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Lactoferrin Production from Bovine Milk or Cheese Whey

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ABSTRACT

Today, the industrial scale production of lactoferrin is carried out in one step by extraction from bovine milk or whey. As the role of lactoferrin in the milk is to protect the liquid against the bacterial contamination binding the lipopolysaccharides (LPS) of those bacteria, it is not surprising that the lactoferrin extracted from milk is covered by bacterial LPS, losing the most part of its biological activities. It is absolutely crucial that the production of Lactoferrin consists to a two steps process. The first step consists to extract from milk or from whey a solution that we called lactenin which contains different molecules including lactoferrin, lactoperoxidase, angiogenin and some other minor components. The second step consists to purify the lactoferrin from the other components including the LPS. Only under such conditions, we could recuperate a high level pure molecule with all its biological activities as it is not done actually.

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Introduction

Lactoferrin (Lf) is a single chain, iron-binding glycoprotein of the transferrin family that is expressed and secreted by glandular cells and found in the secondary granules of neutrophils from which it is released in infected tissues and blood during the inflammatory process.

Initially described as an iron-binding molecule with bacteriostatic properties, Lf is now known to be a multifunctional or multi-tasting protein. It is a major component of the innate immune system of mammals. Its protective effects range from direct anti-microbial activities, against a large panel of microorganisms including bacteria, viruses, fungi, and parasites, to anti-inflammatory and anti cancer activities. While iron chelation is central to some of the biological functions of Lf, other activities involve interactions of Lf with molecular and cellular components of both hosts and pathogens. Combined with *in vitro* and *in vivo* data, the powerful antimicrobial activities, immunomodulatory properties and prevention of septic shock, anti-carcinogenic functions and its growing importance in iron delivery and bone growth, make the Lf a very promising and fascinating molecule for health applications.

Since the first industrial production of lactoferrin and lactoperoxydase built in 1985 by the company Oléofina (Belgium) in collaboration with Dr Prieels and Dr Perraudin, we have noted that the number of research programs devote to the identification of the biological activities of the Lf have particularly increased. In fact, as it progressed with the number of industrial production units was increasing, the manufacturers and other business men appealed to the researchers of Universities to perform researches either by through a thesis, or by through a post-doctorat and that's how several researchers became interested to work on the Lf. That is how since 25 years, more and more scientific papers have been published, believing that each scientist wished to make a contribution about this subject. In 1993, the first International Lf Conference was created and under the control of the scientists from universities, the Congress passes off every two years, showing the synthesis of the scientific works which have been performed during the last two years.

However, in 1985, having few informations about the biological properties of the Lf, it was very difficult to characterize the molecule by specifications. At that time, to establish the first specifications, Dr Prieels, Dr Perraudin (from Oléofina) and Dr Tomita (from Morinaga Milk Industry) had to limit the parameters of the specifications to the purity and to the iron content between 14.5 µg to 30 µg iron per 100 mg Lf. The parameter of purity should have to be superior to 95% in the worry to do not introduce other contaminating molecules and the parameter of iron content was based on the bacteriostatic activity of the molecule as it was described by Bullen in 1975 [1]. So, less iron contained by the Lf, more it had to be active. The other parameters were classic for such product as the protein content, ash content and moisture content and of course the bacteriological level in presence with the molecule in powder form. Although, the results of other experiments had been published in the scientific literature as the synergy between lactoferrin and lysozyme, the first results about the crystallography and on the identification of the amino acids sequence, nothing allowed us to find the link to identify the molecule manufactured industrially versus the molecule produced in the laboratory by different academic institutes [2-4]. Beyond, since 1985 and follow to all results obtained by the research programs, is it not surprising to note that there is a scientific gap is widened during all these years between the industrials and the scientists, not because the interests that they bring to the Lf but on the definition that they combine to the Lf. As we can observe in the figure 1, can we think today that the specifications defined 25 years ago correspond to the biological properties of the Lf, which

have been highlighted during all these years? If the manufacturers have taken advantages of the results of the research to promote their Lf, it is surprising that they have not tried to assess one or more biological properties of their product in order to define their own specifications (figure 1).

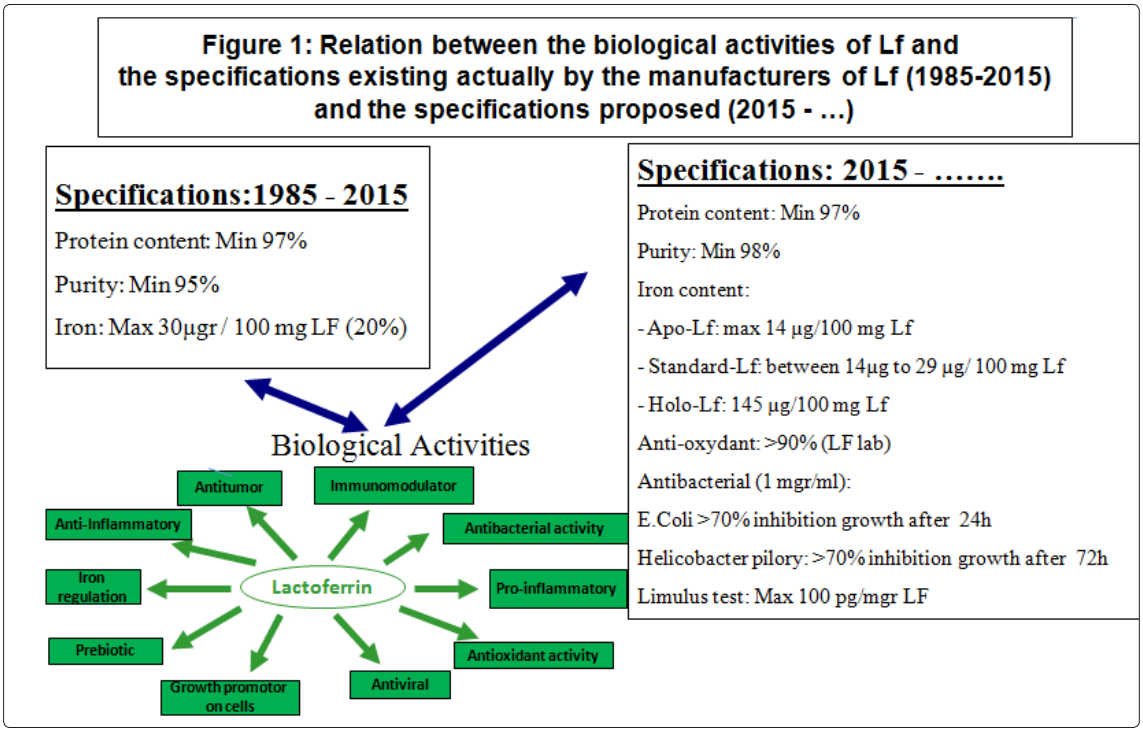

Figure 1: Relation between the biological activities of Lf and the specifications existing actually by the manufacturers of Lf (1985 – 2015) and the specifications proposed (2015 - ….)

In fact, today there are several points of disagreements between the quality of the Lf produced in industry and the Lf purified and studied in the laboratory.

Industrial productions

Taking into account that the Lf concentration in the milk (+/- 150-200 mg/liter) or in the whey (+/- 50-75 mgr /liter), there are mainly the milk cooperatives who present all the advantages for the industrial production of Lf from their raw material. So commercial lactoferrins are usually produced from milk or from whey by one ion-exchange chromatography, especially cation-exchange chromatography follow by a tangential-flow membrane filtration as it is described by Tomita and his collaborators in 2002 and in the figure 2 (red part) [5]. Since you pass the milk or the whey continuously through a chromatography column containing a cationic ion exchange resin, a mixture of milk basic proteins contained in the raw material, will bind on the resin by ion exchange effects. It is certain that the composition of this mixture will depend of the active part of the chromatographic support. Nevertheless, the part which interests all the milk manufacturers is the Lf. So since a certain quantity will be bound on the resin, we will tempt to eliminate all the other components to obtain a Lf with a purity level close to 95%, then we will eliminate the salts and we will dry it.

To obtain a good quality of the molecule, the manufacturers have supposed that the Lf considered as the most basic molecule, has an isoelectric pH sufficiently different to the other basic molecules contained in the milk. That makes easier the production of Lf but the manufacturers have not taken care if there are or not other molecules of which the concentrations, even small, could contaminate the Lf. So only the measure of the activity of one of the biological properties could give us a general idea of the quality of the molecule since we will compare the value of one of the biological activities with the value obtained by the Lf produced in the laboratory. Moreover, another parameter has been highlighted concerning the biological activity of the molecule and which consists to the interaction with the bacterial lipopolysaccharides (LPS) and whose we have to take into account for the quality and the purity of the molecule. It is the main reason that we have to follow the extraction process by a purification process (blue part of the figure 2)

Finally, one of the very important parameters is also the thermal treatment that we have to take in consideration to dry the molecule which can completely perturb the biological activities of the molecule knowing that the temperature is a sensitive parameter of the biological molecules.

Although all treatments of milk necessitate, in priority, a good quality of the raw material, that is proved to be particularly true concerning the production of Lf. What we did not know, 25 years ago, is became an obviousness today. It is very difficult to consider that the production of Lf directly from milk or from whey, that means in one step process as it is performed by all manufacturers, is sufficient to claim a quality of product to which has to be associated all the biological activities. Details of lactoferrin purification using cation-exchange chromatography are given by several authors as Plate in 2006, and van Veen in 2002 but none of these processes, nor any other existing process for commercial-scale purification of lactoferrin, are able to effectively remove other minor components, to remove the bacterial lipopolysaccharides (LPS) and to treat the molecule en function of its physico-chemical characteristics that affect its stability and its activity [6,7].

Materials and Methods

Preparation of bLF-Laboratory (LF-Lab)

Lf was purified from fresh bovine milk by cation-exchange chromatography as previous reported by Mazurier & Spik in 1980 and by Spik and her collaborators in 1982 [8,9]. Homogeneity of the protein was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The Lf was desalted on a PD10 G-25 column equilibrated in 0.01M NaCl. All buffers were prepared with pyrogen-free water. The LPS contamination of this Lf was 12pg of endotoxin/mg protein.

Preparation of bLf-NFQ

Lf was prepared from fresh bovine milk by an industrial cation-exchange chromatography (see the process in red color of the figure 2). The milk basic protein bound to the resin has been eluted with a concentrated NaCl solution. This solution containing the milk basic protein has been concentrated and desalted using an ultrafiltration membrane and has been injected in another ion exchange resin equilibrated in a buffer. For the purification, we applied in stepwise buffers containing different conductivity at different pH (see the process in blue color of the figure 2). As it is described in the figure 3, we eluted:

- some minor components with the first buffer,
- the lactoperoxidase with the second buffer,
- the LPS, protease and angiogenin with the third buffer,
- the lactoferrin with the fourth buffer.

The water which has been used to prepare all the buffers, has been distillated and treated by microfiltration, ozone (O3) and by UV 254nm. This water was pyrogen-free. The LPS contamination of this bLf was 39pg of endotoxin/mg protein. This innovative technology compatible with large-scale manufacturing practices has been developed and patented by the Taradon Laboratory company (Belgium).

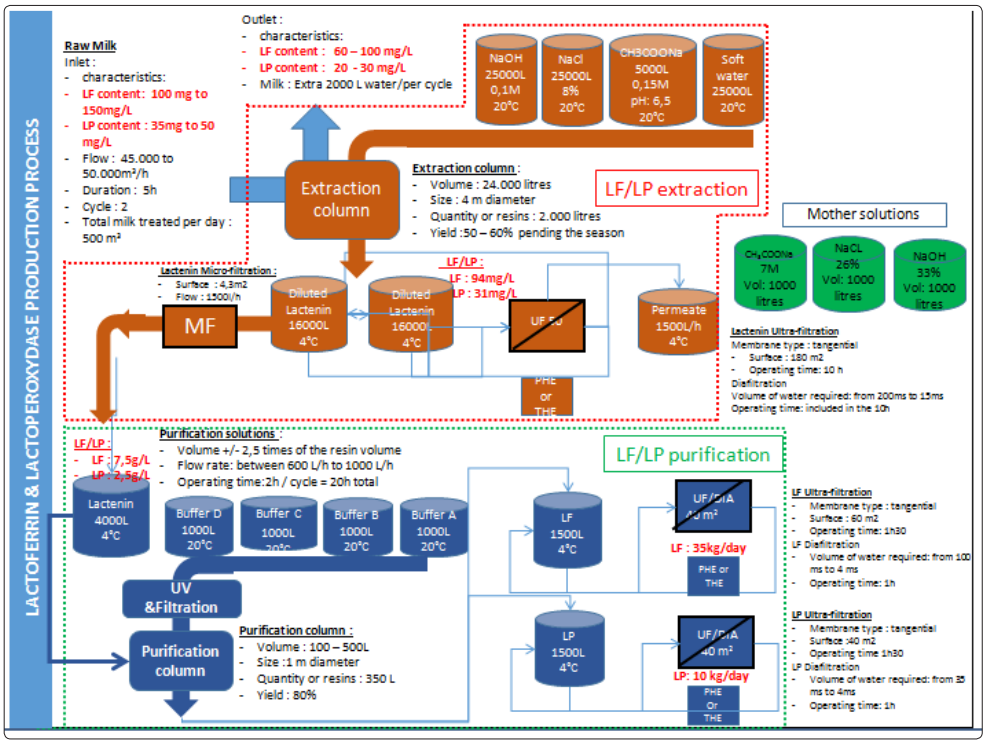

Figure 2

Lactoferrin samples

All the lactoferrin samples which have been tested, represent different commercial lactoferrin sold on the market and which have identified by their LPS content. Lf extracted from milk: 12,000 pg endotoxin/mg protein, Lf extracted from whey: 30,000 pg endotoxin/mg protein, Lf-A: 1650 pg endotoxin/mg protein, Lf-B: 22,000 pg endotoxin/mg protein, Lf-C: 105,000 pg endotoxin/mg protein

Endotoxin assay

Endotoxin levels were determined by *Limulus* Amebocyte Lysate Kit (QCL-1000, Biowhittaker, Walkersville, MD)

Cell culture

Human colon carcinoma Caco-2 cells were growth as semiconfluent monolayer in Dulbecco's modified Eagle's medium supplemented with 1.2 gr of $NaHCO_3$/litre, 2 mmol glutamine/litre, 100 U penicillin/ml, 0.1 mg of streptomycin/ml, and 20% heat inactivated fetal calf serum in a 5% CO_2 incubator at 37°C. Twelve hours before infection, monolayer were washed with PBS without Ca^{2+} and Mg^{2+} and then cultured in fresh media without fetal calf serum to avoid the presence of transferrin during infection.

Infection of host cells with E.coli HB101 (pRI203)

The method has been described by Berlutti in 2006 [10]. Semiconfluent Caco-2 cell monolayer have been infected at multiplicity of infection 100 bacteria per cell with E.coli HB101 (pRI203) either in the absence or presence of LPS free-Lf or in the presence of Lf containing different level of LPS (100 µg protein/ml). After 4 h incubation, cells were extensively washed with PBS, without Ca^{2+} and Mg^{2+}. After washing, fresh medium, containing 100µg of gentamicin/ml, was added to monolayers infected with E.

coli HB101(pRI203) to kill extracellular bacteria, and cells were incubated for a further 2h at 37°C and washed extensively. Then the monolayers were treated with 0.3 ml trypsin-EDTA mixture (0.05% trypsin (1/250) and 0.02% EDTA) for 5 min at 37°C and lysed by the addition of 0.5 ml of 1% deoxycholic acid. Cell lysates were diluted in PBS without Ca^{2+} and Mg^{2+} and plated on agar with ampicillin (100 µg/ml) to quantify the number of viable intracellular *E.coli* HB101(pRI203).

Detection of IL-6, IL-8 and tumor necrosis factor alpha (TNF-α) in Caco-2 supernatants by ELISA

As described by Berlutti in 2006, Semiconfluent Caco-2 cell monolayer were infected as described here above, either in the absence or presence of LPS free-Lf or in the presence of Lf containing different level of LPS (100 µg protein/ml) [10]. After 4h of incubation, cells were extensively washed in PBS, monolayers were added with fresh medium containing 100µg of gentamicin/ml, and cells were incubated for a further 24h at 37°C. At the end, surpernatants were collected for each wells, and the concentration of IL-6, IL-8 and TNF-α were determined using standard ELISA Quantikine kits (R&D Systems, Wiesbaden, Germany) and HBT kits (Holland Biotechnology BV, Firma Bierman, Bad Nauheim, Germany).

Antioxydant activity

The method has been described by Benzie & Strain 1999 [11]. That consists to study at low pH, the reduction of a ferric tripyridyltriazine (Fe3+-TPZ) complex to the ferrous form (Fe^{2+}), which has an intense blue color, and which can be monitored by measuring the change in absorption at 593 nm. The Ferric Reducing/Antioxidant Power Assay (FRAP) reagent has been prepared by mixing 40ml of 0.3 acetate buffer (pH 3.6), 4 ml of 20 mM ferric chloride and 4 ml of 10mM TPTZ (2,4,6-tripyridyl-s-triazine). Different dilutions (0.1 to 10 mM) of 6-OH-2,5,7,8-tetramethyl chroman-2-carboxylic acid (CAS 53188-07-1) were used as FRAP standards. All the reagents have been brought to 37°C prior to the assay. The test has been performed in a 96-well microplate by mixing 20µl of distillated water, 10µl of Lf sample, and 150µl of FRAP reagent. In combination studies 10µl of distillated water and 20µl of Lf samples were mixed with 150 µl of FRAP reagent. After instant incubation at 37°C for 5 min for ascorbic acid and for a time lapse of 5 min to 24h for Lf samples, the absorbance of reaction mixtures was measured at 593 nm. Test compounds were given antioxidant (FRAP) values compared to the FRAP value of ascorbic acid.

Antibacterial activity

Strains

- *Pseudomonas aeruginosa mucoïd and non mucoïd isolated from* Cystic Fibrosis patients obtained with the kindness of Bacteriology Laboratory - EA 3186 - Faculty of Besançon.
- *Burkholderia cepacia* isolated from Cystic Fibrosis patients (*strain LMG 16656 : ATCC BAA-245 from* Belgian Co-ordinated Collections of Micro-organisms)
- *Staphylococcus aureus* Resistant to methicillin and oxacillin *(strain LMG 15975 : ATCC 43300 from* Belgian Co-ordinated Collections of Micro-organisms)
- *Staphylococcus aureus strain* SHY97-4320 was kindly provided by the CER (Centre d'Economie Rurale – Belgium).

All culture media were from Difco Laboratories (Detroit MI) bacteria from frozen stock were streaked onto tryptic soy or brain infusion agar plates and then incubated for 20h at 37°C. Another method which consists to the turbidometric assay has been used to measure the microbial growth. The ability of Lf samples to inhibit microbial pathogens can be measured by micro-scale optical density. 0.1 ml of broth was added to each well followed by inoculation with 0.05 ml microbial cell suspension in order to reach an optical density between 1.0 and 1.5. After inoculation, the plates were incubated at 37°C and the microbial inhibition was monitored at different times as turbidity changes in the medium by measuring optical density at 600 nm using a microplate reader.

HPLC Method

Reverse phase

Resin: Bio-Rad Hi-pore RP-318, 250mm x 4,6mm

Solution A: Measure 50 ml of acetonitrile in a 500 ml flask and adjust the volume up to 500 ml with 0.5 NaCl water solution. Remove 0.5 ml of the solution and add 0.5 ml of trifluoroacetic acid

Solution B: Measure 250 ml of acetonitrile in a 500 ml flask and adjust the volume up to 500 ml with 0.5 NaCl water solution. Remove 0.5 ml of the solution and add 0.5 ml of trifluoroacetic acid

Inject 25µl of a filtered sample-solution (2mg Lf/ml).

Apply a gradient starting with A:50% and B:50% and finishing to A: 0% and B:100% with a flow rate of 2ml/min

The purity of the lactoferrin is calculated by the percentage lactoferrin versus protein content in the powder.

Ion Exchange chromatography

Resin: Mono S (Sulfopropyl: CH_2SO_3-) on a FPLC equipment from Pharmacia

Prepare a stock solution of cytochrome C (2 mgr/ml) in a 240 mM sodium acetate buffer in order to eliminate the error due to the weight and due the injections

Prepare Lf solution (10 mg/ml), diluting the powder in the cytochrome C solution.

Prepare the resin Mono S with the 240 mM sodium acetate buffer

Inject 200µl of the Lf solution

Perform the run with a NaCl gradient solution from 0.0M to 1.5M with a flow rate of 2 ml/min

Results

Lf-Lab has been produced in the laboratory and has been identified by the following parameters: LPS content, antioxidant activity, antibacterial activity, and anti-inflammatory activity. We have compared Lf-Lab with Lf-NFQ manufactured in industrial scale and other commercial Lf that we have called Lf-A, Lf-B, Lf-C, and with Lf extracted from milk and Lf extracted from whey. We have refrained from using all the commercial Lf as references dues to the diversity of their activity. In fact, some of the commercial Lf has been used to demonstrate that their production and the criteria of production are not optimal to characterize the biological functions of the Lf. All commercial Lf have different values for one of its activities and even from the same producer, the different lots of Lf manufactured have different values for the same activity.

Purity

During the industrial process, the Lf is extracted from milk or whey in presence of other Milk Basic Proteins (MBP) such as lactoperoxidase, some immunoglobulins and other components of which the concentration is dependent of the specificity of the cationic ion exchange resin. It is an easy process that consists to extract and to purify the Lf. In fact, we have the advantage that the most part of proteins and enzymes contained in the MBP are colored. The elution of the different components bound on the resin will be performed using different solutions. Using such procedures, the industrial producers consider that a purity between 90 to 92% correspond to a Lf enough pure to be used for the different

applications. However, none of these processes, nor any other existing process for commercial-scale production of lactoferrin, are able to remove totally the minor components that affect the stability and activity of the lactoferrin.

It appears that enzymes part of the minor components are present in currently existing commercial lactoferrin preparation. These enzymes are co-purified during lactoferrin purification from milk or whey. It has been found (diagram of Lf purification described in the figure 3) that existing commercial Lf contains components responsible for protein degradation, decreasing the activity and the stability of the Lf in solution (figure 4a and 4b).

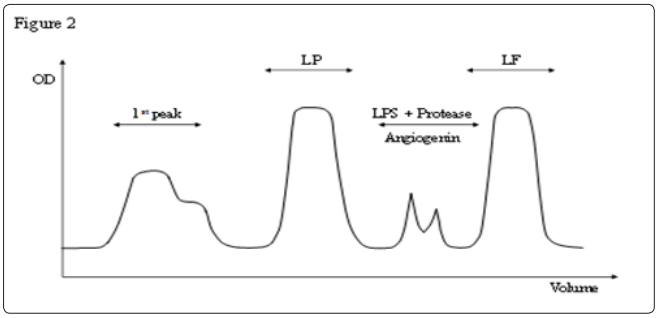

Figure 3: Chromatographic Profile of the purification process of the Lf after its extraction from milk or whey

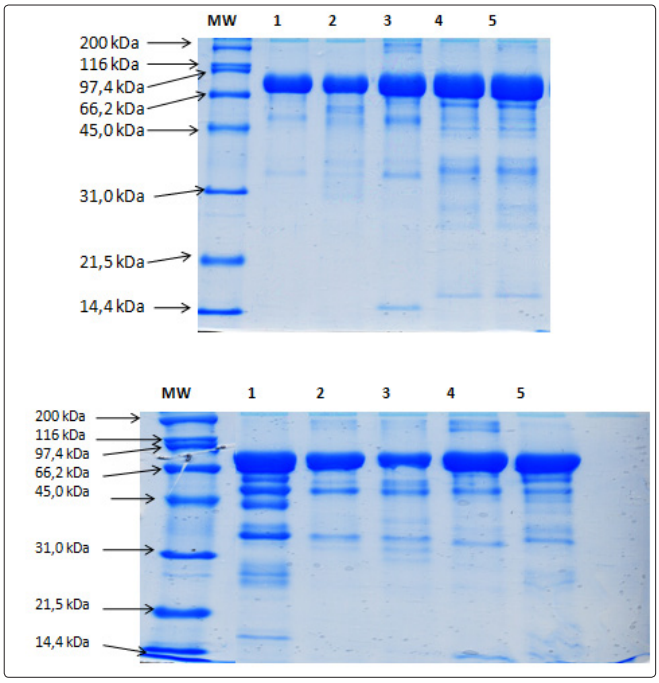

Figure 4a: Analysis at time 0:
- Markers
LF: samples
1,2,3,4 and 5
from commercial Lf

Figure 4b: Analysis after 1 month at 25°C
- Markers
LF: samples
1,2,3,4 and 5
from commercial Lf

Thinking about protein degradation, we have thought to the presence of proteases and we have tested several enzyme inhibitors. Only two of them, the aspartyl protease inhibitor (10µM of Pepstatin A) and the serine protease inhibitor (1mM of AEBSF) have shown an inhibition of the degradation bands at 4°C, at room temperature and at 37°C.

Regarding the minor components, we have also found that the angiogenin can be purified during the purification of the Lf (figure 3). This molecule has a molecular weight of 15 kDa and an isoelectric pH of 9.2 very close to the Lf. This molecule is responsible to the creation of the blood vessel to feed the cancer cells, neo-vascularization indispensable to the growth of tumors and to the development of the metastasis. During the purification of the Lf, this molecule has been concentrated at least 4 times what is certainly not beneficial for the health of the consumers.

Therefore, there is a great need for new purification and stabilization methods of lactoferrin preparations in order to remove minor components, the protein degradation and the LPS to enhance, the activity on bacterial growth and to preserve the protein stability, for a longer period of time.

Thermal treatment

Actually, it has been established by the producers of commercial Lf that the purity of Lf is determined by Reverse Phase HPLC using an acetonitril gradient. Analysing the purity of some commercial Lf, we observe that the proteic components represent around 8 to 9% versus the Lf peak. Nevertheless, diluting the same amount of commercial Lf, adjusted by the ash and moisture content, we have not found the same optical density at 280 nm. That means that some proteins eluting as Lf can increase the optical density. On the other hand, when we analyze the purity of the Lf by ion-exchange chromatography (Mono S resin - Sulfopropyl), we can notice that the peak of Lf is composed of two parts: peak A and peak B, very closed each other and corresponding for the peak A to the presence of one sialic acid content which give to the molecule a less basic behavior compared to the native one which

does not contain sialic acid (figure 5). Anyway, we can consider that the peak A and peak B are parts of the pure Lf. We can also notice in a prominent position the presence of the peak C (shoulder), which is eluted after the Lf peak. The presence of this peak cannot be detected with the use of the Reverse Phase chromatography. To understand the presence of this peak C, we have carried out the complete absorbance spectra from 280 nm to 800 nm and we have observed a band of Soret at 410 nm (figure 5) which is independent of the iron content in the Lf because this band of Soret should have to be present at a wavelength closed to the 465 nm. Moreover, the absorbance of this peak C at 280 nm is almost double to the Lf one.

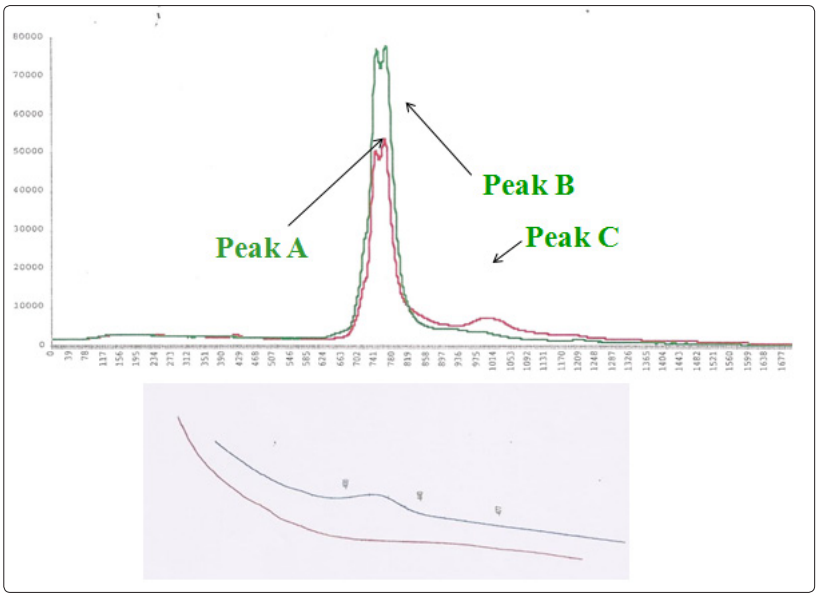

Figure 5: Chromatogram on ion-exchange HPLC chromatography of a no heat treatment native represented by the peak A and peak B compared to the chromatogram of a heat treatment native Lf represented by the peak A, peak B and peak C

Collecting only the peaks A and B, and applying again on the Mono S resin, we can notice that only the peaks A and B are present in the chromatogram without to be contaminated by the peak C. On the other hand, if we submitted the solution containing the peaks A and B to a temperature of 72°C during 5 minutes and that we analyze this solution on the Mono S resin, we observe an important decrease of the surface of the peak A and of the surface of the peak B compared to the original chromatogram but also an appearance of the peak C (figure 5). More long time, we submit Lf to a heat treatment, more the peaks A and B will have a lower surface and more the peak C will be important.

If we compare on the Reverse Phase, the chromatogram of the Lf without heat treatment and the chromatogram of the same Lf but which has been submitted to a heat treatment (72°C) during 5 min, we can notice that the surface of the Lf without heat treatment is lower that the surface of Lf having submitted a heat treatment (figure 6). The peak C has been characterized as Lf polymers having a much higher absorption power. These polymers have been also observed by SDS-PAGE gels.

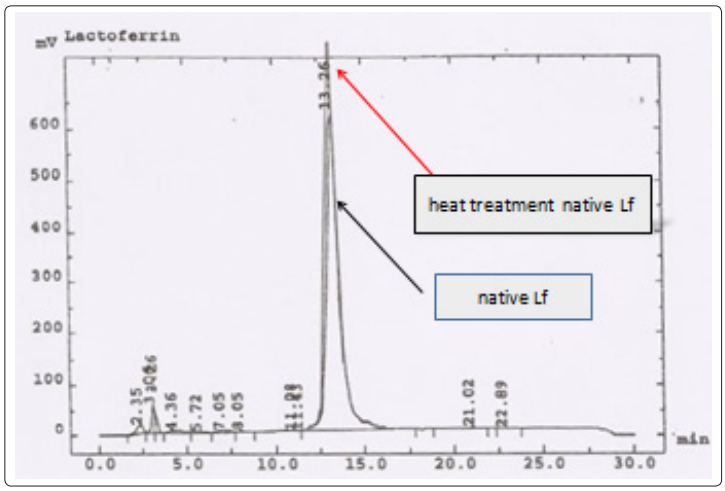

Figure 6: Chromatogram on Reverse Phase chromatography of a no heat treatment native Lf compared to a heat treatment native Lf

Automatically, when you determine the percentage of the Lf peaks compared to the minor components, the non-thermal treatment Lf has a lower purity level compared to the same Lf having been submitted to a thermal treatment and in this case, it is not surprising that the thermal treatment Lf has a much higher than 95% purity compared to the minor contaminants. That is not due to a non presence of minor components, it is due to the presence of the Lf polymers which have a higher absorbance properties than native Lf at 280 nm.

Lipopolysaccharides (LPS)

Endotoxin (lipopolysaccharides, LPS) is a predominant glycolipid in the outer membrane of Gram negative bacteria. LPS stimulates immune responses cytokine production and proinflammatory mediator secretion by monocytes, macrophages and neutrophils, which are recruited into specific host tissues by systemic LPS exposure. The response to the host to LPS is mediated by immune modulator molecules such as tumor necrosis factor α (TNF-α), members of interleukin (IL) family, reactive oxygen species, and lipids. Overproduction of those mediators induces tissue damage that precedes multiple organ failure as described by Morrison & Ryan 1987 and Rietschel in 1996 [12,13]. Structurally, Lf contains a highly basic region (Arg) in the N-Terminal region as its derivative cationic peptide, called lactoferricin which binds to a variety of anionic biological molecules including lipid A of LPS with a high affinity [14]. This LPS-binding property of Lf is considered to be part of the immunomodulatory function of Lf due to the fact that Lf inhibits LPS activity either by direct binding or by competition with lipopolysaccharide-binding-protein (LBP) for the LPS binding and therefore interferes with the interaction of LPS with CD14 [15,16].

So it is important to analyze the anti-inflammatory effect of an industrial Lf purified from milk, and containing LPS arising from the milk bacteria. To analyze this activity of Lf, we have studied its downregulation role on the expression of pro-inflammatory cytokines in infected with E.coli HB101 (pRI203) and none infected intestinal epithelial cells. For this experience, we have followed the protocol described by Berlutti, using Caco-2 cells and the sample Lf-A, Lf-B, and Lf-C having different LPS content and we have compared the results with Lf-NFQ [10]. When we infected the Caco-2 cells without the presence of Lf, we observe a significant increase in the expression of the pro-inflammatory cytokines such as of Il-6, Il-8 and TNF-α compared to the non-infected cells (table 1). In the presence of Lf, the expression of the cytokines is reduced in the case of Lf-NFQ but not in the case of the other Lf samples (table 1). We can conclude that the presence of LPS on the Lf structure inhibits its activity to dowregulate the expression of cytokines by infected cells. What it was surprising, was to observe that in case of non-infected cells in the presence of Lf containing a certain amount of LPS bound on its structure, the cells are able to induce the expression of cytokines and that this expression is dependent of the concentration of the LPS bound on the Lf structure, what was not the case for the Lf-NFQ having only 39pg LPS/mg Lf (table 1). This expression could be due to the fact that it is possible that some LPS are detached from the Lf structure due to the medium used for the cell culture and play a role as pro-inflammatory agent towards the non-infected cells. This role seems more important that the downregulation role of the Lf.

Table 1

None infected cells					
Cytokines (pg/ml)	No Lf	Lf-NFQ	Lf-A	Lf-B	Lf-C
TNF-α	44	40	105	130	165
IL-6	112	87	140	220	430
IL-8	2700	2750	3600	3650	4600
Infected cells with E.coli HB101(pRI203)					
Cytokines (pg/ml)	No Lf	Lf-NFQ	Lf-A	Lf-B	Lf-C
TNF-α	160	48	154	164	160
IL-6	1200	150	1240	1350	1300
IL-8	12250	3200	10700	10800	11500

Lf Activity

Antibacterial activity

We have performed an *in vitro* test regarding the inhibition of the growth of a Staphylococcus aureus strain SHY97-4320 in order to study the influence of contaminants. When we compare the minimal inhibitory concentrations of the Lf-Lab, Lf-NFQ and the different commercial Lf samples from the same suppliers as well as other suppliers available on the market, we do not observe the same activity. Clearly, the Lf-Lab and Lf-NFQ which show the same minimal inhibitory concentrations were more potent and did not lose their activity at higher concentration in the medium. The minimal inhibitory concentration was estimated at 1.2 mg per ml for the *Staphylococcus aureus* SHY-97-4320. None of the commercial Lf preparations available on the market have been able to display a minimal inhibitory concentration. The Lf-Lab and Lf-NFQ at 6.4 mg per ml inhibited in 24h the growth of *Staphylococcus aureus* by 96% compared to control growth. On the other hand, growth inhibition of commercial Lf preparations was observed between 69 to 79%. At higher concentration than 6.4 mg per ml, these differences in favor of Lf-Lab and Lf-NFQ were even more pronounced. Knowing a part of the source of starting material from different commercial suppliers, it seems that the Lf extracted from milk or from whey shown clearly a lost of the growth inhibitory activity at high concentration. This higher re-bounding effect of commercial Lf is due mainly to the presence of a higher amount of contaminants such as the presence of angiogenin, interfering with the activity of Lf on bacterial growth.

Another in vitro tests regarding the antibacterial activity of a culture of *Pseudomonas aeruginosa* mucoïd and non mucoïd, a *Burkhoderia cepacia* and a methicillin and oxacillin resistant *Staphylococcus aureus* have been carried out to study the influence of LPS bound

on their structure compared to Lf-Lab and Lf-NFQ. In these tests, we have observed a similar antibacterial profile activity between the Lf-Lab and the Lf-NFQ, corresponding to a decrease at least by 5 log CFU/ml during the first 5 hours after the contact of the microorganisms with the two Lf. In case of the commercial Lf (Lf-A, Lf-B, Lf-C), we have observed under the same conditions, a decrease between 2 to 3 log CFU/ml corresponding a better activity for the molecule having a less LPS concentration bound on its structure.

Antioxidant activity

When we tested the Lf-Lab and Lf-NFQ at a concentration of 0.1 mM as control, we obtain an antioxidant activity (FRAP units) with a value of 0.42 mM and 0.4 mM at 6h with a gradual increase to 0.990 mM and 0.875 mM respectively. In case of Lf extracted from milk containing 12,000 pg LPS/mg Lf, we obtain an antioxidant activity of 0.15 mM at 6h with a gradual value to 0.5mM at 24h representing 50% of the control. In case of Lf extracted from whey containing 30,000 pg LPS/mg Lf, we have obtain a value of 0.14mM at 6h with a gradual value of 0.39 mM at 24h representing 40% of the control.

Conclusions

Whether in Apo form (lf without iron) or in holo form (lf saturated with iron), we cannot say that there are several Lf, there is only one Lf which is credited with an important list of multifunctional health benefits as it is described in several scientific papers. Its purification from other ingredients contained in the milk has to require necessary several complex steps of protein engineering. On the other hand, it is important that the Lf existing in the raw material is not submitted to a heat treatment and certainly not for the Lf extracted from the raw milk. That means, we have to avoid any heat treatment such as the spray dry process and it is better to use the freeze dry or any other drying process using a low temperature treatment (<50°C). It is also preferably to do not sterilize the LF solution to eliminate the presence of bacteria and/or virus.

In 1985, when we have established the specifications, Morinaga (Dr Tomita) had insisted on the fact that it is important that the Lf had to have a purity level superior at 95%. He wanted to avoid the presence of other molecules which could be co-purified with the Lf and of which the presence could not only inhibit the biological activity of the Lf but also induce secondary effects in the newborn babies feed with baby foods containing this Lf. The presence of angiogenin, molecule having an isoelectric pH very close to the one of Lf, which has been concentrated during the Lf extraction process, is certainly not favorable to the health of the infants. Today, the commercial Lfs have a lower activity due to a too low level of purity (90 to 92%) and nobody has really characterized the identity and the concentration of the other components. The identification of the purity by the Reverse Phase HPLC is not good enough knowing that any heat treatment is going to induce the appearance of Lf polymers, increasing so the absorption power of the molecule. The best way is to combine the ion-exchange FPLC chromatography to the Reverse Phase HPLC. If we cannot detect the peak C using ion-exchange FPLC chromatography, then we could then use the Reverse Phase HPLC to determine the level of purity.

It seems quite realist to think that when Lf is extracted from milk, the molecule having used its antimicrobial activity in the milk, will be covered by bacterial LPS. If these LPS are not eliminated during the purification of the Lf, how can we think that Lf will still bind the lipid A, toxic part of the LPS and works as therapeutic agent to neutralize the effects of endotoxins. Lf could effectively reduce endotoxin influx into the bloodstream while toxins are still inside the intestinal lumen. In this process, if we ingest Lf already covered by LPS, automatically its activity may not be present in sufficient amounts to perform this function if endotoxin is continuously released in large quantities. A protective effect for Lf against lethal shock induced by intravenously administrated endotoxin has been demonstrated. Lf-mediated protection against en endotoxin challenge correlates with both resistance to induction of hypothermia and an overall increase of wellness.

Despite high purity and a low temperature treatment for its drying, the protein may harbor endotoxin contaminants which could compromise Lf functionality, so it is important to enhance the protein quality during its commercial scale production offering the highest standard quality and functional assurance to preserve its biological activities in the different applications.

Besides modulating iron homeostasis during inflammation, there is mounting evidence that Lf could directly regulate various inflammatory responses. This iron-independent mode of action is based on Lf binding to bacterial LPS, which is a major pro-inflammatory mediator during bacterial infections and septic shock. Without LPS bound on its molecular structure, the Lf could play an important role in the modulation of gastric inflammation since the protein interacts with receptors localized on gastric intestinal epithelial cells but this interaction is only possible if the molecule is free LPS. Several in vivo studies have demonstrated that oral administration of Lf could reduce gastritis induced by *Helicobacter pilory* and protect gut mucosal integrity during endotoxemia.

As described by Dr Ashida, a specific receptor for Lf has been identified in the human duodenal brush-border membranes of fetal and infant intestinal [17]. The internalization of Lf has been suggested to contribute to the cellular uptake of iron bound to Lf by intestinal cells and to the binding to a specific DNA sequences activating the transcription of a specific gene [18,19]. Moreover, the N-terminal of Lf, which is rich in basic amino acids, has been identified to be responsible for the ability of the cellular internalization and the nuclear localization of Lf. From then, the presence of LPS, located specifically on the N-terminal of the Lf will disturb, at the time of its ingestion, the binding on its specific receptor and also its internalization avoiding so the activity of the Lf concerning:

- The faster maturation of the gastrointestinal tract in the newborn,
- The cell generation and tissue repair of the intestinal mucosa in conditions as gastroenteritis
- The process of the increasing of the hepatic synthesis in the newborn, suggesting the anabolic function for Lf in the newborn babies.
- The process of the increasing of the iron absorption during the pregnancy and during the neonatal period of the babies.
- The cytotoxic increasing capacity of Lf
- The potential anti-tumor activity of Lf through its specific receptors on macrophages, T and B-lymphocytes and leukeumia cells.

Based on these results, we think that it is imperative today that the Lf industrial producers proceed to a 2nd step which consists to the purification of the Lf extracted either from milk or from whey. As it is shown in the figure 7, the chromatogram (7a) represent a typical chromatogram of commercial Lf. Although the Lf is present only in the last peak, we have demonstrated that re-purifying this Lf (7b), the molecule is totally devoid of angiogenin and of

other components including LPS. This quality is called LF-NFQ. Based on this production process (extraction + purification), it is possible to define new specifications more appropriate to the biological functions of the molecule. So, it is possible to think that the specifications (2015) as it is described in the figure 1 could represent a guarantee to the manufacturers to have produced the Lf with all its biological activities.

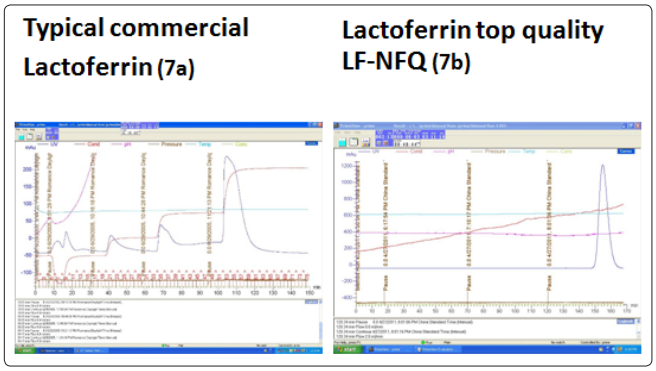

Figure 7: Comparison between the commercial lactoferrin (7a) and the purified Lactoferrin (7b)

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