behavioural symptoms of rats.

Behaviural symptoms	Groups				
Observation	**vehicle treated control**	**negative treated control**	**Lactoferrin**	**Casien**	**Colostrum**
Normal behavior	+	-	-	-	+
Normal morphology	+	-	-	-	+
Normal breathing	+	-	-	+	+
Breathing-	-	-	+	-	-
Exophtalmos	-	-	-	+	-
Endophtalmos	-	-	+	-	-
Pains	-	+++	++++	++++	-
Cramp	-	+++	++	-	-
Normal heart rat	+	-	-	+	+
Heart rat	-	-	-	-	-
Itching	-	-	+++	+++	-
Diarrhea	-	-	+	-	-
Depression	-	+	+	-	-
Swelling	-	+	-	-	-

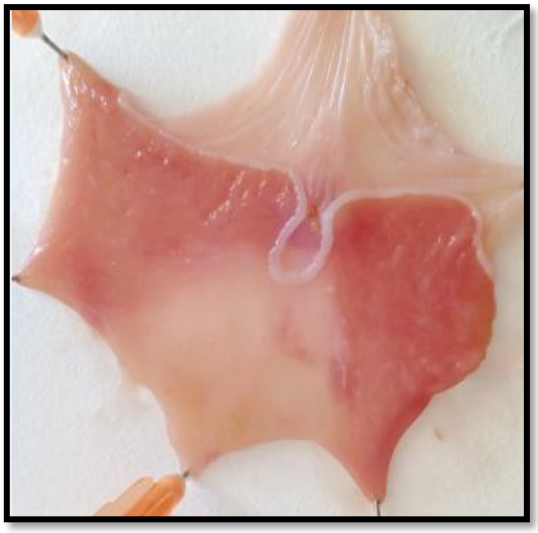

Image 7: Vehicle treated control group

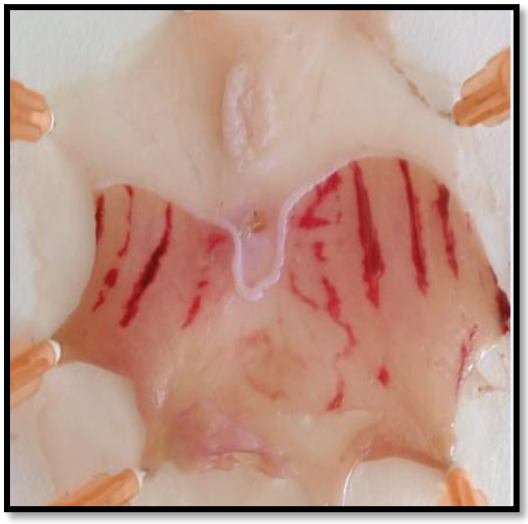

Image 8: Control negative treated group

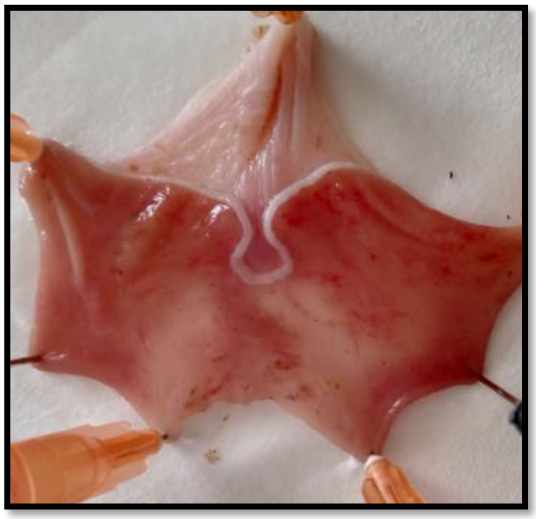

Image 9 : Lactoferrin treated group

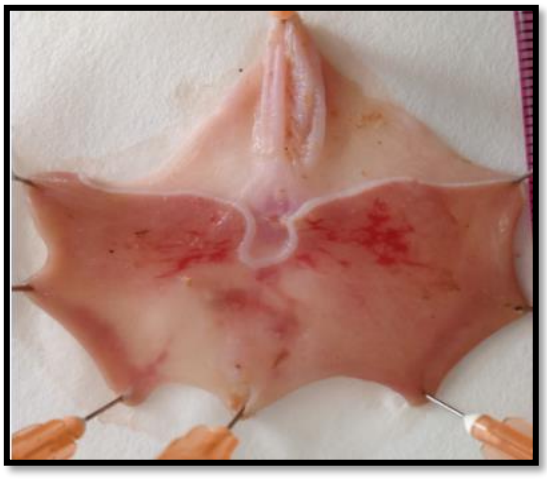

Image 10 : Casien treated group

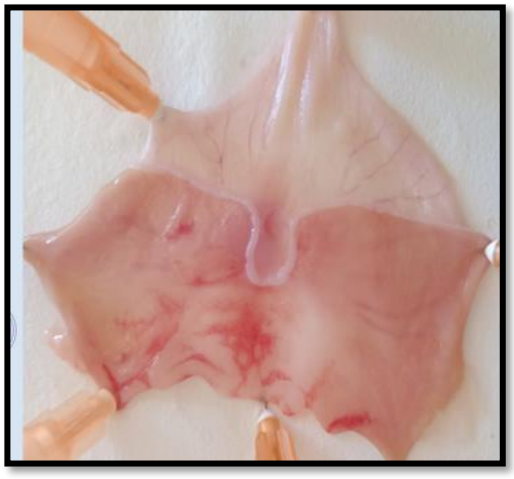

Image 11 : Colostrum treated group

Figure 14: Macroscopic observation of stomachs.

4.3. pH and total acidity

We note that the pH in lactoferrin, casein and colostrum groups are significantly increased ($p< 0.01$) compared to the negative treated control, while they are very close compared to the vehicle treated control.

The total acidity is significantly reduced ($p< 0.001$) in the pre-treated groups compered to the negative treated group. While it is significatively very high ($p< 0.001$) compared to the vehicle treated control.

4.4. Ulcer index

In comparison to the negative treated control group, the lactoferrin, casein, and colostrum group has a significant lower stomach ulcer index ($p< 0.001$).

The stomach ulcer area is less damage ($p< 0.001$) in the lactoferrin, casein, and colostrum groups when compared to negative treated control group.

Table 04: Effect of oral treatment of ethanol on mucus gastric. The values are mean ± standard deviation (n = 3). ** p <0.01; ***p <0.001, compared to control group.

Group	pH	Acidity (%)	Mucosal area (mm)	Ulcer area (mm)	Score	Ulcer index
vehicle treated control	3,50±0,46	35,00	875,77±146,2	0,00	0,00	0,00
negative treated control	1,99±0,53***	82,50±34,06***	948,54±42,61**	128,62±74,98***	3,25***	1,36±0,55***
Lactoferrin	3,93±0,98**a	31,50±4,04***a	800,41±73,14**a	4,74±2,45***a	2,25**a	0,06±0,04**a
Casien	4,08±0,26***a		832,65±91,18**a	17,67±9,77***a	2,00**a	0,22±0,12**a
Colostrum	6,91±0,46***a	42,50±2,89***a	711,86±130,2***a	9,46±4,75***a	2,00**a	0,13±0,08**a

4.5.Percentage of healing and ulcer inhibition

We note that the percentages of stomach ulcers inhibition and Healing are significantly higher ($p< 0.001$) in the three groups treated by lactoferrin, casein and colostrum compared to the negative treated control. Results are mention in table (5).

Table 05: Percentage of healing and ulcer inhibition. The values are mean ± standard deviation (n = 3). ** p <0.01; ***p <0.001, compared to control group.

Group	Inhibition (%)	Healing (%)
vehicle treated control	0,00	100
negative treated control	0,00	86.4***
Lactoferrin treated control	95,47	99.4**a
Casein treated control	84,01	97.87**a
Colostrum treated control	90,27	98.67***a

4.6.Hematological parameters analysis

The results obtained when rats were treated with lactoferrin, casein and colostrum showed a significant increase ($p< 0.01$) in blood parameters as RBC, HB and HCT were compared the negative treated control. We note that the number of PLT was in normal proportions, what was observed significatively mended ($p< 0. 1$) in the lactoferrin and colostrum treated groups compared to the vehicle treated control.

A very significant decrease ($p< 0.01$) was observed in HB and HCT and especially in PLT levels in rats from negative treated control group compered to vehicle treated control group.

Table 06: Hematological parameters analysis. WBC: white blood cells; RBC: red blood cells; HB: hemoglobin; HCT: hematocrit; PLT:platelet. The values are mean ± standard deviation (n = 3). ** p <0.01; ***p <0.001, compared to control group.

	WBC (10^3/µL)	**RBC (10^6/µL)**	**HB (g/dL)**	**HCT (%)**	**PLT (10^3/µL)**
vehicle treated control	n. d	8,65±1,67	16,1±13,47	47±0,057	935±168,58
negative treated control	n. d	7,895±0,43*	14,35±0,86***	40,35±2,48**	747±20.,47***
lactoferrin treated control	4,15±0,40	9,9±1,19***a	20,25±1,67***a	45,2±0,89**a	988±79,67**a
Casein treated control	5±3,117	8,485±0,13**a	15,95±0,057*a	46,75±0,86**a	602±18,47*a
Colostrum treated control	5,3±0,230	9,425±0,109***a	18,6±0,461**a	51,25±0,519***a	1043,5±9,81***a

4.7.Histopathological study

The results of histological photomicrography were clear witness that the stomach is healthy is characterized by normal thickness and histological architecture for the vehicle treated control (image 12), while the negative treated control was characterized by necrosis for mucosa cells (image 13). The results of histological microscopy of the treated vehicle showed that the stomach was in good health and had a normal thickness and tissue structure.

While microscopic imaging of the stomach of the lactoferrin group was characterized by cracks and cavitations (image 14).

The microscopic observation of the stomach sections from indicates that it is distinguished little damage of surface mucous, normal thickness and arrangement in the casein group, it was distinguished crack ulceration of mucosa (image 15), as for the colostrum group (image 16).

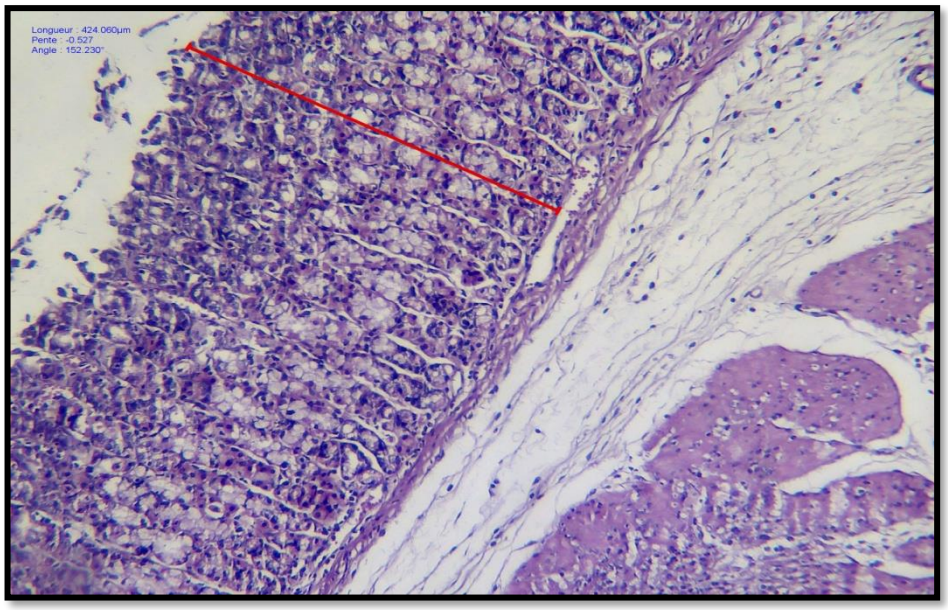

Image 12: Histological photomicrography of vehicle treated control group stomach (X100)

Normal thickness and histological architecture.

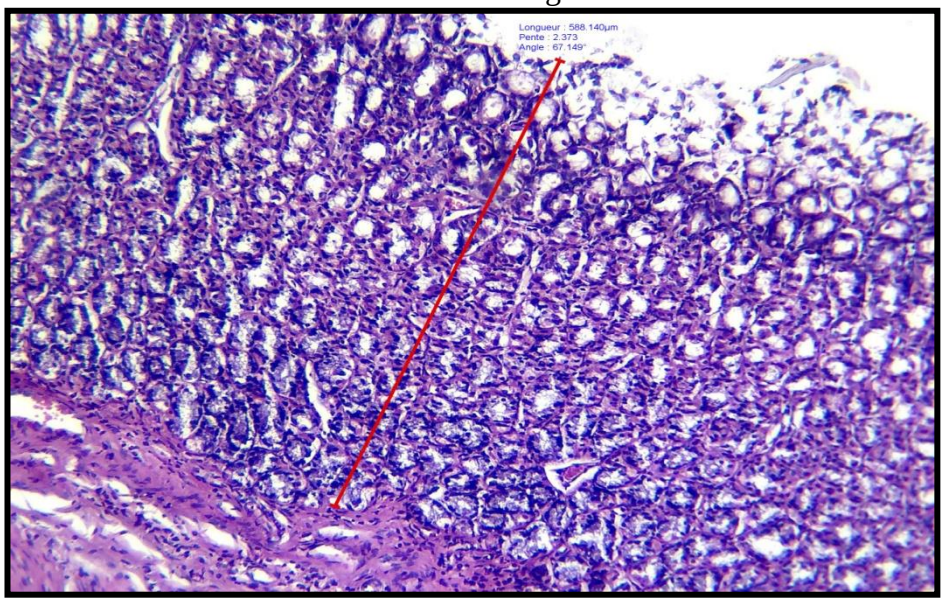

Image 08: Histological photomicrography of negative treated control group stomach (X100)

Necrosis for mucosa cells.

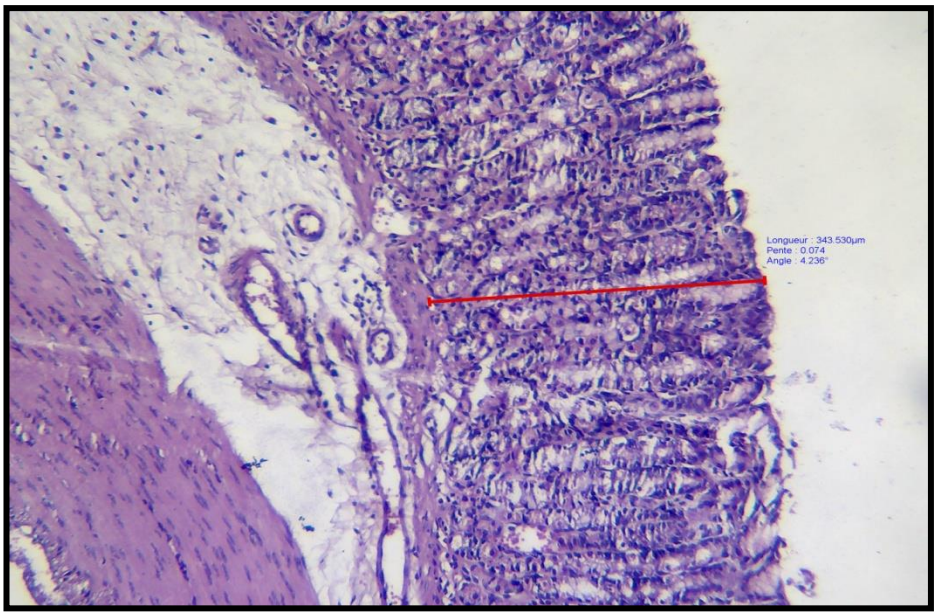

Image 13: Histological photomicrography of lactoferrin treated group stomach (X100)
Crack and cavitations.

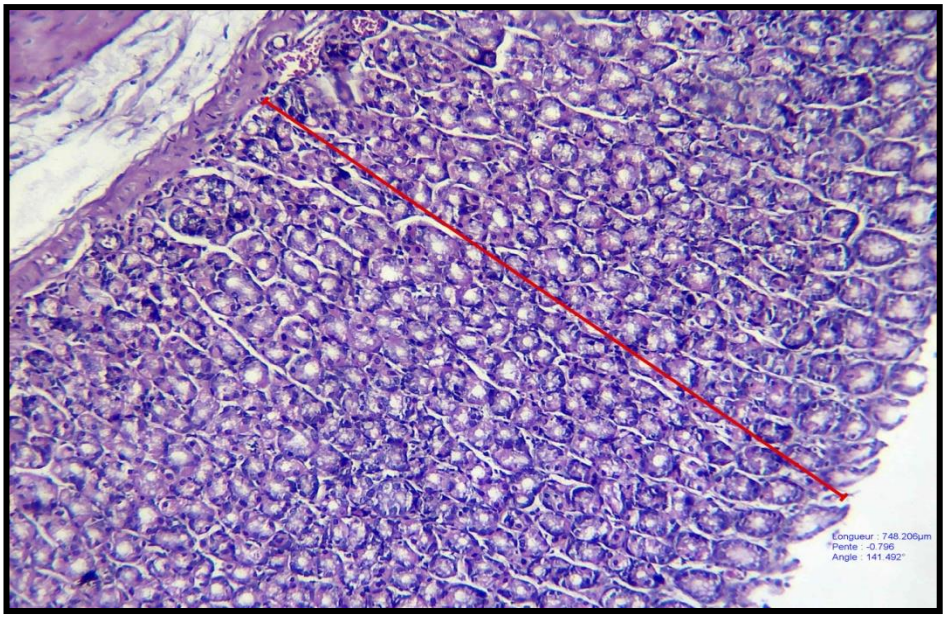

Image 14: Histological photomicrography of casein treated group stomach (X100)
Crack ulceration.

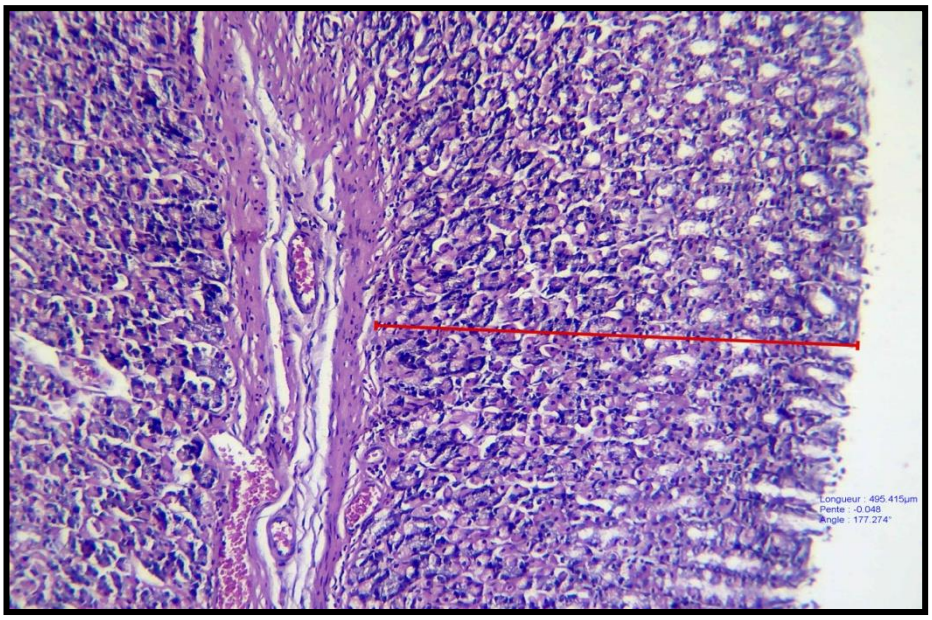

Image 11: Histological photomicrography of colostrum treated group stomach (X100)
Little damage of surface mucous, normal thickness and arrangement.

Conclusion

Conclusion

LF was found to be an ideal nanocarrier for some hydrophobic therapeutics because of its active targeting potential due to overexpression of its receptor on the surface of many cells. Receptors for LF can be found on intestinal tissue, monocytes/macrophages, neutrophils, lymphocytes, platelets, and on certain bacteria.

A wide spectrum of functions is ascribed to LF. These range from a role in the control of iron availability to immune modulation.

Through this work, we were talented to achieve an efficient extraction method and satisfactory results by obtaining a significant yield of LF with a high purity rate.

The study also proved the efficacy of colostrum and its components LF and casein in reducing and inhibing gastric ulcers in adult rats induced by ethanol.

The administration of LF, casein and colostrum during three days results an important enhancement in the hematological parameters specially HB and HCT rate.

Lastly, the histopathological study proofed the capacity of colostrum and its derived LF and casein to protect the stomach architecture and the mucosal thickness against ethanol-induced ulcer.

More researches are necessary however to obtain clarity with regard to the exact mechanism of action of LF.

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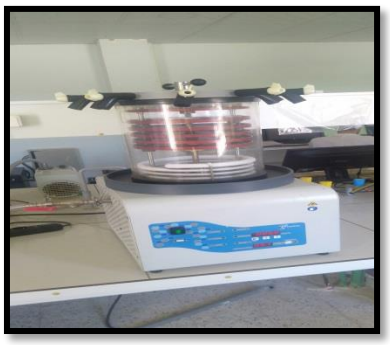
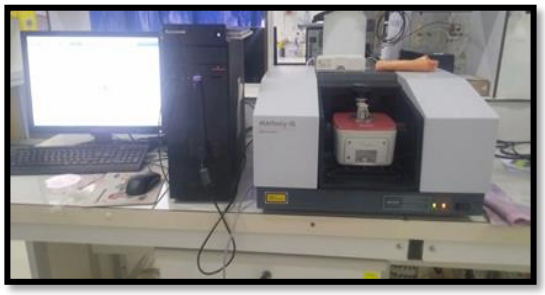
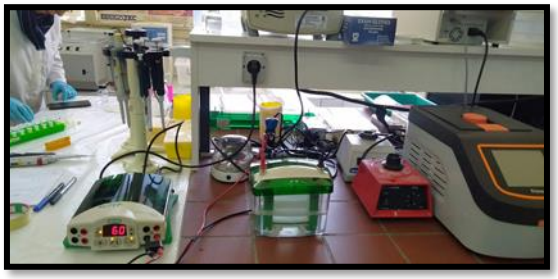
Annex 01

Table represents the used equipments (original photo)

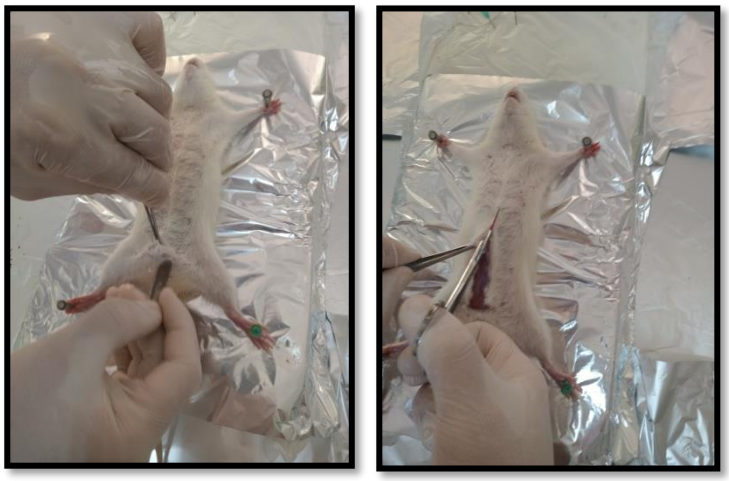
	
Lyophilized (Cryotec ALPHA 1-2LDplus /101521,2006).	**Infrared spectroscopy (FTIR) (Fourier-transform).**
	
SDS- Page electrophoresis (Gelanalyzer software).	**Centrifuge (DLAB).**
	
pH- meter (BANTE).	**Sensitive balance (æADAM).**

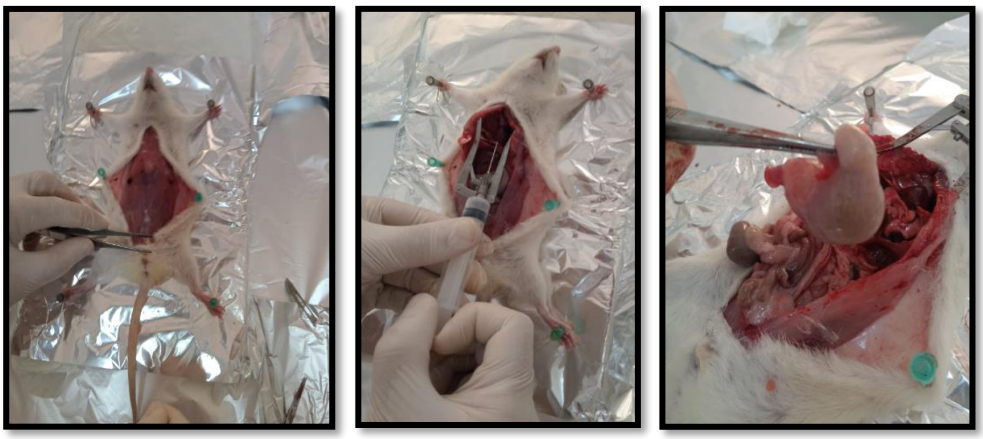
	
Spectrophotometer (Model 4251/50).	Magnifying glass (CARL ZEISS JENA).

Annex 02

***In vivo* (original photo)**

Force feeding technique





Stage of the dissection and gastrectomy process

Annex 03

Histopathological examinations procedure

1. Material

Dehydration machine

Cassettes

The scissors

Knives

The scalpel

The clamps

The tape measure

Slide and coverslip

Refrigerated plate

Coverslip

Slides

Tray

2. Devices

Microtome

Optical microscope

Thermoelectric group (TGE)

3. Products

Alcohol

Paraffin

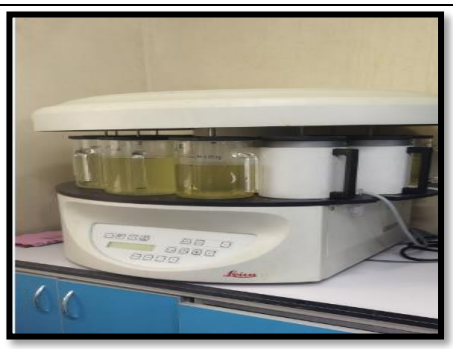
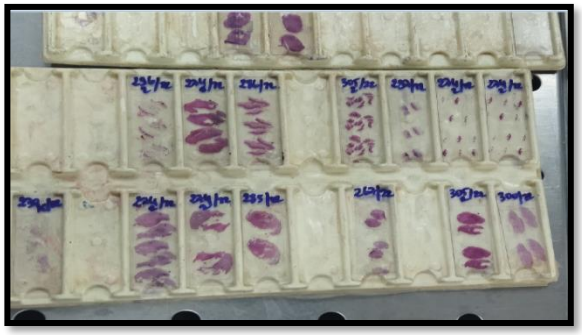
Xylene

Formalin

Blotting paper

Hematoxylin eosin

Optical grade glue

	
Rotary microtom (Leica DM 1000, Germany).	**Preparateur de tissu (Leica DM 1000, Germany).**
	
Dehydration device (Leica DM 1000, Germany).	**Histological sections (Leica DM 1000, Germany).**

University year : 2021-2022	**Presented by:** BENMEZDAD Imene FERNI Ahmed ZOUACHE Afnane

Extraction process, characterization and biological activity evaluation (*in vitro* and *in vivo* studies) of lactoferrin from bovine colostrum coproduct

Thesis presented in view of obtaining the Master's degree in Toxicology

Abstract :

Lactoferrin (LF) is a transferrin family glycoprotein with many biological and functional properties. It has a large range of uses especially in pharmaceutic and cosmetic industry.

The purpose of this research is to develop a process prototype of LF separation from bovine colostrum, and to evaluate its productivity in terms of yield and purity using new technological methods. Correspondingly, this research aimed also to evaluate the colostrum and its derivatives (LF and casein) activity as an antiulcer (*in vivo*) and as an antibacterial (*in vitro*).

Initially, LF is extracted from whole colostrum by sulfate ammonium adhesion followed by adsorption on a solid support, then elution by an acid solution. The product extracted is purified, dia-filtered and then lyophilized.

The characterization analysis of the obtained LF was carried out by electrophoresis (SDS-Page) and spectrophotometric (FTIR) methods, following to the quantification of proteins amount using Bradford's assay.

LF extracted has been tested *in vitro* in terms of its anti-bacterial activity using several clinical strains by disk method.

During work, we have extract casein and conserve colostrum to investigate their biological activity in parallel with the extracted LF. In this context, a complementary *in vivo* study intended to evaluate their anti-ulcer activity in adult female rats, following an ulcer induction by oral dose of ethanol.

Our result shows the quantitative and qualitative efficiency of our developed prototype of LF extraction process. The *in vitro* study results show no anti-bacterial effect of LF at the selected dose.

On the other hand, results show that the extracted LF and casein as well as the whole colostrum have a great gastro-protective activity, proven by their ability to inhibit the ulcer, to normalize the acidity of gastric juice, and to increase the percentage of healing. All the three pre-treatments demonstrate an improvement in hematological parameters Hb, HCT. etc.

Lastly, Macro and microscopic observations prove the anti-ulcer effect of LF, casein and especially colostrum by protecting the mucus gastric against ethanol-induced ulcer.

Other complementary studies are necessary to evaluate the hepatic and nephro-protection of LF by measuring oxidative stress parameters. etc.

Key words: Lactoferrin; Colostrum; Extraction process; Electrophoresis; Ulcer

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