UNIVERSIDAD DE MURCIA

FACULTAD DE VETERINARIA

Functionality of lactoferrin and galactooligosaccharides in infant formulas

Funcionalidad de la lactoferrina y galactooligosacáridos en fórmulas infantiles

D. Esmat Aly Alsayed Aly

2015

UNIVERSIDAD DE MURCIA

FACULTAD DE VETERINARIA

Functionality of lactoferrin and galactooligosaccharides in infant formulas

Esmat Aly Alsayed Aly

Directors:

Gaspar Ros Berruezo

Carmen Frontela-Saseta

Murcia, 2015

Dedication

To all my family: the spirit of my father, my mother, all my brothers and sisters. Especially to my lovely wife Aliaa, my daughter Rofida and my brother Tarek. To everyone who loves the justice, peace, unity, dignity and liberty.

Acknowledgement

First and before all, I would like to thank **Allah** who granted me the ability to perform this work and helped me to pass safety through all the difficulties, I thought impossible to overcome.

I would like to take this opportunity to express my sincere thanks, deepest gratitude and appreciation to my supervisors, Professors Gaspar Ros Berruezo and Carmen Frontela-Saseta, from Murcia University, Department of Food Technology, Nutrition and Food Science. This thesis would not have been possible without their advice, support and encouragement.

Faithful thanks also extended to Professors Carmen Martinez Garcia and Mº-Jesus Periago for their help, personal and scientific compartment with me throughout the present investigation. Also deepest appreciation for my best friends Rubén Lopéz-Nicolas for helping me in this work by many ways as well as for Patricia Peso-Echarri, Carlos A. Gonzales, Carols Gomez-Gallego, Inmaculada Navarro, Victoria Gomez, Rocio Gonzales, Javier Garcia, Guillermo Dominic, Marina and Javier Santaella, Gema Nieto, Amparo Lopez, Lorena Fernandez, Gala, Elvira Samper, Nieves Hidalgo and Sergio Bravo. Special and deepest thanks for Antonia Tovar Madrid (la madre del mundo) for her valuable and continual help.

Special thanks to my teachers and friends that affected my life by their extreme support and encouragement to be the person I am: Professors Ibrahim A. Abd El-Gawad, Abouelsamh M. Mehriz, Saeb A. Hafez, Ahmed M. Abdelsalam, Farag A. Saleh, Hany A. Fahmy, Elsayed Farahat, Abeer Haroun, Hanna Sayed, Mona H. Ahmed, Naglaa Hassanien, Ayman Diab, Hala Thabet, Esraa A. Mohamed, Hanan Fawzy, Mohsen Ahmed, Hassan Abdelhalim, Wafaa Salama, Mahmoud Mailam and many others I cannot count them. Special thanks to my brother Gamal and my close friend Hossam M. Ibrahim for his continuous help. Deepest and faithful thanks for my lovely wife Aliaa for continuous help and support.

Also deepest and faithful thanks for *MAEC-AECID* (*Ministerio de Asuntos Exteriors y de Cooperación- Agencia Española de Cooperación Internacional para el Desarrollo*) for financial support (grant nº 0000595994) and Hero Baby Co. (Alcantarilla, Murcia, Spain) for providing the infant formula.

ABSTRACT

The consumption of milk and dairy products is included as an important element in a healthy and balanced diet. Human milk is the most appropriate choice for newborns and provides all the energy and nutrients needed to ensure proper growth and development. The pattern of the exclusive breastfeeding during the first six months of life is very important to provide the newborn with some immunomodulatory factors and bioactive compounds that are naturally found in human milk and, therefore, it is recommended that breastfeeding continue one or even two years over the course of the introduction of some complementary foods.

Therefore, breastfeeding pattern is critically important for infant health in the early stage of life where it has been demonstrated that breast-fed infants suffer fewer gastrointestinal disorders and respiratory contaminations rather than formula-fed infants. It is scientifically accepted that control of these changes earlier by the nutritional factors may decrease or prevent the extension of these diseases to the adult life.

Moreover, researchers, health and breastfeeding organizations are trying to discover the precise substances in human milk that seem to supply physiological benefits beyond its normal nutritional value which contribute in delay, treatment or prevent some diseases. Thus, these functional ingredients hold a great promise for future trends in human nutrition. Additionally, the relationship between milk consumption and human health requires a deeper understanding to uncover the protective role of some bioactive compounds which naturally present in human milk.

These functional ingredients of human milk, particularly human milk oligosaccharides (HMOs), participate in the promotion of the growth and activity of beneficial bacteria such as *Bifidobacteria* and *Lactobacilli*. HMOs are characterized by its diversity and distinct structure. Although many attempts have been carried out to supplement the infant formulas with different prebiotics and non-digestible oligosaccharides, it could not obtain a similar structure and diversity of that of HMOs. Additionally, the presence of several proteins such as immunoglobulin (IgG), lysozyme, casein,

lactoferrin (Lf), haptocorrin and α-lactalbumin, may improve the defense of breast-fed infants against infection where they are relatively resistant in the gastrointestinal tract. It was found that Lf is characterized by it's largely ability to remain in intact form and be only partially cleaved by gastrointestinal enzymes resulting many bioactive peptides which are positively correlated with the promotion of infant health. Other functional activities were discovered for human Lf including: antibacterial, anti-inflammatory, immunomodulatory activities, and recently, anti-cancer activity.

Infant formulas, as milk substitute, play an indispensable role especially after 4-6 months of infant life where human milk is no longer sufficient to meet all necessary nutritional requirements. Although infant formulas should be similar to mature human milk in terms of its macronutrients and micronutrients, normally it do not have the functional ingredients that are found in human milk, nor do they have the same protein composition and the diversity of oligosaccharides as human milk. So it is critically important adding these ingredients to infant formulas. This evolution of infant formulas manufacturing allow to exert more functionalities in a large group of infants who cannot feed human milk as a primary source and for several physiological as well as social reasons.

For mimicking the structure of human milk, GOS (as prebiotic) and recombinant human Lf (rhLf) are strongly added to infant formulas to obtain similar functionalities for what human milk has.

Thus, the present study is aimed to explore the functionality of rhLf, rhLf hydrolysate and GOS whether alone or added in infant formulas. The present study is divided into four experiments:

- 1th experiment was to *in vitro* evaluate of the role of Lf and/or GOS on iron bioavailability as expressed as ferritin formation by Caco-2 cells
- 2nd experiment was to *in vitro* evaluate of the preventive effect of rhLf and rhLf hydrolysate on LPS-induced inflammation using co-culture gut inflammation model

- 3rd experiment aimed to assess the prebiotic activity of Lf and/or GOS using batch culture fermentation system.
- 4rd experiment aimed to *in vitro* evaluate of rhLf stability and the identification of the generated bioactive peptides as well as determination of long chain fatty acids (LCFAs) profiles before and after *in vitro* simulated gastrointestinal digestion

The obtained findings of the first study on the role of rhLf and/or GOS on Fe bioavailability measured as ferritin formation in Caco-2 model revealed that the addition of rhLf and/or GOS to infant formulas resulted in improvement of iron solubility percentage which in turn may promote iron bioavailability by using the formed ferritin by Caco-2 cells as criteria. It was found that the solely addition of GOS or rhLf improved the iron solubility percentage but non-significant differences were observed between these groups as compared with control group (without any added ingredient). It was also observed that the higher solubility percentage of iron was resulted by a combination of 0.15% rhLf + 10 % GOS (96.13%) followed by 0.15% rhLf + 5% GOS (94.13%), then 0.10% rhLf + 10% GOS (90.01%) and these obtained values significantly differed (P<0.05) as compared with the other treatments.

Regarding with iron bioavailability which was measured by the ferritin levels formed by Caco-2 cells after its exposure to the conditioned digests, the findings showed that the highest value of ferritin was found to the formula which contains 0.15 % rhLf + 5% GOS (45.83) followed by which contains 0.20 % rhLf + 5 % GOS (45.61), 0.20 % rhLf + 3.3 % GOS (43.50), 0.20 % rhLf + 10 % GOS (43.37). These data significantly differed respecting with the rest of treatments. Although ferritin expression is translationally regulated by intracellular iron concentration and its formation by intestinal cells occurs in response to Fe that has been taken up, the presented findings showed that iron solubility is not considered the only determinant factor of ferritin formation by the cultures. Thus, it is possible that another mechanism may participate in ferritin formation rather than mineral solubility. In this manner, many published studies revealed that divalent metal transporter 1 (DMT1) plays a key role in iron bioavailability.

The findings of the second study on the effect of rhLf and rhLf hydrolysate on LPS-induced inflammation demonstrated a preventive effect of rhLf and rhLf hydrolysate. This preventive effect of rhLf and rhLf hydrolysate occurred in a dose-dependent manner and rhLf hydrolysate was more effective than rhLf in prevent the LPS-induced disruption of the cell monolayer leading to decrease the cell permeability and reverse the barrier dysfunction. Findings demonstrated that 2 mg/mL of rhLf hydrolysate caused the major inhibition in TEER, IL-8 and ROS production by the inflamed cells. At the same time, nitric oxide (NO) production by the inflamed cultures did not change after treatment with rhLf or rhLf hydrolysate. It has been demonstrated that rhLf and rhLf hydrolysate can modulate the inflammatory response and oxidative stress in intestinal cells exposed to bacterial endotoxins such as LPS, thus rhLf and rhLf hydrolysate is considered a prominent factors for delay and treatment of the inflammation process. Many previous findings reported that rhLf and rhLf hydrolysate can exert its anti-inflammatory activity through its ability to modulate the production of the inflammatory cytokines as well as via TNF-α inhibition and cytokines modulation. Our findings proposed the importance of rhLf and rhLf hydrolysate where play a critical role in the inflammation treatment and the later findings related with the role of rhLf hydrolysate might start several ideas related with the ability to incorporate it as a new additive in infant formulas to improve its functionality to be close or near to what human milk has.

Respecting with the third study, the prebiotic activity of rhLf and/or GOS showed that rhLf and/or GOS increased the production of acetic acid and total short chain fatty acids (SCFAs) but no significant changes were observed and, as previously reported, acetic acid was the major fermentation end product. Propionic acid was moderately increased while butyric acid was moderately decreased after 24 h of incubation with the tested ingredients. The low level of butyric acid is related with the low numbers of the butyrate-producing bacterial groups found in human feces such as *Clostridium, Enterobacteriaceae* and *Faecalibacterium prausnitzii*

(Fpra655). Thus, rhLf and/or GOS are being able to inhibit the growth and activity of some pathogenic bacteria.

Respecting with the minor SCFAs (isobutyric, isovaleric, n-valeric, isocaproic, n-caproic and heptanoic acids), high variability was reported in its concentration among the different treatments as compared with control group and also among the time of incubation (at 10 and 24 h) with the exception of isobutyric acid which found to be increased at 24 h in all treatment as well as control group. The differences between the flora patterns predominant in the three fecal inoculums may participate in this variability of minor SCFAs concentration.

In the batch culture fermentation system, which was used in this study, pH is one of the most important factors which influences on the growth and/or activity of intestinal microflora, particularly *Bifidobacteria* and *Lactobacilli* and subsequence on the produced SCFAs. The obtained pH values in the present study decreased with the time of fermentation. This decrease in the values of pH is able to induce changes in the gut flora pattern and prevents overgrowth by pH-sensitive pathogenic bacteria like *Enterobacteriacae* and *Clostridia.* Therefore, several benefits were obtained by using rhLf and GOS related with its functionality as bifidogenic factors.

Regarding with the fourth study on rhLf stability against *in vitro* simulated digestion, it was found that rhLf is more stable than human Lf. rhLf treated with pepsin or trypsin seems to be completely degraded and only some small bands were observed by SDS-PAGE analysis. Previous results reported that pectin and soluble soy polysaccharides improved Lf stability and, in the same sense, the present results proposed that the presence of GOS may protect rhLf from the digestive enzymes leading to increase its stability against *in vitro* digestion. Therefore, several studies are needed to discover this hidden character of some prebiotics. In general, Lf stability may be affected by several factors which must be taken into account such as pH, the used enzymes, maturity of the digestive system, the incubation period and the presence of some material such as phospholipid or prebiotics. The use of HPLC technique for determination of the chromatographic analysis of rhLf-derived peptides confirmed the obtained

results of SDS-PAGE analysis where it was found a similar peptidic pattern for rhLf hydrolyzed by trypsin and pepsin. Trypsin has a higher ability to generate fragments detectable by this method than pepsin. It seems that pepsin had the ability to cleavage the protein at various sites of amino acids but trypsin cleaved rhLf at two positions which are arginine (R) and lysine (K). These rhLf-derived peptides might possess many functional activities as the intact protein or even are more active. Respecting with the effect of *in vitro* digestion on long chain fatty acids (LCFAs) profile, the most prominent finding which reflects the importance of breastfeeding pattern rather than bottle feeding pattern concerning with its positive role in supporting the visual and cognitive development in newborns and infants where most of free fatty acids in human milk increased as affected by *in vitro* digestion as compared with infant formula fatty acids which disappeared after *in vitro* digestion.

Overall, the results obtained from this study highlight and confirm the functional activities of rhLf, rhLf hydrolysate and GOS whether as infant formulas additives or alone. Likewise, through the presented findings it was demonstrated that these functional ingredients may be behave as human milk ingredients in the improvement of iron bioavailability, prevent LPS-induced intestinal inflammation and decrease the growth and/or the activity of pathogenic bacteria. As well as, it was found that rhLf is more stable than hLf and more bioactive peptides were generated by trypsin as compared with pepsin. However, similar peptidic pattern was observed for rhLf whether treated with pepsin or trypsin. *In vitro* digestion might increase LCFAs released in human milk rather than infant formula. The presented findings has a great importance at industrial level, these results may open a more specific field of research with the food industry to improve the formulation of infant formulas in order to obtain a better metabolism and development in infants. Taken together, supplementation of infant formulas with rhLf, rhLf hydrolysate and GOS may participate in improvement its functionality which reflects on the bottle-fed infant’s health.

RESUMEN

El consumo de leche y lácteos se incluye como elemento importante en una dieta sana y equilibrada. La leche humana es la elección más adecuada para los recién nacidos y proporciona toda la energía y nutrientes necesarios para garantizar un adecuado crecimiento y desarrollo. El patrón de la lactancia materna exclusiva durante los primeros seis meses de vida es muy importante para proporcionar los recién nacidos con algunos factores inmunomoduladores y compuestos bioactivos que se encuentran naturalmente en la leche materna y, por tanto, se recomienda que la lactancia materna se prolongue uno o incluso dos años a lo largo de la introducción de algunos alimentos complementarios.

Se ha demostrado que los recién nacidos que toman leche materna sufren menos trastornos gastrointestinales y respiratorios que los bebés alimentados con fórmulas infantiles. Está científicamente aceptado que el control que unos adecuados patrones nutricionales durante los primeros meses de vida del niño pueden disminuir o prevenir la extensión de ciertas enfermedades en la vida adulta.

Además, los investigadores y las organizaciones de la salud están tratando de descubrir qué componentes de la leche humana son los que, de un modo específico, suministran beneficios fisiológicos más allá de su valor nutricional normal y que contribuyen en la demora, al tratamiento o a prevenir algunas enfermedades. Así, la investigación en estos ingredientes funcionales es realmente clave para diseñar las futuras tendencias en la nutrición humana en las primeras etapas de la vida.

Los ingredientes funcionales de la leche materna, particularmente los oligosacáridos (HMOs), participan en la promoción del crecimiento y la actividad de las bacterias beneficiosas del intestino como *Bifidobacterias y Lactobacilos*. Las HMO se caracterizan por su diversidad y distinta estructura y a pesar de que la industria alimentaria adiciona prebióticos y oligosacáridos no digeribles en las diferentes fórmulas para bebés que elabora no se ha podido conseguir, por el momento, una estructura similar a la presente en la leche humana. Además, la presencia de distintas proteínas

como inmunoglobulinas (IgG), lisozima, caseína, lactoferrina (Lf), haptocorrina y α-lactoalbúmina, puede mejorar la defensa de los lactantes contra infecciones que son relativamente resistentes en el tracto gastrointestinal. En este sentido, la Lf se caracteriza por permanecer, gran parte de ella, en forma intacta tras la digestión gastrointestinal y sólo es parcialmente hidrolizada a péptidos bioactivos que se correlacionan positivamente con la promoción de la salud. Otras actividades funcionales que son atribuidas a la Lf humana son: antibacteriana, anti-inflamatoria, actividad inmunomoduladora, y recientemente, actividad anti-tumoral.

Las fórmulas infantiles desempeñan un papel indispensable sobre todo después de 4 a 6 meses de vida de un recién nacido ya que la leche materna ya no es suficiente para satisfacer todas las necesidades nutricionales a esta edad. Aunque las fórmulas infantiles deben ser similares a la leche materna madura en términos de sus macronutrientes y micronutrientes, por lo general no tienen, ni cualitativa ni cuantitativamente, los ingredientes funcionales que se encuentran en la leche humana, ni tienen la misma composición de proteínas y la diversidad de oligosacáridos de la leche materna. Por lo tanto, es muy importante agregar estos ingredientes de las fórmulas infantiles. La evolución en la composición las fórmulas infantiles con mejoras en sus procesos de fabricación permiten ejercer más efectos positivos en un grupo grande de los recién nacidos que no pueden ser alimentados con leche materna como fuente primaria por distintas causas.

Por lo tanto, el objetivo del presente estudio ha sido evaluar la funcionalidad de lactoferrina humana recombinante (rhLf), Lf hidrolizada (LfH) y GOS en las fórmulas infantiles.

A este fin, el estudio se dividió en cuatro experimentos:

- 1er experimento, para evaluar *in vitro* la función de rhLf y/o GOS en la biodisponibilidad de hierro expresado como ferritina formada por las células Caco-2
- 2nd experimento, para evaluar *in vitro* del efecto antiinflamatorio de rhLf y el hidrolizado de rhLf en inflamación inducida por LPS mediante co-cultivo celular de un modelo intestinal

- 3rd experimento, encaminado a evaluar la actividad prebiótica de rhLf y/o GOS mediante fermentación de los ingredientes en muestras de heces de lactantes.
- 4rd experimento, evaluar *in vitro* de la estabilidad de rhLf y el perfil de los ácidos grasos de cadena larga (LCFAs) antes y después de la digestión gastrointestinal simulada *in vitro*, así como la identificación de los péptidos bioactivos generados.

Los resultados del primer estudio sobre el papel de la rhLf y/o GOS sobre la biodisponibilidad del Fe a través de la formación de ferritina en las células Caco-2 reveló que la adición de rhLf y/o GOS a las fórmulas infantiles mejora el porcentaje de la solubilidad del hierro que, a su vez, puede promover la biodisponibilidad del hierro mediante la formación de ferritina por la línea celular Caco-2. Se observó que la adición, de manera individual, de la rhLf o GOS mejora el porcentaje de la solubilidad del hierro, pero no se observaron diferencias significativas entre estos grupos en comparación con el grupo control. También se observó que el mayor porcentaje de hierro soluble se obtuvo a partir de los ensayos con una combinación de 0.15 % rhLf + 10 % GOS (96.13 %), seguido por 0.15 % rhLf + 5% GOS (94.13 %) y de 0.10 % rhLf + 10% GOS (90.01 %) y estos valores obtenidos difieren significativamente (P<0.05) en comparación con el resto tratamientos.

En cuanto a biodisponibilidad del hierro medido en función de los niveles de ferritina sintetizados por las células Caco-2 después de su exposición a los digeridos previamente acondicionados, los hallazgos mostraron que el mayor valor de ferritina se encontró en las células expuestas a la fórmula que contiene 0.15 % + rhLf 5% GOS (45.83), seguida por que contiene 0.20 % rhLf + 5 % GOS (45.61), 0.20 % rhLf + 3.3 % GOS (43.50), 0.20 % rhLf + 10 % GOS (43.37). Estos datos difieren significativamente con el resto de los tratamientos. Aunque la expresión de ferritina se forma translacional regulada por la concentración del hierro intracelular y su formación por las células intestinales se produce en respuesta a Fe que ha sido captado, los resultados de este trabajo demostraron que la solubilidad del hierro no es el único factor determinante

de la formación de ferritina por las células. Por lo tanto, es posible que otro mecanismo pueda participar en la formación de ferritina en las células intestinales. De esta manera, muchos estudios publicados han revelado que transportadores de metales divalentes (como DMT1) desempeñan un papel clave en biodisponibilidad de hierro.

Los resultados del segundo estudio sobre los efectos de la lactoferrina intacta e hidrolizada sobre la inflamación inducida por LPS bacteriano demostraron un efecto protector de rhLf y el hidrolizado de rhLf. el hidrolizado de rhLf fue más capaz que rhLf en proteger frente la rotura de la monocapa celular inducida por LPS y que conduce a disminuir la permeabilidad celular y a una disfunción de la barrera. Los resultados demostraron que 2 mg/mL del hidrolizado de rhLf causó una gran disminución en los parámetros de inflamación inducida por la LPS (TEER, y la producción de IL-8 y de ROS por células inflamadas). Al mismo tiempo, la producción del óxido nítrico (NO) por las células inflamadas no cambió después del tratamiento con rhLf o el hidrolizado de rhLf. Se ha demostrado que rhLf y el hidrolizado de rhLf puede modular la respuesta inflamatoria y el estrés oxidativo en las células intestinales expuestas a las endotoxinas bacterianas como LPS, por lo tanto, rhLf y el hidrolizado de rhLf son considerados un importante y destacado factor para el tratamiento del proceso inflamatorio. Muchos de los hallazgos encontrados dicen que rhLf y el hidrolizado de rhLf puede ejercer su actividad anti-inflamatoria a través de su capacidad de modular la producción de las citoquinas inflamatorias, así como mediante el TNF-α y nuestros resultados apuntan en el mismo sentido. Nuestros hallazgos confirman la importancia de rhLf y el hidrolizado de rhLf en ejercer un papel crítico en el tratamiento de inflamación; y los datos relacionados con el papel del hidrolizado de rhLf por lo que su consideración para ser incluida como ingrediente de las fórmulas infantiles es elevada, tiene solidez y debe ser tenida en cuenta.

Respecto al tercer estudio sobre la actividad prebiótica de rhLf y/o GOS), se encontró que rhLf y/o GOS aumentaron la producción del ácido acético y los ácidos grasos de cadena corta (SCFAs), pero no sin poder destacar cambios importantes. El ácido propiónico fue moderadamente

aumentado, mientras que ácido butírico disminuiyó después de 24 horas de incubación con los ingredientes ensayados en el presente estudio. Los bajos niveles del ácido butírico pueden estar relacionados con un bajo número de los grupos bacterianos productores del ácido butírico que se encuentran en las heces humanas, como el *Clostridium*, *Enterobacterias* y *Faecalibacterium prausnitzii* (Fpra655). Por lo tanto, rhLf y/o el GOS pueden estar inhibiendo el crecimiento y la actividad de algunas de estas bacterias patógenas.

Respecto a la menor producción de SCFAs (isobutírico, isovalérico, n-valérico, isocaproico, caproico y n-heptanoico), se observó una gran variabilidad en sus concentraciones en los diferentes tratamientos, en comparación con el grupo control y también para los distintos tiempos de incubación (a las 10 y 24 h), con la excepción del ácido isobutírico que se observó en concentraciones más elevadas a las 24 h, en todos los tratamientos, así como en el grupo control. Las diferencias entre la flora predominante en las heces fecales de los distintos donantes es, probablemente, la responsable de esta variabilidad en la concentración de SCFAs.

El pH es uno de los factores más importantes que influyen al crecimiento y/o la actividad de la microflora intestinal, especialmente sobre las *Bifidobacterias* y *Lactobacilos* y la producción de SCFAs. Los valores de pH obtenidos en el presente estudio disminuyeron durante el tiempo de fermentación. Este pH es capaz de modular los cambios de flora intestinal y evitar el sobrecrecimiento de bacterias patógenas sensibles al pH como *Enterobacteriacae* y *Clostridium*. Por lo tanto, varios beneficios se obtuvieron utilizando rhLf y GOS relacionados con su función como factores bifidogénicos.

En cuanto al cuarto estudio, que trata de la estabilidad de la rhLf frente a la digestión simulada *in vitro*, se encontró que rhLf es más estable que la Lf humana. rhLf tratados con pepsina y tripsina son completamente degradados y sólo algunas pequeñas bandas fueron observados por SDS-PAGE. Resultados de otros autores indican que la pectina y polisacáridos solubles de soja mejoran la estabilidad de rhLf, y en el mismo sentido, los

resultados obtenidos podrían indicar que la presencia de GOS puede proteger la rhLf de las enzimas digestivas contribuyendo a aumentar su estabilidad contra la digestión *in vitro*. En general, la estabilidad de la Lf puede verse afectada por varios factores tales como el pH, las enzimas utilizadas, la madurez del sistema digestivo, el período de incubación y la presencia de fosfolípidos o prebióticos. Por lo tanto, más estudios son necesarios para descubrir nuevas propiedades de algunos prebióticos.

El uso de la técnica de HPLC para la determinación y análisis cromatográfico de Lf-péptidos derivados confirmó los resultados obtenidos en el análisis de SDS-PAGE observándose un perfil peptídico similar para rhLf hidrolizada por tripsina y pepsina. La tripsina tiene una mayor capacidad de generar fragmentos detectables mediante este método que la pepsina. Parece ser que la pepsina tiene capacidad de desdoblamiento de la proteína en diferentes lugares de aminoácidos pero la tripsina escinde la rhLf en dos posiciones que son la arginina (R) y lisina (K). Estos péptidos derivados podrían tener muchas actividades funcionales, como la proteína intacta, o incluso son más activas.

Respecto al efecto de la digestión *in vitro* sobre el perfil de los ácidos grasos de cadena larga (LCFAs), se refleja la importancia de la lactancia materna en lugar de patrón de alimentación con fórmula infantil por su papel positivo en el desarrollo visual y el desarrollo cognitivo en los niños y en los recién nacidos donde la mayoría de los ácidos grasos libres en la leche humana aumenta no son afectados por la digestión *in vitro* en comparación con los ácidos grasos de las formulas infantiles que desaparecieron tras la digestión *in vitro*.

En general, los resultados obtenidos en este estudio ponen de relieve y confirman las actividades funcionales de rhLf, el hidrolizado de rhLf y GOS como aditivos alimentarios a las fórmulas infantiles, de modo conjunto o individual. Del mismo modo, a través de la presentación de los resultados se demostró que estos ingredientes funcionales se pueden comportar como ingredientes de la leche materna para, entre otros efectos más cococidos, mejorar la biodisponibilidad del hierro, evitar inflamación intestinal inducida por LPS y disminuir el crecimiento y/o la actividad de las bacterias

patógenas. Tal y como se ha demostrado, rhLf es más estable que Lf humana y muchos péptidos bioactivos son generados por la tripsina en comparación con la pepsina. Sin embargo, un similar patrón peptídico se observó para rhLf tratada con pepsina o con tripsina. La digestión *in vitro* podría aumentar LCFAs liberados en la leche materna al compararlo con fórmula infantil.

Los resultados de esta tesis tienen una gran relevancia al nivel industrial y pueden abrir un campo de colaboración con la industria de la alimentación para mejorar la formulación de las fórmulas infantiles con el fin de obtener un mejor metabolismo y desarrollo de los bebés. Consideradas en su conjunto, la administración de rhLf, el hidrolizado de rhLf y GOS a las fórmulas infantiles puede contribuir a mejorar la funcionalidad de las mismas en comparación a los niños alimentados con las formulas infantiles convencionales.

List of abbreviations

AAP	American Academy of Pediatrics
ALA	α-Linolenic acid
Apo-Lf	apo-lactoferrin
ARA	Arachidonic acid
B cells	Type of lymphocyte in the humoral immunity of the adaptive immune system, they mature in bone marrow
bLf	Bovine lactoferrin
BSA	Bovine serum albumin
Caco-2	Human Caucasian colon adenocarcinoma
CD14	Cluster of differentiation 14, gene
CpG	CpG are regions of DNA where a cytosine nucleotide occurs next to a guanine nucleotide in the linear sequence of bases along its length. CpG Also " is shorthand for "—C—phosphate—G—", that is, cytosine and guanine separated by only one phosphate
DCFH-DA	2, 7-dichlorofluorescein diacetate
DCT1	Divalent cation transporter 1
Dcytb	Duodenal cytochrome b
DHA	Docosahexaenoic acid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Di-Methyl sulfoxide
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
DP	Degrees of polymerization
DPA	Docosapentaenoic acid
e.g.	exempli gratia, means "for example"
ECACC	European Collection of Cell Culture
EDTA	Ethylenediamine tetra-acetic acid
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
EPA	Eicosapentaenoic acid
ESPGHAN	European Society for Paediatric Gastroenterology, Hepatology and Nutrition
ETE	Eicosatrienoic acid
FAME	Fatty acid methyl ester
FAO	Food and Agriculture Organization
FAO/ILSI	Food and Agriculture Organization of the United Nations and International Life Sciences Institute
FDA	Food and Drug Administration
Fe	Iron
Fe2+	Ferrous iron
Fe3+	Ferric iron

FID	flame-ionization detector
FIF	First infant formulas
FOS	Fructooligosaccharides
Fuc	Fucose
Gal	Galactose
GC	Gas chromatography
GID	Gastrointestinal digestion
Glc	Glucose
GOS	Galactooligosaccharides
GPC	L-α-glycerophosphocholine
HCP1	Hem carrier protein 1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFE	hereditary human hemochromathosis protein
hLf	Human lactoferrin
HMOs	Human milk oligosaccharides
holo-Lf	holo-lactoferrin
HPLC	High pressure liquid chromatography
i. e.	Abbreviation for id est. Latin meaning "that is,"
IDA	Iron deficiency anemia
IFC	International Formula Council
IFN- γ	Interferon gamma
IgA	Immunoglobulin A
IL	Interleukin
ILSI	International Life Sciences Institute
IREG-1	iron transporter iron regulated protein 1 = ferroportin 1
IUPAC	International Union Of Pure and Applied Chemistry
LA	Linoleic acid
LCPUFAs	Long chain polyunsaturated fatty acids
lcFOS	Long chain fructooligosaccharides
Lf	Lactoferrin
LPS	lipopolysaccharides
MEM	Minimum Essential Medium
MOHP	The Egyptian Ministry of Health and Population
NaFeEDTA	sodium ethylenediaminetetraacetate
NARMP2	Resistance-associated macrophage protein 2
NDO	Non-digestible oligosaccharides
NeuAc	N-acetylneuraminic acid
NFP	National Fortification Program
NHMRC	National Health and Medical Research Council
NNI	National Nutrition Institute
NO	Nitric oxide
PBS	Phosphate buffer solution
PGE2	Prostaglandin E2
PUFAs	Polyunsaturated fatty acids
RDI	Recommended dietary intake

rhLf	Recombinant human lactoferrin
RIPA	Radio-Immunoprecipitation Assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCFAs	Short chain fatty acids
scFOS	Short-chain fructooligosaccharides
scGOS	Short chain galactooligosaccharides
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-Poly acrylamide gel Electrophoresis
SLC11A2	Solute carrier family 11, number 2
T cells	Type of white blood cell (T lymphocytes), they mature in thymus
TEER	Transepithelial electrical resistance
TNF-α	Tumor necrosis factor-α
Tf	Transferrin
TfR	Transferrin receptor
TfR1	Transferrin receptor 1
TfR2	Transferrin receptor 2
UNICEF	United Nations General Assembly
USDA	United States Department of Agriculture
UV	Ultra violet light
WHO	World Health Organization

List of Tables

List of Figures

Table of contents

Chapter 1

General Introduction

1.1. Overview

It has been broadly acknowledged that breastfeeding is the best food for newborn during the initial six months of life. Mother's milk gives all the nutritive components to ordinary development and for the digestive states of newborn children notwithstanding build the full of feeling relationship of the mother towards the baby. It additionally contains various defensive and immunoregulatory factors that may have a valuable impact on the advancement of the newborn child's resistant framework. Breastfed babies endure less gastrointestinal and respiratory contaminations, this is particularly highlighted in the lower financial gatherings of creating nations (Cesar et al., 1999), and there is expanding confirmation of a comparable defensive impact of breastfeeding in created nations (Raisler et al., 1999). Subsequently human milk is viewed as the first and the best decision for the baby (Alles et al., 2004).

The bioactive compounds of breast milk are a large group of different kinds of molecules (proteins, peptides, carbohydrates, ...) that are naturally present in human milk which are added to infant formulas for achieving the functional effects that occur in children fed with breastfeeding (Dorca, 2008) and the interest in the presence of these bioactive substances in human milk is reinforced by its almost total absence in infant formulas (Gómez-Gallego et al., 2009).

Human lactoferrin (hLf) is one of the most important components of human milk proteins constituting about 10-15%. This protein has many positive effects on infant health where promotes the absorption of iron, has antimicrobial, antiviral and anti-inflammatory activities, is growth and proliferation factor of the intestinal mucosa, and favors the incorporation of thymidine into DNA (the latter being an independent effect of iron) (Baró et al., 2001), being also immunomodulatory and anticarcinogenic (Korhonen et al., 1998).

Other compounds of human milk are Human Milk Oligosaccharides (HMOs) which have been recognized as a new class of potent bioactive

molecules (Wu et al., 2011). HMOs are the third most abundant component of human milk (Kunz et al. 2000), and also provides functional capacity including prebiotic activity, anti-adhesive and immunomodulators. Various strategies have been used to mimic the structural complexity of HMOs (Hamosh, 1996; Oddy, 2002).

However, breastfeeding is not generally conceivable, attractive or adequate, and is therefore necessary to create infant formulas attempting to replace breastfeeding giving the necessary nutrients for optimal growth and development of infants. In these cases, infant formulas play an indispensable role in infant nutrition (Alles et al., 2004).

The creation of infant formula has developed alongside the knowledge of breast milk composition. Nowadays, companies and research centers is devoted to prepare these formulas focusing their efforts on enhancing the quality of infant formulas, not only adapting the concentration of macronutrients and micronutrients but also the composition of bioactive compounds to make it similar to human milk (Dorca, 2008).

Regarding with infant formulas evolution and how to produce a suitable nutritional substitute for infant and newborns, European Food Safety Authority (EFSA, 2012) recently accepted and approved bovine lactoferrin (bLf) as a new food ingredient. Thus, various types of infant formulas containing Lf are available in the market of many countries such as Japan and Spain (Mulder et al., 2008). Since bLf is truly diverse in several perspectives when compared with hLf (Kawakami and Lönnerdal, 1991), there is significant interest in replacing the use of bLF with hLF in products for human utilization (Conesa et al., 2010). In this way various attempts have been made to produce recombinant human lactoferrin (rhLF) from rice (Nandi et al., 2002; Rachmawati et al., 2005). Currently, supplementation of infant formula with rhLf represents an attractive application (Suzuki et al., 2003).

With the aim to mimic human milk, also prebiotic formulations, which are now added to commercial infant formula, are mixtures of galactooligosaccharides (GOS), fructooligosaccharides (FOS), inulin and polydextrose. These sugars are provided in roughly the same concentration as in human milk, but do not mimic the diversity and complexity of sugar

side chains exhibited in human milk (Sherman et al., 2009). In spite of the tremendous advancement in infant formula industry, human milk is still viewed as the best source of infant nutrition (Leung & Sauve, 2005; American Academy of Pediatrics, AAP, 2012). Many studies needed to assess the functionality of the bioactive components of human milk especially lactoferrin (Lf) and oligosaccharides whether alone or added to infant formulas.

1.2. Infant feeding

1.2.1. The breastfeeding pattern

Good nutrition is essential for the development and improvement that happens during an infant's first year of life. When developing infants are fed the appropriate types and amounts of foods, their health is promoted (United States Department of Agriculture, USDA, 2009). In such manner and as per AAP (2012), the breastfeeding is viewed as the favored decision of feeding for all infants and the exclusive breastfeeding for about the initial 6 months is key for an adequate health, followed by continued breastfeeding with introducing of some complementary foods when breast milk alone is no more adequate to meet the nutritional necessities of infants. Consequently, it has been suggested that breastfeeding ought to be proceeded until one or even two years old (WHO, 2001).

So breastfeeding is without a doubt the best type of feeding for newborns and young infants and its advantage go far beyond nutritional and anti-infective benefits (Mathew, 2004). In this respect, various studies have shown that the breastfeeding at the first months of life can decrease worldwide mortality diarrhea, respiratory illness, and other infectious disease by up to 55% (Chantry et al., 2006), and this is principally because the human milk components that are viewed as major contributors to decrease morbidity rates in breastfed infants (Newburg, 2000a). One of these major active components is Lf which has numerous healthy effects on the newborns such as the antimicrobial effects which add to the protective factor of breast milk (Story & Parish, 2008; Gifford et al., 2005; Jackson & Nazar, 2006).

Additionally, human milk contains vital and multiple immunological and anti-infective agents (Chirico et al., 2008). They include, among many

others, proteins with antimicrobial components such as secretory immunoglobulin A (IgA), lysozyme, and Lf; the last one has immune-modulating properties in addition to its well-known anti-infective properties. Oligosaccharides in human milk inhibit bacterial adhesion, further protecting against pathogens. Nucleotides and cytokines of human milk, also assist with T-cell maturation and immune system modulation, evidenced by, *e.g.*, the more robust immune response that breastfed infants exhibit after vaccination. Human milk also promotes healthful gastrointestinal microbiota (Zivkovic et al., 2011), and can actively stimulate development of the newborn's host defenses to provide continued mucosal protection after breastfeeding. Several components of human milk such as growth factors, interleukin-10 (IL-10) and also Lf can reduce the inflammatory response to stimuli in the newborn intestine (Petit, 2008; Walker, 2010). Lf as a functional human milk ingredient has been demonstrated to increase the resistance of newborns to infections and also has many biological activities that are essential for an adequate health of infants. Recently, Lf has taken more attention in regarding with some healthy activities like its role in the improvement of bone health, cancer prevention and its role as transcription factor. Lf is also able to enter a cell and to activate the transcription of specific DNA sequences and this Lf-DNA interaction is reported to be responsible for antiviral activity (Adlerova et al, 2008).

The benefits of breastfeeding have been well-documented which provides optimal nutrition and prevents common childhood diseases (Abiona et al., 2006). The importance of breastfeeding is not only providing essential nutrients to infants, but it has many health benefits for both children and their mothers (Kramer & Kakuma, 2002). Breastfeeding helps to build up a safe and full of feeling relationship between the mother and her infant and offers numerous other positive advantages. Based on the above-mentioned, breastfeeding should be actively promoted and supported as the most desirable method of infant feeding.

It is scientifically accepted that the feeding pattern can influence the composition of gut flora which differs between breast-fed infant and formula-fed infants with a higher proportion of *Bifidobacteria* species in

breast-fed infants (Harmsen et al., 2000; Alles et al., 2004; Iacono et al., 2005; Granier et al., 2013). Another difference was observed between the two types of feeding which is the higher absorption of iron from human milk as compared with feeding on cow milk or infant formula (Jovani et al., 2001) and this might partly explained by the higher concentration of Lf in human milk than bovine milk (Vorland, 1999). Likewise, the discovery of Lf receptors in the enterocytes of various species and its high affinity for Lf support this hypothesis. These Lf receptors show species and molecular specificities depending of the animal species and this would explain the high bioavailability of iron from human milk, as only hLf releases iron to the enterocyte by this mechanism (Gonzalez-Chavez et al., 2009).

1.2.2. The bottle-feeding pattern

1.2.2.1. Infant formulas: concepts and types

Although breast milk is the optimal source of nutrition for infant, infant formula and milk substitutes are considered as an appropriate alternative for infants nutrition at the first year of life when breast milk is not available, or the mother cannot breastfeed her baby (Alles et al., 2004), or newborns cannot be breastfed or cannot receive human milk (WHO, 1986).

In general, the design of infant formula is based on the composition of human milk and the current trend in infant formulas manufacturing is looking to provide not only nutritional compounds but also similar functional effects than human milk. The final aim of infant formula development is not necessarily to mimic the composition of human milk in every respect, but to achieve physiological effects as in breast fed infants (Gómez-Gallego et al., 2009).

In the European legislation, Commission directive 2006/141/EC of 22 of December 2006 on infant formulas and follow-on formulas and amending the directive of 1999/21/EC, are called "**infant formulas or formula 1**" and defined it as "foodstuff intended for special nutritional use during the first months of life and satisfying by themselves the nutritional requirements of this category of persons", whereas "**follow-on formula or formula 2** " means "foodstuffs intended for special nutritional use by

infants aged over four-six months and young children and constituting the principal liquid element in a progressively diversified diet of this category of persons" (European Commission, 2003). Table 1.1 shows the basic composition of infant formulas 1.

1.2.2.2. The design and current trends of infant formulas composition

Infant formula manufacturers are continuously looking for modifications on composition in an attempt to simulate human milk in function. Nowadays, is normal that infant formulas contain some of the functional ingredients of human milk such as prebiotic, probiotic bacteria, polyunsaturated fatty acids, Lf and nucleotides (Joeckel & Phillips, 2009). Infant milk formula is subjected to strict regulations for composition and hygiene (Koletzko et al., 2005). Nowadays, Lf (Wakabayashi et al., 2006) and GOS (Motil, 2000; Gopal & Gill 2000) are commonly added to infant formulas. The most important compounds added to infant formulas are:

Prebiotic and probiotic

Prebiotics are defined as ¨non-digestible substances in food, such as oligosaccharides, which can stimulate growth and activity of beneficial bacteria in the gastrointestinal tract, they are not digested by human gastrointestinal enzymes, hence, can enter the colon intact serving as fermentable substrates for the colonic microbiota (Gibson & Roberfroid, 1995) preferably *Bifidobacteria* (Roberfroid, 2000). Human milk contains more than one hundred different oligosaccharides structures, comprising a total concentration of 15-23 g/L in colostrum and 8-12 g/L in transitional and mature milk, that together with the other milk components are the major source of prebiotic effect (Kunz et al., 2000; Euler et al., 2005).

They are also considered an important growth-promoting bifidus factor (Kunz et al., 2000). Thus, the prebiotic oligosaccharides play a role in enhancement of the growth and activity of probiotic bacteria and this named “the bifidogenic effect” (Gibson & Roberfroid, 1995) which considered one of the most important biological indicators of the resemblance of infant formulas to human milk (Martinov et al., 2011).

Table 1.1. The basic components of infant formulas.

Components	Units	Minimum	Maximum
Energy	Kcal/100ml	60	70
Protein			
Cows' milk protein	g/100 Kcal	1.8	3
Soy protein isolates	g/100 Kcal	2.25	3
Hydrolyzed cows' milk protein	g/100 Kcal	1.8	3
Lipids			
Total fat	g/100 Kcal	4.4	6.0
Linoleic acid	g/100 Kcal	0.3	1.2
-Linoleic acid	g/100 Kcal	50	NS
Ratio linoleic/a-linolenic acids			
Lauric + myristic acids	% of fat	NS	20
Trans fatty acids	% of fat	NS	3
Erucic acid	% of fat	NS	1
Carbohydrates			
Total Carbohydrates	g/100 Kcal	9.0	14.0
Vitamins			
A	µg RE/100 Kcal	60	180
D3	µg /100 Kcal	1	2.5
E	mg a-TE/100 Kcal	0.5	5
K	µg /100 Kcal	4	25
Thiamin	µg /100 Kcal	60	300
Riboflavin	µg /100 Kcal	80	400
Niacin	µg /100 Kcal	300	1500
B6	µg /100 Kcal	35	175
B12	µg /100 Kcal	0.1	0.5
Pantothenic acid	µg /100 Kcal	400	2000
Folic acid	µg /100 Kcal	10	50
C	µg /100 Kcal	10	30
Biotin	µg /100 Kcal	1.5	7.5
Mineral and trace elements			
Iron (formula based on cows' milk protein and protein hydrolysate)	mg /100 Kcal	0.3	1.3
Iron (formula based on soy protein isolate)	mg /100 Kcal	0.45	2.0
Calcium	mg /100 Kcal	50	140
Phosphorus (formula based on cows' milk protein and protein hydrolysate)	mg /100 Kcal	25	90
Phosphorus (formula based on soy protein isolate)	mg /100 Kcal	30	100
Ratio calcium/phosphorus	mg/mg	1:1	2:1
Magnesium	mg /100 Kcal	5	15
Sodium	mg /100 Kcal	20	60
Chloride	mg /100 Kcal	50	160
Potassium	mg /100 Kcal	60	160
Manganese	µg /100 Kcal	1	100
Fluor	µg /100 Kcal	NS	100
Iodine	µg /100 Kcal	10	50
Selenium	µg /100 Kcal	1	9
Cupper	µg /100 Kcal	35	100
Zinc	mg /100 Kcal	0.5	1.5
Other substances			
Choline	mg /100 Kcal	7	50
Myo-inositol	mg /100 Kcal	4	40
L-carnitine	mg /100 Kcal	1.2	NS
Taurine	mg /100 Kcal	NS	12

NS = not specified, According to Koletzko et al. (2005).

As mentioned above that HMOs may serve as substrates for colonic fermentation, it has been shown that HMOs induce an increase in the number of *Bifidobacteria* of colonic flora in breast-fed infants, accompanied with a significant reduction in the number of potentially pathogenic bacteria (Kunz et al., 2000). Complex oligosaccharides have the ability of inhibiting the binding of pathogens to cell surfaces because they act as competitive receptors on the host cell surface, thereby preventing adhesion of a number of bacterial and viral pathogens (European Commission, 2003). Thus there are many differences in the fecal microbiota between breast-fed infants and formula-fed infants. Harmsen et al. (2000) used a new molecular identification and detection method to compare the fecal flora of breast-fed and formula-fed infants and it was reported that *Bifidobacteria* are dominant in breast-fed infants, while the amounts of *Bifidobacteria* and *Bacteriodes* spp. are similar in the feces of formula-fed infants. In this regards, Solis et al. (2010) reported that the microbiota of formula-fed infants is more diverse and contains substantial quantities of *Bacteriodes*, *Enterobaceriaceae* and *Clostridium species.*

FOS and GOS may be voluntarily added to infant formula (< 0.8 g/100 mL) in a ratio of 90% GOS: 10% FOS. The Food and Agricultural Organization (FAO) of the United Nations supports the supplementation of formula with prebiotics in infants aged five months and older, as these infants will have a mature immune system and intestinal colonization (Ackerberg et al, 2012).

Probiotics are ¨live microbial components that beneficially affect the host by improving its intestinal microbial balance¨. *Bifidobacteria* are predominant in infants fed formulas supplemented with *Bifidobacteria* and was similar to that found for breast-fed infants as compared with the control. Thus probiotic bacteria are promising component and have been used successfully in infant formulas production (Alles et al., 2004). In general, the aim of adding probiotics and prebiotics to preterm infant formula is to improve growth, development and decrease infections by promoting an intestinal microbiota resembling that of breast-fed infants (Underwood et al., 2009).

Nucleotides

Nucleotides are ¨nitrogenous compounds which play and their metabolites derivatives a key role in numerous biochemical and physiological processes, such as energy transfer processes, they are precursors for nucleic acid synthesis (DNA and RNA), and they are key to the synthesis of carbohydrates, lipids and proteins¨. Human milk contains, in free form, ribonucleotides and ribonucleosides, which account for 2-5% of non-protein nitrogen in human milk, may contribute to excellent use of the protein by breast-fed infants (Baró et al., 2001). In addition, human milk contains significant amounts of related compounds: nucleosides, purine and pyrimidine bases, nucleic acids and products derived from them (such as uridine diphosphate galactose) (Gil & Uauy, 1995).

The concentration of free nucleotides in human milk is higher than in bovine milk. Because bovine milk is most often used to formulate infant milk formulas, most milk formulas are supplemented with nucleotides to increase the concentration to a level that is similar to the concentration found in human milk (Pickering et al., 1998).

Recently, legislation allows the addition to infant formulas and follow-on formula, nucleotides in quantities of: 1.5 mg adenosin-5-phosphate/100 kcal, 2.5 cytosine-5-phosphate/100 kcal, 0.5 kcal guanosina-5-phosphate/100 mg, 1.75 mg Uridine-5-phosphate/100 kcal, 1 mg inosin-5-phosphate/100 Kcal, until a total concentration of 5mg/100 kcal, which is similar to the amounts of free ribonucleotides in milk (4-6 mg/100 kcal) (European Commission, 2003). Also in this context, Koletzko et al. (2005) reported that ESPGHAN supports the optional addition of nucleotides in amounts not to exceed 5 mg/100 Kcal as adverse effects have been seen with higher concentrations.

The supplementation of infant formulas with the dietary nucleotides will results in increased the growth of probiotic bacteria in the intestinal tract with a reduction of the pathogenic bacteria population due to the competitive exclusion. It was reported by Gil et al. (1986) that babies fed nucleotide-supplemented infant formula have increased ‘friendly’ *Bifidobacteria* counts in feces compared to infants fed standard formula milk, but counts were still lower than found in breast-fed babies. Infant

studies also suggest those receiving nucleotide supplemented formula have an improved antibody response following immunization (Schaller et al., 2004).

Polyunsaturated fatty acids

In the last two decades, special attention has been paid to the composition and physiological function of the lipid fraction in human milk. Human milk fat is the major source of energy for the breast-fed infants, contributing some 40-55% of the total energy intake. Human milk fat contains essential nutrients which are lipid soluble vitamins and polyunsaturated fatty acids (PUFAs), including linoleic acid of the n-6 series (C18:2 n-6) and α-linolenic acid of the n-3 series (C18:3 n-3). Omega-3 and omega-6 fatty acids are essential fatty acids and are an important component of human milk with a significant role in the overall growth and development of infant (Ganapathy, 2009). The components of human milk that may partly explain the observed differences are the polyunsaturated fatty acids (PUFAs): docosahexaenoic acid (C22-6, n-3; DHA) and arachidonic acid (C20-4, n-6; AA). DHA and AA are derived mainly from their precursors, α-linolenic acid (ALA, an omega-3 fatty acid) and linoleic acid (LA, an omega-6 fatty acid), respectively (Innis, 2008).

Concentrations of PUFAs in human milk are relatively stable during the first year of life: DHA is equivalent to 0.5% in colostrum and 0.25% in mature milk, which is equivalent to 7 to 8 mg/dL; AA is equivalent to 1% in colostrum and 0.5% in mature milk, which is equivalent to 14 - 15 mg/dL (Martinez, 1992). It is well established that breastfeeding pattern is associated with a better neurological, cognitive and behavioral outcome than formula feeding pattern (De Jong et al., 2010), and the prolongation of breastfeeding period was associated with a better cognitive outcome at six years (Kramer et al., 2008), suggesting that the composition of human milk plays a key role in the positive association between breastfeeding and cognitive development.

Nowadays, it is generally accepted that infants should receive at least 0.3% of both DHA and AA in infant feeding (Koletzko et al., 2008), even though higher DHA levels in formulas have been suggested to special group such as preterm infants (Makrides et al., 2009).

1.3. The nutritional needs during infancy

The quantity and quality of nutrient supply during early life modulates the differentiation of tissues and organs and has short- and long-term consequences for health (Koletzko, 2008). Table 1.2 shows the average of nutrient intakes in breast-fed infants and non-breast-fed infants from 6-12 months of age.

The importance of introducing complementary foods and infant formulas after breastfeeding is to cover all the required nutritional needs and to prevent the deficiency of any nutrient. For example, infants with high requirements of specific nutrients such as iron may benefit from the introduction of nutrient sources other than human milk prior to the age of 6 months (ESPGHAN, 2002).

Fats are the main source of energy for infants, and PUFAs especially long-chain polyunsaturated fatty acids (LCPUFAs) are essential for normal growth, development and for maturation of numerous organ systems, most importantly the brain and eye (Mena & Uauy, 2008). Human milk contains varying amounts of LA, ALA, DHA, AA, and other LCPUFAs depending on maternal intake (Koletzko et al., 2008). For infants from birth to six months of age, the adequate intake for total fat is 31 g per day, 4.4 g per day for n-6 polyunsaturated fats and 0.5 g per day for n-3 polyunsaturated fats. For infants aged seven to 12 months, the adequate intake for total fat is 30 g per day, 4.6 g per day for n-6 polyunsaturated fats and 0.5 g per day for n-3 polyunsaturated fats (Ministry of Health of New Zealand, 2008).

Also **protein** is an essential component of the diet required for infant growth. Unlike fat and carbohydrate sources, most protein is used for growth and not for energy generation. Exclusive breastfeeding meets the protein and amino acid requirements during the first 4–6 months of life. During the second 6 months of life, solid foods contribute a significant amount of protein to the infant diet (Michaelsen et al., 2000).

The adequate intake of protein for infants from birth to six months of age is 10 g per day, and for infants aged seven to 12 months it is 14 g per day (National Health and Medical Research Council, NHMRC, 2006). The recommended dietary intake for toddlers aged one to two years is 14 g per

Table 1.2. Average of nutrient intakes in breastfed infants and non-breastfed infants from the 6th to the 12th month of age.

Nutrient	Breastfed infants			Non-breastfed infants		
	6 months of age	**9 months of age**	**12 months of age**	**6 months of age**	**9 months of age**	**12 months of age**
Energy, macronutrients and dietary fiber						
Energy (KJ)	3564	5364	6142	5294	6411	7641
Protein (g)	17	41	50	41	45	61
Carbohydrate	-	-	-	-	-	-
Dietary fibers (g)	5	10	15	6	14	17
Fat (g)	27	39	48	25	34	55
Minerals						
Ca (mg)	672	1209	977	672	1209	977
Zn (mg)	2.2	3.5	6	3	4.1	6.7
Fe (mg)	11	32	16	21	21	12
Mg (mg)	56	125	216	128	209	230
Si (µg)	11	16	25	5	11	26
Sodium (mg)	310	1396	1600	8744	1888	2315
Fat-soluble vitamins						
Vitamin A (µg RE)	1290	1731	1701	943	1858	1168
Vitamin D (µg)	10	19	7	9	10	4
Vitamin E (mg ─TE)	2	4	4	3	4	3
Vitamin K (µg)	-	-	-	-	-	-
Water-soluble vitamins						
Thiamin (mg)	-	-	-	-	-	-
Riboflavin (mg)	-	-	-	-	-	-
Niacin (mg NE)	4	9	16	6	9	19
Vitamin B6 (mg)	-	-	-	-	-	-
Vitamin B12 (µg)	-	-	-	-	-	-
Folate (µg)	47	103	154	78	106	178
Pantothenic acid	-	-	-	-	-	-
(mg)	5	14	23	9	16	30
Biotin (µg)	85	132	162	75	214	131
Vitamin C (mg)	-	-	-	-	-	-
Choline (mg)						

Adapted from Simons (1999). Note: - = not measured.

day, or 1.08 g per kilogram body weight (Ministry of Health of New Zealand, 2008).

Carbohydrates can be classified to digestible and non-digestible carbohydrates and is important to remark that human milk contains both of them. Digestible carbohydrates are one of the main sources of dietary energy in infancy and childhood and are essential for growth and development (Stephen et al., 2012). The main digestible carbohydrate in mature breast milk is lactose which provides about 40% of the energy content (Koletzko et al., 2005), in addition to a large variety of oligosaccharides in concentrations of approximately 5–10 g/L (Kunz et al., 2000).

Non-digestible carbohydrates such as FOS, GOS, inulin, soy polysaccharide, resistant starch, and gums are added to dietary products, enteral formulas and human milk substitutes consumed by infants (Aggett et al., 2003), considering that the adequate intake of carbohydrates for infants from birth to six months of age is 60 g per day; and 95 g per day for infants aged seven to 12 months (Ministry of Health of New Zealand, 2008).

Although the **minerals** and trace elements are very important and play a pivotal role in infant health. The needs and role of iron for infants in the early stage of life are only discussed in this section. Healthy term infants are normally born with plenty of iron where they need a relatively high iron intake because they are growing very rapidly. But after 6 months of age, iron content of human milk is not sufficient to meet many infants' requirements, thus requirement for dietary iron increases to an estimated 0.78 mg/day due to the stepwise depletion of endogenous stores and rapid growth (Institute of Medicine, IOM, 2000). In this regard, where iron-fortified complementary foods are not widely and regularly consumed by young children, infants should routinely receive iron supplements in the first year of life. Where the prevalence of anemia in young children (6–24 months) is 40% or more, supplementation should continue through the second year of life (Stoltzfus & Dreyfuss, 1998).

The recommended dietary intake (RDI) for iron for an infant 7 to 12 months old is 11 mg per day. The recommended daily intake for toddlers aged one to three years is 9 mg per day. Absorption is about 18 percent from

a mixed western diet including animal foods and about 10 percent from a vegetarian diet; so vegetarian infants need higher intakes (NHMRC, 2006).

1.4. Iron as nutrient

1.4.1. The physiological importance and the presence of iron in diet, human and cow milk

Iron is a pivotal and essential trace element for the maintenance of the human health due to its obligate role in a number of the physiological processes (Sharp & Srai, 2007). However, in excess, iron is potentially toxic to cells due to its ability to catalyse the production of reactive oxygen species (ROS) (Steele et al., 2004). Excessive iron accumulation leads to the damage of liver, heart, pancreas and other organs. Beside systemic disorders of iron homeostasis, local mismanagement of iron plays a role in several disorders (Stankowski et al., 2012).

Dietary iron is present in two different forms: **non-heme iron** (found in cereals, vegetables, pulses, beans, fruits as simple iron oxides or complex iron chelates) and **heme iron** (mainly found in meat and meat products). Non-heme iron is predominant in all diets forming some 90-95% (Darshan & Anderson, 2007) and is found as Fe^{2+} bound to insoluble proteins, phytates, oxalates, phosphates and carbonates, and as ferritin (Scientific Advisory Committee on Nutrition, 2010), While heme-iron forms 5-10% of total daily iron intake. However, the heme-iron is the most bioavailable source of iron (20-30%) while the non-heme iron has a low bioavailability amounting of 1-10% of the dietary load (Hallberg et al., 1989).

In human milk, iron content is low 0.2-0.4 mg/L (Domellof et al., 2002) and is mainly bound to Lf (20-45%; Chierici & Vigi, 1994); while in cow milk it is mainly bound to casein (24 %) (Renner et al., 1989), which correlates well with the finding of Makino and Nishimura (1992), which reported that 95% of hLf is in the monoferric and/or apo-lactoferrin form.

1.4.2. Absorption of iron

1.4.2.1. Uptake of iron into enterocytes

Iron absorption occurs mainly in duodenum and upper jejunum, although small amounts may also be absorbed from stomach, ileum and colon (Anderson & Vulpe, 2002). At the cellular level, iron is absorbed through the differentiated epithelial cells (enterocytes) of the mid to upper villus. Iron is provided to the body in various forms through the diet, but is primarily absorbed as either inorganic (non-heme) iron or as heme iron (Lönnerdal, 2010).

The first step is termed mucosal uptake, and iron uptake is defined by Merit et al. (2003) as ¨the transport of dietary iron across the apical membrane of the enterocytes into the intestinal mucosa¨. There are at least two separate mechanisms for the uptake of heme and non-heme iron into the enterocytes (Scientific Advisory Committee on Nutrition, 2010). The passage of iron through the enterocyte into the circulation is depicted in Fig. 1.1.

In the case of the absorption of non-heme iron from the intestinal lumen to the enterocytes, Fe^{3+} (ferric iron) is first reduced to Fe^{2+} (ferrous iron), most likely by duodenal cytochrome b (Dcytb), making it available for transport across the brush border membrane by divalent metal transporter1 (DMT1) into the cytoplasm (Steele et al., 2004).

On the other hand, the absorption of heme iron across the apical membrane occurs more efficiently but the mechanism is still unclear (Steele et al., 2004; West and Oates, 2008; Le Blanc et al., 2012). The passage of heme iron across the apical membrane is facilitated by intestinal heme transporter named heme carrier protein 1 (HCP1) (Dunn et al., 2006). Latunde-Dada et al. (2006) claimed that a heme receptor was identified in piglets and human. Likewise, Dunn et al. (2007) stipulated that a receptor for HCP1 is present in a large concentration in the duodenum. Once heme iron has been taken up by the enterocyte, the heme molecule is degraded by heme oxygenase to release ferrous iron as clarified by Dunn et al. (2007). Inside the enterocyte, heme and non-heme iron enter a common transit pool, where iron may be chelated by low molecular weight compounds or bound to a protein ligand such as ferritin (Steele et al., 2004).

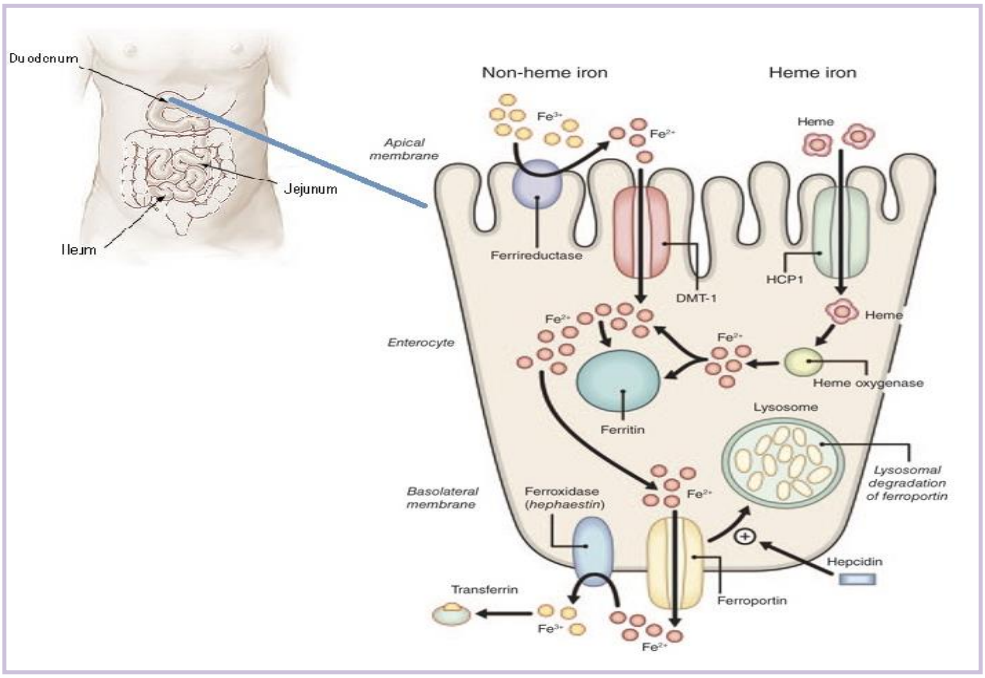


Fig. 1.1. Schematic of intestinal iron absorption (adapted from Rizvi and Schoen, 2011).

1.4.2.2. Export of iron from enterocytes to blood circulation

The second stage of iron absorption is termed basolateral or serosal transfer, where iron is transported from the enterocytes into the intestinal capillaries across the basolateral membrane, most likely via the iron transporter iron regulated protein 1 (IREG-1 or ferroportin 1) (Steele et al., 2004). The ferrous iron delivered for the basolateral membrane of the intestine is exported by ferroprotein, a metal protein transporter 1, to the plasma. The ferrous iron is oxidized to ferric iron by ferroxidase hephaestin (Dunn et al., 2007). The role of hephaestin in association with ferroprotein to oxidize ferrous iron is important before it is exported and bound to transferrin (Mackenzie & Garrick, 2005).

1.4.2.3. Transport of iron

Transferrin (Tf) is the major protein that binds and delivers iron to tissues. Each Tf molecule can transport 2 ferrous iron molecules (Hirose, 2000). Conformation of the binding site is suitable with ferric iron in a delicate manner (Harris, 1986). Transferrin binds to one of the transferrin receptor (TfR) on cell membrane; TfR1 or TfR2 (Casey at al., 1988). TfR1 is expressed in all tissues except mature erythrocytes while TfR2 is primarily expressed in the liver (Kawabata et al., 1999). Although protein

structures of TfR1 and TfR2 have a high degree of homology, their functions and regulation are not the same. Expression of TfR1 is tightly regulated by cellular iron levels through HFE (hereditary human hemochromathosis protein) protein. However, cellular iron levels have no effect on the regulation of TfR2. TfR2 senses the body iron status by sensing the transferrin saturation and regulates hepcidin expression properly (Fleming et al., 2002).

Once Fe^{3+}-Tf complex binds to its receptor at the cell surface, the Tf-TfR1 complex is internalized in clathrin-coated pits that form endocytic vesicles. Inside the cells, the internalized complex in the endosome is acidified by a vacuolar H^{+}-ATPase (V-ATPase) that lowers the luminal pH to about 5.5. This acidification process induces conformational changes in Tf-TfR1 complex with consequent release of iron (Sipe & Murphy, 1991) which transports around the body to various tissues (Ohgami et al., 2005). The main part of the iron carried by Tf is used by the formation of hemoglobin (Hoppe, 2008).

1.5. Dietary factors affecting iron bioavailability

Iron chemical state is one of the most important factors that affect on its absorption. In this context, in inorganic food compounds, iron is normally in the oxidized form (Anderson, 2002), but absorption requires reduction to Fe^{2+}, for iron enters in the mucous cell as a reduced free iron that is more easily absorbed than ferric ion (Sgarbieri, 1987). Also Conrad (1970) reported that iron physical and chemical form affects its absorption that used radioactive markers and concluded that hemoglobinic iron is more efficiently absorbed than inorganic iron. Also Anderson (2002) reported that iron has a greater availability when present in the form of iron sulfate than in salts such as sulfite, bisulfate, phosphate, carbonate, bicarbonate among others. Thus it is notable that iron chemical form has an important effect on its absorption and availability.

There are many differences in absorption of iron in food where foods contain many dietary factors affecting on iron absorption and bioavailability. The iron absorption enhancement agents are vitamins (especially vitamin C or ascorbic acid) and organic acids, meat and fish, meanwhile different compounds (mainly of vegetable origin) such as phytic

acid, soy protein, polyphenols, and calcium are considered as inhibitors of iron absorption. There are also some functional components present in different foods that are considered as iron absorption enhancement agents like Lf and GOS which will be discussed in the section 1.9.

1.6. Absorption and bioavailability: definition and applied methods

An adequate bioavailability is dependent on an optimal digestion and solubility, an optimal transport over the mucosal layer into the circulation, and finally an optimal incorporation into the target organs. Although to assess iron bioavailability all these steps, should be related any of these steps can be used as an isolated measure to estimate bioavailability (Hoppe, 2008). Bioavailability is dependent on digestion, release from the food matrix, absorption by intestinal cells, and transport to body cells (Etcheverry et al., 2012). The concept of bioavailability as applied to nutrients is critically important for understanding nutrient metabolism, homeostasis and ultimately requirements (Krebs, 2001). While utilization is the process of transport, cellular assimilation and conversion to biologically active form(s) (Jackson, 1997).

Throughout the years, *in vitro* screening methods have been developed and refined for determination of nutrient bioaccessibility and bioavailability from foods. These are plentiful number of techniques developed to assess iron absorption and bioavailability that can provide useful information, especially when one considers the vast number of factors that can affect nutrient absorption. There are a several of them can be combined creating a vast number of methods with varying accuracy when it comes to accomplish results relevant in humans. Generally, the principally *in vitro* methods for measuring iron absorption and/or bioavailability are: solubility, dialyzability and the Caco-2 models for bioavailability (Etcheverry et al., 2012).

These *in vitro* methods are useful to provide knowledge on possible interactions between nutrients and/or food components, the effects of luminal factors (including pH and enzymes), food preparation and processing practices, nature of the food matrix…etc., on either micronutrient absorbability (a component of bioavailability) or on the potential for a nutrient to be absorbed (*i.e.*, bioaccessibility). *In vitro*

methods are less expensive, faster, and offer better controls of experimental variables than human or animal studies (Sandberg, 2005). However, *in vitro* studies cannot be substituted for *in vivo* studies, and should be therefore regarded as a screening, ranking, or categorizing tool (Etcheverry et al., 2012).

Because the *in vitro* digestion methods (solubility and dialyzability) has various limitations, these methods have been improved by the incorporation of a human colon carcinoma cell line (Caco-2) which provides many functional and morphological properties of mature human enterocytes (Ekmekcioglu, 2002). So this system is able to mimic and estimates the uptake and/or transport of mineral elements, and has been used to assess iron uptake from infant formulas (Jovaní et al., 2004). In this regard, combining *in vitro* digestion with uptake in Caco-2 cells is a step forward since it predicts both availability and uptake into the enterocyte and at times also the absorption (Fairweather-Tait et al., 2005). Cell culture has been used extensively as an *in vitro* method to assess human iron bioavailability (Pinto et al., 1983). This cell model has been used in a wide variety of nutritional studies, particularly in the study of mechanisms (Han et al. 1995), regulation of iron absorption (Tapia & Nuñez, 1999) and iron bioavailability studies (Glahn et al., 1998). The application of a Caco-2 cell model appears to be promising as a physiological means of measuring mucosal cell iron uptake (Au & Reddy, 2000).

1.7. The nutritional state and prevalence of iron deficiency anemia among the Egyptian infants

Anemia is defined as a state in which the level of hemoglobin or hematocrit is below that which is expected, taking in account both age and sex (Stoltzfus & Dreyfuss, 1998). Although anemia is the most prevalent public health problem with serious consequences for national development, it is ignored in most developing countries (World Bank, 1996; Bashir, 2013). In this regard, the World Bank reported that almost one third of the world population is believed to be anemic and the WHO estimates the number of anemic people worldwide to be about two billions (Stoltzfus, 2001a, b).

Iron deficiency is the main cause of anemia and it is generally assumed that 50% of the cases of anemia are due to iron deficiency (WHO, 2001). In general, iron deficiency occurs when insufficient iron is absorbed to meet the body's needs. This may be due to inadequate iron intake, poor iron absorption, increased iron need or chronic blood loss, and is well known that prolonged iron deficiency leads to iron deficiency anemia (IDA) (Bashir, 2013). Although the most common cause of nutritional anemia is iron deficiency (WHO, 2002), other possible causes include deficiencies of vitamins B-6, B-12, A, and C, folic acid, and riboflavin (Fishman et al., 2000). These micronutrients are known to affect the synthesis of hemoglobin either directly or indirectly by affecting the absorption and/or mobilization of iron (Dreyfuss et al., 2000). Therefore, IDA is a major problem in developing countries especially Egypt (Soliman et al., 2010) and considered to be one of the most contributing factors to the global burden of disease (WHO, 2002).

The causes of nutrition problems in Egypt are a function of many factors: most households are food insecure because of low income, high food prices and low local agricultural production, in addition to poor dietary practices due to lack of awareness, and inadequate health service provision capacities. There are also the problems of environmental pollution and food safety challenges due to lack of enforcement of existing laws. There is an overarching health system challenge that derives from uncoordinated and disjointed planning of nutrition activities; often leading to sub-optimal use of resources and impact on nutrition status (Zawilla, 2013).

Moreover, in the developing countries, the rate of exclusive breastfeeding and complementary feeding is far from optimum, ranging between 30-50%. In Egypt, the rate of exclusive breastfeeding is 79 % for infants under two months of age. This figure drops to 30 % for infants who are 4-5 months of age (El-Zanaty & Way, 2004). At first glance Egypt appears to be doing fairly well and above the range of the rate of exclusively breastfeeding amongst developing countries. However, the observed drop after three months, demonstrates that Egypt is far from optimum. Thus, there is an urgent need to improve breastfeeding practice i.e. increase the duration of exclusive breastfeeding to ensure universal coverage of this

practice (The Egyptian Ministry of Health and Population, MOHP, & UNISEF, 2012).

Also poor eating habits play a major role in the development of IDA which is an important indicator of poor health status (World Bank, 1994). Likewise, the Egyptian rural community has special dietary patterns depend on their culture as drinking tea immediately after meal. In addition, the composition of the typical Egyptian diet consumed daily can inhibit the absorption of iron where it contains low iron-level foods, tea, coffee and a certain type of fiber (Tatala & Svanberg, 1998; Emam et al., 2005). Although the iron deficient intake is one of the major causes of IDA (Aspuru et al., 2011), the poverty and ignorance still primary causal factors of IDA (Odunayo & Oyewole, 2006) especially in the developing counties such as Egypt.

It is worthy to note that the nutritional anemia is the most common type of anemia in Egypt and it is mostly caused by iron deficiency or insufficient intake of folate or vitamin B-12 where the deficiency of these essential nutrients in children may be due to factors such as reduced absorption during meals (*e.g.* due to tannin and phytate in unleavened bread (Verster & van der Pols, 1995). Therefore, the prevalence of IDA is relatively high especially in the rural areas (Emam et al., 2005), and almost 25-27% of the Egyptian infants and young children aged 6-59 months have IDA (El-Beshlawy et al., 2000; Al-Buhairan & Oluboyede, 2001). The anemia was mild in many cases, however, 11% of children had a moderate level of anemia; and a small proportion (less than 1%) were classified as having severe anemia. Children under age of two years were more likely to be anemic than older children. Rural children were more likely to be anemic than urban children (33 and 24%, respectively), and children in rural Upper Egypt and the Frontier Governorates had the highest anemia levels (38%) (El-Zanaty & Way, 2004). Thus and according to WHO criteria, IDA is considered to be a moderate public health problem in Egypt (FAO & Egyptian National Nutrition Institute, NNI, 2003).

El-Sayed et al. (1999) studied the prevalence of anemia in the Upper Egypt area (Minia, Assiut and Sohag cities) and reported that anemia was very highly prevalent among all Egyptian pre-schoolers (69%). Minia City

showed the highest trend in prevalence of anemia, followed by Sohag city and then Assiut city. The prevalence of anemia was slightly higher in most rural sites and was higher among girls (70%) than boys (68%). The highest prevalence was seen in the second year of life. Severe anemia was observed among 5.5% of pre-school children. Breastfeeding pattern, economic status, parasitic load and the anemia state of mothers were all significantly associated with the risk of anemia. Also the prevalence of anemia in children is considered to be a major problem in Qena city as elsewhere in Egypt (Moussa, 1990), but population based data, especially on children, are limited (Ibrahim et al., 1999). Although indicators of child health have improved, the current rates for malnutrition in children are still unacceptably high, especially in rural Upper Egypt (WHO, 2006).

In recognition of its nutritional challenges, Egypt has developed a 10-year Food and Nutrition Policy and Strategy (2007 – 2017). In 2010, UNICEF Egypt Country Office, together with MOHP, commissioned this Landscape Analysis. This was timely to complement Egypt's National Food and Nutrition Policy and Strategy, which had been in existence for four years. Egypt is the first country in the Middle East and North African region to conduct this landscape analysis (MOHP & UNISEF, 2012).

The landscape analysis demonstrates that there are many views on nutrition actions by different public and private stakeholders in Egypt. These actions include, amongst others, development of policies and national nutrition programs such as food fortification, food subsidies and other social assistance, and feeding programs including school and health facility nutrition kitchens; providing technical advice to national and sub-national levels (by UN agencies); developing an innovative community-based nutrition programs in rural and poor settings; supporting baby-friendly facilities; interventions that target pregnant women, infants and children; research, monitoring and evaluation and information dissemination activities; targeted programs for street kids and homeless people in urban settings; child labour and women initiatives; and various training on nutrition. Noteworthy is that none of these activities is operating at scale to meet the current needs in terms of addressing underlying food insecurity issues, there are interventions to improve quality of subsidized food

commodities, support for improved agricultural production, and improved management of water and sanitation services. Once again, these interventions remain inadequate (MOHP & UNISEF, 2012).

Clearly major health benefits could be achieved by choosing appropriate and cost effective strategies that successfully alleviate micronutrient deficiencies in developing countries. Strategies include supplementation to those at risk, food based strategies involving fortification of foods and dietary diversification, and public health actions to reduce infections and promote good health. The food supplementation refers to the addition of a nutritious food to a simple diet (Thompson, 2007).

The WHO is revising global guidelines for controlling IDA. Implementation of anemia control programs in developing countries requires careful baseline epidemiologic evaluation, selection of appropriate interventions that suit the population, and ongoing monitoring to ensure safety and effectiveness. Fortified bread nationwide in Egypt is a goal to compact anemia from 2012. The annual cost of fortifying bread would be US$10.3 million, and the government will supply special mixing machinery to bakeries. The government is also subsidizing folic acid for pregnant women to ensure mothers get the necessary vitamins. Supplementing children after the first year of age with fortified cow's milk and with iron supplements is also important to prevent anemia (Zawilla, 2013). In addition, fortification of infant and follow-up milk-based formula remains a valuable method for delivering iron to infant and young children that use a significant proportion to reduce the incidence of iron deficiency anemia (Ramakrishnan, 2001).

1.8. The common strategies for prevention of iron deficiency and IDA

The prevention and control of IDA is one of the key strategies of the health maintaining which will contribute to reduction of maternal and child mortality and improve health outcomes for population as a whole (Gupta et al., 2013).The three main strategies (Fig. 1.2) that can be implemented to overcome micronutrient malnutrition IDA are dietary diversification, food fortification with iron and supplementation (Huma et al., 2007). Food-based strategies, which include dietary diversification and fortification appears in

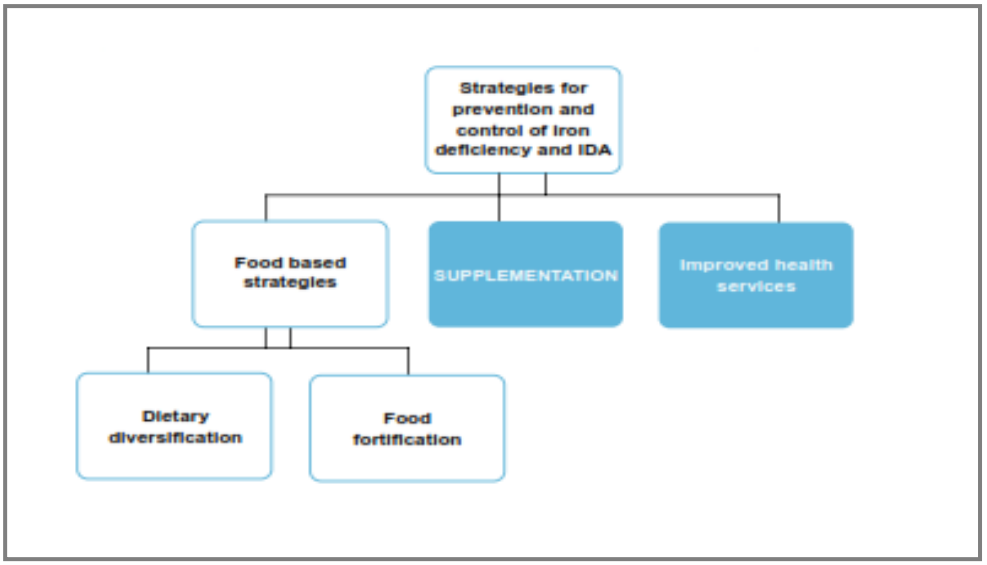


Fig. 1.2. Different strategies to prevent and control IDA and anemia (WHO, 2001).

the most sustainable approaches to increase the iron and other micronutrients status of a population (FAO/ILSI, 1997).

Dietary diversification is encouraging the consumption of micronutrient rich foods-dark green leafy vegetables, lentils and vitamin C rich fruits which may be available but are underutilized by the iron deficient population (Gupta et al., 2013). Dietary modifications for reducing IDA involve increased intake of iron rich food (Graham et al., 1992) or consumption of ascorbic acid rich fruits and vegetables that enhance non-heme iron absorption (Monsen, 1988). Likewise, people with poor iron status should avoid drinking tea with meals as it is likely to inhibit non-heme iron absorption (Nelson & Poulter, 2004).

Techniques such as soaking, germination, and fermentation promote enzymatic hydrolysis of phytic acid in whole grain cereals and legumes by enhancing the activity of endogenous phytase enzyme (Frontela et al., 2009). Even use of non-enzymatic methods such as thermal processing, soaking, and milling for reducing phytic acid content in plant-based staples also have been successfully used (Liang et al., 2008; Schlemmer et al., 2009).

Another approach to overcome the IDA is through supplementation of individuals or communities at risk. This approach would be implemented

for the treatment of individuals with anemia or in situations where at-risk communities of infants and young children do not have ready access to targeted iron-fortified foods (Andres, 1999).

Also foods fortification is an effective public health intervention strategy and it is useful to increase the intake of specific nutrient(s) that have been identified as inadequate in the food (Clydesdale, 1991). Food fortification strategy means the addition of one or more essential nutrients to food for the purpose of preventing or correcting a demonstrated deficiency of one or more nutrients in the population or specific population groups (FAO/WHO, 1999). The first step in a fortification program is the selection of a food that can function as a vehicle for the micronutrient. This food vehicle must be an integral component of the diet of the general population (Arroyave & Dary, 1996). In regard of the vehicle foods, there are two types of fortifications strategies:

1- The fortification of staple foods, such as flour is likely to increase iron intake and

2- The fortification of specific foods, such as infant formula, infant cereals and most breakfast cereals, is suitable to infants and children who have a limited capacity to eat large quantities of food. Targeted fortification (*e.g.*, the fortification of foods typically eaten by infants and children) provides an excellent source of iron (USDA, 2001). The efficacy of iron fortification strategy in the improvement of iron status depends on many factors such as the selected vehicle, the iron compound, and the iron status of the target population group (Hansen et al., 2005). Thus, iron fortification of food is considered a very suitable long-term strategy when selected iron fortificant and food vehicle is safe, acceptable, and consumed by the target population (Huma et al., 2007).

Nowadays in Egypt, controlling micronutrient deficiencies, especially IDA, has been one of the priorities for the Government of Egypt (MOPH/National Nutrition Institute, NNI, 2006).

Iron/folic acid fortification program for wheat flour (82% extraction) used for Baladi bread consumed by Egyptians started for gradual implementation since 2008, as a long-term strategy for prevention and control of IDA (Tawfik et al., 2014). Moreover, the Government of Egypt's

National Food and Nutrition Strategy (2007–2017) report recommended that a nationwide fortification effort must be done to reduce anemia among Egyptian women and children (World Food Program/Cairo Demographic Centre, 2010).

This National Fortification Program (NFP) was based on a pilot Baladi bread fortification project conducted in 2004 and 2005 at El Fayum governorate by NNI representing the MOHP and aimed to participate in reducing micronutrient deficiencies, especially IDA, among the Egyptian populations, especially vulnerable groups (Flour Fortification Initiative Egypt, 2012). In this NFP, Baladi bread was chosen as a vehicle for iron and folic acid fortification because it is the staple food consumed by a majority of the poor Egyptian population and low-income groups (World Food Program/Cairo Demographic Centre, 2010). The subsidized 82% extraction wheat flour was fortified with iron at 30 ppm in the form of ferrous sulfate and folic acid at 1.5 ppm. Ferrous sulfate fortificant was chosen on the basis of previous experience in the pilot project that was implemented in one governorate (Elhakim et al., 2012).

Clear evidence of the reduction of iron deficiency among the Egyptian population through the consumption of wheat flour fortified with iron and folic acid is not yet available and is pending the outcome of an end-line survey (Elhakim et al., 2012). Regarding with the used fortificant, WHO issued new recommendations to use sodium ethylenediaminetetraacetate (NaFeEDTA) in addition to, or instead of, ferrous sulfate for fortification of high-extraction flour such as that used to make Baladi bread. The Egyptian government decided to maintain the same fortificant (ferrous sulfate) and to judge by the outcome of the end-line survey whether and how to proceed in the next 5 years. Depending on the outcome, the government may decide to extend the use of ferrous sulfate for another 5 years, use NaFeEDTA instead, or use a mix of the two (Elhakim et al., 2012).

In addition, the Egyptian Government acknowledges that a single intervention, such as food fortification, will not resolve the problem of iron deficiency. It is scientifically accepted that the best results will be achieved through a multiple intervention strategy like the one currently being

conducted by the Egyptian Government that includes iron supplementation and treatment and prevention of parasitic infestations, as well as activities that are not traditionally micronutrient interventions, such as breastfeeding promotion, family planning, sanitation, and health education (WHO/UNICEF, 2004).

According to Elhakim et al. (2012), a comprehensive communications plan, incorporating an advocacy program and a consumer education campaign, is being conducted. It disseminates messages including the following:

- Malnutrition hinders the future of our children and the development of our country;
- Nutritional deficiencies are a form of "hidden hunger" that affects our body and health without our knowing;
- Eating healthy can be done on a low budget, through a combination of fortified foods (including Baladi bread) and natural, low-cost foods.

1.9. Human milk Lf and oligosaccharides as multifunctional ingredients for infant formulas

1.9.1. Human milk Lf

1.9.1.1. Lf structure and properties

It is well-known that Lf is a glycoprotein with high affinity to bind iron and is secreted in several species, such as human (Baró et al., 2001). It is the second most abundant protein in human milk and belongs to the transferrin family constituting 10-15 % and acting as a first line defense agent against infections in the body (Conneely, 2001). It is found at the highest levels (7 g /L) in human colostrum (Rodriguez et al., 2005) and at a lower level (1-2 g/L) in mature human milk (Nuijens et al., 1996), meanwhile the amount of Lf is lower in bovine colostrum and mature milk to about 1-2 and 0.01-0.1 g/L, respectively, and generally its content varies depending on the species (Wakabayashi et al., 2006; Nuijens et al., 1996). Structurally, bLF is 77 kDa glycoprotein and consists of a single polypeptide chain of about 700 amino acids. Meanwhile, hLf is a glycoprotein with a molecular weight of about 80 kDa and is a polypeptide chain that contains of 703 amino acids distributed as follows: from amino

acid 1 to 332 (lobe N) and from 344 to 703 (lobe C) with a three-turn connecting helix (residues 333-343) and that is sensitive to proteolytic attack. Each lobe contains an iron-binding site (Fe^{3+}) with a high affinity, and a glycan. N and C lobes have very similar conformations but show slight differences in their affinity for iron (Kaim & Schwederski, 1994). The sequence homology between hLf and bLF is about 70% and the 3 D structure of both is very similar but not identical (Steijns et al., 2000). Fig. 1.3. showed the structure of human Lf.

Lf was originally found to be a stable protein (Kuwata et al., 2001) and is only partly digested in newborn alimentary tract and may be absorbed as intact Lf from the infant gut (Chatterton et al., 2004; Artym & Zimecki, 2005). The incomplete development of the digestive system of infants who lesser than 6 months lead to its presence in infant feces where it exhibits as a small percentage (1-6%) of holo-Lf (Britton & Koldovsky, 1987). On the other hand, in the adults where the gastrointestinal tract reach to the maturity status, a decrease in the gastric pH values and an increase in enzymatic secretion are observed thereby enhancing proteolysis (Davidson & Lönnerdal, 1987). The stability of Lf against proteolytic enzymes will be discussed in detail in a next chapter (chapter 5).

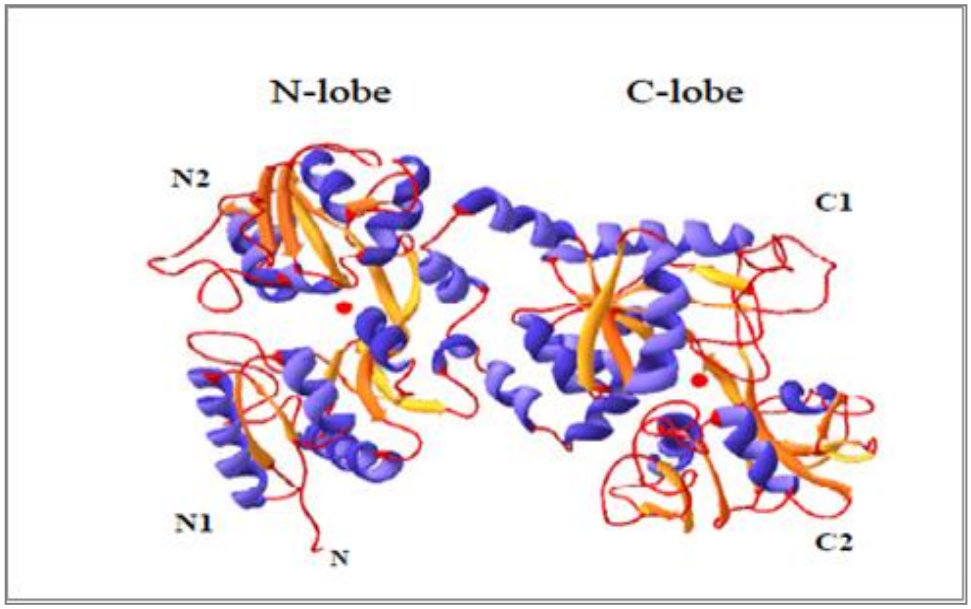


Fig. 1.3. Protein structure of human lactoferrin (van Veen, 2008).

Some of the functional roles exhibited by Lf are iron-dependent, meanwhile there are others reported to be non-dependent of iron (Farnaud & Evans, 2003). The iron-related functions are caused by the competition for iron ions between the protein and receptors of bacterial membranes.

Although the function roles of Lf that are non-dependent with iron binding properties are known to depend mainly on the structural region of amino acid residues 20-37 of the protein, the specific mechanisms still remain unclear (Farnaud & Evans, 2003; Babina et al., 2004).

As previously well-documented that there a quite difference in various aspects between bLf and hLf especially respecting with its lack recognition by Lf receptors which present at surfaces of the intestinal enterocytes of infant intestine and the need to replace the use of bLF with hLF in products for human utilization (Conesa et al., 2010) together with the limited availability of human milk and purified hLf (van Veen, 2008), several attempts have been made to produce rhLf (Conesa et al., 2010). However, some differences between hLf and rhLf were reported such as rhLf has lower carbohydrate content than that of hLf (Nandi et al., 2002) and rhLf from rice has the typical glycans of vegetables (Fujiyama et a., 2004) while hLf has the typical glycan of mammals (Spick et al., 1982). However, biochemical and biophysical analyses indicated that rhLF is similar to native hLF where rhLF has the same isoelectric point, iron binding capacity, pH stability, thermal stability, and antimicrobial activity as hLf (Huang et al., 2008).

Some of the functional activities of Lf related with the aim of this thesis were discussed as following:

1.9.1.2. Functional roles of Lf and its mode of action

1.9.1.2.1 Lf as iron-binding protein

Lf plays a key role in iron homeostasis in the newborn (Sacrino, 2007). Moreover, higher concentration of Lf in human milk than bovine milk raised the hypothesis that it might promote iron absorption in breast-fed infants compared with formulas-fed infants (Vorland, 1999). Likewise, the discovery of Lf receptors in the enterocytes of various species and its high affinity for Lf support this hypothesis. These Lf receptors show species

and molecular specificities depending on the animal species and this would explain the high bioavailability of iron from human milk, as only hLf releases iron to the enterocyte by this mechanism (Gonzalez-Chavez et al., 2009).

It is well-known that Lf has a higher ability to bound iron and retains this metal over a wide pH range and it starts to release the metal below pH 4 and at pH 2 the release of iron is complete allowing to the formation of the apo-form of Lf while hLf is somewhat more resistant with release below pH 3 (Stowell et al., 1991; Steijns et al., 2000). Lf can bind many cations and iron is considered the main cation bound by Lf (Lönnerdal & Iyer, 1995). According to the degree of Lf saturation with iron, three forms of Lf exist: apo-Lf (iron free), monoferric form (one ferric iron), and holo-Lf (binds two Fe^{3+} ions) (Jameson et al., 1998). The apo-Lf molecule tends to be in an open conformation, whereas the holo-Lf molecule is well-known by its closed conformation (Kurokawa et al, 1999; Sharma et al., 1999). In this sense, many researchers reported that holo-Lf form is more stable than apo-Lf form against proteolysis (Gonzalez-Chavez et al., 2009, Baró et al., 2001). So Lf ability to bind iron is playing a central role in its stability against the enzymatic proteolysis.

Lf-iron complex is taken up by the enterocyte, probably by endocytosis, and then release its iron at intracellular level through Lf degradation (Sanchez et al., 1992). Iron seems to be released within the cell where it is quickly complexed by another protein, probably ferritin, and then apo-Lf form comes back again to mucosa surface to start a new transport process (Sigel & Sigel, 1998). In iron absorption process enhanced by Lf, Lf receptors of epithelial cells play a central role in uptake Lf through clathrin-mediated endocytosis (Jiang et al., 2011).

However, the administration of non-human origin Lf involved different pathways of iron absorption with different efficiency compared with Lf of human origin (Jovaní et al., 2001). Although it is technically feasible to add bLf to infant formulas, bLf does not bind consistently to hLf receptors and has not been shown to increase iron absorption. Moreover, the efficacy and safety of adding hLf to infant formulas has not been adequately evaluated (Ben, 2008).

In this regard, there are controversial data respecting the effect of different sources of Lf on iron absorption whether in rats (Fairweather-Tait et al., 1986), mice (Ward et al., 2003), infants (Jovaní et al., 2001), or in piglets (Svoboda et al., 2005). Some of these data confirmed that the use of Lf from different sources could not be the best foods-enrichment way for improving iron absorption in humans (Ward et al., 2003).

1.9.1.2.2 Lf as antibacterial agent

Lf has strong antimicrobial activity against wide spectrum of microorganisms such as bacteria, fungi, yeasts and viruses (Drago, 2006). The antibacterial activity of Lf *in vitro* and *in vivo* has been documented in the past, for Gram-negative bacteria and Gram-positive bacteria and some acid-alcohol resistant bacteria (Garcia-Montoya et al., 2012). Initially it was considered that an iron-binding property is the major mechanism for its antibacterial action. Now it is well-known that iron-independent mechanisms are also responsible for the antibacterial action of Lf such as direct interaction with bacteria leading to membrane destabilization, modulation of bacteria motility, aggregation or endocytosis into host cells, inhibition of adherence and biofilm formation (Harvard & Hancock, 2009). In another words, the antibacterial activity of Lf is mostly due to two mechanisms. The first is the iron chelation which makes the nutrient unavailable for using by the microorganism thereby creating a bacteriostatic effect. The other mechanism is the direct interaction between Lf (the positive amino acids) and the bacterial surfaces (anionic molecules) causing cell breakdown (bactericidal effect) (Gonzalez-Chavez et al., 2009).

However, there are some bacteria in response to iron-limited media has the ability to produce and secrete low molecular weight high affinity chelators, which named siderfores (Yu & Schryvers, 2002). These compounds have a higher affinity for iron chelation than Lf, and then the iron-siderfores complex is taken up into bacteria by siderfores-specific receptors (Farnaud & Evans, 2003). Also other bacteria can produce specific Lf receptors that can stimulate iron removal from the protein (Yu & Schryvers, 2002).

Lf also exerts its antimicrobial action not just in the form of the intact molecule but the monoferric lobes and active peptides of Lf also have

a role in the host defense against microbial disease (Lizzi et al., 2009). These functional peptides are produced from Lf by the action of proteolytic enzymes that are present in the gastrointestinal tract (Sinha et al., 2013).

Lactoferricin, multifunctional cationic peptides, is one of these peptides that are generated by the enzymatic treatment of Lf and has a greater antibacterial activity than the native Lf. There are two forms of lactoferricin: human and bovine lactoferricin. Lactoferricin B consists of 25 amino acids while lactoferricin H is a 47-amino acid peptide. Lactoferricin B is more effective as antibacterial agent than the other peptide. The antibacterial activity of this peptide was attributed to its action of releasing lipopolysaccharide from bacterial strains and, hence, disruption of cytoplasmic membrane permeability after cell binding (Kang et al., 1996), and both lactoferricin (B and H) are derived from the N-terminal region of the N-lobe (Bellamy et al., 1992a).

Although Lf has antibacterial activity for a wide spectrum of microorganisms, it is considered a growth promoter for other organisms and acts as a **bifidogenic factor** for the growth of *bifidobacteria* (Kim et al., 2005). This later effect is named as **"prebiotic activity of Lf".** According to Coppa et al. (2006) hLf supports the predominance of beneficial bacteria which require low concentrations of iron for growth, such as *Lactobacillus* and *Bifidobacteria* of the infant intestinal microflora. Although this mechanism of action is not fully understood, many studies suggest that the growth stimulatory activity of Lf may be related to the presence of Lf-binding proteins on the surface of the bacterial membrane (Kim et al., 2004).

1.9.1.2.3 Lf as immunomodulatory and anti-inflammatory agent

There is a growing awareness of the interaction between the food bioactive constituents and the immune system (Calder and Fritsche, 2006). Lf, among these bioactive constituents, is a well-known natural immune modulator (Teraguchi et al., 2004) and may be considered a marker for inflammation due to the fact that its level is increasing during inflammation (Legrand et al., 2008). A variety of properties including anti-inflammatory (Conneely, 2001), and immunomodulatory agent (Brock, 2002) have been described so far for Lf.

The immunomodulatory activity is one of the very important activities of Lf and this effect was reported on the immune system both *in vivo* and *in vitro* (Brock, 2002), thus Lf is considered a key element in the host defense system (Legrand et al., 2005) and might strengthen the immune response (Teraguchi et al., 2004) as well as mediate anti-inflammatory reactions (Zhong et al., 2003).

Although the cellular and molecular mechanisms accounting for the immunomodulatory effects of Lf are far from being fully elucidated, both *in vitro* and *in vivo* studies suggest the existence of multiple mechanisms that include modulation of cytokine/chemokine production, regulation of ROS production, and of immune cell recruitment. It is now clear that at least some of the Lf biological activities do not merely depend on its iron-binding capacity, but may arise from its interaction with a variety of molecules. In this respect, the capacity of Lf to influence either negatively or positively cytokine production relies, at least in part, on its ability to bind and sequester both lipopolysaccharides (LPS) and its receptor CD14, as well as CpG bacterial DNA, thus preventing the downstream activation of pro-inflammatory pathways, septic shock and tissue damage (Britigan et al., 2001). The membrane glycoprotein CD14 (molecular mass of 55 kDa), is the main receptor for LPS and is expressed predominantly on the surface of monocytes and macrophages (Jiang et al., 2011). However, Lf can also favor the activation, differentiation, and proliferation of immune cells and this promoting activity has been related to a direct effect of Lf on immune cells through the recognition of specific Lf binding sites (Legrand et al., 2006). Beside its direct effects in host defense on bacteria, fungus and parasites, it were reported possible roles in the modulation of the immune response and it activates the innate and acquired immunities. These effects may resulted by the association between Lf's positive charge and the negatively charged molecules on the surface of various cells of the immune system (Baker & Baker, 2005).

In general, the anti-inflammatory effects of Lf have been shown by the inhibition of pro-inflammatory cytokine production (Kruzel et al., 2002) and the up regulation of anti-inflammatory cytokines (Togawa et al., 2002). On the other hand, Lf may enhance directly or indirectly the immune

response (*in vitro* and *in vivo*) by regulating the proliferation, differentiation and activation of both T and B cells (Zimecki et al., 1995).

Particularly, Lf has the potency to enhance the expression of various types of cytokines in intestinal mucosa such as IL-18, IFN-γ, IL-12, interferons (IFNs) and IL-7 and these cytokines has a role in activation of immune cells (Yang et al., 2009). As well as both *in vitro* and *in vivo* studies suggest that the effect of Lf involves an inhibition of production of several cytokines, named pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and IL-1β that are key mediators of the inflammatory response leading to death from toxic shock (Thompson et al., 1990; Machnicki et al., 1993). Also it was reported that Lf has the capacity to inhibit the production of LPS-induced pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1 in *in vitro* studies (Håversenet al., 2002). Conneely (2001) reported that the anti-inflammatory activity of Lf has two mechanisms through its ability in inhibition of binding of LPS to inflammatory cells, as well as through interaction with epithelial cells at local sites of inflammation to inhibit inflammatory cytokine production. Cytokines are defined as soluble factors which are mostly generated by immune cells and in turn play crucial roles in the differentiation, maturation, and activation of various immune cells (Su et al., 2012).

The identification of receptors for Lf on the surface of myeloblasts (Birgens et al., 1983), monocytes (Van Snick & Masson, 1976), macrophages (Mazurier et al., 1989), and lymphocytes (Cumberbatch et al., 2000), in addition to epithelial cells involved in local production of TNF-α (Iyer & Lönnerdal, 1993), suggests that Lf may have a direct effect on regulation of cytokine production by these cells via receptor mediated signaling pathways (Conneely, 2001). However, the molecular mechanisms that Lf mediates its anti-inflammatory effects are not well-known (Inubushi et al., 2012).

Bovine Lf supplements have the ability to support the immune system and influence immune cell activity (Mulder et al., 2008). The exogenous Lf has the ability to transport as intact form from the intestine to the blood circulation through the enterocytes by endocytosis and this it was reported *in vitro* (Fischer et al., 2007) and *in vivo* (Hutchens et al., 1991)

studies. The intact Lf form can stimulate intestine-associated immune functions and thereby enhance the immunocompetence during the postnatal period (Kuhara et al., 2006).

It worthy to note that lactoferricin has shown to contain the bactericidal domain (this due to the specific amino acid sequence that contains, resiudes 18-40 of hLf) (Bellamy et al., 1992b) and has cytokine-inhibiting activity of the protein, thus has a high affinity-binding site to LPS (Elass-Rochard et al., 1995).

Finally, it is known that using of naturally occurring ingredient and food bioactive components such as carotenoids, flavonoids, phenolic compounds, fiber and oligosaccharides have many therapeutic effects and recently Lf is considered a prominent active protein (Aly et al., 2013).

Given the emerging knowledge of the biological importance of hLf in infant nutrition, EFSA (2012), regarded the notion of Lf supplementation as worthy of consideration. However, clinical studies will be essential to demonstrate the efficacy and safety of such addition. With this background, it should be considered that Lf has enough beneficial properties on human health to can be considered as a functional ingredient if it is added to some foods such as infant formulas (Aly et al., 2013).

1.9.1.3. Lf as a functional component in infant formulas

Nutritional efficacy and safety are not the only challenge of the infant nutrition research and the infant formula development. When infants are bottle-fed should intake a food with similar properties to mother´s milk for its optimal growth and development (Alles et al., 2004). This fact, far to be easy to achieve, requires a deep knowledge of human milk properties and to identify which are the responsible compounds of the beneficial effects on the health of infant's breastfed (Aly et al., 2013).

Attempts are in progress to supplement infant formulas with protective and trophic factors so far unique only to human milk. The final aim is not necessarily to mimic the composition of human milk in every respect, but to achieve physiological effects as in breast-fed infants (Gómez-Gallego et al., 2009). Since human milk contains a considerable amount of Lf, special attention is paid to its functional role. Many of those functions are directly related to its ability to bind iron, which influence on iron

absorption, and its bacteriostatic and antioxidant activities. Based on this, the addition of Lf to infant formulas seems to be reasonable; nevertheless, the supplementation of infant formulas should be discussed intensively because there has to be a scientifically proven advantage for the infant to get this protein by daily formula (Sawatzki, 1997). Recently, EFSA (2012) accepted and approved bLf as a new food ingredient. Nowadays, there are many infant formulas supplemented with Lf available in the market (Mulder et al., 2008). From results obtained by different authors, it can be concluded that the addition of Lf, usually bovine, to infant formulas, does not affect iron absorption. However, given its ability to bind iron, its use in infant formulas could be useful for protecting the gut of infants against infections from microbial-requiring iron, its ability to reduce interelemental interactions and especially to protect infant formulas supplemented with iron and ascorbic acid against free radical formation.

The proposed concentration of bLF is 100 mg/100 mL for infant formulas. It was reported that the estimated intake of bLf for infants with an age of 0 - 6 months is approximately 200 mg per kg bodyweight and 1.2 g bLF per day assuming that the mean intake is 1.2 litres of infant formula per day. While the mean estimated intake of bLF by infants of 8 - 10 months of age would amount to 1.9 g per day (EFSA, 2012).

In this context, Raiten et al. (1998) and Wakabayashi et al. (2006) reported that it is possible to enrich infant formulas with bLf or rhLf. The application of rhLf to infant formulas represents an attractive issue (Suzuki et al., 2003).

In this regard, it must be taken into account that the enrichment of infant formulas with hLf would probably lead to an improvement in their amino acidic profile making it more similar to that of human milk (Jovaní et al., 2001).

EFSA (2012) considered that bLf is an essential protein constituent of cow milk and is considered a novel food ingredient. Bovine Lf is present in the novel food ingredient mostly as non-denatured protein. It must be noted that Lf is a normal constituent of human milk, and that the intended consumption of bLf is within the levels of hLf consumed in breast milk by infants; hLf is also non-denatured.

Currently, bLf is added as a supplement to several products in Japan, including infant formula and yoghurt (Wakabayashi et al., 2006). Similarly, infant formulas enriched with bLf are also available in other countries, including Indonesia, South Korea and Spain (Conesa et al., 2010).

1.9.2. Human milk oligosaccharides (HMOs)

Traditionally, oligosaccharides are defined as polymers of monosaccharides with degrees of polymerization (DP) between 2 and 10 (3 and 10 according to the IUB-IUPAC nomenclature) but DPs up to 20–25 are often assimilated with them. Prebiotic oligosaccharides are non-cariogenic, non-digestible (NDO) and low caloric compounds which stimulate the growth and development of gastrointestinal microflora described as probiotic bacteria. It is claimed that these bacteria belonging to *Bifidobacteria* and *Lactobacilli* have several health-promoting effects (Zopf & Roth, 1996; Rastall et al., 2005).

HMOs are considered the third most abundant component of human milk (Kunz et al., 2000), and also provides functional activities (Hamosh, 1996; Oddy, 2002).

1.9.2.1. HMOs structure, composition and variation

The composition and content of milk oligosaccharides, as well as other milk components, differs among mammalian species and also during the course of lactation and HMOs are characterized by an enormous structural diversity (Chaturvedi et al., 2001). In this regard, it was reported that HMOs content and composition of breast milk has many variations between different women and also depending on the stage of lactation. The total amount of HMOs is highest in colostrum and decreases through transitional to mature milk. Colostrum, the thick, yellowish fluid secreted by the mammary gland a few days before and after parturition, contains as much as 20-25 g/L HMO (Gabrielli et al., 2011), while HMOs concentrations decline to 5-20 g/L of mature human milk (Bao et al., 2007; Gabrielli et al., 2011), which still exceeds the concentration of total milk protein. On the other hand, the concentration of oligosaccharides in milk of the most relevant domestic mammals is smaller by a factor of 10 to 100 (Boehm & Stahl, 2003). Moreover, inter- and intrapersonal variations in

HMO synthesis determine the composition and relative abundance of individual HMOs in given milk sample (Bode & Jantscher-Krenn, 2012).

HMO-like structures are also found as components of glycolipids and glycoproteins (Newburg, 1999). So there are approximately 200 known compositions incorporating ≥3 carbohydrate monomers via 13 possible glycosidic linkages (Kunz et al., 2000; Niñonuevo et al., 2006), and each one has a structurally unique and structure often determines biological function (Bode & Jantscher-Krenn, 2012).

1.9.2.2. Physiological function of HMOs in infants

HMOs are believed to have many roles in a developing infant in addition to putative prebiotic functions. HMOs may possess anti-adhesive effects that reduce the binding of pathogenic bacteria to colonocytes (Lane et al., 2010). HMOs have modulating effects on immunologic processes at the level of gut-associated lymphoid tissue (Guarner, 2009) and may also decrease intestinal permeability in preterm infants in a dose-related manner in the first postnatal month (Taylor et al., 2009). Others have suggested that HMOs are an important source of N-acetyl-neuraminic acid (NeuAc; sialic acid), an essential monosaccharide during the period of neonate brain development and myelination (Wang et al., 2001). Also HMOs are thought to be the main contributors to the predominance of *Bifidobacterium* species in the infant gut (Schell et al., 2002). Thus there is a broad consensus that breast-fed infants grow and develop differently than infants with artificial feeding (Davis, 2001). Consequently, some physiological functions HMOs in infant related with this thesis are outlined as following:

1.9.2.2.1. HMOs as prebiotics

One of the suggested hypotheses regarding the bioactive function of HMOs is its role as "prebiotic" (Kunz & Rudloff, 1993). Most of HMOs are indigestible due to lack of luminal enzymes able to cleave most glycosidic linkages in the gastrointestinal tract (Engfer et al., 2000; Gnoth et al., 2000). However, bacteria in the colon express glycosidases and metabolize HMOs (Sela & Mills, 2010) leading to stimulate the growth of the "beneficial" bacteria including *Bifidobacteria*, genus generally predominant in the intestinal microbiota of the breast-fed infants (Favier et al., 2003).

It was reported that the neonatal gastrointestinal tract undergoes pronounced structural and functional changes in response to feeding (Donovan, 2006). Thus, it was noted that there are different in the composition of the microbiota between breast-fed infants and formula-fed infants with a higher proportion of *Bifidobacteria* species in breast-fed infants (Yoshioka et al., 1983).

This bifidogenic effect is likely attributed to both the protein and carbohydrate components in human milk. For example, growth of *Bifidobacteria* is promoted by Lf both *in vitro* (Rahman et al., 2009) and *in vivo* (Roberts et al., 1992). In addition, peptides produced by *in vitro* proteolytic digestion of Lf and secretory component are bifidogenic (Liepke et al., 2002). However, Coppa et al. (2006) reported that among all of the components, such as proteins, lactose, and nucleotides, HMOs is the only component that has been demonstrated to play a significant role in stimulation of the growth of specific bacteria. Also, most recent studies have focused on HMO as the primary bifidogenic components of human milk (Sela & Mills, 2010). Also the ability of selected *Bifidobacteria* to consume prebiotic oligosaccharides from human milk is likely an essential trait enabling these genera to be one of the most abundant colonizers of the breast-fed infant gut (LoCascio et al., 2007, 2009).

Thus the complex array of sugars present in HMOs have evolved to provide the newborn infant with a rich source of nutrient and also serve as substrate for specific microbes promoting the growth of selected enteric bacteria (Zivkovic et al., 2011), particularly *Bifidobacteria* and selected lactic acid-producing bacteria, that are present in high abundance in the gut microbiota of the exclusively breast-fed infant (Koropatkin et al., 2012).

1.9.2.2.2. HMOs as immunomoduladors

HMOs may affect the infant's immune system indirectly by its modulating role on the infant's microbiota composition or intestinal epithelial cell response (Rudloff et al., 2011; Gnoth et al., 2001). HMOs also act as receptor analogs to inhibit the adhesion of pathogens on the epithelial surface and this evidence is seen as a passive defense of the host (Boehm & Stahl, 2003; Barthelson et al., 1998).

Presently, results from *in vitro* studies suggest that HMOs can also directly modulate immune responses. HMOs may either act locally on cells of the mucosa-associated lymphoid tissues or on a systemic level (Rudloff et al., 2011). Because HMOs are resistant to digestion, they can pass the intestinal wall in small amounts and reach the systemic circulation (~1% of intake) (Rudloff et al., 2011; Eiwegger et al., 2004; Coppa et al., 2001) and can be detected in the urine of breast-fed infants (Coppa et al., 2001).

In an *in vitro* study, human white blood cells -which separated from cord blood- were incubated with fractions of neutral and acidic HMOs, which were separated from pooled human milk (Finke et al., 2002; Geisser et al., 2005). It was found that acidic HMOs led to a decrease of activated or regulatory T cells (Eiwegger et al., 2004).

1.9.2.3. Alternative sources of HMOs-like prebiotic in infant formula

HMOs are structurally very complex and have a huge diversity (Boehm & Stahl, 2003; Bode, 2006); thus, identical structures are not available for use in infant formulas (Boehm et al., 2003). So, several researchers suggested supplementing infant formula with oligosaccharides similar to those found in human milk (Motil, 2000). Given that human milk is obviously not amenable to large-scale production, there is an urgent demand for alternative, yet functionally comparable, oligosaccharide sources from which to obtain sufficient amounts to perform clinical studies and examine the potential for use in infant nutrition (Gopal & Gill, 2000).

In this context, various strategies have been used to mimic the structural complexity of HMOs; much simpler structures, including FOS and GOS, so far have been used in dietary products (Boehm & Moro, 2008). Bovine milk, and in particular colostrum, is also considered a source of simple as well as complex oligosaccharides that resemble HMOs (Tao et al., 2008, 2009; Barile et al., 2010).

1.10. Objectives of thesis

Given the great importance of human milk, which due to the large number of functional components that are positively correlated with infant's health, many studies have been carried out to identify these beneficial effects in breast-fed infants. Based on these studies and evidences, mature human milk is the reference. Therefore, infant formulas, as human milk substitute, should resemble that composition. Consequently, it is necessary to include some ingredients to infant formulas and thus it may be able to exert its functionalities in a large group of infants who cannot feed human milk as a primary source and for several physiological as well as social reasons. In view of this and to examine this concept, lactoferrin and GOS, as components of human milk, were selected to explore its health benefits using batch culture fermentation, Caco-2 model, western blotting and co-culture inflammation gut system.

The following points were investigated:

1. *In vitro* assessment of the bioavailability of iron from infant formulas supplemented with different concentrations of rhLf and/or GOS, using the amount of ferritin synthesized as criteria
2. *In vitro* evaluation of the anti-inflammatory effect of different concentrations of rhLf and rhLf hydrolysate
3. *In vitro* evaluation of the prebiotic activity of rhLf and/or GOS
4. *In vitro* evaluation of the effect of simulated gastrointestinal digestion on the added rhLf fractionation and long chain fatty acids (LCFAs) profile

1.11. References

Abiona, T. C., Onayade, A. A., Ijadunola, K. T., Obiajunwa, P. O., Aina O. I., & Thairu, L. N. (2006). Acceptability, feasibility and affordability of infant feeding options for HIV-infected women: a qualitative study in south-west Nigeria. Maternal and Child Nutrition, 2: 135-144.

Ackerberg, T. S., Labuschagne, I. L., & Lombard, M. J. (2012). The use of prebiotics and probiotics in infant formula. South Africa Fam. Pract., 54 (4): 321-323.

Adlerova, L., Batroskova, A., & Faldyna, M. (2008). Lactoferrin: a review. Veterinari Medicina, 53(9): 457-468.

Aggett, P. J., Agostoni, A., Axelsson, I., Edwards, C. A., Goulet, O., Hernell, O. et al. (2003) Non-digestible carbohydrates in the diets of infants and young children: a commentary by the ESPGHAN Committee on Nutrition. Journal of Pediatric Gastroenterology and Nutrition 36: 329–337.

Al-Buhairan, A. M. & Oluboyede, O. A. (2001). Determination of serum iron, total iron binding capacity and serum ferritin in Saudi adults. Annals of Saudi medicine. 21: 100-106.

Alles, M. S., Scholtens, P. A. S. M., & Bindles, J. (2004). Current trends in the composition of infant milk formulas. Current Pediatric, 14 (1), 51-63.

Aly, E., Ros, G. & Frontela, C. (2013). Structure and functions of lactoferrin as ingredient in infant formulas. Journal of Food Research, 2 (4): 25-36.

American Academy of Pediatrics, AAP (2012). Breastfeeding and the Use of Human Milk. Pediatrics, 129, e827-e841.

Anderson, G. J. and Vulpe, C. D. (2002). Molecular and Cellular Iron Transport (Templeton, D.M., ed), pp. 559-598.

Anderson, J. J. B. (2002). Minerals. In: Mahan KL, Stump SE. Krause Alimentos, nutrição & dietoterapia. 10. ed. São Paulo: Rocca; Cap 5, p. 120-6.

Andres, N. C. (1999). Disorders of iron metabolism. N Engl J Med; 341: 1986-1995.

Anticarcinogenesis pathways activated by bovine lactoferrin in the murine small intestine. Biochemie, 91(1), 86-101.

Arroyave, G., & Dary, O. (1996). Manual for sugar fortification with vitamin A, Part 1: Guidelines for the development, implementation, monitoring and evaluation of a vitamin A sugar fortification program. USA: OMNI/USAID, Arlington.

Artym, J., & Zimecki, M. (2005). The role of lactoferrin in the proper development of newborns. Postepy Higieny i Medycyny Doswiadczalnej, 59: 421-432.

Aspuru, K., Villa, C., Bermejo, F., Herrero, P. & López, S. G. (2011). Optimal management of iron deficiency anemia due to poor dietary intake. International Journal of General Medicine. 4: 741–750.

Au, A. P. & Reddy, M. B. (2000). Caco-2 Cells Can Be Used to Assess Human Iron Bioavailability from a Semipurified Meal. J. Nutr., 130 (5): 1329-1334.

Babina, S. E., Kanyshkova, T. G., Buneva, V. N., & Nevinsky, G. A. (2004). Lactoferrin is the major deoxyribonuclease of human milk. Biochemistry (Moscow), 69(9): 1006-1015.

Baker, E. N., & Baker, H. M. (2005). Lactoferrin molecular structure, binding properties and dynamics of lactoferrin. Cell Molecular and Life Science, 62: 2531-2539.

Bao, Y., Zhu, L. & Newburg, D. S. (2007). Simultaneous quantification of sialyloligosaccharides from human milk by capillary electrophoresis. Anal Biochem, 370: 206-214.

Barile, D., Marotta, M., Chu, C., Mehra, R., Grimm, R., Lebrilla, C. B. & German, J. B. (2010). Neutral and acidic oligosaccharides in -Friesian colostrum during the first 3 days of lactation measured by high performance liquid chromatography on a microfluidic chip and time-of-flight mass spectrometry. J Dairy Sci., 93:3940–3949.

Baró, L., Jiménez, J., Martínez-Férez, A. and Boza, J. J. (2001). Bioactive compounds derived from human milk. Ars Pharmaceutica. 42(1): 21-38.

Barthelson, R., Mobasseri, A., Zopf, D. & Simon, P. (1998). Adherence of Streptococcus pneumoniae to respiratory epithelial cells is inhibited by sialylated oligosaccharides. Infect Immun. 66: 1439–1444.

Bashir, R. (2013). Prevalence of iron deficiency Anaemia among adolescent girls and impact of Health and Nutrition Education Programme in changing their dietary behavior. Faculty of Applied Science and Technology University of Kashmir, Srinagar, Pakistan, 2012.

Baveye, S., Elass, E., Mazurier, J., Spik, G., & Legrand, D. (1999). Lactoferrin: a multifunctional glycoprotein involved in the modulation of the inflammatory process. Clinical Chemistry and Laboratory Medicine, 37: 281-286.

Bellamy, W., Takase, M., Wakabayashi, H., Kawase, K. & Tomita, M. (1992a). Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. Journal of Applied Bacteriology, 73(6): 472-479.

Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K. & Tomita, M. (1992b). Identification of the bactericidal domain of lactoferrin. Biochimica et Biophysica Acta, 1121(1-2):130-136.

Ben, X-M. (2008). Nutritional management of newborn infants: Practical guidelines. World Journal of Gastroenterology, 14(40): 6133–6139.

Birgens, H. S., Hansen, N. E., Karle, H. & Kristensen, L. O. (1983). Receptor binding of lactoferrin by human monocytes. British Journal of Hematology, 54: 383-391.

Blais, A., Malet, A., Mikogami, T., Martin-Rouas, C., & Tomé, D. (2009). Oral bovine lactoferrin improves bone status in ovariectomized mice. The American Journal of Physiology-Endocrinology and Metabolism, 296, E1281-1288.

Bode L. (2006). Recent advances on structure, metabolism, and function of human milk oligosaccharides. J. Nutr. 136:2127–2130.

Bode, L. (2012). Human milk oligosaccharides: every baby needs a sugar mama. Glycobiology 22 (9): 1147-1162.

Bode, L. & Jantscher-Krenn, E. (2012). Structure-Function Relationships of Human Milk Oligosaccharides, Adv. Nutr. 3: 383S–391S.

Bode, L., Rudloff, S., Kunz, C., Strobel, S., Klein, N. (2004). Human milk oligosaccharides reduce platelet-neutrophil complex formation leading to a decrease in neutrophil beta 2 integrin expression. J Leukoc Biol. 76:820–6.

Boehm, G., Fanaro, S., Jelinek, J., Stahl, B. & Marini, A. (2003). Prebiotic concept for infant nutrition. Acta Paediatr., 91: 64–67.

Boehm, G. & Moro, G. (2008). Structural and functional aspects of prebiotics used in infant nutrition. J. Nutr. 138: 1818S–1828S.

Boehm, G. & Stahl, B. (2003). Oligosaccharides. In: Mattila-Sandholm T, editor. Functional dairy products. Cambridge: Woodhead Publishing, p. 203–243.

Britigan, B. E., Lewis, T. S., Waldschmidt, M., McCormick, M. L. & Krieg, A. M. (2001). Lactoferrin binds CpG-containing oligonucleotides and inhibits their immunostimulatory effects on human B cells. Journal of Immunology, 167: 2921-2928.

Britton, J. R., & Koldovsky, O. (1987). Luminal digestion of lactoferrin in suckling and weanling rats. The American Journal of Physiology, 253: G397-G403.

Brock, J. H. (2002). The physiology of lactoferrin. Biochemistry and Cell Biology, 80: 1-6.

Calder, P. C. & Fritsche, K. (2006). n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. Fatty acids as modulators of the immune response. American Journal of Clinical Nutrition, 83 (6 Suppl): 1505S–1519S.

Casey, J. L., Di Jeso, B., Rao, K., Klausner, R. D. & Harford, J. B. (1988). Two genetic loci participate in the regulation by iron of the gene for the human transferin receptor. Proc Natl Acad Sci USA, 85:1787-1791.

Cashman, K. D. (2002). Trace elements, Nutritional Significance. In U. Roginski, H. Fuquay & J. W. Fox (Eds.), Encyclopedia of Dairy Sciences, 3, (pp. 2059-2065). London: Academic Press.

Cesar J.A., Victora C.G., Barros F.C., Santos I.S. & Flores J.A. (1999). Impact of breastfeeding on admission for pneumonia during post neonatal period in Brazil: nested case–control study. British Medical Journal 318, 1316–1320.

Chantry, C. J., Howard, C. R., & Auinger, P. (2006). Full breastfeeding duration and associated decrease in respiratory tract infection in US children. Pediatrics, 117: 425-432.

Chatterton, D. E. W., Rasmussen, J. T., Heegaard, C. W., Sorensen, E. S., & Petersen, T. E. (2004). In vitro digestion of novel milk protein ingredients for use in infant formulas: Research on biological functions. Trends in Food Science Technology, 15:_373-383.

Chaturvedi, P., Warren, C. D., Altaye, M., Morrow, A. L., Ruiz-Palacios, G., et al. (2001). Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. Glycobiology 11: 365–372.

Chierici, R., & Vigi, V. (1994). Lactoferrin in infant formula. Acta Paediatrica, 402: 83-88.

Chirico, G., Marzoll, R., Cortinovis, S., Fonte, C., & Gasparino, A. (2008). Anti-infective properties of human milk. Journal of Nutrition, 138: S1801-S1806.

Clydesdale, F. M. (1991). Mineral additives. In: Bauernfeind, J.C., Lachance, P.A., and Trumbull, C.T., Eds. Nutrients Addition to Food: Nutriotional, Technological and Regulatory Aspects. Food Nutrition Press, Inc., pp. 87–107.

Conesa, C., Calvo, M., & Sánchez, L. (2010). Recombinant human lactoferrin: A valuable protein for pharmaceutical products and functional foods. Biotechnology Advances, 28: 831-838.

Conneely, O. M. (2001). Anti-inflammatory activities of lactoferrin. Journal of the American College of Nutrition, 2(5): 389S-395S.

Coppa, G. V., Pierani, P., Zampini, L., Bruni, S., Carloni, I. & Gabrielli, O. (2001). Characterization of oligosaccharides in milk and feces of breast-fed infants by high performance anion exchange chromatography. Adv Exp Med Biol., 501: 307–14.

Coppa, G. V., Zampini, L., Galeazzi, T., & Gabrielli, O. (2006). Prebiotics in human milk: a review. Digestive Liver Disease, Amsterdam, 38(2): 291-294.

Cornish, J. (2004). Lactoferrin promotes bone growth. Biometals, 17, 331-335.

Cornish, J., & Naot, D. (2010). Lactoferrin as an effector molecule in the skeleton. Biometals, 23, 425-430.

Cumberbatch, M., Dearman, R. J., Uribe-Luna, S., Headon, D. R., Ward, P. P., Conneely, O. M. & Kimber, I. (2000). Regulation of epidermal Langerhans cell migration by lactoferrin. Immunology, 100 (1): 21-28.

Darshan, D., & Anderson, G. J. (2007). Liver-gut axis in the regulation of iron homeostasis. World Journal of Gastroenterology, 13: 4737-4745.

Davidson, L. A., & Lönnerdal, B. (1987). Persistence of human milk proteins in the breast-fed infant. Acta Paediatrica Scandinavica, 76: 733-740.

Davis, M. K. (2001). Breastfeeding in chronic disease in childhood and adolescence. Pediatr Clin North Am. 48: 125–141.

De Jong, C., Kikkert, H. K., Fidler, V. & Hadders-Algra, M. (2010). The Groningen LCPUFA study: no effect of postnatal long-chain polyunsaturated fatty acids in healthy term infants on neurological condition at 9 years. British Journal of Nutrition, 104 (4): 566-572.

Domellof, M., Lonnerdal, B., Abrams, S. A., & Hernell, O. (2002). Iron absorption in breast-fed infants: effects of age, iron status, iron supplements, and complementary foods. The American Journal of Clinical Nutrition, 76: 198-204.

Donovan, S. M. (2006). Role of human milk components in gastrointestinal development: current knowledge and future needs. J. Pediatr., 149: S49–S61.

Dorca, J. (2008). Ingredientes funcionales en las fórmulas infantiles. BOL PEDIATR, 48: 347-352.

Drago, S. M. E. (2006). Actividades antibacterianas de la lactoferrina. Enfermedades Infecciosas y Microbiología, 26: 58-63.

Dreyfuss, M. L., Stoltzfus, R. J., Shrestha, J. B., Pradhan, E. K., LeClerq, S. C., et al. (2000) Hookworms, malaria and vitamin A deficiency contribute to anemia and iron deficiency among pregnant women in the plains of Nepal. J Nutr., 130: 2527-2536.

Dunn, L. L., Suryo Rahmanto, Y. & Richardson, D. R. (2007). Iron uptake and metabolism in the new millennium. Trends in Cell Biology, 17 (1): 93-100.

Dunn, L. L., Sekyere, E. O., Suryo Rahmanto, Y. & Richardson, D. R. (2006). The function of melanotransferrin: a role in melanoma cell proliferation and tumorigenesis. Carcinogenesis 7: 2157–2169.

EFSA (2012). Panel on Dietetic Products, Nutrition and Allergies (NDA); Scientific Opinion on bovine lactoferrin. EFSA Journal 2012; 10(5), 26 pp.

Eiwegger, T., Stahl, B., Schmitt, J. J., Boehm, G., Gerstmayr, M., Pichler, J., Dehlink, E., Loibichler, C., Urbanek, R. & Sze´pfalusi, Z. (2004). Human milk derived oligosaccharides and plant derived oligosaccharides stimulate cytokine production of cord blood T-cells in vitro. Pediatr Res., 56: 536–540.

Ekmekcioglu, C. A. (2002). A physiological approach for preparing and conducting intestinal bioavailability studies using experimental systems. Food Chemistry, 76: 225-230.

Elass-Rochard, Roseanu, A., Legrand, D., Trif, M., Salmon, V., Motas, C., Montreuil, J. and Spik, G. (1995). Lactoferrin-lipopolysaccharide interaction: involvement of the 28-34 loop region of human lactoferrin in the high-affinity binding to Escherichia coli 055B5 lipopolysaccharide. The Biochemical Journal, 15 (312): 839–845.

El-Beshlawy, A., Emara, A., Hanna, T., Moustafa, A., Ragab, L., Youssry, I. et al. (2000). Microcytic hypochromic anemia in Egyptian children. Egyptian Journal of Hematology, 25: 585-596.

Elhakim, N., Laillou, A., El Nakeeb, A., Yacoub, R., & Shehata, M. (2012). Fortifying baladi bread in Egypt: Reaching more than 50 million people through the subsidy program. Food and Nutrition Bulletin, 33 (4): S260-S271.

Ella, E. E., Ahmed, A. A., Umoh, V. J., Ogala, W. N., & Balogun, T. B. (2009). Lactoferrin levels in human breast milk among lactating mothers with sick and healthy babies in Kaduna state, Nigeria. International Journal of Medicine and Medical Science, 1(11), 495-500.

El-Sayed, N., Gad, A., Nofal, L., Zeid, H. A., El-Morshedy, H., El-Waseef, S. (1999). Assessment of the prevalence and potential determinants of nutritional anaemia in Upper Egypt. Food and Nutrition Bulletin, 20: 417-421.

El-Zanaty, F. & Way, A. A. (2004). Egypt Interim Demographic and Health Survey 2003 (2003 EIDHS). Cairo, Egypt: Ministry of Health and Population [Egypt], National Population Council, E1-Zanaty and Associates, and ORC Macro, 179 p.

Emam, S., Mostafa, R., Wassef, O., et al. (2005). Assessment of Nutritional Status of Some Primary School Children. Their Awareness in Slum Areas. Alexandria Journal of Pediatrics, 19: 113-119.

Engfer, M. B., Stahl, B., Finke, B., Sawatzki, G. & Daniel, H. (2000). Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. Am J Clin Nutr. 71: 1589–1596.

ESPGHAN (2002). Committee on Nutrition: Aggett, P. J., Agostoni, C., Axelsson, I., Bresson, J. L., Goulet, O., Hernell, O., Koletzko, B., Lafeber H. L., Michaelsen, K. F., Micheli, J. L., Rigo, J., Szajewska, H., Weaver, L. T. Iron metabolism and needs in early childhood: do we know enough? J Pediatr Gastroenterol Nutr., 34: 337-345.

Etcheverry, P., Grusak A. M., & Fleige, L. E. (2012). Application of in vitro bioaccessibility and bioavailability methods for calcium, carotenoids, folate, iron, magnes, polyphenols, zinc, and vitamins B6, B 12 ,D, and E. Frontiers in Physiology, 3, 1-22.

Euler, A., Mitchell, D., Kline, R. & Pickering, L. (2005). Prebiotic effect of fructo-oligosaccharide supplemented term infant formula at two concentrations compared with un-supplemented formula and human milk. J. Pediatr. Gastroenterol. Nutr. 40: 157-164.

European Commission (2003). Report of the Scientific Committee on Food on the Revision of Essential Requirements of Infant Formulae and Follow-on Formulae. April, 2003. 213p.

Fairweather-Tait, S. J., Wright, A. G. A., & Piper, Z. (1986). The influence of human and bovine lactoferrin on iron absorption from ferrous sulphate in rats. Proceedings of the Nutrition Society. 45, 40A.

Fairweather-Tait, S., Lynch, S., Hotz, C., Hurrell, R., Abrahams, L., Beebe, S., Bering, S., Bukhave, K., Glahn, R., Hambidge, M., Hunt, J., Lonnerdal, B., Miller, D., Mohktar, N., Nestel, P., Reddy, M., Sandberg, A.-S., Sharp, P., Teucher, B. & Trinidad, P. (2005). The usefulness of in vitro Models to Predict the Bioavailability of Iron and Zinc: A Consensus Statement from the Harvest Plus Expert Consultation. Int. J. Vit. Nutr. Res., 75: 371 – 374.

FAO/Egypt National Nutrition Institute (2003). Nutrition country profiles: Egypt. Pp. 54.

FAO/ILSI (1997). Preventing Micronutrient Malnutrition: A Guide to Food-based Approaches. ILSI Press, Washington, D.C.

FAO/WHO (1999). Evaluation of certain food additives and contaminants (Forty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 884, p 7.

Farnaud, S., & Evans, R. W. (2003). Lactoferrin: a multifunctional protein with antimicrobial properties. Molecular Immunology, 40: 395-405.

Favier, C. F., de Vos, W. M. & Akkermans, A. D. (2003). Development of bacterial and bifidobacterial communities in feces of newborn babies. Anaerobe, 9: 219–229.

Finke, B., Stahl, B., Pritschet, M., Facius, D., Wolfgang, J. & Boehm, G. (2002). Preparative continuous annular chromatography (P-CAC) enables the large-scale fractionation of fructans. J Agric Food Chem., 50:4743–4748.

Fischer, R., Debbabi, H., Blais, A., Dubarry, M., Rautureau, M., Boyaka, P. N., & Tome, D. (2007). Uptake of ingested bovine lactoferrin and its accumulation in adult mouse tissues. International Immunopharmacology, 7(10): 1387-1393.

Fishman, S. M., Parul Christian, P. & West, K. P. Jr. (2000). The role of vitamins in the prevention and control of anaemia, Public Health Nutrition: 3(2): 125-150.

Fleming, R. E., Ahmann, J. R., Migas, M. C., Waheed, A., Koeffler, H. P., Kawabata, H., Britton, R. S., Bacon, B. R. & Sly, W. S. (2002). Targeted mutagenesis of the murine transferrin receptor-2 gene produces hemochromatosis. Proc Natl Acad Sci USA, 99:10653-10658.

Flour Fortification Initiative Egypt (2012). Wheat facts. Available at: http://www.sph.emory.edu/wheatflour/IEMT/ IEMT_files/Country_summaries/egypt.pdf.

Frontela, C., Scarino, M. L., Ferruzza, S., Ros, G. & Martínez, C. (2009). Effect of dephytinization on bioavailability of iron, calcium and zinc from infant cereals assessed in the Caco-2 cell model. World Journal of Gastroenterology, 28; 15(16): 1977-1984.

Fujita, K., Matsuda, E., Sekine, K., ligo, M., & Tsuda, H. (2004). Lactoferrin enhances Fas expression and apoptosis in the colon mucosa of azoxymethane-treated rats. Carcinogenesis, 25, 1961-1966.

Fujyama, K., Sakai, Y., Misaki, R., Yanagihara, I., Honda, T., Anzai, H. et al. (2004). N-linked glycan structures of human lactoferrin by transgenic rice. Biosci. Biotechnol. Biochem., 68: 2565-2570.

Gabrielli, O., Zampini, L., Galeazzi, T., Padella, L., Santoro, L., Peila, C., Giuliani, F., Bertino, E., Fabris, C. & Coppa, G. V. (2011). Preterm milk oligosaccharides during the first month of lactation. Pediatrics, 128: 1520-1531.

Ganapathy, S. (2009). Long chain polyunsaturated fatty acids and immunity in infants. Indian Pediatrics, 46 (17): 785-790.

Garcia-Montoya, I. A., Cendon, T. S., Arevalo-Gallegos, S., & Rascon-Cruz, Q. (2012). Lactoferrin a multiple bioactive protein: an overview. Biochemica et Biophysica Acta, 1820(3): 226-236.

Geisser, A., Hendrich, T., Boehm, G. & Stahl, B. (2005). Separation of lactose from human milk oligosaccharides with simulated moving bed chromatography. J Chromatogr A, 1092:17–23.

Gibson, G. & Roberfroid, M. (1995). Dietary modulation of the Human Colonic Microbiota: Introducing the Concept of Prebiotics. J. Nutr. 125: 1401-1412.

Gifford, J. L., Hunter, H. N., Vogel, H. J. (2005). Lactoferricin: a lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties. Cell Mol Life Sci., 62: 2588–2598

Gil, A. & Uauy, R. (1995). Nucleótides and related compounds in human and bovine milks. 1995. In: Jensen, R. G. ed. Handbook in milk composition, Academic Press, New York. 436-464.

Gil, A., Pita, M., Martinez, A., Molina, J. A. & Sanchez Medina, F. (1986) Effect of dietary nucleotides on the plasma fatty acids in at-term neonates. Hum. Nutr. Clin. Nutr. 40:3, 185-195

Giuffre`, G., Barresi, V., Skliros, C., Barresi, G., & Tuccari, G. (2007). Immuno-expression of lactoferrin in human sporadic renal cell carcinomas. Oncology Reports, 17, 1021-1026.

Glahn, R. P., Lai, C., Hsu, J., Thompson, J. F., Guo, M. & Van Campen, D. R. (1998) Decreased citrate improves iron availability from infant formula: application of an in vitro digestion/Caco-2 cell culture model. J. Nutr., 128: 257-264.

Gnoth, M. J., Kunz, C., Kinne-Saffran, E. & Rudloff, S. (2000). Human milk oligosaccharides are minimally digested in vitro. J. Nutr., 130: 3014–3020.

Gnoth, M. J., Rudloff, S., Kunz, C. & Kinne, R. K. (2001). Investigations of the in vitro transport of human milk oligosaccharides by a Caco-2 monolayer using a novel high performance liquid chromatography-mass spectrometry technique. J Biol Chem. 276: 34363–34370.

Gómez-Gallego, C., Pérez-Conesa, D., Cava, B., Periago-Castón, M. J., Ros-Berruezo, G. (2009). Compuestos funcionales de la leche materna. Enfermería Global, 16: 1-14.

Gonzalez-Chavez, S., Arevalo-Gallegos, S., & Rascon-Cruz, Q. (2009). Lactoferrin: structure, function and applications. International Journal of Antimicrobial Agents, 33: 301.e1-301. e8.

Gopal, P. K. & Gill, H. S. (2000). Oligosaccharides and glycoconjugates in bovine milk and colostrum. Br. J. Nutr. 84: S69–S74.

Graham, R.D., Acher, J.S., & Hynes, S.C. (1992). Selecting Zinc-efficient cereal genotypes for soils of low zinc status. Plant Soil, 140: 241–250.

Granier, a., Goulet , o., Hoarau, c. (2013). Fermentation products: immunological effects on human and animal models. Pediatric Research, 74: 238–244.

Guarner F. (2009). Prebiotics, probiotics and helminths: the "natural" solution? Dig. Dis. 27: 412–417.

Gupta, A., Kumar, R., Salhotr, V. S., Mohan, A. & Rahi, S. (2013). Guidelines for Control of Iron Deficiency Anaemia. National Iron+ Initiative towards Infinite Potential in an Anemia Free India. Adolescent Division Ministry of Health and Family Welfare Government of India, 54 p.

Hallberg, L., Brune, M., & Rossander, L. (1989). Iron absorption in man: ascorbic acid and dose-dependent inhibition by phytate. American Journal of Clinical Nutrition, 49: 140-144.

Hamosh M. (1996). Breastfeeding: Unravelling the mysteries of mother's milk. Medscape Womens Health. 16:4–9.

Han, O., Failla, M., Hill, A., Morris, E. & Smith, J. Jr. (1995) Reduction of Fe(III) is required for uptake of nonheme iron by Caco-2 cells. J. Nutr. 125: 1291– 1299.

Hansen, M., Bach, S. B., Thomsen, A. D., Tetens, I. & Sandstrom B. (2005). Long-term intake of iron fortified whole meal rye bread appears to benefit iron status of young women. J. Cereal Sci., 42:165–171.

Harmsen, H. J. M., Wideboer-Veloo, A. C., Grijpstra, J., Knoll, J., Degener, J. E., Welling, G. W. (2000). Development of 16S rRNA-based probes for the Coriobacterium group and the Atopobium cluster and their application for enumeration of Coriobacteriaceae in human feces from volunteers of different age groups. Appl. Environ. Microbiol, 66: 4523-4527.

Harris, W. R. (1986). Estimation of the ferrous-transferrin binding constants based on thermodynamic studies of nickel (II)-transferrin. J Inorg Biochem, 27:41-52.

Harvard, J., & Hancock, R. E. W. (2009). Antimicrobial properties of lactoferrin. Biochimie, 91: 19-29.

Håversen, L., Ohlsson, B. G., Hahn-Zoric, M., Hanson, L. A. & Mattsby-Baltzer, I. (2002). Lactoferrin down-regulates the LPS-induced cytokine production in monocytic cells via NF-κB. Cellular Immunology, 220 (2): 83–95.

Hayashida, K., Kaneko, T., Takeuchi, T., Shimizu, H., Ando, K., & Harada, E. (2004). Oral administration of lactoferrin inhibits inflammation and nociception in rat adjuvant-induced arthritis. The Journal of Veterinary Medical Science, 66, 149-154.

Heaney, R. P., Abrams, S., Dawson-Hughes, B., Looker, A., Marcus, R. Matkovic, V., & Weaver, C. (2000). Peak bone mass. Osteoporosis International, 11(12), 985-1009.

Hirose, M. (2000). The structural mechanism for iron uptake and release by transferrins. Biosci Biotechnol Biochem, 64:1328-1336.

Hoppe, M. R. (2008). Iron absorption in man. Diet modification and fortification. Chalmers Reproservice, Göteborg, Sweden. 67 p.

Huang, N., Bethell, D., Card, C. Cornish, J., archbank, T. M., Wyatt, D., Mabery, K., Playford, R. (2008). Bioactive recombinant human lactoferrin, derived from rice, stimulates mammalian cell growth. In Vitro Cell.Dev.Biol. Animal, 44:464-471

Huma, N., Rehman, S. U., Anjum, F. M., Murtaza, M. A., & Sheikh, M. A. (2007). Food Fortification Strategy—Preventing Iron Deficiency Anemia: A Review, Critical Reviews in Food Science and Nutrition, 47: 259-265

Hutchens, T. W., Henry, J. F., Yip, T. T., Hachey, D. L., Schanler, R. J.,, Motil, K. J., & Garza, C. (1991). Origin of intact lactoferrin and its DNA-binding fragments found in the urine of human milk-fed preterm infants. Evaluation by stable isotopic enrichment. Pediatric Research, 29(3): 243-250.

Iacono G , Merolla R., D'Amico, D., Bonci, E., Cavataio, F., Di Prima, L., Scalici, C., Indinnimeo, L., Averna, M. R. & Carroccio, A. (2005). Gastrointestinal symptoms in infancy: a population-based prospective study. Dig Liver Dis., 37: 432-438.

Ibrahim, B., Sallam, S., El Tawila, S., El Gibaly, O., El Sahn, F., Lee, S. M., Mensch, B., Wassef, H., Bukhari, S. & Galal, O. (1999). Transition to adulthood. A national survey of Egyptian adolescents. Cairo, Population Council, New York, Population Council, 218 p.

Iigo, M., Alexander, N., Long, J., Xu, K., Fukamachi, M., Futakuchi, Takase, M., & Tsuda, H. (2009). Anticarcinogenesis pathways activated by bovine lactoferrin in the murine small intestine. Biochemie, 91(1), 86-101.

Innis, S. M. (2008). Dietary omega 3 fatty acids and the developing brain. Brain Res., 1237: 35–43.

Institute of Medicine (IOM) (2000). Dietary reference intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc. National Academy Press: Washington DC.

Inubushi, T., Kawazoe, A., Miyauchi, M., Kudo, Y., Ao, M., Ishikado, A., Makino, T. & Takata, T. (2012). Molecular mechanisms of the inhibitory effects of bovine lactoferrin on lipopolysaccharide-mediated osteoclastogenesis. The Journal Of Biological Chemistry, 287 (28): 23527–23536.

Iyer, S. & Lönnerdal, B. (1993). Lactoferrin, lactoferrin receptors and iron metabolism. European Journal of Clinical Nutrition, 47 (4): 232-241.

Jackson, K. M. & Nazar, A. M (2006). Breastfeeding, the Immune Response, and Long-term Health . The Journal of the American Osteopathic Association, 106: 203-207.

Jackson, M. J. (1997). The assessment of bioavailability of micronutrients: introduction. European Journal of Clinical Nutrition, 51: 1-2.

Jameson, G. B., Anderson, B. F., Norriss, G. E., Thomas, D. H., & Baker, E. N. (1998). Structure of human apolctoferrin at 2.0 a resolution. Refinement and analysis of ligand-induced conformational change. Acta Crystallographica, D54: 1319-1335.

Jiang, R., Lopez, V., Kelleher, S. L., & Lönnerdal, B. (2011). Apo- and holo-lactoferrin are both internalized by lactoferrin receptor via clathrin-mediated endocytosis but differentially affect ERK-signaling and cell proliferation in Caco-2 cells. Journal of Cellular Physiology, 226(11): 3022-3031.

Joeckel, R. J. and Phillips, S. K. (2009). Overview of infant and pediatric formulas. Nutr Clin Pract., 24(3): 356-362.

Jovaní, M., Barbera, R., & Farré, R. (2001). Lactoferrin and its possible role in iron enrichment of infant formulas. Food Science and Technology International, 7(2): 97-103.

Jovaní, M., Viadel, B., Laparra, M., Barberá R. & Farré, R. (2004). Improvement of analytical conditions of mineral Caco-2 cells uptake assays. Food Science and Technonlogy International, 10: 197-201.

Kaim, W., & Schwedereski, B. (1994). Transport and storage of iron. In John Wiley & Sons (Eds.), Bioinorganic Chemistry: Inorganic Elements in the Chemistry of Life (pp. 162-165). England: Bookcraft (Bath) Ltd., Midsomer Norton, Somerset.

Kang, J. H., Lee, M. K., Kim, K. L., & Hahm, K. S. (1996). Structure biological activity relationships of 11-residue highly basic peptide segment of bovine lactoferrin. International Journal of Peptide and Protein Research, 48(4), 357-363.

Kanyshkova, T. G., Buneva, V. N., & Nevinsky, G. A. (2001). Lactotrferrin and its biological functions. Biochemistry (Moscow), 66(1), 1-7.

Kawabata, H., Yang, R., Hirama, T., Vuong, P. T., Kawano, S., Gombart, A. F., Koeffler, H. P. (1999). Molecular cloning of transferrin receptor 2. A new member of the transferin receptor-like family. J Biol Chem, 274: 20826-20832.

Kawakami H & Lonnerdal B. (1991). Isolation and function of a receptor for human lactoferrin in human fetal intestinal brush-border membranes. Am J Physiol; 261:G841–6.

Kim, W. S., Ohashi, M., Tanaka, T., Kumura, H., Kim, G. Y., Kwon, I. K., … Shimazaki, K. (2004). Growth-promoting effects of lactoferrin on L. acidophilus and Bifidobacterium spp. Biometals (Oxford), 17(3): 279-283.

Kim, W. S., Rahman, M. M., kumura, H., & Shimazaki, K. (2005). Comparison of growth promoting effects on bifidobacteria spp. by bovine lactoferrin hydrolysates. Bioscience Microflora, 24(4): 119-123.

Koletzko, B. (2008). Basic concepts in nutrition: Nutritional needs of children and adolescents. e-SPEN. the European e-Journal of Clinical Nutrition and Metabolism, 3: e179–e184.

Koletzko, B., Baker, S., Cleghorn, G., Neto, U. F., Gopalan, S., Hernell, O., et al. (2005). Global standard for the composition of infant formula: recommendations of an ESPGHAN coordinated international expert group. Journal of pediatric gastroenterology and nutrition 41: 584–599.

Koletzko, B., Lien, E., Agostoni, C., Böhles, H., Campoy, C., Cetin, I., et al. (2008). The roles of long-chain polyunsaturated fatty acids in pregnancy, lactation and infancy: review of current knowledge and consensus recommendations. Journal of Perinatal Medicine 36: 5–14.

Korhonen, H., Pihlanto-Leppälä, A., Rantamäki, P. and Tupasela, T. (1998). Impact of processing on bioactive proteins and peptides. Trends Food Sci Technol. 9: 307-319.

Koropatkin, N. M., Cameron, E. A & Martens, E. C. (2012). How glycan metabolism shapes the human gut microbiota. Nat Rev Microbiol., 10: 323–335.

Kramer, M. S., & Kakuma, R. (2002). Optimal duration of exclusive breastfeeding. Cochrane Database Systematic Review, 1: 1-106.

Kramer, M. S., Aboud, F., Mironova, E., Vanilovich, I., Platt, R. W., Matush, L., Igumnov, S., Fombonne, E., Bogdanovich, N., Ducruet, T., Collet, J. P., Chalmers, B., Hodnett, E., Davidovsky, S., Skugarevsky, O., Trofimovich, O., Kozlova, L., Shapiro, S. (2008). Promotion of Breastfeeding Intervention Trial (PROBIT) Study Group. Breastfeeding and child cognitive development: new evidence from a large randomized trial. Arch. Gen. Psychiatry, 65: 578-584.

Krebs, N. F. (2001). Bioavailability of dietary supplements and impact of physiologic state: infants, children and adolescents. Journal of Nutrition, 131: 1351-1354.

Kruzel, M. L., Harari, Y., Mailman, D., Actor, J. K. & Zimecki, M. (2002). Differential effects of prophylactic, concurrent and therapeutic lactoferrin treatment on LPS-induced inflammatory responses in mice. Clinical and Experimental Immunology, 130 (1): 25–31.

Kuhara, T., Yamauchi, K., Tamura, Y., & Okamura, H. (2006). Oral administration of lactoferrin increases NK cell activity in mice via increased production of IL-18 and type I IFN in the small intestine. Journal of Interferon Cytokine Research, 26(7): 489-499.

Kunz, C. & Rudloff, S. (1993). Biological functions of oligosaccharides in human milk. Acta Pediatric, 82: 903–912.

Kunz, C., Rudloff, S., Baier, W., Klein, N. & Strobel, S. (2000). Oligosaccharides in human milk: structural, functional, and metabolic aspects. Annual Review of Nutrition 20: 699–722.

Kurokawa, H., Dewan, J. C., Mikami, B., Sacchettini, J. C. & Hirose, M. (1999). Crystal structure of hen apoovotransferrin. Both lobes adopt an open conformation upon loss of iron. J. Mol. Biol. 274: 28445- 28452.

Kuwata, H., Yamauchi, K., Teraguchi, S., Ushida, Y., Shimokawa, Y., Toida, T., & Hayasawa, H. (2001). Functional fragments of ingested lactoferrin are resistant to proteolytic degradation in the gastrointestinal tract of adult rats. The Journal of Nutrition, 131(8): 2121-2127.

Lane, J. A., Mehra, R. K., Carrington, S. D. & Hickey, R. M., (2010). The food glycome: a source of protection against pathogen colonization in the gastrointestinal tract. Int. J. Food Microbiol. 142: 1–13.

Latunde-Dada, G., Simpson, R., McKie, A. (2006). Recent advances in mammalian hem transport. Trends in Biochemical Science, 31 (3), 182-188.

Le Blanc, S., Garrick, M. D. & Arredondo, M. (2012). Heme carrier protein 1 transports heme and is involved in heme-Fe metabolism. Am J Physiol-Cell Ph, 302:C1780–1785.

Legrand, D., Elass, E., Carpentier, M. & Mazurier, J. (2005). Lactoferrin: a modulator of immune and inflammatory responses. Cellular and Molecular Life Science, 62: 2549-2559.

Legrand, D., Elass, E., Carpentier, M. & Mazurier, J. (2006). Interactions of lactoferrin with cells involved in immune function, Biochemistry and Cell Biology, 84: 282-290.

Legrand, D., Pierce, A., Elass, E., Carpentier, M., Mariller, C. & Mazurier, J. (2008). Lactoferrin Structure and Functions. Advance in Experimental Medicine and Biology, 606: 163–194.

Leung, A. K. & Sauve, R. S. (2005). Breast is best for babies. Journal of the National Medical Association, 97(7):1010-1019.

Liang, J., Han, B. Z., Nout, M. J. R. & Hamer, R. J. (2008). Effects of soaking, germination and fermentation on phytic acid, total and *in vitro* soluble zinc in brown rice. Food Chem., 110: 821-828.

Liepke, C., Adermann, K., Raida, M., Mägert, H. J., Forssmann, W. G. & Zucht, H. D. (2002). Human milk provides peptides highly stimulating the growth of bifidobacteria. Eur J Biochem., 269: 712–718.

Lizzi, A. R., Carnicelli, V., Clarkson, M. M., Di Giulio, A., & Oratore, A. (2009). Lactoferrin derived peptides: mechanisms of action and their perspectives as antimicrobial and antitumoral agents. Mini-Reviews in Medicinal Chemistry, 9(6): 687-695.

LoCascio, R. G., Niñonuevo, M., Kronewitter, S., Freeman, S. L., German, J. B., et al. (2009). A versatile and scalable strategy for glycoprofiling bifidobacterial consumption of human milk oligosaccharides. Microb. Biotechnol., 2333–2342.

LoCascio, R. G., Niñonuevo, M. R., Freeman, S. L., Sela, D. A., Grimm, R., et al. (2007). Glycoprofiling of bifidobacterial consumption of human milk oligosaccharides demonstrates strain specific, preferential consumption of small chain glycans secreted in early human lactation. J. Agric. Food Chem., 55: 8914–8919.

Lonnerdal, B. (1989). Dietary factors affecting trace elements absorption in infants. Acta paediatrica scandinavica, S351, 109-113.

Lönnerdal, B. (2010). Alternative pathways for absorption of iron from foods. Pure Appl. Chem., 82 (2): 429–436.

Lonnerdal, B., & Iyer, S. (1995). Lactoferrin: molecular structure and biological function. Annual Review of Nutrition, 15: 93-110.

Lorget, F., Clough, J., Oliveira, M., Daury, M. C., Sabokbar, A., & Offord, E. (2002). Lactoferrin reduces in vitro osteoclast differentiation and resorbing activity. Biochemical and Biophysical Research Communications, 296, 261-266.

Losnedahl, K. J., Wang, H., Aslam, M., Zou, S., & Hurley, W. L. (1998). Antimicrobial Factors in Milk. Illini Dairy Net Papers, University of Illinois.

Machnicki, M., Zimecki, M. & Zagulski, T. (1993). Lactoferrin regulates the release of tumour necrosis factor alpha and interleukin 6 in vivo. International Journal of Experimental Pathology, 74: 433-439.

Mackenzie, B. & Garrick, M. (2005). Iron imports. II Iron uptake at the apical membrane in the intestine. American Journal of Physiology Gastrointestinal and liver Physiology, 289: G981-G986.

Makino, Y., & Nishimura, S. (1992). High performance liquid chromatographic separation of human apolactoferrin and monoferric and diferric lactoferrin. Journal of Chromatography, 579: 346-349.

Makrides, M., Gibson, R. A., McPhee, A. J., Collins, C. T., Davis, P. G., Doyle, L. W., Simmer, K., Colditz, P. B., Morris, S., Smithers, L. G., Willson, K. & Ryan, P. (2009). Neurodevelopmental outcomes of preterm infants fed high-dose docosahexaenoic acid: a randomized controlled trial. JAMA. 301(2):175-82.

Martinez, M. (1992). Tissue levels of polyunsaturated fatty acids during early human development. J. Pediatr., 120: S129–S138.

Martinov, O. B., Spasic, S. D., Lugonja, N. M., Gojgic-Cvijovic, G. D. J. G. D. J., & Vrvic, M. M., (2011). Infant formula as a substrate for bifidogenesis: An in vitro investigation. African Journal of Biotechnology, 10 (12): 2302-2307.

Mathew, J. L. (2004). Effect of maternal antibiotics on breast feeding infants. Postgraduate Medical Journal, 80: 196-200.

Matsuda, Y., Saoo, K., Hosokawa, K., Yamakawa, K., Yokohira, M., Zeng, Y., … Imaida, K. (2006). Post-initiation chemo-preventive effects of dietary bovine lactoferrin on 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis in female A/J mice. Cancer Letters, 246, 41-46.

Mazurier, J., Legrand, D., Hu, W. L., Montreuil, J. & Spik, G. (1989). Expression of human lactotransferrin receptors in phytohemagglutinin-stimulated human peripheral blood lymphocytes. Isolation of the receptors by antiligand-affinity chromatography. European Journal of Biochemistry, 179: 481-1989.

Mena, P. & Uauy, R, (2008). Fats. In: Pediatrics Nutrition in Practice (Berthold Koletzko, Peter Cooper, Maria Makrides, R. Uauy, Weiping Wang, Eds). Reinhardt Druck, Basel, Switzerland, 299 p.

Merit, S., Simpson, R., & McKie, A. (2003). Physiological and molecular biology of dietary iron absorption. Annual Reviews of Nutrition, 23: 283-301.

Michaelsen, K. F., Weaver, L., Branca, F. & Robertson, A. (2000). Feeding and nutrition of infants and young children: guidelines for the WHO European Region, with emphasis on the former Soviet countries. WHO Regional Publications, European Series, No. 87.

Ministry of Health and Population/National Nutrition Institute of the Government of Egypt. (2006). Egypt National Food and Nutrition Strategy (2007–2017). Cairo: A Ministry of Health Report.

Ministry of Health of New Zealand (2008). Food and Nutrition Guidelines for Healthy Infants and Toddlers (Aged 0–2): A background paper: Wellington: Ministry of Health. New Zealand, 246 p.

Monsen, E. (1988). Iron nutrition and absorption: Dietary factors which impact iron bioavailability. In: Nestel, P. Ed., Proceedings: Interventions for Child Survival, OMNI/USAID, Arlington, V. A.

Motil, K. J. (2000). Infant feeding: a critical look at infant formulas. Curr. Opin. Pediatr., 12:469–476.

Moussa, W. A. (1990). Nutritional status in Egypt. Final report of the Health Examination Survey. Cairo, Ministry of Health.

Mulder, A. M., Connellan, P. A., Oliver, C. J., Morris, C. A., & Stevenson, L. M. (2008). Bovine lactoferrin supplementation supports immune and antioxidant status in healthy human males. Nutrition Research, 28: 583-589.

Mundy, G. R. (1999). Bone remodeling. In M. J. Favus (Ed.), Primer on the Metabolic Bone Disease and Disorders of Mineral Metabolism (pp. 30-38). (Fourth edition). Philadelphia, Lippincot Williams & Welkins.

Nandi S, Suzuki YA, Huang J, Yalda D, Pham P, Wu L, et al. (2002). Expression of human lactoferrin in transgenic rice grains for the application in infant formula. Plant Sci., 163:713–722.

Naot, D., Grey, A., Reid, L. R., & Cornish, J. (2005). Lactoferrin: a novel bone growth factor. Clinical Medicine & Research, 3(2), 93-101.

National Nutrition Institute of the Government of Egypt. Available at: http://www.nni.org.eg/Html/Bread_Ar.html. Accessed 18 July 2012.

Nelson, M. & Poulter, J. (2004). Impact of tea drinking on iron status in the UK: a review. J Hum Nutr Dietet, 17: 43–54

Newburg, D. S. (1999). Human milk glycoconjugates that inhibit pathogens. Curr. Med. Chem. 6(2):117–127.

Newburg, D. S. (2000). Oligosaccharides in human milk and bacterial colonization. Journal of Pediatric and Gastroenterology Nutrition, 30, S8-S17. http://dx.doi.org/10.1097/00005176-200003002-00003.

Newburg, D. S. (2000a). Are all human milks created equal? Variation in human milk oligosaccharides. J Pediatr Gastroenterol Nutr. 30:131–133.

NHMRC (National Health and Medical Research Council). (2006). Nutrient Reference Values for Australia and New Zealand including Recommended Dietary Intakes. Canberra: NHMRC, Wellington: Ministry of Health.

Niñonuevo, M. R., Park, Y., Yin, H., Zhang, J., Ward, R. E., et al. (2006). A strategy for annotating the human milk glycome. J. Agric. Food Chem. 54: 7471–7480.

Norrby, K., Mattsby-Baltzer, I., Innocenti, M., & Tuneberg, S. (2001). Orally administered bovine lactoferrin systemically inhibits VEGF (165)-mediated angiogenesis in the rats. International Journal of Cancer, 91, 236-240.

Nuijens, J. H., van Berkel, P. H. C. & Schanbacher, F. L. (1996) Structure and biological actions of lactoferrin, Journal of Mammary Gland Biology and Neoplasia, 1: 285-295.

Oddy, W. H. (2002). The impact of breast milk on infant and child health. Breastfeed Rev. 10: 5–18.

Odunayo, S. I. & Oyewole, A. O. (2006) Risk Factors for Malnutrition among Rural Nigerian Children. Asia Pacific Journal of Clinical Nutrition, 15: 491-495.

Ohgami, R. S., Campagna, D. R., Greer, E. L., Antiochos, B., McDonald, A., Chen, J., Sharp, J. J., Fujiwara, Y., Barker, J. E. & Fleming, M. D.. (2005). Identification of a ferrireductase required for efficient transferrin-dependent iron uptake in erythroid cells. Nat Genet, 37: 1264-1269.

Pan, Y., Rowney, M., Guo, P., & Hobman, P. (2007). Biological properties of lactoferrin: an overview. Australian Journal of Dairy Technology, 62(1), 31-42.

Petit, A. I. (2008). Perception and knowledge on exclusive breastfeeding among women attending antenatal and postnatal clinics. A study from Mbarara Hospital, Uganda (pp. 27-30). Tanzania Medical Students' Association.

Pickering, L. K., Granoff, D. M., Erickson, J. R., Masor, M. L., Cordle, C. T., Schaller, J. P., Winship, T. R., Paule, C. L., & Hilty, M. D. (1998). Modulation of the immune system by human milk and infant formula containing nucleotides. Pediatrics. 101:242-249.

Pinto, M., Robine-Leon, S., Appay, M.-D., Kedinger, M., Triadou, N., Dussaulx, E., LaCroix, B., Simon-Assmann, P., Haffen, K., Fogh, J. & Zweibaum, A. (1983). Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol. Cell., 47: 323-330.

Rahman, M. M., Kim, W. S., Ito, T., Kumura, H. & Shimazaki, K. (2009). Growth promotion and cell binding ability of bovine lactoferrin to Bifidobacterium longum. Anaerobe, 15:133–137.

Raisler J, Alexander C, O'Campo P. (1999). Breast-feeding and infant illness: a dose–response relationship? Am J Public Health 89(1):25–30.

Raiten, D. J., Talbot, J. M., & Waters, J., H. (1998). Assessment of nutrient requirement for infant formulas. The Journal of Nutrition, 128(11S): 2059S-2294S.

Ramakrishnan, U. (2001). Functional consequences of nutritional anemia during pregnancy and early childhood. In U Ramakrishnan (Ed.). Nutritional Anemia's. Boca Raton FL., CRC Press. pp 43–68.

Rachmawati, D., Mori, T., Hosaka, T., Takaiwa, F., Inoue, E. & Anzai, H. (2005). Production and characterization of recombinant human lactoferrin in transgenic javanica rice. Breed Sci., 55:21322.

Rastall, R. A., Gibson, G. R., Gill, H. S., Guarner, F., Klaenhammer, T. R., Pot, B., Reid, G., Rowland, I. R. & Sanders, M. E. (2005). Modulation of the microbial ecology of the human colon by probiotics, prebiotics and synbiotics to enhance human

health: An overview of enabling science and potential applications, FEMS Microbiol. Ecol. 52: 145–152.

Redel, C. A., & Shulman, R. J. (1994). Controversies in the composition of infant formulas. Pediatric Clinics of North America, 41, 909-923.

Renner, E., Scchaafsma, G., & Scott, K. J. (1989). Micronutrients in milk. In E. Renner (Ed.), Micronutrients in milk and milk-based products. (pp. 1-60). New Yourk: Elsevier Applied Science.

Rizvi, S. and Schoen, R. E. (2011). Supplementation With Oral vs. Intravenous Iron for Anemia With IBD or Gastrointestinal Bleeding: Is Oral Iron Getting a Bad Rap?. American Journal of Gastroenterology, 106:1872–1879

Roberfroid M. (2007). Prebiotics: The concept revisited. J. Nutr., 830S–837S.

Roberfroid, M. (2000). Prebiotics and probiotics: are they functional foods. Am. J. Clin. Nutr. 71(suppl): 1682S-1687S.

Roberts, A. K., Chierici, R., Sawatzki, G., Hill, M. J., Volpato, S. & Vigi, V. (1992). Supplementation of an adapted formula with bovine lactoferrin: 1. Effect on the infant faecal flora. Acta Paediatr., 81: 119–124.

Rudloff, S., Pohlentz, G., Borsch, C., Lentze, M. J. & Kunz, C. (2011). Urinary excretion of in vivo 13C-labelled milk oligosaccharides in breastfed infants. Br J Nutr., 107:957–963.

Rodriguez, D. A., Vázquez, L. & Ramos, G. (2005). Antimicrobial mechanisms and potential clinical application of lactoferrin [in Spanish]. Rev Latinoam Microbiol., 47:102–11

Sacrino, M. L. (2007). A sideways glance: Take it or leave it? The role of lactoferrin in iron sequestration and delivery within the body. Genes Nutrition, 2: 161-162.

Sanchez, L., Calvo, M., & Brock, J. H. (1992). Biological role of lactoferrin. Archives of disease in childhood, 67: 657- 661.

Sandberg, A. S. (2005). Methods and options in vitro dialyzability; benefits and limitations. Int. J. Vitam. Nutr. Res., 75: 395–404.

Sawatzki, G. (1997). Lactoferrin in infant formulas. Experimental Biology and Medicine, 28: 389-397.

Schaller, J. P., Kuchan, M. J., Thomas, D. L., Cordle, C. T., Winship, T. R., Buck, R. H., Baggs, G. E. and Wheller, J. G. (2004). Effect of dietary ribonucleotides in infant immune status. Part 1: Humoral responses. Pediatr Res., 56:6, 883-890

Schell, M. A., Karmirantzou, M., Snel, B., Vilanova, D., Berger, B., Pessi, G., Zwahlen, M. C., Desiere, F., Bork, P. & other authors (2002). The genome sequence of Bifidobacterium longum reflects its adaptation to the human gastrointestinal tract. Proc Natl Acad Sci U S A 99: 14422–14427.

Schlemmer, U., Frølich, W., Prieto, R. M. & Grases, F. (2009). Phytate in foods and signifi cance for humans: Food sources, intake, processing, bioavailability, protective role and analysis. Mol Nutr Food Res., 53: S330-S375.

Scientific Advisory Committee on Nutrition (2010). Iron and health. TSO print, 374p.

Sela, D. A. & Mills D. A. (2010). Nursing our microbiota: molecular linkages between biofidobacteria and milk oligosaccharides. Trends Microbiol. 18: 298–307.

Sgarbieri, V. C. (1987). Alimentação e nutrição-fator de saúde e desenvolvimento. Campinas: UNICAMP; Cap 10, p. 213-42.

Sharma, A. K., Rajashankar, K. R., Yadav, M. P. & Singh, T. P. (1999). Structure of mare apolactoferrin: the N and C lobes are in the closed form. Acta Crystallog. sect. D, 55: 1152-1157..

Sharp, P., & Srai, S. K. (2007). Molecular mechanisms involved in intestinal iron absorption. World Journal of Gastroenterology, 21(35): 4716-4724.

Sherman, P. M., Cabana, M., Gibson, G. R., et al. (2009). Potential roles and clinical utility of prebiotics in newborns, infants, and children: proceedings from a global prebiotic summit meeting, J Pediatr, 155: S61–S70.

Sigel, A., & Sigel, H. (1988). Transferrin, the transferring receptor, and the uptake of iron by cells. In A. Y. Sigel & H. Sigel (Eds.), Metal ions in Biological Systems (Vol. 35, pp. 586-631). New York: Marcel Dekker.

Simons, M. S. L. (1999). Feeding Practices, Nutrient Intakes and Iron Deficiency Anemia in a Select Group of Infants from Dunedin, New Zealand. Thesis submitted for the degree of Master of Science at the University of Otago, Dunedin New Zealand, 17 June.

Sinha, M., Kaushik, S., Kaur, P., Sharma, S., & Singh, T. P. (2013). Antimicrobial Lactoferrin Peptides: The Hidden Players in the Protective Function of a Multifunctional Protein. International Journal of Peptides, 3: 1-12.

Sipe, D. M. & Murphy, R. F. (1991). Binding to cellular receptors results in increased iron release from transferrin in mildly acidic pH. J Biol Chem., 266: 8002-8007.

Soliman, G. Z., Mahfouz, M. H. & Emara, L. A. (2010). Effect of different types of oral therapy used for the treatment of iron deficiency anemia and their effects serum on some hormones and mineral in anemia rats. Journal of American Science, 6(6): 109-118.

Solis, G., Reyes-Gavilan, C. G., Fernandez, N., Margolles, A. & Gueimonde, M. (2010). Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breast milk and the infant gut. Anaerobe, 16(3): 307-310.

Spike, G., Strecker, G., Fournet, B., Bouquelete, S., Montreuil, J., Dorland, L. et al. (1982). Primary structure of the glycans from human lactotransferrin. European Journal of Biochemistry, 121: 413-419.

Stankowski, J. N., Dawson, V. L. & Dawson, T. M. (2012). Ironing out tau's role in Parkinsonism. Nat Med., 18: 291-295.

Steele, T. M., Frazer, D. M. & Anderson, G. J. (2004). Systemic regulation of intestinal iron absorption. Australian Biochemist, 35 (3): 9-12.

Steijns, J. M., & van Hooijdonk, C. M. (2000). Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. British Journal of Nutrition, 84(1): S11-S17.

Stephen, A., Alles, M., de Graaf, C., Fleith, M., Hadjilucas, E., Isaacs, E., Maffeis, C., Zeinstra, G., Matthys, C., & Gil, A. (2012). The role and requirements of digestible dietary carbohydrates in infants and toddlers. European Journal of Clinical Nutrition, 66: 765 – 779.

Stoltzfus, R. J. (2001a). Defining Iron-Deficiency Anemia In Public Health Terms: A Tome For Reflection. J Nutr., 131: 565S–567S.

Stoltzfus, R. J. (2001b). Iron-Deficiency Anemia: Reexamining the Nature and Magnitude of the Public Health Problem. Summary: Implications for Research and Programs. J. Nutr., 131: 697S–701S.

Stoltzfus, R. J. & Dreyfuss, M. L. (1998). Guidelines for the Use of Iron Supplements to Prevent and Treat Iron Deficiency Anemia. International Nutritional Anemia Consultative Group (INACG). International Life Sciences Institute 1126 Sixteenth Street, N. W. Washington, D. C. ILSI PRESS, 46 p.

Story, L., & Parish, T. (2008). Breastfeeding helps prevent two major infant illnesses. The Internet Journal of Allied Health Science and Practice, 6(3): 1-5.

Stowell, K. M., Rado, T. A., Funk, W. D., & Tweedie, J. W. (1991). Expression of cloned human lactoferrin in baby hamster kidney cells. Journal of Biochemistry, 276: 349-355.

Stransky, M., & Rysava, L. (2009). Nutrition as prevention and treatment of osteoporosis. Physiological Research, 1, S7-S11.

Su, D. L., Lu, Z. M., Shen, M. N., Li, X. & Sun, L. Y. (2012). Roles of Pro- and Anti-Inflammatory Cytokines in the Pathogenesis of SLE. Journal of Biomedicine and Biotechnology, 1-15

Suzuki YA, Kelleher SL, Yalda D, Wu L, Huang J, Huang N, et al. (2003). Expression, characterization, and biologic activity of recombinant human lactoferrin in rice. J Pediatr Gastroenterol Nutr., 36:190–9.

Svoboda, M., Drábek, J., & Ficek, R. (2005). Effect of bovine lactoferrin on utilization of orally administered iron in suckling piglets. Bulletin of the veterinary institute in pulawy, 49: 471-474.

Tao, N., DePeters, E. J., Freeman, S., German, J. B., Grimm, R. & Lebrilla, C. B. (2008). Bovine milk glycome. J. Dairy Sci., 91:3768–3778.

Tao, N., DePeters, E. J., German, J. B., Grimm, R. & Lebrilla, C. B. (2009). Variations in bovine milk oligosaccharides during early and middle lactation stages analyzed by high-performance liquid chromatography-chip/mass spectrometry. J Dairy Sci.92:2991–3001.

Tapia, V. & Nuñez, M. T. (1999). Transferrin stimulates iron absorption, exocytosis and secretion in cultured intestinal cells. Am. J. Physiol., 276: C1085-C1090.

Tatala, S., & Svanberg , U., (1998). Low dietary iron availability is a major cause of anemia: a nutrition survey in the Lindi District of Tanzania. Am .J. clinical Nutr., 68(1), 171-178.

Tawfik, A. A., Hanna, E. T. & Freig, S. A. (2014). Folate Status in Egypt. Journal of Nursing and Health Science, 3 (2): 32-36.

Taylor, S. N., Basile, L. A., Ebeling, M. & Wagner, C. L. (2009). Intestinal permeability in preterm infants by feeding type: mother's milk versus formula. Breastfeed. Med. 4:11–15.

Teraguchi, S., Wakabayashi, H., Kuwata, H., Yamauchi, K. & Tamura, Y. (2004). Protection against infections by oral lactoferrin: evaluation in animal models. BioMetals, 17(3): 231–234.

The Egyptian Ministry of Health and Population and UNISEF Egypt office (2012). EGYPT Nutrition Landscape Analysis Report, 116p.

Thompson, B. (2007). Food-based approaches for combating iron deficiency..Nutritional Anemia. Sight and Life Press, Switzerland, pp. 22.

Thompson, A. B., Bohling, T., Payvandi, F. & Rennard, S. I. (1990). Lower respiratory tract lactoferrin and lysozyme arise primarily in the airways and are elevated in association with chronic bronchitis. The Journal of Laboratory and Clinical Medicine, 115: 148-158.

Togawa, J., Nagase, H., Tanaka, K., Inamori, M., Nakajima, A., Ueno, N., Saito, T. & Sekihara, H. (2002). Oral administration of lactoferrin reduces colitis in rats via modulation of the immune system and correction of cytokine imbalance. Journal of Gastroenterology and Hepatology, 17 (12):1291–1298.

Tsuda, H., Kozu, T., Iinuma, G., Ohashi, Y., Saito, Y., Saito, D., … Iigo, M. (2010). Cancer prevention by bovine lactoferrin: from animal studies to human trial. Biometals, 23, 399-409.

Tsuda, H., Sekine, K., Fujita, K., & Ligo, M. (2002). Cancer prevention by bovine lactoferrin and underlying mechanism-a review of experimental and clinical studies. Biochemistry and Cell Biology, 80, 131-136.

Underwood, M. A., Salzmand, N. H, Bennett, S. H., Barman, M., Mills, D. A., Marcobal, A., Tancredi, D. J., Bevins, C. L. & Sherman, M. (2009). A randomized placebo-controlled comparison of 2 prebiotic/probiotic combinations in preterm infants: Impact on weight gain, intestinal microbiota and fecal short chain fatty acids. J Pediatr Gastroenterol Nutr, 48:216–225.

USDA (2001). Nutrient database for standard reference: release 14. Beltsville (MD) Nutrient Data Laboratory, Human Nutrition Research Center of the Agricultural Research Service (ARS); Available: www.nal.usda.gov/fnic/foodcomp/Data/SR14/sr14.html (accessed 2002 Nov 18).

USDA (2009). Infant nutrition and feeding: a guide for use in the WIC and CSF programs. Washington. 221p.

Van Snick, J. L. & Masson, P. L. (1976). The binding of human lactoferrin to mouse peritoneal cells. The Journal of Experimental Medicine, 144: 1568-1580.

Van Veen, H. (2008). Production and characterization of recombinant human lactoferrin. Ph.D thesis, Leiden University, Netherlands, 113p.

Verster, A., & van der Pols, J. C. (1995). Anemia in the Eastern Mediterranean Region. Eastern Mediterranean health journal, 1: 64–79.

Vorland, L. H. (1999). Lactoferrin: a multifunctional glycoprotein. Acta pathologica, Microbiologica et Immunologica Scandinavica (APMIS), 107: 971-981.

Wakabayashi, H., Yamauchi, K., & Takase, M. (2006). Lactoferrin research, technology and applications. International Dairy Journal, 16: 1241-1251.

Walkar, A. (2010). Breast milk as the gold standard for protective nutrients. Journal of Pediatrics, 156(2): S3-S7.

Wang, B., Brand-Miller, J., McVeagh, P. & Petocz, P. (2001). Concentration and distribution of sialic acid in human milk and infant formulas. Am. J. Clin. Nutr., 74: 510–515.

Wang, W. P., Iigo, M., Sato, J., Sekine, K., Adachi, I., & Tsuda H. (2000). Activation of intestinal mucosal immunity in tumor-bearing mice by lactoferrin. Japan Journal of Cancer Research, 91, 1022-1027.

Wang, X. Y., Guo, H. Y., Zhang, W., Wen, P. C., Zhang, H., Guo, Z. R., & Ren, F. Z. (2013). Effect of iron saturation level of lactoferrin on osteogenic activity in vitro and in vivo. Journal of Dairy Science, 96(1), 33-39.

Ward, P. P., Mendoza-Meneses, M., Cunningham, G. A., & Conneely, O. M. (2003). Iron status in mice carrying a targeted disruption of lactoferrin. Molecular and cellular biology, 178-185.

West, A. R. & Oates, P. S. (2008). Mechanisms of heme iron absorption: Current questions and controversies. World J Gastroenterol, 14(26): 4101-4110.

Whiting, S. J., Vatanparast, H., Baxter-Jones, A., Faulkner, R. A., Mirwald, R., & Bailey, D. A. (2004). Factors that affect bone mineral accrual in the adolescent growth spurt. Journal of Nutrition, 134(3S), 696S-700S.

WHO (1986). Thirty-Ninth World Health Assembly. Guidelines concerning the main health and socioeconomic circumstances in which infants have to be fed on breast-milk substitutes. A39/8 Add. 1. 10 April 1986.

WHO (2001). Iron deficiency anaemia: assessment, prevention and control: a guide for programme managers. Document WHO/NHD/01.3. Geneva: 2001./publications/enWHO,(http://www.who.int/nutritionassessment_prevention_control.pdf).

WHO (2002). The World Health Report, Reducing risks, promoting healthy life. Geneva, World Health Organization.

WHO (2006). WHO child growth standards: length/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age: methods and development. Geneva: World Health Organization.

WHO/UNICEF (2004). Focusing on anemia: Towards an integrated approach for effective anemia control. Geneva: World Health Organization.

World Bank (1994). Enriching lives, Overcoming Vitamin and Mineral malnutrition in developing countries. Washington DC, World Bank, 1994.

World Bank (1996). Micronutrient Interventions, HDD Flash. Washington, DC: World Bank, 1996.

World Food Programme/Cairo Demographic Centre (2010). Analysis of consumer profiles and behavior patterns of food subsidy recipients: an approach to targeting. Cairo: WFP.

Wu S, Grimm R, German JB, Lebrilla CB. (2011). Annotation and structural analysis of sialylated human milk oligosaccharides. J Proteome Res. 10:856–68.

Yang, D., de la Rosa, G., Tewary, P., & Oppenheim, J. J. (2009). Alarmins link neutrophils and dendritic cells. Trends in Immunology, 30(11): 531-537.

Yoshioka, H., Iseki, K. & Fujita, K. (1983). Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. Pediatrics, 72:317–321.

Yu, R. H., & Schryvers, A. B. (2002). Bacterial lactoferrin receptors: insight from characterizing the Moraxella bovis receptors. Biochemistry and Cell Biology, 80: 81-90.

Zawilla, N. (2013). Iron deficiency anemia in Egypt. The Health, 4 (3): 58-59.

Zhong, Z., Wheeler, M. D., Li, X., Froh, M., Schemmer, P., Yin, M., Bunzendaul, H., Bradford, B. & Lemasters, J. J. (2003). L-Glycine: a novel anti-inflammatory, immunomodulatory, and cytoprotective agent. Current Opinion in Clinical Nutrition and Metabolic Care, 6 (2): 229–240.

Zimecki, M., Mazurier, J., Spik, G. & Kapp, J. A. (1995). Human lactoferrin induces phenotypic and functional changes in murine splenic B cells. Immunology, 86 (1):122–127.

Zivkovic, A. M., German, J. B., Lebrilla, C. B., & Mills, D. A. (2011). Human milk glycobiome and its impact on the gastrointestinal microbiota. Proceedings of the National Academy of Science of USA, 108(1): 4653-4658.

Zopf, D. & Roth, S. (1996). Oligosaccharide anti-infective agents, Lancet, 347: 1017–1021.

Chapter 2

The role of rhLf and GOS on Fe bioavailability: Formation of ferritin by Caco-2 cell model

2.1. Introduction

Recently, it is accepted that feeding during childhood is among the most important factors affecting immediate or short-term growth and body composition and function. Moreover, feeding at this critical period of life has many long term effects on different physiological and metabolic processes that may play a key role in reducing the incidence of different diseases (Gómez-Gallego et al., 2009).

Part of the functional components of human milk are an overabundance of complex oligosaccharides that cannot be digested by human but are fermented by the human gut microflora, and proteins with antibacterial activity such as Lf (Yeung et al., 2005; González-Chávez et al., 2009) and these ingredients have a role in enhancement the absorption of Fe (Scholz-Ahrens et al., 2001; Legrand et al., 2008).

Therefore, the current trend for infant formulas production is that containing the functional ingredients of human milk such as Lf and GOS (Alles et al., 2004). HMOs are one of the functional ingredients among many others of human milk (Niers et al., 2007). GOS has a wide spectrum of functionalities, among them the facilitation of mineral absorption (Kaur & Gupta, 2002).

2.2. Aims of this chapter

This study aimed to explore the functional activity of infant formulas supplemented with different concentrations of rhLf and/or GOS on Fe absorption throughout the determination of the synthesized ferritin by Caco-2 cell line exposed to samples after *in vitro* simulated digestion.

2.3. Materials and methods

2.3.1. Infant formula

First infant formula (FIF) was provided by Hero Baby Co. (Alcantarilla, Murcia, Spain). This formula was used as a base for create various formulas by supplementation with different concentrations of rhLf (0.10, 0.15 or 0.20 g/100 mL of reconstituted infant formula) and/or different concentrations of GOS (3.3, 5 or 10 g/100 mL of reconstituted infant formula). Thus Lf content so obtained was similar to that of mature human milk ranging from 1 to 2 g/L (Nuijens et al., 1996). For the infant formula reconstitution, 100 mL of deionized water were mixed with 15 g of powder according to the manufacturer's instructions. The basic composition of the sample is reported in Table 2.1.

2.3.2. Materials and reagents

- **4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)**, H7006 (100 g) (Sigma Chemical Co., St. Louis, MO, USA).
- **D (+)-Glucose monohydrate** (Merck, Darmstadt, Germany).
- **Di-Methylsulfoxide (DMSO)** (Sigma Chemical Co., St. Louis, MO, USA).
- **Enzymes and bile salts** were purchased from Sigma Chemical Co. (Sigma Chemical Co., St. Louis, MO, USA): **Pepsin** (Porcine: cat. No. P-7000), **Pancreatin** (Porcine: cat. no. B-8756) and **bile salt** (Porcine: cat. no. P-1750).
- **Fetal bovine serum** (Sigma Chemical Co., St. Louis, MO, USA)
- **Glutamine** (Sigma Chemical Co., St. Louis, MO, USA).
- **Mixture of protease/phosphatase inhibitor cocktail and EDTA solution (100X)** (Thermo Scientific, cat. no. 78440, USA)
- **MEM** (Minimum Essential Medium, Gibco, Life Technologies, UK).
- **Mixture of antibiotics** ″penicilin-estreptomicin″ (Sigma Chemical Co., St. Louis, MO, USA).
- **Non-essential amino acid solution** (Sigma Chemical Co., St. Louis, MO, USA).

Table 2.1. The nutritional composition of the infant formula (control) for 100 g dry weight and for 100 mL reconstituted formula.

		For 100 g formula (dry weight)	For 100 mL reconstituted formula
Energy value	KJ/Kcal	2184/522	285/68
Nutrients			
Proteins	G	10.2	1.3
Caseins	G	5.1	0.7
Whey protein	G	5.1	0.7
Carbohydrates	G	55.2	7.2
Lipids	G	29.0	3.8
Linoleic acid	Mg	4510.0	586.3
Alpha-linolenic acid	Mg	430.0	55.9
Ratio		10.5	10.5
Minerals			
Sodium	Mg	130.0	16.9
Potassium	Mg	497.0	64.6
Colure	Mg	366.0	47.6
Calcium	Mg	392.0	51.0
Phosphor	Mg	220.0	28.6
Relation Ca/P		1.8	1.8
Magnesium	Mg	42.0	5.5
Fe	Mg	6.3	0.8
Zinc	Mg	4.2	0.5
Cupper	µg	314.0	40.8
Iodine	µg	78.0	10.1
Selenium	µg	7.0	0.9
Vitamins			
A	µg	523.0	68.0
D	µg	7.8	1.0
E	Mg	7.8	1.0
K	µg	52.0	6.8
C	Mg	52.0	6.8
B1	µg	523.0	68
B2	µg	785.0	102.0
Niacin	Mg	785.0	0.7
B6	µg	78.0	10.1
Folic acid	µg	2.1	0.3
Biotin	µg	16.0	2.1
Pantothenic acid	µg	2353.0	305.9
Others			
L-Carnitine			
Taurine	Mg	7.8	1.0
Inositol	Mg	42.0	5.5
Colin	Mg	26.1	3.4

- **Recombinant human lactoferrin (rhLf).** Expressed in rice, Fe saturated, >90% (SDS-PAGE)], purchased from Sigma Chemical Co. (cat. no. L1294, St. Louis, MO, USA).
- **RIPA** (Radio-Immunoprecipitation Assay) lysis buffer was purchased from Sigma Co. USA (cat. no. R0278). RIPA buffer enables efficient cell lysis and protein solubilization while avoiding protein degradation and interference with the proteins' immunoreactivity and biological activity. RIPA buffer also results in low background in immunoprecipitation and molecular pull-down assays. Sigma's RIPA buffer formulated as follows: 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0.
- **Tripan blue**, CI 23850 (Merck, Darmstadt, Germany).
- **Tripsin-EDTA solution** (0, 25 mg/mL) (Sigma St Louis, MO, USA).
- **Vivinal GOS syrup** (dry matter 75 % of which GOS 59 %, lactose 21 %, glucose 19 % and galactose 1 %) was provided by Hero Baby Co. (Alcantarilla, Murcia, Spain).

2.3.3. Cleaning of laboratory materials (glassware, tubes, porcelain)

Glassware, bottles, tubes and porcelain used throughout the *in vitro* digestion experiment and throughout the determination of mineral were immersed at least for 24 hours in 10% nitric acid to eliminate any traces of minerals that may be trapped in the material, and then rinsed three times thoroughly with distilled-deionized water before use.

2.3.4. Preparation of working enzymes solutions

Pepsin solution was prepared by dissolving 1.6 g of pepsin in 10 ml of 0.1 M HCl while the pancreatic-bile extract solution was prepared by dissolving 0.2 g of pancreatin and 1.25 g of bile extract in 50 ml of 0.1 M $NaHCO_3$. It is important to be taking into account that working enzyme solutions were prepared immediately before use and Milli-Q distilled-deionized water (Millipore Ibérica S.A., Barcelona, Spain) was used throughout the experiments.

2.4. *In vitro* digestion of infant formulas

The different infant formulas reconstituted with deionized distilled water according to the manufacturer instructions and they were exposed to *in vitro* simulated gastrointestinal digestion using *in vitro* method described by Miller et al. (1981) and modified by (Bosscher et al., 2001; Jovaní et al., 2001) to decrease the amount of used enzymes where it found that the gastrointestinal tract of newborns and infants is not completely mature. The method consisted of two phases: gastric and intestinal. Prior to the gastric stage, the pH of 4.5 g of each infant formula homogenized with 30 mL of deionized-distilled water was lowered to pH 4 with 6 mol/L HCl. Then, freshly prepared pepsin solution to provide 0.02 g of pepsin/g of sample was added, and then incubated in a shaking water bath at 37ºC and 120 strokes/min for 2 h to allow pepsin digestion. The pepsin digest was then placed in ice bath for 10 min to inactivate the pepsin enzyme. For intestinal digestion, the pH of the gastric digests was raised to 5.0 by dropwise addition of $NaHCO_3$ 1 mol/L. Then, a freshly prepared pancreatin-bile extract solution to provide 0.005 g of pancreatin and 0.03 g of bile salts/g of sample was added, and incubation was continued for 2 h at the same conditions. To stop intestinal digestion, the sample was kept for 10 min in an ice bath. Then, the gastrointestinal digests were centrifuged at 3500 x g for 1 h at 4 ºC and the supernatants were kept at -20ºC until analysis.

2.4.1. Determination of Fe content in samples and their digests

Fe concentration in samples (before digestion) and in the soluble fractions of digests was determined from the ashes obtained after incineration of commercial slurries in muffle furnace at 525 °C for 32 h. These ashes were dissolved by adding 2 mL of 65% HNO_3 and 5 mL of 37% HCl to the porcelain crucibles, gently stirring with the application of heating until completely evaporation. Then, the concentration of Fe of the supernatant was measured by atomic absorption spectrophotometer (Perkin-Elmer, Norwalk, USA). The Fe solubility % was calculated according to Pyanert et al. (2006):

Solubility % = (soluble Fe (mg/100g)/total Fe (mg/100g) × 100

2.4.2. Caco-2 cell management, growth and viability

All tests performed for this work were carried out in the tissue culture laboratory of SACE (Servicio de Apoyo a las Ciencias Experimentales de la Universidad de Murcia). Experiments were run on laminar flow in vertical direction type II cabins, where we obtained sterile conditions for handling cell line. Prior to starting work with Caco-2 cell line, the cabin was ready about 15 minutes before to get a sterile working environment, lighting light lamp UV, and turning it off about 15 minutes before, leaving only the fan hood to remove contaminants introduced material work and the work area. Cabin surface was cleaned with 70% alcohol. Only the necessary material should be introduced to the cabin immediately. Generally, it must be take all the precautions to prevent all using materials away from any contaminants and follow all instructions to protect yourself and get satisfactory results before and after work.

Caco-2 cell line was obtained from the European Collection of Cell Culture (ECACC; number 86010202, Salisbury, UK) and used in assays at passages 15-25. Caco-2 cells are adherent cells with epithelial morphology with the feature that when they reach the confluence stop proliferating, they differentiate spontaneously developing monolayers of polarized cells in which the edge stresses brush own intestinal mucosa.

MEM with red phenol was used through this experiment and changed on alternate days and cells were incubated at 37 °C, at a partial pressure of 5% CO_2 and a relative humidity of 95%. The culture was maintained until 80% confluence and then subcultured. To detach the cells from the flask was used trypsin-EDTA solution (0.25%), the enzyme acts digesting adherence proteins that are dependent on calcium and magnesium that is where it intervenes EDTA sequestering free divalent cations in the joints involved. Viability assessed by trypan blue staining became demonstrate those cells with ruptured membranes and therefore were dead. This test was performed each time the subculture is conducted, before freezing and after thawing to determine the cells viability %. In all cases, cell viability was over 85%. The Caco-2 cell line was maintained in 75 cm^2 flasks until reaching 70- 80% confluence where was the time to performs subculture. To

know the changes of cell growth, the culture was observed at 2, 4, 8 and 20th day post seeding using a phase contrast microscope (Fig. 2.1). The cell line used in this study showed to be free of mycoplasma in tests carried out (Fig. 2.2).

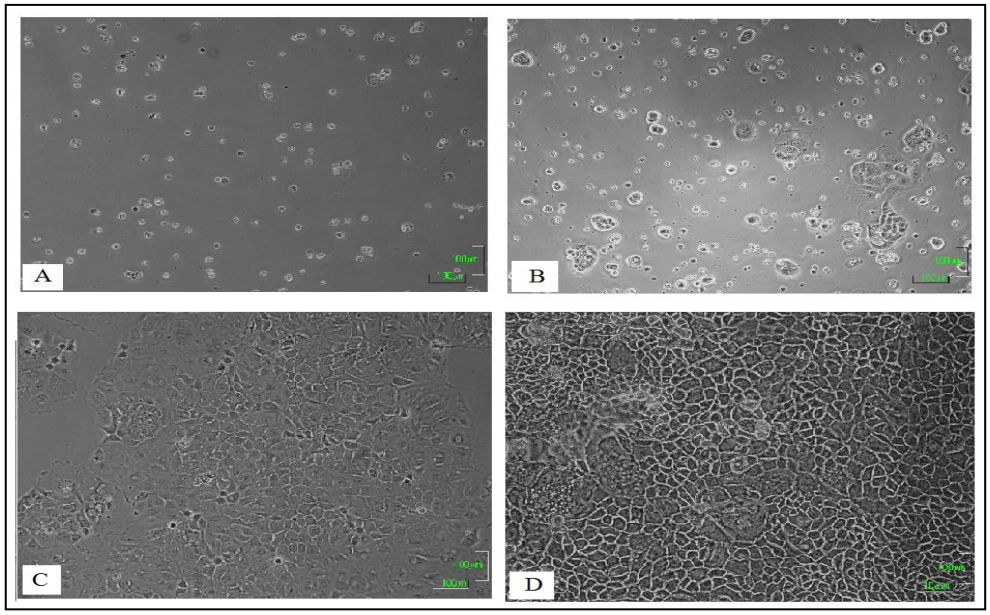


Fig. 2.1. Image of Caco-2 cell line in different days post-seeding by contrast microscope observation of the test phases. A) 2 days crop, B) 4 days of culture, C) 8 days of culture, D) 20 days post-seeding.

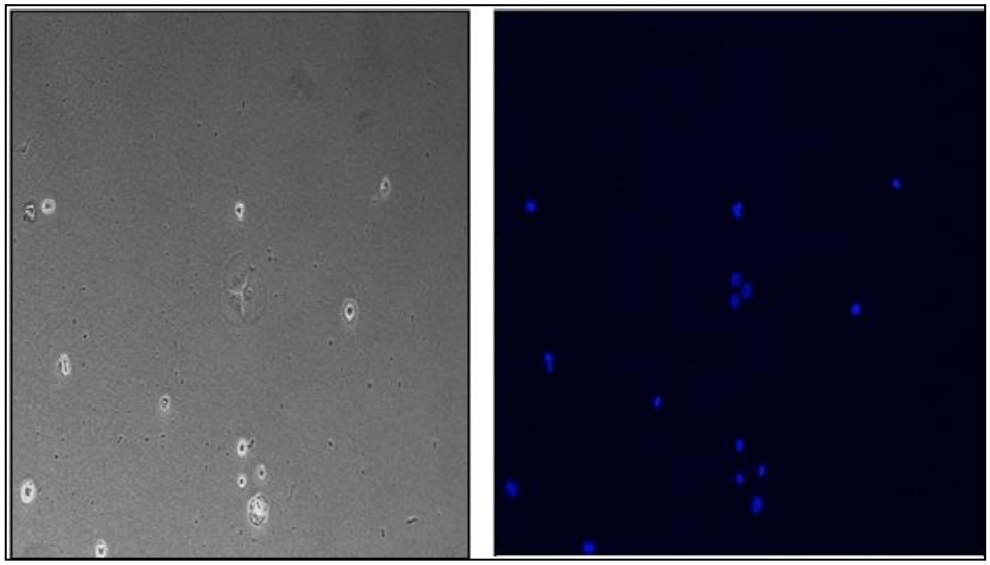


Fig. 2.2. Image of Caco-2 cell line presented the absence of mycoplasma in the cultured Caco-2 cell line. A) image using phase contrast microscope and B) image using the fluorescence microscope.

The contamination of cell cultures with mycoplasmas can induce cytogenetic effects, decreased effects on nutrient concentrations; alter the cellular morphology, modulation of the immune response and even disruption of cellular metabolism (Rivera et al., 2011).

Respecting with Lf toxicity on Caco-2 cell line, Yecta et al. (2010) reported that none of the Lf concentrations tested (zero. 0.001. 0.005. 0.01. 0.05. 0.1. 0.5. 1.0. 5.0 and 10 mg/mL) were cytotoxic to Caco-2 cells, as compared to untreated control cells. Thus, the different concentrations of rhLf used in this study were not toxic for Caco-2 cells.

2.5. Fe bioavailability *in vitro* by Caco-2 cell line model (Ferritin synthesis)

Caco-2 cell culture model is used as a good tool for determination of mineral bioavailability *in vitro* especially Fe. In this study it was assessed the effect of the supplementation of infant formula with rhLf in three concentrations (0.10, 0.15, 0.20%) and/or GOS also in three concentrations (3.3, 5, 10%) on Fe bioavailability. To perform these assays, cells were seeded into flasks of 25 cm^2. The seeding was 50 000 cells/cm^2, where they were kept for up to their differentiation (21 days), the medium was changed in alternate days.

2.5.1. Conditioning of the soluble fraction for addition to cell monolayer

In this assay, the different soluble fractions of infant formulas obtained by *in vitro* digestion were used. Prior to addition of the soluble fractions to the cell monolayer, glucose (5 mmol/L final concentration), HEPES (50 mmol/L final concentration) were added to make the soluble fractions similar to the culture media, and finally, the pH was adjusted to be between 7.1-7.2 by using 0.5 mol/L NaOH (Crison pH-meter, EEC), and finally water was added to adjust the osmolarity to 310 ± 10 mOsm/kg (Vapor pressure osmometer 5520, Wescor, USA) according to Ekmekcioglu (2002).

The conditioned soluble fraction was combined with an equal volume of basal growth medium (Fe depleted-MEM). After removing the old media from flasks and washing cell monolayers three times with suitable volumes of PBS (37ºC), an appropriate volume of the conditioned solution,

previously sterilized by 0.22 µm filter, was allowed to remain on the top of cell monolayer for 2 h at 37°C in 5% CO_2 with 95% relative humidity. After 2 h of incubation, cells were washed and a new media were added. At the same conditions, the cultures were incubated for an additional 22 h.

It is very important to note that at the day of experiment cells were maintained in culture medium deficient in Fe. For this it was demineralized the fetal bovine serum to eliminate a high amount of its Fe following the procedure described by Alvarez-Hernández et al. (1991). Fifty milliliters (50 mL) of serum fetal bovine demineralized by stirring with 15g of Chelex-100 (Na form) for 2 h at pH 4.5. Subsequently, pH value was increased to 7.4 with NaOH, allowed to stand overnight and the mixture was filtered through paper filter (Whatman 1 or 2). The filtrate was sterilized by a membrane filter of 0.22 µm pore diameter.

2.5.2. Ferritin extraction and measurement

For ferritin extraction from cell cultures, cell monolayers were washed three times with PBS (37ºC) to remove non-specifically bound mineral and residual medium and detached with trypsin-EDTA solution. Cells were collected with 1 mL of deionized water at 4 ºC and homogenized at 17000 rpm for 3 min at 4 °C (Polytron PT 2000, Kinematica AG). 50 microlitre aliquots of the sonicated Caco-2 monolayer were used in ferritin determination (AssayMax human ferritin ELISA kit, Catalog No. EF2003-1, Assaypro LLC, USA), where ferritin acts as the main intracellular store of cytosolic Fe (Glahn et al., 1998).

This assay was operated according to human ferritin ELISA kit instructions and it employs a quantitative sandwich enzyme immunoassay technique, which measures ferritin in less than 4 hours. A polyclonal antibody specific for ferritin has been pre-coated onto a 96-well microplate with removable strips. Ferritin in standards and samples is sandwiched by the immobilized antibody and biotinylated polyclonal antibody specific for ferritin, which is recognized by a streptavidin-peroxidase conjugate. All unbound materials were then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured by reading the absorbance on a microplate reader

(Fluostar Omega, BMG Labtech, USA) at a wavelength of 450 nm immediately.

2.5.3. Extraction and quantification of cell proteins

The Bradford protein assay is a simple and accurate procedure for determining the concentration of protein in solution and it is based on the classic method of Bradford (1976). The Bradford assay is a protein determination method that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins. When the dye binds to protein, it is converted to a stable unprotonated blue form that can be measured easily at 595 nm using a microplate reader (Fluostar Omega, BMG Labtech, USA). The quick start Bradford protein assay is a compatible method with some reagents such as acetone (10%), acetonitrile (10%), PBS, DMSO (5%), MEM, HEPES (0.1 M), and methanol (10%). To determine the total protein concentration present in a sample of a line pattern is used bovine serum albumin (BSA) as a protein standard.

For the extraction of protein from cells, RIPA was used, and a mixture of protease/phosphatase inhibitor cocktail and EDTA solution which protects proteins from degradation by endogenous proteases, phosphatases and metalloprotease released during protein extraction and purification. Briefly, the medium was removed by centrifugation at 450 x g for 5 min and cells were washed 2 times with cold PBS. Then, the mixture of RIPA lysis buffer, Halt protease/phosphatase inhibitor cocktail and EDTA solution were added to the cell pellet, and mix or vortex briefly to resuspend the cells completely. Incubation on ice or in a refrigerator (2–8 °C) for 5 min. Vortex briefly to resuspend and lyse residual cells. The lysate can either be used immediately or quick frozen in liquid nitrogen or stored at -70 °C for future use.

2.6. Statistical analysis

Results were expressed as mean ± SD of three experiments. After testing for normality and equal variances, the mean solubility percentages of Fe from different infant formulas were compared by one-way analysis of variance (ANOVA) including the Duncan Multiple Range Test in the data

treatment to determine significant differences among means ($P<0.05$). A Pearson correlation analysis was performed to investigate the possible correlation between mineral solubility (%) and ferritin formation by Caco-2 cells. Values of $P<0.05$ were considered significant. All statistical analyses were performed with the Statistical Package for the Social Sciences (version 14.0; SPSS).

2.7. Results and Discussion

Fe bioavailability of some infant formulas supplemented with some functional ingredients of human milk such as rhLf and/or GOS was determined. These formulas were *in vitro* digested, the obtained soluble fractions were conditioned and added to the cell monolayers and finally the ferritin content was determined espectrophotometrically by ELISA method. Ferritin acts as the main intracellular store of cytosolic Fe, thus it was used as indirect indicator of Fe absorption. In this regard, Glahn et al. (1998) reported that the use of intracellular ferritin formation as an indicator of Fe uptake by Caco-2 cells gives a highly sensitive and accurate measure of the availability of Fe from foods, and eliminates the need for extrinsic or intrinsic labeling of food Fe in availability assays.

Total Fe content (mg/100 g dry weight of formula) and Fe solubility % are shown in Table 2.2. Findings indicated that control formula (without any added ingredient) had the low concentration of total Fe. The total Fe content of control formula was slightly lower than the data provided by the manufacturer (~6.3 mg/100 g dry weight). As well as formulas supplemented with 3.3, 5 or 10% GOS had the same content of Fe. The results revealed that there is a positive linear correlation between formula Fe content and the increasing rhLf added where *e.g.* Fe content was 6.86, 7.04 and 7.36 (mg/100 g powder) for the formulas contain 0.10, 0.15 or 0.20 % rhLf, respectively. Findings showed that all treatments had a Fe solubility % higher than control formula. Respecting with infant formulas supplemented with GOS (3.3, 5 or 10%), it was found that formula with 3.3 % GOS had the highest value of Fe solubility percentage (78.65%) followed by formula

Table 2.2. Total Fe content (mg/100 g dry weight) and Fe solubility (%).

Treatment	Total Fe	Fe solubility (%)
Control	6.23 ± 0.20	66.37 ± 1.77 ^{c}
3.3 % GOS	6.23 ± 0.20	78.65 ± 8.30 ^{abc}
5 % GOS	6.23 ± 0.20	77.69 ± 4.11 ^{abc}
10 % GOS	6.23 ± 0.20	76.48 ± 7.68 ^{bc}
0.10 % rhLf	6.86 ± 0.29	83.35 ± 8.79 ^{abc}
0.10 % rhLf + 3.3 % GOS	6.86 ± 0.29	82.78 ± 5.35 ^{abc}
0.10 % rhLf + 5 % GOS	6.86 ± 0.29	84.41 ± 1.54 ^{abc}
0.10 % rhLf + 10 % GOS	6.86 ± 0.29	90.01 ± 3.51 ^{ab}
0.15 % rhLf	7.04 ± 0.10	84.97 ± 2.72 ^{abc}
0.15 % rhLf + 3.3 % GOS	7.04 ± 0.10	88.94 ± 11.53 ^{ab}
0.15 % rhLf + 5 % GOS	7.04 ± 0.10	94.13 ± 2.95 ^{ab}
0.15 % rhLf + 10 % GOS	7.04 ± 0.10	96.13 ± 5.74 ^{a}
0.20 % rhLf	7.36 ± 0.29	80.56 ± 3.66 ^{abc}
0.20 % rhLf + 3.3 % GOS	7.36 ± 0.29	78.27 ± 0.84 ^{abc}
0.20 % rhLf + 5 % GOS	7.36 ± 0.29	79.69 ± 3.20 ^{abc}
0.20 % rhLf + 10 % GOS	7.36 ± 0.29	80.71 ± 0.82 ^{abc}

Values are mean ± SD of three independent experiments. Means of the same column with different superscripts are statistically different (P<0.05). Abbreviations: rhLf: recombinant human lactoferrin, GOS: galactooligosaccharides.

contains 5% GOS (77.69%), then 10% GOS (76.48%); however, the differences between these values were very low and not differ significantly ($P<0.05$) (Table 2.2).

These experimental data agreed with a study reported that supplemental inulin promotes Fe absorption in piglets (Yasuda et al., 2006) as well as agreed with previous findings from animal studies (Lopez et al., 2000; Santos et al., 2010). Furthermore, Scholz-Ahrens et al. (2001) reported that prebiotics such as oligofructose, inulin, glucooligosaccharide, and GOS promoted Fe, Ca and Mg absorption and retention. Results obtained were in line with findings obtained by Christides and Sharp (2013) who reported that sugars had a positive effect on non-heme Fe absorption in Caco-2 cell line. However, they appear to conflict with findings of a study on non-anemic humans indicating that non heme-Fe absorption does not enhanced by using the indigestible prebiotics (Van den Heuvel et al., 1998).

Regarding with formulas contain rhLf, findings revealed that all these formulas offered high values of Fe solubility % as compared with that of formulas supplemented with different concentrations of GOS or control formula (Table 2.2). The data revealed that a combination of 0.15% rhLf + 10 % GOS has provided the major positive effect on Fe solubility % (96.13%) followed by 0.15% rhLf + 5% GOS (94.13%), then 0.10% rhLf + 10% GOS (90.01%) and these obtained values significantly differed ($P<0.05$) as compared with all treatments.

The high percentage of Fe solubility obtained (ranging from 66.37 to 96.13%) was in a good agreement with those reported by Perales et al. (2007) who found even a higher range of Fe solubility (31.9-99.1%). The formulas composition and the type of potent promotor of Fe absorption (Lf vs ascorbic acid) in both experiments may explained the slightly difference in Fe solubility percentage. Interestingly, Perales et al. (2007) reported the difficulty of comparing their results with others due to differences in methodology, amount of enzyme, pH and time incubation in gastric digestion and intestinal and even variations in speed centrifugation in the case of solubility. In this sense, Bosscher et al. (2001b) conducted a study

on conditions similar to ours and concluded that the mineral availability varies pH used in the gastric and intestinal digestion and incubation time.

These findings suggested that rhLf and GOS have an Fe absorption increasing property and they are in line with those found by Kawakami et al. (1993) that demonstrated that intact Lf could solubilizing up to a 70-fold molar equivalent of Fe under neutral conditions in the intestine, which is much higher than the Fe-binding property of Lf. It seems likely that the excess Fe was electrostatically associated with Lf. In this sense, Lf may be useful as a natural solubilizer of Fe for food products and it was suggested that Lf, orally administered, could solubilize ferric Fe in the intestine. Interestingly, under neutral conditions, pepsin-digested Lf retained its capability to solubilize dietary Fe. This finding proposed that restricted breakdown of Lf accounted for intestinal Fe solubility (Ushida et al., 2006). Also the addition of Fe-Lf complex to complementary foods may stimulate the Fe status (Lönnerdal & Bryant, 2006).

Likewise, Fe absorption positively influenced by the presence of inulin and FOS (Yeung et al., 2005) and this be partly explained by the colonic bacterial fermentation of these substances (Scholz-Ahrens et al., 2007) which participate in a lowering of the luminal pH which in turn may enhance mineral solubility (Yeung et al., 2005). In the same manner, Kamasakaa et al. (1997) demonstrated that oligosaccharides could solubilize Fe in the intestine. Thus the Fe absorption is improved by the presence of Lf and oligosaccharides (Jovaní et al., 2003; Etcheverry et al., 2004; Uchida et al., 2006).

Furthermore, some bioactive peptides produced during *in vitro* digestion process could improve the Fe bioavailability through its high chelating characteristics for Fe. This effect on Fe bioavailability was demonstrated for synthetic β-casein (Bæch et al., 2003; Argyri et al., 2007) and soy protein hydrolysate (Lv et al., 2014) as well as for certain amino acids (Swain et al., 2002). These compounds may bind Fe, forming soluble complexes and thus improving its bioavailability (Glahn et al., 1997; Storcksdieck et al., 2007; Lv et al., 2014). Some published studies, using

Caco-2 model, revealed that milk peptides increased Fe dialyzability and Fe uptake (Argyri et al., 2007; 2009). Recently, it was reported that the chelating peptides have received more attention because it facilitate the conversion of ferric Fe to ferrous which enters to the enterocytes through its DMT1 receptors. Moreover, enterocytes may uptake the Fe-peptides complexes through a specific peptides transporter located on the surface of the brush border membrane and subsequently improve Fe bioavailability (Torres-Fuentes et al., 2012).

Ferritin levels formed by Caco-2 cells exposed to soluble fractions of formulas are shown in Table 2.3. As stated above, the increase of ferritin formation in cells is an indicator that Fe has entered the cell because the intracellular ferritin formation increased in response to Fe that has been entered to the cells (Reymond et al., 1996, 1998; Cook et al., 2003; Laparra et al.; 2008). Therefore, the intracellular ferritin formation was used as an indicator of Fe bioavailability. Fe uptake into intracellular medium was expressed as a ratio of ferritin and cell protein (ng ferritin/mg cell protein), because the factors that promote multiplication of a cell such as Lf were involved in whey (Schottstedt et al., 2005). The highest value of ferritin was detected in the infant formula with 0.15 % rhLf + 5% GOS (45.83) followed by the one that contained 0.20 % rhLf + 5 % GOS (45.61), 0.20 % rhLf + 3.3 % GOS (43.50), 0.20 % rhLf + 10 % GOS (43.37). These findings were significantly ($P<0.05$) differed respecting with control and other treatments. It is interesting to mention that addition of both of rhLf and GOS caused a 5-7.5-fold increase in ferritin concentration quantified in these cultures. In the present study, Caco-2 ferritin levels ranging from 6.13 to 45.83 ng ferritin/mg cell protein were obtained, the lowest value corresponding to the formulas without any added ingredient and the highest to formulas containing 0.15 % rhLf + 5% GOS. The low ferritin levels obtained in our study, when compared to those reported by Etcheverry et al. (2004), can be mainly explained by the differences in the formula composition. There were also differences in the applied methodology to measure ferritin (ELISA versus immunoradiometric).

Table 2.3. Ferritin level of Caco-2 cell exposed to different digests of infant formulas.

Treatment	Fe added to the monolayer (µg)	Ferritin/cell protein (ng/mg)
Control	16.10 ± 0.84	6.13 ± 1.40 ^{f}
3.3 % GOS	20.18 ± 2.15	12.18 ± 1.57 ^{def}
5 % GOS	20.51 ± 0.03	11.45 ± 0.60 ^{ef}
10 % GOS	19.30 ± 2.93	12.11 ± 1.37 ^{def}
0.10 % rhLf	21.13 ± 0.73	20.51 ± 3.84 ^{cde}
0.10 % rhLf + 3.3 % GOS	19.26 ± 1.75	24.32 ± 2.86 ^{cde}
0.10 % rhLf + 5 % GOS	18.43 ± 0.57	20.95 ± 0.22 ^{cde}
0.10 % rhLf + 10 % GOS	19.12 ± 0.86	32.11 ± 10.21 ^{bc}
0.15 % rhLf	24.73 ± 0.21	24.94 ± 1.89 ^{cd}
0.15 % rhLf + 3.3 % GOS	25.45 ± 3.22	33.81 ± 5.61 ^{abc}
0.15 % rhLf + 5 % GOS	26.88 ± 0.79	45.83 ± 8.91 ^{a}
0.15 % rhLf + 10 % GOS	26.88 ± 1.52	31.97 ± 3.12 ^{bc}
0.20 % rhLf	25.70 ± 1.28	31.74 ± 3.59 ^{bc}
0.20 % rhLf + 3.3 % GOS	27.67 ± 0.3	43.50 ± 4.77 ^{ab}
0.20 % rhLf + 5 % GOS	27.09 ± 1.00	45.61 ± 6.76 ^{a}
0.2 % rhLf + 10 % GOS	26.83 ± 0.20	43.37 ± 7.45 ^{ab}

Values are mean ± SD of three independent experiments. Means of the same column with different superscripts are statistically different (P<0.05). Abbreviations: rhLf: recombinant human lactoferrin, GOS: galactooligosaccharides.

These low levels of ferritin obtained in the presented study are in line with those obtained by Viadel et al. (2007) who used the same technique to measure ferritin level (ELISA method). However, the ferritin level of our study still lower and this may be explained by the differences in the formula composition.

The obtained results revealed that addition of GOS and/or rhLf to the infant formulas enhanced the ferritin formation as compared to control formula. It is important to mention that ferritin formation in cultures exposed to formulas containing different concentration of GOS did not correspond to the higher Fe solubility % and this it was be supported by the findings of Laparra et al. (2008) study, which indicated that supplementation with inulin led to a 2-fold increase in dialyzable Fe and cell Fe content, without differences in ferritin concentration evaluated in the cultures. Although ferritin expression is translationally regulated by intracellular Fe concentration (Pantopoulos, 2004; Rouault, 2006) and its formation by intestinal cells occurs in response to Fe that has been taken up (Laparra et al., 2008), the presented findings showed that Fe solubility is not considered the only determinant factor of ferritin formation by the cultures. Thus, it is possible that another mechanism may participate in ferritin formation rather than mineral solubility. In this sense, Laparra et al. (2008) proposed that Fe solubility is needed to be absorbed; however, not all soluble forms of Fe are available to Caco-2 cells as concluded from ferritin formation values quantified. In this regard, Zhu et al. (2006) reported that DMT1 may facilitate the Fe uptake via a proton-coupled mechanism.

Although the mineral solubility is not the only factor which affect on ferritin formation, the increase in ferritin formation observed could be due to the effect of rhLf and GOS on Fe solubilization occurred during *in vitro* digestion. In this sense, *in vivo* study (Chierici et al., 1992) has noted an increase in serum ferritin of infants fed formula supplemented with bLf as compared with non-supplemented formula. Likewise, an increase in ferritin formation was observed by Caco-2 in the presence of casein phosphopeptides, bioactive peptides derived from milk casein by proteolytic

digestion (Yeung et al., 2002; Argyri et al., 2007). Moreover, Etcheverry et al. (2004) reported that a low molecular-weight factor in human and bovine milk whey, named Lf, enhanced Fe bioavailability by Caco-2 cells. Similarly, it was proposed that prebiotics, *e.g.* inulin, FOS and GOS, had an enhancing activity on the absorption of Fe (Yeung et al., 2005). Furthermore, Lf plays an effective role in Fe homeostasis in newborns (Sacrino, 2007) and this suggestion was supported by the higher content of Lf in human milk than bovine milk (Vorland, 1999).

Despite the many and various studies have carried out to identify the different functionalities of Lf, particularly which by promote the Fe absorption, the role of Lf in intestinal Fe absorption is still controversial and it is not fully understood the mechanism by which this protein may participate in that process (Uchida et al., 2006).

For evaluating the interactions might be occurred between Fe solubility and the formation of ferritin by the different cultures, Pearson Correlation Test was performed where significant differences were noted. In this sense, the presented findings are in a good agreement with those reported by Zhu et al. (2006) where a positive linear correlation between Fe bioavailability (expressed as ferritin level) and its intestinal solubility was observed; thus, mineral solubility can be used to establish trends in the bioavailability or relative bioavailability of Fe, because the chance for Fe uptake increases with a higher solubility. Fe bioavailability can be affected by the matrix composition of the samples and the presence of Fe enhancers or inhibitors. Components of milk (casein and Lf) or fruit juices components (ascorbic, citric and malic acids) and the Fe salt used for enrichment could explain differences among samples (Perales et al., 2007).

In conclusion, the supplementation of infant formulas with both rhLf and GOS could promote the Fe solubility almost 1.5 times, which may turn into an improvement of Fe (bio)-availability (expressed as ferritin formation by the cultures) 5-7.5 fold increase.

2.8. References

Alles, M. S., Scholtens, P. A. S. M. & Bindles, J. (2004). Current trends in the composition of infant milk formulas. Current Pediatric, 14 (1): 51-63.

Alvarez-Hernandez, X, Nichols, G. & Glass, J (1991). Caco-2 cell line: a system for studying Fe transport across epithelial cell monolayers. Biochem Biophys Acta, 110: 205-208.

Argyri, K., Miller, D. D., Glahn, R. P., Zhu, L. &Kapsokefalou, M. (2007). Peptides isolated from *in vitro* digests of milk enhance Fe uptake by Caco-2 cells. J Agric Food Chem, 55: 10221-10225.

Argyri, K., Tako, E., Miller, D. D., Glahn, P. R., Komaitis, M. and Kapsokefalou, M. (2009). Milk peptides increase Fe dialyzability in water but do not affect DMT-1 expression in Caco-2 cells. J Agric Food Chem, 57: 1538-1543.

Bæch, B. S., Hansen, M., Bukhave, K., Jensen, M., Sørensen, S. S., Kristensen, L., Purslow, P. P., Skibsted, H. L. & Sandström, B. (2003). Non heme-Fe absorption from a phytate-rich meal is increased by the addition of small amounts of pork meat. Am. J. Clin. Nutr., 77: 173-179.

Bosscher, D., Lu Z, Cauwenbergh, R. V., Van Caillie-Bertrand, M., Robberecht, H. & Deelstra, H. (2001a). A method for *in vitro* determination of calcium, Fe and zinc availability from first age infant formula and human milk. Int. J. Food Sci. Nutr., 52: 173- 182.

Bosscher, D., Van Caillie-Bertrand M, Robberecht H, Van Dyck K, Cauwenbergh RV, Deelstra H. (2001b). In vitro availability of calcium, Fe and zinc from first-age infant formulae and human milk. J. Pediatr. Gastroenterol. Nutr., 32: 54-58.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

Chierici, R., Sawatzki G, Tamisari, L., Volpato, S. & Vigi, V. (1992). Supplementation of an adapted formula with bovine lactoferrin. 2. Effects on serum Fe, ferritin and zinc levels. Acta Paediatr., 81: 475–479.

Christides, T. & Sharp, P. (2013). Sugars increase non-heme Fe bioavailability in human epithelial intestinal and liver cells. Plos One, 8(12): e830-831.

Cook, J. D., Flowers, C. H. & Skikne, B. S. (2003). The quantitative assessment of body Fe. Blood, 101: 3359–3363.

Ekmekcioglu, C. A. (2002). A physiological approach for preparing and conducting intestinal bioavailability studies using experimental systems. Food Chemistry, 76: 225-230.

Etcheverry, P., Wissler, J., Wortley, G. & Glahn, R. (2004). Caco-2 cell Fe uptake from human milk and infant formula. Nutrition Research, 24: 573–579.

Glahn, R. P., & Van Campen, D. R. (1997). Fe uptake is enhanced in Caco-2 cell monolayers by cysteine and reduced cysteinyl glycine. The Journal of Nutrition, 127: 642–647.

Glahn, R. P., Olivia, A. L., Yeung, A., Goldman, M. I., & Miller, D. D. (1998). Caco-2 cell ferritin formation predicts non-radiolabeled food Fe availability in an *in vitro* digestion/Caco-2 cell culture model. Journal of Nutrition, 128: 1555–1561.

Gómez-Gallego, C., Pérez Conesa, D., Bernal Cava, M. J., Periago Castón, M. J. & Ros, G. (2009). Compuestos funcionales de la leche materna. Enfermería Global, 1-14.

Gonzalez-Chavez, S., Arevalo-Gallegos, S., & Rascon-Cruz, Q. (2009). Lactoferrin: structure, function and applications. International Journal of Antimicrobial Agents, 33: 301-308.

Jovaní, M., Barbera, R. & Farre, R. (2001). Lactoferrin and its possible role in Fe enrichment of infant formulas. Food Science and Technology International, 7: 97-103.

Jovaní, M., Barbera, R. & Farre, R. (2003). Effect of lactoferrin addition on the dialysability of Fe from infant formulas. J. Trace. Elem. Med. Biol., 17: 139-142.

Kamasaka, H., To-o, K., Kusaka, K., Kuriki, T., Kometani, T. & Okada, S. (1997). Effect of Phosphoryl Oligosaccharides on Fe Solubility under Neutral Conditions, Bioscience, Biotechnology and Biochemistry, 61: 1209-1210.

Kaur, N. & Gupta, A. K. (2002). Applications of inulin and oligofructose in health and nutrition. Journal of Bioscience, 27 (7): 703-714.

Kawakami, H., Dosakoa, S. I. & Nakajima, I. (1993). Effect of lactoferrin on Fe solubility under neutral conditions. Bioscience, Biotechnology, and Biochemistry, 57 (8): 1376-1377.

Laparra, J.-M., Tako, E., Glahn, R. P. & Miller, D. D. (2008). Supplemental inulin does not enhance Fe bioavailability to Caco-2 cells from milk- or soy-based, probiotic-containing, yogurts but incubation at 37 ºC does. Food Chemistry, 109: 122–128.

Legrand, D., Pierce, A., Elass, E., Carpentier, M., Mariller, C. & Mazurier, J. (2008). Lactoferrin structure and functions. Advance in Experimental Medicine and Biology, 606: 163–194.

Lönnerdal, B. & Bryant, A. (2006). Absorption of Fe from recombinant human lactoferrin in young US Women. Am. J. Clin. Nutr., 83: 305–309.

Lopez, H. W., Coudray, C., Levrat-Verny, M. A., Feillet-Coudray, C., Demigné, C. & Rémésy, C. (2000). Fructooligosaccharides enhance mineral apparent absorption and counteract the deleterious effects of phytic acid on mineral homeostasis in rats. J. Nutr. Biochem., 11: 500–508.

Lv, Y., Shuntang Guo, Elad Tako, Raymond P. & Glahn, R. P. (2014). Hydrolysis of soybean protein improves Fe bioavailability by Caco-2 cell. Journal of Food and Nutrition Research, 2 (4): 162-166.

Miller, D. D, Schricker, B. R., Rasmussen, R. R. & Van Campen, D. (1981). An *in vitro* method for estimation of Fe availability from meals. Am. J. Clin. Nutr., 34: 2248-2256.

Niers, L., Stasse-Wolthus, M., Rombouts, F. M. & Rijkers, G.T. (2007). Nutritional support for the infant's immune system. Nutr. Rev., 65: 347–360.

Nuijens, J. H., van Berkel, P. H. C. & Schanbacher, F. L. (1996). Structure and biological actions of lactoferrin, Journal of Mammary Gland Biology and Neoplasia, 1: 285-295.

Pantopoulos, K. (2004). Fe metabolism and the IRIE/IRP regulatory system- An update. Annals of the New York Academy of Sciences.

Perales, S., Barbera´, R., Lagarda, M. J. & Farré, R. (2007). Availability of Fe from milk-based formulas and fruit juices containing milk and cereals estimated by *in vitro* methods (solubility, dialysability) and uptake and transport by Caco-2 cells. Food Chemistry, 102: 1296–1303.

Pyanert, I., Armah, C., Fairweather-Tait, S., Kolsteren, P., van Camp, J. & De Henauw, S. (2006). Fe solubility compared with *in vitro* digestion-Caco-2 cell culture method for the assessment of Fe bioavailability in a processed and unprocessed complementary food for Tanzanian infants (6 to12 months). British Journal of Nutrition, 95(4): 721-726.

Raymond, P. G., Elizabeth, M. W., Darrell, R. V. C. & Miller, D. D. (1996). Caco-2 cell Fe uptake from meat and casein digests parallels *in vivo* studies: use of a novel *in vitro* method for rapid estimation of Fe bioavailability. J. Nutr., 126: 332-339.

Raymond, P. G., Lee, O. A., Andrew, Y., Matthew, I. G. & Miller, D. D. (1998). Caco-2 cell Ferritin formation predicts non-radiolabeled food Fe availability in an *in vitro* digestion/Caco-2 cell culture model. J. Nutr., 128: 1555-1561.

Rivera. J. A., Castillo. L. V. & Sánchez, J. A. (2011). Detección de micoplasmas en cultivos celulares. Enf. Inf. Microbiol., 31 (2): 60-62.

Rouault, T. A. (2006). The role of Fe regulatory proteins in mammalian Fe homeostasis and disease. Nature Chemical Biology, 2: 406–414.

Sacrino, M. L. (2007). A sideways glance: take it or leave it? the role of lactoferrin in Fe sequestration and delivery within the body. Genes Nutrition, 2: 161-162.

Santos, E. F., Tsuboi, K. H., Araújo, M. R., Falconi, M. A., Ouwehand, A. C., Andreollo, N. A. & Miyasaka, C. K. (2010). Ingestion of polydextrose increase the Fe absorption in rats submitted to partial gastrectomy. Acta Cir Bras., 25(6): 518-524.

Scholz-Ahrens, K. E, Schaafsma, G., Ellen, G. H., van den Heuvel, M. & Schrezenmeir, J. (2001). Effects of prebiotics on mineral metabolism, Am. J. Clin. Nutr., 73: 459S-464S.

Scholz-Ahrens, K. E., Ade, P., Marten, B., Weber, P., Timm, W., Acil, Y., Gluer, C. C. & Schrezenmeir, J. (2007). Prebiotics, probiotics, and symbiotics affect mineral absorption, bone mineral content, and bone structure. J. Nutr., 137: 838S-846S.

Schottstedt, T., Muri, C., Morel, C., Philipona, C., Hammonand, H. M. & Blum, J. W. (2005). Effects of feeding vitamin A and lactoferrin on epithelium of lymphoid tissues of intestine of neonatal calves. J. Dairy Sci., 88: 1050-1061.

Storcksdieck, S., Bonsmann, G., & Hurrel, R. F. (2007). Fe-binding properties, amino acid composition, and structure of muscle tissue peptides from in vitro digestion of different meat sources. Journal of Food Science, 72: S19–29.

Swain, J. H., Tabatabai, L. B. & Reddy, M. B. (2002). Histidine content of low-molecular-weight beef proteins influences non-heme Fe bioavailability in Caco-2 cells. The Journal of Nutrition, 132: 245–251.

Torres-Fuentes, C., Alaiz, M. & Vioque, J. (2012). Fe-chelating activity of chickpea protein hydrolysate peptides, Food Chemistry, 134 (3): 1585–1588.

Uchida, T., Oda, T., Sato, K. & Kawakami, H. (2006). Availability of lactoferrin as a natural solubilizer of Fe for food products. Int. Dairy J., 16, 95-101.

van den Heuvel, E.G.H.M., Schaafsma, G., Muys, T. & van Dokkum, W. (1998). Non-digestible oligosaccharides do not interfere with calcium and nonheme-Fe absorption in young, healthy men. American Journal of Clinical Nutrition, 67:445–451.

Viadel, B., Perales, S., Barberá, R., Lagarda, M. J. & Farré, R. (2007). Ferritin synthesis by Caco-2 cells as an indicator of Fe bioavailability: Application to milk-based infant formulas. Food Chemistry, 102, 925–931.

Vorland, L. H. (1999). Lactoferrin: a multifunctional glycoprotein. Acta pathologica, Microbiologica et Immunologica Scandinavica (APMIS), 107: 971-981.

Yasuda, K., Ronecker, K. R., Miller, D., Welch, R. M. & Lei, X. G. (2006). Supplemental dietary inulin affects the bioavailability of Fe in corn and soybean meal to young pigs. J. Nutr. 2006, 136, 3033–3038.

Yekta, M. A., Verdonck. F., Van Den Broeck, W., Goddeeris, B. M., Cox. E. & Vanrompay, D. (2010). Lactoferrin inhibits *E. coli* O157:H7 growth and attachment to intestinal epithelial cells. Veterinarni Medicina, 55(8): 359-368.

Yeung, A. C., Glahn, R. P. & Miller, D. D. (2002). Effects of Fe source on Fe availability from casein and casein phosphopeptides. J. Food Sci., 67: 1271-1275.

Yeung, C. K., Glahn, R. P., Welch, R. M. & Miller, D. D. (2005). Prebiotics and Fe Bioavailability- Is there a Connection?. J. Food Sci., 70: 88-92.

Zhu, L., Glahn, R. P., Yeung. C. K. & Miller, D. D. (2006). Fe uptake by Caco-2 cells from NaFeEDTA and FeSO4: Effects of ascorbic acid, pH, and a Fe (II) chelating agent. J. Agric. Food Chem., 54: 7924-7928.

Chapter 3

The effect of rhLf and rhLf hydrolysate on LPS-induced inflammation

3.1. Introduction

One of the most important factors in inducing the intestinal inflammation is the bacterial infection (O'Byrne et al., 2000; O'Byrne and Dalgleish, 2001). Pathogens produce some enterotoxins such as lipopolysaccharides (LPS) whose increase the cellular permeability, dysregulate the mucosal immune system and decrease the barrier function, resulting in generation of an inflammatory response (Wells *et al.* 1996; Robertson & Sandler 2001; McGuckin et al. 2009). Although inflammatory response is self-balanced (Gorczyniski & Stanley, 1999) and helps to restore homeostasis at the infected sites (Fiocchi, 2003), excessive and uncontrolled inflammatory changes often lead to chronic diseases (Davidge et al., 2001; Fiorucci et al., 2004). At the inflamed sites, large proportion of chemokines (IL-8) and pro-inflammatory cytokines (TNF-α and IL-1β), are secreted by the epithelial cells and macrophages (Arai et al., 1999). As well as the overproduction of ROS (reactive oxygen species) at the inflammation sites stimulates high oxidative stress (Zhang, 2010), which promotes IL-8 production in Caco-2 cells (Yamamoto et al., 2003), and other mediators of inflammation associated with immune dysregulation (Conner & Grisham, 1996). The levels of these mediators amplify the inflammatory response being destructive and contributing to clinical symptoms (Romier et al., 2008).

Given the undesirable side effects of anti-inflammatory and anti-oxidant drugs currently used (Waldner & Neurath, 2009; Chakrabarti et al., 2014); there is a growing interest for the using of the dietary nutrients to modulate the inflammatory process, or at least relieve its symptoms (Romier-Crouzet et al., 2009; Sergent et al., 2010). Due to its multifunctional properties as antibacterial, anti-inflammatory and immunomodulatory, Lf and its hydrolysate are thought to be able to

suppress or decrease the development of inflammation (Håversen, et al., 2002). This hypothesis was supported by the confirmation of its endotoxin-neutralizing capacity (Zhang et al., 1999) and its preventive effects on infection and inflammation (Lönnerdal, 2009). Also Lf is markedly increased in the nidus of inflammation (Roseanu & Brock, 2006, Legrand et al., 2008) and at infection sites (Actor et al., 2009, Ahmad et al. 2011) showing that it plays a central role in the inflammation treatment.

Thus the addition of Lf to infant formulas or other food types or even Lf-orally administration may plays a prominent role in inflammation treatment. Many studies are needed to explore Lf functionality regarding with its ability to reduce or modify the inflammation process in intestinal models and to discover the molecular mechanisms by which participate in diseases treatment and prevention.

3.2. Aims of this chapter

The present study aimed to explore the anti-inflammatory effect of different concentrations of rhLf and rhLf hydrolysate (1, 1.5 or 2 mg/mL) *in vitro* by using an *in vitro* gut inflammation model.

3.3. Materials and methods

3.3.1. Chemicals:

- **Lipopolysaccharides (LPS)** from *E.coli* 0127:B8 were purchased from Sigma Chemical Co. (Sigma, St. Louis, MO, USA). LPS was diluted in sterile phosphate buffer solution (PBS) to give 3 mg/ml.
- **Recombinant human lactoferrin (rhLf).** Expressed in rice, Fe saturated, >90% (SDS-PAGE)], was purchase from Sigma Chemical Co. (cat. no. L1294, St. Louis, MO, USA). Three different solutions of rhLf and rhLf hydrolysate (1, 1.5 and 2 mg/mL) were prepared.
- **Vivinal GOS syrup** (dry matter 75 % of which GOS 59 %, lactose 21 %, glucose 19 % and galactose 1 %) was provided by Hero Baby Co. (Alcantarilla, Murcia, Spain).

3.3.2. Proteolytic digestion of rhLf

rhLf was hydrolyzed using pepsin according to the method of Bellamy et al. (1992). Briefly, rhLf was dissolved in distilled water at a concentration

of 5% (w/v) and the pH was adjusted to 3.0 by addition of 1 M HCl. Pepsin was added to a final concentration of 3% (w/w of substrate) and hydrolysis was performed at 37°C for 4 h. The reaction was stopped by heating at 80°C for 15 min. The solution was then cooled until 20ºC and adjusted to pH 7.00 by dropwise addition of 1 M NaOH. Then, the solution was centrifuged at 4 ºC for 15 min at 17000 x g. The pellet was discarded and the supernatant was kept for using in the inflammation study.

3.3.3. Caco-2 and RAW 264.7 cell lines and culture conditions

The human colon adenocarcinoma **Caco-2 cell** line was obtained from the European Collection of Cell Cultures (ECACC; number 86010202, Salisbury, UK) and used in assays at passages 29-35. Murine macrophage **RAW 264.7** cell line was obtained from the European Collection of Cell Cultures (ECACC; number TIB-71, Salisbury, UK) and used in assays at passages 5-10. These two cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL Life Technologies, Paisley, Scotland), supplemented with 10% (v/v) fetal bovine serum (heat inactivated 60 °C, 45 min), 100 µg/mL streptomycin and 100 U/mL penicillin, 4.5 % glutamine, 1% Pyruvate at 37 °C in a 5% CO_2 humidified atmosphere (Cell culture CO2 incubator, Thermo Scientific, USA).

For anti-inflammatory assay, Caco-2 cells were seeded onto 6 Transwell-insert plates (polycarbonate membrane chamber inserts: 24 mm diameter, 0.4 µm pore size; Transwell, Costar Corp.) at a density of 4×10^5 cells/insert and allowed to grow until the monolayer formation for 14 day. The medium was changed every 2 day. One day before the experiment, Raw 264.7 cells were seeded at the bottom of 6-well plates at density of 8×10^5 cells/well and allowed to grow in the same growth media as well as Caco-2 cells.

3.3.4. Intestinal epithelium/immune co-culture model (Caco-2/RAW 264.7 cells)

An intestinal epithelium/immune co-culture model (Caco-2/RAW 264.7) (Tanoue et al., 2008) was established to imitate inflammation events in the gastrointestinal tract. This model system allowed co-cultivation of Caco-2 cells (in the apical compartment) and murine RAW 264.7 cells (in

the basolateral compartment) in separate compartments of a Transwell culture (the Transwell system including an 24 mm insert with a 0.4 µm pore polycarbonate membrane, and a companion 6-well plate tissue culture treated sterile, polystyrene plates, Corning, NY, USA) to simulate intestinal inflammation-associated events and to study the potential anti-inflammatory activity of rhLf and rhLf hydrolysate. For studying the anti-inflammatory activity of rhLf and rhLf hydrolysate; 1 mL of rhLf or rhLf hydrolysate solution (1, 1.5 or 2 mg/mL) was applied at the apical side for 5 h at two days before the experimental day as well as the day of experiment.

At the day of experiment and after replacing the old media, the Transwell insert Caco-2 cells had been cultured were translated into multiple plate wells preloaded with RAW 264.7 cells as shown in Fig. 3.1. 30 µl of LPS solution (3 mg/mL) was added to the basolateral side in this model, thus, the tested concentration is 50 µg/mL. After an additional incubation of 5 h with LPS solution, the integrity of the monolayer was assessed by measuring the transepithelial electrical resistance (TEER) according to the method of Okada et al. (2000), then the culture supernatants from the basolateral side and the apical side were collected and stored at -80 ºC for cytokines determination by flow cytometry and NO determination by ELISA kit. Then Transwell insert where Caco-2 cell monolayer grow was translated a new 6-well plate, the monolayer was cleaned by PBS and then picked up by 1 mL of PBS and scraper in an eppendorf which was centrifuged at 1000 rpm at 6 min at ambient temperature.

Finally, the cell pellet was stored at -80 ºC for the detection of ROS by flow cytometry. Along the experiment, it was used negative control (where LPS solution and the anti-inflammatory agent were not added) as well as positive control (where LPS solution was added while the anti-inflammatory agent was not added). The pro-/anti-inflammatory cytokines such as: IL-8, IL-1B, IL-10, IL-6, IL-12p70 and TNF-α were measured using flow cytometry by using BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit (BD Biosciences, USA).

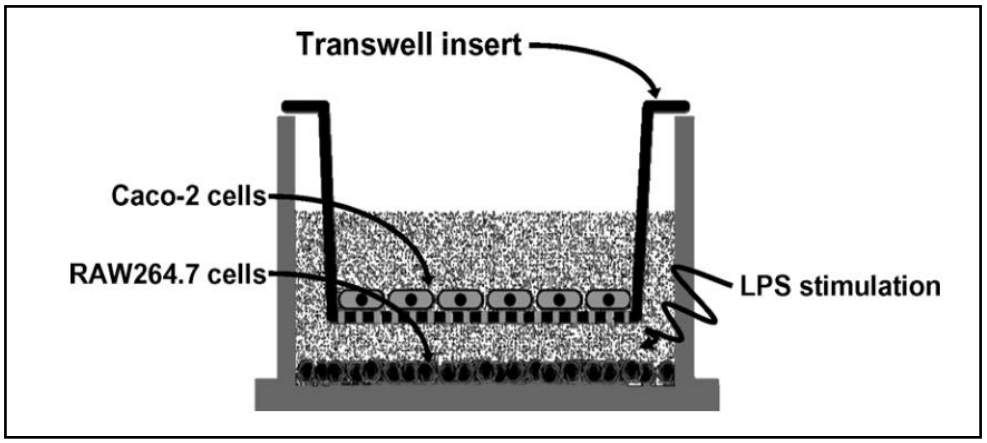


Fig. 3.1. Co-culture system constructed with Caco-2 cells and RAW 264.7 cells. Transwell inserts on which Caco-2 cells had been cultured were inserted into 6-well plate containing RAW 264.7 cells. To imitate the gut inflammation, LPS was added to the basolateral compartment of this co-culture system (Tanoue et al., 2008).

3.3.5. TEER measurement

TEER is an indicator of cell confluence, monolayer integrity and the formation of tight junctions between cells (Lu et al., 1996; Guillén Gómez, 2006) which serve as barriers to paracellular diffusion. TEER value reflects the tightness of the junctions between epithelial cells and is expressed the integrity of the Caco-2 monolayer (Hidalgo et al., 1989).

In the presented study, TEER value was determined immediately after LPS application (before incubation, time = 0 h) and after incubation (time = 5 h) and the units in which the obtained values is expressed as $\Omega.cm^2$. TEER value was measured by using Millicell-ERS instrument (Millipore, Eschborn, Germany).

3.3.6. Cytokine detection (Flow Cytometry)

The culture supernatants collected from the basolateral side and the apical side and stored at -80 ºC were thawed at 4 ºC. These samples did not expose to any type of dilution. The cytokines were determined according to the manufacturer's protocol (BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit, BD Biosciences, USA).

To perform the assay:

1. Vortex the mixed Capture Beads and add 50 µL to all assay tubes.

2. Add 50 µL of the Human Inflammatory Cytokine Standard dilutions to the control tubes.
3. Add 50 µL of each unknown sample to the appropriately labeled sample tubes.
4. Incubate the assay tubes for 1.5 hours at room temperature, protected from light. Note: If you have not yet performed cytometer setup, you may wish to do so during this incubation, or during the incubation in step 8.
5. Add 1 mL of Wash Buffer to each assay tube and centrifuge at 200g for 5 minutes.
6. Carefully and consistently aspirate and discard the supernatant, leaving approximately 100 µL of liquid in each assay tube.
7. Add 50 µL of the Human Inflammatory Cytokine PE Detection Reagent to all assay tubes. Gently agitate the tubes to resuspend the pellet.
8. Incubate the assay tubes for 1.5 hours at room temperature, protected from light.
9. Add 1 mL of Wash Buffer to each assay tube and centrifuge at 200g for 5 minutes.
10. Carefully aspirate and discard the supernatant from each assay tube.
11. Add 300 µL of Wash Buffer to each assay tube to resuspend the bead pellet. Then acquire the standards from the lowest to the highest concentration, followed by the test samples.

3.3.7. Nitric oxide determination

Nitric oxide (NO), which was present in the culture medium as nitrite and nitrate, was assayed using the colorimetric NO assay (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's protocol.

Total NO contributed by nitrate and nitrite was measured as nitrite from a nitrite standard curve provided with the kit, after converting all nitrate to nitrite. Briefly, the nitrate present in the supernatant was enzymatically transformed into nitrite by nitrate reductase, and the total nitrite concentration was measured photometrically at 530 nm using the Griess reagent (solution of sulfanilamide in 2 M HCl and N-(1-napthyl) ethylenediamine dihydrochloride in 2 M HCl). Results were expressed in

relative terms to the positive control (Caco-2 cells co-cultured with LPS-stimulated RAW 264.7 in the absence of rhLf or rhLf hydrolysate). The experiments were performed three times, with each individual treatment being run in triplicate.

3.3.8. Intracellular accumulation of ROS

The intracellular accumulation of ROS in the Caco-2 cells was measured using the oxidant-sensitive fluorescent probe, DCFH-DA. DCFH converted from DCFHDA deacetylase within the cells was oxidized by a variety of intracellular ROS to DCF, a highly fluorescent compound. After incubation with the samples (3 h), the monolayers were washed twice with PBS. The cells were then harvested and stained with 12.5 l M of DCFH-DA for 20 min in darkness at room temperature. A FACSort flow cytometer (BD Biosciences, San Jose, CA, USA) was used to analyze the intracellular ROS production and measure the fluorescent intensities of DCFH-DA (λ ex = 488 nm and λ em = 530 nm). Approximately 10 000 counts were made for each sample.

3.4. Statistical analysis

Results were expressed as mean ± SD of three replicates. After testing for normality and equal variances, the mean from three experiments were compared by analysis of variance (ANOVA) including the Duncan Multiple Range Test in the data treatment to determine significant differences among means ($P<0.05$). Values of $P<0.05$ were considered significant. All statistical analyses were performed with the Statistical Package for the Social Sciences (version 14.0; SPSS).

3.5. Results and Discussion

Many physiological effects for whey proteins have been identified including anti-oxidant and anti-inflammatory activities (Kano et al., 2002; Kimber et al., 2002; Ward et al., 2002; Beaulieu et al., 2007; Piccolomini et al., 2012). In particular, Lf exerts anti-inflammatory properties in animal models (Shimizu et al., 2006), *in vitro* (Håversen et al., 2002; Hirotani et al., 2008) and humans (Conneely, 2001). Under inflammatory conditions, it is well-known that the intestinal mucosa, in inflammatory bowel disease (IBD) patients, produced various pro-inflammatory cytokines (MacDermot et al.,

1999) such as IL-8 (Strufy et al., 2005) and TNF-α (Zareie et al., 2001), and then the newly secreted TNF-α could reduce the monolayer integrity leading to barrier dysfunction and finally increased the monolayer permeability (Sunaert et al., 2002) which reflected in reduction of TEER values (Dongmei et al., 2006). A shown in Fig. 3.2, TEER value of Caco-2 cells stimulated with LPS (positive control) highly (P<0.05) decreased (from 599.33 to 366.33 Ohm × cm^2) at 5 h. This result indicated that LPS led to the disruption of the monolayer of Caco-2 cells. Similar trends were reported in other *in vitro* gut inflammation models (Satsu et al., 2006; Tanoue et al., 2008) and in IBD patients (Suenaert et al., 2002). The present findings revealed that rhLf and rhLf hydrolysate prevented the permeability of the monolayers exposed to LPS and therefore LPS-induced barrier dysfunction was reduced or prevented and this effect was correlated with TEER values which kept higher than 500 Ω.cm^2 in the inflamed Caco-2 cells. Similar results proposed that Lf is able to protect the intestinal mucosa and improve the barrier function of intestinal cells (Hirotani et al., 2008).

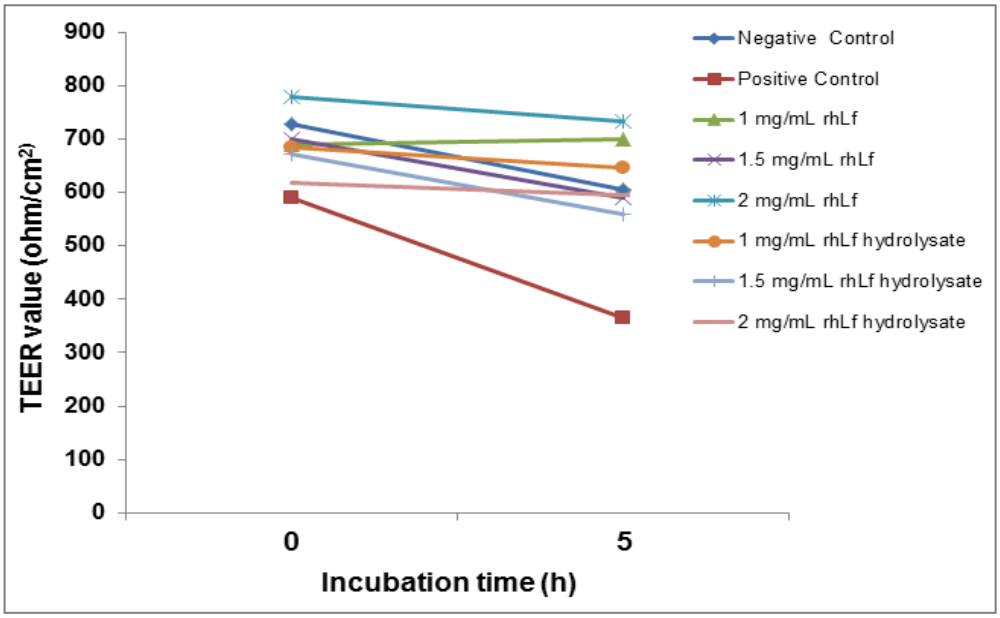


Fig. 3.2. TEER values of Caco-2 monolayers measured at 0 and 5 h of incubation with LPS with/without rhLf or rhLf hydrolysate. The results are expressed as Ohm (resistance) × cm^2 (surface area of the monolayer) vs. Time (min). Negative control corresponded to Caco-2 cells incubated with DMEM alone. Positive control corresponded to Caco-2 cells stimulated with 50 µg/mL LPS. Abbreviations: TEER: Transepithelial Electric Resistance, rhLf: recombinant human lactoferrin. Values are mean ± SD of three independent experiments.

In Fig. 3.3, IL-8 secreted in the co-culture system constructed with the Caco-2 cells and the RAW 264.7 cells is shown. IL-8 is the major cytokine measured in Caco-2 with respect the inflammatory response (Van De Walle et al., 2010). Our findings indicated that IL-8 production was minimal at 5 h for control cultures (3.61 ± 0.32 pg/mL) but was markedly increased in the apical compartment of LPS-treated cultures (75.37 ± 3.24 pg/mL). As can be seen in Fig. 3.3, a lower IL-8 production (P<0.05) was observed in the inflamed cultures treated with rhLf and rhLf hydrolysate and this effect of both rhLf and rhLf hydrolysate was almost dose-dependent. rhLf hydrolysate was more effective in reducing IL-8 production. IL-8 production was highly inhibited (P<0.05) by using 2 mg/mL rhLf hydrolysate and these cultures had the lowest value of IL-8 (10.9 ± 3.87 pg/mL) and it is worthy to mention that there is no significant difference (P<0.05) between this value as compared with that of non-inflamed cultures.

The obtained results confirmed previous findings reported that rhLf (Florian et al., 2012) and native hLf (Håversen et al., 2002; Mattsby-Baltzer et al., 1996) are able to decrease the secretion of LPS-induced IL-8. The mechanism proposed for this anti-inflammatory effect of Lf may involves the interference of Lf with nuclear factor (NF)-B activation (Håversen et al., 2002) or reducing the production of TNF-α (Choe & Lee, 1999). Likewise, the anti-inflammatory effect of Lf could partly explained by its ability to bind with LPS (van der Velden et al., 2008). Therefore, high Lf concentrations lead to decrease free LPS (Ward et al., 2002). Moreover, it blocks the binding of LPS with its protein receptors (Drago-Serrano et al., 2012). Thus, Lf ultimately modulates LPS-induced inflammation (van der Velden et al., 2008; Puddu et al., 2010; Latorre et al., 2010; Legrand and Mazurier, 2010).

As well as, it well-known that stimulated macrophages able to produce NO (Stuehr & Marletta, 1985, 1987). NO concentration (µM) of the cultures shown in Table 3.1. There is no clear trend of NO production by the different cultures treated with rhLf or rhLf hydrolysate.

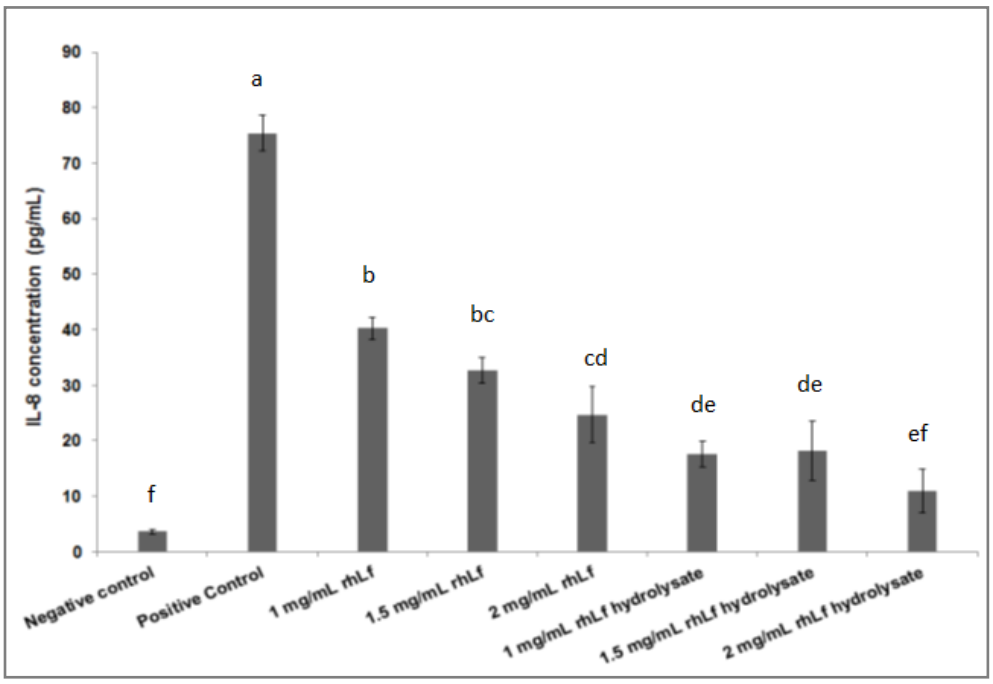


Fig. 3.3. Effect of rhLf and rhLf hydrolysate on IL-8 produced by Caco-2 cells. Negative control corresponded to Caco-2 cells incubated with DMEM alone. Positive control corresponded to Caco-2 cells stimulated with 50 µg/mL LPS. Abbreviations: IL-8: interleukin-8, rhLf: recombinant human lactoferrin. Values are mean ± SD of three independent experiments. Means with different superscripts are statistically different (P<0.05).

Non-significant differences (P<0.05) were observed for NO production between all treated cultures as compared with negative or positive control cultures. Interestingly, although the cultures incubated with 1 mg/mL rhLf hydrolysate showed an inhibitory effect on IL-8 secretion after 5 h, high amount of NO (23.28 ± 7.17) secreted by these cultures as compared to the positive control samples. These findings confirmed previous findings found by Choe and Lee (1999) showing that Lf has no effect on NO production showing that the inhibitory role of Lf does not interfere the other functions of stimulated macrophages, at least NO production. This finding leads us to conclude that rhLf function does not occur through the interference of the macrophage activation, but possible through the TNF-α-specific regulatory mechanism such as transcriptional regulation of TNF-α. Likewise, the measurement of the intracellular accumulation of ROS by FACS flow cytometry is of interest where it was thought that ROS produced in the injured cells via the penetration of

neutrophils and macrophages may stimulate the inflammation process (Naik & Dixit, 2011).

Fig. 3.4 showed the percentage of ROS produced by Caco-2 cells. These findings revealed that non-inflamed cultures had the minimal value of ROS (1.17 ± 0.06%) while the LPS-treated cultures highly produced ROS (77.80 ± 4.36%). The Caco-2 cells stimulated with LPS had reduced (P<0.05) ROS concentrations when treated with rhLf or rhLf hydrolysate and it was found that 2 mg/mL rhLf hydrolysate is the more effective dose in reducing ROS production. This inhibitory effect on ROS production was more effective for rhLf hydrolysate than rhLf and these findings are in a good agreement with previously reported findings showing that hLf and rhLf has the ability to reduce the secretion of ROS (Mulder et al., 2008; Kruzel et al., 2010) and this effect was in a dose-dependent manner (Tsubota et al., 2008 Kruzel et al., 2007, 2013).

Table 3.1. Effect of rhLf and rhLf hydrolysate on NO secretion in Caco-2 monolayers.

Treatment	NO content (µM)
Negative control	13.85 ± 5.56 ^{ab}
Positive control	14.27 ± 3.89 ^{ab}
1 mg/mL rhLf	14.77 ± 4.57 ^{ab}
1.5 mg/mL rhLf	13.51 ± 5.11 ^{ab}
2 mg/mL rhLf	11.42 ± 2.08 ^{b}
1 mg/mL rhLf hydrolysate	23.28 ± 6.17 ^{a}
1.5 mg/mL rhLf hydrolysate	14.45 ± 8.53 ^{ab}
2 mg/mL rhLf hydrolysate	12.48 ± 2.94 ^{b}

Negative control corresponded to Caco-2 cells incubated with DMEM alone. Positive control corresponded to Caco-2 cells stimulated with 50 µg/mL LPS. Abbreviations: NO: nitric oxide, rhLf: recombinant human lactoferrin. Values are mean ± SD of three independent experiments. Means with different superscripts are statistically different (P<0.05).

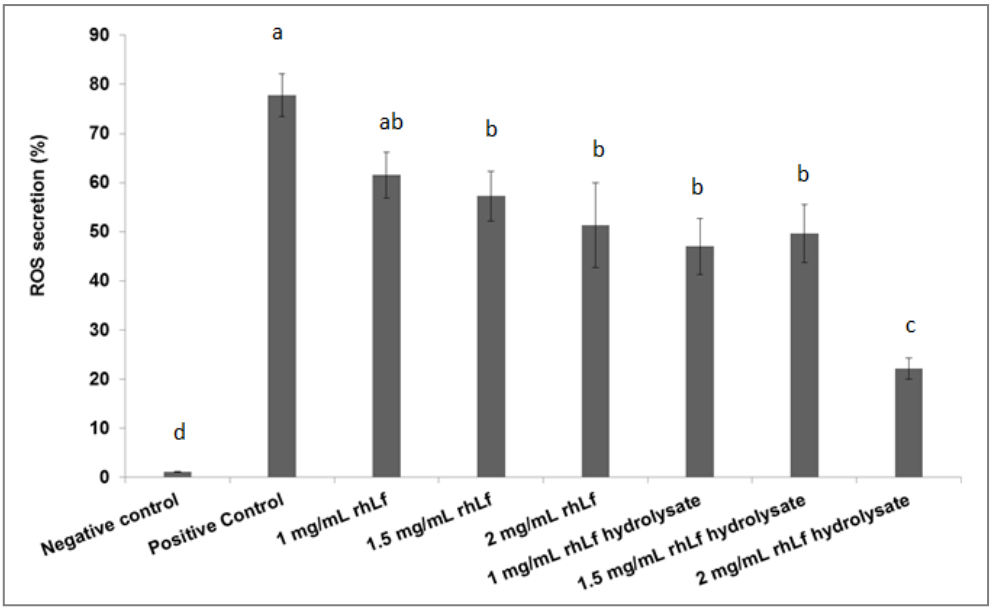


Fig. 3.4. Effect of rhLf and rhLf hydrolysate on ROS production in Caco-2 monolayers. Negative control corresponded to Caco-2 cells incubated with DMEM alone. Positive control corresponded to Caco-2 cells stimulated with 50 µg/mL LPS. Abbreviations: ROS: reactive oxygen species, rhLf: recombinant human lactoferrin. Values are mean ± SD of three independent experiments. Means with different superscripts are statistically different (P<0.05).

In this sense, Kruzel et al. (2007) reported that the intracellular ROS levels decreased up to 50% in the cultures treated with 32 µg/mL of Lf. Moreover, ROS production decreased to the minimal levels, which reported for non-inflamed cultures, in the presence of higher concentrations of Lf. The obtained findings revealed that ROS production by the cultures treated with 2 mg/mL of rhLf hydrolysate decreased up to 71.5% (~3.5 fold decrease). The failure in reducing of ROS production may induce oxidative stress (Kruzel et al., 2013) which promotes the pro-inflammatory cytokine IL-8 production by caco-2 cells (Yamamoto et al., 2003).

It could be concluded that rhLf and rhLf hydrolysate exert its anti-inflammatory activity through its ability to bind LPS and subsequently modulate cytokine production, *e.g.* the reducing of IL-8 production. The mechanism of Lf's action is also largely dependent on its ability to influence early responses, including the modulation of intracellular ROS production (Kruzel et al., 2007).

3.6. References

Actor, J. K., Hwang, S. A. & Kruzel, M. L. (2009). Lactoferrin as a natural immune modulator. Curr. Pharm. Des., 15: 1956-1973.

Ahmad, G., Sial, G. Z., Ramadori, P., Dudas, J., Batusic, D. S. & Ramadori, G. (2011). Changes of hepatic lactoferrin gene expression in two mouse models of the acute phase reaction. Int. J. Biochem. Cell Biol., 43: 1822-1832.

Arai, F., Takahashi, T., Furukawa, K., Matsushima, K. & Asakura, H. (1999). Mucosal expression of interleukin-6 and interleukin-8 messenger RNA in ulcerative colitis and Crohn's disease, Dig. Dis. Sci., 43: 2071-2079.

Beaulieu, J., Dupont, C. & Lemieux, P. (2007). Anti-inflammatory potential of a malleable matrix composed of fermented whey proteins and lactic acid bacteria in an atopic dermatitis model. Journal of Inflammation, 4(6): 1-10.

Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K. & Tomita, M. (1992b). Identification of the bactericidal domain of lactoferrin. Biochim. Biophys. Acta, 1121: 130-136.

Chakrabarti, S., Jahandideh, F. & Wu, J. (2014). Food-Derived Bioactive Peptides on Inflammation and Oxidative Stress. BioMed Research International, 1-11.

Choe, Y.-H. & Lee, S. W. (1999). Effect of lactoferrin on the production of tumor necrosis factor-α and nitric oxide. Journal of Cellular Biochemistry, 76:30-36.

Conneely, O. M. (2001). Anti-inflammatory activities of lactoferrin. J. Am. Coll. Nutr., 20: 389S–395S; discussion 396S–397S.

Conner, E. M. & Grisham, M. B. (1996). Inflammation, free radicals, and antioxidants. Nutrition, 12: 274–277.

Davidge, S. T. (2001). Prostaglandin H synthase and vascular function, Circulation Research, 89(8): 650–660.

Dongmei, Y., Iris, M. & Thomas, Y. M. (2006). Molecular mechanism of tumor necrosis factor-α modulation of intestinal epithelial tight junction barrier, Am. J. Physiol. Gastrointest. Liver Physiol., 290: 496-504.

Drago-Serrano, M. E., Garza-Amaya. M., Jesús Serrano Luna, J. S. & Campos-Rodríguez, R. (2012). Lactoferrin-lipopolysaccharide (LPS) binding as key to antibacterial and antiendotoxic effects. International Immunopharmacology, 12: 1–9.

Fiorucci, S., Distrutti, E., Mencarelli A., Rizzo, G., Di Lorenzo, A. R., Baldoni, M., del Soldato, P., Morelli, A. & Wallace, J. L. (2004). Cooperation between aspirin-triggered lipoxin and nitric oxide (NO) mediates antiadhesive properties of 2-(acetyloxy)benzoic acid 3(nitrooxymethyl)phenyl ester (NCX-4016) (NO-aspirin) on neutrophil-endothelial cell adherence. Journal of Pharmacology and Experimental Therapeutics, 309(3): 1174-1182.

Florian, P. E., Moisei, M., Trif, M., Evans, R. W. & Roseanu, A. (2012). Anti-inflammatory and anti-tumoral activit of human recombinant lactoferrin. Rom. J. Biochem., 49 (2): 163–171.

Gorczyniski, R. M. & Stanley, J. (1999). Clinical immunology. Landes Bioscience; Austin, TX.

Guillen-Gómez, E., (2006). Distribución tisular de transportadores de fármacos en tejidos normales y transformados. Papel en el sistema nervioso central. Tesis doctoral. Facultad de Biología. Universidad de Barcelona.

Håversen, L. A., Ohlsson, B. G., Hahn-Zoric, M., Hanson, L. A. & MattsbyBaltzer, I. (2002). Lactoferrin down-regulates the LPS-induced cytokine production in monocytic cells via NF-B. Cell Immunol., 220: 83–95.

Hidalgo, I. J., Raub, T. J. & Borchardt, R. T. (1989). Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability, Gastroenterology, 96: 736–749.

Hirotani, Y., Ikeda, K., Kato, R., Myotoku, M., Umeda, T., Ijiri, Y. & Tanaka, K. (2008). Protective effects of lactoferrin against intestinal mucosal damage induced by lipopolysaccharide in human intestinal Caco-2 cells. Journal of the Pharmaceutical Society of Japan, 128(9):1363-1368.

Kano, H., Mogami, O. & Uchida, M. (2002). Oral administration of milk fermented with Lactobacillus delbrueckii ssp. Bulgaricus OLL1037R-1 to DBA/1 mice inhibits secretion of pro-inflammatory cytokines. Cytotechnology, 40: 67-73.

Kimber, I., Cumberbatch, M., Dearman, R. J., Headon, D. R., Bhushan, M. & Griffiths, C. E. (2002). Lactoferrin: influences on Langerhans cells, epidermal cytokines, and cutaneous inflammation. Biochem. Cell. Biol., 80(1): 103-107.

Kruzel, M. L., Actor, J. K., Boldogh, I. & Zimecki, M. (2007). Lactoferrin in Health and Disease. Postepy Hig Med Dosw., 61: 261-267.

Kruzel, M. L., Actor, J. K., Radak, Z., Bacsi, A., Saavedra-Molina, A. & Boldogh, I., (2010). Lactoferrin decreases LPS-induced mitochondrial dysfunction in cultured cells and in animal endotoxemia model. Innate Immun., 16: 67–79.

Kruzel, M. L., Actor, J. K., Zimecki, M., Wisee, J., Płoszaj, P., Mirzac, S., Kruzel, M., Hwang, S-A., Ba, X. & Boldogh, I. (2013). Novel recombinant human lactoferrin: Differential activation of oxidative stress related gene expression. Journal of Biotechnology, 168: 666–675.

Latorre, D., Puddu, P., Valenti, P. & Gessani, S. (2010). Reciprocal interactions between lactoferrin and bacterial endotoxins and their role in the regulation of the immune response. Toxins, 2: 54–68.

Legrand, D. & Mazurier, J. (2010). A critical review of the roles of host lactoferrin in immunity. Biometals, 23: 365–376.

Legrand, D., Pierce, A., Elass, E., Carpentier, M., Mariller, C. & Mazurier, J. (2008). Lactoferrin structure and functions. Adv. Exp. Med. Biol., 606: 163–194.

Lönnerdal, B. (2009). Nutritional roles of lactoferrin. Curr. Opin. Clin. Nutr. Metab. Care, 12: 293-297.

Lu, S., Gough, A. W., Bobrowski, W. F. & Stewart, B. H. (1996). Transport properties are not altered across Caco-2 cells with heightened TEER despite underlying physiological and ultrastructural changes. Journal of Pharmaceutical Sciences, 85: 3.

MacDermott, R. P. (1999). Chemokines in the inflammatory bowel diseases, J. Clin. Immunol., 19: 266–272.

Mattsby-Baltzer, I., Roseanu, A., Motas, C., Elverfors, J., Engberg, I. & Hanson, L. A. (1996). Lactoferrin or a fragment thereof inhibits the endotoxin-induced interleukin-6 response in human monocytic cells. Pediatr. Res., 40: 257-262.

McGuckin, M. A., Eri, R., Simms, L. A., Florin, T. H. & Radford-Smith, G. (2009) Intestinal barrier dysfunction in inflammatory bowel diseases. Inflamm Bowel Dis., 15: 100-113.

Mulder, A. M., Connellan, P. A., Oliver, C. J., Morris, C. A. & Stevenson, L. M. (2008). Bovine lactoferrin supplementation supports immune and antioxidant status in healthy human males. Nutrition Research, 28 (9): 583–589.

Naik, E. & Dixit, V. M. (2011). Mitochondrial reactive oxygen species drive pro-inflamatory cytokine production. J. E. M., 3: 417–420.

O'Byrne, K. J. & Dalgleish, A. G. (2001). Chronic immune activation and inflammation as the cause of malignancy. Br. J. Cancer, 85: 473-483.

O'Byrne, K. J., Dalgleish, A. G., Browning, M. J., Steward, W. P. & Harris, A. L. (2000). The relationship between angiogenesis and the immune response in carcinogenesis and the progression of malignant disease. Eur. J. Cancer, 36: 151-169.

Okada, T., Narai, A., Matsunaga, S., Fusetani, N. & Shimizu, M. (2000). Assessment of the marine toxins by monitoring the integrity of human intestinal Caco-2 cell monolayers. Toxicol. In Vitro, 14: 219–226.

Piccolomini, A. F., Iskandar, M. M., Lands, L. C. & Kubow, S. (2012). High hydrostatic pressure pre-treatment of whey proteins enhances whey protein hydrolysate inhibition of oxidative stress and IL-8 secretion in intestinal epithelial cells. Food & Nutrition Research, 56: 1-10.

Puddu, P., Latorre, D., Valenti, P. & Gessani, S. (2006). Immunoregulatory role of lactoferrin-lipopolysaccharide interactions. Biometals, 23: 387–397.

Robertson, D. J. & Sandler, R. S. (2001). Measles virus and Crohn's disease: a critical appraisal of the current literature. Inflamm Bowel Dis., 7: 51–57.

Romier, B., Van De Walle, J., During, A., Larondelle, Y. & Schneider, Y. J. (2008). Modulation of signaling NF-kB activation pathway by polyphenols in human intestinal Caco-2 cells. Br. J. Nutr., 100: 542–551.

Romier-Crouzet, B., Van De Walle, J., During, A., Joly, A. & Rousseau, C. (2009). Inhibition of inflammatory mediators by polyphenolic plant extracts in human intestinal Caco-2 cells. Food Chem. Toxicol., 47: 1221-1230.

Roseanu, A. & Brock, J. H. (2006). What are the structure and the biological function of lactoferrin in human breast milk?. IUBMB Life, 58: 235–237.

Satsu, H., Ishimoto, Y., Nakano, T., Mochizuki, T., Iwanaga, T. & Shimizu, M. (2006). Induction by activated macrophage-like THP-1 cells of apoptotic and necrotic cell death in intestinal epithelial Caco-2 monolayers via tumor necrosis factor alpha. Exp. Cell Res., 312: 3909-3919.

Sergent, T., Piront, N., Meurice, J., Toussaint, O. & Schneider, Y. (2010). Anti-inflammatory effects of dietary phenolic compounds in an in vitro model of inflamed human intestinal epithelium. Chem. Biol. Interact. 188: 659–667.

Shimizu, N., Dairiki, K., Ogawa, S. & Kaneko, T. (2006). Dietary whey protein hydrolysate suppresses development of atopic dermatitis-like skin lesions induced by mite antigen in NC/Nga mice. Allergol. Int., 55(2): 185-189.

Struyf, S., Gouwy, M., Dillen, C., Proost, P., Opdenakker, G. & Damme, J. V. (2005). Chemokines synergize in the recruitment of circulating neutrophils into inflamed tissue. European Journal of Immunology, 35: 1583–1591.

Stuehr, D. J. & Marletta, M. A. (1985). Mammalian nitrite biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide. Proc. Natl. Acad. Sci., USA82: 7738.

Stuehr, D. J. & Marletta, M. A. (1987). Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-gamma. J. Immunol., 139: 518-525.

Suenaert, P., Bulteel, V., Lemmens, L., Noman, M., Geypens, B., Assche, G.V., Geboes, K., Ceuppens, J. L. & Rutgeerts, P. (2002). Anti-tumor necrosis factor treatment restores the gut barrier in Crohn's disease. American Journal of Gastroenterology. 97: 2000–2004.

Tanoue, T., Nishitani, Y., Kanazawa, K., Hashimoto, T. & Mizuno, M. (2008). *In vitro* model to estimate gut inflammation using co-cultured Caco-2 and RAW 264.7 cells. Biochemical and Biophysical Research Communications, 374: 565-569.

Tsubota, A., Yoshikawa, T., Nariai, K., Mitsunaga, M., Yumoto, Y., Fukushima, K., Hoshina, S. & Fujise, K. (2008). Bovine lactoferrin potently inhibits liver

mitocondrial 8-OHdG levels and retrieves hepatic OGG1 activities in Long-Evans Cinnamon rats. Journal of Hepatology, 48: 486-493.

Van De Walle, J., Hendrickx, A., Romier, B., Larondelle, Y. & Schneider, Y.-J. (2010). Inflammatory parameters in Caco-2 cells: Effect of stimuli nature, concentration, combination and cell differentiation. Toxicol in Vitro, 24: 1441-1449.

van der Velden, W. J. F. M., Blijlevens, N. M. A. & Donnelly, J. P. (2008). The potential role of lactoferrin and derivatives in the management of infectious and inflammatory complications of hematology patients receiving a hematopoietic stem cell transplantation. Transpl Infect Dis., 10: 80-89.

Waldner, M. & Neurath, M. (2009). Novel cytokine-targeted therapies and intestinal inflammation. Curr. Opin. Pharmacol. 9: 702–707.

Ward, P. P., Uribe-Luna, S. & Coonnely, O. M. (2002). Lactoferrin and host defense. Biochem. Cell Biol., 80: 95-102.

Wells, C. L., van de Westerlo, E. M., Jechorek, R. P., Feltis, B. A., Wilkins, T. D. & Erlandsen, S. L. (1996). Bacteroides fragilis enterotoxin modulates epithelial permeability and bacterial internalization by HT-29 enterocytes. Gastroenterology 110: 1429-1437.

Yamamoto, K., Kushima, R., Kisaki, O., Fujiyama, Y. & Okabe, H. (2003). Combined effect of hydrogen peroxide induced oxidative stress and IL-1 alpha on IL-8 production in CaCo-2 cells (a human colon carcinoma cell line) and normal intestinal epithelial cells. Inflammation, 27(3):123-128.

Zareie, M., Singh, P. K., Irvine, E. J., Sherman, P. M., McKay, D. M. & Perdue, M. H. (2001). Monocyte/macrophage activation by normal bacteria and bacterial products: implications for altered epithelial function in Crohn's disease, Am. J. Pathol., 158: 1101–1109.

Zhang, G. H., Mann, D. M. & Tsai, C. M. (1999). Neutralization of endotoxin in vitro and in vivo by a human lactoferrin-derived peptide. Infect Immun., 67: 1353-1358.

Zhang, K. (2010). Integration of ER stress, oxidative stress and the inflammatory response in health and disease. Int. J. Clin. Exp. Med., 3: 33-40.

Chapter 4

The prebiotic activity of rhLf and GOS. Batch culture fermentation study

4.1. Introduction

In the recent decades, the growing awareness of consumption of food products containing some functional ingredients beyond to the nutritional components due to its positive impact on consumer health. In this context, the more demanded functional ingredients by consumers are those positively affecting the intestinal flora (Saarela et al., 2002). Among these ingredients: human milk oligosaccharides (HMOs) and human lactoferrin (hLf) that are considered as prebiotics with an interesting effect on the growth of intestinal microbiota, especially *Bifidobacteria* and *Lactobacilli* (Kim et al., 2004; Boehm et al. 2005; Rahmarn et al., 2006; MacFarlane et al., 2008).

Many previously published studies confirmed that the presence of these ingredients in human milk may influences the composition of the gut microbiota leading to an increase on the *Bifidobacteria* proportion of breast-fed infants as compared to those fed on infant formulas (Harmsen et al., 2000; Alles et al., 2004; Iacono et al., 2005; Granier et al., 2013). Thus, the addition of these functional ingredients to infant formulas is considered an adequate and recent trend to produce milk substitute in order to highly resemble the human milk composition (Alles et al., 2004). Since Lf has been added as a new bioactive component in infant formulas (IFSA, 2012), many formulas containing Lf are commercialized in the markets (Wakabayashi et al., 2006; Mulder et al., 2008).

HMOs are resistant to digestion and reach the colon in a high proportion, where they prevent the attachment of enteropathogens and serve as prebiotics protecting the breast-fed infant against infections and diarrhea by different mechanisms. HMOs stimulate the growth of *Bifidobacteria* and *Lactobacilli*, thus decreasing the fecal pH and reducing the presence of pathogens (Boehm et al. 2005). However, many *in vitro*, *in vivo* and

preclinical studies are needed to discover the functionality of human milk components especially Lf and HMOs in the gut of infants.

4.2. Aims of this chapter

The present study aimed to evaluate the role of rhLf and GOS on the profile of short chain fatty acids (SCFAs) and the evolution of infant fecal bacteria using batch culture fermentation.

4.3. Materials and methods

4.3.1. Chemicals:

- **Recombinant human lactoferrin (rhLf).** Expressed in rice, Fe saturated, >90% (SDS-PAGE)], purchased from Sigma Chemical Co. (cat. no. L1294, St. Louis, MO, USA).
- **Vivinal GOS syrup** (dry matter 75 % of which GOS 59 %, lactose 21 %, glucose 19 % and galactose 1 %) was provided by Hero Baby Co. (Alcantarilla, Murcia, Spain).

4.3.2. Proteolytic digestion of rhLf

It was used the same method described in section 3.3.2.

4.3.3. Preparation of the fecal inoculum

Fecal samples were obtained from three healthy babies (aged 2-4 months) without any known metabolic or gastrointestinal disorders and were not taken antibiotics before fecal sample donation. Fresh fecal samples were immediately placed in anaerobic jars and transported to the laboratory within 2 h of collection. Fecal samples available at each time were diluted (1:10, w: v) with phosphate buffer (PBS composition was as following: 8 g/L NaCl, 0.2g/L KCl, 1.15 g/L Na_2HPO_4, 0.2g/L KH_2PO_4, pH 7.3) and homogenized in a stomacher for 2 min at normal speed. Subsequently, the fecal homogenates of each donor (10 mL) were added to 90 mL of the Minimal Basal medium (MBM) and allowed to stabilize by being kept for 4 h at 37 ºC under anaerobic conditions.

4.3.4. *In vitro* batch culture fermentations

Three independent small scale fecal batch cultures, each of them corresponding to samples from three different babies. Briefly, the medium Minimal Basal Medium (MBM) contained the following ingredients (g/L): peptone water (2); yeast extract (2); NaCl (0.1); K_2HPO^4 (0.04); KH_2PO^4 (0.04); $MgSO_4.7H_2O$ (0.01); $CaCl_2.6H_2O$ (0.01); $NaHCO_3$ (2); L-Cysteine (0.5); bile salts (0.5); Tween 80 (2 ml); 4 ml of 0.025% (w/v) Resazurin solution. All ingredients were reconstituted with bidistilled water. The medium was adjusted to pH 7.0 by using 1 mol/L HCl and was autoclaved at 121 ºC for 15 min. After autoclaving, 10 µL of vitamin K1 and 1 mL of hemin solution (50 mg/mL) per liter were added, and the two later solutions were filter sterilized (0.20 µm pore size). First of all, 1% (w/v) lactose was added to the media before autoclaving because it is the main sugar in milk. The fecal slurry and autoclaved MBM were prepared at the same day and maintained overnight under conditions of anaerobiosis at 37 °C, before use.

For each batch, MBM was distributed into different glass vessels (5 mL per vessel) along with 5, 7.5 or 10 mg of rhLf or mixture of 1% GOS and 5, 7.5 or 10 mg rhLf. 1 % GOS was used as positive control. Likewise, one additional tube was kept without adding any substrate and it was used as a negative control.

The next day, ingredients assayed (rhLf and GOS) (at the different concentrations) were added to each vessel just prior to the inoculation with the fecal slurry (50 µL of the fecal slurry was added to each vessel). The different treatments were incubated at 37 ºC in anaerobic conditions that were maintained throughout the batch culture experiments. Batch culture study was run for 24 h, which is the typical incubation time for batch system when simulating the large intestine of monogastrics. Samples were taken at 0, 10 and 24 h of incubation and pH was measured at each time. In the different points of sampling, approximately 1.5 mL of each tube were taken and put into sterile vials, then centrifuged at 12000 x g for 15 min. The pellet was immediately freezed at -80 ºC, and subsequently used for analysis of bacterial populations by Q-PCR while the supernatant was filtered

through 0.2 µm filter, then freezed at -80ºC for SCFAs analysis by gas chromatography (GC).

The different treatments used in this study are the following:

1. Negative control (without substrate) + Fecal slurry	2. Positive control (1 % GOS)	3. 0.10 % rhLf
4. 0.15 % rhLf	5. 0.20 % rhLf	6. 0.1 % rhLf + 1 % GOS
7. 0.15 % rhLf + 1% GOS	8. 0.20 % rhLf + 1 % GOS	

4.3.5. Short chain fatty acids (SCFAs) analysis by GC

SCFAs analysis in fecal cultures, was determined by GC according to Mateo-Anson et al. (2011) and Salazar et al. (2008). For SCFAs analysis, 1.5 mL of batch culture fermentation sample was centrifuged at 12000 rpm for 10 min at room temperature. 100 µL of supernatant was added to 650 µL of a mixture of formic acid (20%), methanol and 2-ethyl butyric acid (previously prepared internal standard, 2 mg/mL in methanol) at a ratio of 1:4.5:1 (v/v/v). Then mixtures were exposed to ultrasonic sound for 5 min. After centrifugation at 12000 rpm for 10 min, supernatants were filtered through a 13 mm (diameter), 0.22 µm (pore size) polytetrafluoroethylene (PTFE) filter into a GC vial and the cap applied. 2 µL of samples were injected into GC (GC system Agilent 7890A) equipped with a flame-ionization detector and a Nukol TM column (30 × 0.25 mm × 0.25 µm, Supelco, USA). More details about the chromatographic conditions of the applied analytical method are described in Table 4.1.

The different organic acids were identified according to the retention times, and the concentrations were calculated by using calibration curve for each fatty acid.

The chromatographic data were processed using the software ALS Controller Utility of Agilent Technologies. Fig. 4.1 showed the chromatogram of the different SCFAs analyzed using the multi-standard solution of 10 mM concentration.

Table 4.1. Chromatographic conditions for SCFAs analysis by GC.

Instrumental parameters	Conditions
Elution method	Pressure injection constant
Flow	Helium 25 ml/min (elution gas) Air 400 ml/min Hydrogen 30 ml/min
Injection method	Splitless
Injection volume	2 µL
Oven temperature	80 ºC
Temperature ramp	80 ºC/5 min 5 ºC/min to 185 ºC
Injector temperature	22 ºC
Detector temperature	220 ºC
Injector pressure	58.99 kPa

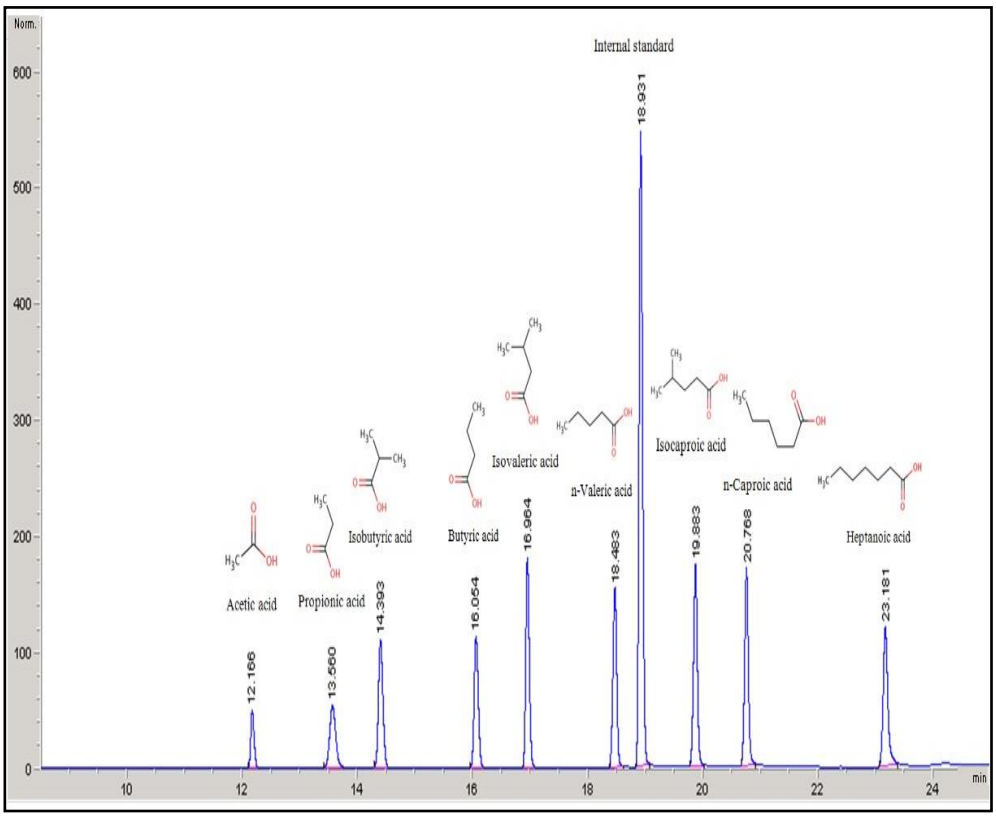


Fig. 4.1. The chromatogram of the different SCFAs analyzed using the multi-standard solution of 10 mM concentration.

Two calibration curves were prepared, one with different concentrations of a volatile acids standard mix (initial concentration 10 mM, Supelco, USA) which considered as an internal standard and other with acetic acid in order to increase its concentration respect the concentration of the first one (Table 4.2).

Table 4.2. The different standard of fatty acids used for doing the calibration curve.

	St. 1	St. 2	St. 3	St. 4	St. 5	St. 6
Multi-standard	10 mM	5 mM	2 mM	1 mM	0.5 mM	0.25 mM
Acetic acid	200 mM	100 mM	50 mM	10 mM	5 mM	2 mM

4.3.6. Measurement of pH

pH of batch culture samples was measured immediately after taking the aliquots to analyze the SCFAs and Q-PCR. This measurement was done in triplicate directly by using the pH-meter (Crison, Barcelona, Spain) in the tubes in which the bacterial growth occurred.

4.4. Statistical analysis

Results were expressed as mean ± SD of three experiments (three donors). After testing for normality and equal variances, the mean of the major and minor SCFAs from three experiments were compared by Two-way analysis of variance (ANOVA) including the Duncan Multiple Range Test in the data treatment to determine significant differences among means ($P<0.05$). A Pearson Correlation Analysis was performed to investigate the possible correlation between pH variation and SCFAs concentration using the batch culture fermentation system. Values of $P<0.05$ were considered significant. All statistical analyses were performed with the Statistical Package for the Social Sciences (version 14.0; SPSS).

4.5. Results and Discussion

The findings obtained in this study regarding SCFAs production by the fecal bacterial population are presented in Table 4.3 for the major SCFAs (acetic, propionic, butyric acids) and in Fig. 4.2 for the minor SCFAs (isobutyric, isovaleric, n-valeric, isocaproic, n-caproic and heptanoic acids).

These SCFAs are organic acids with 1 - 6 carbon atoms and are one of the main anions that resulted by the bacterial fermentation of polysaccharides, oligosaccharides, proteins, peptides and glycoprotein precursor in the colon (Cummings & Macfarlane, 1991). Now it is accepted that SCFAs production has beneficial effects on human health including: the enhancement of water transport, sodium absorption and bicarbonate excretion. Furthermore, SCFAs functionality has expanded to include their role as nutrients for the colonic epithelium, as modulators of colonic and intracellular pH, and other functions related with Fe transport, and as regulators of proliferation, differentiation, and gene expression (Hijova & Chmelarova, 2007). Also the accumulation of SCFAs is related with the pH decrease, which influences indirectly on the colonic microflora pattern, and indirectly increases the minerals absorption such as calcium and iron (Jenkins et al., 1987; Bougle et al., 2002), as well as lowering the bioavailability of the toxic amines and thus protecting from the carcinogenic progress (Puccio et al., 2007).

As can be seen in Table 4.3, the total SCFAs levels increased after 24 h of incubation of fecal microbiota with the tested ingredients. The highest values of total SCFAs were detected after fermentation in the presence of 0.10 % rhLf + 1% GOS followed by 0.15% rhLf + 1% GOS as compared with control group (without any ingredient added). The obtained findings revealed, as unexpected, that total SCFAs in the presence of 1% GOS showed the lowest value after 24 h as compared with control group. Non-significant differences (P<0.05) were observed between total SCFAs in all treatments. Among the various detected SCFAs, acetic acid was the most abundant, followed by similar amounts of both propionic acid and butyric

acid. After 24 h of fermentation, an elevated production of acetic acid was reported and its higher value was found for 0.10 % rhLf + 1 % GOS followed by 0.15 % rhLf + 1 % GOS as compared with control group. Unexpectedly, in the presence of 1% GOS, the acetic acid production was the lowest compared with control after 24 h. It is worthy to note that the highest acetic acid value after 10 h was reported in presence of 1 % GOS as compared with all treatments with exception of control sample. However, non-significant (P<0.05) changes in the acetic acid production by the tested ingredients was observed at the different points of sampling and this may be resulted by the high differences between acetic acid produced by the three donors.

Similar results in line with the present findings were found by Miller and Wolin (1996) revealed that acetic acid was the major product of the bifidobacteria pathway following fermentation of glucose purified from cabbage by human fecal suspensions. Also Velazquez et al. (2000) found that the incubation with glucose gave rise to more acetic acid but less propionic and butyric acid production than other fermentable substrates. In this sense, Rycroft et al. (2001) found that acetic acid and total SCFAs were highly produced by lactulose and GOS fermentation as compared with other oligosaccharides after short-term incubations. Likewise, Macfarlane et al. (2008) reported that the colonic fermentation of GOS induced the production of acetic acid, which correlated positively with the increase of *Bifidobacteria* populations, as acetate formation is consistent with *Bifidobacteria* and *Lactobacilli* metabolism. Similar results have been reported showing that the increasing production of acetic acid was directly associated with the increasing in *Bifidobacteria* counts (Cardelle-Cobas et al., 2009). Many published studies remarkably reported that prebiotics are the main responsible for the marked increasing in the counts of *Bifidobacteria*, *Lactobacilli* and subsequently the higher production of acetic acid and total SCFAs (Ben et al., 2004; Knol et al., 2005; Cai et al., 2008), and these changes may be responsible for promoting the defense functions of the host and thereby protecting the host from severe infection (Fukuda et al., 2011, 2012).

Table 4.3. SCFAs concentration (mM), acetic/propionic ratio and total SCFAs at 0, 10 and 24 h of incubation with rhLf and/or GOS in batch culture fermentation.

	Time (h)	Negative C	GOS (1%)	0.10 % rhLf	0.15% rhLf	0.20 % rhLf	0.10 % rhLf + 1% GOS	0.15% rhLf + 1% GOS	0.20 % rhLf + 1% GOS
Acetic acid	**0 h**	3,68 ± 1.69	3,68 ± 1.69	3,68 ± 1.69	3,68 ± 1.69	3,68 ± 1.69	3,68 ± 1.69	3,68 ± 1.69	3,68 ± 1.69
	10 h	10.35 ± 3.51	10.03 ± 1.36	9.45 ± 1.92	9.54 ± 1.43	8.72 ± 1.46	9.45 ± 0.99	9.74 ± 0.74	9.91 ± 1.25
	24 h	10.97 ± 3.97	9.26 ± 3.15	15.20 ± 6.08	11.32 ± 2.43	13.80 ± 7.01	16.01 ± 7.03	15.80 ± 6.12	15.92 ± 2.48
Propionic acid	**0 h**	0,24 ± 0.06	0,24 ± 0.06	0,24 ± 0.06	0,24 ± 0.06	0,24 ± 0.06	0,24 ± 0.06	0,24 ± 0.06	0,24 ± 0.06
	10 h	0.06 ± 0.02	0.13 ± 0.14	0.46 ± 0.07	0.38 ± 0.07	0.09 ± 0.03	0.36 ± 0.28	0.26 ± 0.26	0.19 ± 0.13
	24 h	0.62 ± 0.34	0.35 ± 0.14	2.05 ± 2.81	1.31 ± 1.44	0.90 ± 0.98	3.44 ± 5.51	3.27 ± 5.22	1.96 ± 2.70
Butyric acid	**0 h**	0,14 ± 0.09	0,14 ± 0.09	0,14 ± 0.09	0,14 ± 0.09	0,14 ± 0.09	0,14 ± 0.09	0,14 ± 0.09	0,14 ± 0.09
	10 h	0.08 ± 0.01	0.07 ± 0.01	0.08 ± 0.02	0.08 ± 0.04	0.07 ± 0.01	0.08 ± 0.02	0.09 ± 0.01	0.09 ± 0.02
	24 h	0.11 ± 0.01	0.08 ± 0.02	0.09 ± 0.04	0.09 ± 0.01	0.09 ± 0.03	0.10 ± 0.05	0.09 ± 0.01	0.12 ± 0.02
Acetic acid/Propionic	**0 h**	17.91 ^{c} ± 13.70	17.91 ^{c} ± 13.70	17.91 ^{c} ± 13.70	17.91 ^{c} ± 13.70	17.91^{c} ± 13.70	17.91^{c} ± 13.70	17.91^{c} ± 13.70	17.91^{c} ± 13.70
	10 h	186.07 ^{a} ± 88.68	151.30 ^{ab} ± 3.71	21.25 ^{c} ± 6.02	25.12 ^{c} ± 1.16	102.4 ^{bc} ± 37.12	38.92 ^{c} ± 22.94	89.00 ^{bc} ± 97.37	93.13b^{c} ± 94.40
	24 h	22.51^{c} ± 14.05	31.98 ^{c} ± 17.40	23.99 ^{c} ± 21.28	17.01 ^{c} ± 11.49	34.28^{bc} ± 34.78	31.64^{c} ± 25.29	53.70 ^{c} ± 63.69	29.02 ^{c} ± 25.04
Total SCFAs	**0 h**	4.06 ± 1.73	4.06 ± 1.73	4.06 ± 1.73	4.06 ± 1.73	4.06 ± 1.73	4.06 ± 1.73	4.06 ± 1.73	4.06 ± 1.73
	10 h	10.49 ± 3.52	10.23 ± .141	9.98 ± 1.90	10.00 ± 1.53	8.87 ± 1.45	9.88 ± 0.75	10.05 ± 0.52	10.18 ± 1.15
	24 h	11.70 ± 3.73	9.68 ± 3.05	17.33 ± 6.89	12.73 ± 2.83	14.78 ± 6.72	19.55 ± 12.52	19.16 ± 11.21	18.00 ± 4.93

Values are mean ± SD of three independent experiments. Means of the same column with different superscripts are statistically different (P<0.05). Abbreviations: rhLf: recombinant human lactoferrin, GOS: galactooligosaccharides, SCFAs: short chain fatty acids.

In the case of propionic acid, the findings revealed a moderately increasing of its level for all the tested ingredients after 24 h of fermentation. After 10 h of fermentation, in the group of samples containing 0.20 % rhLf showed the lowest value as compared with the rest of treatments. While after 24 h, the highest value of propionic acid was found in the presence of 0.10 % rhLf + 1 % GOS followed by 0.15 % rhLf + 1 % GOS while the lowest value was observed in the presence of 1 % GOS respecting with control group. The results revealed that there are non-significant (P<0.05) differences between the values of propionic acid among the different treatments. It was previously reported that propionate may form soluble complex with iron, thereby maintaining the solubility of iron in the lumen of the colon, as well as facilitating the transfer across endosome membranes of the enterocytes (Bougle et al., 2002).

The contrary occurred for butyric acid, which decrease moderately after 24 h of fermentation as compared to its content at zero time of incubation. The highest value of butyric acid was observed at 24 h in the presence of 0.20 % rhLf + 1 % GOS followed by control sample but it is not significantly (P<0.05) differed with the values of different treatments. Finally, it was observed a small variability between the other groups in their content of butyric acid whether at 10 h or 24 h. The low concentration of butyric acid could be related with the decrease of *Clostridium* and *Enterobacteriaceae* numbers; the major butyrate-producing bacterial groups found in human feces (Barcenilla et al., 2000) as well as related with the decrease of *Faecalibacterium prausnitzii* (Fpra655) populations determined by Fluorescence In Situ Hybridization (FISH) as reported in a recently study (Rodriguez-Colinas et al., 2013). Furthermore, butyric acid production was not observed in these mostly acidic cultures when pH values of fecal cultures fall below 6.0 as indicated to occur by Walker et al. (2005). Likewise, many studies revealed that acetic acid is produced at higher levels than propionic and butyric acids (Velazquez et al., 2000; Bruck et al., 2003; Delgado et al., 2006; Al-Tamimi et al., 2006). By contrast, Rossi et al. (2005) revealed that SCFAs production in fecal fermentation highly affected by the presence of FOS and inulin. Acetic and lactic acids were the major

fermentation end products in the presence of FOS while inulin fermentation gave rise butyric acid level. Thus, in view of the previous reported data we can report that the type of dietary components, its composition, polymerization degree and intestinal flora pattern of infants and the age of the infant may greatly influence on the produced SCFAs.

Interestingly, as a consequence of SCFAs production pattern mentioned above, the acetic acid/propionic acid ratio highly increased at 10 h of fermentation then this ratio decreased moderately or highly depending on the added compound where this ratio of control group achieved the highest value at 10 h while was the lowest value at 24 h. Our findings reported that negative control and positive control led to a significant changes (P<0.05) in the acetic/propionic acid ratio. Respecting with this, Delzenne & Kok (2001) reported that the decrease in acetic acid/propionic acid ratio has been suggested to be a possible marker of the hypolipidemic effect of prebiotics (as noted by the inhibition of cholesterol and fatty acids biosynthesis in liver, which finally results in a decrease in lipid levels in blood). Remarkably, the highest decrease on this ratio observed in the present study was for the 0.15 % rhLf group.

It is well-established that the feeding pattern of infant has an effect on the predominant microflora (type, composition and activity) in the infant gastrointestinal tract and subsequently the gut microflora of infants who received human milk have been reported to differ from infants on standard infant formula (Iacono et al., 2005; Granier et al., 2013). This difference may partly explained by the presence of HMOs. Several mechanisms can explain these effects, such as the selective fermentation and growth of *Lactobacillus* and *Bifidobacteria* species and the subsequent production of SCFAs (Cummings, 1984; Roberfroid, 1993). Many published studies have shown that acetic acid was the main fermentation end-product in infants that received human milk while propionic and butyric acids were the predominance metabolites in the gut of bottle-fed infants (Edwards & Parret, 2002; Vasallo, 2010). Various studies confirmed that beneficial effect of prebiotics added to infant formulas which strongly influenced SCFAs pattern providing a fecal SCFAs profile that is close to that of

breastfed infants, with high level of acetate and lower level of propionate and butyrate (Knol et al., 2005; Boehm & Moro, 2008).

In short, SCFA (acetate, propionate or butyrate) are the main end products results by microbial fermentation of these indigestible oligosaccharides and they are well-known to possess beneficial effects on the host (Cummings, 1981; Topping & Clifton, 2001; Macfarlane & Macfarlane, 2003). Enhanced SCFAs production and increased delivery of these compounds in the distal colon, especially butyrate, may have a role in preventing colon cancer and other intestinal disorders (Wong et al. 2006). Respecting with the effect of these products of prebiotics fermentation related with Fe absorption, it has been proposed that these acids enhanced mineral absorption by decreasing the pH of colon contents (Coudray et al., 1997). This decrease in pH will presumably promote the release of iron bound to proteins, thereby increasing its bioavailability (Cook et al., 1964). Another possible mechanism, these short-chain fatty acids may stimulate the proliferation of epithelial cells, thereby increasing absorptive surface area in the colon. Likewise, prebiotics or products of their fermentation may create an environment in the colon that promotes the reduction of Fe^{3+} to Fe^{2+} (Yeung et al., 2005); and prebiotics or products of their fermentation may stimulate the expression of iron regulatory genes, thereby increasing iron absorption such as calbindin-D9K which positively affect Ca absorption (Ohta et al., 1998a) and DMT1 (Gunshin et al., 1997; Zoller et al., 2001), hemochromatosis protein (HFE) (Zhou et al., 1998), duodenal cytochrome b (Dcytb) (McKie et al., 2001), and ferroprotein-1 (Frazer et al., 2001) which positively influenced Fe absorption.

Respecting with the minor SCFAs, Fig. 4.2 showed the concentration of isobutyric, isovaleric, n-valeric, isocaproic, n-caproic and heptanoic acids. It was found great variability in the concentration of these SCFAs among the different treatments as compared with control group and also among the time of incubation (at 10 and 24 h). The differences between the flora patterns predominant in the three fecal inoculums may participate in this variability of SCFAs concentration.

In the case of isobutyric acid, the highest value at 24 h was found in presence of 0.10 % rhLf + 1 % GOS (0.09 ± 0.06) followed by 0.10 % rhLf (0.08 ± 0.02). Non-significant (P<0.05) difference was noted between the values of isobutyric acid among the different treatments whether at 10 or 24 h of fermentation. For isovaleric acid, 1 % GOS gave rise the concentration of this SCFA for the highest value (0.37 ± 0.04) at 10 h of incubation while at 24 h, the presence of 0.10 % rhLf + 1 % GOS had the highest values (0.39 ± 0.08) which significantly (P<0.05) differed as compared with all treatments. Addition of 0.20 % rhLf led to a significant (P<0.05) decrease in isovaleric content at 24 h (0.09 ± 0.01). The obtained findings indicated that 1 % GOS had the highest concentration of valeric acid at 10 h (0.23 ± 0.07), as well as the findings indicated that high variability was found whether by moderately increasing or decreasing for valeric acid. Non-significant differences (P<0.05) were reported for the effect of the tested ingredients on the production of valeric acid.

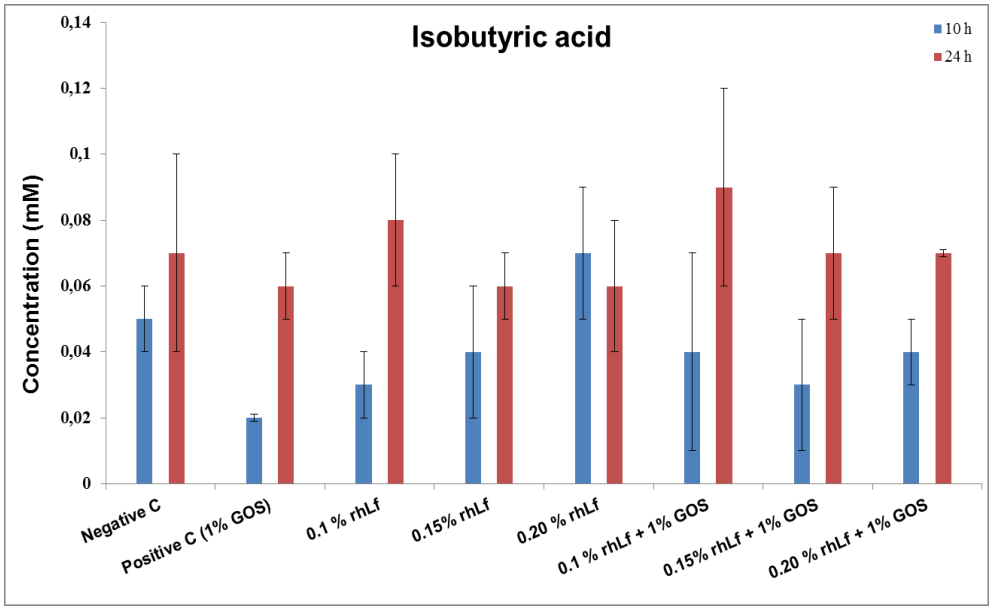
Isocaproic acid has significantly been increased and the highest obtained value (0.40 ± 0.09) was in presence of 0.15 % rhLf at 10 h but its content decreased after 24 h (0.24 ± 0.03). The maximum values of caproic acid obtained at 10 h were for the groups containing 0.20 % rhLf + 1 % GOS then 0.10 % rhLf + 1 % GOS (1.13 ± 0.18 and 1.02 ± 0.12, respectively) while for heptanoic acid, the maximum value at 10 h was for 0.20 % rhLf group (0.82 ± 0.19).

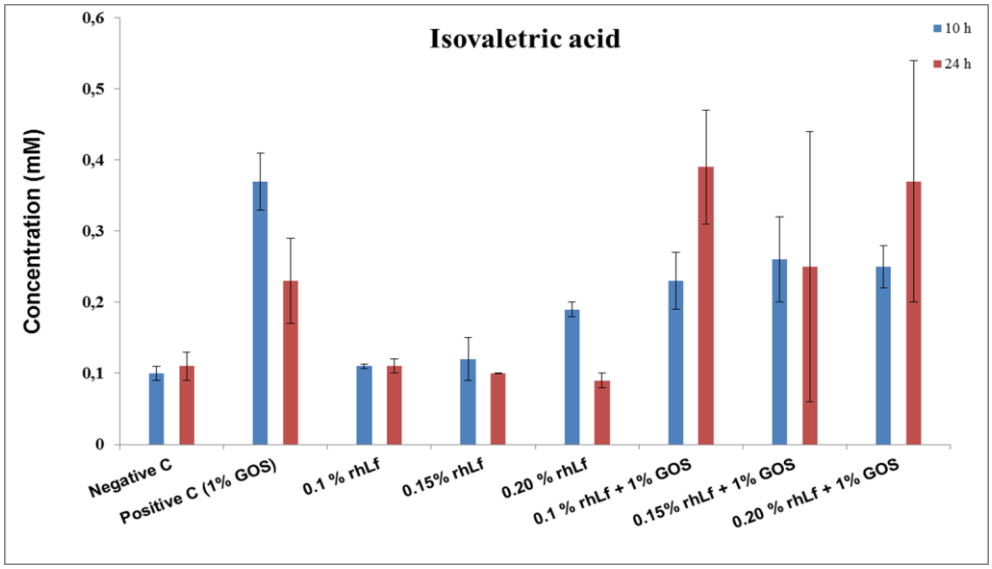
In view of the obtained findings (Fig. 4.2), it worth noting that there was no clear trend related with the effect of rhLf and/or GOS on the production of minor SCFAs in fecal cultures fermentation with the exception of isobutyric acid which found to be increased at 24 h in all treatment as well as control group.

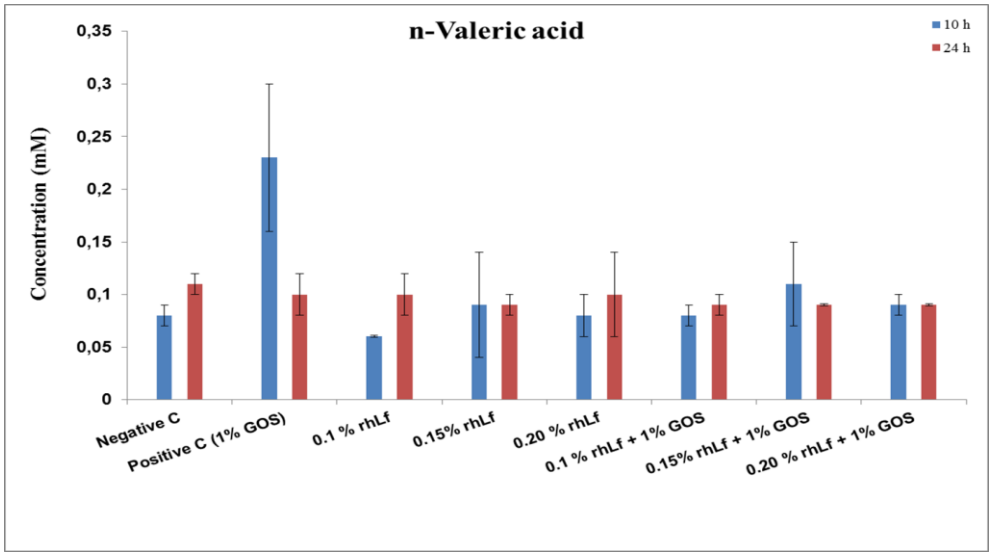
One of the most important factors in this topic is the pH which influences on the growth and/or activity of intestinal microflora, particularly *Bifidobacteria* and *Lactobacilli* and subsequence on the produced SCFAs. More recently, Peso-Echarri (2012) indicated that decreasing of pH means an increase in acidity due to an increased production of SCFAs by the fecal

microbiota and reported a negative significant relationship between the pH and acetic acid or total SCFAs in all sampling points.

Respecting with the pH variation of our study (Fig. 4.3), pH values decreased with the time of fermentation. At 10 h of incubation, we obtained the lowest value of pH in presence of 1 % GOS which similar to the value of control group whereas 0.10 % rhLf followed by 0.20 % rhLf had the maximum values of pH. The values of pH continued to decrease at 24 h of fermentation and the lowest value of pH was for control and 1 % GOS groups while the major value was obtained for 0.15 % rhLf group. Recently, the drop in pH from the ileum to the cecum due to the higher SCFAs concentrations has two effects. First, both *in vitro* and animal studies showed that lower pH values change gut microbiota composition and secondly, it prevents overgrowth by pH-sensitive pathogenic bacteria like *Enterobacteriacae* and *clostridia* (Duncan et al., 2009).


Isobutyric acid
10 h
24 h
Concentration (mM)
0,14
0,12
0,1
0,08
0,06
0,04
0,02
0
Negative C
Positive C (1% GOS)
0.1 % rhLf
0.15% rhLf
0.20 % rhLf
0.1 % rhLf + 1% GOS
0.15% rhLf + 1% GOS
0.20 % rhLf + 1% GOS


Isovaletric acid
10 h
24 h
Concentration (mM)
0,6
0,5
0,4
0,3
0,2
0,1
0
Negative C
Positive C (1% GOS)
0.1 % rhLf
0.15% rhLf
0.20 % rhLf
0.1 % rhLf + 1% GOS
0.15% rhLf + 1% GOS
0.20 % rhLf + 1% GOS


n-Valeric acid
10 h
24 h
Concentration (mM)
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
Negative C
Positive C (1% GOS)
0.1 % rhLf
0.15% rhLf
0.20 % rhLf
0.1 % rhLf + 1% GOS
0.15% rhLf + 1% GOS
0.20 % rhLf + 1% GOS

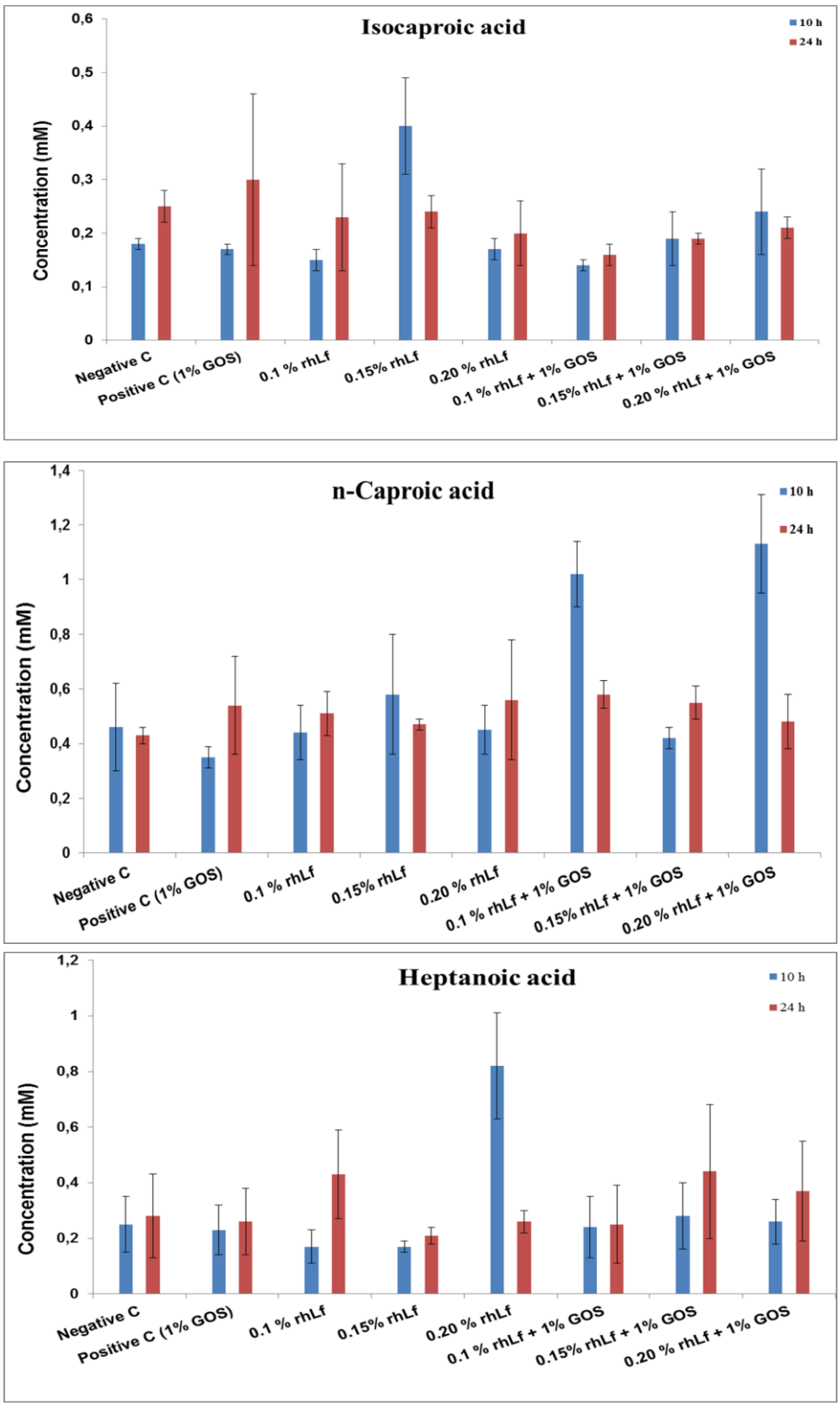


Fig. 4.2. Minor short chain fatty acids concentration produced at 10 and 24 h of incubation with rhLf and/or GOS in batch culture fermentation vessels.

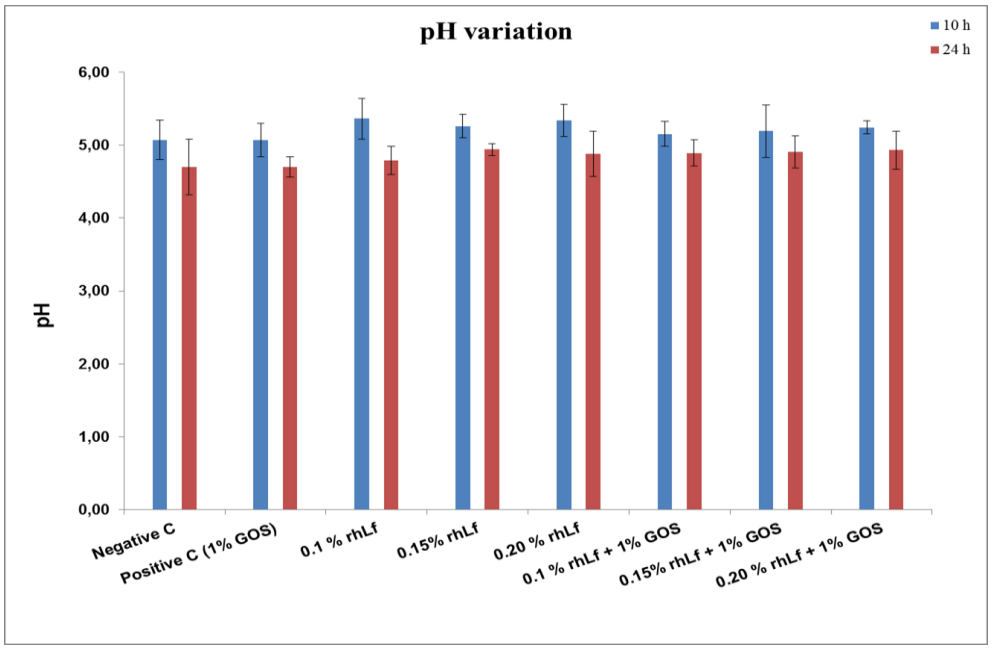


Fig. 4.3. pH variation at 10 and 24 h of incubation with rhLf and/or GOS in batch culture fermentation vessels.

4.6. Reference

Alles, M. S., Scholtens, P. A. S. M. & Bindles, J. (2004). Current trends in the composition of infant milk formulas. *Current Pediatric, 14* (1): 51-63.

Al-Tamimi, M. A. H. M., Palframan, R. J., Cooper, J. M., Gibson, G. R. & Rastall, R. A. (2006). *In vitro* fermentation of sugar beet arabinan and arabinooligosaccharides by the human gut microflora. J. Appl. Microbiol., 100: 407-414.

Barcenilla, A., Pryde, S. E., Martin, J. C., Duncan, S. H., Stewart, C. S., Henderson, C. & Flint, H. J. (2000). Phylogenetic relationships of butyrate-producing bacteria from the human gut. Applied and Environmental Microbiology, 66: 1654e1661.

Ben, X., Zhou, X., Zhao, W., Yu, W., Pan, W., Zhang, W., Wu, S., Van, M., Beusekom, C. M. & Schaafsma, A. (2004). Supplementation of milk formula with galacto-oligosaccharides improves intestinal micro-flora and fermentation in term infants. Chin Med J (Engl)., 117: 927–931.

Boehm, G., Stahl, B., Jelinek, J., Knol, J., Miniello, V. & Moro, G. E. (2005). Prebiotic carbohydrates in human milk and formulas. Acta Paediatr, 94:18–21.

Boehm, G. & Moro, G. (2008). Structural and functional aspects of prebiotics used in infant nutrition. The Journal of Nutrition, 138: 1818S-1828S.

Bouglé, D., Vaghefi-Vaezzadeh, N., Roland, N., Bouvard, G., Arhan, P., Bureau, F., Neuville, D. & Maubois, J. L. (2002). Influence of short-chain fatty acids on iron absorption by proximal colon. Scand. J. Gastroenterol., 37: 1008–1011.

Brück, W. M., Kelleher, S. L., Gibson, G. R., Nielsen, K. E., Chatterton, D. E. & Lönnerdal B. (2003). rRNA probes used to quantify the effects of glycomacropeptide and a-lactalbumin supplementation on the predominant groups of intestinal bacteria of infant rhesus monkeys challenged with enteropathogenic *Escherichia coli*. Journal of Pediatric Gastroenterology and Nutrition, 37 (3): 273–280.

Cai, J. W., Luo, Y. D. & Ben, X. M. (2008). Effects of infant formula containing galacto-oligosaccharides on the intestinal microflora in infants. Zhongguo Dang Dai Er Ke Za Zhi, 10(5): 629-632.

Cardelle-Cobas, A., Fernández, M., Salazar, N., Martınez-Villaluenga, C., Villamiel, M., Ruas-Madiedo, P. & de los Reyes-Gavilan, C. G. (2009). Bifidogenic effect and stimulation of short chain fatty acid production in human fecal slurry cultures by oligosaccharides derived from lactose and lactulose. Journal of Dairy Research, 76: 317–325.

Cook, J. D., Brown, G. M., Valberg, L. S. (1964). The effect of achylia gastrica on iron absorption. J. Clin. Invest., 43: 1185–1191.

Coudray, C., Bellanger, J., Castiglia-Delav, C., Remesy, C., Vermorel, M. & Rayssignuier, Y. (1997). Effect of soluble or partly soluble dietary fibres supplementation on absorption and balance of calcium, magnesium, iron and zinc in healthy young men. Eur. J. Clin. Nutr., 51: 375-380.

Cummings, J. H. (1981). Short chain fatty acids in the human colon. Gut, 22: 763-779.

Cummings, J. H. (1984). Constipation, dietary fibre and the control of large bowel function. Postgrad. Med. J., 60: 811-819.

Cummings, J. H., & MacFarlane, G. T. (1991). The control and consequences of bacterial fermentation in the human colon. J. Appl. Bacteriol., 70: 443–459.

Delgado, S., Ruas-Madiedo, P., Suárez, A. & Mayo, B. (2006). Inter-individual differences in microbial counts and biochemical-associated variables in the feces of healthy Spanish adults. Digest. Dis. Sci., 51: 737-743.

Delzenne, N. M. & Kok, N. (2001). Effects of fructan-type prebiotics on lipid metabolism. Am. J. Clin. Nutr., 73: 456S–458S.

Duncan, S. H., P. Louis, Thomson, J. M. & Flint, H. J. (2009). The role of pH in determining the species composition of the human colonic microbiota. Environ. Microbiol., 11: 2112-2122.

Edwards, C. A. & Parret, A. M. (2002). Intestinal flora during the first months of life: new perspectives. British Journal of Nutrition, 88: S11–S18.

EFSA (2012). Panel on Dietetic Products Nutrition and Allergies. (2012). Scientific opinion on bovine lactoferrin. EFSA Journal, 10: 1-26.

Frazer, D. M., Vulpe, C. D., McKie, A. T., Wilkins, S. J., Trinder, D., Cleghorn, G. J. & Anderson, G. J. (2001). Cloning and gastrointestinal expression of rat hephaestin: relationship to other iron transport proteins. Am. J. Physiol. Gastrointest. Liver Physiol., 281: G931–G939.

Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., Tobe, T., Clarke, J. M., Topping, D. L., Suzuki, T., Taylor, T. D., Itoh, K., Kikuchi, J., Morita, H., Hattori, M. & Ohno, H. (2011). *Bifidobacteria* can protect from enteropathogenic infection through production of acetate. Nature, 469: 543-547.

Fukuda, S., Hidehiro, Toh., Todd, D., Taylor, A., Ohno, H. & Hattori, M. (2012). Acetate-producing bifidobacteria protect the host from enteropathogenic infection via carbohydrate transporters. Gut Microbes, 3:5: 449-454.

Granier, A., Goulet , O. & Hoarau, C. (2013). Fermentation products: immunological effects on human and animal models. Pediatric Research, 74: 238–244.

Gunshin, H., Mackenzie, B., Berger, U. V., Gunshin, Y., Romero, M. F., Boron, W. F., Nussberger, S., Gollan, J. L. & Hediger, M. A. (1997). Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature, 388:482–488.

Harmsen, H. J. M., Wideboer-Veloo, A. C., Grijpstra, J., Knoll, J., Degener, J. E. & Welling, G. W. (2000). Development of 16S rRNA-based probes for the *Coriobacterium* group and the Atopobium cluster and their application for enumeration of *Coriobacteriaceae* in human feces from volunteers of different age groups. Appl. Environ. Microbiol, 66: 4523-4527.

Hijova, E. & Chmelarova, A. (2007). Short chain fatty acids and colonic health. Bratisal Lek Listy, 108 (8): 354-358.

Iacono, G., Merolla, R., D'Amico, D., Bonci, E., Cavataio, F., Di Prima, L., Scalici, C., Indinnimeo, L., Averna, M. R. & Carroccio, A. (2005). Gastrointestinal symptoms in infancy: a population-based prospective study. Dig. Liver Dis., 37: 432-438.

Jenkins, D. J., Wolver, T. M., Collier, G. R., Ocana, A., Rao, A. V., Buckley, G., Lam, Y., Mayer, A. & Thompson, L. U. (1987). Metabolic effects of a low-glycemic-index diet. American Journal of Clinical Nutrition, 1431S-1433S.

Kim, W. S., Ohashi, M., Tanaka, T., Kumura, H., Kim, G. Y., Kwon, I. K., Goh, J. S. & Shimazaki, K. (2004). Growth-promoting effects of lactoferrin on L. acidophilus and Bifidobacterium spp. Biometals, 17(3): 279-283.

Knol, J., Sholten, P., Kafka, C., Stemmbakkers, J., Grob, S., Helm, K., Klarczyk, M., Schöpfer, H., Böckler, H. M. & Wells, J. (2005). Colon microflora in infants fed formula with galacto-oligosaccharides: More like breast-fed infants. J. Ped. Gastroenterol. Nutr., 40: 36-42.

Macfarlane, S. & Macfarlane, G. T. (2003). Regulation of short-chain fatty acid production. Proceedings of the Nutrition Society, 62: 67-72.

MacFarlane, G. T., Steed, H. & MacFarlane, S. (2008). Bacterial metabolism and health related effects of galacto-oligosaccharides and other prebiotics. Journal of Applied Microbiology, 104: 305-344.

Anson, N. M., Havenaara, R., Vaesa, W., Couliera, L., Venemaa, K., Selinheimoc, E., Bastb, A. & Haenenb, G. R.M.M. (2011). Effect of bioprocessing of wheat bran in whole meal wheat bread on the colonic SCFA production *in vitro* and postprandial plasma concentration in men. Food chemistry, 128: 404-409.

McKie, A. T., Barrow, D., Latunde-Dada, G. O., Rolfs, A., Sager, G., Mudaly, E., Mudaly, M., Richardson, C., Barlow, D., Bomford, A., Peters, T. J., Raja, K. B., Shirali, S., Hediger, M. A., Farzaneh, F. & Simpson, R. J. (2001). An iron-regulated ferric reductase associated with the absorption of dietary iron. Science 291:1755–1759.

Miller, T. L. & Wolin, M. J. (1996). Pathways of acetate, propionate, and butyrate formation by the human fecal microbial flora. Appl. Environ. Microbiol., 62: 1589-1592.

Mulder, A. M., Connellan, P. A., Oliver, C. J., Morris, C. A. & Stevenson, L. M. (2008). Bovine lactoferrin supplementation supports immune and antioxidant status in healthy human males. Nutrition Research, 28: 583-589.

Ohta, A., Motohashi, Y., Ohtsuki, M., Hirayama, M., Adachi, T. & Sakuma, K. (1998a). Dietary fructooligosaccharides change the concentration of calbindin-D9k differently in the mucosa of the small and large intestine of rats. J. Nutr., 128: 934–939.

Peso-Echarri, P. (2012). Ingredientes funcionales en fórmulas infantiles que afectan al equilibrio de la microbiota intestinal del lactante y a la absorción y disponibilidad de minerales. PhD Thesis, Faculty of veterinary, University of Murcia. 267p.

Puccio, G., Cajozzo, C., Meli, F., Rochat, F., Grathwohl, D. & Steenhout, P. (2007). Clinical evaluation of a new starter formula for infants containing live *Bifidobacterium longum* BL999 and prebiotics. Nutrition, 23: 1-8.

Rahmarn, M., Kim, W. S., Tanaka, T., Kumura, H., & Shimazaki, K. (2006). Lactoferrin effects on the growth of bifidobacteria. Foods Food Ingredients, 211 (9): 1.

Roberfroid, M. (1993). Dietary fiber, inulin, and oligofructose: a review Comparing their physiological effects. Crit. Rev. Food Sci. Nutr., 33: 103-148.

Rodriguez-Colinas, B., Kolida. S., Baran, M., Ballesteros, A. O., Rastall, R. A. & Plou, F. J. (2013). Analysis of fermentation selectivity of purified galactooligosaccharides by *in vitro* human fecal fermentation. Appl. Microbiol. Biotechnol., 97: 5743-5752.

Rossi, M., Corradini, C., Zanoni, S., Amaretti, A., Nicolini, M. & Matteuzzi, D. (2005). Fermentation of Fructooligosaccharides and Inulin by *Bifidobacteria*: a Comparative Study of Pure and Fecal Cultures. Applied and Environmental Microbiology, 6150–6158

Rycroft, C. E., Jones, M. R., Gibson, G. R. & Rastall, R. A. (2001). A comparative *in vitro* evaluation of the fermentation properties of prebiotic oligosaccharides. J. Appl. Microbiol., 91: 878–887.

Saarela, M., Lähteenmäki, R., Crittenden, R., Salminen, S. & Mattila-Sandholm, T., (2002). Gut bacteria and health foods - the European perspective. International Journal of Food Microbiology, 78: 99–117.

Salazar, N., Gueimonde, M., Hernández-Barranco, A. M., Ruas-Madiedo, P. & De los Reyes-Gavilán, C. G. (2008). Exopolysaccharides Produced by Intestinal Bifidobacterium Strains act as Fermentable Substrates for Human Intestinal Bacteria. Appl. Environ. Microbiol., 74: 4737-4745.

Topping, D. L. & Clifton, P. M. (2001). Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiological Reviews, 81: 1031–1064.

Vasallo, I. (2010). Estudio del efecto nutricional y bifidogénico de una formula infantil suplementada con nucleótidos, alfa-lactoalbúmina y ácidos grasos poliinsaturados de cadena larga en niños recién nacidos sanos. Universidad de Murcia.

Velázquez, M., C., Davies, R., Marett, J., Slavin, L. & Feirtag. J. M. (2000). Effect of Oligosaccharides and Fibre Substitutes on Short-chain Fatty Acid Production by Human Faecal Microflora. *Anaerobe* 6: 87-92.

Wakabayashi, H., Yamauchi, K. & Takase, M. (2006). Lactoferrin research, technology and applications. International Dairy Journal, 16: 1241-1251.

Walker, A. W., Duncan, S. H., McWilliam, E. C., Child, L., M. W. & Flint, H. J. (2005). pH and Peptide Supply Can Radically Alter Bacterial Populations and Short-Chain Fatty Acid Ratios within Microbial Communities from the Human Colon. Appl. Environ. Microbiol., 71: 3692-3700.

Wong, J. M. W., Souza, d. R., Kendall, C. W. C., Emam, A. & Jenkins, D. J. A. (2006). Colonic health: fermentation and short chain fatty acids. Journal of Clinical Gastroenterology, 40: 235-243.

Yeung, C. K., Glahn, R. P., Welch, R. M. & Miller, D. D. (2005). Prebiotics and Iron Bioavailability-Is There a Connection?. Journal of Food Science, 70(5): 88-92.

Zhou, X. Y., Tomatsu, S., Fleming, R. E., Parkkila, S., Waheed, A., Jiang, J., Fei, Y., Brunt, E. M., Ruddy, D. A., Prass, C. E., Schatzman, R. C., O'Neill, R., Britton, R. S., Bacon, B. R. & Sly, W. S. (1998). HFE gene knockout produces mouse model of hereditary hemochromatosis. Proc. Nat. Acad. Sci., 95:2492–4297.

Zoller, H., Koch, R. O., Theurl, I., Obrist, P., Pietrangelo, A., Montosi, G., Haile, D. J., Vogel, W. & Weiss, G. (2001). Expression of the duodenal iron transporters divalent-metal transporter1 and ferroportin-1 in iron deficiency and iron overload. Gastroenterology, 120: 1412–1419.

Chapter 5

Effect of *in vitro* gastrointestinal digestion on the profile of rhLf and long chain fatty acids in human milk as compared with different infant formulas

5.1. Introduction

Lactoferrin (Lf) is the second most abundant human milk protein component (Gonzalez-Chavez et al., 2009) constituting about 10-15% (Baró et al., 2001) and belongs to the transferrin family (Xavier et al., 2010). Since Lf has many functional effects (e.g. enhancement of iron absorption, anti-inflammatory and antibacterial effect,… etc) (Pan et al., 2007; Zimecki et al., 2007; Lönnerdal, 2009; Zimecki et al., 2009) and is acting as first line defense agent against infections in the body (Iigo et al., 2009), it is accepted as functional ingredient by EFSA in infant formulas (EFSA, 2012) and are now marketed in many countries (Mulder et al., 2008, Wakabayashi et al., 2006).

One of the most important characteristics of Lf, which defines its effects, is its resistance to different conditions along the gastrointestinal digestion process, where it is slightly degraded and the produced bioactive peptides have many functional effects (Bellamy et al., 1992). In view of this, now the research in this topic is focused on the Lf degradation and its bioactive peptides released during the gastrointestinal digestion (GID) process.

There is very limited in vivo information whether Lf is digested in the stomach or in the intestine (Britton & Koldovsky, 1989), since most of studies about Lf stability or degradation have been tested with in vitro digestion models using pure proteolytic enzymes (Yao et al., 2013, 2014). Moreover, there are few studies about the resistance of Lf during the *in vitro* simulated GID of human milk or infant formulas containing this protein. In general, stability of Lf added to infant formulas exposed to in vitro digestion is not fully understood. Thus, there was an increasing interest in improving Lf stability in foods by using some dietary components. Regarding with this,

Lf was more stable by using pectin (Bengoechea et al., 2011) and soluble soy polysaccharides in the aqueous solution (Ueno et al., 2012). In this sense, the presence of indigestible prebiotics, e.g. GOS, which are well-known to be quite resistant to different environments and enzymatic digestions along the digestive tract (Hernandez-Hernandez et al., 2012), in infant formulas may protect Lf against the proteolytic enzymes activity leading to higher degree of stability.

To reinforce the functionality of infant formulas, the addition of long chain fatty acids (LCFAs), such as docosahexaenoic acid (DHA) and arachidonic acid (ARA), to infant formulas is well reported, where it have a positive role in supporting the visual and cognitive development in newborns and infants. Recently, the use of LCFAs as a novel food additive in infant formulas has been supported by many scientific agencies such as FDA, EFSA, FAO, WHO (International Formula Council, IFC, 2010). However, there is no scientific literature about the stability of these fatty acids along the gastrointestinal tract or its availability at intestinal level.

5.2. The aims of this chapter

The present study aimed to investigate the effect of *in vitro* simulated gastrointestinal digestion (consisted in two phases: gastric and intestinal) on the profile of rhLf and LCFAs of human milk as compared to different infant formulas containing various concentrations of rhLf (0.1 or 0.15%) and/or GOS (3.3, 5 or 10%).

5.3. Materials and methods

5.3.1. Infant formulas and chemicals:

- **First infant formula (FIF):** was provided by Hero Co. (Murcia, Alcantarilla, Spain), and experimentally supplemented with different concentrations of rhLf (0.1 and 0.15%) and/or different concentrations of GOS (3.3, 5 and 10%) and those were used in SDS-PAGE analysis and western blotting and LCFAs determination. To compare these infant formulas with human milk, samples from three Egyptian mothers were used.

- **Recombinant human Lf (rhLf)** [(expressed in rice, iron saturated, >90% (SDS-PAGE)], as well as enzymes used in this work (pepsin, trypsin and bile salts, from porcine gastric mucosa), were purchased from Sigma Chemical Co. (St. Louis, MO, USA).
- **Vivinal GOS syrup** (dry matter 75 % of which GOS 59 %, lactose 21 %, glucose 19 % and galactose 1 %) was provided by Hero Baby Co. (Alcantarilla, Murcia, Spain).

5.3.2. *In vitro* digestion model of infant formulas

It was used the same method described in the above section 2.4.

5.3.3. Proteolytic digestion of rhLf

It was used the same method described in section 3.3.2. The obtained supernatant was retained for analysis by SDS-PAGE and western blotting (rhLf semi-quantification) and used for studying rhLf fractionation by HPLC-Mass (see section 5.3.5).

5.3.4. *SDS-PAGE* analysis and western blotting

Western blotting is often used for semi-quantification of protein levels of samples. In this study, it was used for semi-quantification of rhLf in some infant formulas and human milk (before and after *in vitro* digestion), as well as pepsin- or trypsin-treated rhLf. Samples of different digests were separated by electrophoresis (Bio-Rad, California, USA) on 12 % SDS-PAGE. After loading standard (5 µL/lane) and samples (5µl of milk or infant formula previously mixed with Lammeli Buffer 4X and heated during 5 min at 95ºC), electrophoresis was started with 90 V for 20 min, which is considered enough time until samples run the stacking gel and go into resolving gel. Then the voltage was increased to 180 and the electrophoresis was run for additional 60 min (until the end of resolving gel). After gel electrophoresis, the transfer phase was carried out using BioRad Trans-blot turbo (Bio-Rad, USA) and appropriate transfer buffer according manufacturer instructions (the roller was used to remove any air trapped between the blotting layers).

Thus proteins transferred electrophoretically to a nitrocellulose membrane (0.2 µm of pore). Then the membrane was blocked with blocking solution (2% BSA/TBST) for 1 h at least, followed by overnight incubation at 4º C with rabbit polyclonal human lactoferrin antibody diluted in block

solution (1:200) (Santa Cruz Biotechnology, USA. Ref. sc-25622). The next day and after three times 10 min washes shaking at room temperature with Tris-Buffered Saline and Tween 20 (TBST), the membranes were incubated with block solution for 25 min followed by incubation the membranes with horseradish peroxidase (HRP)-conjugated secondary antibody diluted in block solution (goat anti-rabbit IgG, 1:10000) (Santa Cruz Biotechnology, USA. Ref. sc-2030). The membrane was washed as previously and protein bands were detected using ECL prime and ImageQuant LAS500 (GE Healthcare Europe, Germany) according manufacturer instructions. The optical densities of proteins were analyzed with software ImageQuant TL (GE Healthcare, Amersham Biosciences, Germany).

5.3.5. Chromatographic separation of rhLf-derived peptides by HPLC-MS

The separation and analysis of the protein digests of the samples were also performed with a HPLC-MS system consisting of an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a μ-wellplate autosampler and a capillary pump, and connected to an Agilent Ion Trap XCT Plus Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA) using an electrospray (ESI) interface.

Samples (40 μl) were injected at a flow rate of 10 μl/min onto a Waters BEH C18 HPLC column (5 μm, 150 × 0.5 mm) for peptide separation and analysis, which was thermostatted at 40°C. After the injection, the column was washed for 10 min with buffer A (water/acetonitrile/formic acid, 94.9:5:0.1) and the digested peptides were eluted using a linear gradient 0-80% B (buffer B: water/acetonitrile/formic acid, 10:89.9:0.1) for 150 min. The column was coupled online to an Agilent Ion Trap XCT Plus Mass spectrometer using an electrospray interface. UV/Vis detector was used, specifically, the absorbance at 210/280 nm was measured.

The Mass spectrometer was operated in the positive mode. The nebulizer gas pressure was set to 15 psi, whereas the drying gas was set to a flow of 5 l/min at a temperature of 350 °C. The capillary spray voltage was 3500 V, whereas the scan speed was set to 8100 (m/z)/sec from 200-2200

m/z, with a target mass of 1000 m/z, and 3 spectra averaging. Smart ion target was set to 150.000, whilst the maximum accumulation time was 20 ms. MS/MS data were collected in an automated data-dependent mode (AutoMSn mode). The three most intense ions were sequentially fragmented using helium collision-induced dissociation (CID) with an isolation width of 2 and relative collision energy of 35%.

Data processing was performed with DataAnalysis program for LC/MSD Trap Version 3.3 (Bruker Daltonik, GmbH, Germany) and Spectrum Mill MS Proteomics Workbench (Rev A.03.02.060B, Agilent Technologies, Santa Clara, CA, USA).

Briefly, raw data were extracted under default conditions as follows: unmodified cysteines; sequence tag length >1; [MH] +100–8000 m/z; maximum charge +6; minimum signal-to-noise (S/N) 25; finding 12C signals. The MS/MS search against the sequence of human Lactoferrin from NCBInr database with the following criteria: identity search mode; digestion with pepsin or trypsin with 5 maximum missed; peptide charge +1, +2, +3; monoisotopic masses; peptide precursor mass tolerance 2.5 Da; product ion mass tolerance 0.7 amu; ESI ion trap instrument; and minimum matched peak intensity 50%.

5.3.6. LCFAs profile of digested samples

Total lipid in infant formulas and human milk (both, before and after digestion) was extracted according to the method of Bligh & Dyer (1959) with minor modifications. A 60 µL of sample was taken in a 15-ml screw-top culture tube with Teflon cap and mixed with water to a total volume of 1 mL; then 2.5 mL of methanol and 1.25 mL of chloroform were added in the same tube and vortexed. The contents of the culture tube were kept at room temperature for 60 min with vortexing every 10 min. After 1 h, 1.25 mL of chloroform, 1.15 of water and 100 µL of 3 M HCl were added (to ensure the pH of the extract was acidic), vortexed and centrifuged at 1200 x g at room temperature. The chloroform layer (bottom phase), containing lipid, was removed using 2 Pasteur pipettes, one inserted into the other. The methanol-water phase was extracted with an additional 1.25 mL of chloroform and both chloroform phases were combined, dried over anhydrous Na2SO4,

filtered, and then transferred into a 4-mL vial. Chloroform was removed from the vial under a stream of N_2, and 3 drops of benzene were added and vortexed. The lipid content in the vial was methylated by adding 200 µL of NaOCH3 (0.5 M solution in methanol, Cruz-Hernandez et al., 2004 and Muller et al., 2005). The vials were kept at room temperature for 24 min. Then, 1 mL of 1 N methanolic sulphuric acid (2.8 mL of 96% sulphuric acid in 100 mL methanol) was added. After vortexing, the vials were heated at 50º C for 15 min and cooled at -20º C for 3 min. Then, 1 mL of water and 1 mL hexane were added, vortexed, and centrifuged at 1200 x g for 3 min at room temperature. The upper portion (hexane layer) containing FA methyl ester (FAME) was transferred into an specific vial for GC analysis.

FAME analysis was performed using an Agilent 7890A GC equipped with a HP-88 112-8867 column (60 m x 0.25 mm x 0.20 µm), flame-ionization detector (FID) and automatic injector. Peaks were routinely identified by comparison of retention times with FAME standards.

5.4. Results and discussion

This study was designed to examine the effect of the *in vitro* simulated gastrointestinal digestion on rhLf degradation added to infant formulas as compared to Lf of human milk. The first step of the gastrointestinal digestion of food proteins is the gastric phase (low pH and pepsin treatment), followed by stomach emptying and further digestion simulating the upper part of duodenum by pancreatic and brush border enzymes (pancreatin and bile salts), the later named intestinal digestion phase (Inglingstad et al., 2010).

Animal origin-purified commercial enzymes (consist mainly of pepsin, trypsin or chymotrypsin, and pancreatin) are used in the simulation of infant gastrointestinal digestion process (David-Birm et al., 2013, Liu et al., 2013); however, the information about the degradation of Lf by various commercial enzymes is controversial (Gonzalez-Chavez et al., 2009, Baldi et al., 2005). In the presented study, it was used an *in vitro* digestion model employing freshly prepared pepsin solution (the gastric phase) followed by the intestinal phase (freshly pancreatin-bile extracts solution).

Several factors must be taken into account when considering the variability of results about Lf degradation. Among these factors: gastric pH, age of the consumer (maturity of the digestive system), the transit time in the stomach (incubation time in *in vitro* model) and enzyme treatments are the most important ones (Kalantzi et al., 2006). Therefore, this topic is very controversial and the obtained data vary depending on the conditions used.

rhLf stability in digested samples

Degradation patterns of pepsin- or trypsin-treated rhLf were checked by Western blot (Fig. 5.1) and also SDS-PAGE pattern stained with Coomassie blue, Fig. (5.2). Pepsin- or trypsin-treated rhLf showed completely degradation and very small peptides were observed, whereas in the untreated rhLf solution only one large band was observed, corresponding to the intact protein. Regarding with human milk, a main band of 80 kDa (corresponding to Lf) and many small peptides bands were observed, whereas after gastrointestinal digestion, Lf from human milk was highly degraded, only remaining around 7.3% of undigested protein and some small peptides bands of lower molecular weight (Fig. 5.3). These obtained findings revealed that Lf of human milk was highly sensitive to degradation during *in vitro* digestion and might be partially explained by the possibility of proteolysis occurred after samples collection and during samples transport and storing (Fig. 5.3) which human milk samples were frozen at -18ºC after collection in Egypt, then were transported to Spain and then stores at -80ºC. It is known that freezing could cause protein denaturation (Chang et al., 1996; Cao et al., 2003).

In the same manner, Rollo et al. (2014) showed that long-term freezing significantly reduces Lf concentrations of human milk as well as some of its biological activities could be influenced. Likewise, Lf concentration in human milk significantly decreased over time.

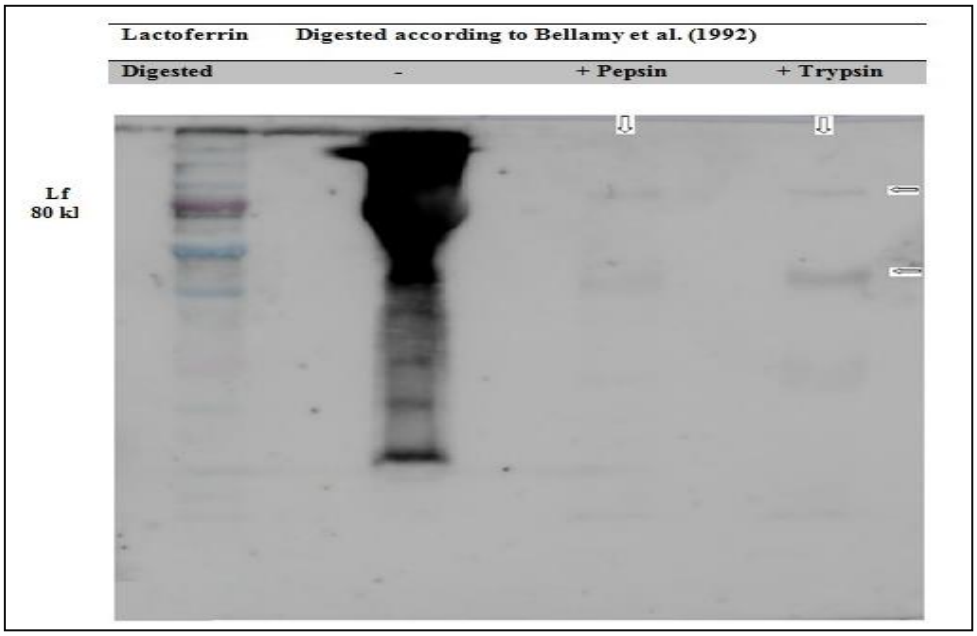


Fig. 5.1. Western Blot of rhLf-untreated and treated with pepsin or trypsin.

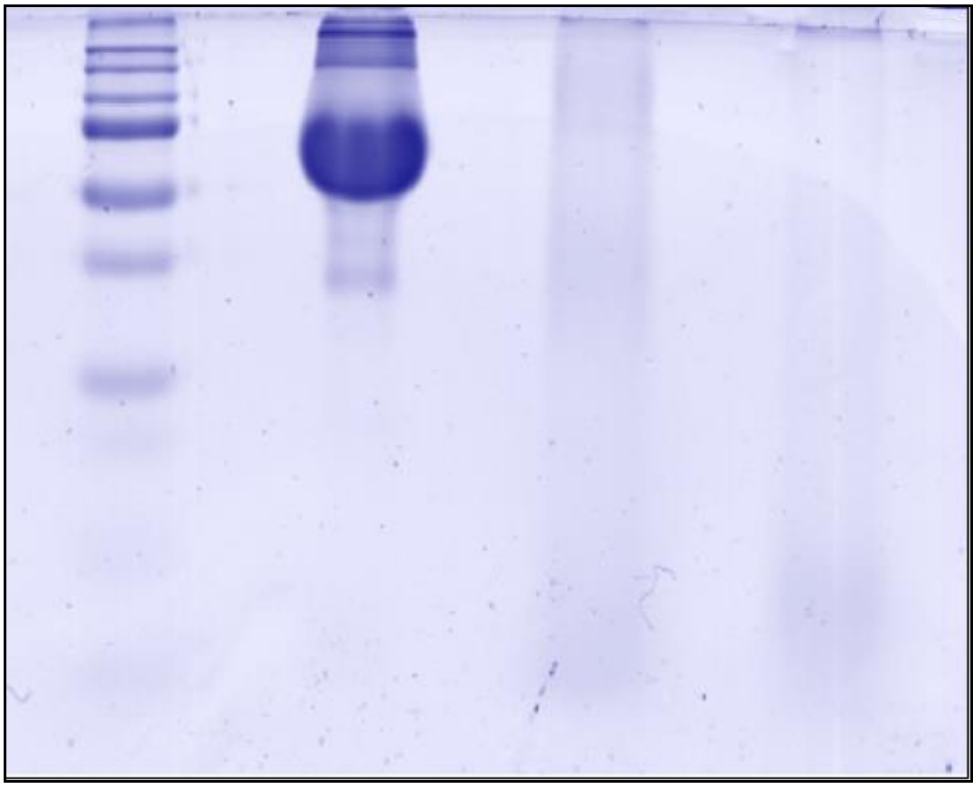


Fig. 5.2. SDS-PAGE pattern of rhLf stained with Coomassie blue. Line 1: Standard; line 2: untreated rhLf; line 3: pepsin-treated rhLf; line 4: trypsin-treated rhLf. Abbreviation: rhLf: recombinant human lactoferrin

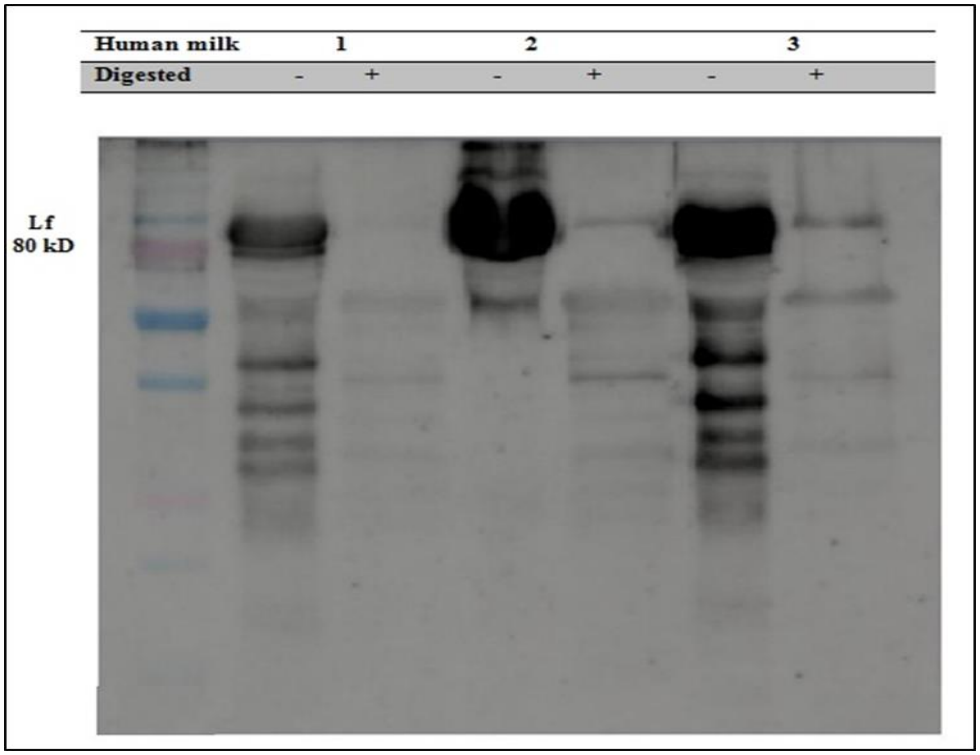


Fig. 5.3. Western Blot of digested human milk.

In the case of infant formulas, as it is shown in Table 5.1, rhLf was poorly digested, but the stability depends on the amount of rhLf added. When the infant formula was enriched with 0.10% of this protein, ~40.9% of whole protein after gastrointestinal digestion process was found in intact form, whereas in 0.15% enriched formula, the undigested protein level was higher preserving 59 % of the initial rhLf (Fig. 5.4. a, b). As it has been mentioned in the introduction, GOS seems to protect rhLf from the enzymatic treatment along the gastrointestinal tract. In the presented study, both infant formulas (supplemented with 0.10 and 0.15% of rhLf) were enriched with three different amounts of this prebiotic substance. As indicated in Table 5.1, infant formula supplemented with 0.10% of rhLf + 3.3% of GOS, showed that ~65.5% of this antimicrobial protein was intact after the digestion process, while 45% and 63% is still in intact form in formulas supplemented with 5 and 10% GOS, respectively (Fig. 5.4. c and d).

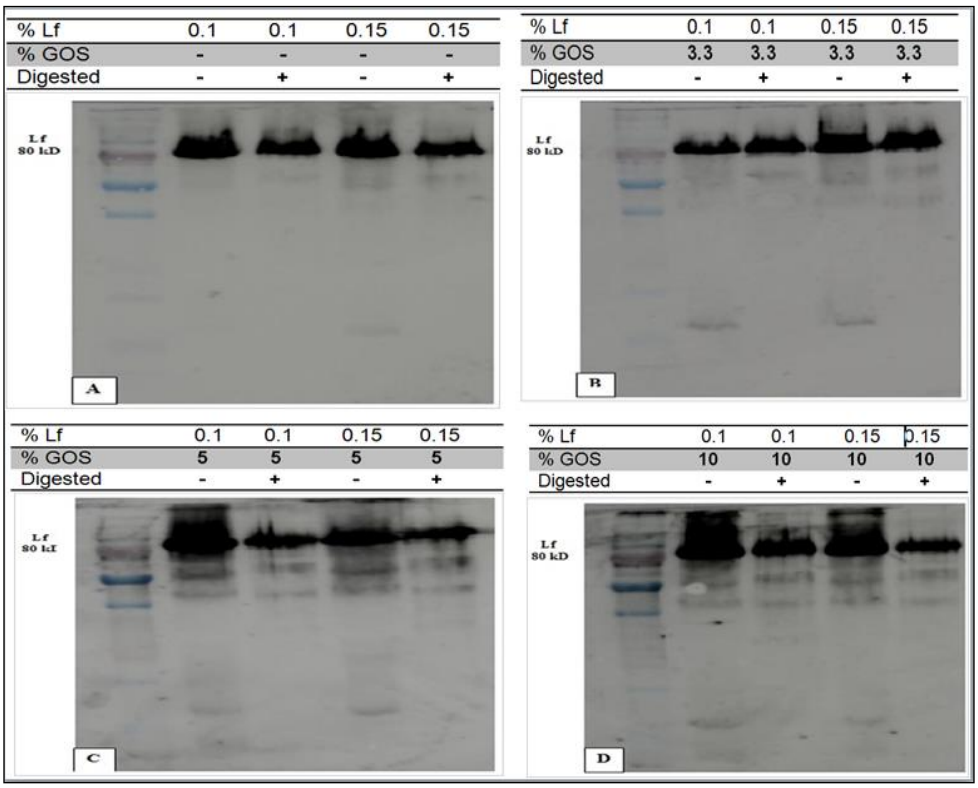


Fig. 5.4. Western Blot of digested infant formulas. Formulas without adding GOS (A); with 3.3% of GOS (B); 5% GOS (C) and 10% GOS (D).

Table 5.1. Recombinant human lactoferrin (rhLf) intact percentage after gastrointestinal digestion.

Treatment		Intact rhLf (%)
0.10 % rhLf		40.9 ± 0.35
0.10% rhLf + 3.3 % GOS		65.5 ± 2.3
0.10% rhLf + 5 % GOS		45 ± 1.5
0.10% rhLf + 10 % GOS		63 ± 5.8
0.15% rhLf		59 ± 1.6
0.15% rhLf + 3.3% GOS		48 ± 1.9
0.15% rhLf + 5 % GOS		76 ± 4.1
0.15% rhLf + 10 % GOS		44 ± 7.4
Human milk		7.3 ± 1.8
rhLf	Pepsin-treated	0.20
	Trypsin-treated	0.15

In the digested infant formulas enriched with 0.15% rhLf, an opposite effect was observed. The results revealed that there is a high level of undigested rhLf (59%) while in enriched formula with 3.3, 5 or 10% GOS, the level of intact rhLf found after being digested was 48%, 76% and 44%, respectively (Fig. 5.4. c and d).

The presented results showed that in 0.10% rhLf enriched formulas; the addition of 3.3% and 10% of GOS was more effective preserving rhLf from proteolysis and subsequently increase its stability than using 5% rhLf. Meanwhile in the case of the digested formulas enriched with 0.15% rhLf, it was found that using 5% GOS was more effective in increasing the stability of rhLf against the digestion process.

Overall, in infant formulas the greatest stability of rhLf was observed in samples containing 0.15% rhLf + 5% GOS while the greatest degradation was observed in those containing 0.10% rhLf and no GOS. Regarding human milk, we found very low level of this protein after digestion process. However, the lowest amount was found in pepsin- or trypsin-treated rhLf, where it was degraded completely. The presented findings showed that rhLf added to infant formulas seemed to be highly resistant and major proportion were detected as intact protein in digested samples after 4 h of the simulated gastrointestinal digestion, while most of small peptides generated were not detected (Fig. 5.3, 5.4 a, b, c and d). As well as the findings indicated that the presence of GOS in infant formulas supplemented with rhLf could raise its stability against simulated digestion. Depending on the amount of rhLf added to infant formulas (0.10 or 0.15%), the presence of GOS can increase, decrease or remain at similar concentration of this protein after digestion. The most stable rhLf was found in infant formulas supplemented with 0.15 % of rhLf + 5 % of GOS (Table 5.1 and Fig. 5.4 c), and all these data suggest that the protective effect of GOS to rhLf degradation during gastrointestinal digestion may be dose-dependent and requires future research to be confirmed. In this sense, it was found that pectin (Bengoechea et al., 2011) and soluble soy polysaccharides (Ueno et al., 2012) would enhance Lf stability. rhLf ability to remain stable under the digestion conditions is extremely important, since intact rhLf could be available to be transferred across the intestinal epithelium (Tomita et al.,

2009), playing its effective roles as iron transporter and immune modulator. On the other hand, various studies inform that the partial digestion of Lf may be beneficial for the host, since some biologically active peptides named lactoferricin and others generated fragments play their roles, such as antibacterial, anti-oxidant, anti-inflammatory and immunomodulatory activities, more effectively than intact protein (Bellamy et al., 1992b, Yamauchi et al., 1993). Thus, the partial degradation of Lf may play a dual role; where Lf as intact protein plays many beneficial roles, and after its partial degradation derived peptides that retains the activities of the native protein, being in many cases even more active.

Another factor that affects Lf stability is pH, even if its variation is slow or fast (Furlund et al., 2013). At pH above 4.0, Lf is a resistant protein as reported Troost et al. (2001), indicating that the Lf conformation is different to native one and pepsin is not fully active (Roberts, 2006, Sreedhara et al., 2010), what could reduce or delay protein digestion (Furlund et al., 2013). While at very low pH values (1.5-2.5), Lf releases its linked iron (Mazurier & Spik, 1980), thus Lf becomes more susceptible to degradation (Brock et al., 1976, Brines & Brock, 1983). These findings are in accordance with the results obtained in the present work, where pH must be adjusted at 4 before the addition of pepsin solution (starting point of gastric phase) and at 5 before the addition of pancreatin-bile extract solution (starting point of the intestinal phase). Therefore, the data showed in this study revealed that rhLf was highly resistant at high intestinal pH and the highest degradation of the protein occurred in the gastric phase when pH decreased below 4.0. The presented findings are in a good agreement with that reported by Britton & Koldovsky (1987); Kuwata et al. (2001) who confirmed that Lf is not degraded in the stomach of infants because they have a low pepsin secretion and its pH values is usually around 4, being it a value too high for significant pepsin activity. Moreover, in infants the secretion of pancreatic enzymes is minor, limiting the proteolytic activity in the small intestine. It was previously reported that the oral administration of liposomal bLf has enhanced its resistance to gastric digestion and was able to achieve the gut and deliver the protein to all tissues via the circulation (Yamano et al., 2010). By contrast, in adults, most of the orally ingested

proteins are degraded by digestive enzymes, such as pepsin or trypsin. In this regard, Kuwata et al. (1998; 2001) reported that the presence of Lf in the stomach of the adults confirm that major proportion of Lf is degraded in this part of the gastrointestinal tract. In view of this, in early stages of life, as newborns and infants, the digestive system is not fully developed (Bosscher et al., 2001, Jovani et al., 2001) and it is thought that Lf remains undigested preserving its bioactivity. On the other hand, in adults the digestive system is completely mature, so proteins are completely digested in the upper gastrointestinal tract (Brock, 2012).

These findings are in line with previous reports on *in vitro* digestion (Eriksen et al., 2010) and also are well correlated with the findings of Chatterton et al. (2004) that indicated that many milk proteins, particularly bLF, remain stable to degradation when milk is ingested by neonates. In the same context, studies simulating adult conditions (gastric pH was adjusted to 2.5 before the addition of human gastric juice) showed that bLf was completely digested (Furlund et al., 2013). However, it also was reported that *in vivo* intragastric Lf digestion occurs at a pH higher than 4, suggesting a difference between the results of *in vitro* and *in vivo* experiments (Troost et al., 2001). One of these differences is the presence of phospholipids in the gastrointestinal tract (*in vivo* studies) which probably affect to the milk protein digestion while they are absent in *in vitro* digestion models using simple commercial enzymes (Macierzanka et al., 2009).

The role of glycosylation in protecting the protein from protease digestion allowing its continued bioactivity is well-known. Thereby, the loss of the glycan part could make hLf more degradable by digestive enzymes, generating small peptides such as lactoferricin (Bellamy et al., 1992a, b, Wei et al., 2007). However, contrary to these works, other researches highlight the presence of intact whey protein in human jejunum after milk ingestion (Mahé et al., 1991). In this regard, Lf was highly resistant and 60–80% was detected as intact protein, suggesting the stability of this protein under the adult stomach conditions (Troost et al., 2001). In neonates, hLf is only partially digested and may be absorbed in intact form in gut (Chatterton et al., 2004). In contrast, other researcher reported that bLf is

totally degraded in the upper gastrointestinal tract (Kuwata et al., 1998, Yao et al., 2014).

It is also worthy of note that along the digestive system of human (infants and adults), the protein-linked glycans are not degraded, since the specialized enzymes required are absent partially or totally in the upper gastrointestinal tract, mainly in infants where the digestion system is not completely developed. However, these enzymes are abundantly produced by colonic bacteria leading to the most degradation of glycoproteins. The differences in bacterial populations and species may alter degradation of glycoproteins and therefore its functionality (Dallas et al., 2011). Stability of Lf treated with trypsin and trypsin-like enzymes is well-known and it is related to the iron saturation (Iyer & Lönnerdal, 1993). Hence, the level of Lf saturation and subsequently, the iron binding, stabilizes bLf and holo-Lf molecules (Troost et al., 2002). In this regard, holo-Lf was more resistant to degradation than apo-Lf when were incubated with proteolytic enzymes (Brines & Brock, 1983). In our study, rhLf was iron saturated and our findings indicated that rhLf was more stable to degradation and more intact rhLf was remained after 4 h of *in vitro* digestion. Similar results were obtained by Spik et al. (1982) who showed that a minor degradation of Lf *in vivo* in the entire digestive tract of newborns, as well as similar results were obtained by *ex vivo* of gastric juice in preterm infants although some degradation was found in the later study (Britton & Koldovsky, 1982). In the same direction, patterns of SDS-PAGE revealed that free iron-rhLf was totally degraded in human by *in vivo* studies during stomach and small intestine phases. Therefore, the free iron-rhLf may be more susceptible to be digested than Lf of animal origin (Troost et al., 2002). These results may partially explain the higher degradation of Lf from human milk than infant formula, since a major proportion (~90%) of human milk Lf is in the form of apo-Lf (Lönnerdal, 2010), and is partially iron saturated (5-8%) (Yen et al., 2011), thus hLf is being more sensitive to gastrointestinal enzymes.

Chromatographic separation of rhLf-derived peptides

The trypsin or pepsin digested rhLf was run in SDS-PAGE observing that protein was completely degraded into small peptides with low and very low molecular weights. In the case of trypsin, much more

peptides were generated, all of them with lower size than in pepsin treatment. The effect of proteolytic enzymes (pepsin or trypsin) on the integrity of rhLf and the generated peptides was determined by HPLC-MS. The data indicated, as expected after SDS-PAGE, that rhLf treated with pepsin or trypsin was completely degraded and many small peptides were produced. A mass spectrum of the major peak of the UV-chromatogram is shown in Fig. (5.5, 5.6). The findings revealed that an increase in peptides content was observed after hydrolysis of rhLf by trypsin over pepsin (Table 5.2). However, a similar peptidic pattern was obtained for rhLf hydrolyzed with pepsin and trypsin. As seen in Fig. 5.5; 5.6 and Table 5.2, a total of 10 and 14 chromatographic peaks were considered for peptide identification by MS/MS analysis of pepsin-or trypsin-treated rhLf, respectively. Trypsin has a higher ability to generate fragments detectable by this method than pepsin. It appears from these findings that pepsin had the ability to cleavage the protein at various sites of amino acids but trypsin cleaved rhLf at two positions, which are arginine (R) and lysine (K). Rastogi et al. (2014) found that hydrolysis of bLf by trypsin produced three major functional molecules of sizes approximately 21 kDa, 38 kDa and 45 kDa with producing minor molecular peptides with a low molecular weight, below 14 kDa. Also, in all the three sites the residues usually attacked by trypsin are arginine and lysine. Likewise, Legrand et al. (1986) demonstrated that the moderate treatment of Lf with trypsin led to producing two iron-binding Lf fragments of 30 kDa and 50 kDa. In our experiment, the rhLf-derived peptides have molecular weight ranged between ~ 8-15 kDa. Many published studies reported the efficacy of Lf-derived peptides produced by pepsin or trypsin treatment. Among these well-known activities, antimicrobial activity (Roşeanu et al., 2010; Rastogi et al., 2014), anti-inflammatory capacity (Andrä et al., 2005) and angiotensin converting enzyme-inhibitory activity (Lee et al., 2006; Hernandez-Ledesma et al., 2007). Therefore, many *in vitro* and *in vivo* studies are required to clarify these activities of Lf-derived peptides. Although the functional role of intact Lf is well-documented, the hydrolyzed functional fragments of Lf continue to function in a similar and sometimes more potent manner (Rastogi et al., 2014).

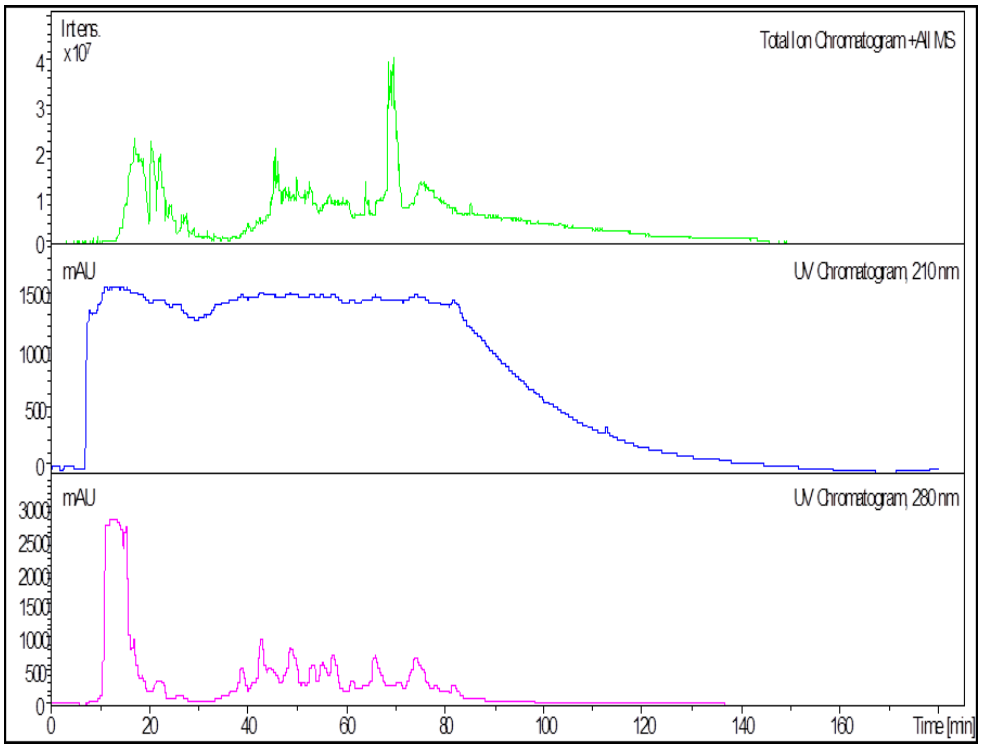


Fig. 5.5. Fractionation of rhLf-derived peptides by pepsin.

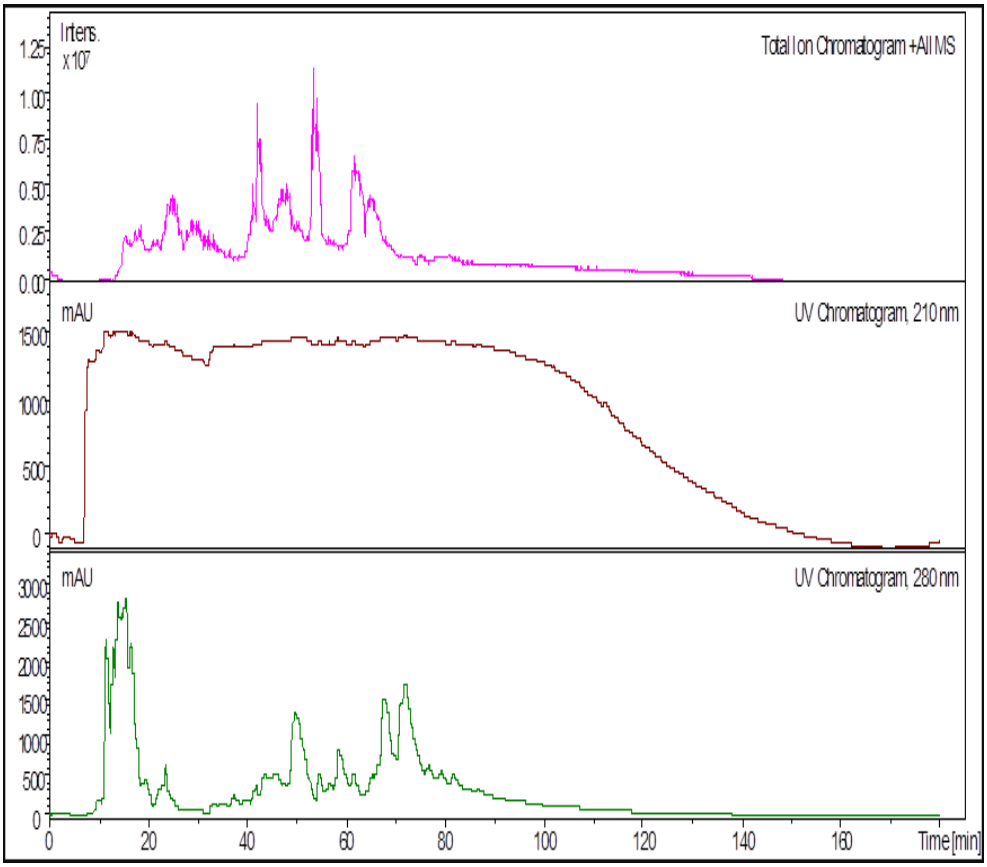


Fig. 5.6. Fractionation of rhLf-derived peptides by trypsin.

Table 5.2. HPLC-MS analysis of peptide separation of pepsin- or trypsin-treated rhLf. Retention time (RT, min), *m/z*, and rhLf-derived peptides sequence.

Sample	Peak	RT	*m/z*	Peptide sequence
Pepsin-treated rhLf	1	15.29	1306.53	(R)LKQVLLHQQAK(F)
	2	24.73	1501.24	(G)LLFNQTGSCKFDE(Y)
	3	29.69	1548.82	(A)IAENRADAVTLDGGF(I)
	4	41.53	1306.75	(L)LCLDGKRKPVTEARSCHLAMAPNHAVVSR MDKVER(L)
	5	46.48	1144.22	(R)IDSGLYLGSGY(F)
	6	60.81	802.30	(L)YLGSGYFTAIQNLR(-)
	7	61.43	501.81	(L)YLGSGYFTAIQNLR(-)
	8	61.52	886.111	(A)IAENRADAVTLDGGFIYEAGLAPYK(L)
	9	63.15	1537.07	(K)FQLFGSPSGQKDLL(F)
	10	66.73	801.81	(A)FRCLAENAGDVAFVKDVTVLQNTDGNNMR HG(L)
Trypsin-treated rhLf	1	15.44	533.81	(K)LRPVAAEVYGTER(Q)
	2	15.47	1460.11	(K)QVLLHQQAK(F)
	3	19.15	1018.4	(R)DGAGDVAFIR(E)
	4	19.81	1239.17	(R)SDTSLTWNSVK(G)
	5	24.56	1195.62	(K)FQLFGSPSGQK(D)
	6	24.80	1150.43	(R)THYYAVAVVK(K)
	7	43.49	802.58	(-)MKLVFLVLLFLGALGLCLAGRR(R)
	8	45.40	1390.36	(K)GGSFQLNELQGLK(S)
	9	48.84	1536.85	(K)YLGPQYVAGITNLK(K)
	10	52.78	773.06	(R)DSPTQCIQAIAENR(A)
	11	62.12	1129.67	(K)EDAIWNLLR(Q)
	12	63.17	1129.87	(K)EDAIWNLLR(Q)
	13	73.74	1045.72	(R)IDSGLYLGSGYFTAIQNLR(K)
	14	152.19	1036.87	(R)ADAVTLDGGFIYEAGLAPYK(L)

Release of fatty acids by *in vitro* digestion

It was studied the effect of *in vitro* gastrointestinal digestion on the profile or the evolution of polyunsaturated fatty acids in different infant formulas as compared with human milk. Fatty acids compositions are shown in Table 5.3.

The obtained findings revealed that *in vitro* digestion led to an increase in the contents of lauric, myristic, myristoleic, palmitoleic, elaidic, oleic and linoleic acids in the digested human milk samples. The data also showed that oleic acid increased after digestion from 710 to 761 (µg/mL) while the content of gamma-linolenic acid and eicosapentanoic acid in digested human milk has been decreased. Although the effect of *in vitro* digestion on profile of fatty acids content of the mature human milk, this effect was slightly whether in the case of the increasing or decreasing.

Unlike, in first infant formulas (suitable for newborns and up to 6 months) it was oberved that the content of lauric and myristoleic acids decreased with *in vitro* gastrointestinal digestion (103.6 and 4.23 µg/ml) in comparison with their undigested formulas (355.79 and 600.09 µg/ml). Some fatty acids of infant formulas were detected only before digestion (palmitoleic, α-linolenic acid and gamma-linolenic acid). On the other hand, myristic, oleic and eicosapentanoic acids were not detected whether before or after *in vitro* digestion. In the presented study, it was detected a similar trend (data not shown) of the effect of *in vitro* digestion on the fatty acids profile with other formulas marketed in Egypt (*e.g*. Isomil 2, NAN 2 and Aptamil 2). All fatty acids were not significantly ($P<0.05$) different, in all samples. These findings reflect the importance of the breastfeeding pattern rather than the bottle-feeding pattern respecting with its positive role in supporting the visual and cognitive development in newborns and infants. As far as we know, this is the first study exploring the effect of *in vitro* digestion on the fat fractionation in infant formulas compared to mature human milk. Thus, many difficulties we faced for interpreting this topic. Hur et al. (2009) reported that the amount of free fatty acids dramatically increased after *in vitro* digestion in beef patty samples.

Table 5.3. Relative changes of LCFAs content in different infant formulas as affected by *in vitro* gastrointestinal digestion.

LCFAs (µg/ml)		FIF		Human milk	
		Not digested	Digested	Not digested	Digested
C12:0	Lauric acid	355,79	103.96	411,47 ± 52,65	493,09 ± 5.96
C14:0	Myristic acid	ND	ND	109,95 ± 7.80	123.97 ± 13.61
C14:1	Myristoleic acid	600,09	4.23	42.59 ±7.46	52.84 ± 2.07
C16:1	Palmitoleic acid	50,16	ND	785.98 ± 16.68	805.97 ± 84.41
C18:1	Cis-9-Oleic acid	ND	ND	710.15 ± 76.54	761.47 ± 27.82
C18:2n-6	Linoleic acid (Cis-9, 12-……)	7,30	ND	65.46 ± 8.51	100.09 ± 34.14
C18:3n-6	ɣ-Linolenic acid ɣ-LA	663,76	ND	2024.70 ± 446.01	1872.31 ± 661.29
C20:5n-3	Cis-5, 8, 11, 14, 17-Eicosapentanoic	ND	ND	24.52 ± 2.52	18.92 ± 1.36

LCFAs: long chain fatty acids.
FIF: First Infant formula.
ND: not detected.

Based on the results of the current research it seems that the *in vitro* digestion stimulates the lipid digestion of FIF and almost no free fatty acids were not detected. The lack of free fatty acids in the digested FIF could be due to the vegetable fats which may be more susceptible to be digested *in vitro*. On the other hand, it has been identified the increase of free fatty acids content after the digestion of human milk, partially due to the capacity of enzymes to release digestest the structure of mature human milk. It is well-documented that there is a difference between the lipid fraction of human milk and infant formulas, including lipid composition, structure, digestion and ultimately absorption, which may partially explain the different effect of *in vitro* digestion on fatty acids release in both human milk and infant formulas. Generally, dietary fat digestion in the gastrointestinal tract into fatty acids is essential to be absorbed by the epithelial cells. Fat digestion occurs in the stomach by gastric lipase

producing diacylglycerol and free fatty acids (Thomsom et al., 1989), and continues in the gut by pancreatic lipase (Small, 1991; Carriere et al., 1993). However, at birth the ability of lipid digestion is incomplete but rapidly develops the capacity to digest fat, but during the first months of life quickly develops to normal levels (Manson et al., 1999). As mentioned above, this is the first study to evaluate the *in vitro* digestion of human milk and infant formula respecting with its effect on fatty acids profile and many studies may be required for discover this unknown-effect.

It could be concluded that rhLf is a highly stable protein against the simulated *in vitro* digestion. Prebiotics might protect rhLf form the digestive enzymes and this hidden property needs more investigation to be confirmed. The obtained results confirmed the importance of breast-feeding pattern related with the released LCFAs and its role in the visual and cognitive development.

Overall, the obtained findings revealed various functionalities for rhLf and its hydrolysate and GOS which play a prominent role in the development of newborn and infants health.

5.5. References

Andrä, J., Lohner, K., Blondelle, S. E., Jerala, R., Moriyon, I., Koch, M. H., Garidel, P. & Brandenburg, K. (2005). Enhancement of endotoxin neutralization be coupling of a C12-alkyl chain to a lactoferricin-derived peptide. Biochem. J., 385: 135-143.

Baldi, A., Ioannis, P., Chiara, P., Eleonora, F., Roubini, C. & Vittorio, D. (2005). Biological effects of milk proteins and their peptides with emphasis on those related to the gastrointestinal ecosystem. J. Dairy Res., 72: 66–72.

Baró, L., Jiménez, J., Martínez-Férez, A. & Boza, J. J. (2001). Bioactive compounds derived from human milk. Ars Pharmaceutica., 42(1): 21-38.

Bellamy, W., Takase, M., Wakabayashi, H., Kawase, K. & Tomita, M. (1992a). Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. J. Appl. Bacteriol., 73: 472–479.

Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K., & Tomita, M. (1992b). Identification of the bactericidal domain of lactoferrin. Biochim. Biophys. Acta, 1121: 130–136.

Bengoechea, C., Jones, O. G., Guerrero, A. & McClements, D. J. (2011). Formation and characterization of lactoferrin/pectin electrostatic complexes: Impact of composition, pH and thermal treatment. Food Hydrocoll., 25: 1227–1232.

Bligh, E. G. & Dyer, W. J. (1959). A rapid method for total lipid extraction and purification. Can. J. Biochem. Physiol., 37: 911-917.

Bosscher, D., Lu, Z., Janssens, G., Van Caillie-Bertrand, M., Rubberiest, H., De Rycke, H., De Wilde, R. & Deelstra, H. (2001). *In vitro* availability of zinc from infant foods with increasing phytic acid contents. British Journal of Nutrition, 86(2): 241–247.

Brines, R. D. & Brock, J. H. (1983). The effect of trypsin and chymotrypsin on the in vitro antimicrobial and iron-binding properties of lactoferrin in human milk and bovine colostrums: unusual resistance of human apolactoferrin to proteolytic digestion. Biochim. Biophys. Acta, 759: 229–235.

Britton, J. R. & Koldovsky, O. (1987). Luminal digestion of lactoferrin in suckling and weanling rats. Am. J. Physiol., 253:G397-G403.

Britton, J. R. & Koldovsky, O. (1989) Gastric luminal digestion of lactoferrin and transferrin by preterm infants. Early Hum. Dev., 19: 127–135.

Brock J. H. (2012) Lactoferrin-50 years on. Biochem Cell Biol., 90: 245 –251.

Brock, J. H., Arzabe, F., Lampreave, F. & Pineiro, A. (1976). The effect of trypsin on bovine transferrin and lactoferrin. Biochim. Biophys. Acta, 446: 214–225.

Cao, E., Chen, Y., Cui, Z. & Foster, P. R. (2003). Effect of freezing and thawing rates on denaturation of proteins in aqueous solutions. Biotechnol. Bioeng., 82(6): 684-690.

Carriere, F., Barrowman, J. A., Verger, R. & Laugier, R. (1993). Secretion and contribution to lipolysis of gastric and pancreatic lipases during a test meal in humans. Gastroenterology, 105: 876–888.

Chang, B. S., Kendrick, B. S. & Carpenter, J. F. (1996). Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. J. Pharm. Sci., 85(12): 1325–1330.

Chatterton, D. E. W., Rasmussen, J. T., Heegaard, C. W., Sorensen, E. S. & Petersen, T. E. (2004). *In vitro* digestion of novel milk protein ingredients for use in infant

formulas: Research on biological functions. Trends Food Sci. Technol., 15: 373–383.

Dallas, D. C., Sela, D., Underwood, M. A. German, J. B., & Lebrilla, C. (2011). Protein-Linked Glycan Degradation in Infants Fed Human Milk. Journal of Glycomics Lipidomics, S1-S10.

David-Birm T., Mackie A. & Lesmes U. (2013) Impact of dietary fibers on the properties and proteolytic digestibility of lactoferrin nano-particles. Food Hydrocoll., 31: 33–41.

EFSA (2012). Panel on Dietetic Products, Nutrition and Allergies (NDA); Scientific Opinion on bovine lactoferrin. EFSA Journal, 10(5): 2701. 26 pp.

Eriksen, E. K., Holm, H., Jensen, E., Aaboe, R., Devold, T. G., Jacobsen, M. & Vegarud, G. E. (2010). Different digestion of caprine whey proteins by human and porcine gastrointestinal enzymes. Br. J. Nutr., 104: 374–381.

Furlund, C. B., Ulleberg, E. K., Devold, T. G., Flengsrud, R., Jacobsen, M., Sekse, C., Holm, H. & Vegarud, G. E. (2013). Identification of lactoferrin peptides generated by digestion with human gastrointestinal enzymes. J. Dairy Sci., 96: 75–88.

Gonzalez-Chavez, S. A., Arevalo-Gallegos, S. & Rascon-Cruz, Q. (2009) Lactoferrin: structure, function and applications. Int. J. Antimicrob. Agents, 33: 301 e301-308.

Hernandez-Hernandez, O., Marín-Manzano, M. C., Rubio, L. A., Moreno, F., J., Sanz, M. L. & Clemente, A. (2012). Monomer and linkage type of galacto oligosaccharides affect their resistance to ileal digestion and prebiotic properties in rats. Journal of Nutrition, 142: 1232–1239.

Hernandez-Ledesema, B., Quirós, A., Amigo, L. & Recio, I. (2007). Identification of bioactive peptides after digestion of human milk and infant formula with pepsin and pancreatin. International Dairy Journal, 17: 42-49.

Hur, B. O., Lim, S. J., Parak, G. B. & Joo, S. T. (2009). Effects of various fiber additions on lipid digestion during *in vitro* digestion of beef patties. Journal of Food Science, 74 (9): C653-C657.

IFC (2010). International Formula Council: Infant Formula Council (IFC) statement on DHA/ARA and infant formula. Infant Feeding & Nutrition. http://www.infantformula.org/news-room/press-releases-and-statements/infant-formula-and-dha/ara.

Iigo, M., Alexander, N., Long, J., Xu, K., Fukamachi, M., Futakuchi, Takase, M. & Tsuda, H. (2009). Anticarcinogenesis pathways activated by bovine lactoferrin in the murine small intestine. Biochemie, 91(1): 86-101.

Inglingstad, R. A., Devold, T. G., Eriksen, E. K., Holm, H., Jacobsen, M., Liland, K. H., Rukke, E. O. & Vegarud, G. E. (2010). Comparison of the digestion of caseins and whey proteins in equine, bovine, caprine and human milks by human gastrointestinal enzymes. Dairy science & technology, 90: 549-563.,

Iyer, S. & Lönnerdal B. (1993). lactoferrin, lactoferrin receptors and iron metabolism. European Journal of Clinical Nutrition, 47: 232–241.

Jovaní, M., Barbera, R. & Farré, R. (2001). Lactoferrin and its possible role in iron enrichment of infant formulas. Food Science and Technology International, 7: 97-103.

Kalantzi, L., Goumas, K., Kalioras, V., Abrahamsson, B., Dressman, J. B. & Reppas, C. (2006). Characterization of the human upper gastrointestinal contents under

conditions simulating bioavailability/bioequivalence studies. Pharm. Res., 23:165-176.

Kuwata, H., Yamauchi, K., Teraguchi, S., Ushida, Y., Shimokawa, Y., Toida, T. & Hayasawa, H. (2001). Functional fragments of ingested lactoferrin are resistant to proteolytic degradation in the gastrointestinal tract of adult rats. J. Nutr., 131: 2121–2127.

Kuwata, H., Yip, T., Tomita, M. & Hutchens, T. W. (1998). Direct evidence of the generation in human stomach of an antimicrobial peptide domain (lactoferricin) from ingested lactoferrin. Biochim. Biophys. Acta, 1429: 129-141.

Lee, N-Y., Cheng, J-T., Enomoto, T. & Nakamura, I. (2006). The Antihypertensive Activity of Angiotensin-Converting Enzyme Inhibitory Peptide Containing in Bovine Lactoferrin. Chinese Journal of Physiology, 49(2): 67-73.

Legrand, D., Mazurier, J., Aubert, J. P., Marie-Henriette, Loucheux-Lefebvre, M. H., Montreuil, J. & Spik, G. (1986). Evidence for interactions between the 30 kDa N- and 50 C-terminal tryptic fragments of human lactotransferrin. Biochem. J., 236: 839-844.

Liu, W., Ye, A., Liu, W., Liu, C. & Signh, H. (2013). Stability during in vitro digestion of lactoferrin-loaded liposomes prepared from milk fact globule membrane-derived phospholipids. J. Dairy Sci., 96: 2061–2070.

Lönnerdal, B. (2010). Bioactive proteins in human milk: mechanisms of action. J Pediatr, 156: S26-30.

Lönnerdal, B. (2009) Nutritional roles of lactoferrin. Curr. Opin. Clin. Nutr. Metab. Care, 12: 293-297.

Macierzanka, A., Sancho, A. I., Mills, E. N. C., Rigby, N. M. & Mackie, A. R. (2009). Emulsification alters simulated gastrointestinal proteolysis of β-casein and β-lactoglobulin. Soft Matter, 5: 538–550.

Mahé, S., Messing, B., Thuillier, F. & Tome, D. (1991). Digestion of bovine milk proteins in patients with a high jejunostomy, Ami. J. Cli. Nuir., 54:534-8.

Manson, W. G., Coward, W. A., Harding, M. & Weaver, L. T. (1999). Development of fat digestion in infancy. Arch. Dis. Child. Fetal Neonatal Ed., 80: F183–F187.

Mazurier, J. & Spik, G. (1980) Comparative study of the iron-binding properties of human transferrins: complete and sequential iron saturation and desaturation of the lactotransferrin. Biochim. Biophys. Acta, 629: 399-408.

Mulder, A. M., Connellan, P. A., Oliver, C. J., Morris, C. A. & Stevenson, L. M. (2008). Bovine lactoferrin supplementation supports immune and antioxidant status in healthy human males. Nutrition Research, 28: 583-589.

Pan, Y., Rowney, M., Guo, P. & Hobman, P. (2007). Biological properties of lactoferrin: an overview. Australian Journal of Dairy Technology, 62(1): 31-42.

Rastogi, N., Nagpal, N., Alam, H., Pandey, S., Gautam, L., Sinha, M., Shin, K., Manzoor, N., Virdi, J. S., Kaur, P., Sharma, S. & Singh, T. P. (2014). Preparation and antimicrobial action of three tryptic digested functional molecules of bovine lactoferrin. PLoS One, 9(3): e90011: 1-12.

Roberts, N. B. (2006). Human pepsins-their multiplicity, function and role in reflux disease. Aliment. Pharmacol. Ther., 24: 2–9.

Rollo, D. E., Radmacher, P. G., Turcu, R. M., Myers, S. R. & Adamkin, D. H. (2014). Stability of lactoferrin in stored human milk. Journal of Perinatology, 1–3.

Roşeanu, A., Damian, M. & Evans, R. W. (2010). Mechanisms of the antibacterial activity of lactoferrin and lactoferrin-derived peptides. Rom. J. Biochem., 47 (2): 203-209.

Small, D. M. (1991). The effects of glyceride structure on absorption and metabolism. Annu. Rev. Nutr., 11: 413-434.

Spik, G., Brunet, B., Mazurier-Dehaine, C., Fontaine, G. & Montreuil, J. (1982) Characterization and properties of the human and bovine lactotransferrins extracted from the faeces of newborn infants. Acta Paediatr. Scand., 71: 979–985.

Sreedhara, A., Flengsrud, R., Langsrud, T., Kaul, P., Prakash, V. & Vegarud. G. E. (2010). Structural characteristic, pH and thermal stabilities of apo and holo forms of caprine and bovine lactoferrins. Biometals, 23:1159-1170.

Thomsom, A. B. R., Keelan, M., Garg, M. L. & Clandinin, M. T. (1989). Intestinal aspects of lipid absorption: in review. Can. J. Physiol. Pharmacol., 67:179-191.

Tomita, M., Wakabayashi, H., Shin, K., Yamauchi, K., Yaeshima, T. & Iwatsuki, K. (2009) Twenty-five years of research on bovine lactoferrin application. Biochimie, 91: 52–57.

Troost, F. J., Saris, W. H. M. & Brummer, R.-J. M. (2002). Orally ingested human lactoferrin is digested and secreted in the upper gastrointestinal tract *in vivo* in women with ileostomies. Journal of Nutrition, 132: 2597–2600.

Troost, F. J., Steijns, J., Saris, W. H. M. & Brummer, R.-J. M. (2001). Gastric digestion of bovine lactoferrin *in vivo* in adults. Journal of Nutrition, 131(8): 2101-2104.

Ueno, H. M., Ueda, N., Morita, M., Kakehi, Y. & Kobayashi, T. (2012). Thermal stability of the iron–lactoferrin complex in aqueous solution is improved by soluble soybean polysaccharide. Food Biophysics, 7 (3): 183-189.

Wakabayashi, H., Yamauchi, K., & Takase, M. (2006). Lactoferrin research, technology and applications. International Dairy Journal, 16: 1241-1251.

Wei, Z., Nishimura, T. & Yoshida, Y. (2001). Characterization of glycans in a lactoferrin isoform, lactoferrin-a. J. Dairy Sci., 84: 2584–2590.

Xavier, P. L., Chaudhari, K., Verma, P. K., Palc, S. K. & Pradeep, T. (2010). Luminescent quantum clusters of gold in transferrin family protein, lactoferrin exhibiting FRET. Nanoscale, 2: 2769–2776.

Yamano, E., Miyauchi, M., Furusyo, H., Kawazoe, A., Ishikado, A., Makino, T., Tanne, K., Tanaka, E. & Takata, T. (2010). Inhibitory effects of orally administrated liposomal bovine lactoferrin on the LPS-induced osteoclastogenesis. Lab. Invest., 90: 1236–1246.

Yamauchi, K., Tomita, M., Giehl, T. & Ellison, R. (1993). Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment. Infect. Immun., 61: 719-728.

Yao, X., Bunt, C., Cornish, J., Quek, S. Y. & Wen, J. (2013). Improved RP-HPLC method for determination of bovine lactoferrin and its proteolytic degradation in simulated gastrointestinal fluids. Biomed Chromatogr., 27: 197-202.

Yao, X., Bunt, C., Cornish, J., Quek, S. Y. & Wen, J. (2014). Stability of bovine lactoferrin in luminal extracts and mucosal homogenates from rat intestine: a prelude to oral absorption. Chem Biol Drug Des., 1-9.

Yen, C. C., Shen, C. J., Hsu, W. H., Chang, Y. H., Lin, H. T., Chen, H. L. & Chen, C. M. (2011). Lactoferrin: an iron-binding antimicrobial protein against *Escherichia coli* infection. Biometals, 24: 585-594.

Zimecki, M., Artym, J. & Kocieba, M. (2009) Endogenous steroids are responsible for lactoferrin-induced myelopoiesis in mice. Pharmacol. Rep., 61: 705-710.

Zimecki, M., Artym, J., Chodaczek, G., Kocieba, M., Kuryszko, J., Houszka, M. & Kruzel M. L. (2007) Immunoregulatory function of lactoferrin in immunosuppressed and autoimmune animals. Postepy. Hig. Med. Dosw., 61: 283-287.

Chapter 6

Conclusions and future work/Conclusiones y trabajo del futuro

1. Beside heme and non heme specific transporters for the absorption of iron in duodenal cells, it has been proved that rhLf has a specific site for the absorption of ferrous and ferric forms of iron. The obtained results of the study carried out using Caco-2 cell model confirmed the importance of the supplementation of infant formulas with rhLf and GOS where it led to increase ferritin formed by the cell cultures. The results confirmed that solubility is not the only factor correlated with improvement of iron bioavailability, therefore it is useful investigate the role of some genes related with iron bioavailability such as DMT1.

   1. Además de los transportadores específicos de hierro hemo y no-hemo para la absorción de hierro en las células duodenales, se ha demostrado que rhLf tiene un sitio específico para la absorción de formas ferrosos y férricos de hierro. Los resultados obtenidos del estudio llevado a cabo con células Caco-2 modelo confirmó la importancia de la suplementación de las fórmulas infantiles con rhLf y GOS que llevó a aumentar ferritina formada por los cultivos celulares. Los resultados confirmaron que la solubilidad no es el único factor correlacionado con la mejora de la biodisponibilidad del hierro, por lo tanto, es útil investigar el papel de algunos genes relacionados con la biodisponibilidad del hierro como DMT1.

2. rhLf and rhLf hydrolysate exert their anti-inflammatory activity through its ability to bind LPS and subsequently modulate cytokine production. The mechanism of Lf's action is also largely dependent on its ability to influence early responses, including the modulation of intracellular ROS production. Between rhLf and rhLf hydrolysate, rhLf hydrolysate is more effective and is considered a prominent therapeutic agent, which must to be taken into account during infant formulas design.

2. rhLf y su hidrolizado ejercen su actividad anti- inflamatoria a través de su capacidad para unirse a LPS y posteriormente modular la producción de citoquinas. El mecanismo de acción de Lf también depende en gran medida de su capacidad para influir en las respuestas tempranas, incluyendo la modulación de la producción de ROS intracelular. La actividad anti-inflamatoria de rhLf y del hidrolizado de rhLf, esta última se ha mostrado más eficaz y es considerado como un destacado agente terapéutico que se debe tener en cuenta durante el diseño de las fórmulas infantiles.

3. The results of study carried out using batch culture fermentation system proposed that rhLf and/or GOS influenced the fermentation end products such as SCFAs. This fermentation process lead to a decreasing of the pathogenic bacteria and complementary an increase of the bifidogenic ones.

3. Los resultados del estudio llevado a cabo con cultivos por lotes utilizando como sistema de fermentación ha mostrado que tanto la rhLf y/o GOS influyen en la obtención de productos finales de fermentación como los ácidos grasos de cadena corta, Este proceso fermentativo además lleva a la disminución de bacterias patógenas y de forma complementaria el incremento de las bifidogénicas.

4. The obtained results confirmed that rhLf is more stable than human Lf and thus it is considered a prominent factor for infant formulas supplementation. Although many studies are needed to discover this characteristic, the results proposed a role for GOS in protect rhLf against the digestive enzymes. Likewise, the results confirmed the importance of the breastfeeding pattern concerning with the released free fatty acids by digestion.

4. Los resultados obtenidos confirmaron que rhLf es más estable que Lf humana, por lo que se considera un factor importante para la suplementación de fórmulas infantiles. Aunque se necesitan estudios complementarios para descubrir y detectar esta característica, los

resultados propuestos un papel de GOS a proteger rhLf contra las enzimas digestivas. Del mismo modo, los resultados obtenidos confirman la importancia de la lactancia materna relacionada a los ácidos grasos liberados por la digestión.

5. Overall, the obtained findings confirm and support the urgently need to supplementation with rhLf and/or GOS of infant formulas. The results demonstrated the importance of the rhLf hydrolysate as a new additive and may be in the near future new formulas contain the hydrolysate of rhLf will be seen in the markets.

5. En conjunto, los resultados obtenidos confirman y apoyan la urgente necesidad de suplementación con rhLf y/o GOS de las fórmulas infantiles. Los resultados demostraron la importancia del hidrolizado de rhLf como un nuevo aditivo y puede ser en un futuro próximo nuevas fórmulas contienen el hidrolizado de rhLf será visto en los mercados.