UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Anti-Regurgitation Infant Formulas: *in-vitro* Study About the Effect of Different Thickening Ingredients on their Physicochemical Characteristics Across a Digestive Process, Mineral Availability and Infant Microbiota

Formulas Infantiles Antirregurgitación: Estudio *in-vitro* del Efecto de Diferentes Ingredientes Espesantes sobre sus Características Fisicoquímicas a lo Largo de un Proceso Digestivo, Disponibilidad Mineral y Microbiota del Lactante

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2017

ANTI-REGURGITATION INFANT FORMULAS: IN-VITRO STUDY ABOUT THE EFFECT OF DIFFERENT THICKENING INGREDIENTS ON THEIR PHYSICOCHEMICAL CHARACTERISTICS ACROSS A DIGESTIVE PROCESS, MINERAL AVAILABILITY AND INFANT MICROBIOTA

FORMULAS INFANTILES ANTIRREGURGITACIÓN: ESTUDIO IN-VITRO DEL EFECTO DE DIFERENTES INGREDIENTES ESPESANTES SOBRE SUS CARACTERÍSTICAS FISICOQUÍMICAS A LO LARGO DE UN PROCESO DIGESTIVO, DISPONIBILIDAD MINERAL Y MICROBIOTA DEL LACTANTE.

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2017

ABREVIATIONS

AAS → Atomic Absorption Spectrometry

AF4 → Asymmetrical Flow Field-Flow Fractionation

AR- → Anti-Regurgitation

CNRQ → Calibrated and Normalized Relative Quantification

DMT-1 → Divalent Metal Transporter-1

dRI → Differential refractive index

ESPGHAN → European Society for Pediatric Gastroenterology, Hepatology, and Nutrition

FGID → Functional Gastrointestinal Disorders

FOS → Fructo-Oligosaccharides

FTL → Ferritin Light Chain

GC → Gas Cromatography

GER → Gastroesophageal Reflux

GERD → Gastroesophageal Reflux Disease

GOS → Galacto-Oligosaccharides

gRS → Pregelatinized rice starch.

ICP-OES → Inductively Coupled Plasma Optical Emission Spectrometry

LBG → Locust Bean Gum

LOS → Lower Oesophageal Sphincter

MALS → Multiangle light scattering

MBM → Minimum Basal Medium

Mhdp → Maize hydroxypropylated distarch phosphate

MT1M → Metallothionein-1M

ZNT-1 → Zinc Transporter 1

NASPGHAN → North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition

PCA → Principal Components Analysis

qPCR → Real Time Polimerase Chain Reaction

SCFAs → Short Chain Fatty Acids

TRLES → Transient Relaxationt of Lower Esophageal Sphincter

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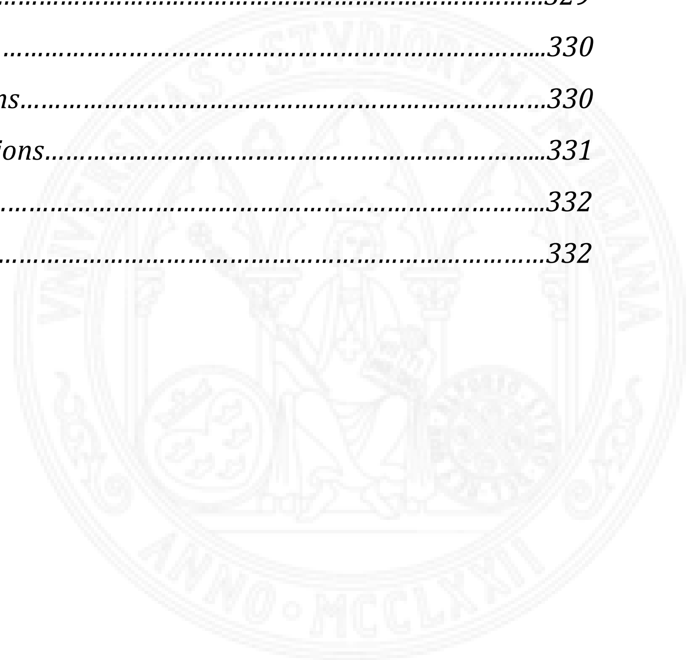


SUMMARY

Gastroesophageal reflux in infants, accompanied or not by regurgitation, is a frequently described process, associated with a lack of maturity in the physiological antireflux barrier. In the specific case of newborns, gastroesophageal refluxes/regurgitation are frequently associated with transient lower oesophageal sphincter relaxation during food intake periods. Their origin seems to be related to a vagal reflex promoted by the activation of distension receptors, located in the proximities of cardiac sphincter and gastric fundus. During transient lower oesophageal sphincter relaxation episodes, the gastric contents would be allowed to return to the distal esophagus, and even to or out of the mouth (regurgitation). As neonate grows and the gastrointestinal system develops, the frequency of these episodes varies from a prevalence of 40-65% before the first 4 months to a prevalence of 4-5% after the first year of life. Unless these episodes are acompanied by issue damage and/or failure to thrieve (gastroesophageal reflux disease), infant gastroesophageal reflux should be considered as a physiological process that accompanies the development of gastrointestinal tract.

Despite being considered as a physiological process, gastroesophageal reflux, especially when it is accompanied by regurgitation, is a cause of concern for parents, representing the second cause of pediatric consultation, after infant colic, within mild gastrointestinal disorders. In this regard, the North American and European Association of Pediatrics, Gastroenterology, Hepatology and Nutrition (NASPGHAN and ESPGHAN) published in 2009 a clinical practice guideline for the management of pediatric gastrointestinal disorders. For the specific case of non-pathological gastrointestinal

reflux, this guidelines mainly focus on postural and nutritional therapies, through different advices for parental education and modification of infant's feeding habits. With regard to frequent reflux/regurgitation episodes, this guideline proposes the use of "anti-reflux formulas or AR-formulas".

AR-formulas are specially formulated to increase gastric retention time and prevent the return of the content to the esophagus. Among these ingredients, AR-formulas include thickening agents, such as modified maize or rice starches, as well as vegetable gums, including locust bean gum or guar gum. Although their use seem to be associated with a reduction in the reflux/regurgitation episodes, controversy exists arround the possible side effects that these ingredients could have in the physiological function and development of infant digestive system. At these respect, some authors have proposed that, thickening ingredients, could negatively affect mineral bioavailability. Likewise, the addition of vegetable starches or gums would be a source of fermentiscible substrates, whose impact on the infant microbiota development is still unknown.

On the basis of this background, this dissertation delves deep into the *in-vitro* study of the possible effect of modified maize and rice starches, as well as carob flour, on mineral availability (calcium, iron and zinc) and the development of the intestinal microbiota in infants younger than 4 months of age. With this aim, three thickening ingredients were selected: Locust Bean Gum (LBG), Hydroxypropylated Maize Distarch Phosphate (Mhdp), and pre-Gelatinized Rice Starch (gRS), toghether with a standard infant formula (Hero Baby® 1). All ingredients were donated by Hero Spain S.A. (Alcantarilla, Murcia, Spain). This research has been divided into three chapters:

In the first chapter, the physicochemical characteristics of thickeners (LBG, Mhdp and gRS), were analyzed during an *in-vitro* gastrointestinal digestion process, after previous resuspension in MilliQ® water. *In-vitro* gastrointestinal digestión consisted of two stages, a gastric digestion phase (pH = 4, pepsin solution) followed by an intestinal digestion phase (pH = 5, bile salts solution and pancreatic extract). The digestive process was carried out in a previously tempered at 37 ºC water shaking bath. The oral digestión phase was omitted since, in infants, this phase is practically non-existent, being the food rapidly transported into the oesophagus and the stomach. The gastrointestinal enzymes concentrations used, as well as the pH levels, were adjusted to the conditions described for infants, in which the pH-buffering capacity and the enzymatic production are limited. After the ingredient resuspension, as well as after the gastric phase and intestinal phase of the digestive process, the following parameters were determined: apparent amount of solubilized ingredient (by dehydration and gravimetry), relative dynamic viscosity, properties (Asymetric Flow Field Flow Fractionation, AF4). This phase of the research was carried out through a 6-months scientific mission, at the Department of Food Technology, Engineering and Nutrition, Faculty of Chemistry, University of Lund (Sweden), under the supervision of the Dr. Professor Lars Nilsson.

In the second chapter of this doctoral thesis, the effect of the addition of different concentrations of LBG, Mhdp and gRS to a standard infant formula (Hero Baby® 1), on the availability of Ca, Fe and Zn, was analyzed after a Process of in-vitro digestion. The concentrations of thickeners used were established according to the current legislation that regulates the addition of these thickeners to infant foods. For this purpose, three types of experiments were carried out:

- Solubility and dialysis of Ca, Fe and Zn after an *in-vitro* digestion process. Basically, the previously described *in-vitro* digestion process was followed. The soluble fraction was determined by centrifugation of the intestinal content, while the dialyzable fraction was determined by the introduction of a dialysis membrane during the intestinal digestion phase. The mineral content (Ca, Fe and Zn) in each of the fractions, were determined by Atomic Absorption Spectrophotometry (AAS). In order to determine the possible presence of antinutrients associated with plant ingredients, a quantification of phosphate inositols and derivatives was carried out.

- Capture and transport of Ca, Fe and Zn by a Caco-2 cells culture. For this experiment, previously differentiated cultures of Caco-2 were exposed to the soluble intestinal fractions obtained after the in-vitro digestion of the different ingredients (LBG, Mhdp and gRS). As control, $FeSO_4$, $ZnSO_4$ and $CaCl_2$ solutions were evaluated. Assays were performed through a bicameral culture system, which consisted in two chambers, one basal and one apical, both separated by a semipermeable membrane on which the cells were grown. Treatments were added in the apical chamber. After an exposure period of 2h, the contents of the apical and basolateral chamber, as well as the the cell monolayer, were separately collected. The mineral content in each of the fractions was quantified by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-OES). From these results, transport and uptake efficiency of the system was calculated.

- Mineral transporters encoding gene expression en Caco-2 cells. From the previous experiment, but after a 6 hour exposure to the different treatments, cell monolayers were collected and lysed. The extracted mRNA was subsequently reverse transcribed into DNA and transporter expression quantified by qPCR. The mineral transporters analyzed were:

Calbindin D-9k, Divalent Mineral Conveyor-1 (DMT-1), Ferritin (heavy chain and light chain; F), Metallothionein-1M (MT1M), Zip-4 and ZnT1 zinc transporters. For this purpose, a calibrated and normalized quantification of the coding genes expression for each transporter was performed. Normalization was calculated with respect to the basal expression of different reference genes (B2M, SDH-A, YWHAZ, HPRT1). In addition, after the treatments, a quantification of the ferritin cellular content was carried out by ELISA immunoassay. This part of the study was developed through a three-months scientific mission at the Environment and Agro-biotechnologies (EVA) Department, Gabriel Lippman Public Research Center (Luxembourg) under the supervision of Dr. Danièle Evers.

The last chapter of the present work deals with the study of the effect of the addition of LBG, Mhdp and gRS to a basal culture medium, on the fecal microbiota of 3 healthy and mixed feeding infants younger than 4 months of age. For this purpose, a static and non-pH controlled batch experiment was carried out. The evolution of different microbial groups presented in the fecal microbiota of the infant (Atopobium, Bacteroides-Prevotella, Enterobacteriaceae, Enterococaceae, Lactobacillus, Bifidobacterium, Clostridia IV and Clostridia XIVa) were cuantified at 5, 8, 10, 24 and 48 h of 37 ºC incubation. This determination was carried out by microbial DNA extraction and qPCR quantification. As indicators of microbial fermentation activity, the determination of acetate, propionate and butyrate by gas chromatography, total gas production, as well as the evolution of pH during fermentation, were carried out. The results obtained for LBG, Mhdp and gRS were compared with those obtained for D-glucose (negative prebiotic control) and inulin (positive prebiotic control).

In relation to the results obtained, LBG was the ingredient that presented a higher relative dynamic viscosity throughout the digestive process, being also resistant to enzymatic digestion. According to this results, LBG would be a more efficient thickener, resisting the disgestion process. In this respect, LBG would reach the distal part of gastrointestinal tract without modifications, acting as a posible fermentiscible substract for colonic microbiota. On the contrary, despite starches (Mhdp and gRS) thickening capacity was lower than the one described for LBG, it was limmited to gastric stage, being the stage in wich gastroesophageal reflux episodes occurs. During the digestión process, modified starches were completely degraded to glucose and oligosaccharides, acting as a possible extra caloric source.

In relation to the effect of thickeners on mineral availability, the addition of LBG negatively affected the *in-vitro* Ca, Fe and Zn availability, decreasing both, mineral solubility and dialyzability. In contrast, modified starches (Mhdp and gRS) only affected Ca solubility and dialyzability. The inositol phosphate content was higher in LBG, compared to Mhdp and gRS. However, its presence was not related to the previously described negative effect on mineral solubility and dialyzability, since the critical relationship between inositol phosphate and mineral concentration described by other authors was not reached. The effect of LBG, Mhdp and gRS on solubility and mineral dialyzability was extensible to the retention capacity of each mineral by the Caco-2 monolayer. However, there was no relation between *in-vitro* solubility/dialyzability and transport/ uptake of minerals by Caco-2 cells. In this regard it was assumed the intervention of other factors such as interactions with food matrix components, as well as the integrity of the cellular monolayer. Concerning the Ca, Fe and Zn transporters gene encoding expression in Caco-2 cells, only the metallothionein-1M (MT1M) and Zn

transporter ZnT-1 encoding genes were specifically stimulated by the exposure to Zn sources (control of zinc sulfate and soluble intestinal fractions of the formulas with the different added thickeners). However, no differences were found between the different treatments. For the rest of the mineral transporters, their expression was constant and independent of the type of treatment, either controls or formulas with added thickening ingredients. These results were attributed to the fact that exposure time (6 h) was insuficient to modify the gene expression. Under the fixed experimental conditions, a higher exposure time was discarded in order to prevent cells damage. In the case of ferritin encoding genes, they showed an unvariable expression, being compatible with a post-transcriptional regulation. In this regard the quantification of ferritin by immunoassay-ELISA techniques was a better indicator of Fe availability. In fact , Ferritin concentration was strongly increased after the exposure to $FeSO_4$ control, but no differences were described for the rest of the treatments.

Regarding to the effect of the different thickeners on the infant fecal microbiota, LBG showed a similar behavior to that described by Inulin. In this regard, both ingredients showed a slow fermentability, causing a retarded pH drop and a moderate total gas production. In relation to the fatty acid profile, in spite of the fact that acetate was the predominant short chain fatty acid, as the fermentation process progressed, the propionate content was increased, decreasing the acetate: propionate ratio. In contrast to LBG and Inulin, Mhdp and gRS, were rapidly fermented by fecal microbiota, with a similar behavior to that described for D-glucose. In this regard, Mhdp, gRS and D-glucose caused an early and marked decrease in pH after 5h of incubation, as well as a high total gas production. In relation to the production of short chain fatty acids, the fermentation of Mhdp, gRS and D-glucose resulted in a large increase in acetate production, while

propionate production remained constant during the whole fermenting assay. When the evolution of the different bacterial groups throughout the trial was analyzed, only a clear trend was observed in the development of Bacteroides and Atopobium. In fact, when LBG and inulin were added, both groups experienced a time dependent growth. However, this effect was not observed after the addition of Mhdp, gRS or D-glucose, which resulted in a low or undetectable growth for both bacterial groups. Thus, the addition of LBG as thickening ingredient, could result in the development of a more varied microbiota than that obserbed after the addition of Mhdp or gRS.

Based on the previously exposed results and considering the experimental conditions of the studies carried out, it can be concluded that the adition of LBG as thickning agent results in a greater viscosity than the one provided by modified starches (Mhdp and gRS), being resistant to the in-vitro digestive process. In this regard, meanwhile LBG decreases Ca, Fe and Zn *in-vitro* solubility and dialisability, Mhdp and gRS only affect Ca availability. These effects are extended to Caco-2 cell mineral retention, but not to the uptake or transport efficiency, with no effect on celular mineral transporters molecules. Attending to its fermentability, LBG can be defined as a "*slow fermented ingredient*", resulting in a moderate drop of pH, a moderate total gas production, as well as a decreasing in the acetate:propionate ratio. These properties are related to the development of a varied fecal microbiota, increasing *Atopobium* and *Bacteroides* populations. On the contrary, modified starches, Mhdp and gRS are classified as "*rapidly fermented ingredients*", resulting in a high total gas production, a sudden drop of pH and a majority production of Acetate. These properties promote the development of a less varied fecal microbiota, with absence or minimal development of *Atopobium* and *Bacteroides* groups.

RESUMEN

El reflujo gastroesofágico en lactantes, acompañado o no de regurgitación, es un proceso frecuentemente descrito, asociado a una falta de madurez en los mecanismos fisiológicos que integran la barrera antirreflujo. En el caso concreto de recién nacidos, estos reflujos se asocian, en la mayoría de casos, con una relajación transitoria del esfínter esofágico inferior, la cual acompaña a la ingesta de alimentos. El origen de estos episodios de relajación transitoria, parece estar relacionado con un reflejo vagal promovido por la activación de receptores de distensión localizados en las proximidades del cardias y fundus gástrico. Durante los mismos, se permitiría el retorno del contenido gástrico al esófago distal, e incluso, hacia la boca o fuera de ella, lo cual se define como regurgitación. Esto se ve favorecido por una posición en decúbito supino predominante. Conforme el neonato avanza en el desarrollo y en la maduración del sistema gastrointestinal, la frecuencia de estos episodios remite, pasando de una prevalencia del 40 – 65% antes de los primeros 4 meses, a una prevalencia del 4 – 5% tras el primer año de vida. Es por ello que, siempre que estos episodios no se acompañen de daño tisular o alteraciones en el desarrollo (enfermedad por reflujo gastroesofágico), el reflujo gastroesofágico infantil se debe considerar como un proceso fisiológico que acompaña al desarrollo del tracto gastrointestinal del lactante.

Pese a ser un proceso considerado fisiológico, el reflujo gastroesofágico, sobre todo cuando se acompaña de regurgitaciones, supone un motivo de preocupación para los padres, representando la segunda causa de consulta pediátrica, después del cólico del lactante, dentro de los denominados trastornos gastrointestinales no severos. A este respecto, la Asociación Norte Americana y Europea de Pediatría, Gastroenterología,

Hepatología y Nutrición (NASPGHAN y ESPGHAN), publicaron en el año 2009 una guía de práctica clínica para el abordaje de trastornos gastrointestinales pediátricos. Para el caso concreto del reflujo gastrointestinal no patológico, la guía establece unas pautas de educación parental y una modificación en los hábitos de alimentación del lactante, centrados sobre todo en terapias posturales durante la alimentación. Para casos de reflujos/regurgitaciones frecuentes, estas guían proponen el empleo de las denominadas "fórmulas anti-reflujo o fórmulas-AR".

Las fórmulas-AR están especialmente formuladas para aumentar el tiempo de retención gástrico y evitar el retorno del contenido al esófago. Entre sus ingredientes, estas fórmulas incluyen agentes espesantes, tales como los almidones modificados de maíz o arroz, así como gomas vegetales, entre las que destacan la harina de semilla de algarrobo o la goma de guar. Pese a que su empleo parece ir asociado a una reducción en los episodios de reflujo/regurgitación, existe controversia en relación a los posibles efectos que dichas fórmulas podrían tener en el normal funcionamiento del sistema digestivo del lactante. Así, algunos autores han propuesto que, estos ingredientes, podrían repercutir negativamente sobre la biodisponibilidad mineral. Del mismo modo, la adición de almidones o gomas vegetales, supondría una fuente de sustratos fermentiscibles, cuyo impacto sobre el desarrollo de la microbiota del lactante se desconoce.

En base a estos antecedentes, la presente tesis doctoral ahonda en el estudio in-vitro de los posibles efectos que, el empleo de almidones modificados de maíz y arroz, así como de harina de algarrobo, podrían tener sobre la disponibilidad mineral (calcio, hierro y zinc) y el desarrollo de la microbiota intestinal en el lactante de menos de 4

meses de edad. Con este objetivo principal, se seleccionaron tres ingredientes espesantes: Harina de algarrobo (LBG), fosfato dialmidón hidroxipropilado de maíz (Mhdp) y almidón pregelatinizado de arroz (gRS) y una formula infantil estándar (Hero Baby® 1). Todos los ingredientes fueron cedidos por la empresa Hero España S.A. (Alcantarilla, Murcia, Spain). El presente trabajo de investigación se ha dividido en tres capítulos:

En el primer capítulo, se analizaron las características fisicoquímicas de los tres espesantes (LBG, Mhdp y gRS), previamente resuspendidos en agua MilliQ®, durante un proceso de digestión gastrointestinal *in-vitro*, el cual consistió en una fase de digestión gástrica (pH=4; solución de pepsina) seguido de una fase de digestión intestinal (pH=5; solución de sales biliares y extracto pancreático). El proceso digestivo fue llevado a cabo en un baño de agitación previamente atemperado a 37 ºC. La fase de digestión bucal fue omitida ya que en lactantes esta fase es prácticamente inexistente, pasando el alimento rápidamente al esófago y estómago. Las concentraciones de enzimas gastrointestinales empleadas, así como los valores de pH fueron ajustados a las condiciones descritas para lactantes. En ellos, la capacidad amortiguadora de pH y la producción enzimática es limitada. Tras la resuspensión del ingrediente, así como tras la fase gástrica y la fase intestinal del proceso digestivo, se determinaron los siguientes parámetros para cada uno de los espesantes seleccionados: cantidad aparente de ingrediente solubilizada (mediante deshidratación y gravimetría), viscosidad dinámica relativa, propiedades moleculares (Fraccionamiento en Flujo mediante Campo de Flujo Asimétrico, AF4). Esta fase de la investigación fue llevada a cabo mediante una estancia de investigación de 6 meses de duración, en el Departamento de Tecnología de los Alimentos, Ingeniería y

Nutrición, de la Facultad de Química, Universidad de Lund (Suecia), bajo la supervisión del Dr. Profesor Lars Nilsson.

En el segundo capítulo de la presente tesis doctoral, se analizó el efecto de la adición de diferentes concentraciones de LBG, Mhdp y gRS a una formula infantil estándar (Hero Baby® 1), sobre la disponibilidad de Ca, Fe y Zn, tras un proceso de digestión *in-vitro*. Las concentraciones de espesantes empleadas fueron establecidas de acuerdo a la legislación vigente que regula la adición de estos espesantes a alimentos infantiles. Con este objetivo se llevaron a cabo tres tipos de experimentos:

- Solubilidad y diálisis de Ca, Fe y Zn tras un proceso de digestión in-vitro: Básicamente se siguió el proceso de digestión descrito para el apartado anterior. La fracción soluble se determinó mediante centrifugación del contenido intestinal, mientras que la fracción dializable se determinó mediante la introducción de una membrana de diálisis durante la fase de digestión intestinal. El contenido mineral en cada una de las fracciones se determinó mediante espectrofotometría de absorción atómica. Con la finalidad de determinar la posible presencia de antinutrientes asociados a ingredientes vegetales, una cuantificación de inositoles fosfato y derivados fue conjuntamente llevada a cabo.

- Captación y transporte de Ca, Fe y Zn por un cultivo de Caco-2. Para este experimento, cultivos previamente diferenciados de Caco-2, fueron expuestos a las fracciones solubles intestinales de los distintos ingredientes, así como a soluciones control para los distintos minerales evaluados ($FeSO_4$, $ZnSO_4$ y $CaCl_2$). Para este experimento se empleó un sistema de cultivo bicameral consistente en dos cámaras, una basal y una apical, ambas separadas por una membrana semipermeable sobre la que se cultivan las células. Los tratamientos fueron añadidos en la cámara apical. Tras un periodo de exposición de 2h, el contenido de la cámara

apical, la monocapa celular y el contenido de la cámara basal fueron recogidos por separado. El contenido mineral en cada una de las fracciones fue cuantificado mediante espectrometría de emisión atómica con plasma acoplado inductivamente (ICP-OES). A partir de estos resultados se calculó la eficiencia de transporte y la eficiencia de captación del sistema.

- Expresión génica de transportadores minerales en Caco-2. A partir del experimento anterior pero tras una exposición de 6 horas a los distintos tratamientos, las monocapas celulares fueron lisadas y el mRNA extraído y posteriormente transcrito de forma reversa a DNA. Los transportadores analizados fueron: Calbindina D-9k, Transportador Mineral Divalente-1 (DMT-1), Ferritina (cadena pesada y cadena ligera; F), Metalotioneina-1M (MT1M) y transportadores de zinc Zip-4 y ZnT1. Para ello se llevó a cabo una cuantificación calibrada y normalizada de la expresión de los genes codificantes para cada transportador, respecto a la expresión basal de diferentes genes de referencia (B2M, SDH-A, YWHAZ, HPRT1). Junto a ello, tras los tratamientos, se llevó a cabo una cuantificación del contenido celular en ferritina mediante inmunoensayo ELISA. Esta parte del estudio fue llevado a cabo mediante una estancia de investigación de tres meses, en el Departamento de Medio Ambiente y Agrobiotecnología (EVA) del Centro de Investigaciones Públicas Gabriel Lippman (Luxemburgo), bajo la supervisión de la Dra. Danièle Evers.

El último capítulo del presente trabajo, aborda el estudio del efecto de la adición de LBG, Mhdp y gRS a un medio de cultivo basal, sobre la microbiota fecal de 3 lactantes menores de 4 meses, sanos y con alimentación mixta. Para ello, un ensayo de fermentación estático y con pH no controlado, se llevó a cabo. Durante el mismo, la evolución de diferentes grupos microbianos presentes en la microbiota fecal del lactante (*Atopobium, Bacteroides-Prevotella, Enterobacteriaceae, Enterococaceae, Lactobacillus,*

Bifidobacterium, Clostridia IV and Clostridia XIVa) fueron determinados a las 5, 8, 10, 24 and 48h de incubación (37ºC). Esta determinación se llevó a cabo mediante la extracción de DNA microbioano y cuantificación por qPCR. De forma paralela, la determinación de acetato, propionato y butirato mediante cromatografía de gases, así como la evolución del pH o la producción total de gas, durante la fermentación, fueron determinados como indicadores de la fermentación microbiana. Los resultados obtenidos para LBG, Mhdp y gRS fueron comparados con los obtenidos para D-glucosa (control prebiótico negativo) e inulina (control prebiótico positivo).

En relación a los resultados obtenidos, LBG fue el ingrediente que presentó una mayor viscosidad dinámica relativa en todo el proceso digestivo, siendo también resistente a la digestión enzimática. Según estos resultados, LBG sería un espesante más eficiente, resistiendo el proceso de degradación enzimática. En este sentido, este ingrediente alcanzaría la parte distal del tracto gastrointestinal sin modificaciones, actuando como posible substrato fermentiscible para la microbiota colónica. Por el contrario, a pesar de que la capacidad espesante de los almidones (Mhdp y gRS) fue inferior a la descrita para LBG, esta se limitó a la etapa gástrica, siendo la etapa en la que se producen episodios de reflujo gastroesofágico. Durante el proceso de digestión, los almidones modificados se degradaron completamente a glucosa y oligosacáridos, actuando como una posible fuente extra de calorías.

En relación al efecto de los espesantes sobre la disponibilidad mineral, la adición de LBG afectó negativamente la disponibilidad *in-vitro* de Ca, Fe y Zn, disminuyendo tanto la solubilidad mineral, como la diálizabilidad. Por el contrario, los almidones modificados (Mhdp y gRS) solo afectaron a la solubilidad y dializabilidad del Ca. Pese a

que el contenido en inositoles fosfato fue mayor en LBG en comparación con Mhdp y gRS, su presencia no se relaciono con el efecto negativo sobre la solubilidad y diálizabilidad mineral, ya que no se alcanzó la relación crítica entre el contendo en inositol fostato y la concentración mineral descrita por otros autores. El efecto de LBG, Mhdp y gRS sobre la solubilidad y diálizabilidad mineral fue extensible a la capacidad de retención de cada mineral por la monocapa de Caco-2. Sin embargo, no se observó relación entre la solubilidad y la dializabilidad *in-vitro* y el transporte/captación de minerales por las células Caco-2, asumiendo la intervención de otros factores tales como las interacciones con los componentes de la matriz alimentaria, así como la integridad de la monocapa celular. Con respecto a la expresión génica de transportadores relacionados con la captación y transporte de Ca, Fe y Zn en células caco-2, sólo los genes que codifican para la metalotioneína-1M (MT1M) y el transportador de Zn ZnT-1, fueron específicamente estimulados por la exposición a fuentes de Zn (control de sulfato de zinc y fracciones solubles intestinales de las formulas con los distintos espesantes añadidos). Sin embargo, no se encontraron diferencias entre los distintos tratamientos. Para el resto de transportadores de minerales, su expresión fue constante e independiente del tipo de tratamiento, ya sean controles o fórmulas con ingredientes espesantes añadidos, achacando este echo a un tiempo de exposición (6 h) insuficiente para modificar la expresión génica. Bajo las condiciones experimentales planteadas en este capítulo, un tiempo mayor de exposición fue descartado para prevenir la alteración del cultivo celular. En el caso de los genes que codifican la ferritina, estos mostraron una expresión constante y compatible con una regulación postranscripcional, por lo que la cuantificación de ferritina por técnicas de inmunoensayo-ELISA fue un mejor indicador de la disponibilidad de Fe. De hecho, la concentración de Ferritina se vio fuertemente

incrementada tras la exposición al control de sulfato de hierro, pero no se encontraron diferencias respecto al resto de tratamientos.

En relación al efecto de los diferentes espesantes sobre la microbiota fecal del lactante, LBG mostró un comportamiento similar al de la Inulina. Así, ambos ingredientes mostraron una lenta fermentabilidad, provocando una caída de pH tardía y una producción de gas moderada. En relación al perfil de ácidos grasos, pese a que el Acetato fue el acido graso de cadena corta mayoritario, conforme avanzó el proceso fermentativo, el contenido en propionato fue en aumento, disminuyendo el ratio acetato:propionato. Por el contrario, Mhdp y gRS, fueron rápidamente fermentados por la microbiota fecal, con un comportamiento similar al de la D-glucosa. Así, Mhdp, gRS and D-glucosa porvocaron un acusado descenso en el pH y una elevada producción total de gas. En relación a la producción de ácidos grasos de cadena corta, la fermentación de Mhdp, gRS y D-glucosa resultó en un gran incremento en la producción de acetato, mientras que la producción de propionato se mantuvo constante. Cuando la evolución de los diferentes grupos bacterianos a lo largo del ensayo fue analizada, solo se observó una tendencia clara en el desarrollo de *Bacteroides* and *Atopobium*. De hecho, cuando LBG e inulina fueron añadidos, ambos grupos experimentaron un crecimiento dependiente del tiempo de fermentación. Este efecto no se observó tras la adición de Mhdp, gRS o D-glucosa, los cuales resultaron en una ausencia o un mínimo crecimiento de ambos grupos bacterianos. Así, la adición de LBG como ingrediente espesante, podría resultar en el desarrollo de una microbiota más variada que la observada tras la adición de Mhdp o gRS.

En base a los resultados previamente expuestos, y considerando las condiciones experimentales de los estudios realizados, se puede concluir que la adición de LBG como agente espesante resulta en una viscosidad mayor que la proporcionada por los almidones modificados (Mhdp y gRS), siendo resistente al proceso digestivo *in-vitro*. En este sentido, mientras que LBG disminuye la solubilidad y dialisability de Ca, Fe y Zn, Mhdp y gRS sólo afectan a la disponibilidad de Ca. Estos efectos son extensibles a la retención mineral por cultivos de células Caco-2, pero no a la absorción o eficiencia de su transporte, sin efecto sobre las moléculas de los transportadores de minerales celulares. Atendiendo a su fermentabilidad, LBG puede definirse como un "*ingrediente de fermentación lenta*", dando como resultado una moderada producción de gas, una caída moderada del pH, así como una relación decreciente de acetato: propionato. Estas propiedades están relacionadas con el desarrollo de una microbiota fecal variada, aumentando las poblaciones de *Atopobium* y *Bacteroides*. Por el contrario, los almidones modificados, Mhdp y gRS se clasifican como "*ingredientes de fermentación rápida*", dando como resultado una gran producción de gas, una caída repentina del pH y una producción mayoritaria de acetato. Estas propiedades promueven el desarrollo de una microbiota fecal menos variada, con ausencia o desarrollo mínimo de los grupos *Atopobium* y *Bacteroides*.

Gastroesophageal Reflux and Regurgitation in infants.
GENERAL INTRODUCTION

1. DEFINITIONS AND ETIOLOGY

Gastroesophageal reflux (GER) is being defined as the passage of gastric content into the oesophagus. When the refluxed content goes into the farinx, the mouth or out of the mouth, is considered as regurgitation (Vandenplas *et al.*, 2011; Lightdale *et al.*, 2013).

The etiology of GER, could be understood as an imbalance in the interaction between antireflux physiological mechanisms and different predisposing factors (Badriul & Vandenplas, 1999; Price 2007; Martín-de Capri 2009; Loots *et al.*, 2012). The Lower Oesophageal Sphincter (LOS), which maintains a basal pressure <5 mmHg, mainly integrates the antireflux physiological mechanisms. This pressure is normally enough to avoid the reflux of gastric content to the oesophagus in infants. However, LOS cannot be considered as an anatomical sphincter, as it is formed by the confluence of muscle fibres from the oesophagus and the stomach. In this regard, to act as an effective sphincter, LOS needs the cooperation of oesophageal peristalsis, diaphragm muscles and phrenoesophageal ligament, which reinforce the gastro-oesophageal junction. Together with the lack of maturity of the previously described components, a delayed gastric emptying and/or a frequent supine position have been described as the most relevant GER predisposing factors in children.

Omari *et al.*, (2002) studied the patterns of LOS motility in 36 neonates by a micro-manometric technique. According to their results, Transient Lower Oesophageal Sphincter Relaxation (TLOSR) was described as the predominant mechanism of GER in infants. TLOSR is a vagal reflex that allows the alimentary bolus to pass into the stomach

whereas facilitates the gas to be released. During these TLOSR episodes, gastric content can return to the oesophagus, resulting in GER episodes (Omari *et al*., 2002; Kessing *et al*., 2011; Loots *et al*., 2012). There are different factors that could act as a trigger for TLOSR, being the activation of tension receptors located in close proximity to LOS the most relevant described (Penagini *et al*., 2004).

2.- CLINICAL RELEVANCE OF PAEDIATRIC GER AND REGURGITATIONS: PREVALENCE AND EVOLUTION.

GER is considered as a normal physiologic process occurring several times per day in healthy infants. But, despite being considered as a developmental issue instead of a disease, many parents seek for guidance from their paediatrician, especially when GER episodes are followed by regurgitation. Frequently, GER/regurgitation episodes result in an increase of parental anxiety, paediatrician referral and overtreatment (Martín-de Capri 2009; Nel 2012; Hegar & Vandenplas, 2013; Lightdale *et al*., 2013). However, when GER episodes occurs repeatedly over a long period of time, and they are associated with tissue damage (for instance esophagitis, respiratory aspiration or swallowing difficulties) and/or failure to thrive, it is then clasified as GER Disease (GERD). (Hyman *et al*., 2006; Vandenplas *et al*., 2009; Hegar & Vandenplas, 2013). The presence of GERD has bee related to chronic illness as feeding difficulties, dysphagia, Barrett oesophagus, adult GERD or even oesophageal adenocarcinoma later in life (Nelson *et al*., 1998; Chak *et al*., 2006; Gold 2006). Fortunately, GERD is not frequently described. Several studies have defined a GERD prevalence of 8.5% in Eastern-Asia countries (Jung, 2011), reaching an estimated prevalence of 10 – 20% in Western-Europe and North-America (Dent *et al*., 2005). Nevertheless, GERD is currently considered as an underdiagnosed

world-wide disease, due to its unspecific symptoms and the non availability of specific diagnostic techniques in many health care centres (Hegar & Vandenplas, 2013; Lightdale 2013).

In Spain, Infante-Pina *et al*., (2008), developed a cross-sectional epidemiological study including 285 paediatricians and 3487 children, from 0 to 4 months of age, seen during a period of one week. One of the aims of this study was to asses the prevalence of different mild gastrointestinal disorders in paediatric practice. According to the result obtained (Figure 0.1), 27.8% of total paediatrician consultations corresponded to mild gastrointestinal disorders (MGDs), which included colic (9.2%), regurgitation (6.1%), constipation (7.8%) and diarrhoea (4.6%). As can be seen, infant regurgitation was the second cause for paediatrician consultation after colic.

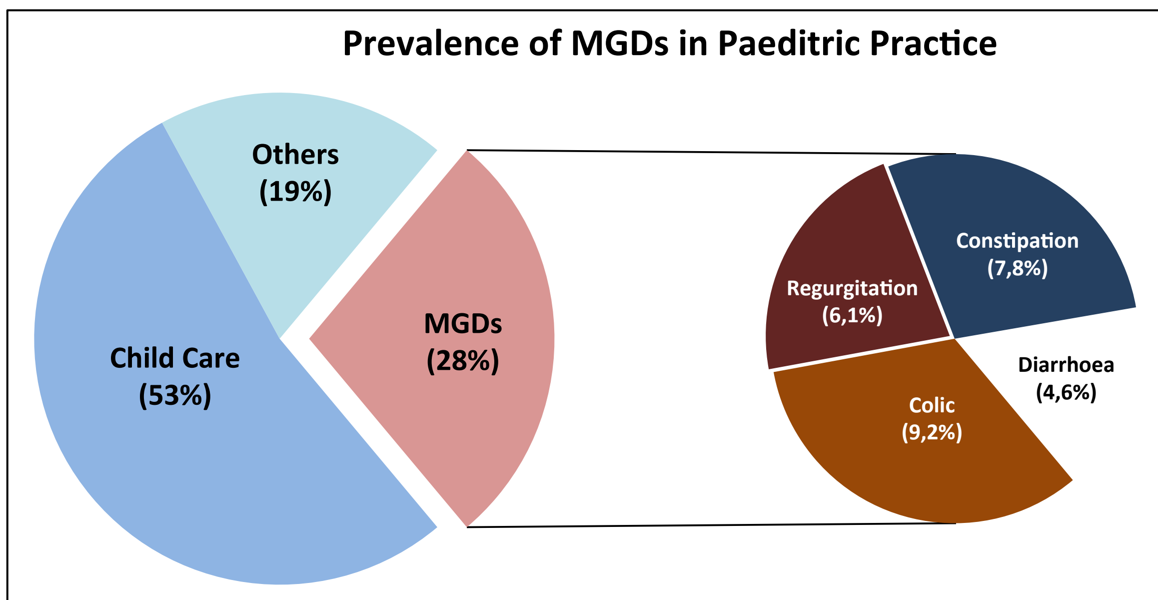


Figure 0.1.- *Prevalence of mild gastrointestinal disorders (MGDs) in paediatric practice. Adapted from Infante-Pina et al., (2008).*

In the same way, Campanozzi *et al*., (2009) published a paediatric prospective survey with the aim of evaluates the prevalence of infant regurgitation in Italian children during the first 2 years of life. 313 children (147 girls) were included, 12% of which received the diagnosis of infant regurgitation. According to the results, regurgitation boy:girl ratio was 166:147, discarding sex influence in the diagnosis.

Regurgitation prevalence in paediatrician consultations reported by Infante-Pina *et al.*, (2008) and Campanozzi *et al.*, (2009) were lower than the ones reported by other authors (Nelson *et al.*, 1997; Vandenplast *et al.*, 1998; Kostovsky, 2006; Hyman *et al.*, 2006), which was of 40 – 65% in infants younger than 4 months of age. Differences in prevalence depend on parent's perception. In this way, when regurgitations are recurrent or they are associated to poor weight gain and discomfort, for instance, crying or fussiness, parents conceive regurgitation as a problem and seek for guidance (Nelson *et al.*, 1997; Rudolph *et al.*, 2001).

Regarding evolution of GER and regurgitation, a variation of prevalence has been described across healthy infants development. Osatakul *et al*., (2002) reported a 86.9% prevalence at 2 months of age. In the study developed by Martin *et al*., (2002), spilling of most feeds each day was frequent during firsts months, showing a prevalence of 41% between 3 and 4 months of age. Hegar *et al*., (2009) found that in a sample of 130 infants, 73% showed a high rate of daily spilling. Despite this variability, all of them reported that regurgitation resolves spontaneously in most healthy infants by 12 to 14 months of age, reaching a prevalence of 4-5% after the first year of life. This decreasing trend has been associated to the digestive track maturation (Hegar *et al*., 2009).

3.- INTRODUCTION TO THE CLINICAL MANAGEMENT OF GER: DIAGNOSIS AND TREATMENT.

Due to its prevalence and possible health consequences, GER and GERD are specially relevant in paediatric patients, needing not only a clinical diagnosis, but also an evaluation of the impact of symptoms in the family (Hyman *et al.*, 2006).

As GER cannot be explained by structural or biochemical abnormalities, it has been classified as a functional gastrointestinal disorder (FGID) in infants/toddler by the Rome III Criteria (Drossman 2006). This classification has involved committees of 87 internationals investigators from 17 different countries, with the aim of classify and establish diagnosis criteria for different process under the label of FGID. In this classification, other infants/toddler gastrointestinal processes have been included (Table 0.1). These functional symptoms are often related to normal development or rise from maladaptive behavioural responses to internal or external stimuli (Hyman *et al.*, 2006). According to the Rome III Criteria, the infant regurgitation diagnostic criteria for children of 3 – 12 months of age, must include both, medical findings (regurgitation two or more times per day for 3 or more weeks), and no presence of clinical signs as retching, hematemesis, aspiration, apnea, failure to thrive, swallowing difficulties, or abnormal posturing (Drossman, 2006). During the diagnostic approach, it is very important to determine which children have GERD in order to offer an optimal treatment (Hegar & Vandenplas, 2013).

***Table 0.1**.- Roma III Criteria. Functional Gastrointestinal Disorders*

G. Funtional Disorders: Infants and Toddlers
G1. Infant Regurgitation
G2. Infant Rumination Syndrome
G3. Cycling Vomiting Syndrome
G4. Infant Colic
G5. Functional Diarrhoea
G6. Infant Dyschezia
G7. Functional Constipation.

With the aim of providing pediatricians with a common resource for the evaluation and management of patients with GER and GERD, the North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition (NASPGHAN) and the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) published a clinical practice guideline (Vandenplas *et al.*, 2009). In this guideline, different tests are included: history and clinical examination, oesophageal pH monitoring, motility studies, endoscopy and biopsy, contrast radiography or oesophageal/gastric ultrasonography. In these guidelines, treatment recommendations and management options are also included (Vandenplas 2009; Hegar & Vandenplas 2013; Lightdale *et al.*, 2013). In case of uncomplicated GER, lifestyle changes, including feeding and positioning interventions, are emphasized, since they can effectively reduce symptoms with a minimal intervention. When GERD is presented, the use of medication as buffering agents, acid secretion suppressants, or promoters of gastric emptying and motility; together with surgical approaches, could be necessary. In order to clarify the diagnostic and management recommendations different flow diagrams have been published. In the case of infants presenting recurrent regurgitations and vomiting the following flow diagram has been proposed (figure 0.2).

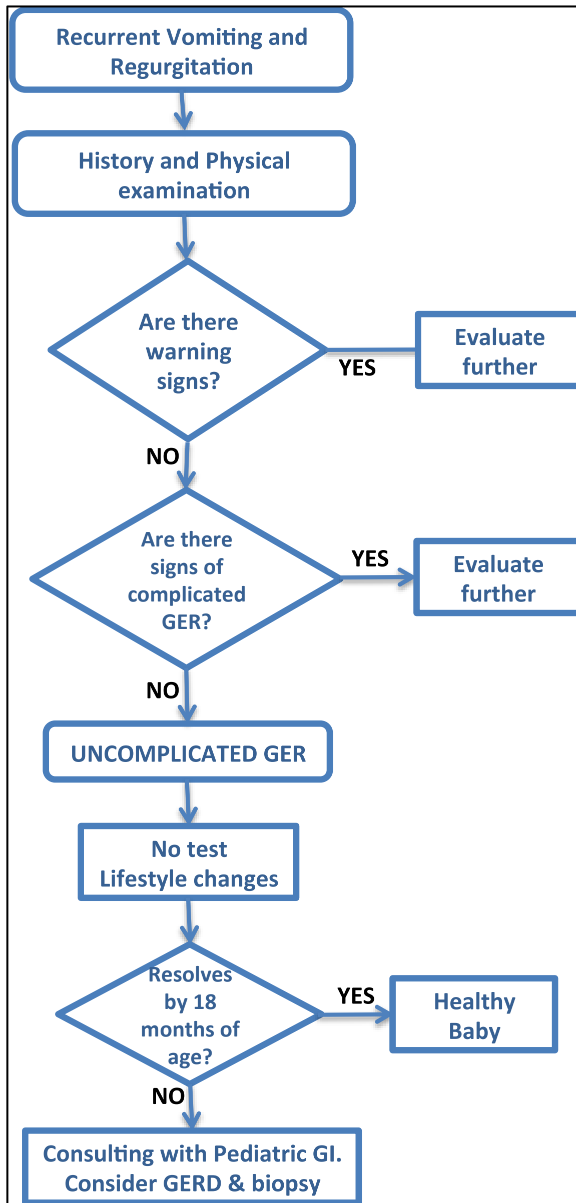


Figure 0.2.- *Flow Diagram for approaching to the infant with recurrent regurgitation and vomiting. Adapted from Lightdale et al., (2013).*

4.- LIFESTYLE CHANGES FOR GER MANAGEMENT.

Lifestyle changes in infants include parental education and feeding/positioning modifications. According to the diagram previously exposed (Figure 0.2), these conservative or non-pharmacological strategies are always the first option for GER management, being usually sufficient to control regurgitations in healthy infants (Vandenplas *et al.*, 2009)

4.1. Positional therapy

Body positioning has been defined as an effective strategy to manage GER in infants (Loop *et al.*, 2012; Nel 2012; Corvaglia *et al.*, 2013; Lightdale *et al.*, 2013). Different studies that used pH-monitoring and impedance have described that, flat prone and left lateral positions, seems to reduce GER episodes, whereas supine and right lateral positioning seems to increase them (Bhat et al., 2007; Corvaglia *et al.*, 2007; van Wijk *et al.*, 2007). However, it has to be considered that prone positioning has been linked to the risk of sudden infant death syndrome (Moon *et al.*, 2011). For this reason, the guidelines consider prone positioning only if the infants are under vigilance and awake (Vandenplas *et al.*, 2009). In order to decrease the risk of sudden infant death syndrome, Corvaglia *et al.* (2013), consider that, positional therapy, should be limited to hospitalized babies under cardiorespiratory monitoring. What is more, prone positioning should be only adopted in children older than 1 year, as this risk is greatly decreased in older age groups (Lightdale *et al.*, 2013).

4.2. Feeding modifications

Feeding modifications include either, modifying maternal diet in breastfed infants, or modified formulas in formula-fed ones. Together with these interventions, reducing the feeding volume, as well as increasing the frequency of feedings, are also included (Lightdale *et al.*, 2013).

According to the practice guidelines published by the ESPGHAN and NASPGHAN (Vandenplas *et al.*, 2009), milk protein intolerance/allergy can also manifest as

unexplained recurrent vomiting in infants. It has been described that, GER and cow's milk protein allergy may co-exist in 42-58 % of infants (Czinn & Blanchard, 2013). In the case of breastfed infants, a 2 to 4week trial of a milk and eggs restricted maternal diet is recommended. In formula-fed infants, an extensively hydrolysed protein may be appropriated (Isolauri *et al.*, 2009; Vandeplas *et al.*, 2009; Czinn & Blanchard, 2013; Lightdale *et al.*, 2013)

For infants who are not cow's milk protein intolerant, the use of thickened feedings is being proposed as an option for GER management. In this regard, two possibilities have been described by ESPGHAN and NASPGHAN (Vandenplas *et al.*, 2009). On one hand, thickening agents such as rice cereal can be added to standard formula; on the other hand, different commercial thickened formulas are currently available (Vandenplas *et al.*, 2009). The basis for this therapy is that the addition of thickeners increases the stickiness and weight of the feeds, which are retained in the stomach, preventing the return into the oesophagus (Huang *et al.*, 2002).

With regard to the first possibility, it has been reported that, the addition of thickeners to standard formulas decreases the frequency of regurgitation (Khoshoo *et al.*, 2000; Chao & Vandenplas, 2007; Orenstein & McGowan, 2008). However, it has to be taken into account that this option could increase the calorie intake by 25% (Chao & Vandenplas, 2007). In this regard, Vandenplas *et al.*, (2011) estimated that, thickening a 20 kcal/oz (~0.65 Kcal/ml) infant formula with one tablespoon of rice cereal per once (30 ml) of formula, increases the caloric density to ~34 kcal/oz (~1.1 kcal/ml). Thickening with one tablespoon per two ounces (60 ml) of formula increases the caloric

density to ~27 kcal/oz (~0.95 kcal/ml). Despite this increase in caloric intake, no relationship between the use of cereals as thickeners and childhood obesity has been reported (Lightdale *et al.*, 2013). Another inconvenience is that, due to the formula viscosity increment, standard nipples are not adequate, being necessary to cross-cut the nipple in order to provide an adequate flow, minimizing the shucking effort (Rudolph *et al.*, 2001).

Nowadays, commercial thickened formulas are available in Europe, Asia and United States under the name of Anti-Regurgitation (AR) infant formulas. The most commonly thickeners used are modified corn and rice starches, potato starch, guar gum and locust bean gum (Vandenplas *et al.*, 2009). Compared with the addition of thickeners to a standard infant formula, commercial AR-formulas contain almost similar caloric density (72 cal/100 ml) as other infant formulas (Czinn & Blanchard, 2013). As for the rest of ingredients, the addition of thickeners to infant formulas is regulated by the effective legislation. In Europe, different maximum concentrations have been established for each ingredient. In this way, modified starches may be added to infant formulas up to either, 30% of total carbohydrates or 2 g/100 ml. In the case of locust bean gum, it may be added up to a maximum level of 10 g/l from birth onwards (European Parliament and Council, 1995; European Parliament and Council, 2006). These maximum levels are similar to those recommended by ESPGHAN in its Global Standard for the Composition of Infant Formula (Koletzko *et al.*, 2005) and by the European Commission Scientific Committee for Food (1997).

Different studies have shown the efficacy of AR-infant formulas on GER management. In this regard, Vandenplas *et al.*, (1994) studied 20 infants from 1 week to

4 months age with regurgitations in a double-blind prospective study that included an AR-infant formula. This study concluded that milk thickening products contributed to GER clinical remission. Wenzl *et al.*, (2003) performed a randomized, placebo-controlled crossover study in 14 infants with recurrent regurgitation. The aim of the study was to examine the influence of formula thickened with locust bean gum on GER. According to its results, thickened feeding show a significant effect on the reduction of regurgitation frequency and amount, but was ineffective against acid GER. In other study, Vanderhoof *et al.*, (2003), evaluated a commercial pre-thickened formula with a multicentre, double-blind, randomized placebo controlled parallel group trial, which included 104 infants during 5 weeks. The group treated with the thickened formula showed a reduction in the total regurgitation volume, and in the presentation of feedings with choke-gag-cough. Horvath *et al.*, (2008) published a review of 14 randomized controlled trials concluding that, thickening feeds, does not seem to reduce measurable reflux, but decreases the frequency of over regurgitation and vomiting

Despite the benefits reported and being legally allow, different in-vitro studies have reported a decrease in the availability of minerals related to the addition of thickeners to infant formulas (Bosscher *et al.*, 2000; Bosscher *et al.*, 2001; Bosscher *et al.*, 2003). Other authors have aware about a possible association between thickened foods and necrotizing enterocolitis (Clarke & Robinson, 2004), the potential allergenicity of commercial thickening agents (Vandenplas *et al.*, 2005; Vandenplas *et al.*, 2009; Lightdale *et al.*, 2013), and possible digestive difficulties as abdominal pain, colic or diarrhoea due to colonic fermentation of undigested carbohydrates (Vandenplas *et al.*, 2005). As consequence, the ESPGHAN Committee on Nutrition (Aggett *et al.*, 2002) recommends that AR-infants formulas should be used only in selected infants with

failure to thrive caused by excessive regurgitations and nutrient losses. Due to these possible negative effects, the need to explore the effect of these ingredients on the nutrition and health of infants has been indicated by different authors (Aggett *et al.*, 2002; Vandenplas *et al.*, 2009; Vandenplas *et al.*, 2011). In the same way, concern is raised about the fact that, AR-infant formulas are currently available in pharmacy without need of prescription or medical supervision (Sievers & Schaub, 2003; Agostoni, 2004).

5.- BIBLIOGRAPHY.

Aggett, P. J., Agostoni, C., Goulet, O., Hernell, O., Koletzko, B., Lafeber, H. L., et al. (2002). Antireflux or antiregurgitation milk products for infants and young children: a commentary by the ESPGHAN Committee on Nutrition. Journal of Pediatric Gastroenterology and Nutrition; 34(5): 496 – 498.

Agostoni, C. (2004). Antireflux or antiregurgitation milk products for infants and young children: a commentary by the ESPGHAN Committee on Nutrition. Acta Paediatrica; 93(4): 456.

Badriul H., & Vandenplas Y. (1999). Gastroesophageal relux in infancy. Journal of Gastroenterology and Hepatology; 14: 13 – 19.

Bhat, R.Y., Rafferty, G.F., Hannam, S., & Greenough, A. (2007). Acid gastroesophageal reflux in convalescent preterm infants: effect of posture and relationship to apnea. Pediatric Research; 62(5): 620 – 623.

Bosscher, D., Van Caillie---Bertrand, M., Van Dyck, K., Robberecht, H., Van Cauwenbergh, R., & Deelstra, H. (2000). Thickening infant formula with digestible and indigestible carbohydrate: availability of calcium, iron, and zinc in-vitro. Journal of Pediatric Gastroenterology and Nutrition, 30(4), 373 – 378.

Bosscher, D., Van Caillie-Bertrand, M., & Deelstra, H. (2001). Effect of thickening agents, based on soluble dietary fiber, on the availability of calcium, iron, and zinc from infant formulas. Nutrition; 17(7), 614-618.

Bosscher, D., Van Caillie-Bertrand, M., Van Cauwenbergh, R., & Deelstra, H. (2003). Availabilities of calcium, iron, and zinc from dairy infant formulas is affected by soluble dietary fibers and modified starch fractions. Nutrition; 19(7): 641 – 645.

Bosscher, D., Van Caillie-Bertrand, M., & Deelstra, H. (2003). Do thickening properties of locust bean gum affect the amount of calcium, iron and zinc available for absorption from infant formula? In-vitro studies. International Journal of Food Sciences and Nutrition; 54(4): 261 – 268.

Campanozzi, A., Boccia, G., Pensabene, L., Panetta, F., Marseglia, A., Strisciuglio, P., *et al.* (2009). Prevalence and natural history of gastroesophageal reflux: pediatric prospective survey. Pediatrics; 123(3): 779 – 783.

Czinn, S. J., & Blanchard, S. (2013). Gastroesophageal reflux disease in neonates and infants. Pediatric Drugs, 15(1), 19-27.

Chak A, Faulx A, Eng C, Grady, W., Kinnard, M., Ochs-Balcom, H., & Falk, G. (2006). Gastroesophageal reflux symptoms in patients with adenocarcinoma of the esophagus or cardia. Cancer;107(9): 2160 – 2166.

Chao, H. C., & Vandenplas, Y. (2007). Effect of cereal-thickened formula and upright positioning on regurgitation, gastric emptying, and weight gain in infants with regurgitation. Nutrition, 23(1): 23 – 28.

Clarke, P., & Robinson, M. (2004). Thickening milk feeds may cause necrotising enterocolitis. Archives of Disease in Childhood Fetal and Neonatal Edition; 89(3): 280.

Corvaglia, L., Martini, S., Aceti, A., Arcuri, S., Rossini, R., & Faldella, G. (2013). Nonpharmacological management of gastroesophageal reflux in preterm infants. BioMed research international; 2013: 1 – 7.

Corvaglia, L, Rotatori, R., Ferlini, M., Aceti, A., Ancora, G., & Faldella, G. (2007). The effect of body positioning on gastroesophageal reflux in premature infants: evaluation by combined impedance and pH monitoring. Journal of Pediatrics; 151(6): 591 – 596.

Dent, J., El-Serag, H.B., Wallander, M., & Johansson, S. (2005). Epidemiology of gastro-oesophageal reflux disease: a systematic review. Gut; 54(5): 710 – 717.

Drossman, D.A. (2006). The functional gastrointestinal disorders and the Rome III process. Gastroenterology; 130(5): 1377 – 1390.

European Commission Scientific Committee for Food (1997). Opinion on certain additives to foods for infants and young children in good health and in foods for special medical purposes for infants and young children: Locust bean gum. Document XXIV/1270/97. Annex II.

European Parliament and Council. Directive 95/2/EC of February 1995 on food additives other than colours and Sweeteners. (http://ec.europa.eu/food).

European Parliament and Council. Directive 2006/141/EC on infant formulae and follow-on formulae. (http://ec.europa.eu/food).

Gold, B. D. (2006). Is Gastroesophageal Reflux Disease Really a Life-Long Disease: Do Babies Who Regurgitate Grow up to Be Adults with GERD Complications & quest. The American journal of gastroenterology; 101(3): 641 – 644.

Hegar, B., Dewanti, N.R., Kadim, M., Alatas, S., Firmansyah, A., & Vandenplas, Y. (2009). Natural evolution of regurgitation in healthy infants. Acta pædiatrica; 98(7): 1189 – 1193.

Hegar, B., & Vandenplas, Y. (2013). Gastroesophageal reflux: natural evolution, diagnostic approach and treatment. The Turkish Journal of Pediatrics; 55(1): 1 – 7.

Horvath, A., Dziechciarz, P., & Szajewska, H. (2008). The effect of thickened-feed interventions on gastroesophageal reflux in infants: systematic review and meta-analysis of randomized, controlled trials. Pediatrics; 122(6): 1268 – 1277.

Huang, R. C., Forbes, D., & Davies, M. W. (2002). Feed thickener for newborn infants with gastro-oesophageal reflux. The cochrane library.

Hyman, P.E., Milla, P.J., Benninga, M.A., Davidson, G.P., Fleisher, D.F., & Taminiau, J. (2006). Childhood functional gastrointestinal disorders: neonate/toddler. Gastroenterology; 130(5): 1519 – 1526.

Isolauri, E., Tahvanainen, A., Peltola, T., Arvola, T. (1999). Breast-feeding of allergic infants. J Pediatrics; 134(1): 27 – 32.

Jung, H.K. (2011). Epidemiology of gastroesophageal reflux disease in Asia: a systematic review. Journal of neurogastroenterology and motility; 17(1): 14 – 27.

Kessing, B.F., Conchillo, J.M., Bredenoord, A.J., Smout, A.J., &Masclee, A.A.M. (2011). Review article: the clinical relevance of transient lower oesophageal sphincter relaxations in gastroesophageal reflux disease. Alimentary pharmacology & therapeutics; 33(6): 650 – 661.

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(5): 584 – 599.

Kostovsky A. (2006). Gastro-oesophageal reflux disease. Paediatria Croatica; 50(1): 93 – 102.

Lightdale, J. R., Gremse, D. A., Heitlinger, L. A., Cabana, M., Gilger, M. A., Gugig, R., & Hill, I. D. (2013). Gastroesophageal reflux: management guidance for the pediatrician. Pediatrics; 131(5): 1684 – 1695.

Loots, C.M., Benninga, M.A., &Omari T.I. (2012) Gastroesophageal reflux in pediatrics; (patho) physiology and new insights in diagnostics and treatment. Minerva Pediatrica; 64(1): 101 – 119.

Martin, A.J., Pratt, N., Kennedy, J.D., Ryan, P., Ruffin, R.E., Miles, H., & Marley, J. (2002). Natural history and familial relationships of infant spilling to 9 years of age. Pediatrics; 109(6): 1061 – 1067.

Martín-De Capri, J. (2009). Reflujo gastro-esofágico. Boletin de la Sociedad de Pediatria de Aragon, la Rioja y Soria; 39: 20-25.

Moon, R.I., Darnall, R.A., & Goodsteinetal, M.H. (2011). SIDS and other sleep-related infant deaths: expansion of recommendations for a safe infant sleeping environment. Pediatrics, 128(5): 1030 – 1039.

Nel, E.D. (2012). Gastro-oesophageal reflux in infants and children.South African Family Practice; 54(5): 414 – 417.

Nelson, S.P., Chen, E.H., Syniar, G.M., & Christoffel, K.K. (1997). Prevalence of symptoms of gastroesophageal reflux during infancy: a pediatric practice-based survey. Archives of pediatrics & adolescent medicine; 151(6): 569 – 572.

Nelson, S.P., Chen, E.H., Syniar, G.M., & Christoffel, K.K. (1998). One-year follow-up of symptoms of gastroesophageal reflux during infancy. Pediatrics; 102(6): 67.

Omari, T.I., Barnett, C.P., Benninga, M.A., Lontis, R., Goodchild, L., Haslam, R.R., Dent, J., & Davidson, G.P. (2002). Mechanisms of gastro-oesophageal reflux in preterm and term infants with reflux disease. Gut; 51(4): 475 – 479.

Orenstein, S. R., & McGowan, J. D. (2008). Efficacy of conservative therapy as taught in the primary care setting for symptoms suggesting infant gastroesophageal reflux. The Journal of pediatrics, 152(3): 310 – 314.

Osatakul, S., Sriplung, H., Puetpaiboon, A., Junjana, C.O., & Chamnongpakdi, S. (2002). Prevalence and natural course of gastroesophageal reflux symptoms: a 1-year cohort study

in Thai infants. Journal of pediatric gastroenterology and nutrition; 34(1), 63 – 67.

Penagini, R., Carmagnola, S., Cantù, P., Allocca, M., & Bianchi, P.A. (2004). Mechanoreceptors of the proximal stomach: role in triggering transient lower esophageal sphincter relaxation. Gastroenterology; 126(1): 49 – 56.

Price, F.N. (2007). Gastroesophageal Reflux Disease.Recognition and management. In Browne, N.T., Flanigan, L.M., McComiskey, C.A., & Pieper, P. "Nursing Care of the Pediatric Surgical Patient". 2nd Edition. London, United Kingdom. Jhonnes and Barlett Publishers International: 325 – 332.

Rudolph, C.D., Mazur, L.J., Liptak, G.S., Baker, R.D., Boyle, J.T., Colletti, R.B., et al. (2001). Guidelines for evaluation and treatment of gastroesophageal reflux in infants and children: recommendations of the North American Society for Pediatric Gastroenterology and Nutrition. Journal of pediatric gastroenterology and nutrition; 32: 1 – 31.

Sievers, E., & Schaub, J. (2003). Antireflux or antiregurgitation milk products. Journal of Pediatric Gastroenterology and Nutrition; 36(3): 418.

Vandenplas, Y., Hachimi-Idrissi, S., Casteels, A., Mahler, T., & Loeb, H. (1994). A clinical trial with an "anti-regurgitation" formula. European Journal of Pediatrics; 153(6): 419 – 423.

Vandenplas, Y., Hauser, B., Devreker, T., Mahler, T., Degreef, E., & Wauters, GV. (2011). Gastro-esophageal reflux in children: Symptoms, diagnosis and treatment. Journal of Pediatric Sciences; 3(4): 101.

Vandenplas, Y., Lifshitz, J.Z., Orenstein, S., Lifschitz, C.H., Shepherd, R.W., Casaubón, P.R., & Lifshitz, F. (1998). Nutritional management of regurgitation in infants.Journal of the American College of Nutrition; 17(4): 308 – 316.

Vandenplas, Y., Rudolph, C. D., Di Lorenzo, C., Hassall, E., Liptak, G., Mazur, L., *et al*. (2009). Pediatric gastroesophageal reflux clinical practice guidelines: joint recommendations of the North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition (NASPGHAN) and the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN). Journal of pediatric gastroenterology and nutrition; 49(4): 498 – 547.

Vandenplas, Y., Salvatore, S., & Hauser, B. (2005). The diagnosis and management of gastro-oesophageal reflux in infants. Early human development; 81(12): 1011 – 1024.

Vanderhoof, J. A., Moran, J. R., Harris, C. L., Merkel, K. L., & Orenstein, S. R. (2003). Efficacy of a Pre-thickened Infant Formula: A Multicenter, Double-Blid, Randomized, Placebo-Controlled Parallel Group Trial in 104 Infants with Symptomatic Gastroesophageal Reflux. Clinical pediatrics; 42(6), 483 – 495.

vanWijk, M. P., Benninga, M. A., Dent, J., Lontis, R., Goodchild, L., McCall, L. M., et al. (2007). Effect of body position changes on postprandial gastroesophageal reflux and gastric emptying in the healthy premature neonate.The journal of pediatrics; 151(6): 585 – 590.

Wenzl, T. G., Schneider, S., Scheele, F., Silny, J., Heimann, G., & Skopnik, H.

(2003). Effects of thickened feeding on gastroesophageal reflux in infants: a placebo-controlled crossover study using intraluminal impedance. Pediatrics; 111(4): 355 – 359.

Physicochemical characterization of thickeners across an *in-vitro* digestion. CHAPTER 1

1.-INTRODUCTION

Thickeners are commonly used in infant nutrition in a wide range of foods, such as AR-infant formulas, baby purees or cereal based foods. Some of these products are specifically intended for infants with swallowing issues, as dysphagia or gastroesophageal reflux. As thickening agents, Locust Bean Gum (LBG) and modified corn and rice starches, are the most frequently used (Agget. *et al*., 2002; Cichero *et al*., 2013).Based in their physicochemical properties, thickeners are added with the aim of increasing the stickiness and weight of the feeds, being retained in the stomach and preventing the return of the content into the oesophagus (Huang *et al*., 2002).

1.1.-Characteristics of LBG as food additive (E410).

LBG, also known as carob bean gum, is a neutral galactomannans obtained from the seeds of Carob tree (*Ceratonia siliqua*), a common legume tree in the Mediterranean region (Battle & Tous, 1997; Dakia *et al*., 2008; Haddarah *et al*., 2014). Main carob bean producer and exporter countries are Spain, Italy, Portugal, Morocco, Greece, Cyprus and Turkey (Karababa & Coşkuner, 2013). As is has been represented in figure 1.1, the seeds are composed by the husk, the endosperm and the germ, obtaining LBG by cruising the endosperm (Dakia *et al*., 2008).

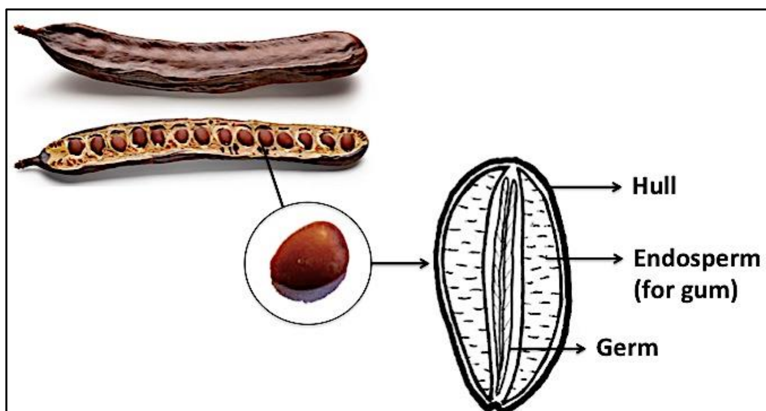


Figure 1.1*-Carob seed scheme. (Adapted from Dakia et al., 2008. Pictures obtained from http://www.lbg.it/seedgum.html)*

1.1.1.- Molecular structure of LBG.

Molecularly, LBG is a highly polydisperse polysaccharide, consisting of a linear chain of (1→4)-linked β-D-mannopyranosyl units with randomly distributed (1→6)-linked-d-galactopyranosyl residues as side chains. The average degree of branching is approximately 25% (about 1galactose residues for every 4 manose), its molar mass (M) is in a range of 0.3 – 2.0·10^6g/mol, and its weight–average molecular weight 535–826 kDa (McCleary, 1980; Daas Piet et al., 2000; Sittikijyothin *et al.*, 2005; Haddarah *et al.*, 2014).

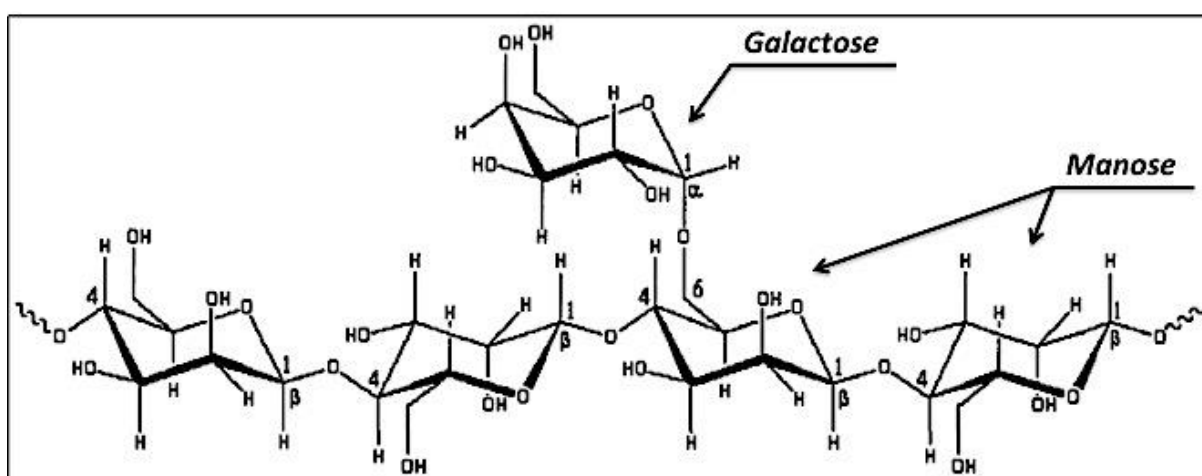


Figure 1.2.- *Molecular structure of Locust Bean Gum (LBG). (Adapted from Sittikijyothin et al., 2005)*

1.1.2.- Physicochemical properties of LBG

Physicochemical properties of LBG are strongly influenced by the galactose content and distribution of the galactose units along the β-D-mannopyranosyl backbone, which in turn, is determined by different factors such as plant source, growing conditions and manufacturing processes (Coviello, *et al.*, 2007; Dakia *et al.*, 2008, Prajapati *et al.*, 2013; Barak & Mudgil, 2014, Haddarah *et al.*, 2014).

Historically, LBG was the first galactomannan used in food industry (Dakia *et al*., 2008). As industrial thickener (E410), LBG is widely used due to its ability to form very viscous solutions at relatively low concentration, being only slightly affected by pH or heat treatment because it is non ionic (Batlle & Tous, 1997; Dakia, *et al*., 2008; Prajapati, *et al*., 2013; Haddarah, *et al*., 2014). To these properties it has to be mentioned that LBG does not provide color, aroma or taste to feeds (SamilKök, 2007).

- ***Nutritional composition***.

Dakia *et al*., (2008), investigated the nutritional composition of locust bean gum extracted with two different methods (boiling water and acidic pre-treatment) and compare it with the nutritional composition declared by other authors. The results obtained were quite similar each other and closer to those declared by the literature. These results are show in table 1.1.

Table 1.1- *Composition (%) of LBG (adapted from Dakia et al., 2008).*

	LBGw	LBGa
Moisture	6.5 ± 0.6	5.9 ± 0.1
Ashes	1.5 ± 0.1	0.7 ± 0.2
Total proteins	7.4 ± 0.7	5.2 ± 0.4
Lipids	1.5 ± 0.1	1.3 ± 0.1
Nitrogen free extract	89.6	92.8
Total sugars	73.3	87.3

- ***Digestive enzyme resistance***

Regarding enzyme resistance, LBG has been classified as a non-digestible carbohydrate, remaining quite resistant to hydrolysis under a wide range of pH (from 3 to 11). In this regard, Fabek *et al*., (2014), performed a two step *in-vitro* digestion simulating gastric and small intestine phases on six different hydrocolloids, including guar gum, locust bean gum, fenugreek gum, xanthan gum, soluble flaxseed gum, and DA-100 variety soy soluble polysaccharides. The results obtained lead to conclude that the structure of these hydrocolloids was not affected by hydrolytic enzymes during the simulated gastric and small intestinal digestion. According to Dey *et al*., (2012), linear polysaccharides are resistant to the digestive action of the gastrointestinal enzymes and retain their integrity in the upper gastrointestinal tract.

- ***Water solubility***

Concerning water solubility of galactomannan, Silveira & Bresolin (2011) described that solubility increases with increasing the content of galactose, as these residues inhibit the packaging of the mannan chains. These residues also contribute to the solution stability by freedom of rotation about the (1→6) linkages. In order to prevent precipitation, a galactose substitution level of 10% is necessary. Regarding to the dissolution temperature effect on equilibrium solubility, Pollard et al., (2007) found that about 50% of the galactomannan polysaccharide contained in crude carob endosperm flour, was readily soluble at 5 ºC. The remaining polysaccharide dissolved with increasing temperature, reaching 95% solubility at 85 ºC. These authors related the temperature effect to the content of galactose residues. In this way, readily soluble components presented a galactose degree of substitution higher than 0.35. On the

contrary, poorly soluble components had a galactose degree of substitution lower than 0.35, increasing their solubility with temperature.

- ***Rheological properties: Viscosity*.**

Viscosity could be considered as the most important physicochemical property of galactomannans, including LBG, as this is directly related to their thickening capacity when used as thickener in foods. As a consequence, different researches on rheological behavior of galactomannans have been published. LBG has the ability to form very viscous solutions at relatively low concentration (Prajapati, *et al*., 2013; Haddarah, *et al*., 2014). However, viscosity is a dynamic property and depends on different parameters as the molecular weight, the mannose/galactose ratio, the shear stress applied, concentration or the temperature.

Wu et al., (2009) analyzed the rheological properties of four types of galactomannans, including guar gum, tara gum, fenugreek gum and LBG. These authors described that the mannose/galactose ratio and the molecular weight played an essential role on the rheological properties. In this way, as the ratio and the molecular weight increases, intrinsic viscosity increased as well. For this reason, LBG with the lowest molecular weight led to the lesser viscous solutions. On the contrary, guar gum show the highest viscosity. These findings were also reported by Haddarah *et al*., (2014). Wang & Cui (2005) related the relationship between viscosity and molecular weight to the conformation of the molecules in such away that higher values of intrinsic viscosity represent a more extended structure at a given chain length.

Haddarah et al., (2014) analyzed the dynamic viscosity of solutions with different varieties of LBG at different concentrations. A non Newtonian behavior was reported for all of them, which means that shear viscosity or resistance to flow will be different depending on the shear rate applied, at a given temperature and pressure (Whittingstall, 2001). In figure 3, the dynamic viscosity behavior of a Newtonian and a non-Newtonian fluid has been represented. As can be seen, in a Newtonian fluid, dynamic viscosity remains stable. On the contrary, in a non-Newtonian fluid, dynamic viscosity decreases as the shear rate increases.

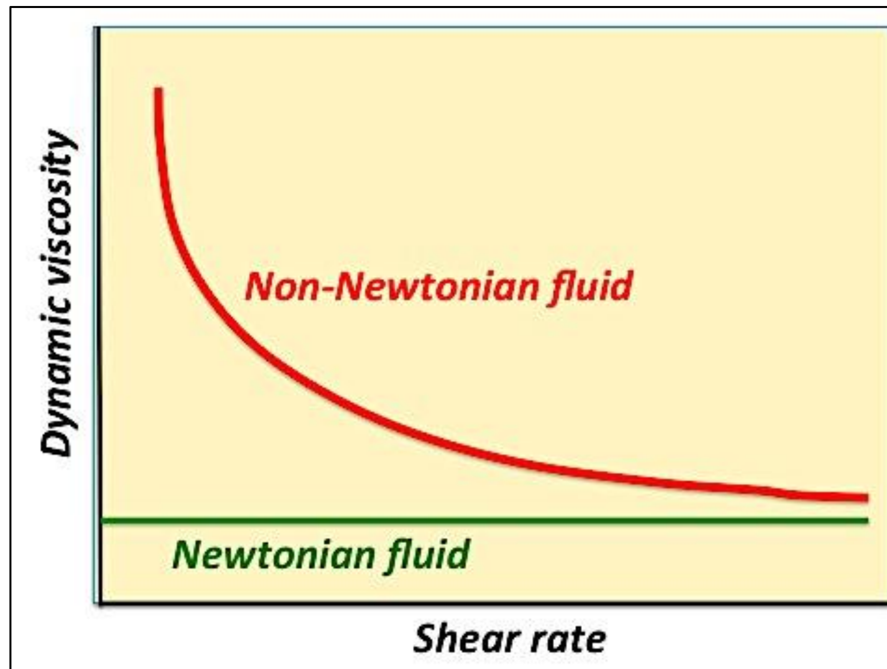


Figure 1.3.*-Newtonian vs non-Newtonian dynamic viscosity comparison.*

Haddarah *et al*., (2014) reported that, when LBG was used in a low concentration (0.1% w/v), the solution presented a nearly Newtonian behavior. When higher concentrations were analyzed (0.5 – 2%) a nearly Newtonian behavior was reported in a shear-thinning region. However, at higher shear rates, viscosity decreases. These findings have been also reported by other authors (Mao & Chen, 2006; Dakia *et al*., 2008; Wu *et al*., 2009). The explanation given by these authors is that, as the shear rates

increases, a disruption of molecular entanglements occurs. When the shear rate is low, the viscosity remains constant as the disruption of the entanglements is compensated by the reformation of new ones. On the contrary, when high shear rates are applied, the disruption of entanglements dominates, leading to a decrease in dynamic viscosity. The disruption of entanglements by the imposed shear stress, made that molecules align in the direction of flow and the dynamic viscosity decreases with increasing shear rate

Regarding the effect of temperature on viscosity, Dakia *et al*., (2008) reported that the viscosity values provided by LBG were higher as temperature increased, explaining this behavior with the increment of solubility at high temperatures. According this author, as the temperature increases, new molecules are solubilized, increasing in aggregates and entanglement.

1.2.-Characteristics of native and modified starches as food additives.

Native starches are carbohydrate polymers organized as tiny white granules located in different sites of plants, as for instance, in cereal grains, roots, tubers, stems or legume seeds. The main sources of commercial starches are wheat, maize, rice, potato and cassava (Swinkels, 1985). Nowadays, starches are being used as food additives in many products, as contribute greatly to their textural properties. Due to their physicochemical characteristics, starches are widely used as thickeners, colloidal stabilizers, gelling agents, bulking agents and water retention agents. However these properties varies with the chemical composition and physical characteristics of starches, being decisive the botanical source of starches (Swinkels, 1985; Singh *et al*., 2007).

1.2.1.- Molecular structure of native starches.

Chemically, native starches consist of two polysaccharides, amylose and amylopectin. Amylose is a mainly linear molecule of α-(1→4)-linked D-glucopyranosyl units with relatively low M, whereas amylopectin has high M and highly branched structures consisting of α-(1→4)-linked D-glucopyranosyl chains linked with 5-6% non-randomly distributed α-(1→6)-D-glucopyranosyl branch points (Fernandez, *et al.*, 2011).

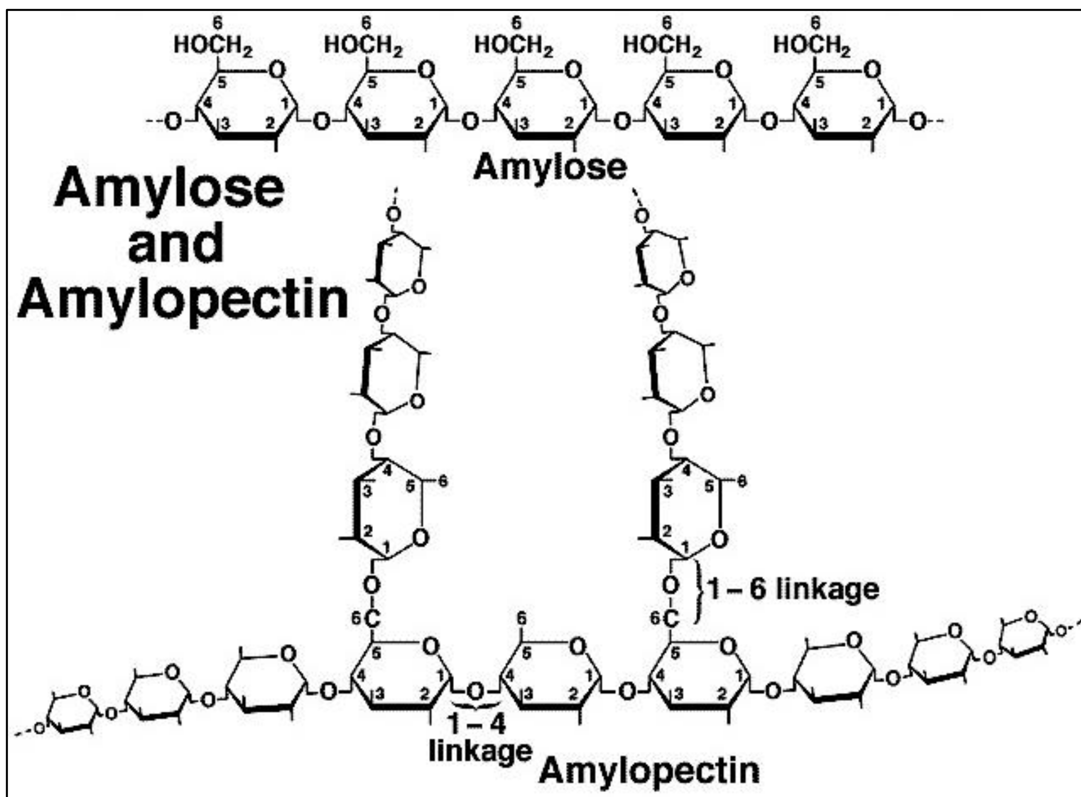


Figure 1.4.*-Amylose and amylopectin structure. How both structures are bounded have been represented. (Figure obtained from Randy Moore, Dennis Clark, and Darrell Vodopich, Botany Visual Resources Library © 1998 The McGraw-Hill Companies)*

The ratio of amylose to amylopectin depends on the botanical source and is commonly between 10-30% (Hizukuri, 1985; Vandeputte, *et al*., 2003). Apart from botanical source, this ratio is also affected by the climatic conditions and soil type during growth (Singh *et al*., 2003). These factors seem to influence the activity of the enzymes involves in starch biosynthesis, determining the amylose content between different starches (Krossmann & Lloyd, 2000). Singh *et al*., 2003, compiled the amylose content in different native starches declared by different authors (table 1.2).

Table 1.2.*-Amylose content (%)*
(Adapted from Singh et al., 2003)

	Amylose (%)
Potato starch	20.1 – 31.0
Corn starch	22.4 – 32.5
Rice starch	5 – 28.4
Wheat starch	24.5 – 28.4

In nature, starch granules structure is semi-crystalline, with a crystalline and an amorphous region. The crystallinity is exclusively associated with the amylopectin component, while the amorphous regions mainly represent amylose as it has been represented in figure 5 (Singh *et al*., 2003). In this way, the extent of crystallinity of native starch granules ranges from about 15% for high amylose content starches to about 45 – 50% for low amylose content (also known as waxy starches) (Copeland *et al*., 2009). Properties and functionality of starches are influenced by the amylose to amylopectin ratio and the crystalline structure (Chung *et al*., 2011). In this way, amylose has a high tendency to retrograde and produce tough gels and strong films. In contrast, amylopectin, is more stable and produces soft gels and weak films when dissolves in water (Ashogbon et al., 2014). Amylopectin stability varies with the length of its

branches. Long branch chains, form long double helices that provide stability, whereas short branch chains destabilize the crystalline structure (Chung *et al*., 2011).

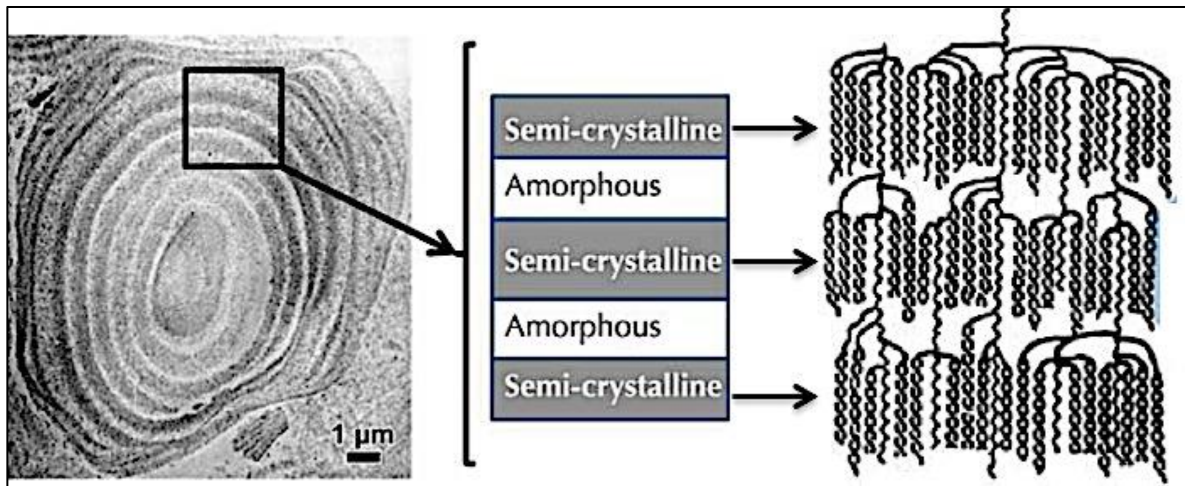


Figure 1.5.- *Semi-crystalline structure of starch granules. As can be seen, crystalline regions correspond with the amylopectin ramifications. (Adapted from http://www.scienceinschool.org/2010/issue14/starch).*

Phosphorus is also present in starches structure as non-carbohydrate constituent, varying from 0.003 to 0.09% depending on the source. Phosphorus is present as phosphate monoesters or phospholipids. The first form is covalently bound to the amylopectin fraction and provides clarity when pastes are formed. The second is proportional to the amylose content of the starch, resulting in opaque pastes. Phosphates content significantly affects the functional properties of the starches (Singh *et al*., 2003).

1.2.2.-Physicochemical properties of native starches

- ***Swelling power and solubility*.**

When starches are in contact with water, the starch granules swell. due to water percolation in their semi-crystalline structure. Water molecules will percolate in their semi-crystalline structure,being bounded to the exposed hydroxyl groups of amylose and amylopectin. These properties vary with the botanical source, chemical composition, and with the exposition to different factors, mainly by the temperature of the system (Tester & Morrison, 1990; Singh *et al*., 2003; Singh *et al*., 2007; Copeland 2009).

Tester & Morrison (1990) studied the effect of temperature on swelling power of different starches by measuring the granule volumes by a counter coulter. They reported that swelling of granules increased when temperature raised, reaching a maximum. After this point, higher temperatures resulted in a decrease in swelling, as the swollen granules became almost completely permeable and began to disintegrate. Thermal influence on swelling power and its mechanism have been explained by different authors (Tester & Morrison, 1990; Jenkins & Donald 1998; Singh et al., 2003; Copeland 2009; Chung *et al*., 2011). According to these authors, water first enters the amorphous regions, transmitting a disruptive stress from the amorphous to the crystalline regions. When heating, the crystalline structure is disrupted and water molecules become linked by hydrogen bonding to the exposed hydroxyl groups of amylose and amylopectin. This will lead to an increase in granule swelling. The maximum swelling capacity is followed by the rupture of starch granules (starch breakdown) and, the dispersion of starch molecules in the aqueous phase.

Swelling power is being related to the amylopectin content. Starches with high amylopectin content, especially with tangled long amylopectin branches, will present a very stable crystalline structure, being resistant to swelling. A high temperature would be needed in order to destabilize the crystalline structure and reach a maximum swell power. Therefore, a great amount of long amylopectin branch chain length would lead to a reduction in swelling. On the contrary, short branch chains destabilize the crystalline structure and facilitate swelling (Chung *et al*., 2011). Against amylopectin, amylose is being defined as an inhibitor of swelling (Tester & Morrison, 1990). Amylose content is proportional to the presence of lipids in starch, which have a reducing effect on the swelling of starch granules. This effect could be explained by the formation of insoluble and hydrophobic lipid-amylose complexes, which prevent water percolation and amylose from leaching. In order to dissociate amylose-lipid complexes, temperatures above 95 ºC are needed (Tester & Morrison, 1990; Singh *et al*., 2003; Chung *et al*., 2011).

Regarding to phosphorous content, it has been proposed that, a high content of phosphate monoesters covalent bounded to the amylopectin fraction, would result in an increase in swelling power. Phosphate groups on adjacent amylopectin chains will increase hydration by weakening the crystalline structure because of repulsions between phosphate groups (Singh *et al*., 2003).

Starches solubility is being related to the swelling power. As a result of water percolation, the amorphous regions swelling, and the disruptive stress transmitted to the crystalline regions, amylose and polysaccharide molecules begin to leach from the granules, which results in a solubility increase (Tester & Morrison 1990; Jenkins & Donald 1998).

- ***Rheological properties of native starches.***

Starch rheological behavior depends on temperature. This characteristic behavior has been described by different authors (Singh *et al.*, 2003; Singh *et al.*, 2007; Copeland *et al.*, 2009). Initially, an increase in viscosity as temperature rises has been described. This increase could be attributed to the degree of granular swelling and the inter-granule contact, forming a three-dimensional network of swollen granules. Swelling of starch granules during thermal treatment is accompanied by leaching of amylose and others polysaccharides. Viscosity increases to a peak (peak viscosity), which correspond to the point when the number of swollen but still intact starch granules is at a maximum. If temperature continues increasing, a decrease in viscosity will be observed due to the starch granules rupture and the dispersion of their component in the aqueous phase. As the starch paste cools, leached amylose molecules rapidly aggregate forming a network composed by molecular interactions between released amylose and amylopectin molecules. As a result, the viscosity of the solution will increase. Typical rapid viscosity profile of starches has been represented in figure 6.

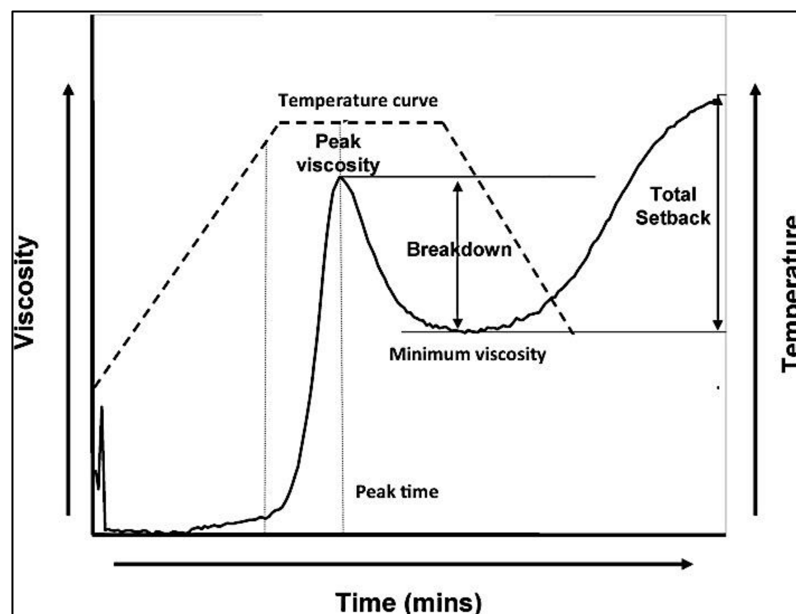


***Figure 1.6.**- Typical RVA profile of starches (Adapted from Copeland et al., 2009).*

Rheological properties of starches are influenced by amylose content, lipid content and amylopectin branch chain length distribution (Jane *et al*., 1999). As it has been previously explained, amylose and lipid suppress welling and maintain the integrity of the swollen starch granules. On the contrary, amylopectin mainly contributes to swelling of starch granules (Tester & Morrison, 1990; Singh *et al*., 2003; Chung *et al*., 2011). Chung *et al*., (2011) determined that waxy rice starch, mainly containing amylopectin with an absence of lipid-amylose complexes, swelled rapidly at a low temperature, providing a great peak viscosity. However, due to the low content of amylose, final viscosity after cooling was lower than for higher amylose content starches. In the same study, Chung *et al*., (2011), evaluated the influence of amylopectin branch chain length. At this respect, these authors found that starches consisting of a low proportion of short amylopectin branches needed a very high temperature for swallowing and increasing viscosity. As a result, the viscosity peak was lower than for other starches with high proportions of short amylopectin branches. These findings were explained by the high stability provided by the long amylopectin branches to the crystalline structure, leading to a high resistance to swelling.

- ***Enzymatic resistance of starches.***

Starches contain a rapidly digested portion, a slowly digested portion and a portion that is resistant to digestion. This last portion receives the name of resistant starch (Singh et al., 2010). According to their digestibility, starches have been classified in different fractions by measuring the released glucose at different times during an *in-vitro* enzymatic digestion (Englyst *et al*., 1992):

- *Rapidly digestible starch*: amount of glucose release after 20 min.
- *Slowly digestible starch*: amount of glucose released between 20 and 120 min.
- *Resistant starch*: Total starch minus the amount of glucose released within 120 min of *in-vitro* digestion.

Resistance of starches to enzymatic digestion is mainly due to their intrinsic physicochemical properties (Topping & Clifton, 2001). The digestion process start at the granular surface, creating holes in the susceptible zones that become into channels towards the center of the granule (Copeland, 2009). Digestion of starches in the upper gut is effected by α-amylase, which cleave the α-(1 → 4) links and release maltodextrins. These are then hydrolyzed by isomaltases associated to the enterocyte membrane (Topping & Cliffton, 2001; Copeland *et al*., 2009). α-amylase is produced in abundance by salivary glands and pancreas, but this enzyme can be also produced by the jejunum and mammary gland (Butterworth *et al*., 2011).

Starch granules susceptibility to α-amylase digestion depends on treatment and on different characteristics, which include crystallinity, granule size and amylose to amylopectin ratio among others (Copeland *et al*., 2009). Distinctly hydrolysis degree has been related to the granules morphology. In this way, different authors described that small granules has a greater surface area, leading to a higher degree of enzyme digestion when compared with larger granules (Tester *et al*., 2004; Tester & Karkalas 2006; Kaur *et al*., 2007; Chung *et al*., 2011).

Regarding to crystallinity, amorphous regions are more susceptible of being digested by α-amylase than crystalline regions. In crystalline starch, the packing of the double helices are stabilized by hydrogen bonding between glycan residues. For optimal amylase action, a run of glucan residues needs to fill the five sites that form the active region of the enzyme. Therefore, amylopectin chains that are intimately associated in forming crystallites are unlikely to bind effectively to the active region of the enzyme. Hydrothermal treatment, as has been previously explained, cause the granules to swell by absorption of water and crystalline structures are disrupted with, increasing amorphous material and digestibility (Copeland *et al*., 2009; Butterworth *et al*., 2011).

Amylose to amylopectin ratio also influence digestibility of starches. Amylose content in starches has been negatively correlated with amylase digestibility. In this way, digestion resistance has been reported for high amylose starches. On the contrary, waxy starches showed a greater digestibility when compared with their normal starch counterparts (Noda *et al*., 2003; Tester *et al*., 2004; Srichuwong & Jane, 2007; Chung *et al*., 2011).

Another parameter that has been related to digestibility of starches is the lipid content. In this way, the presence of lipid-amylose complexes could impaired the accessibility of amylose chains to the active sites of α-amylase, resulting in a prolonged hydrolysis profile (Tester *et al*., 2006).

1.2.3.-Modified starches and their physicochemical properties

Despite being a good texture stabilizer in food systems, native starches have technical limitations as low shear resistance, low thermal resistance and sensibility to thermal and enzymatic treatments. These weaknesses limit the use of starches in some foods applications (Singh *et al.*, 2007). Nowadays, numerous modifications of native starches are available in the food industry. These modifications improve some physicochemical characteristics of starches in order to cover new specific foods applications demanded by the industry(Singh *et al.*, 2007; Kaur *et al.*, 2012). Some modifications available in food industry have been summarized in table 1.3 (BeMiller 1997; Singh *et al.*, 2007; Chung et al., 2011; Kaur *et al.*, 2012):

Table 1.3.*- Methods of starch modification*

Chemical modifications
▪ Derivatization (Hydroxypropilation by etherification, esterification and cross-linking).
▪ Acid/enzymatic hydrolysis
▪ Dextrinization
▪ Oxidation
Physical modifications
▪ Gelatinization/Pregelatinization
▪ Deep freezing
▪ Micronization
▪ Thermal inhibition or dry heating
▪ Superheated starch
Genetic modifications
▪ Amylose content modification (Low amylose or waxy starches, High amylose starches)
▪ Alteration of amylopectin structure
▪ Phosphate content modifications
▪ Granule number and size modifications

- ***Chemical modifications commonly used in food industry.***

Common types of chemical modifications are derivatization, which include hydroxypropilation, etherification, esterification or cross-linking (BeMiller 1997; Singh, *et al*., 2007; Kaur 2012). Gray & BeMiller (2005) proposed the hypothesis that during derivatization, reagent enters the amorphous region of starch granules easily. The derivatization in these regions causes the granules to swell, pulling the crystalline regions. As the reaction proceeds, derivatization becomes more facile. In this way, derivatization process can be defined as a cooperative process that starts in the more accessible amorphous regions and proceeds until reaching the most crystalline regions of the granule.

Hydroxypropilation of starches by etherification, also known as hydroxyetherification, is commonly used in food industry. This modification is generally obtained with propylene oxide in an alkaline environment. During this reaction, hydroxyl groups are capable of disrupting the inter- and intra-molecular hydrogen bonds between the amylose and amylopectin chains, rendering a hydrophilic character and weakening the granular structure of starches (Pal *et al*., 2002; Singh *et al*., 2007). Hydroxypropilation of starches results in a water holding capacity and mobility of amylose chains increment. These modifications would lead to a higher swelling power than native starches (Pal *et al*., 2002). The inclusion of hydrophilic groups and the increment of swelling power caused loosening on the molecular network, allowing additional water to enter and remaining the starch intact during the thermal hydration treatment. This higher resistance to deformation results in a higher peak viscosity respect the native starches (Pal *et al*., 2002; Singh *et al*., 2007).

Cross-linking treatment consists in adding intra and intermolecular bonds at different locations in the starch granules in order to stabilize and strengthen their structure. This is especially useful for minimizing granule rupture and loss of viscosity during extended cooking times, as well as for increasing the resistance to amylases, resulting in a reduction of the extent of enzyme hydrolysis (Han & BeMiller, 2007; Singh *et al*., 2007). Cross-linked starches are obtained in the presence of a cross-linking reagent, such as phosphoryl chloride, sodium trimetaphosphate or sodium trypolyphosphate. This reagents are capable of forming ether or ester linkages between the hydroxyl groups presented in the molecular structure of starches (Guo *et al*., 2015).

- ***Physical modifications commonly used in food industry.***

One of the most commonly physically modified starch used in food industries are pregelatinized starches. They are basically prepared by gelatinizing an aqueous solution of starch granules during a mild heating treatment and, once gelatinization is reached, the paste is instantaneously dehydrated by drum-drying (Anastasiades et al., 2002; Lawal *et al*., 2015). This modification results in a product that rapidly dissolves in water, forming stable and viscous solutions without the need for high temperatures treatments (Anastasiades *et al*., 2002).

2.-AIM OF THE STUDY

Thickeners are commonly used as additives in infant products, which includes AR-infant formulas. However, no studies have been found about the effect of infant digestion process on physicochemical properties of these ingredients. With this background, the aim of this study was to analyze the evolution of apparent viscosity, solubility and molecular properties of LBG and modified corn and rice starches during an *in-vitro* digestion process adapted to infant conditions.

3.-MATERIALS AND METHODS

3.1.-Experimental design.

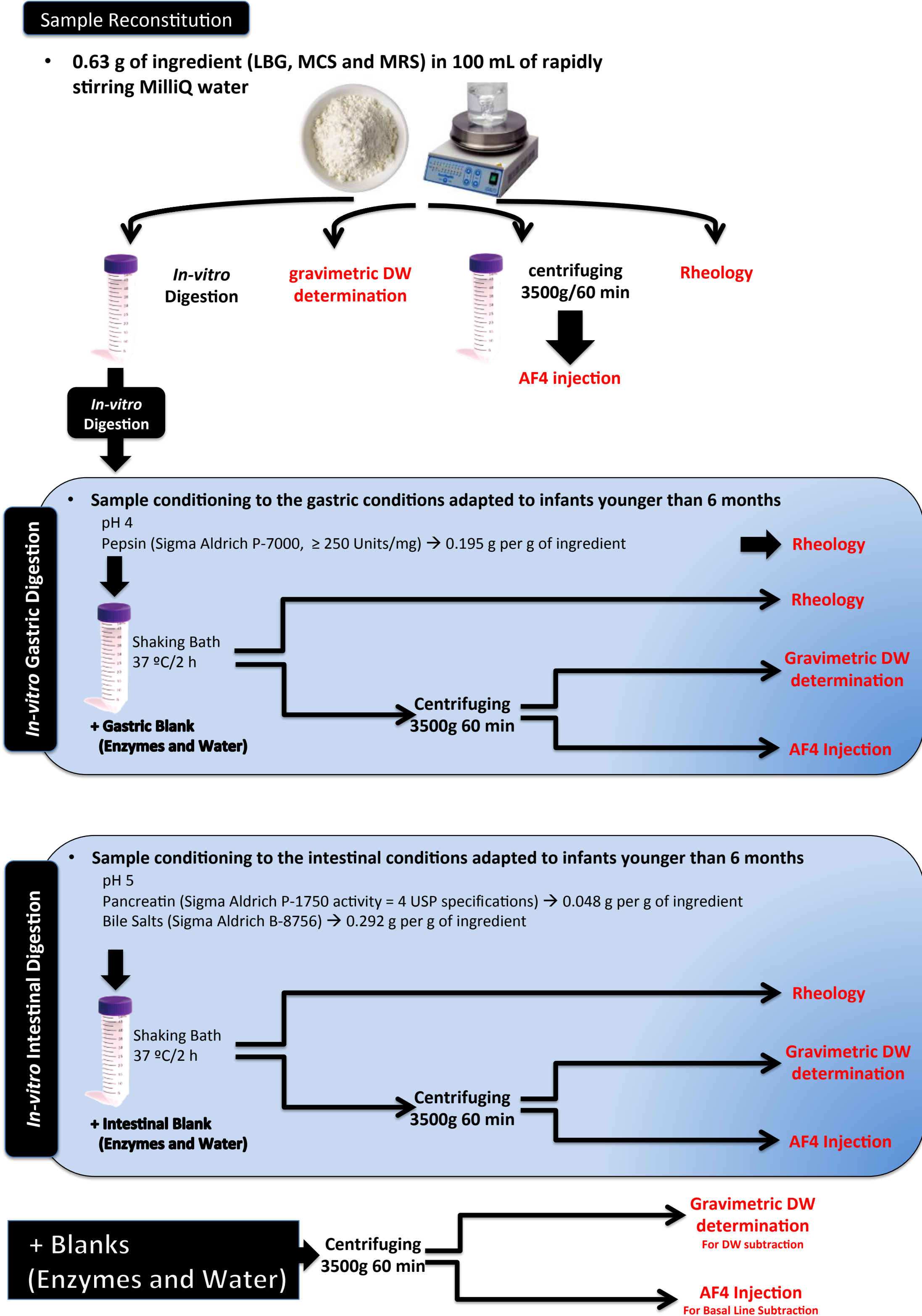


Figure 1.7.- *Workflow. It has been summarized the experiments carried out.*

3.2.- Samples and reconstitution.

LBG (Grindsted LBG 860, Danisco, Portugal), cross-linked maize hydroxypropyl distarch phosphate (Mhdp) (MCS; Multi-Thick®, Abbott Nutrition, Spain) and pre-gelatinized rice starch (gRS) (MRS; Beneo-Remy Industries, Belgium) were used as thickeners. Each ingredient was dispersed with rapid stirring in pre-heated deionised water (37 ºC) at a concentration of 0.63 g/100ml of thickeners with minerals added. Thickener concentrations were established according to the European legislation (European Parliament and Council, 1995; European Parliament and Council, 2006).

3.3- *In-vitro* digestion adapted to infant gastrointestinal conditions.

An *in-vitro* digestion method (Miller *et al*., 1981;Boato, *et al*., 2002; Minekus *et al*., 2014) adapted to the gastrointestinal conditions in infants younger than 6 months of age (Frontela, *et al*., 2008; Frontela, *et al*., 2009) was performed. The *in-vitro* digestion process consisted in a gastric and an intestinal stage. According to the standardized digestion protocol proposed by international consensus (Minekus *et al*., 2014) and, due to the fact that liquid samples were used, oral phase was skipped. The modifications for adapting the digestion to the gastrointestinal conditions of infants were related to the amount of enzymes added, as well as modifications in the pH values for each stage.

Before each phase, freshly enzyme solutions were prepared. To simulate the gastrointestinal conditions of children less than 6 months of age, a pepsin solution was prepared by dissolving 1.6 g of pepsin from porcine stomach mucosa (P-7000, Sigma-Aldrich, St. Louis, MO, USA) in 10 ml of 0.1 N HCl. The pancreatin-bile salts solution was

prepared by dissolving 0.2 g of pancreatin from porcine pancreas (P-1750, Sigma-Aldrich, St. Louis, MO, USA) and 1.25 g of bile salts (B-8756, Sigma-Aldrich, St. Louis, MO, USA) in 50 ml of 0.1 M $NaHCO_3$.

After samples reconstitution, pH was lowered to 4 using 1M HCL. After pH adjustment, pepsin solution was added in order to have 0.195 g of pepsin per gram of ingredient. Samples were incubated in a shaking water bath set at 37°C for 90 min. After incubation, *in-vitro* gastric digestion was stopped by soaking samples in an ice bath for 10 min. Previously to intestinal digestion, pH was raised to 5 by adding 6M $NaHCO_3$ and pancreatin-bile salts solution was added until a concentration of 0.048 g of pancreatin and 0.292 g of bile salts was reached. Samples were then maintained for 120 min in a shaking water bath set at 37 ºC. After the digestion process, samples were maintained under – 20 ºC until use. Figure 1.7 shows a scheme of the *in-vitro* digestion process.

3.4- Amount of ingredient solubilized during the *in-vitro* digestion process.

The apparent amount of ingredient solubilized during the different stages of the *in-vitro* digestion process was stablished according to a modification of the protocol described by Pollard *et al.*, (2007) and Nakorn *et al.*, (2009). Dry weight (DW) after dehydration (100 °C for 24 h) was measured by gravimetric techniques for each thickener without reconstitution, and after different stages within the *in-vitro* digestion process after centrifugation (3500g, 60 min, 4ºC). The selected stages were:

- Samples after reconstitution,
- samples after *in-vitro* gastric digestion and
- samples after *in-vitro* intestinal digestion.

The apparent dissolved amount of each ingredient for each stage was expressed as the percentage in weight of the dried supernatant (DW_f) respect to the initial weight of dry ingredient before reconstitution (DW_0). The gastric and intestinal enzymes dry weight (DW_e) were subtracted from the DW_f.

$$Apparent\ Disolved\ amount\ of\ ingredients\ (\%) = \frac{(DW_f - DW_e)}{DW_0}\ x\ 100$$

A laboratory incubator HORO (DrIng A Hofman, Germany) and a precision balance Mettler AE 160 (Mettler Germany) were used for determinations.

3.5.-Relative dynamic viscosity determination at different stages in the *in-vitro* digestion process:

A Rheometer Kinexus Pro (Malvern instruments, UK) equipped with ar Space data acquisition software was used. Analyses were carried out using a cup (2.7 cm of diameter) and bob (2.5 cm of diameter). Viscosity determinations were performed with a shear rate linear ramp method in a shear rate range of 0.00–1000 s-1 at 37ºC. For each sample, four different measurements were done:

- Samples after reconstitution (initial dispersion),
- samples just before *in-vitro* gastric digestion (pH 4 and gastric enzymes solution added),
- samples after *in-vitro* gastric digestion and
- samples after *in-vitro* intestinal digestion.

3.6.-Characterization of molecular properties by asymmetrical Flow Field Flow Fractionation (AF4)

The molecular properties were characterized using asymmetrical flow field-flow fractionation (AF4) coupled to multiangle light scattering (MALS) and differential refractive index (dRI) detection. AF4 is a chromatography-like technique that allows to fractionate and characterize macromolecules, and colloidal particles, in a very wide size range (2 nm – 1 µm). However, AF4 does not relay on an stationary phase, minimizing shear forces during separation. The principles of AF4 are described elsewhere (Wahlund & Giddings, 1987; Wahlund & Nilsson, 2012; Nilsson 2013). Briefly, this technique consists in the injection of samples in a channel composed by two clamping blocks and one spacer (figure 8a). The lower clamping block has an inset frit panel that allows a crossflow stream in a perpendicular direction respect to the axial or main flow stream. The name of Asymmetrical refers to the morphology of the channel. Once injected in the channel, all the components will be driven toward an ultrafiltration membrane or accumulation wall (placed inside the channel over the frit panel) by the crossflow, forming a thin concentrated layer. The accumulation membrane is permeable to the carrier liquid, but not the sample. This crossflow-induced transport will be compensated

by an opposite diffusional transport, yield by a Brownian motion of the particles (random moving of particles suspended in a fluid). After some time (focusing), the different components in the sample will became relaxed in relation to the cross-flow, being reorganized according to their diffusion capacity, in a steady-state concentration distribution. As the diffusion capacity depends on the size of the particles, the concentration average distance from the accumulation wall for the smallest components will be longer than for the larger ones. Once established the sample relaxation, a longitudinal flow will be applied. This is a laminar flow, with a parabolic front profile, being the velocity higher in the center of the channel and lower as the accumulation wall is approached. In this way, as the smallest sample components will be located far from the retention wall, they will be eluted more rapidly along the channel than the largest components (Figure 1.8b). Because the components emerge sequentially, they can be individually detected.

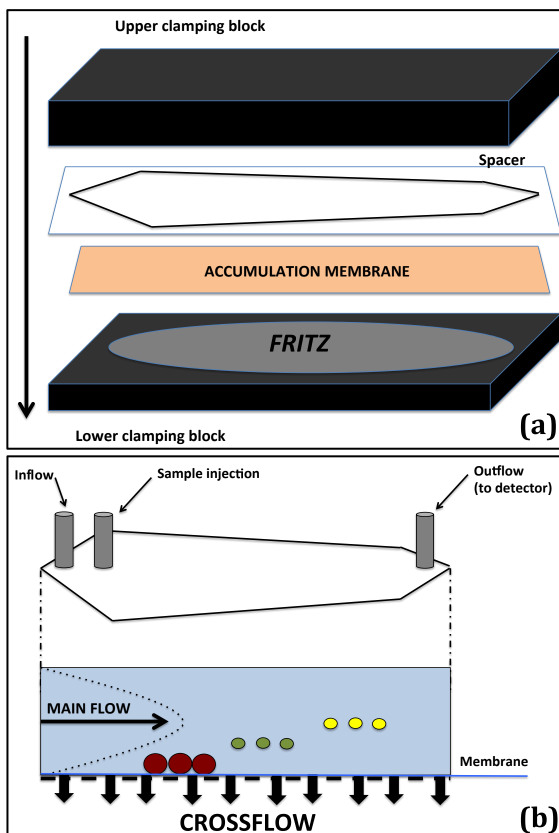


Figure 1.8.*-(a) The components of the AF4 channels have been represented. (b) The basis of molecules fractionation in which is based the AF4 method has been represented.*

AF4-MALS-RI has been shown to be a versatile tool for the characterization of food macromolecules (Nilsson, 2013).

After reconstitution and at the end of the gastric and intestinal stages, samples were centrifuged at 3500g for 1 h at 4 ºC. (Eppendorf 5804-R Centrifuge, Hamburg, Germany). The carrier used for AF4 was deionized water containing 10mM $NaNO_3$ (p.a. grade, Applichem, Darmstadt, Germany) together with 0.02% (w/v) NaN_3 (S-8032, Sigma-Aldrich, Steinheim, Germany). Before the analyses, the carrier liquid was filtered through a 0.20 µm pore-size cellulose acetate filter (Whatman OE 66, Whatman, Dassel, Germany) to remove possible particulate impurities.

The analyses were carried out using an Eclipse 3+ System (Wyatt Technology, Santa Barbara, CA, USA) serially connected to a MALS detector (Dawn Heleos II ,Wyatt Technology) and a differential refractive index (dRI) detector (Optilabt Rex, Wyatt Technology). Both detectors operated at a wavelength of 658 nm. A short AF4 channel (SC-050) having a tip-to-tip length of 17.4 cm (Wyatt Technology) assembled with a 350 µm spacer and an ultrafiltration membrane (regenerated cellulose) with a 10-kD cut-off (Millipore, Billerica, MA, USA) was used. The channel thickness was determined, using ferritin, to be 309 µm (Håkansson *et al.*, 2012; Magnusson *et al.*, 2012). The carrier liquid was delivered by an Agilent 1100 Series Isocratic HPLC Pump (Agilent Technologies, Germany) with an inline vacuum degasser. Injected amounts were optimized to ensure no overloading of the channel while still maximizing the signal-to-noise ratio in the detectors. This resulted in injected amounts of approximately 20-100 µg injected material for the ingredients, as has been detailed in Table 1.3.

Table 1.3.- *Concentrations and amount of samples injected for each in-vitro digestion stage selected for the AF4 analyses.*

Sample	LBG		Mhdp		gRS	
	Concent (g/mL)	**Injected (g)**	**Concent (g/mL)**	**Injected (g)**	**Concent (g/mL)**	**Injected (g)**
Without digestion	$4.4\ 10^{-3}$	$2.18\ 10^{-5}$	$5.18\ 10^{-4}$	$5.18\ 10^{-5}$	$9.04\ 10^{-4}$	$9.04\ 10^{-5}$
Gastric digestion	$4.4\ 10^{-3}$	$2.18\ 10^{-5}$	$8.32\ 10^{-4}$	$8.32\ 10^{-5}$	$1.02\ 10^{-3}$	$1.02\ 10^{-4}$
Intestinal digestion	$4.4\ 10^{-3}$	$2.18\ 10^{-5}$	$2.55\ 10^{-2}$	$1.70\ 10^{-3}$	$1.53\ 10^{-2}$	$1.02\ 10^{-3}$

Each sample was injected at a flow of 0.2 mL/min. The total time for the injection/relaxation step was 6 min. The crossflow during the relaxation step was 2 mL/min. During elution, crossflow decaying with time were used in order to speed up analysis and to avoid excessive retention in the channel. The exponentially decaying crossflow was calculated according to the following expression:

$$Q_c(t) = Q_o(0)e^{\left(-\frac{ln2}{t_{1/2}}\right)t}$$

where Q_c is the volumetric crossflow Q_0 is the initial volumetric crossflow, $t_{1/2}$ is the half-life of the decay and t is the time. In the present case Q_0=2mL/min and $t_{1/2}$=6min. The flow from the channel to the detectors was 0.5 mL/min. After 25 min of elution, a constant Q_c of 0.09 mL/min was maintained for 19 min after which Q_c was turned off. After elution, the channel was flushed without crossflow for 3 min before the next injection. Processing of light scattering data was performed with the Astra software, version 5.3.4.20 (Wyatt Technology). The M and the root-mean-square radius (r_{rms}) were obtained by fitting the light scattering data with the Berry method (Andersson *et al.*, 2003; Berry 1966) fitting a straight line to data obtained at 50.0-126.0° scattering

angle. A dn/dc value of 0.146 $mL \cdot g^{-1}$ was used and the second virial coefficient was assumed to be negligible. Differential distributions where obtained from fitting of the relevant data with a 3rd degree exponential fit with the Astra software (Wyatt Technology).

The hydrodynamic radius (r_h) was obtained from the Stokes-Einstein equation (Einstein, 1905)

$$r_{h,i} = \frac{k_b T}{6\pi\eta D_i}$$

where k_b is the Boltzmann constant, T is the temperature, η is the dynamic viscosity of the solvent and D_i is the diffusion coefficient. In turn, the diffusion coefficient was obtained from AF4 retention times (Håkansson *et al*., 2012; Magnusson *et al*., 2012).

The apparent densities were obtained from the M and r_h assuming a homogeneous distribution of mass and a spherical shape. Hence, the apparent density is an apparent property. The apparent density, ρ_i, for component I of the sample is calculated from

$$\rho_{h,i} = \frac{M_i}{V(r_h)_i N_A}$$

where$M$ is the molar mass, $V(r)$ is the volume of a sphere with radius r_h and N_A is the Avogadro constant. The mass-weighted average apparent density was obtained from

$$\bar{\rho} = \frac{\sum m_i \cdot \rho_i}{\sum m_i}$$

where m_i is the mass flow in each class (i).

3.7.- Statistical analysis:

Previously to Statistical analysis, data normality using the Kolmogorov-Smirnov test and homoscedasticity by the Levene test was confirmed. All the experiments were carried out six times per sample (n=6), and the results were reported as means ± SD. Data were compared for the same sample between digestion stages, and between different samples within the same digestion stage, by one-way analysis of variance (ANOVA) and a Tukey post-test for multiple comparisons to determine the significance of the effects of the different thickening agents used as well as the effect of the digestion process ($p<0.05$). All statistical analyses were performed with the Statistical Package for the Social Sciences (IBM SPSS Statistics version 19; SPSS Inc., Chicago, IL, USA).

4.-RESULTS AND DISCUSSION

4.1.-Amount of ingredient dissolved during the *in-vitro* digestion process:

Table 1.4 shows the apparent solubility percentages obtained for LBG, Mhdp and gRS after three different stages of the *in-vitro* digestion process: (1) After reconstitution, (2) after gastric digestion and (3) after intestinal digestion.

Table 1.4.- *LBG, Mhdp and gRS apparent solubility percentages obtained during different stages of an in-vitro digestion process, adapted to the gastrointestinal conditions in infants younger than 6 months of age. Values are displayed as mean ± S.D. Different superscripts (a, b, c) denote significant differences (p <0.05) for each ingredient between the different stages of in-vitro digestion process.*

	Solubility %		
	LBG	**Mhdp**	**gRS**
After reconstitution	$64.84 \pm 3.09^{a,b}$	3.40 ± 0.54^{c}	5.94 ± 0.54^{b}
After gastric digestion	69.83 ± 4.48^{a}	5.46 ± 0.42^{b}	6.67 ± 0.27^{b}
After intestinal digestion	61.61 ± 4.50^{b}	74.35 ± 6.67^{a}	67.19 ± 3.71^{a}

As can be seen in table 1.4, under the conditions of the study, LBG showed the highest apparent solubility after reconstitution (higher than 60%) remaining quite stable during the *in-vitro* digestion process. However, apparent solubility of LBG after intestinal digestion was significantly lower than the apparent solubility after gastric digestion. No differences were found between the initial solubility (after reconstitution) and after both stages, gastric and intestinal digestion.

Solubility values obtained for LBG were in concordance with the results showed by Dakia *et al*., (2008), who obtained a partial solubility (50%) in water at 25 ºC/1h, becoming more soluble as the temperature increased (70 – 85% at 80 ºC/30 min). Pollard, *et al*., (2007) obtained similar results using similar approaches to determine apparent solubility. These studies also showed a dependence of solubility on M. About 50% of LBG was soluble at 5 ºC, corresponding to the lower M fraction (M ≈ 1060 Kg/mol) and a higher degree of galactose substitution (≈ 0.35). According to them, the solubility dependence on temperature was stronger as M increased and the degree of substitution decreased, reaching the maximum solubility (70–85%) at 80ºC. Constant apparent solubility values across all the process could be due to the resistance of LBG to the *in-vitro* digestion process, being classified as an indigestible carbohydrate (Ikegami, *et al*., 1990; Bosscher *et al*., 2000; Fabek *et al*., 2014). If the ingredient remains intact across the digestion process, the water solution stability showed across all the process could be related to the residue of galactose. In this regard Silveira & Bresolin (2011) described that these galactose residues could be responsible of water suspension stability by inhibiting the packaging of the mannan chains and by their freedom rotation about the (1 → 6) linkages. The statistical differences described between gastric and intestinal stage could be attributed to the pH values. However, LBG is a nonionic polysaccharide and the solubilization process should be independent of pH (Venkataraju *et al*., 2007).

Compared to LBG, both starches (Mhdp and gRS) showed a very low apparent solubility after reconstitution and gastric digestion, (3.4 – 6.7%). Solubility of starches is intimately correlated to swelling by water percolation. Water molecules first enter the amorphous regions causing it to swell. This result in a disruptive stress transmitted to

the crystalline regions and in the leaching of amylose from the starch granules. These modifications will result in a solubility increase (Tester & Morrison 1990; Jenkins & Donald 1998; Singh *et al*., 2003; Copeland 2009; Chung *et al*., 2011). All this process will be facilitate by thermal treatment and physicochemical modifications. Increasing temperature results in a disruption of crystalline structure, increasing swelling power and solubility (Tester & Morrison, 1990; Singh et al., 2003; Chung et al., 2011). In this regard, it has to be taken into account that the experiments have been performed at 37 ºC, which means a mild thermal treatment. Regarding modification, chemically and physically modified starches (Mhdp and gRS respectively) were considered in the experiments performed.

About Mhdp, it has been described that hydroxypropilation mainly occurs in amorphous regions, which promotes swelling and thus, facilitates the disruption of the crystalline structure. As consequence, when compare with native starches, a lower temperature for destabilization would be needed (Singh *et al.*, 2007). In this regard, Liu *et al*., (1999) analyzed the physical properties of different hydroxipropilated maize starches and compare them with the native maize starch. These authors reported that hydroxypropilation resulted in an increment of swelling power and solubility. However, the crystalline transition temperatures, considered as the temperature needed for melting the crystalline structure (Liu *et al*., 2009), for Hydroxypropilated (59 – 75 ºC) and for native starch (65 – 81 ºC) were remarkably higher than the temperature used in our experiment (37 ºC). Waliszewski *et al*., (2003) compared the solubility of native and hydroxypropilated starches. These authors reported similar solubility between both kinds of starch in a temperature range of 10 to 60 ºC. Nevertheless, a more than double

difference between solubility of native and hydroxypropilated starch was reported when the temperature increasedto 70 ºC.

Despite the low solubility reported, statistical differences were obtained when compared Mhdp apparent solubility after reconstitution (3.40 % ± 0.54) and after gastric digestion (5.46 ± 0.42). These differences could be attributed to the hydration and swelling of starch molecules during the water incubation. This dependence between time and starch solubilization, have been reported by other authors (Yeh & Yeh, 1993).

With regard to pregelatinization, it has been defined as a physical modification of starches that provides a high solubility in water without the need for high temperatures treatments (Anastasiades *et al*., 2002). However, the apparent solubility obtained after reconstitution and after gastric digestion were surprisingly low, (5.94 % ± 0.54) and (6.67 % ± 0.27) respectively, with no statistical differences between them. These results are in agreement with those reported by Nakorn *et al*., (2009). These authors studied water solubilization at 30 ºC for four rice starches, which included native rice starch and three different kinds of pregelatinized starches. Differences consisted in their amylose content: high amylose content (20.2%), medium amylose content (15%) and low amylose content (1.4%). According to their results, pregelatinized starches showed, in all cases, a significantly higher solubility than native rice starch. However, solubility values for pregelatinized starches with a high and medium amylose content remained under 10%. Only pregelatinized rice starch with a low amylose content show solubility values higher than 20%. It has been described that, an increase in amylose content causes a decreased destruction of starch granules during pregelatinization process and

hence, decreases water solubility (Tester & Morrison, 1990; Singh *et al*., 2003; Nakorn *et al*., 2009; Chung *et al*., 2011).

Compared to the previous stages (after reconstitution and after gastric digestion), apparent solubility significantly increased ($p<0.05$) during the intestinal digestion, reaching values of about 70% for both kind of modified starches analyzed, gRS and Mhdp. These findings most likely reflect that the starches are being degraded during the *in-vitro* intestinal digestion process being in agreement with previous studies that indicate that starches are mainly digested in the small intestine by α-amylase (Frei *et al*., 2003; Lehmann & Robin, 2007; Singh *et al*., 2007).

4.2.-Relative dynamic viscosity during the *in-vitro* digestion process:

The viscosity of the LBG samples, as a function of the increasing shear rate, is shown in figure 9. According to the results obtained, LBG showed a non-Newtonian behavior when shear viscosity was determined at 37ºC, over a shear rate range from 0 to 1000 s^{-1}. This rheological behavior defined is in agreement with the results reported by Haddarah *et al*., (2014).

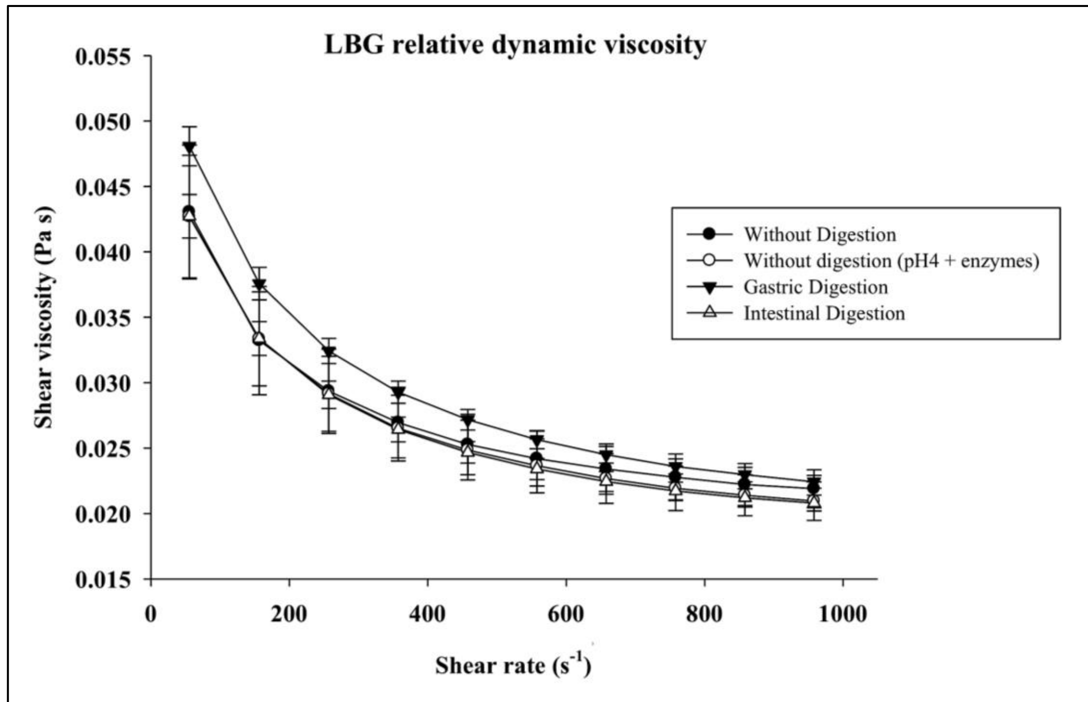


Figure 1.9.- *Locust Bean Gum shear viscosity (Pa s) against Shear rate (s-1) measured at 37 ºC during the different phases of the in-vitro digestion process.*

No significant differences ($p<0.05$) between the apparent viscosity values obtained for the different digestion phases included in the study were observed. These values remained quite stable during digestion, which reflects the resistance of LBG to the digestion process i.e. changes in pH, digestive enzymes, and possible influence of bile salts. This resistance has been also reported by other authors (Dey *et al*., 2012; Fabek *et al*., 2014). Although not significant differences in LBG viscosity during the in-vitro digestion process were found, at shear rates<400$s^{-1}$the average viscosity values of LBG during gastric digestion were slightly higher than the average values obtained during the intestinal stage. These slight differences could be due to a dilution effect by the addition of bile salts and pancreatin solution as it has been previously explained by Fabek *et al*., (2014).

The relative dynamic viscosity values obtained for modified starches are shown in table 1.5. Under the experimental conditions used in this work, both gRS and Mhdp behaved as Newtonian fluids in a range from 0 to 50 s^{-1}. In this shear rate range, viscosity values were independent of shear rate.

Table 1.5*. Relative dynamic viscosity (Pa s) respect the dynamic viscosity for MilliQ® water (37 ºC), for modified starches. Shear rate range 0.00 – 50 s^{-1}. Values are displayed as mean ± S.D. Values with different superscript (a,b) denote significant differences (p <0.05) for each ingredient during the different phases of in-vitro digestion process.*

	Relative Dynamic Viscosity (Pa s)	
***In-vitro* digestion phase**	**Mhdp**	**gRS**
After reconstitution	$1.07\ 10^{-4} \pm 0.05\ 10^{-4b}$	$1.07\ 10^{-4} \pm 0.01\ 10^{-4b}$
Before digestion (pH4 + enzymes)	$1.06\ 10^{-4} \pm 0.02\ 10^{-4b}$	$1.07\ 10^{-4} \pm 0.03\ 10^{-4b}$
After gastric digestion	$1.20\ 10^{-4} \pm 0.06\ 10^{-4a}$	$1.25\ 10^{-4} \pm 0.05\ 10^{-4a}$
After intestinal digestion	$1.07\ 10^{-4} \pm 0.03\ 10^{-4b}$	$1.08\ 10^{-4} \pm 0.03\ 10^{-4b}$
Dynamic viscosity at 37ºC MilliQ water (Pa s)	$5.97\ 10^{-4} \pm 1.88\ 10^{-5}$	

As can be seen in table 1.5, the relative dynamic viscosity values reported for both starches during the in-vitro digestion process were lower than those reported for LBG, and similar for both kind of starches analyzed (Mhdp and gRS). In both cases, relative dynamic viscosity remained stable after reconstitution and after pre-gastric stage conditioning (pH adjustment to 4 and addition of pepsin solution), with values above 1.07 10^{-4} Pa s^{-1}. However, the values obtained after the *in-vitro* gastric digestion were significantly higher (p<0.05) than the values reported for the previous stages (above 1.20 10^{-4} Pa s^{-1}). After the *in-vitro* intestinal stage, the apparent dynamic viscosity decreased, reaching values similar to the ones obtained for at the beginning of the process.

The viscosity of starch suspensions basically depends on the temperature treatment, swelling capacity of the starch granules and the amount of polysaccharide leached during this process. Increasing the temperature, results in an increment of starch granules swelling and leaching of amylose. During this process, the viscosity of the suspension will increase due to the formation of a tridimensional network of swollen granules. (Singh *et al*., 2003; Singh et al., 2007; Copeland *et al*., 2009). This mechanism, together with the results obtained for starch solubility reported in table 1.4, supports the relative dynamic viscosity values show in table 1.5. In this way, a low solubility value during samples reconstitution should be related to a reduced swelling of starch granules, explaining the low relative dynamic viscosity values obtained. The significant viscosity increment reported after gastric digestion could be due to the progressive hydration and swelling of starch molecules during the water incubation, what is supported by the significant higher values previously reported for Mhdp during gastric digestion when compared with the other stages (table 1.4). After *in-vitro* intestinal digestion, the reduction of relative dynamic viscosity reported in table 1.5 could be explained by the enzyme degradation of starches (Topping & Cliffton, 2001; Copeland *et al*., 2009), causing in the same time a solubility increment as has been reported in table 1.4.

Under the experimental condition previously defined, LBG showed a higher thickening capacity than the starches when was added at the same concentration level. The higher thickening capacity in respect to starch, has been also reported by other authors (Fabek *et al*., 2014). In order to increase the relative dynamic viscosity provided by Mhdp and gRS, a higher thermal treatment should be performed during the ingredients reconstitution (Singh et al., 2007). With the same aim, a reduction of

amylose content could be effective as has been reported by different authors (Tester & Morrison, 1990; Singh *et al.*, 2003; Nakorn *et al.*, 2009; Chung *et al.*, 2011).

4.3.- Asymmetrical Flow Field Flow Fractionation (AF4):

Thickeners were analyzed with AF4-MALS-dRI in order to determinate the M distribution and conformational properties before and after digestion. Regarding LBG, it was disqualified for AF4 analysis as it proved to be highly aggregated to a large extend under the conditions of the study. In order to be analyzed by AF4 a more extensive dissolution should be needed, for instance, by increasing temperatures during reconstitution (Pollard & Fischer, 2006; Pollard *et al.*, 2007).

Modified starches (Mhdp and gRS) were analyzed by AF4 at three different sampling times across the digestion process: (1) after reconstitution, (2) after gastric digestion and (3) after intestinal digestion.

The results obtained for the first sampling time, after reconstitution, can be seen in figures 10a and 10b. For Mhdp (figure 10a) and gRS (figure 10b), M ranges were between approximately $3·10^4$ to $2·10^7$ g/mol and between approximately $3·10^4$ to 10^7 g/mol, respectively. The similarities may reflect that a similar M range is “dissolved” for both starches under the reconstitution conditions used (rapidly stirring deionized water at 37 ºC). This may also be reflected in the similar averages of M and hydrodynamic radius (r_h) obtained for both starch samples (Table 6). The mass recovery from the separation channel was also rather low 46-49% (Table 6). It has to be taken into account that, the mass recovery determination is based on a gravimetric analysis as thus most

likely contains an error from low M species which are flushed out through the membrane in the separation channel. A further observation indicates that low M analytes could be lost is the fact that the lower end of the M detected is approaching the cut-off of the membrane (i.e. 10^4 g/mol). Hence, the recovery values are highly apparent but they do give a rough estimate of differences/similarities between the samples.

Regarding the results obtained after gastric digestion stage, at short retention times, a large dRI-peal was observed for both starches. However, it was not present in the sample before gastric digestion, corresponding to the pepsin added in the digestion protocol. This deduction was supported by analyzes of a blank sample not containing starch. After gastric digestion the MALS-peak was shifted towards longer retention times (i.e. larger hydrodynamic radius) (Figure 1.10 a-b).Similarly, the presence of higher M analytes were observed after digestion, which was more pronounced for gRS. It can also be noted that late eluting analytes (elution time > 25min), in the digested samples, appear to have a higher M at the same elution time in compared to the sample before digestion. The result reflects a higher apparent density of the analytes i.e. higher M confined in a similar r_h, which is also reflected in a higher average apparent density after digestion (Table 6). One possibility is that the starch is slowly dissolving to a higher extent during the gastric digestion step solely as a result of longer hydration time. In order to investigate this a longer reconstitution time (the same time as reconstitution + gastric digestion would take), without gastric digestion, was investigated. No effect of this longer reconstitution time was observed and the resulting M distribution was identical to that of the short reconstitution time. Thus, this explanation could be ruled out. It is possible that, the gastric digestion, results in some type of aggregation of starches but it is, however, difficult to understand what would be the origin of this effect.

The increased size after digestion does, on the other hand, correlate to the increase in viscosity observed as a result of gastric digestion (Table 6).

Table 1.6. *Average molar mass (M), hydrodynamic radius (r_h), apparent density (ρ_{app}) and separataion channel mass recovery for Mhdp and gRS before and after gastric digestion.*

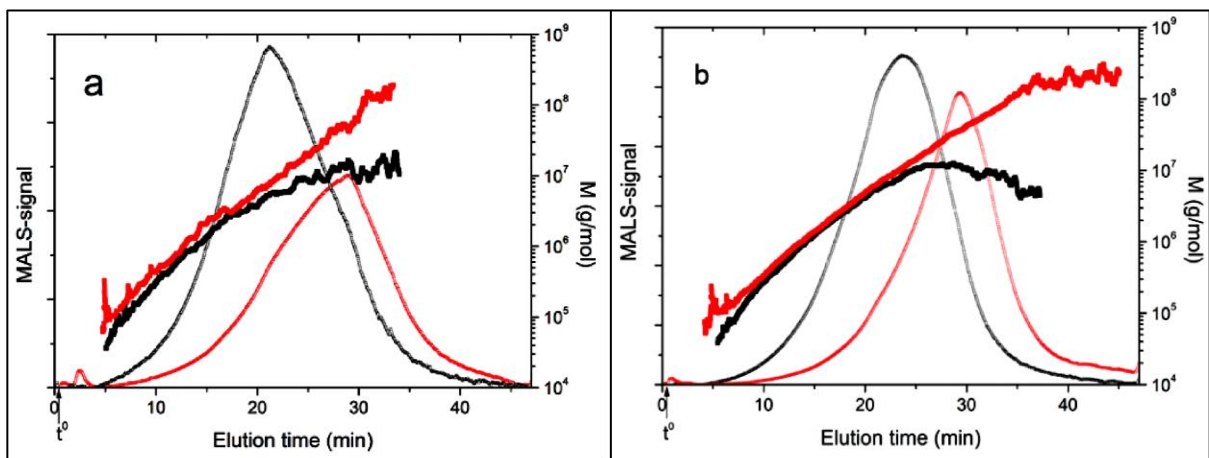
Sample	M^a (g/mol)/10^6	r_h^b (nm)	ρ_{app}^a (kg/m^3)	Recovery^c (%)
Mhdp after reconstitution	3.7	34	49	46
Mhdp after gastric digestion	19	34	73	53
gRS after reconstitution	3.5	34	37	49
gRS after gastric digestion	20	50	41	62

[a]*Weight average.*

[b]*z-average.*

[c]*based on the integrated dRI signal in relation to the injected mass determined gravimetrically.*

Figure 1.10*.-AF4 fractograms (MALS-signal at 90° scattering angle) and molar mass vs. elution time of Mhdp (a) and gRS (b) before (black) and after (red) gastric digestion. t^0 indicates the void time.*



Figures 11a-b and 12a-b show the conformational properties (apparent density and the ratio of the root-mean-square radius, r_{rms}, and r_h) of Mhdp and gRS, over the distribution of M, before and after digestion. In the figures, r_{rms}/r_h cannot be obtained for the lower end of the M distribution due to the theoretical limitations in determining r_{rms} at wavelength at which the MALS-detector operates (i.e. the analytes are too small to obtain a reliable determination of r_{rms}). The results show that, before digestion, the high M species in the distribution had an r_{rms}/r_h of approximately 0.6-1 (Figure 1.11a and 1.12a). The lower values are, however, rather inaccurate as the error is high (±15-50%) at an M of approximately $1\text{-}2{\cdot}10^6$ g/mol which, in turn, depends on the weak angular dependence of the light scattering in this size range. Hence, the ratios for $M<2{\cdot}10^6$ should most likely be considered erroneous and not interpreted. At higher M this problem is, however, not present. At $M>2{\cdot}10^6$ g/mol, r_{rms}/r_h is approximately 0.9-1.1 which corresponds to a hyperbranched structure (Burchard, 1999). Similar values obtained by various methods have previously been reported for amylopectin (Galinsky & Buchard, 1995; Roger *et al.*, 1999). The apparent density decreases with increasing M, what correlates well with branched species growing in size. After gastric digestion the distribution of M shifts upwards to higher values (as discussed above) (Figure 1.11b and 12b). The apparent density for the starches after gastric digestion also decreased with increasing M. However, for low M species (approximately 10^5 g/mol) the apparent density was considerably higher in both starches (Figure 1.11b and 1.12b). The r_{rms}/r_h remains between approximately 0.9 - 1.1, suggesting that the analytes are hyperbranched. Similarly to what was discussed above for the non-digested starches the error in the determination of r_{rms} increases considerably for lower M and, thus, r_{rms}/r_h at $M<2{\cdot}10^6$ shows unreliable values

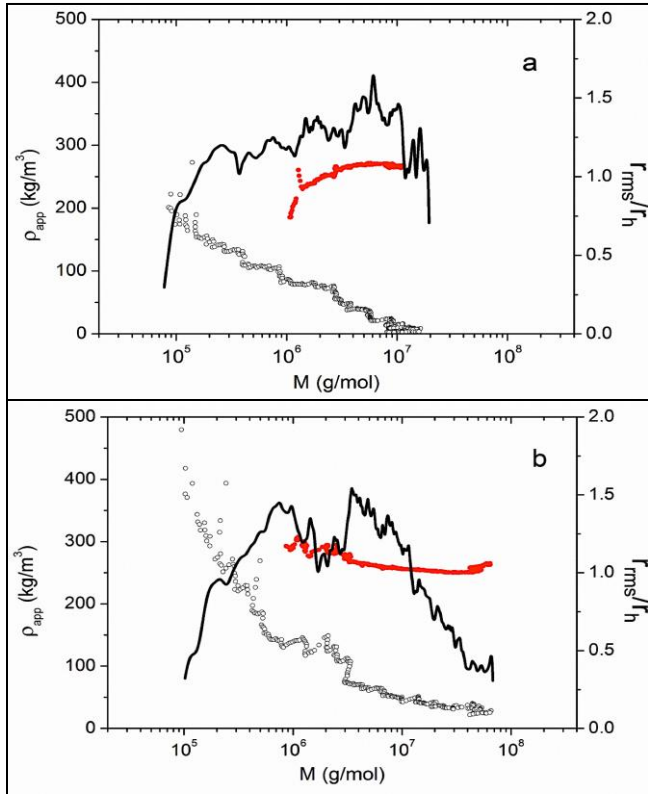


Figure 1.11.- *Differential molar mass distribution, apparent density (ρapp, open circles) and rrms/rh (red dots) obtained by AF4-MALS-dRI for Mhdp after reconstitution (a) and after gastric digestion (b).*

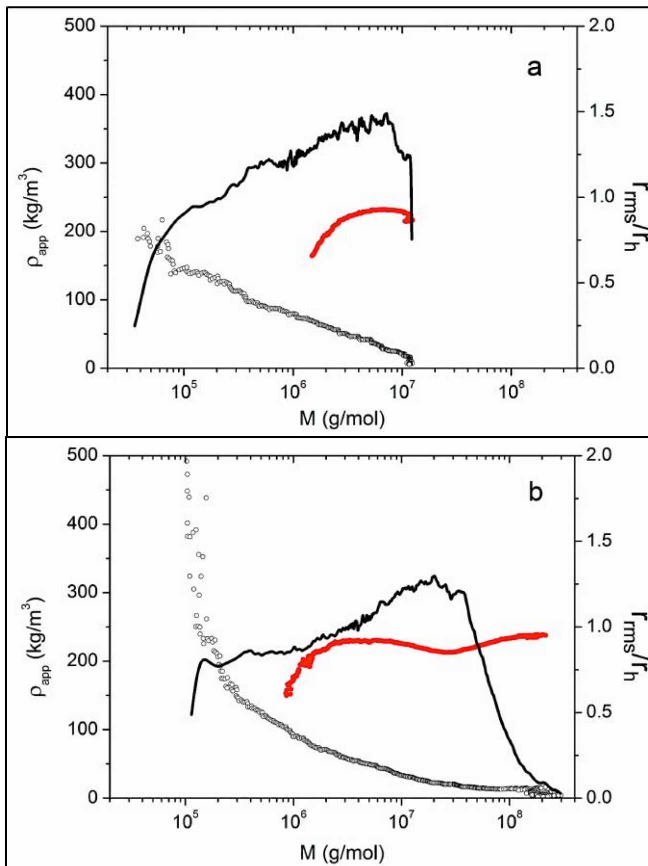


Figure 1.12.-*Differential molar mass distribution, apparent density (ρapp, open circles) and rrms/rh (red dots) obtained by AF4-MALS-dRI for gRS after reconstitution (a) and after gastric digestion (b)*.

After intestinal digestion the samples were heavily degraded and no detector signals were observed in the AF4-analysis.Most likely the starches were degraded into oligosaccharides and glucose, smaller than the cut-off of the accumulation wall membrane in the AF4 separation channel and were, thus, lost. A representative example fractogram of a digested sample (Mhdp) is shown in figure 13.

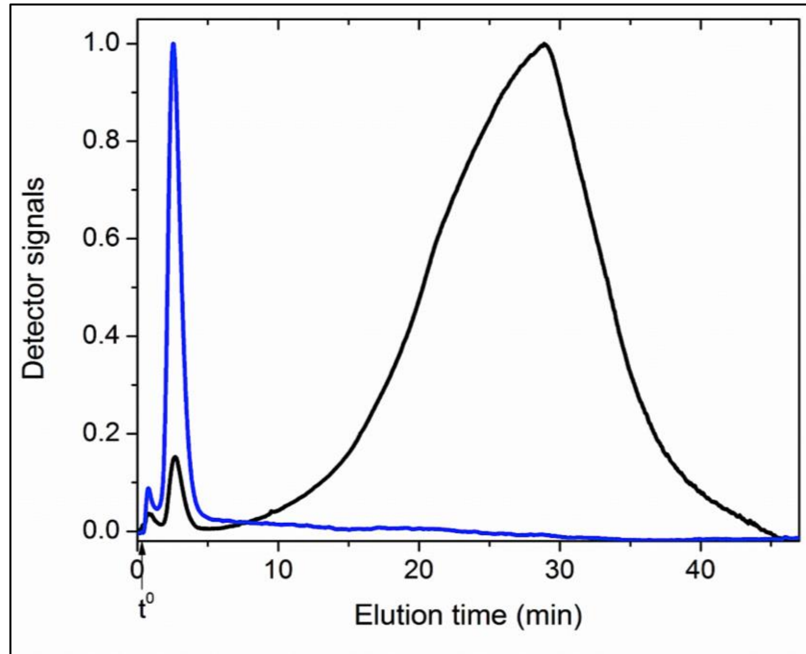


***Figure 1.13**. AF4 fractograms of Mhdp after intestinal digestion. Black trace: MALS-signal at 90° scattering angle, blue trace: dRI-signal.t0 indicates the void time.*

5.-CONCLUSIONS

Under the experimental conditions detailed, LBG seems to be a more effective thickener than modified starches due to the higher viscosity values reported and its resistance to the in-vitro digestion process. In this regards, modified starches, Mhdp and gRS, provide a lower viscosity, which is confined to the gastric environment, being completely degraded after an intestinal digestion. In order to increase thickening capacity of starches a high temperature treatment and long hydration time during initial reconstitution, combined with a modification in the amylose content could be effective. However, it has to be taken into account that this thickeners are added, among other uses, to infant food, in which high heat treatments or genetic modifications are not recommended or permitted.

6. BIBLIOGRAPHY

Aggett, P.J., Agostoni, C., Goulet, O., Hernell, O., Koletzko, B., Lafeber, H.L., *et al.* (2002). Antireflux or Antiregurgitation Milk Products for Infants and Young Children: A Commentary by the ESPGHAN Committee on Nutrition. Journal of Pediatric Gastroenterology & Nutrition; 34(5): 496 – 498.

Andersson, M., Wittgren, B., & Wahlund, K. G. (2003). Accuracy in multiangle light scattering measurements for molar mass and radius estimations.Model calculations and experiments.Analytical Chemistry; 75(16): 4279 – 4291.

Anastasiades, A., Thanou, S., Loulis, D., Stapatoris, A., & Karapantsios, T. D. (2002). Rheological and physical characterization of pregelatinized maize starches. Journal of Food Engineering; 52(1): 57 – 66.

Ashogbon, A. O., & Akintayo, E. T. (2014). Recent trend in the physical and chemical modification of starches from different botanical sources: A review. Starch ‐ Stärke; 66(1-2): 41-57.

Barak S & Mudgil D. (2014). Locust bean gum: Processing, properties and food applications—A review. International Journal of Biological Macromolecules; 66: 74 – 80.

Batlle, I., & Tous, J. (1997). Carob tree. Ceratoniasiliqua L. Promoting the conservation and use of underutilized and neglected crops. 17.Institute of Plant Genetics andCrop Plant Research, Gatersleben/International Plant Genetic ResourcesInstitute: Rome, Italy.

Bemiller, J. N. (1997). Starch modification: challenges and prospects. Starch ‐ Stärke; 49(4): 127 – 131.

Berry, G. C. (1966). Thermodynamic and conformational properties of polystyrene. I. Light-scattering studies on dilute solutions of linear polystyrenes. Journal of Chemical Physics; 44(12): 4550 – 4564.

Boato, F., Wortley, G.M., Liu, R.H. & Glahn, R.P. (2002). Red grape juice inhibits iron availability: application of an in-vitro digestion/Caco-2 cell model. Journal of Agricultural and Food Chemistry; 50: 6935 – 6938.

Bosscher, D., Van Caillie-Bertrand, M., Van Dyck, K., Robberecht, H., Van Cauwenbergh, R., & Deelstra, H. (2000). Thickening infant formula with digestible and indigestible carbohydrate: Availability of calcium, iron, and zinc in-vitro. Journal of Pediatric Gastroenterology and Nutrition; 30: 373–378

Butterworth, P. J., Warren, F. J., & Ellis, P. R. (2011). Human α- amylase and starch digestion: An interesting marriage. Starch ‐ Stärke; 63(7): 395 – 405.

Burchard, W. (1999). Solution properties of branched macromolecules. Advances in Polymer Science, 143: 113 – 194.

Chung, H.J., Liu, Q., Lee, L. & Wei, D. (2011). Relationship between the structure, physicochemical properties and in-vitro digestibility of rice starches with different amylose contents. Food Hydrocolloids; 25: 968 – 975.

Cichero, J.A.Y., Nicholson, T.M. & September, C. (2013). Thickened Milk for the Management of Feeding and Swallowing Issues in Infants: A Call for Interdisciplinary Professional Guidelines. Journal of Human Lactation; 20(10): 1 – 4.

Coviello, T., Alhaique, F., Dorigo, A., Matricardi, P. & Grassi, M. (2007). Two galactomannans and scleroglucanas matrices for drug delivery: Preparation and release studies. European Journal of Pharmaceutics and Biopharmaceutics; 66: 200 – 209.

Copeland, L., Blazek, J., Salman, H., & Tang, M. C. (2009). Form and functionality

of starch.Food Hydrocolloids; 23(6): 1527 – 1534.

Dakia, P.A., Blecker, C., Robert, C.,Wathelet B., Paquot M. (2008). Composition and physicochemical properties of locust bean gum extracted from whole seeds by acid or water dehulling pre-treatment. Food Hydrocolloids; 22: 807–818.

Daas Piet, J. H., ScholsHenk, A., & De JonghHarmen, H. J. (2000). On the galactosyl distribution of commercial galactomannans.Carbohydrate Research; 329(3): 609 – 619.

Dey P, Maiti S, Biswanath SA. (2012). Locust bean gum and its application in pharmacy and biotechnology: an overview. International Journal of Current Pharmacological Research;4:7 – 11.

Englyst, H. N., Kingman, S. M., & Cummings, J. H. (1992). Classification and measurement of nutritionally important starch fractions.European journal of clinical nutrition; 46: 33 – 50.

European Parliament and Council. Directive No 95/2/EC of 20 February 1995 on food additives other than colours and sweeteners. **(http://eurlex.europa.eu/en/index.htm)**

EuropeanParliamentandCouncil. Directive 2006/141/EC on infant formulae and follow-on formulae. **(http://ec.europa.eu/food).**

Fabek H., Messerschmidt S., Brulport V. & Goff H.D. (2014). The effect of in-vitro digestive processes on the viscosity of dietary fibres and their influence on glucose diffusion.Food Hydrocolloids; 35: 718 – 726.

Fernandez, C., Rojas, C.C. & Nilsson, L. (2011). Size, structure and scaling relationships in glycogen from various sources investigated with asymmetrical flow field-flow fractionation and 1H NMR. International Journal of Biological Macromolecules; 49: 458 – 465.

Frei, M., Siddhuraju, P. & Becker, K. (2003). Studies on the in-vitro starch digestibility and the glycemic index of six different indigenous rice cultivars from the Philippines. Food Chemistry, 83(3); 395 – 402.

Frontela, C., Haro, J.F., Ros, G. & Martinez, C. (2008). Effect of Dephytinization and Follow-on Formula Addition on in-vitro Iron, Calcium, and Zinc Availability from Infant Cereals.Journal of Agricultural and Food Chemistry; 56: 3805 – 3811.

Frontela, C., Ros, G. & Martinez, C. (2009). Iron and calcium availability from digestion of infant cereals by Caco-2 cells.European Food Research and Technology; 228: 789 – 797.

Galinsky, G., & Burchard, W. (1999). Starch fractions as examples for non-randomly branched macromolecules. 1. Dimensional properties. Macromolecules; 28: 2363 – 2370.

Gray, J. A., & BeMiller, J. N. (2005). Influence of reaction conditions on the location of reactions in waxy maize starch granules reacted with a propylene oxide analog at low substitution levels. Carbohydrate polymers; 60(2): 147 – 162.

Guo, L., Liu, R., Li, X., Sun, Y., & Du, X. (2015). The physical and adsorption properties of different modified corn starches. Starch-Stärke; 67(3-4): 237 – 246.

Haddarah A., Bassal A., Ismail A., Gaiani C., Ioannou I., Charbonnel C., *et al.* (2014). The structural characteristics and rheological properties of Lebanese locust bean gum. Journal of Food Engineering; 120: 204 – 214.

Håkansson, A., Magnusson, E., Bergenståhl, B., & Nilsson, L. (2012). Hydrodynamic radius determination with asymmetrical flow field-flow fractionation using decaying cross-flows. Part I. A theoretical approach.Journal of Chromatography A; 1253: 120 – 126.

Han, J.A. & BeMiller, J.N. (2007). Preparation and physical characteristics of slowly digesting modified food starches. Carbohydrate Polymers; 67: 366 – 374.

Hizukuri, S. (1985). Relationship between the distribution of the chain length of amylopectin and the crystalline structure of starch granules.Carbohydrate Research; 141: 295 – 306.

Huang, R.C., Forbes,D., & Davies M.W.(2002). Feed thickener for newborn infants with gastro-oesophageal reflux. The Cochrane library.

Ikegami S, Tsuchihashi F, Harada H, Tsuchihashi N, Nishide E, Innami S. (1990). Effect of viscous indigestible polysaccharides on pancreatic-biliary secretion and digestive organs in rats.The Journal of Nutrition; 120(4): 353 – 360.

Jane, J., Wong, K. S., & McPherson, A. E. (1997). Branch-structure difference in starches of A- and B-type X-ray patterns revealed by their Naegelidextrins.Carbohydrate Research; 300: 219 – 227.

Jenkins, P. J., & Donald, A. M. (1998). Gelatinisation of starch: a combined SAXS/WAXS/DSC and SANS study. Carbohydrate research; 308(1): 133 – 147.

Karababa, E., & Coşkuner, Y. (2013). Physical properties of carob bean (Ceratoniasiliqua L.): An industrial gum yielding crop.Industrial crops and Products; 42: 440 – 446.

Kaur, B., Ariffin, F., Bhat, R., & Karim, A. A. (2012). Progress in starch modification in the last decade.Food Hydrocolloids; 26(2): 398 – 404.

Kossmann, J., & Lloyd, J. (2000). Understanding and influencing starch biochemistry.Critical Reviews in Plant Sciences; 19(3): 171 – 226.

Lawal, M. V., Odeniyi, M. A., & Itiola, O. A. (2015). Material and rheological properties of native, acetylated, and pregelatinized forms of corn, cassava, and sweet potato starches. Starch ‐ Stärke; 67: 1 – 12.

Lehmann, U., & Robin, F. (2007). Slowly digestible starch – its structure and health implications: a review. Trends in Food Science & Technology; 18(7): 346 – 355

Liu, P., Yu, L., Liu, H., Chen, L., & Li, L. (2009). Glass transition temperature of starch studied by a high-speed DSC.Carbohydrate polymers; 77(2): 250 – 253.

Liu, H.,Ramsden, L., & Corke, H. (1999). Physical properties and enzymatic digestibility of hydroxypropylatedae, wx and normal maize starch.Carbohydrate Polymers; 40: 175–182.

Magnusson, E., Håkansson, A., Janiak, J., Bergenståhl, B., & Nilsson, L. (2012). Hydrodynamic radius determination with asymmetrical flow field-flow fractionation using decaying cross-flows. Part II. Experimental evaluation. Journal of Chromatography A; 1253: 127 – 133.

Mao, C.F., & Chen, J.C.(2006). Interchain association of locust bean gum in sucrose solutions: an interpretation based on thixotropic behavior. Food Hydrocolloids;20 (5): 730– 739.

McCleary, B. (1980). Hydrolysis of galactomannans by α-d-galactosidase and β-d-mannanase.J. John Marshall (Ed.), Mechanisms of saccharide polymerization and depolymerization, Academic Press, New York: 285–300.

Miller, D. D., Schricker, B. R., Rasmussen, R. R., & Van Campen, D. (1981). An in-vitro method for estimation of iron availability from meals.The American Journal of Clinical Nutrition; 34(10): 2248 – 2256.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., *et al*. (2014). A standardised static in-vitro digestion method suitable for food–an international consensus.Food & function, 5(6), 1113-1124.

Nakorn, K. N., Tongdang, T., & Sirivongpaisal, P. (2009). Crystallinity and rheological properties of pregelatinized rice starches differing in amylose content. Starch ‐ Stärke; 61(2): 101 – 108.

Nilsson, L. (2013). Separation and characterization of food macromolecules using field-flow fractionation: A review. Food Hydrocolloids, 30(1): 1-11.

Noda, T., Nishiba, Y., Sato, T., & Suda, I. (2003). Properties of starches from several low-amylose rice cultivars. Cereal Chemistry; 80: 193 – 197.

Pal, J., Singhal, R. S., & Kulkarni, P. R. (2002). Physicochemical properties of hydroxypropyl derivative from corn and amaranth starch.Carbohydrate Polymers; 48(1): 49 – 53.

Pollard, M. A. & Fischer, P. (2006). Partial aqueous solubility of low-galactose-content galactomannans. What is the quantitative basis? Current Opinions in Colloid & Interface Science, 11(2-3): 184 – 190.

Pollard, M.A., Kelly, R., Wahl, C., Fishcher, P., Windhab, E., Eder, B. & Amado, R. (2007). Investigation of equilibrium solubility of a carob galactomannan.Food Hydrocolloids; 21: 683 – 692.

Prajapati, V.D., Jani, G.K., Moradiya, N.G., Randeria N.P. & Nagar, B.J.(2013). Locust bean gum: A versatile biopolymer. Carbohydrates Polymer; 94: 814 – 821.

Roger, P., Bello-Perez, L. A., & Colonna, P. (1999). Contribution of amylose and amylopectin to the light scattering behaviour of starches in aqeoussolution.Polymer, 40: 6897 – 6909.

SamilKök, M., (2007). A comparative study on the compositions of crude and refinedlocust bean gum in relation to rheological properties.Carbohydrate Polymers; 70: 68 – 76.

Silveira, J.L.M., Bresolin, T.M.B., 2011. Pharmaceutical use of galactomannans. Quimica Nova; 34 (2): 292 – 299.

Singh, N., Singh, J., Kaur, L., Sodhi, N. S., & Gill, B. S. (2003). Morphological, thermal and rheological properties of starches from different botanical sources.Food Chemistry; 81(2): 219 – 231.

Singh, J., Kaurb, L., & McCarthy O.J. (2007). Factors influencing the physico-chemical, morphological, thermal and rheological properties of some chemically modified starches for food applications—A review. Food Hydrocolloids; 21: 1–22.

Sittikijyothin, W., Torres, D. & Gonçalves, M.P. (2005). Modelling the rheological behavior of galctomannan aqueous solutions. Carbohydrate Polymers; 59: 339 – 350.

Srichuwong, S., & Jane, J. (2007). Physicochemical properties of starch affected by molecular composition and structure: a review. Food Science and Biotechnology; 16: 663 – 674.

Swinkels, J. J. M. (1985). Composition and properties of commercial native starches.Starch ‐ Stärke, 37(1), 1-5.

Tester, R. F., Qi, X., & Karkalas, J. (2006). Hydrolysis of native starches with amylases.Animal Feed Science and Technology; 130(1): 39 – 54.

Tester, R.F., Karkalas, J. & Qi X. (2004). Starch structure and digestibility Enzyme-Substrate relationship. World's Poultry Science Journal; 60: 186 – 195.

Tester, R.F., & Morrison, W. R. (1990). Swelling and gelatinization of cereal starches.Cereal Chemistry; 67: 558–563.

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(3): 1031 – 1064.

Vandeputte, G.E., Vermeylen, R., Geeroms J. & Delcour, J.A. (2003). Rice starches. I. Structural aspects provide insight into crystallinity characteristics and gelatinization behaviour of granular starch. Journal of Cereal Science; 38: 43 – 52.

Venkatarajua, M. P., Gowdaa, D. V., Rajeshb, K. S., & Shivakumara, H. G. (2007). Xanthan and locust bean gum (from Ceratonia siliqua) matrix tablets for oral controlled delivery of propranolol hydrochloride. Asian Journal of Pharmaceutical Sciences; 2(6): 239 – 248.

Wahlund, K. G. & Nilsson, L. (2012). Flow FFF - basics and key applications. In Field-flow fractionation in biopolymer analysis, Williams, S. K. R.; Caldwell, K., Eds. Springer Verlag: Wien: 1-21.

Wahlund, K. G. & Giddings, J. C. (1987). Properties of an asymmetrical flow field-flow fractionation channel having one permeable wall. Analytical Chemistry; 59(9): 1332 – 1339.

Waliszewski, K. N., Aparicio, M. A., Bello, L. A., & Monroy, J. A. (2003). Changes of banana starch by chemical and physical modification. Carbohydrate polymers; 52(3): 237 – 242.

Whittingstall, P. (2001). Measuring the Viscosity of Non - Newtonian Fluids. Current Protocols in Food Analytical Chemistry. Available on: http://onlinelibrary.wiley.com/doi/10.1002/0471142913.fah0102s00/full

Wu, Y., Cui, W.,Eskin, N.A.M. & Goff, H.D. (2009). An investigation of four commercial galactomannans on their emulsion and rheological properties.Food Research International; 42: 1141 – 1146.

Yeh, A. I., &Yeh, S. L. (1993). Some characteristics of hydroxypropylated and cross-linked rice starch. Cereal Chemistry; 70: 596 – 596.

Effect of different thickening agents on Calcium, iron and Zinc *in-vitro* availability. CHAPTER 2

1.- INTRODUCTION

Calcium (Ca), iron (Fe) and Zinc (Zn), together with iodine and Vitamin A, have been defined as the most important micronutrients in infant feeding, being related to severe symptoms of infant malnutrition (UNICEF, 2010). The composition and structure of breast milk ensure an optimal mineral bioavailability. In this regard, minerals contained in human milk are mostly bound to proteins which act as carriers, improving their intestinal absorption (Bosscher *et al*., 2003[b]; Almeida *et al*., 2008).

Bioavailability can be defined as the proportion of nutrients that can be "used" by the organism. In the case of micronutrients, they must be released from the food matrix and solubilized in the intestine, allowing absorption to happen efficiently. However, this process is very complex, depending on multitude of physiological factors, along with others related to the food matrix itself (Bosscher *et al*., 2003[b]).

AR infant formulas include in their composition non-digestible carbohydrates such as Locust Bean Gum (LBG). The purpose of AR formulas is to prevent the return of gastric content to the oesophagus (Agostoni, 2004; Kirmemis, 2017). Associated to their molecular structure during digestion, and the presence of ionisable groups (hydroxyls and carboxyls), the addition of these ingredients has been related to a potential decrease in the intestinal absorption of Ca, Fe and Zn (Bosscher *et al*., 2003[a]; Bosscher *et al*., 2003[b]; Horvath *et al*., 2008; Ferrer-Lorente *et al*., 2009). Other studies have also indicated the possibility of hypersensivity associated to LBG oral exposition (Savino *et al*., 1999; Alarcon *et al*., 2011). However, these allergies studies are based on isolated cases, with a lack of generalized evidence, which indicates a low probability of LBG

hypersensibility in general population (Hegar *et al.,* 2008; Meunier *et al*., 2014). Due to the lack of consistent results about these possible side effects, different professionals have questioned the addition of thickeners to AR infant formulas (Aggett *et al*., 2002[a]; Birch & Newell, 2009;). At this respect, Levtchenko *et al.,* (1997) indicated normal growth and normal nutritional parameters when children where fed with AR infant formulas.

Considering these findings and the fact that "*The use of AR formulae and formulae with added thickener results in a decrease of observed regurgitation*", the European and North American Societies for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN–NASPGHAN) published the guidelines for management of infant anti-regurgitation (Vandeplas, *et al*., 2009[b]). According to these guidelines, the use of AR infant formulas should be only prescribed in healthy term infants with uncomplicated gastroesophageal reflux, avoiding their use in premature and low birth weight infants.

1.1.- Composition of human milk

Brest milk has been defined as the optimal food for infant nutrition during the first years of life. On this point, it covers the newborn's nutritional requirements during the first months of life, providing bioavailable forms of essential macro and micronutrients. Apart from its nutritional profile, human milk contains antimicrobial and immunological factors that improves the resistance of newborns to microorganisms and diseases, as well as it facilitates the establishment of a healthy microbiota (Pronczuk *et al*., 2004; WHO, 2009; Ballard & Morrow, 2013).

1.1.1.- General Composition of Human Milk.

Defining a standard for human milk composition is difficult, as it is subjected to longitudinal and transversal variations. This variation attends not only to environmental conditions, but also to stages of lactation, defining breast milk as a dynamic fluid, which adapts its composition to the nutritional requirements of child (Mitoulas *et al*., 2002; Ballard & Morrow, 2013; Martin *et al*., 2016). Despite these variations a general composition has been defined for human milk, which consist in 87 % water, 3.8 % fat, 1.0 % protein, and 7 % lactose (Guo, 2014). With regard to longitudinal variations, human milk composition changes through the lactation period (Nommsen *et al*., 1991; Picciano, 2001; Mitoulas *et al*., 2002; Bauer & Gerss, 2011; Ballard & Morrow, 2013; Andreas *et al*., 2015; Martin *et al*., 2016):

- During the first days postpartum, a secretion with a high content in proteins and low content in lactose is produced. This secretion is named "Colostrum", being specially rich in immunological (IgA, lactoferrin or leukocytes) and trophic factors (epidermal growth factor, neurotrophic factors, insulin-like growth factor, Erythropoietin, or Vascular endothelial growth factor). By 5 – 6 weeks, this secretion will be gradually replaced by "mature breast milk".
- A constant breast milk production has been described during the first 6 months after birth (≈400 mL/24h), progressively decreasing as lactation progresses (≈250 mL/24h after 12 months).
- Lactose content remains constant after 21 days postpartum (61.4 g/L), being related with the mammary gland acinus osmotic pressure maintenance.
- When energy content is analyzed, it decreases at 2 months and then increases after 9 months of lactation. Due to the fact that lactose content remains constant, changes in energy content have been associated with the fat content variations (ranging from 3.5 % to 4.5 %), decreasing between 1 and 4 months (39 to 35 g/L) but increasing after 9-12

months (40 g/L). When analyzing lipid profile, triglycerides are the major fraction, representing 95 % of total content.

- About protein content, it decreases by 6 months of lactation (from 16 mg/L to 8 mg/L), remaining constant for the rest of the first year. Proteins in human milk can be divided in two groups, casein and whey proteins, with a ratio ranging from 70:30 to 80:20 during early lactation, and a a 50:50 ratio during late lactation.
- Apart from macronutrients, breast milk is an important source of vitamins (except for vitamin D and K), minerals, bioactive compounds (lactoferrin, cytokines, hormones, oligosaccharides and glycans, mucins, etc…) and microorganisms (human milk microbiota).

Other than these “longitudinal variations”, human milk composition is also affected by environmental factors. Between these factors, weeks of gestation (preterm or term infants) (Bauers & Gerss, 2011; Gidrewicz & Fenton, 2014; Khodayar-Pardo *et al*., 2014); mode of delivery (Dizdar *et al*., 2014; Khodayar-Pardo *et al*., 2014) age of the mother or even ethnicity (Andreas *et al*., 2015) have been defined.

1.1.2.- Minerals in Human Milk.

Among other nutrients, human milk is an important source of minerals, which play a role in an adequate neonate development. As the rest of nutrients, mineral composition of breast milk is also exposed to variations (Yamawaki *et al*., 2005; Bauer & Gerss, 2011). Depending on their concentration in human milk, minerals can be classified as major and minor minerals (Picciano, 2001). According to this classification whereas Ca (Ca), phosphorous (P), magnesium (Mg), sodium (Na) or potassium (K) are

classified as major minerals; iron (Fe), Zn (Zn), copper (Cu) or manganese (Mn) are included in the minor group.

During lactation, Mg and Ca average concentrations, remain quite stable, ranging from 30 and 260 mg/L at 3 weeks of lactation, to 33 and 248 mg/L at 26 weeks, respectively. On the contrary, P concentration decreases from 147 mg/L at 3 weeks to 107 mg/L at 26 weeks of lactation (Greer *et al*., 1982; Picciano, 2001; Yamawaki *et al*., 2005).

With regard to Na and K, their concentrations in human milk are related to the tight junction permeability in the secretory epithelium and the production of lactose (Picciano, 2001; Pang & Hartmann, 2007; Ballard and Morrow, 2014). Lactose content in colostrum is lower than later in lactation. During its secretion, lactose draws water to the secretory alveoli, determining the potential difference needed for maintaining electrolyte concentration. Such increment in lactose concentration is accompanied by a tight junction closure and a decrease in Na:K ratio. According to Yamawaki *et al*., (2005), Na and K average concentrations in mature human milk are, respectively, 13.5±8.7 mg/100 mL and 47.0±12.1 mg/ 100mL, defining a high individual variation.

Compare to others elements, Cu, Fe and Zn present a low concentration, being classified as minor minerals. Regarding Fe and Cu, their concentrations are higher in colostrum (700 and 400 µg/L respectively) than in mature milk, decreasing rapidly until an average concentration of 0.3 and 0.1 mg/L respectively. In respect of Zn, its decrease is more progressive than Ca and Cu, varying from a concentration of 4 mg/L in

calostrum, to final values of 0.7 – 0.4 mg/L in mature milk (Picciano, 2001; Domellöf *et al*., 2004; García-López, 2011).

Influence of maternal mineral status on mineral concentration in human milk seems to be different depending on the element. In this way, Domellöf *et al*., (2004) evaluated milk samples from 191 women from different countries (Honduras and Sweden) concluding that Fe, Cu and Zn concentration in breast milk, is maintained by an active membrane transport process, being independent of mother mineral status. In the same way, Jarjou *et al*., (2006) analyzed the effect of Ca supplementation (1500 mg Ca carbonate/d) versus placebo on its concentration in human milk (n=125), but no significant results were reported. However, other minerals as Iodine (I) have shown a clear dependence between mother intake and its concentration in breast milk. In this way, breast milk from areas with high prevalence of I deficiency contents a 10 times-lower I concentration than breast milk from I sufficient areas (Picciano, 2001).

It has to be taken into account that minerals in human milk are frequently bound to others compounds, mainly protein. The formation of these mineral complexes is essential for their adequate biological function. In this way, different minerals act as cofactor in catalytic reaction or stabilize protein structures. The formation of organic-metallic compounds has been also related to a better inorganic elements bioavailability (Pozzi *et al*., 2015). Using high performance liquid chromatography-inductively coupled plasma atomic emission spectrometry, Bocca *et al*., (2000) determined the binding pattern of different minerals in human milk. According to this report, Zn seems to be linked to different compounds, highlighting not only α-lactalbumin, but also citrates, casein, albumin or lactoferrin. Apart from caseins, Ca seemed to bind preferably to low

molecular weights compounds as non-proteic ones. Regarding Fe, it showed an homogeneous distribution across different organic compounds, with special abundance in caseins and Immunoglobulin fractions. Apart from these forms, lactoferrin is considered to be bound to a 20 – 30 % of soluble Fe, being considered as an important antimicrobial agent, as well as a high bioavailable Fe form (Picciano, 2001; Pozzi et al., 2015).

1.2.- AR-Infant formulas.

1.2.1.- Breastfeeding. Prevalence and recommendations.

As it has been previously explained, human milk is a dynamic and complete food that adapts to the nutritional needs of the infant during the first 4-6 months of life. In addition to an adequate nutrient supply, human milk provides living cells (microorganisms, lymphocytes and macrophages), digestive enzymes, immunomodulators and growth factors, which are essential for proper feeding and development of the newborn (Pronczuk *et al*., 2004; Hernandez-Aguilar & Aguayo-Maldonado, 2005; Infante-Pina *et al*., 2008; WHO, 2009; Ballard & Morrow, 2013).

In 1990, the global initiative known as "*The Innocenti Declaration on the Protection, Promotion and Support of Breastfeeding*", proposed by the World Health Organization (WHO) and United Nations Children's Fund (UNICEF), was accepted by more than 30 countries (UNICEF/WHO, 1990). According to this declaration, exclusive breastfeeding is recommended until 4 to 6 months of age. After this period, complementary feeding combined with breastfeeding should be maintained up to two years of age. Despite these recommendations, breastfeeding prevalence is lower than

expected. According to the research published by Victoria *et al*., (2016), prevalence of breastfeeding at 12 months is higher in low incomes countries (>70 %) than in high incomes countries (<20 %), finding a negative relation of ten percentage point between, each doubling in gross domestic product per head, and breastfeeding prevalence at 12 months. In the same study, France, Spain, and the USA showed the lower rates of exclusive breastfeeding at 0 - 5 months (<80 %). Gage *et al*., (2010) analyzed the infant feeding intentions of 2071 new mothers from different European countries. In the case of Spain (404 mothers), an 87.6 % of new mothers manifested the intention to exclusively breastfeed, against a 3.7 % who were intended to bottle-feed. In this intention, the advices of healthcare professionals were decisive.

When breastfeeding is not possible, the use of infant formulas is recommended. These formulas are specially formulated to mimic human breast milk (Joeckel & Phillips, 2009). According to the ESPGHAN, infant formulas can be defined as "*a product based on milk of cows or other animals and/or other ingredients which have been proven to be suitable for infant feeding*" (Koletzko *et al*., 2005). Some of these formulas have been specially designed for special medical purposes, being classified as special infant formulas. In this group, AR-infant formulas are included. This kind of formulas are specially formulated with thickeners in order to prevent the return of gastric content into the oesophagus during gastric digestion (Agostoni, 2004; Ferrer-Lorente et al., 2009; Kirmemis, 2017).

1.2.2.- General Compositions of Infant formulas and Thickeners.

Due to its relevance, composition of infant formulas is strongly regulated by a normative. As it has been mentioned, infant formulas are specially formulated to mimic

human breast milk (Joeckel & Phillips, 2009). Considering that breast milk composition and nutrition requirements of infants varies with stage of lactation, different kind of formulas have been designed. These formulas are generally designed as “infant formulas” (intended for particular nutritional use by infants during the first months of life and satisfying by themselves their nutritional requirements until the introduction of appropriate complementary feeding) and “follow-on formulas” (intended for particular nutritional use by infants when appropriate complementary feeding is introduced and constituting their principal liquid element in a progressively diversified diet) (Commission Directive 2006/141/EC). In 2005, an ESPGHAN coordinated international expert group published a global standard for the composition of infant formula (Koletzko *et al*., 2005). Together with these recommendations, Commission Directive 2006/141/EC and ulterior modifications constitute the legal basis for infant formulas production. In table 2.1., macronutrient and minerals (Ca, Fe and Zn) specifications have been respectively compiled.

Table 2.1.*- Macronutrients and minerals (Ca, Fe and Zn) legal specifications for Infant and follow-on formulas (adapted from Commission Directive 2006/141/EC)*

	Infant Formulas		**Follow-on Formulas**	
	Minimum	**Maximum**	**Minimum**	**Maximum**
Energy	60 Kcal/100 mL	70 Kcal/100 mL	60 Kcal/100 mL	70 Kcal/100 mL
Protein	1.8 g/100 Kcal	2.5 g/100 Kcal	2.25 g/100 Kcal	2.8 g/100 Kcal
Lipids	4.4 g/100 Kcal	6.0 g/100 Kcal	4.4 g/100 Kcal	6.0 g/100 Kcal
Carbohydrates	9 g/100 Kcal	14 g/100 Kcal	9 g/100 Kcal	14 g/100 Kcal
Lactose	4.5 g/100 Kcal	-	4.5 g/100 Kcal	-
Ca	50 mg/100 Kcal	140 mg/100 Kcal	50 mg/100 Kcal	140 mg/100 Kcal
Fe	0.3 mg/100 Kcal	1.3 mg/100 Kcal	0.6 mg/100 Kcal	2 mg/100 Kcal
Zn	0.5 mg/100 Kcal	1 mg/100 Kcal	0.5 mg/100 Kcal	1 mg/100 Kcal

With regard to thickeners, the addition of gelatinized or pre-cooked starches, as well as LBG to infant formulas were respectively authorized by, the Commission of the European Communities, Scientific Committee for Food (1992) and the Commission of the European Communities, Scientific Committee for Food (1997). Current legislation establishes a maximum legal limit for the addition of these ingredient. According to it, pre-cooked starch and/or gelatinized starch can be added up to a máximum legal limit of 2 g/100 ml, or 30 % of the total carbohydrate content. Regarding to LBG, its addition is regulated by the European Parliament and Council Directive 95/2/EC (1995) and its modifications. As stated to it, LBG can be added under a maximum legal limit of 1 g/L in products intended to reduce infant gastroesophageal reflux.

1.3.- Mineral Requirements in infant nutrition.

Recommended Daily Intake (RDI) can be defined as "*the average daily dietary intake level sufficient to meet the nutrient requirements of nearly all (97–98 %) healthy individuals in a group*". According to the National Institutes of Health (NIH) from the U.S. Department of Health & Human Services, in a range of 0 – 6 months of age, no sufficient information is available to establish an adequate mineral RDI. In these cases, an Adequate Intake (AI) has been established (NIH, 2017). In compliance with this information, Ca, Fe and Zn AI for infants between 0 and 6 months of age are respectively, 200, 0.27 and 2 mg/day.

Different authors have reported the difficult for establishing minerals RDI during the first months of age. At this respect, the ESPGHAN Committee of Nutrition (Aggett *et al*., 2002[b]), explained that, in order to provide an adequate Fe RDI, a reference threshold

should be extrapolated from a global hematologic and biochemical study. These data could be useful for determining the relationship between Fe supply and stores, and subclinical Fe deficiency/overdose. With regard to Ca, the RDI establishment during the first months of life, is influenced by the varying physiologic requirements during development and bioavailability of Ca in food (breast milk and/or formula) (Greer & Krebs, 2006).

1.4.- Ca in infant nutrition and development.

Ca is the most abundant mineral in the human body (Bass & Chan, 2006), as well as an essential nutrient required for critical biological functions, such as nerve conduction, muscle contraction, mitotic division of cells, blood coagulation or an adequate development and maintenance of the bone mass (Miller *et al*., 2001). With regard to Ca overdose, a damage to kidneys or the interference of Mg, Fe or Zn intestinal absorption has been proposed (Molska *et al*., 2014). Due to its relevance for the human organism, a complex homeostasis and regulation is stablished.

1.4.1.- Basis of Ca homeostasis in infants.

Distribution of Ca in the human body can be explained through an equilibrium model with different compartments and a central plasmatic pool of soluble or ionic Ca. Between these compartments, an homeostatic equilibrium will be established, which will be mainly regulated by parathyroid hormone, calcitonin, and 1,25-dihydroxyvitamin-D (Bronner & Pansu, 1999; Bass & Gary, 2006; Greer & Krebs, 2006).

Approximately a 99 % of total body Ca is stored in bones as hydroxyapatite, constituting the main Ca deposit in human organism (Greer & Krebs, 2006). Between this deposit and plasma, a narrow equilibrium will be established, maintaining an average normal plasma concentration of 2.5 mmol/L (Bronner & Pansu, 1999). With regard to infant serum Ca concentration, Greer *et al*., (1982) described a concentration increment associated to lactation progression (from 9 mg/dL at 3 weeks to 10 mg/dl at 6 months). This increment was correlated with a decrease in P concentration (from 8 mg/dl at 3 weeks to 6 mg/dl at 6 months). These findings have been associated with skeletal remodeling (Greer et al., 1982; Picciano *et al*., 2001). Indeed, according to the study published by Bronner & Pansu (1999), bone Ca mass max was minimum in the newborn infant, reaching a maximum at 35 – 45 years old. During this remodeling process, the rate of Ca removal from the skeleton was correlated with the Ca deposition rate, what could explain the increasing amounts of Ca in infant serum.

Exogenous Ca is provided by food digestion and absorption. At the same time, the digestive tract will produce Ca losses associated with the continuous intestinal epithelium renewal, as well as the secretion of digestive fluids and enzymes. At the renal level, approximately half of the circulating Ca ions are filtered. After filtration, approximately 70 % of Ca will be reabsorbed in different portions of the nephron, excreting the remaining amount in the urine. Finally, there is a decrease in endogenous Ca in other processes such as tissue remodeling, enzyme synthesis and other cellular components (Bronner & Pansu, 1999).

Due to the relevance of Ca for different biochemical process, acute Ca deficiencies can cause hiperexcitability of neurons, parathyroid hyperplasia, and even decalcification-alteration of bone synthesis. On the contrary, an excessive supply of Ca, may lead to hypercalcemia, metastatic calcification of soft tissues and severe kidney damage (Molska *et al*., 2014)

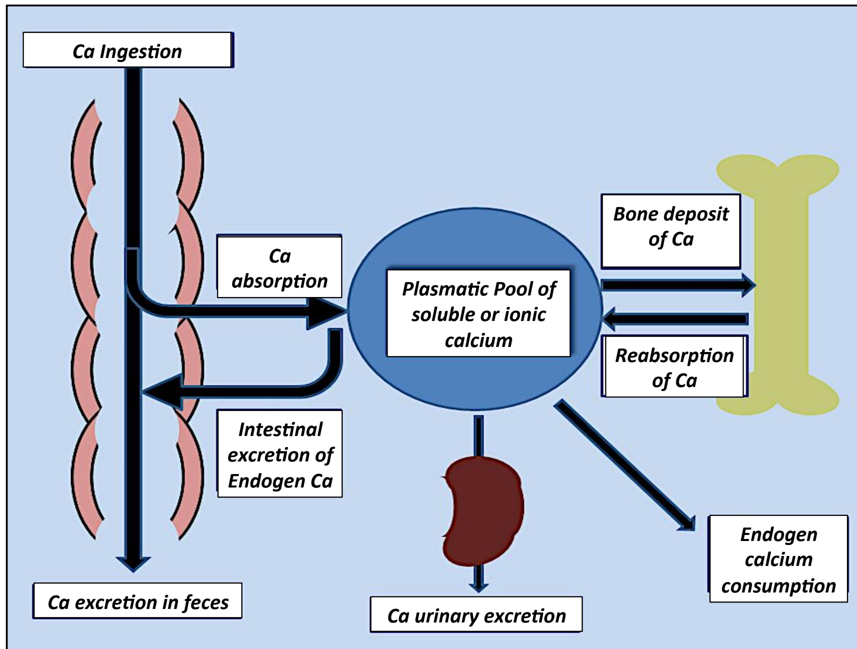


Figure 2.1.- *Scheme of Ca homeostasis in human organism. Adapted from Bonner & Pansu (1999).*

1.4.2.- Introduction to Ca intestinal absorption.

During fetal development, placenta is responsible for delivering all the nutrients, including Ca, to the fetus. As a result, fetal serum will be kept in a state of hypercalcemia, with 1 mg of Ca/ dL above the maternal serum levels. After birth, this exogenous supply of Ca is abruptly interrupted, resulting in a 5 % decrease of neonate plasmatic Ca levels at 2 hours of birth. This depression of plasmatic Ca is accentuated in the following hours, stabilizing by action of hormonal and breast milk intake (Bass & Chan, 2006). Breast milk is able to cover Ca nutritional requirements during the first months of life. With this purpose, Ca content in human milk varies from 38 to 41 mg/100 Kcal during the first year of lactation (Neville *et al.*, 1991).

To be absorbed at the intestinal level, Ca must be released from food matrix and solubilized. However, it has been described that Ca association with organic molecules as casein phosphopeptides or non-protein-low molecular weight substances, facilitates its intestinal absorption (Bronner & Pansu, 1999; Bocca *et al*., 2000; Picciano, 2001; Pozzi *et al*., 2015. Ca intestinal absorption is also influenced by Ca: phosphorus ratio, which must be 2:1 to facilitate its absorption (Bronner & Pansu, 1999).

Two different pathways have been described for intestinal Ca absorption, a paracellular and a transcellular transport (Hoenderop *et al*., 2005) (Figure 2.2). In general, the transport of nutrients through the enterocyte membrane (transcellular transport), is regulated by different molecular transporters and channels coupled to a Na/K-ATPase pump. On the contrary, paracellular transport results from the passive movement of substances by an electrochemical gradient, which was previously imposed by the transcellular transport, the plasmatic concentration of different substances and the ingestion of solutes (Van Itallie & Anderson, 2006).

- ***<u>Paracellular transport of Ca</u>***: One of the main functions of the epithelium is to separate body compartments and regulate the exchange of substances between them. Therefore, the epithelium can be understood as a continuous layer composed of individual cells, and narrow intercellular spaces that allow the passage of certain substances, mainly small molecules and ions. Proteins involved in tight unions between cells, as claudins, act as a dynamic barrier to the passage of substances, modifying the intercellular flow in relation to the functional requirements. This regulation can be assessed through transepithelial electrical resistance (TER), and the ionic charge and size of the solutes (Van Itallie & Anderson, 2006; de Barboza *et al*., 2015). In this way, narrow epithelial junctions with a high TER are able to generate and maintain a high transepithelial electrical potential,

resulting in a clear compartmentalization with different ionic composition between the extracellular space, and the interstitium. On the contrary, porous epithelia with a low TER, maintain a low transepithelial potential, resulting in an iso-osmotic fluids flow between both compartments (Reuss, 2001). Paracellular transport of Ca^{+2} transport is a non-saturable and passive process which depends on both, Ca^{+2} concentration in the intestinal lumen and plasma, and the electrochemical gradient across the epithelium (Bronner, 2003; Hoenderop *et al*., 2005; de Barboza *et al*., 2015).

- ***Transcellular transport of Ca***: Transcellular transport of Ca can be understood as a three stages process, including the entry of Ca into the enterocyte, its translocation to the basolateral membrane and, an active transport from the cell to the circulatory system (Hoenderop *et al*., 2005).
  - Different selective channels (TRPV5 and TRPV6), situated in the enterocyte apical membrane, regulate intestinal Ca^{+2} absorption. TRPV5 and TRPV6 are ionic channels, included in the transient receptor potential vanilloid (TRPV) family, and formed by different protein subunits. They are capable of regulating Ca^{+2} transport by varying their kinetics or size, depending on the electric gradient and the activity of associated proteinkinases. TRPV5 and TRPV6 expression is regulated by parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D3. Both factors affect TRPV gene transcription and translation, determining its presence in the apical membrane (Singer *et al*., 1991; Montell *et al*., 2002; Hoenderop *et al*., 2005; Christakos *et al*., 2011; van Goor *et al*., 2016).
  - Once in the cytoplasm, Ca^{+2} ions are chelated by Calbindin-D9k, being transported to the basolateral membrane without increasing intracellular free Ca^{+2}. Calbindin-D9k is a member of the S100 family of Ca-binding proteins. Interaction between Ca^{+2} and Calbindin binding is weak allowing its release after reaching the

basolateral membrane. This transport is mediated by myosin filaments. As with membrane proteins, 1,25-dihydroxyvitamin-D3 and PTH will regulate their synthesis and cytoplasmic concentration. (Hoenderop *et al*., 2005; Christakos *et al*., 2011). In addition, an endomembrane Ca-transporters system has been described. This system is mediated by endocytosis vesicles that capture Ca^{+2}, being stimulated by a high concentration of this ion in the apical portions of the cell, and ceasing once the apical concentration of Ca^{+2} decreases. These vesicles are transported to the basolateral membrane by cytoskeleton microtubules (Larson & Nemere, 2002).

- After reaching basolateral membrane, Ca is released into the blood stream by two specific transporters: a Na^{+}/Ca^{+2} exchanger (NCX), which depends on membrane potential and ion gradients; and a Ca-ATPase pump (PMCA). Meanwhile PMCA has been defined as the main responsible for Ca^{+2} active excretion from enterocytes, NCX is only responsible for about 20 % of Ca^{+2} extrusion. The expression and activity of these exchangers seem to be dependent on the active form of vitamin D (1,25-dihydroxyvitamin D3) (Wasserman *et al*, 1992; Hoenderop *et al*., 2005; de Barboza *et al*., 2015).

Based on the presence of molecules associated with intracellular Ca transport, transcellular transport has been primarily described in the proximal portions of the duodenum and, to a lesser extent, in cecum and ascending colon. Paracellular transport of Ca occurs throughout the small intestine, mainly in jejunum and ileum. When comparing both, paracellular and transcellular pathways, the former is the predominating process during Ca^{+2} over-intake, since a negative feedback regulation of transporter expressions and/or a transport capacity saturation have been described

(Bronner & Pansu, 1999; Bronner, 2003; Hoenderop *et al.*, 2005; de Barboza *et al.*, 2015).

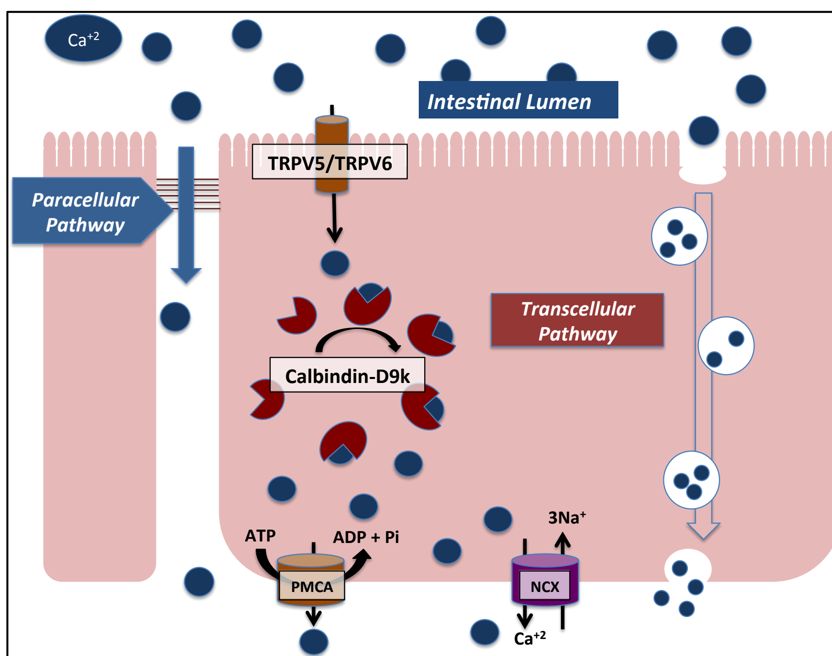


Figure 2.2.- *Scheme of trans-cellular and para-cellular pathways for Ca enterocyte absorption. Adapted from Larsson & Nemere, (2002)*; de Barboza *et al.*, (2015)

1.5.- Fe in infant nutrition and development.

Fe is essential for the cells metabolism and maintenance. Two stable oxidation forms of Fe can be found in aqueous solutions, ferrous Fe (Fe^{+2}) or ferric Fe (Fe^{+3}), which can respectively donate or accept electrons. This property makes it able to participate in many biochemical reactions, being an important cofactor for many redox enzymes (Ponka, 2000). In this way, there are proteins with a prosthetic heme-Fe group, consisting of an Fe^{+2} (ferrous) ion in the center of a heterocyclic organic compound or porphyrin. These proteins are involved in oxygen metabolism (oxidases, peroxidases, catalases and hydroxylases), electron transference (cytochromes) or oxygen transport (hemoglobin). There are other enzymes that use non-heme or inorganic Fe as cofactor, which play a large role in oxidative and aminoacid metabolism, or DNA synthesis (Perez

et al., 2005). Heme and non-heme Fe, constitute the functional form of Fe in the organism, constituting more than 70 % of total body Fe (Ponka et al., 2000; Perez *et al*., 2005). Apart from its enzymatic and biochemical functions, Fe plays a major role on erythropoiesis, hemoglobin formation and oxygen transport (Collard, 2014, Lopez *et al*., 2016).

Due to their rapid growth rate and the low Fe concentration in human milk (0.2-0.4 mg/L), infants and young children are especially susceptible to Fe deficiency (ID), being the most common micronutrient deficiency worldwide (Domellöf *et al*., 2004; Domellöf *et al*., 2014; Lopez *et al*., 2016). Apart from other systemic alteration, Fe deficiency and a low body Fe store leads to a deficient erythropoiesis and anemia, reducing blood O_2 transport. Because of its rapid development, during the first months of life, nervous system would be especially susceptible to oxygen deficiency. Consequently, infant anemia could result in an irreversible alteration of neurodevelopment (Collard *et al*., 2009; Carter *et al*., 2010; Lopez *et al*., 2016).

Together with gastrointestinal infections, Fe deficiency is the main cause of infant anemia. The prevalence of anemia in children younger than 5 years is, respectively 42 % in developing countries and 17 % in industrialized countries, being even higher during rapid growth periods (6–24 months of age). However, during the first 4 – 6 months of life, healthy term infants show no signs of Fe deficiency or Fe stores depletion (Aggett *et al*., 2002). In contrast with its high bioavailability, Fe concentration in breast milk is under infant Fe requirements (Domellöf *et al*., 2004). Apart from the low Fe intake, the rapid growth and restoration of erythropoiesis, determine the mobilization of infant Fe stores, which have been estimated at 75 mg/Kg after birth. Considering an Fe

requirements increment of 0.78 mg per day, Fe stores reach their minimum between 4 and 6 months of age (Aggett *et al*., 2002; Gil-Hernández *et al*., 2006; Domellöf *et al*., 2014). For this reason, before 6 months of age, the ESPGHAN Committee on Nutrition (Domellöf *et al*., 2014), establishes a low but unspecific theoretical Fe requirements. Between 6 – 12 months, these theoretical requirements increase to 0.9–1.3 mg/kg. For these reason, the introduction of supplementary feeding should be carried out between 4 and 6 months to avoid Fe deficiency (Muñoz-Hoyos & Molina-Carballo, 2005). Fe recommendations for follow-on formulas are equal or greater than 1 mg/100 Kcal, with a total intake lower than 15 mg/day (Reilly, 2007).

1.5.1.- Basis of Fe homeostasis in infants.

After delivery, healthy full-term neonates have sufficient Fe stores for covering their requirements during the first 4 – 6 months. These stores have been estimated at 75 mg/Kg of body weight. During the third trimester of pregnancy, an estimated Fe accumulation of 1.35 mg per kg of fetal weight has been proposed. At term, 70–80 % of fetal Fe is present in red-blood cells as Hemoglobin, 10 % in tissues as myoglobin and cytochromes. The remaining 10–15 % is stored in reticuloendothelial and parenchymal tissues as ferritin, an heteropolymeric protein with 24 subunits (heavy and light subunits). Due to the fact that maternal Fe metabolism is adapted to ensure an adequate Fe supply, fetal Fe status will be only affected during severe Fe deficiencies (Agget *et al*., 2002[b], Siddappa *et al*., 2007). Between the first 6 – 8 weeks of life, body Fe reserves are increased by the physiological hemolysis of erythrocytes and the reduction of erythropoiesis rates. The reason is that, at the moment the infant breaths autonomously the arterial oxygen saturation increases, fetal polyglobulia is no longer necessary leading to an Fe recirculation from senescent erythrocytes. This Fe is transferred from

hemoglobin to Fe stores, which thereby increase in size (Muñoz -Hoyos & Molina-Carballo, 2010; Domellöf *et al.*, 2014).

The oxidizing-reducing activity of Fe could result in the formation of free radicals, becoming dangerous for cells and tissues. Because of this potential toxicity, Fe is bound to transport and storage molecules, forming soluble non-toxic complexes. For this reason, Fe in plasma or in extravascular fluids is transported by the Fe-binding glycoprotein transferrin, which consits of a single polypeptide chain with two Fe-binding sites. In order to be bound by transferrin, Fe need to be converted in ferric ion (Fe^{+3}) by the enzyme ceruloplasmin. Apart from transferrin, seric albumin can bind Fe, specially in its ferrous form (Fe^{+2}) (Ponka, 2000; Collard, 2009; Brissot *et al.*, 2012).

Plasmatic Fe concentration and transferrin saturation is correlated to Fe storage and tissue requirements. This equilibrium is controlled by the hormone hepcidin, produced by hepatocytes. Indeed, hepcidin decreases Fe intestinal absorption through the interactions with a basolateral transporter called ferroportin. This interaction results in the destruction of ferroportin by endocytosis and lysosomal proteolysis (Ganz, 2013). Tissue Fe requirement is mainly determined by bone marrow and erythropoiesis. When Fe requirements increases, hepcidin expression decreases, leading to an increment of Fe intestinal absorption, and plasmatic Fe concentration/transferrin saturation (Forrellat-Barrios *et al.*, 2012). Under physiological conditions, mean plasma Fe and transferrin concentrations are 20 μmol/L and 30 μmol/L, respectively, corresponding to a transferrin saturation of approximately 30 %. Due to the reduced synthetic capacity in term infants, transferrin and ceruloplasmin plasmatic

concentrations are lower in healthy newborns than in adults, resulting in a high transferrin saturation (≈48 %) (Milman *et al.*, 1987; Collard, 2009; Brissot *et al.*, 2012).

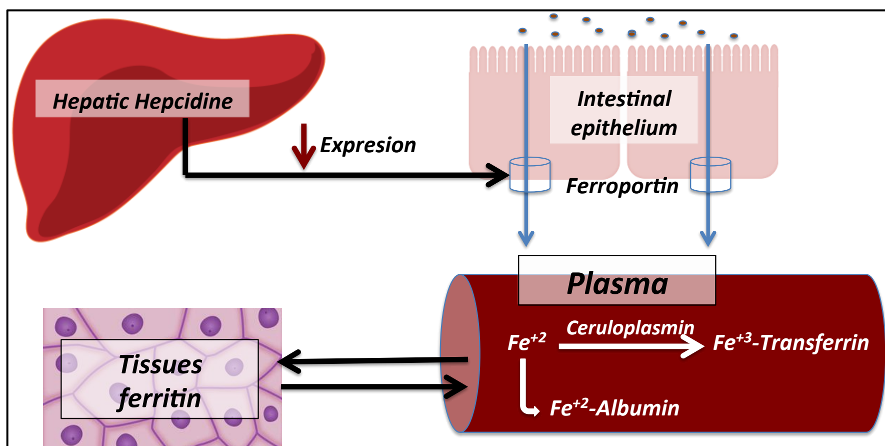


Figure 2.3.- *Scheme of Fe homeostasis*

Apart from transferrin, serum ferritin is the standard biomarker for the evaluation of body Fe stores as it has been shown to closely parallel the size these stores in adults. In this population, an equivalence of 8 – 10 mg of storage Fe per each 1 μg/l of serum ferritin is established (Siddappa *et al.*, 2007; Domellöf *et al.*, 2014). According to Carter *et al.*, (2010) transferrin saturation <12 %, and ferritin <12 μg/L can be considered as reference levels for Fe deficiency in infants. However, according to the ESPGHAN Committee on Nutrition (Domellöf *et al.*, 2014), no validation studies have been performed in infants, being the ratio between serum Fe and transferrin a more reliable marker (transferrin saturation ratio >12 – 15 %). Indeed elevated ferritin concentrations in infants could be a consequence of neonatal hemochromatosis, infection, inflammation or neoplasia, masking the diagnosis of Fe deficiency (Siddappa *et al.*, 2007).

1.5.2.- Introduction to Fe intestinal absorption.

Dietary Fe can be found in different forms, being generally classified as non-heme or inorganic Fe, which mainly appears as ferric Fe (Fe^{+3}); and organic or heme Fe. Depending on its form, intestinal absorption process and bioavailability will be different (Amaro & Camara, 2004). Between both forms, heme Fe is ready for its absorption, presenting a high bioavailability. However, the absorption of non-heme Fe is best understood (Amaro & Camara, 2004; Hallberg & Hulthén., 2007; Fuqua *et al*., 2012).

Fe absorption from intestinal lumen and its transference to the blood stream, takes place through the apical and basolateral membrane of enterocytes (Figure 2.4); being regulated by different proteins and transporters presented in the membrane and the cytoplasm (Andrews, 2000; Fuqua *et al*., 2012). This absorption process mainly occurs in the upper part of the intestine (duodenum and proximal jejunum) (Fuqua *et al*., 2012). In order to summarize Fe intestinal absorption, it will be divided in an apical and a basolateral-plasma transference process:

- ***Apical absorption of heme or organic Fe***: During digestion organic Fe is released from the hemoprotein as a result of proteolytic digestion by pancreatic enzymes. It is subsequently incorporated by the enterocytes as an intact metaloporphyrin. This transport is regulated by receptor-mediated endocytosis through the apical membrane of the enterocyte, but the complete process remains unclear (Andrews 2000; Fuqua *et al*., 2012). Once inside the cell, heme group is degraded by a hemoxygenase, releasing inorganic Fe (Perez *et al*., 2005). A small part of heme group can be directly transferred to the portal blood, although (Reddy *et al*., 2006).
- ***Apical Absorption of non-heme or inorganic Fe***: The hydrochloric acid present in the gastric secretion, reduces ferric ion (Fe^{+3}) released from food matrix to a more soluble

ferrous state (Fe^{+2}), allowing its absorption through the membranes of the enterocytes. As intestinal content acidity decreases across the intestinal track, most of the absorption of Fe occurs in the proximal region of the duodenum, near the gastric pylorus. To make Fe absorption more effective, a Duodenal Cytochrome-B protein (DcytB) is presented on the apical surface of the enterocytes. DcytB reduces ferric ions (Fe^{+3}) to Fe^{+2}. Due to the action of HCl and DcytB, Fe^{+2} is the major form in intestinal lumen, but a low amount of Fe^{+3} also reach the apical membrane of the enterocyte. Depending on the Fe form, the absorption process will be different (Pérez *et al*., 2005; Andrews 2000; Boudry *et al*., 2010; Fuqua *et al*., 2012; Kiela & Ghishan, 2016):

- The absorption of ferrous ions (Fe^{+2}) is mediated by a divalent metal transporter (DMT1), which has a broad substrate range, including Fe^{+2}, Zn^{+2}, Mn^{+2}, Co^{+2}, Cd^{+2}, Cu^{+2}, Ni^{+2}, and Pb^{+2}.
- Ferric ions (Fe^{+3}) are absorbed across a membrane integrin (β3-integrin) and transferred to the protein mobilferrin. Once inside the enterocyte, the absorbed ferric ion (Fe^{+3}) is reduced to its ferrous state (Fe^{+2}) thanks to a cytoplasmic complex called NADPH-dependent paraferritin.

- **Basolateral membrane-plasma transference of Fe**: In order to minimize oxidizing-reducing activity of Fe^{+2} ions, they are attached to a cytoplasmatic protein paraferritin. Fe^{+2} can be stored in the cytoplasm attached to ferritin, or reach the basolateral membrane, where a multipass trans-membrane protein (ferroportin-Fpn1) transferee them to the plasma. Fpn-1 is the only described Fe exporter protein in mammalian, and the main target for Fe absorption regulation by hepcidin. Once in plasma, ceruloplasmin promotes the oxidation of Fe^{+2} Fe to Fe^{+3}, facilitating its incorporation into circulating transferrin (Andrews, 2000; Perez *et al*., 2005; Fuqua *et al*., 2012, Ganz, 2013; Kiela & Ghinhan, 2016).

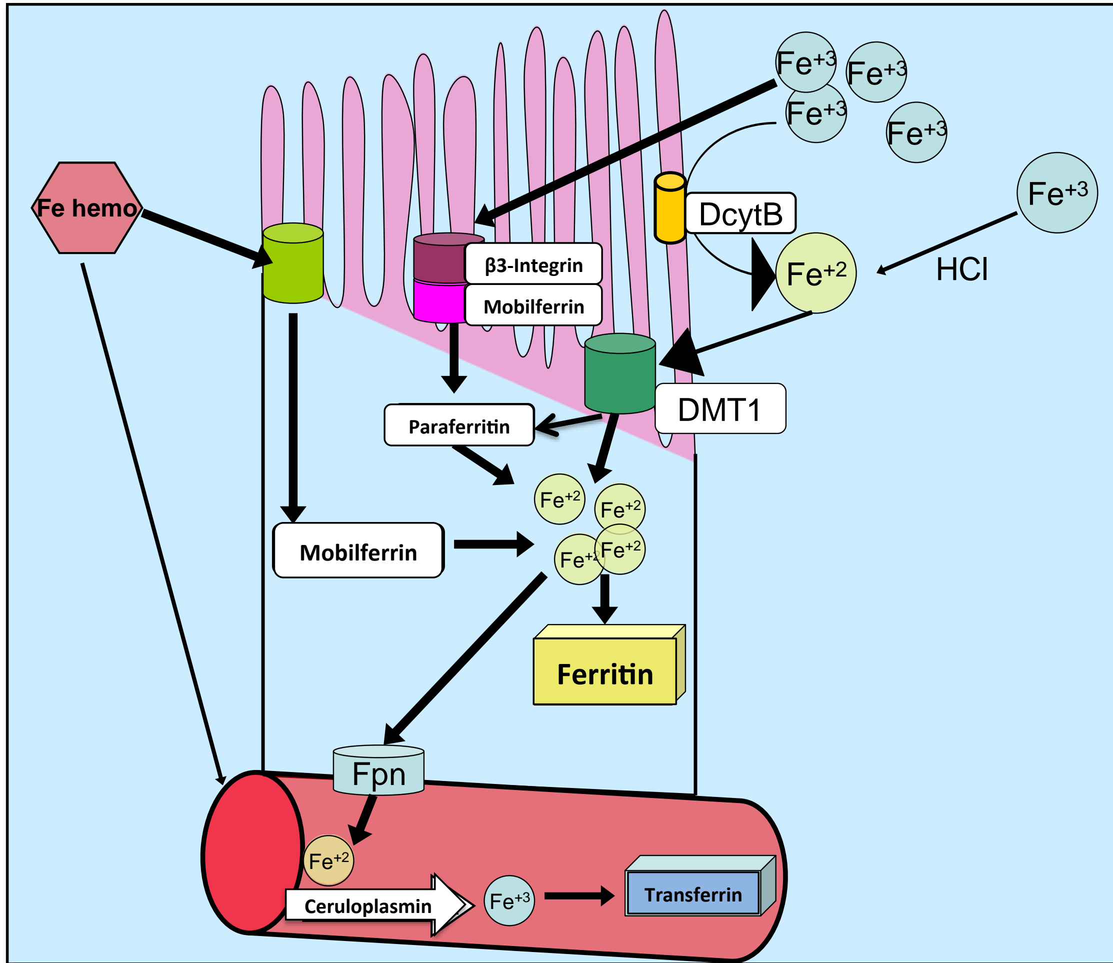


Figure 2.4.- *Non-heme and heme Fe intestinal absorption scheme*

Considering that breast milk and formulas do not contain heme Fe, non-heme Fe is the main source of dietary Fe in newborn. Non-heme Fe is bound to proteins or other low molecular weight proteins presented in milk (Leong, 2003; Collard, 2009). Among these compounds, lactoferrin is the most important protein, with a concentration of 1 to 6 mg/ml and a 10 % of Fe saturation. In addition to Fe, lactoferrin is able to bind other divalent ions, such as Zn or copper, being also involved in its transport and absorption (Kanyshkova *et al*., 2001).

As non-heme Fe is the main form in breast milk, the major Fe-transport proteins in the newborn are the divalent metal transporter 1 (DMT-1) and ferroportin (Collard,

2009). During the first 6 months of age, infants can not regulate Fe absorption depending on Fe intake, however, during intestinal maturation, infant and toddlers gains the ability of upregulating Fe absorption when its stores decreases (Boudry *et al*., 2010; Domellöf *et al*., 2014).

1.6.- Zn in infant nutrition and development.

Zn is an important trace element that plays an essential role during development and growth, forming part of organic structures or regulating the catalytic activity of different enzymes (Salgueiro *et al*., 2002). In particular, this mineral is a cofactor of different enzymes involved in the synthesis of DNA and RNA. Zn also contributes to the stabilization of neuronal proteins structure, being essential for the development and correct functioning of the central nervous system. In addition, Zn interacts with important enzymes involved in bone growth, such as somatomedin-C, osteocalcin, testosterone, thyroid hormone or insulin. Apart from these functions, Zn actively participates in the metabolism of lipids, carbohydrates and proteins (Sandestead *et al*., 2000; Brown *et al*., 2001; Salgueiro *et al*., 2002; Prado & Dewei, 2014).

There is a lack of information about prevalence of global Zn deficiency. It has been estimated as the total daily per capita amount of Zn in the national food supply in relation to the population's theoretical Zn requirements. According to this method, in Europe and North American a 1 – 3 % population is at risk for low Zn intake. Higher values (68 – 95 %) have been described for Southest Asia and Africa. Globally speaking, nearly half of the world's population is at risk for low Zn intake (Brown *et al*., 2001). Due to its essential role on brain development and function, Zn deficiency in animals has

been associated with deficits in activity, attention, learning, and memory (Prado & Dewey, 2014). Yasuda *et al*., (2011) found that, low hair concentration of Zn, was commonly described in children diagnosed with autistic spectrum disorders. In full term and preterm infants, Zn deficiency has been also associated with dermatitis and other cutaneous symptoms, which appears at about 3 months of age (Dorea, 2000).

Krebs (1998) reported that, during the first weeks postpartum, Zn excretion in breast milk ranges from 30-40 μM/day, decreasing to 15 μM/day at 2 or 3 months postpartum. Other authors have reported a high variability between mothers (Dorea, 2000). In the majority of cases, this Zn is bound to carrier proteins, especially α-2-macroglobulin, lactoferrin, casein, albumin and methalothionein, ensuring a high bioavailability for the infant and a suitable supply of Zn (Dorea, 2000; Salgueiro *et al*., 2002).

1.6.1.- Basis of Zn homeostasis in infants.

Body's Zn content in adults has been estimated at 2 – 3g. Skeletal muscle contains the major Zn storage (60 %), followed by bones (30 %), liver and skin (5 %) and other tissues (2-3 %). According to these data, serum Zn represents a very low percentage of the total body stores (0.1 %), being mainly bound to albumin (80 %) and α-2-macroglobulin (20 %). Body stores are highly regulated by Zn intestinal absorption, which increases when Zn intake is deficient. Main excretion pathways are constituted by endogen intestinal excretion (sloughing mucosal cells and intestinal secretion), and renal excretion. These mechanisms are able to maintain a constant content of Zn over a 10-fold change in intake (King *et al*., 2000; Kambe *et al*., 2015). In infants, serum Zn concentration is comparable to serum concentrations in adults. During the first months

of life, liver stores maintain serum concentration, which accumulates during the last trimester of pregnancy (Picciano, 2001). For this reason, breastfed infants younger than 6 months of age, are assumed to be able to satisfy Zn requirements from human milk (Brown *et al*., 2001)

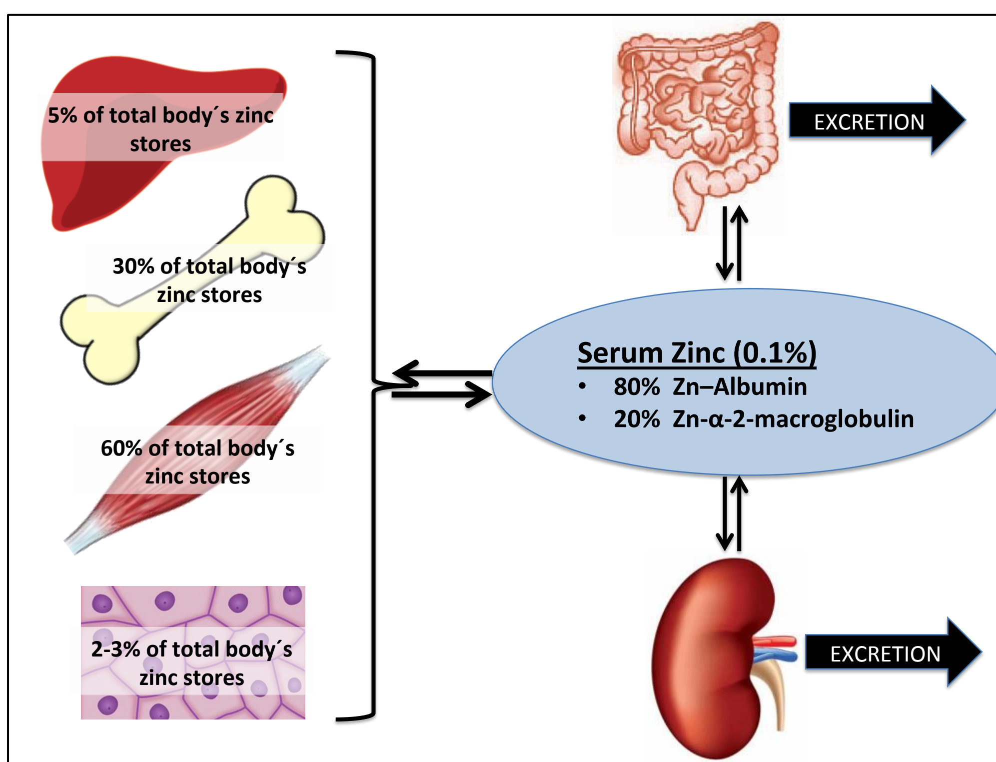


Figure 2.5.- *Body's Zn homeostasis scheme. % of the total body's Zn stores per organ has been represented. Adapted from Kambe et al., (2015).*

Gastrointestinal tract is the main mechanism for controlling Zn homeostasis, as it regulates Zn absorption and endogenous excretion in feces (King *et al*., 2000). The basis of Zn regulation mechanism is not very well known, but it seems that a deficient intake leads is compensated by an increment in Zn intestinal absorption and a significant reduction in fecal/urine excretion. When comparing both, intestinal and urinary regulation of Zn homeostasis, urinary Zn excretion seems to be less effective than intestinal excretion, contributing with endogenous fecal excretion after high intakes (King *et al*., 2000; Cousins, 2010).

1.6.2.- Introduction to Zn intestinal absorption.

As it was explained for Ca, Zn absorption process occurs by two different mechanisms: a saturable carrier-mediated process which is mainly described in the proximal small intestine; and a passive paracellular transport of Zn by diffusion between tight junctions, which involves small intestine and colon. Whereas the former is more efficient under low Zn concentrations, the latter efficiency is directly proportional to Zn concentrations in intestinal lumen (Cousins, 2010; Gopalsamy *et al*., 2015).

Zn membrane transporters are divided in two families, encoded by the solute linked carrier (SLC) gene family: the Zn/Fe-regulated transporter-like protein (Zip) family, and the Zn Transporter family (ZnT) (Boundry *et al*., 2010). Their classification in the SLC family attends to the fact that hydrolysis is not required for Zn membrane transport. With regard to Zn homeostasis, Zip and ZnT families seem to have opposite functions (Liuzzi & Cousins, 2004; Boundry *et al*., 2010; Kambe *et al*., 2015; Kiela & Ghishan, 2016):

- On one hand, Zip transporters promote Zn transport into cytoplasm, increasing its intracellular concentrations.
- On the other hand, ZnT transporters reduce intracellular Zn concentrations by favoring its efflux through basolateral membrane to portal circulation, or by moving Zn into cytoplasmic vesicles/Golgi apparatus.

14 different types of Zip transporters have been defined in humans, of which Zip4 is the main responsible for Zn intestinal absorption from intestinal lumen. The biochemical basis of Zip functionality still remains unclear (Liuzzi & Cousins, 2004; Boundry *et al*., 2010; Kambe *et al*., 2015). Zip4 expression in apical enterocyte

membrane, is strictly regulated by Zn concentration. Under Zn deficiency, its expression increases, being rapidly internalized and destroyed when Zn organic concentration increases (Kambe *et al*., 2015). Transcription of Zip4 encoding genes seems to be regulated by Kruppel-like factor 4 (KLF4) (Cousins, 2010).

Once in the cytoplasm, Zn is distributed in the three cellular compartments: cytoplasm (50 %), nucleus (30–40 %), and endomembrane system (10 %), maintaining a total cellular concentration ranged between tens and hundreds µM. Due to the fact that Zn plays a crucial role in cellular signaling, a cytosolic protein named metallothionein chelates it. Zn-metallothionein complexes represent about 5 – 15 % of total cytosolic Zn pool. Apart from being an important Zn storage molecule, metallothionein is also an intermediate product in intracellular Zn trafficking and signaling, carrying it to the basolateral membrane for being exported to the portal circulation. When Zn concentrations are excessive, metallothionein gene expression is upregulated by the metal-response element-binding transcription factor-1 (MTF-1) (Cousins, 2010; Kambe *et al*., 2015).

With regard to ZnT family, 9 different types have been described in Humans. These transporters regulate intracellular concentrations of Zn, facilitating its excretion or their storage in intracellular vesicles. Biochemically, ZnT act as Zn^{+2}/H^{+} exchanger (Kambe *et al*., 2015). When transcellular Zn trafficking in enterocyte is analyzed, the main ZnT transporter involved seems to be ZnT7. To be concrete, ZnT7 is localized at Golgi apparatus membrane, being associated to to the secretory pathway. Other ZnT transporters such as ZnT2 and ZnT4-6 have been described in the endomembrane system of enterocytes (Cousins, 2010; Kambe *et al*., 2015). Apart from ZnT in

intracytosolic vesicles, ZnT1 mediates transference of intracellular Zn into portal circulation being the main controller of enterocyte Zn efflux. ZnT1 appears at the basolateral membrane of enterocyte (Cousins, 2010). As it has been described for metallothionein, ZnT expression is regulated by MTF-1. Together with KLF4 for transcription of Zip4, MTF-1 is the only described transcription factor described in humans that plays a central role in Zn homeostasis (Cousins., 2010; Kambe *et al*., 2015).

Huang *et al*., (2006) analyzed the expression of Zn transporters in the developing mouse intestine. According to these authors, ZIP4 encoding mRNA increases by 15 days of life, being able to upregulate the presence of Zip4 on the apical membrane of enterocytes under dietary deficiency. With regard to ZnT1, its expression on basolateral membrane increases during development (Liuzzi *et al*., 2003).

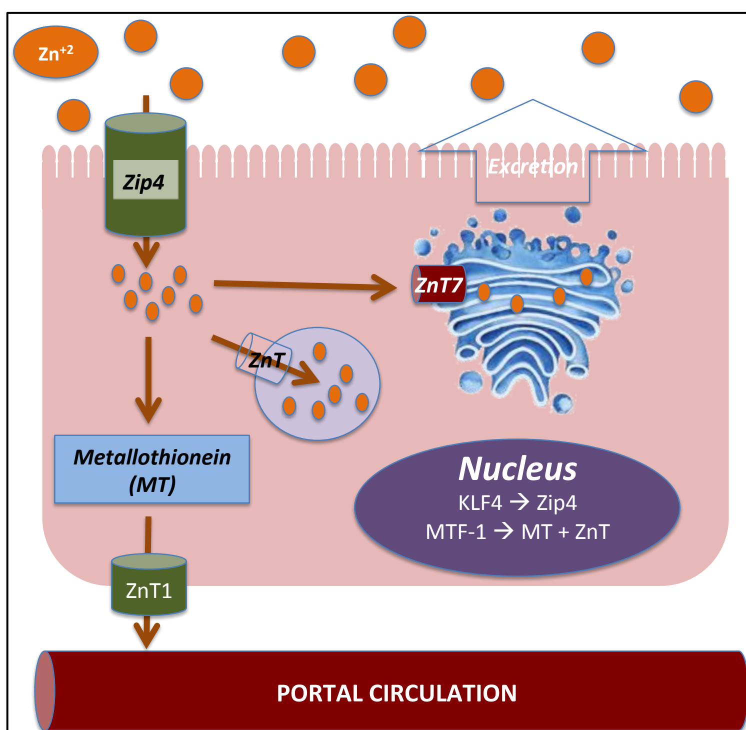


Figure 2.6.- *Scheme of Zn intestinal absorption and its regulation.*

1.7.- AR-infant formulas and mineral availability.

Nowadays, infant formulas are specially formulated to mimic human breast milk, being mainly made from modified cow's milk. When comparing both, whey proteins:casein ratio is higher in human's milk than in cow's milk (Fomon, 2001). Whereas in human's milk whey proteins:casein ratio varies from 90:10 in early lactation to 60:40 in mature milk (Kunz & Lönnerdal, 1992), a 20:80 ratio have been described in cow's milk (Malacarne *et al*., 2002). Due to the high casein concentration in cow's milk, this protein tends to form clots of curds in the stomach after the association with Ca and phosphorous, making it harder to digest. In comparison, human's milk is more easily digested as whey proteins remains in a soluble form. As a consequence, Ca and other ions bioavalability are lower in cow's milk than in human's milk (Malacarne *et al*., 2002; Martin *et al*., 2016). In order to make infant formulas similar to human's milk composition, since 1990's humanized infant milk formulations are used, in which casein concentration is reduced to 30-40 % of the total protein content (Fomon, 2001). However, aminoacidic profile from cow's milk proteins, still remains quite different from human's milk, existing a possible relationship between this composition and food allergies development (Lara-villoslada *et al*., 2005)

With regard to AR infant formulas, they are specially formulated for preventing the return of gastric content to the oesophagus in non-complicated infant gastro-oesophageal reflux. With this purpose, Locust bean gum or modified starches have been added as thickening ingredients (Agostoni, 2004; Vandeplas, *et al*., 2009[b]; Kirmemis, 2017). Despite being available for consumer purchase, several authors have indicated the need to explore further the effect of these ingredients on the nutritional status and

health of infants. These advises are based on different *in-vitro* studies which suggest that, the bioavailability of Ca, Fe and Zn may be affected by the addition of thickening agents. Negative effect of thickeners in mineral availability could be associated with the presence of ionisable groups, their resistance to the digestion process and/or the presence of antinutrients such as phytic acid *(*Bosscher *et al*., 2000; Bosscher *et al*., 2003[a]; Bosscher *et al*., 2003[b]; Frontela *et al*., 2008; Vandenplas, *et al*., 2013). Due to the previously exposed findings, the European and North American Societies for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN–NASPGHAN) published the guidelines for management of infant anti-regurgitation (Vandeplas, *et al*., 2009[b]). According to these guidelines, the use of AR infant formulas should be only prescribed in healthy term infants with uncomplicated gastroesophageal reflux, avoiding their use in premature and low birth weight infants.

2.- AIM OF THE STUDY

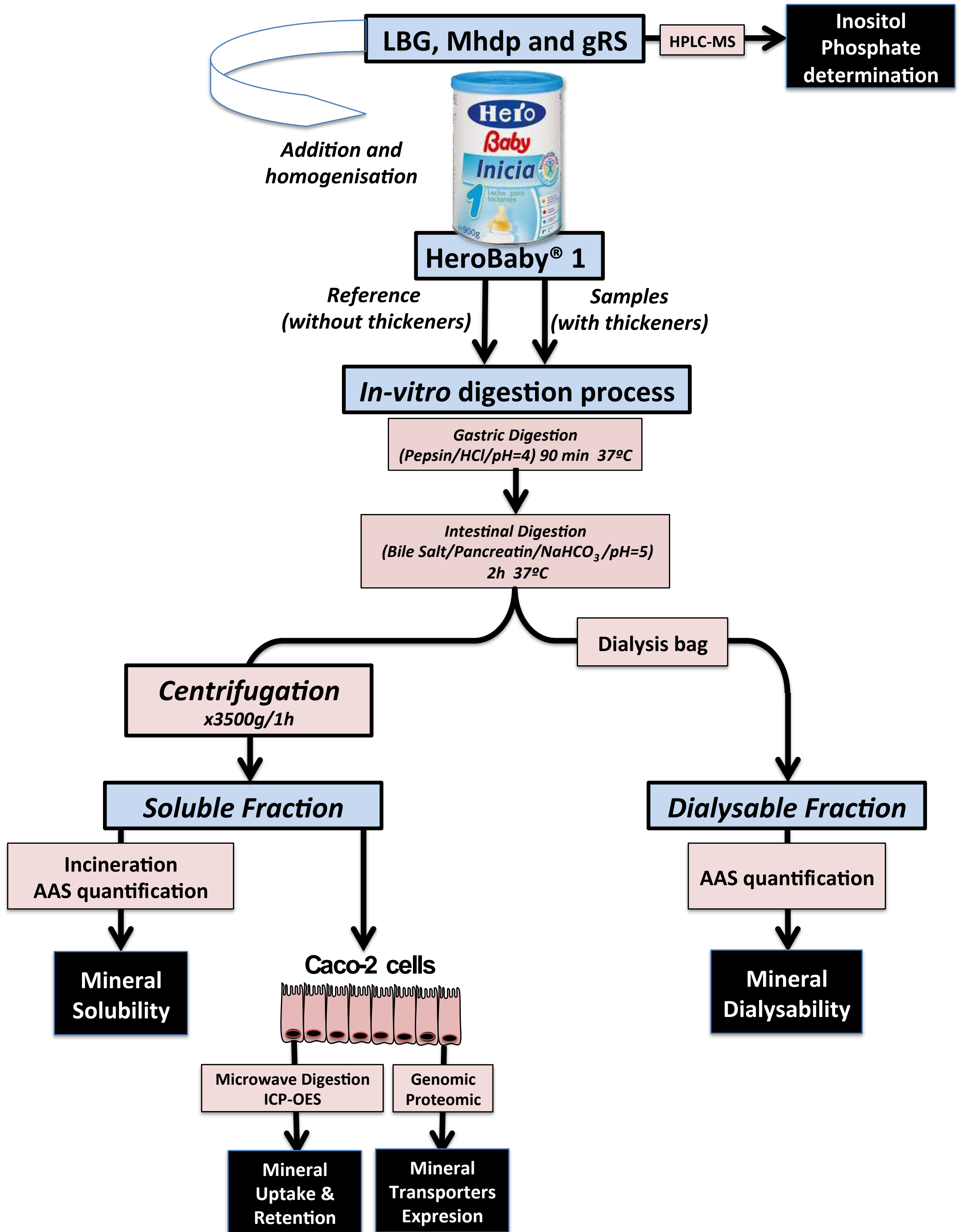
As it has been explained before, no concluding results about the effect of adding thickener agents on mineral availability are currently available. At this respect, the ESPGHAN highlighted the need to explore further their possible interference on infant mineral availability.

In order to clarify this effect, the main objective of this chapter was to determine the effect of the addition of three different agents, Locust Bean Gum (LBG), Maize distarch phosphate (Mhdp) and pre-gelatinized Rice Starch (gRS) to an infant formula, on Ca, Fe and Zn *in-vitro* availability after an *in-vitro* digestion process. With this aim, three different specific objectives have been proposed:

- To determine the effect of LBG, Mhdp and gRS on Ca, Fe and Zn solubility and dialysability after an *in-vitro* digestion process. (This specific objective was completed with the quantification of inositol phosphates in LBG, Mhdp and gRS, as possible antinutrients).
- To analyze the effect of LBG, Mhdp and gRS on Ca, Fe and Zn uptake and intracellular transport, using a human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial absorption, after an *in-vitro* digestion process.
- To clarify the effect of LBG, Mhdp and gRS on Ca, Fe and Zn cell's transporters expression, on a human carcinoma cell line (Caco-2) model, after an *in-vitro* digestion process.

3.- MATERIAL & METHODS

3.1.- Experimental design



3.2.- Samples & Thickening Ingredients.

Standard infant formula (Hero Baby® 1) was provided by Hero España S.A. (Alcantarilla, Murcia, Spain). Locust bean gum (Grinsted LBG 860, Danisco, Portugal), Maize distarch phosphate (Mhdp; Multi-Thick®, Abbott Nutrition, Spain) and pre-gelatinized Rice Starch (gRS; Beneo-Remy Industries, Belgium) where used as thickening agents.

3.3.- Samples preparation and reconstitution.

Different concentrations of each thickening agent were added to 15 g of the standard infant formula (Hero Baby® 1) (Table 2.2). These concentrations were fixed as 7.5 %, 15 %, 50 % and 100 % respect the maximum legal limit per each thickener. In order to get as homogenous mixture a VH-5 high-efficiency mixer (Comecta SA, Barcelona, Spain) was used. (European Parliament and Council, 1995; European Parliament and council, 2006). Formula without thickener added was used as reference. Once prepared, each sample was reconstituted according manufacturer recommendations (30 g/200 mL). For reconstitution, MilliQ water (Millipore, Bedford, MA, USA) was added.

Table 2.2.- *Concentration of each thickening agent (LBG, Mhdp and gRS) per each 15 g of standard infant formula. These concentrations were fixed as 7.5 %, 15 %, 50 % and 100 % respect the maximum legal limit.*

Infant Formula		Ingredient	7.5%	15%	50%	100%
		LBG	7.5 mg	15 mg	50 mg	100 mg
15 g	+	Mhdp	0.15 mg	0.3 mg	1 mg	2 mg
		gRS	0.15 mg	0.3 mg	1 mg	2 mg

3.4.- Quantification of Inositol Phosphates in thickening agents

In order to determine/discard the possible presence of antinutrient in LBG, Mhdp and gRS, an Inositol Phosphate (IPs) extraction and measurement method was carried on as follows:

Inositol phosphates (IPs), including phytic acid (myo-inositol hexaphosphoric acid), were extracted from the different ingredients before mixing with infant formula. This extraction was performed with 0.5 N HCl at room temperature for 2 h. Each extract was then centrifuged and the supernatant frozen overnight, followed by thawing and centrifugation. An aliquot of supernatant was poured onto an anion exchange (SAX) column (500 mg; Supelco, Bellefonte, PA, USA) connected to a vacuum manifold set at 20 mmHg. The resin-bound inositol polyphosphates were eluted with 2 mL of 2 M HCl. Eluted samples were evaporated to dryness under vacuum at 40ºC, and then dissolved in 1 mL of deionized water.

Inositol phosphates were determined by HPLC-MS (Liu, *et al*., 2009) using a reverse-phase chromatography Agilent 1100 series (Agilent Technologies, Santa Clara, CA, USA) equipped with a thermostated micro-well plate auto-sampler, and a quaternary pump. The equipment was connected to an Agilent Ion Trap XCT Phis Mass Spectrometer (Agilent Technologies) using an electro-spray interface (ESI). Samples and standards (40 µL) were injected into a C18 reverse-phase HPLC column (Agilent Technologies), thermostated at 40ºC, and eluted at a flow rate of 200 µL/min throughout the separation. Samples were passed through 0.22 µm HPLC filters before

injection. The mobile phase consisted of two solvents: solvent A, 0.1 % formic acid in water; and solvent B, 0.1 % formic acid in acetonitrile. Inositol phosphates were eluted as follows: from 10 to 100 % B in 30 min; from 100 to 10 % B in 15 min; an isocratic elution of 10 % was maintained from 45 to 60 min to equilibrate the column under the initial conditions. Mass spectrometer was operated in a negative ion model with a capillary spray voltage of 3,500 V, and a scan speed of 2,200 (m/z)/sec at 50–750 m/z. The nebulizer gas pressure, drying gas flow rate and drying gas temperature were respectively set at 30 psi, 8 L/min and 350ºC. HPLC-MS equipment control and data acquisition was performed with Agilent Chemstation Rev B.01.03.SR2. Data were processed using the data analysis software for LC/MSD Trap version 3.3 (BrukerDaltomik, GmbH, Bremen, Germany) provided by the manufacturer.

3.5.- In-vitro digestion process.

After samples and reference (formula without thickeners) reconstitution, they were digested using a non-dynamic *in-vitro* method (Miller *et al*., 1981; Boato, *et al*., 2002; Minekus *et al*., 2014). Considering that the gastrointestinal tract in the early stages of life is not yet fully developed, the method was modified for, reducing the amounts of enzymes used, and varying the original pH values (Frontela, *et al*., 2008; Frontela, *et al*., 2009). This *in-vitro* digestion process consisted in a gastric and an intestinal stage, which where performed at 37ºC in a shaking water bath. In order to obtain a soluble and a dialyzable fraction, two different kind of intestinal digestions were performed. A in-vitro digestion flow diagram can be seen in Figure 2.7.

According to the standardized digestion protocol proposed by international consensus (Minekus *et al*., 2014), and due to the fact that liquid samples were digested, the oral digestion phase was skipped.

3.5.1.- Digestive enzyme solutions preparation.

Before each digestion phase, freshly enzyme solutions were prepared. To simulate the gastrointestinal conditions of children younger than 6 months of age, a pepsin solution was prepared by dissolving 1.6 g of pepsin from porcine stomach mucosa (P-7000, Sigma-Aldrich, St. Louis, MO, USA) in 10 ml of 0.1 N HCl. The pancreatin-bile salts solution was prepared by dissolving 0.2 g of pancreatin from porcine pancreas (P-1750, Sigma-Aldrich, St. Louis, MO, USA) and 1.25 g of bile salts (B-8756, Sigma-Aldrich, St. Louis, MO, USA) in 50 ml of 0.1 M $NaHCO_3$.

3.5.2.- Gastric Digestion.

After each samples reconstitution, pH was lowered to 4 adding 1M HCL, and pepsin solution added until reaching a concentration of 0.195 g of pepsin per gram of dry sample. Samples were incubated in a shaking water-bath set at 37°C for 90 min. After incubation, *in-vitro* gastric digestion was stopped by soaking samples in an ice bath for 10 min.

3.5.3.- Intestinal digestion for soluble fraction.

Previously to the intestinal digestion, pH was raised to 5 by adding 6M NaHCO3. After pH adjustment, pancreatin-bile salts solution was added until a concentration of 0.048 g of pancreatin and 0.292 g of bile salts per gram of dry sample was reached. Samples were then maintained for 120 min in a shaking water bath set at 37 ºC.

At the end of the intestinal stage, aliquots of each sample were transferred to 50 mL polypropylene centrifuge tubes (Costar Corning Europe, Badhoevedorp, Netherlands) and then centrifuged (Eppendorf 5804-R Centrifuge, Hamburg, Germany) at 3,500 x g for 1 h at 4ºC. Supernatant (soluble fraction) was maintained under -20ºC until use

3.5.4.- Intestinal digestion for dialyzable fraction.

Dialysis comprised the gastric stage, followed by an intestinal step in which, a 12,000- 14,000 MW cut-off dialysis membrane, previously rehydrated, was introduced in a flask, together with a 20 mL aliquot of sample. Each dialysis bag contained 50 mL of a $NaHCO_3$ dissolution with a concentration equivalent to the titratable acidity.

Titratable acidity can be defined as the total concentration of free protons and un-dissociated acids in a solution, which can react with a strong base and thus, be neutralized. With this purpose, 20g aliquots of each gastric digestion were isolated and 0.5M NaOH dropwise added until reaching a pH value of 5. The amount of $NaHCO_3$ needed for increasing gastric content pH from 4 to 5 was determined as follows:

$$g\ NaHCO3 = \frac{(mL\ of\ 0.5M\ NaOH)\ x\ 2\ x\ 84}{1000}$$

In order to let $NaHCO_3$ diffuse, and the pH increase until stabilization, samples were incubated in a shaking water bath for 15 minutes. After stabilization, pancreatin-bile salts solution was added until a concentration of 0.048 g of pancreatin and 0.292 g

of bile salts per gram of dry sample. After the digestion, dialysis membrane content was collected and preserved under -20ºC until use.

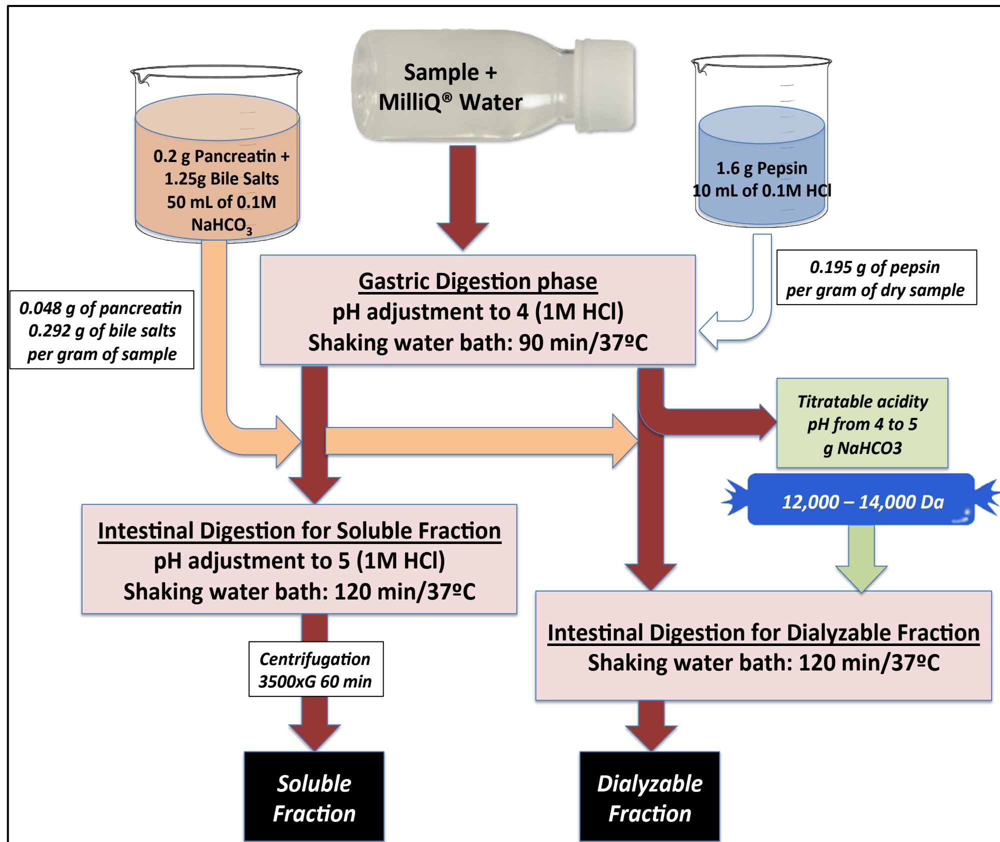


Figure 2.7.- *In-vitro digestion process flow diagram. Two different intestinal digestion were performer for obtaining soluble and dialyzable fractions.*

3.6.- Mineral solubility and dialysability determination.

Despite the fact that, *in-vitro* digestion models based in solubility and dialysability determinations are limited in the way that they skip different factors involved in *in-vivo* intestinal digestion and absorption, they are useful method to understand factors that may affect subsequent mineral absorption (Sandberg, 2005). In this way, minerals contained in the supernatant represent the percentage of total mineral content that are released from food matrix during the digestion process, solubilized and, therefore, are available for being absorbed by the intestinal epithelium.

With regard to the mineral dialyzed through the semipermeable membrane, they represent the bio-available fraction (expressed as a percentage) of the total mineral present in the sample (Etcheverry, *et al*., 2012; Frontela, *et al*., 2008). As no membrane transporters are involved, dialysability could be compared to the passive mineral absorption through paracellular pathways. As it has been previously explained in the introduction section, paracellular pathways transport is especially efficient when there are high concentrations of minerals in the intestinal lumen (Hoenderop *et al*., 2005; Cousins, 2010; de Barboza *et al*., 2015; Gopalsamy *et al*., 2015). With regard to Fe, dialysability can be used to predict availability of non-heme Fe, which is the main source of dietary Fe in newborn.

3.6.1.- Soluble fraction conditioning.

After *in-vitro* digestion and centrifugation, organic matter contained in supernatants was destroyed by ashing in a temperature-programmed furnace (Heraeus M1100/3, Hanau, Germany) at 525°C for 24 h. After incineration, an acidic digestion process was performed adding 3 mL of HNO_3 (specific gravity: 1.38 g/mL) and heating until dryness. Inorganic matter was then resuspended by adding 1 mL of HCl (specific gravity: 1.19 g/mL). The solution was transferred to a volumetric flask and volume adjusted by adding MilliQ® water. Samples were in this way ready for mineral determination.

3.6.2.- Mineral Solubility and dilysability determination by AAS.

Ca, Fe and Zn concentrations in soluble and dialyzable fractions, were determined by flame atomic absorption spectrometry (AAS) (Thermo Scientific AA Spectrometer S Series, USA). Mineral content in the conditioned soluble and dialyzable

fractions was determined against Fe, Ca, and Zn standard solutions. For Ca determination, lanthanum chloride was added to obtain a final content of 0.1 % to suppress phosphate interferences. Calibration curve was obtained between 1 and 5 ppm for Fe and Ca, and 0.1 and 1 ppm for Zn, showing correlations coefficients grater than 0.997. Solubility and dialysability were calculated as follows:

$$\text{Solubility (\%)} = \frac{\text{Soluble mineral content (mg/100 g)}}{\text{Total mineral content of the sample (mg/100 g)}} \times 100$$

$$\text{Dialysability (\%)} = \frac{\text{Dialysable mineral content (mg/100 g)}}{\text{Total mineral content of the sample (mg/100 g)}} \times 100$$

In order to discard matrix interferences in mineral quantification, and establish AAS measurements accuracy, a validation criteria was previously performed. With this pourpose, Ca, Fe and Zn content were analyzed in both, a reference material BRC-380R (whole-milk powder) (Sigma Aldrich, Germany) and samples. The measured mean values (n=3) for Fe, Ca and Zn were 2.04, 13,125 and 37.98 µg/g respectively, which were in accordance with the certified range of 2.56 ± 1.28 µg/g for Fe, 12,855 ± 445 µg/g for Ca, and 39.45 ± 2.52 µg/g for Zn. The obtained values, expressed as a coefficient of variation (%), were 0.97 for Fe, 1.2 for Ca, and 1 for Zn.

3.7.- Caco-2 cells: Mineral uptake and transport analysis.

Caco-2 cells is a human epithelial cell line, derived from a Human colonic adenocarcinoma (Caco-2). When these cells are under *in-vitro* culture and reach confluence, they differentiate to human intestinal cells, behaving structurally and functionally mostly as mature enterocytes (Etcheverry *et al*., 2012). The combination of

Caco-2 cell cultures with *in-vitro* digestion process is a useful method for analyzing mineral availability in humans, regardless of type of transport: transcellular or paracellular (Yee, 1997; Fairweather *et al*., 2005).

3.7.1.- Caco-2 cells stock maintenance.

Caco-2 cells line (ECACC number 86010202) was obtained from the European Collection of Cell Cultures, ECACC (Salisbury, UK) at passage 16. Cells were cryopreserved in liquid nitrogen until use (4 – 6 x 10^6 cells/ml), using a 10 % dilution of dimetil-sulfoxide (DMSO, Sigma-Aldrich, St Louis, MO, USA) in Dubelco's minimum essential medium (DEMEM, Gibco BRL life technologies, Rockville, MD, USA) as preserver medium.

Cells were seed in polypropylene 75 cm^2 culture flask (Sarstedt Inc., NC USA) at a density of 40,000 viable cells/cm^2. Cell concentration and viability was assessed by direct cell counting in a Neubauer chamber (Neubauer, Marienfeld, Germany) after staining with 0.4 % trypan blue (Colour Index, 23850; Merck KGaA, Darmstadt, Germany). Viability > 85 % was considered as optimum. During the growing period, cells were maintained in DMEM + Glutamax®, previously supplemented with 10 % v/v heat-inactivated fetal bovine serum, FBS (Gibco BRL life technologies, Rockville, MD, USA), 1 % v/v non-essential amino acids solutions (Sigma-Aldrich, Germany), 1 % penicillin-streptomycins solution (Sigma-Aldrich, Germany). Cells were incubated in an incubator (Binder, Tuttlingen, Germany) under the following environmental conditions: 37 °C, 10 % CO_2, 90 % atmospheric air, and 95 % relative humidity. The medium was changed every 2 days. Before reaching a 75 % of confluence, cells were trypsinized with a

trypsin-EDTA solution (0.25 mg/mL) and then re-seeded to avoid cells differentiation (see Figure 2.8). Experiments were conducted between passages 25 – 39.

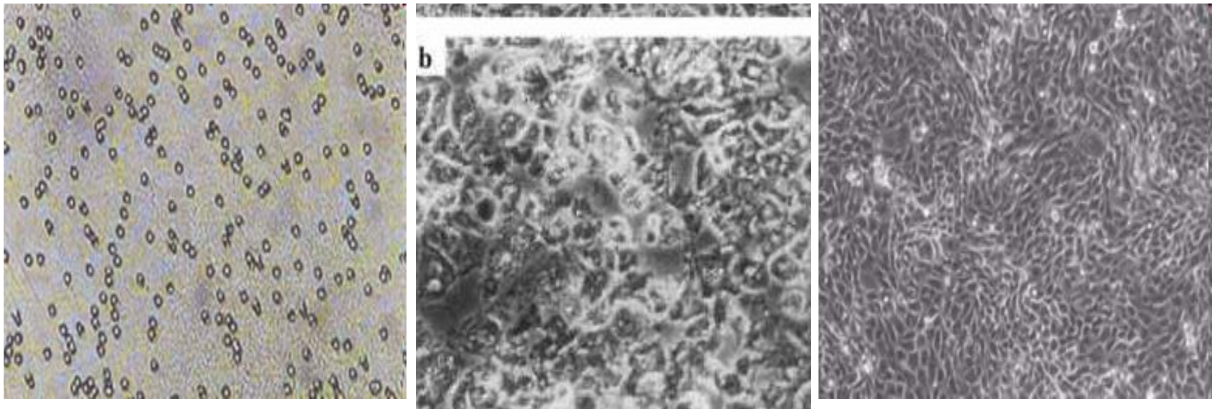


Figure 2.8.- *Microscopic differentiation of Caco-2 cell culture.*

Before the experiments, Caco-2 cells were routinely tested to discard mycoplasma contamination, as they have been described to affect cells culture growth and metabolism (Rivera-Tapia *et al*., 2010). The detection method consisted of a nuclear-DNA stain by Hoechst® 33342 and fluorescence microscopy (Chen, 1997). Negative (a) and positive (b) Hoechst test are shown in Figure 2.9.

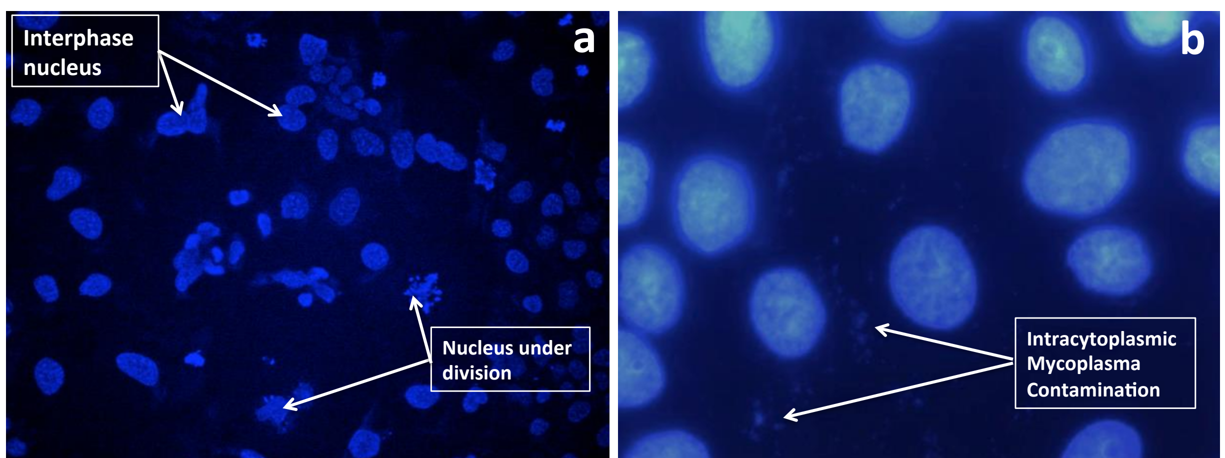


Figure 2.9.- *Mycoplasma detection by Hoechst® 33342 nucleic-acid tinction. Negative (a) and positive (b) mycoplasma contamination.*

3.7.2.- Mineral uptake and transport assay.

For *in-vitro* mineral uptake and transport determination by Caco-2 cells monolayer, samples with a maximum legal limit concentration of thickening agents were chosen. Formula without thickening agent added, and Serum Free Medium (SFM) (DMEM+Glutamax® without FBS added) were respectively used as reference and Control.

For the experiment, Caco-2 cells (passage 25 – 39), were trypsinized and seeded onto polycarbonate membrane chamber inserts (24 mm diameter, 0.4 µm pore size; Transwell, Costar Corp) at a density of 50,000 cells/cm^2. Inserts were placed in 6 wells plates (Multiwell-TM6 wells Falcon®, Dutscher, France), allowing cells to differentiate for 14 d. During this period, cells were maintained in DMEM+Glutamax®, previously supplemented with 10 % v/v heat-inactivated FBS, 1 % v/v non-essential amino acids solutions, and 1 % penicillin-streptomycin solution. 1 mL and 1.5 mL of medium were respectively added to the apical and to the basolateral chambers. Cells were incubated at 37 °C, 10 % CO_2, 90 % atmospheric air, and 95 % relative humidity. On day 14, in order to avoid interferences by minerals presented in FBS, apical and basolateral chamber were washed with 37 ºC pretemperated phosphate-buffered saline (PBS) (3 washes x 1mL) (catalog number P3813-10PAK, Sigma-Aldrich, St Louis, USA), and medium was substituted by SFM.

After differentiation, cells monolayers were exposed to the previously obtained soluble fractions (see explanation at section 3.5.3.). In order to avoid cell damage by residual activity of gastric and intestinal enzymes, soluble fractions were diluted in SFM at a 3:1 ratio. Exposition to diluted soluble fractions was maintained for 2h. With the aim

of ensuring monolayer integrity, Trans-Epithelial Electrical Resistance (TEER) was measured every 30 minutes, using a Millicell® ERS-2 Volt-Ohm meter (Merck Millipore, Darmstadt, Germany). TEER (Ω/cm^2) was calculated according to the manufacturer recommendations, considering an average basal resistance of cell-free filters ≈ 0.3 Ω/cm^2. Monolayers with a TEER $< 500\ \Omega/cm^2$ were discarded.

After 2h of exposition, each fraction was separately collected (apical chamber, basolateral chamber and cell monolayer). For Cell monolayers collection, each membrane was carefully washed with 1 mL of 37 ºC pre-temperated PBS to remove any residual soluble fractions. Cells were subsequently scrapped and lysed by the addition of 1 mL of pre-cooled deionized water. Each fraction was stored under -80ºC until mineral determination. Ca, Fe and Zn content in apical chamber, basolateral chamber and cell monolayer were determined by Inductively Coupled Plasma-Optical Emission Spectrometry (iCAP 6500 ICP-OES Duo, Thermo Scientific, Waltham, MA, USA), previous organic matter destruction by microwave acid digestion (Analysis were performed in the Ionomic Service; CEBAS–CSIC, Murcia, Spain). In Figure 2.10 an scheme of Caco-2 cells assay can be seen.

Mineral uptake and transport through Caco-2 cells monolayer were estimated through the calculation of the percentages described by Perales *et al.*, (2005). As it has been previously explained, serum free medium (SFM) was used as control, and its values were respectively substracted from the results obtained for each sample and reference.

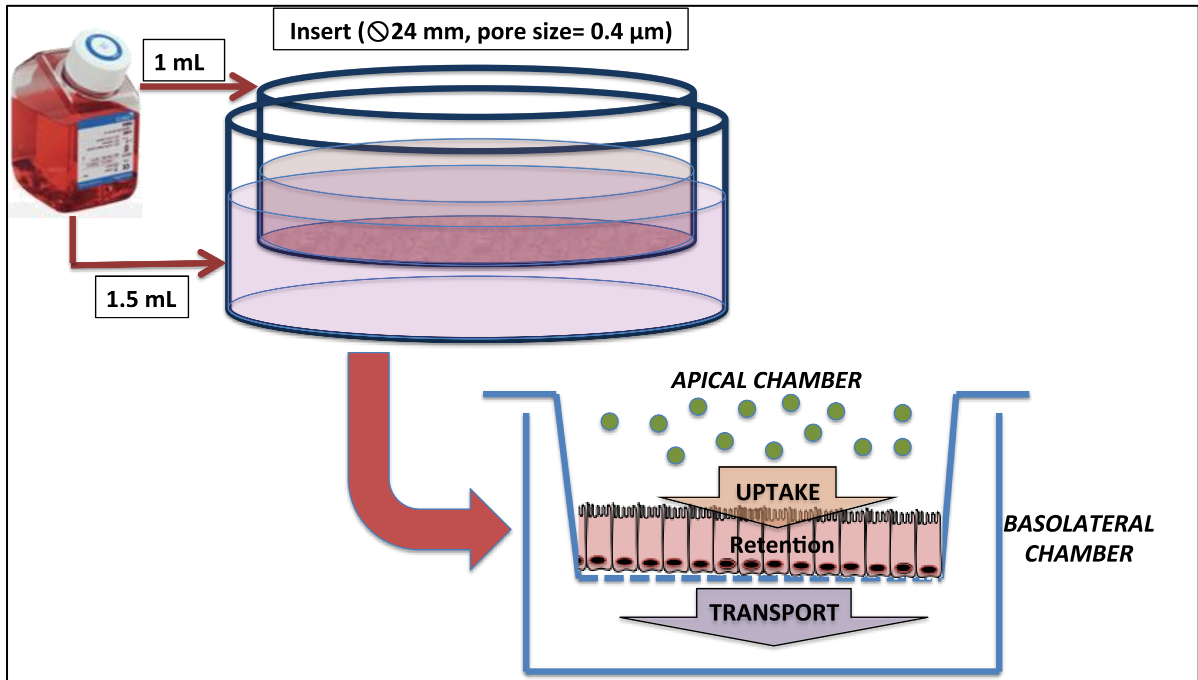


Figure 2.10.- *Scheme of Caco-2 cells uptake and transport assay.*

- ***Mineral Transport through cells monolayer***: Total amount of solubilized mineral that reaches the basolateral chamber across the cells monolayer.

$$Transp\ (\%) = \frac{Mineral\ content\ in\ basolateral\ fraction - mineral\ content\ with\ SFM}{Total\ mineral\ added\ in\ apical\ chamber}\ x\ 100$$

- ***Mineral Retained by cells monolayer***: Total amount of solubilized mineral retained by cells monolayer. In order to avoid variations in cells density, mineral contents were standardized as µg of mineral per mg of cells protein. Protein content in cells monolayer were calculated according to the Bradford Coomassie brilliant blue assay (Bradford, 1976). With this aim, the Coomassie (Bradford) Protein Assay Kit, (Thermo Scientific, Dreieich, Germany) was used.

$$Retention\ (\%) = \frac{Mineral\ content\ in\ cell\ monolayer - Mineral\ content\ in\ SFM\ treated\ cells}{Total\ calcium\ added\ in\ apical\ chamber}\ x\ 100$$

- ***Mineral Uptake***: Total amount of mineral that is captured by the system.

$$Uptake\ (\%) = Transport\ (\%) + Retention\ (\%)$$

- ***Transport (%) Efficiency:*** Total amount of solubilized mineral that reaches the basolateral chamber across the cells monolayer.

$$Transport\ Efficiency\ (\%) = \frac{(Solubility\ (\%) x\ Transport\ (\%))}{100}$$

- ***Uptake (%) Efficiency***: Relationship between the total amount of solubilized mineral captured by the system.

$$Transport\ Efficiency\ (\%) = \frac{(Solubility\ (\%) x\ Uptake\ (\%))}{100}$$

3.8.- Caco-2 cells: Mineral transporters expression.

In order to evaluate the effect of thickening agents on *in-vitro* mineral availability, expression of different mineral transporters encoding genes has been evaluated by qPCR. With this aim, the following mineral transporters have been chosen:

- ***Calbindin-D9K (CalD9K)***: After apical absorption, CalD9k chelates cytoplasmic free Ca^{+2} ions and transports them to the basolateralmembrane, maintaining a cytoplasmic low free Ca^{+2} concentration (Hoenderop *et al*., 2005; Christakos *et al*., 2011).
- ***Divalent Metal Transporter-1 (DMT-1)***: Is the major apical transporter involved in non-heme ferrous Fe (Fe^{+2}) intestinal absorption in infants. Apart from Fe, DMT-1 has a broad substrate range, including Zn^{+2}, Mn^{+2}, Co^{+2}, Cd^{+2}, Cu^{+2}, Ni^{+2}, and Pb^{+2} (Collard, 2009; Boudry *et al*., 2010; Domellöf *et al*., 2014).

- ***Ferritin***: Ferritin is a cytoplasmic store for intracellular ferrous Fe (Fe^{+2}). Ferritin is a heteropolymeric protein composed by two types of 24 subunits, light chain and heavy chain subunits (Ganz, 2013).
- ***Metallothionein-1M (MT1M)***: Metallothionein is a cytosolic protein, which chelates intracellular Zn. Apart from being a storage molecule, metallothionein is an intermediate product in intracellular Zn trafficking and signaling, carrying it to the basolateral membrane for being exported to the portal circulation (Cousins, 2010; Kambe *et al.*, 2015).
- ***Zip-4***: Is a Zn transporter presented in apical membrane, being responsible for Zn intestinal absorption (Liuzzi & Cousins, 2004; Boundry *et al.*, 2010; Kambe *et al.,* 2015).
- ***Zn Transporter 1 (ZnT1)***: ZnT-1 appears at the basolateral membrane of enterocytes, mediating Zn transference into the portal circulation (Cousins, 2010).

With regard to ferritin expression, it has been proposed that ferritin heavy and light chains are regulated on a post-transcriptional level (Sammarco *et al.*, 2008). For this reason, apart from the analysis of encoding genes expression, a ferritin direct quantitative determination by Inmunoassay ELISA was performed.

3.8.1.- Caco-2 cultures for mineral transporters gene expression.

Caco-2 cells were seeded in 6 wells plates at a density of 40,000 viable cells/cm^2. During the growing period, cells were maintained in DMEM + Glutamax®, previously supplemented with 10 % v/v heat-inactivated fetal bovine serum (FBS), 1 % v/v non-essential amino acids solutions, and 1 % penicillin-streptomycin solution. Cells were incubated at 37 ºC, 10 % CO_2, 90 % atmospheric air, and 95 % relative humidity. The medium was changed every 2 days. After microscopic examination of the cultures, the

experiments were conducted on day 14, once confluence and differentiation signs of the cells were observed.

3.8.2.- Time-Course experiment to determine optimal time of exposure.

Before samples evaluation, a time course experiment was conducted to determine the optimum time of exposure that will ensure a measurable effect on the mineral transporters genes expression after the treatment exposition. Once differentiation (14 days), Caco-2 cells were exposed to the soluble fraction of infant formula without thickeners added (reference), previously diluted 3:1 in Serum Free Media (SFM). Furthermore, cells were exposed to three mineral dissolutions of $CaCl_2$ (5 mM), $FeSO_4$ (200 µM) and $ZnSO_4$ (50 µM) in SFM, as positive controls. Based on previous bibliography, mineral concentrations were chosen to induce a positive mineral transporters expression without having toxic effects (Zödl *et al*., 2003; Frontela *et al*., 2009). As negative control, SFM was used. Five different times of exposition were analyzed (0h, 2h, 4h, 8h, 16h). For each exposition time, cell viability was assessed by the “Resazurin Reduction test” or “Alamar Blue test” (O’brien *et al*., 2009). Resazurin reduction was measured by fluorescence, using a Fluostar Optima spectrophotometer (BMG labtechnologies, Offenburg, Germany) at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Results were expressed as Absorbance increments between sample and control (resazurin solution without cells). According to the results summarized in section 4.4.1., an exposition time of 6h was chosen. An scheme of the time course experiment has been shown in Figure 2.11,

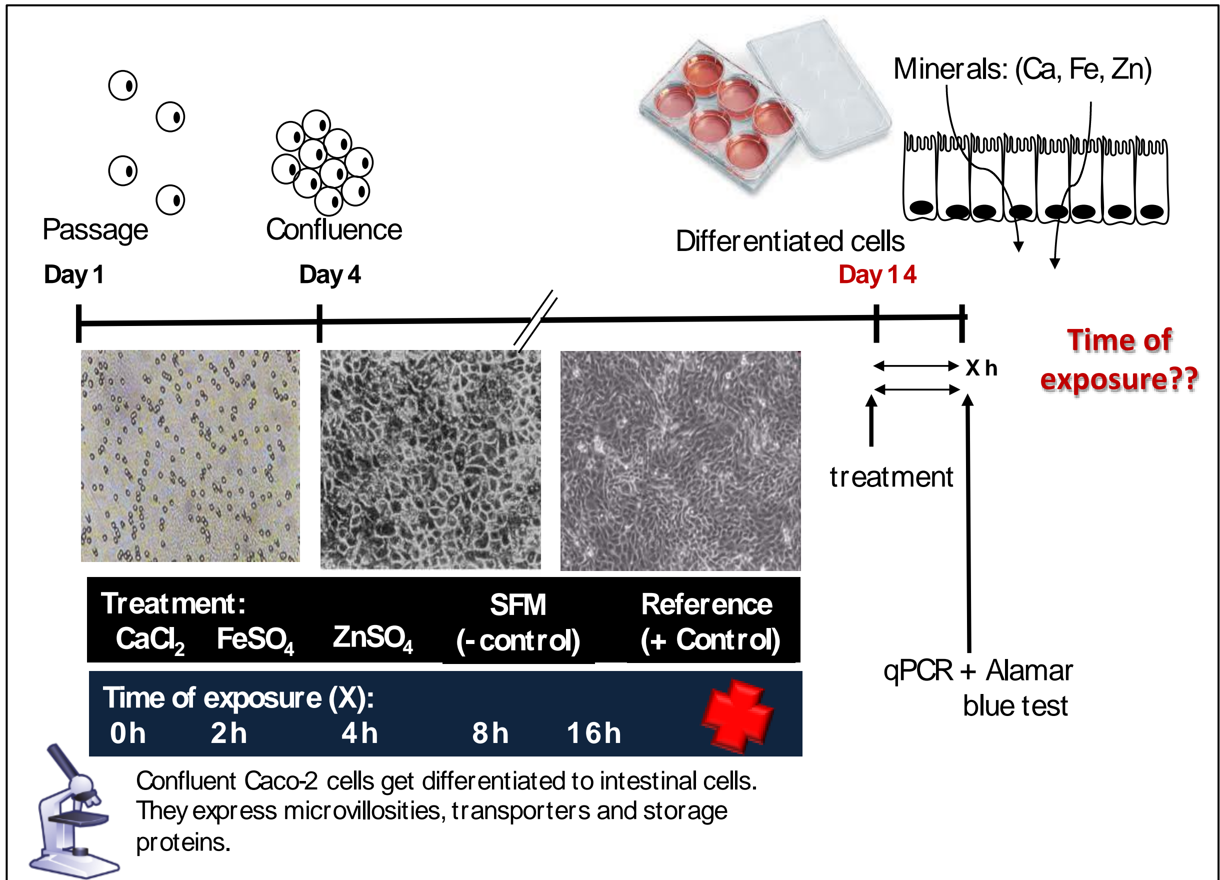


Figure 2.11.*-Time course Scheme to determine the optimum time of exposure for measuring the effect on the mineral transporters genes expression. As positive and negative controls, Caco-2 cells were exposed to infant formula without thickeners (Reference), and serum free media (SFM) respectively.*

3.8.3.- Samples exposition to treatments.

After 14 days of growth and differentiation (see section 3.8.1.), medium was discarded and Caco-2 monolayers were carefully washed 3 times with pre-tempered PBS. Caco-2 cells were then exposed to each soluble fraction obtained after the *in-vitro* digestion (explained in section 3.5.). Previously, in order to minimize the possible negative effects associated to residual enzyme activity, soluble fractions were diluted 3:1 in Serum Free Media (SFM). Cells were also exposed to different dissolutions of $CaCl_2$ (5 mM), $FeSO_4$ (200 µM), $ZnSO_4$ (50 µM) in SFM as positive controls. As Reference and negative controls, formula without thickening agents added and SFM were respectively added. In order to discard the effect of the enzymes added during *in-vitro* digestion, cells

were also exposed to the enzymes solution diluted 3:1 in SFM (Blank). Time of exposure (6h) was chosen according to the previously explained time course experiment (section 3.8.2). In Figure 2.12, a scheme of Caco-2 cells exposition experiment has been shown.

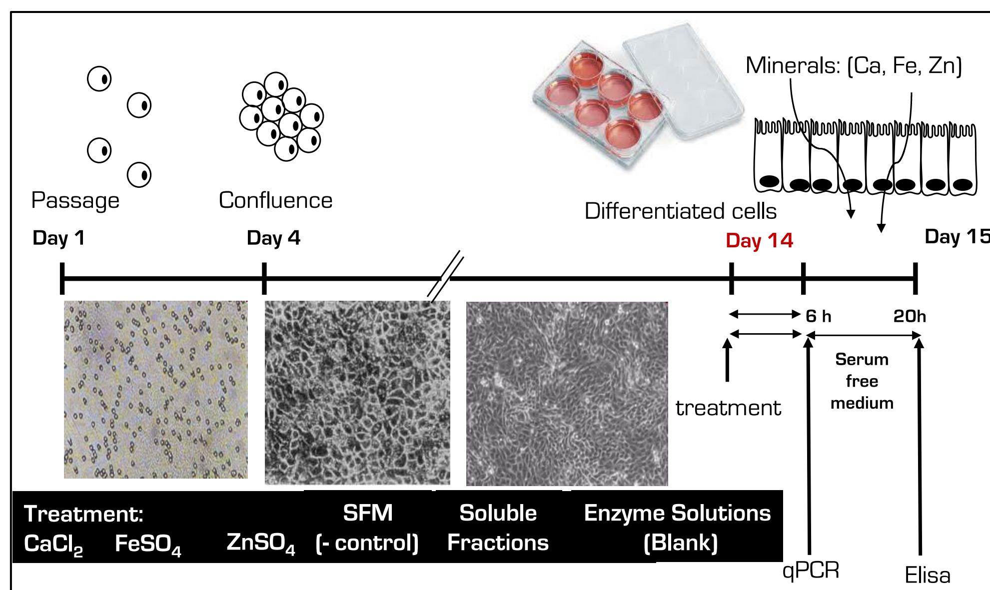


Figure 2.11.- *Scheme of Caco-2 cells exposition experiment. After exposition qPCR experiments were performed. For Ferritin determination by immunoassay ELISA, cells were maintained in SFM for 20 additional hours before being collected.*

As it can be seen in Figure 2.12, after exposition, qPCR gene expression quantification was performed. For ferritin determination by immunoassay ELISA, in order to let the mRNA being translate into proteins, treatments were discarded and cells were maintained in SFM for 20 additional hours before being collected (Glahn *et al*., 1998[a]).

3.8.4.- RNA extraction and Reverse Transcription.

After the exposition to the different treatments, Caco-2 cells were harvested by trypsinization and washed three times with DPBS (Gibco BRL life technologies, Rockville, MD, USA). RNA extraction was performed with the RNeasy extraction kit (Qiagen). RNA purity and concentration were measured at 260/280 nm using a

spectrophotometer (NanoDrop-1000, Thermo Scientific, Villebonsur Yvette, France) after RNA cleaning. RNA integrity was measured on the Agilent 2100 Bioanalyzer with the RNA 6000 Nano LabChip kit (Agilent, Diegem, Belgium). Extracted RNA was maintained at – 80 °C until use. From each sample, cDNA was synthesized from 1 μg of total RNA using SuperScript® II Reverse Transcriptase kit (Invitrogen, Karlsruhe, Germany) and random hexamers (Invitrogen, Karlsruhe, Germany). A Veriti 96 well Thermal Cycler (Applied Byosistem, Foster CA) was used for the reaction with the following parameters:

- Incubation: 25 °C for 10 min,
- RT: 42 °C for 50 min,
- enzyme inactivation: 70 °C for 15 min). cDNA was kept at – 20 °C until use.

3.8.5.- Primers design for minerals transporters gene expression.

As can be seen in Table 2.3., primers for CalD9k, Zip-4 and ZnT1 encoding genes, were obtained from previously published bibliography (Wang *et al*., 2004; Jou *et al*., 2010). Primers for DMT1, Ferritin Light and Heavy chain (FTH1 and FTL) and MT1M were designed using Primer3 software (http://frodo.wi.mit.edu/) according to the following criteria: 18 – 25 bp primer length, GC content between 30 % and 75 %, amplicon size between 50 – 200 bp and annealing temperature between 59 °C and 61 °C. Matching primer sets were validated using Net-Primer (http://www.premierbiosoft.com/netprimer/index.html) for unexpected secondary structures ($\Delta G < 5$ Kcal/ mol). Primers were purchased from Sigma–Aldrich (Taufkirchen, Germany)

Table 2.3.- *Primers list used for mineral transporters genes expression quantification*.

Gene name	Gene product	Accesion Nb	Primer	Reference	Amplicon Size
CalD9k	Calbindin D9-k	X65869	fw: ATGAGTACTAAAAAGTCTCCT	Wang *et al*. 2004	226
			rv: CTGGGATATCTTTTTTACTAA		
DMT-1	Divalent Metal ion Transporter-1	NM_001174130.1	fw: AACCCAGCCAGAGCCAGGTA		391
			rv: CCCCCTTTGTAGATGTCCAC		
FTH1	Ferritin Heavy Chain	NM_002032.2	fw: ATGAGCAGGTGAAAGCCATC		120
			rv: CACTGTCTCCCAGGGTGTG		
FTL	Ferritin Light Chain	AA304832.1	fw: CAGTGTTTGGACGGAACAGA		180
			rv: AGGTCCCAGAAGCAGGAGAT		
MT1M	Metallothionein 1M	NM_176870.2	fw: AGCAGTCGCTCCATTTATCG		226
			rv: AGCTGCAGTTCTCCAACGTC		
			p: CAAGTGTGCCCACGGCTGTGTCTG		
ZIP-4	Zinc Transporter Zip-4		fw: CCAGTGTGTGGGACACGGTAT	Jou *et al*. 2010	
			rv: TGTTCCGACAGTCCATATGCA		
ZnT-1	Zinc Transporter 1		fw: GAAGAAGATAGGGCTGGACAACTT	Jou *et al*. 2010	
			rv: CCCAAGGCATCTCCAAGGA		

3.8.6.- qPCR for minerals transporters gene expression.

qPCR reaction was carried out in a thermal cycler 7500 Fast Real Time PCR System (Applied-Biosystem, Foster City, CA, USA). Reactions were made in 25 µl containing 100 nM of primers (forward and reverse), 5µl of cDNA (10ng cDNA equivalent RNA), and 12.5 µl 29 SYBR MESA GREEN MasterMix Plus, Low ROX (Eurogentec, Belgium). Cycling conditions included:

- an initial denaturation at 95 °C for 5 min
- followed by 45 cycles of denaturation at 95 °C for 15 s
- and primer annealing-extension at 60 °C for 1 min.
- A final dissociation-melting curve was done at the end of each run to check the specificity of the primers.

To ensure reliability of the results, PCR efficiency was determined. To this end, 5µl were taken from each cDNA sample and pooled. Five serial ten-fold dilutions (from 10 to 0.001 ng/µl) of pooled cDNAs were made using DNase free water. These dilutions

were amplified with the corresponding primer pairs and realtime RT-Mix. The raw Ct values were plotted against log-transformed concentrations of the serial dilutions to obtain the slope from the following equation ($E=10^{(-1/slope)} - 1$). No-template controls were included for each primer pair. PCR efficiency per each primer pair is shown in table 2.4.

Table 2.4.- *qPCR efficiency for each primer pair analyzed*

Primer Pair	CalD9k	DMT1	FTH1	FTL	MT1M	ZIP-4	ZnT1
qPCR Efficiency	**1.89**	**1.91**	**2.05**	**1.97**	**1.87**	**1.98**	**1.96**

*Efficiency was calculated as $E=10^{(-1/slope)} - 1$

3.8.7.- Calibrated and Normalized Relative gene expression quantification (CNRQ).

In order to analyse mineral transporters encoding gene expression, a calibrated relative normalized quantification (CNRQ) was performed (Hellemans & Vandesompele, 2011). For gene expression normalization, different reference genes were used. Housekeeping or reference genes, can be defined as genes which expression remains invariable without influence of experimental conditions (Vandesompele *et al*., 2002). In the case of Caco-2 cells (Piana *et al*., 2008), four reference genes were chosen, being their respective primers shown on Table 2.5.

Table 2.5.- *Housekeeping or reference genes chosen for gene expression normalization*.

Gene name	Gene product	Accesion Nb	Primer	Reference	Amplicon Size
B2M	β – 2- Microglobuline	NM_004048	fw: TGCTGTCTCCATGTTTGATGTATCT rv: TCTCTGCTCCCCACCTCTAAGT	Wang *et al*. 2004	86
SDH-A	Succinate dehydrogenase complex (Sub. A)	NM_004168	fw: TGGGAACAAGAGGGCATCTG rv: CCACCACTGCATCAAATTCATG	Vandesompele *et al*. 2002	86
YWHAZ	Tyrosine-3-monoxygenase/tryptophan 5-monoxigenase activation protein, zeta polypeptide	NM_003406	fw: ACTTTTGGTACATTGTGGCTTCAA rv: CCGCCAGGACAAACCAGTAT	Vandesompele *et al*. 2002	94
HPRT1	Hypoxanthine phosphoribosyl-transferase 1	NM_000194	fw: TGACACTGGCAAAACAATGCA rv: GGTCCTTTTCACCAGCAAGCT	Vandesompele *et al*. 2002	94

Reference genes were tested by qRT-PCR and ranked using two different softwares (geNorm and NormFinder). With regard to geNorm software [http://medgen.ugent.be/~jvdesomp/genorm/], it was used to determine the stability of expression as well as the optimal number of reference genes to be used for normalization. Normfinder [http://www.mdl.dk/publicationsnormfinder.htm] allowed assessing the stability of expression as well as the combination of the best reference genes. A normalization factor based on the best housekeeping genes was used to calculate the gene expression.

The expression levels of mineral transporters encoding genes were analyzed relative to the geometric average of the best normalizing genes as determined previously. The normalized relative amount of expression for blank (dilution 3:1 of gastric and intestinal enzymes solution in SFM) was subtracted from the normalized relative amount of expression for each sample, positive and negative controls.

3.8.8.- Immunoassay ELISA for cytoplasmic ferritin quantification

After treatments, cells were carefully washed with pre-tempered DPBS, and scraped off with a rubber in 150 µL of Lysis Buffer (50 mM KCl, 50 Mm Hepes, 50 mM NaF, 5 mM NaPPi, 5 mM EDTA, 5 mM EGTA, 0,1 % (v/v) Triton X-100 and 0,1 % Protease Inhibitor Cocktail). Samples were centrifuged 20 min, 16,000 x g at 4 °C, and the supernatant was stored at – 20 °C until use.

Direct quantitative determination of cytoplasmic ferritin was performed using the Ferritin ELISA kit (Biovendor Gmbh, Heidelberg, Germany) according to the supplier's manual instructions. The results were expressed as ng of Ferritin per mg of protein. Previously, total protein concentration in each sample was assessed by Bradford assay (Bradford, 1976).

3.9.- Statistical analyses

Statistical analyses were performed using the IBM Statistical Package for the Social Sciences (IBM-SPSS) v.19.0 Inc., (Chicago, IL, USA). Data normality and homoscedasticity were confirmed by the Shapiro-Wilk/Kolmogorov-Smirnov test and Levene test, respectively, (significance level $p < 0.05$).

All experiments were carried out by triplicates (n=3), and the results reported as means ± SD. In order to determine the significance of the effects of different thickeners (LBG, Mhdp and gRS) on mineral *in-vitro* availability and mineral transporters expression, a one-way analysis of variance (ANOVA) and a Tukey post-test for multiple comparisons ($p<0.05$) were performed. Depending on the experiments, the results were compared with the standard formula without thickeners added (Reference) and/or minerals solutions ($CaCl_2$, $FeSO_4$ and $ZnSO_4$) as controls. Pearson's correlation test was performed to investigate the relationship between the concentration of each thickening agent and *in-vitro* mineral solubility and dialysability. For all the analysis, a level of significance of $p<0.05$ was established.

4.- RESULTS & DISCUSSION

In order to analyze the effect of locust bean gum (LBG), maize distarch phosphate (Mhdp) and pre-gelatinized rice starch (gRS), on mineral availability during an *in-vitro* digestion process, Ca, Fe and Zn solubility and dialysability, as well as uptake and transport by Caco-2 cells culture were determined. The study was completed with the analysis of mineral transporters expression (CalD9K, DMT-1, Ferritin, MT1M, Zip-4 and ZnT1) by qPCR and immunoassay ELISA.

4.1.- Determination of inositol phosphates in thickening agents.

Prior to the analysis of the effect of LBG, Mhdp and gRS on mineral solubility and dialysability, content of inositol phosphates (IP_S), including phytic acid (IP_6) (mg/100g of dry thickener), was determined by HPLC-MS. Results can be seen in table 2.6.

Table 2.6.- *Inositol and Inositol phosphates (IPs) content (mg/100 g) in LBG, Mhdp and gRS*

	LBG	Mhdp	gRS
IP_6	47.31 ± 1.05	19.16 ± 0.50	17.48 ± 0.39
IP_5	61.70 ± 1.21	17.01 ± 0.52	23.91 ± 0.91
IP_4	13.18 ± 0.63	12.80 ± 0.34	20.03 ± 0.73
IP_3	77.52 ± 0.95	11.73 ± 0.25	21.33 ± 0.72
IP_2	13.43 ± 0.31	13.52 ± 0.44	18.40 ± 0.50
IP_1	9.98 ± 0.20	4.27 ± 0.52	14.07 ± 0.61
Total IP	3.61 ± 0.43	3.62 ± 0.31	3.53 ± 0.53

(IP = Inositol Phosphates)

4.2.- Effect of LBG, Mhdp and gRS on Ca, Fe and Zn availability.

With the purpose of analyzing the effect of LBG, Mhdp and gRS on Ca, Fe and Zn solubility and dialysability after an *in-vitro* digestion process, different concentrations of thickeners were fixed with respect to their maximum legal limit: 7.5 %, 15 %, 50 % and 100 % (Table 2.2.). The three measured minerals (Ca, Fe and Zn) assessed in infant formula were aligned to the levels recommended by infant formula regulation (Commission Directive, 2006). Differences observed in mineral content between samples can be considered negligible and attributable to the different proportions of thickening agents added (Table 2.7).

***Table 2.7.**- Thickening agent concentration (mg of thickener/100 g of dry infant formula) and mineral content (mg/100g of dry infant formula) per sample*

Thickening agent (%)	LBG (mg/100g)	Mhdp (mg/100g)	gRS (mg/100g)	Ca (mg/100 g)	Fe (mg/100 g)	Zn (mg/100g)
0	-	-	-	397.64 ± 27.86	4.98 ± 0.27	3.81 ± 0.14
7.5	50	-	-	373.88 ± 24.38	4.80 ± 0.15	3.81 ± 0.15
7.5	-	1	-	294.23 ± 17.16	4.97 ± 0.28	3.92 ± 0.18
7.5	-	-	1	303.18 ± 10.63	5.22 ± 0.11	4.59 ± 0.02
15	100	-	-	338.23 ± 6.36	5.34 ± 0.13	4.56 ± 0.03
15	-	2	-	296.23 ± 24.39	4.62 ± 0.12	4.01 ± 0.08
15	-	-	2	301.47 ± 5.11	4.76 ± 0.15	4.19 ± 0.14
50	333.33	-	-	342.26 ± 6.19	4.85 ± 0.36	3.98 ± 0.07
50	-	6.67	-	393.65 ± 2.37	5.11 ± 0.07	4.04 ± 0.08
50	-	-	6.67	301.42 ± 22.01	4.48 ± 0.13	3.99 ± 0.05
100	666.67	-	-	278.92 ± 5.37	5.11 ± 0.06	4.22 ± 0.11
100	-	13.33	-	380.63 ± 12.75	4.82 ± 0.04	4.23 ± 0.06
100	-	-	13.33	362.26 ± 30.49	5.18 ± 0.11	4.18 ± 0.05

After the *in-vitro* digestion process, soluble and dialyzable fractions were collected, and mineral content was analyzed by AAS according to the method previously explained. Mineral solubility and dialysability were calculated as percentages over the mineral content in each sample before digestion (Table 2.7). The sample without added thickeners was used as reference. The effect of each thickening agent concentration on Ca, Fe and Zn solubility and dialysability percentages are shown in Figures 2.12 and 2.13, respectively.

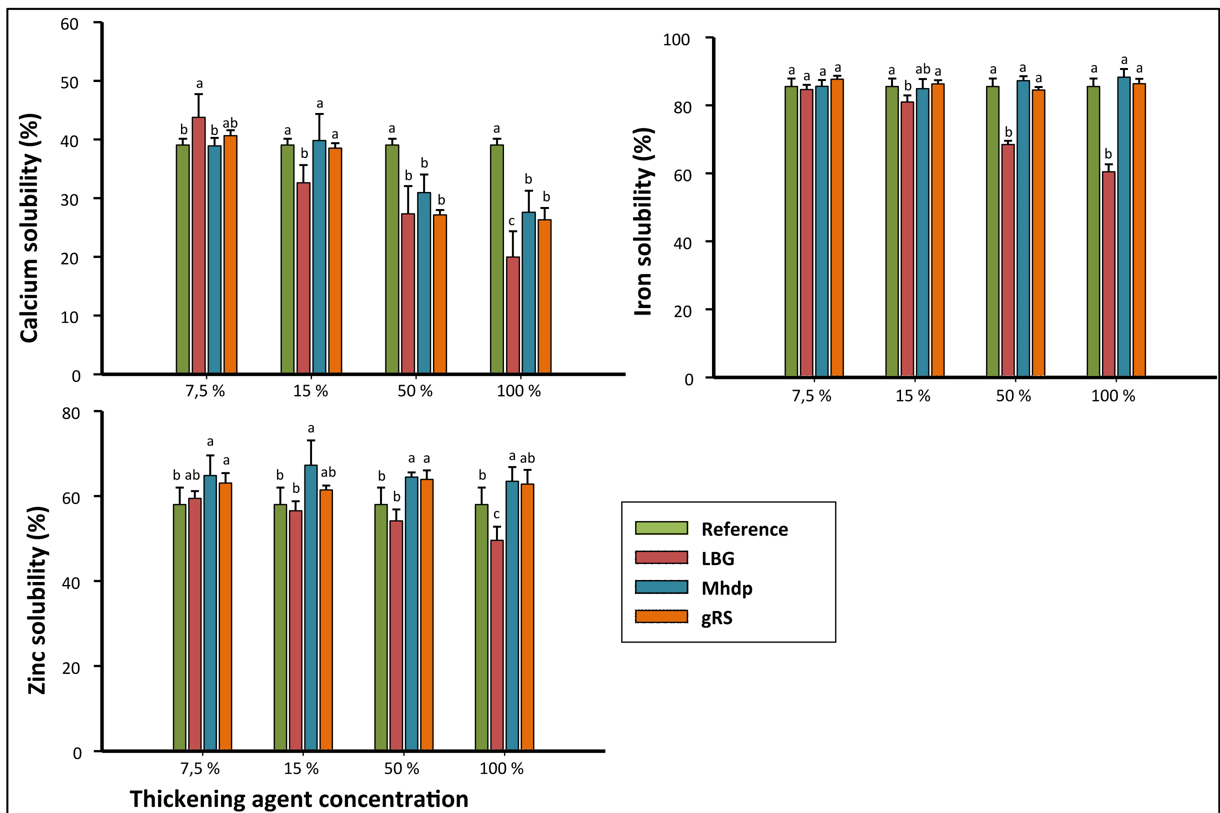


Figure 2.12.- *Effect of different thickening agent concentrations (LBG, Mhdp, gRS), on Ca, Fe and Zn solubility. Different superscripts (a-c) indicate significant differences (p<0.05) between Ca, Fe or Zn solubility within the same concentration level. Data are expressed as mean ± SD*

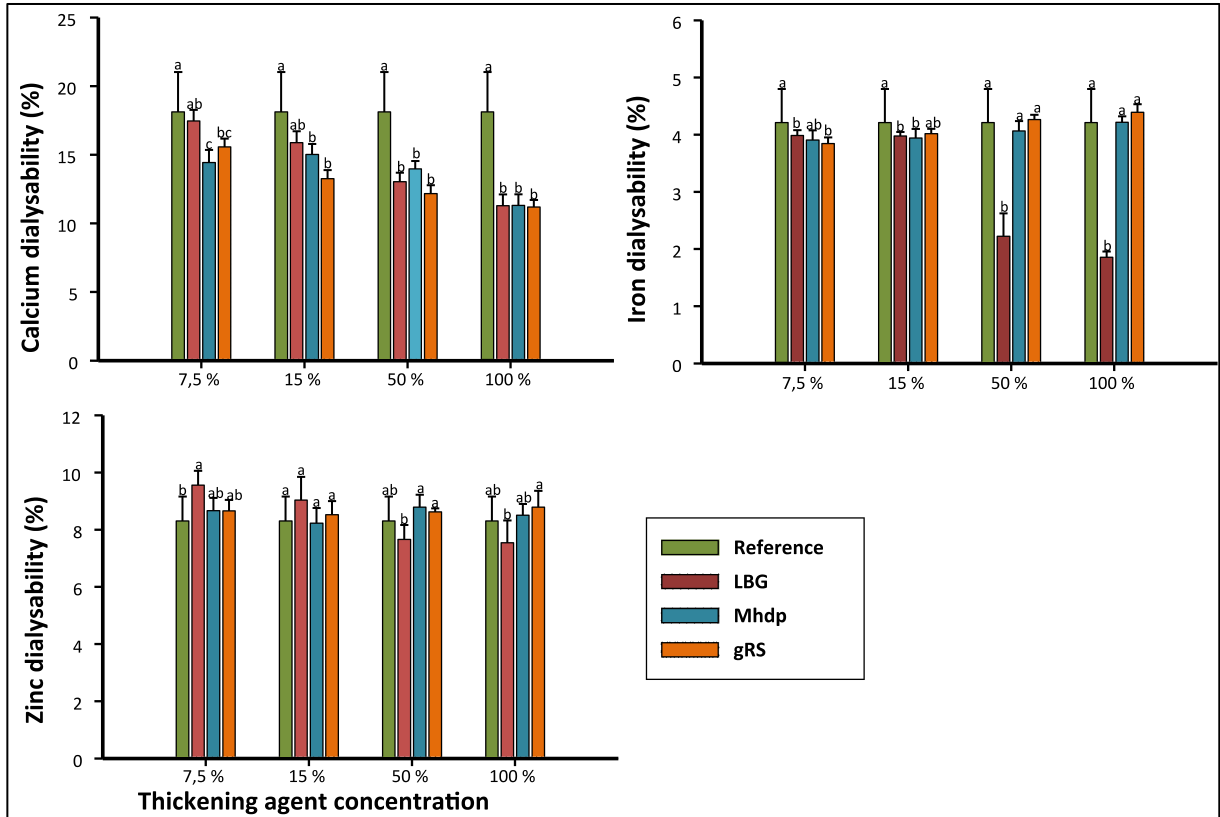


Figure 2.13.- *Effect of different thickening agent concentrations (LBG, Mhdp, gRS), on Ca, Fe and Zn dialysability. Different superscripts (a-c) indicate significant differences (p<0.05) between Ca, Fe or Zn dialysability within the same concentration level. Data are expressed as mean ± SD*

In order to analyze a concentration dependent effect on Ca, Fe and Zn solubility and dialysability per each ingredient (LBG, Mhdp and gRS), a Pearson's correlation test was performed. Results are shown in table 2.8.

Table 2.8.- *Pearson's correlation coefficients between different concentration of each thickening agent (LBG, Mhdp and gRS), and Ca, Fe and Zn solubility and dialysability percentages.*

	Solubility			Dialisability		
	LBG	**Mhdp**	**gRS**	**LBG**	**Mhdp**	**gRS**
Ca	-0.909*	-0.793*	-0.866*	-0.953*	-0.733*	-0.861*
Fe	-0.912*	0.483	0.437	-0.912*	0.632*	0.837*
Zn	-0.834*	-0.187	0.710	-0.783*	0.016	0.279

*p<0.05

4.3.- Caco-2 cells: Ca, Fe and Zn uptake and transport.

Ca, Fe and Zn uptake and transport by Caco-2 cells were analyzed through a bicameral assay, consisting of an apical and a basolateral chamber separated by a semipermeable membrane. Caco-2 cells were seeded and growth on the apical surface of the membrane. After differentiation, cells were exposed to the different soluble fractions obtained after *in-vitro* digestion, previously diluted 3:1 in SFM. In order to characterize the effect of LBG, Mhdp and gRS on mineral uptake and transport through cell monolayer, the maximum legal limit for each thickener was chosen. Formula without thickeners added was used as reference. Results for Ca, Fe and Zn transport (%), retention (µg/mg of protein) and Uptake (%) are shown in Figures 2.14, 2.15, and 2.16. With regard to Ca, Fe and Zn transport efficiency and uptake efficiency, results can be seen in table 2.9.

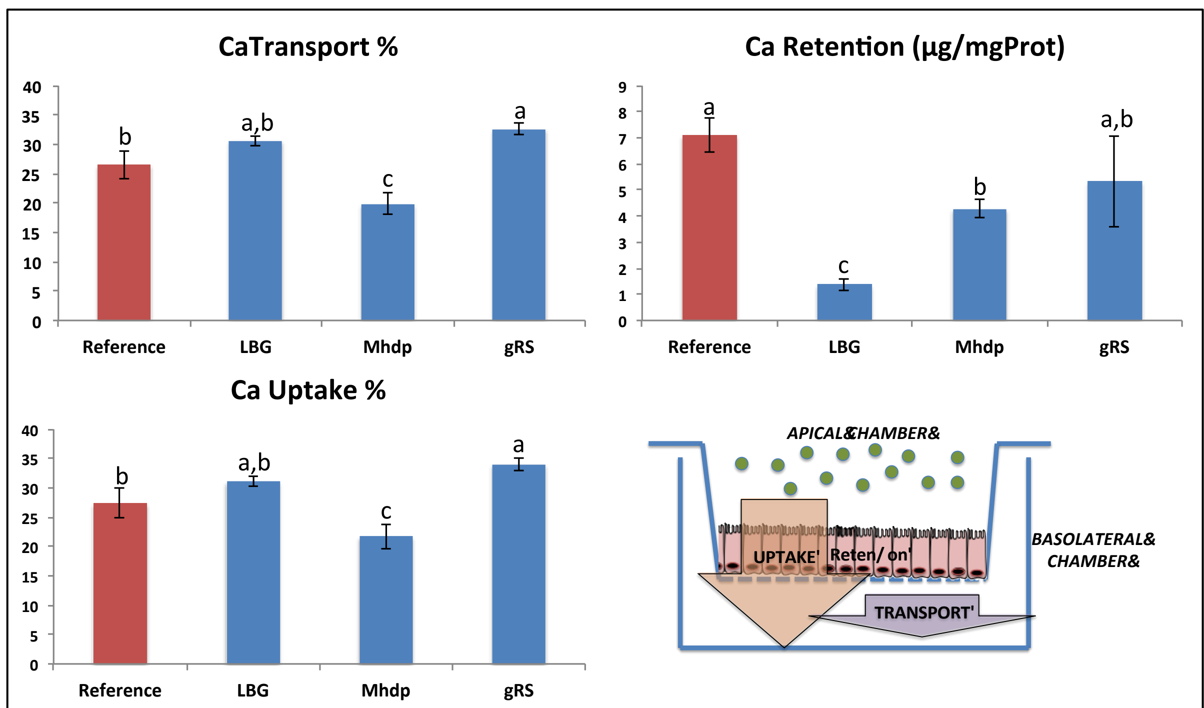


Figure 2.14.- *Effect of LBG, Mhdp, gRS on Ca transport (%), retention (µg/mg of protein) and uptake (%). Different letters (a-c) indicate significant differences (p<0.05) between ingredients. Formula without thickeners was used as Reference. Data are expressed as mean ± SD*

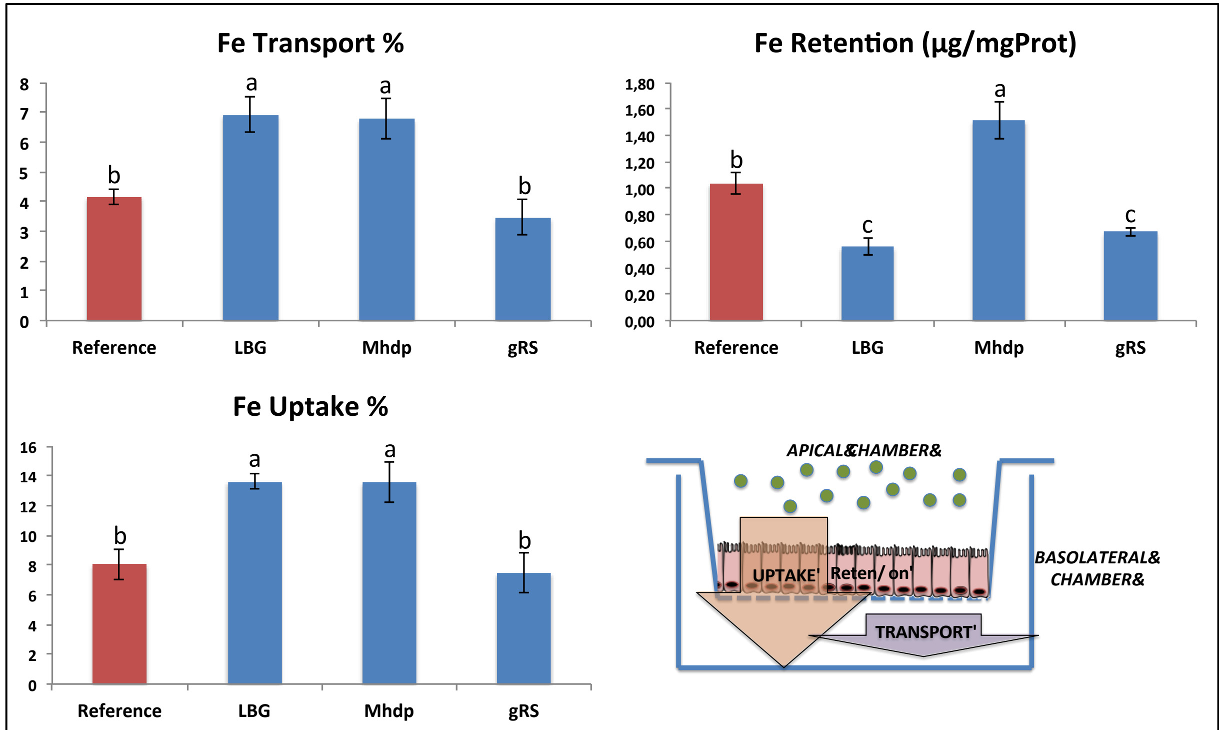


Figure 2.15.- *Effect of LBG, Mhdp, gRS on Fe transport (%), retention (µg/mg of protein) and uptake (%). Different letters (a-c) indicate significant differences (p<0.05) between ingredients. Formula without thickeners was used as Reference. Data are expressed as mean ± SD*

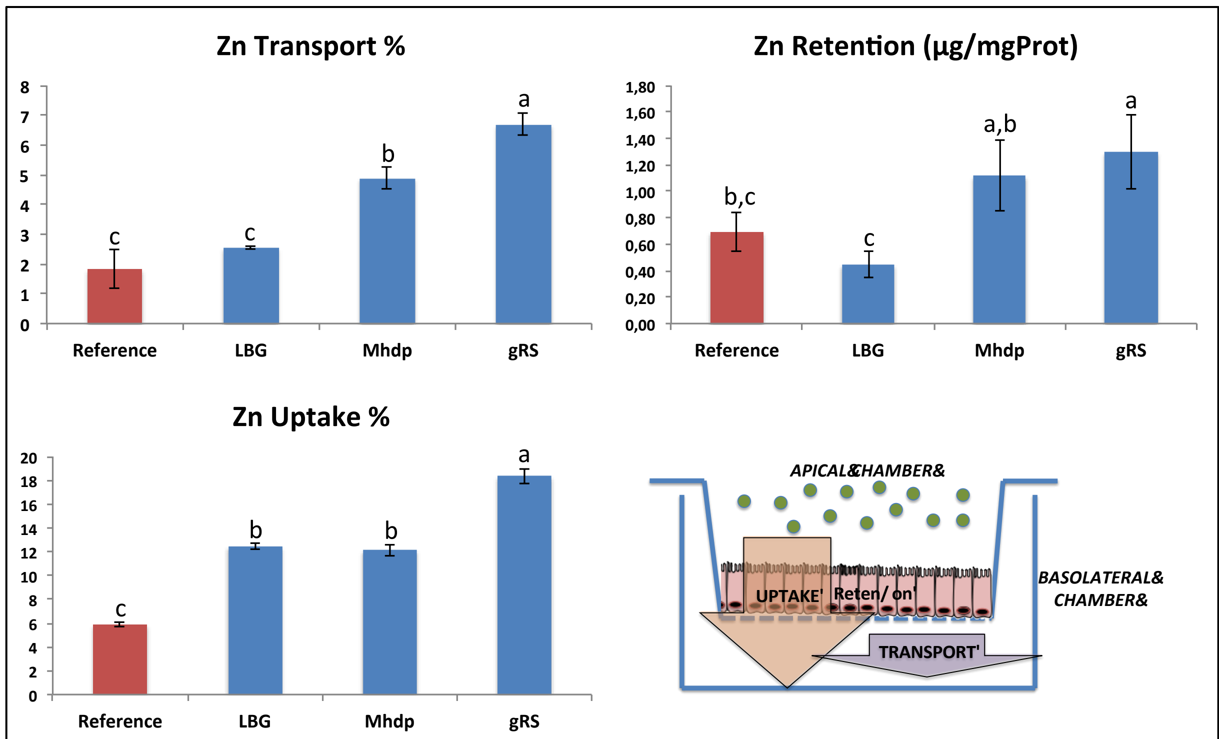


Figure 2.16.- *Effect of LBG, Mhdp, gRS on Zn transport (%), retention (µg/mg of protein) and uptake (%). Different letters (a-c) indicate significant differences (p<0.05) between ingredients. Formula without thickeners was used as Reference. Data are expressed as mean ± SD*

Table 2.9.- *Ca, Fe and Zn transport and uptake efficiency. Within the same mineral, different superscripts (a-c) indicate significant differences (p<0.05) between ingredients. Data are expressed as mean ± SD*

	CALCIUM						IRON						ZINC					
	Transport Efficiency %			Uptake Efficiency %			Transport Efficiency %			Uptake Efficiency %			Transport Efficiency %			Uptake Efficiency %		
	Mean	±	S.D.	Mean	±	S.D.	Mean	±	S.D.	Mean	±	S.D.	Mean	±	S.D.	Mean	±	S.D.
Reference	27.74^{a}	±	2.36	28.63^{a}	±	2.44	5.26^{b}	±	0.32	10.22^{b}	±	1.29	1.95^{c}	±	0.67	6.31^{c}	±	0.14
LBG	20.05^{b,c}	±	1.12	20.33^{b,c}	±	1.14	4.98^{b}	±	0.59	9.78^{b}	±	0.48	1.26^{c}	±	0.07	6.14^{c}	±	0.34
Mhdp	15.96^{c}	±	2.45	17.47^{c}	±	2.72	8.05^{a}	±	0.94	16.11^{a}	±	1.88	4.32^{b}	±	0.32	10.73^{b}	±	0.49
gRS	22.65^{b}	±	0.20	23.62^{b}	±	0.24	3.86^{b}	±	0.72	8.33^{b}	±	1.69	5.59^{a}	±	0.41	15.34^{a}	±	1.34

4.4.- Caco-2 cells: mineral transporters expression.

4.4.1.- Time-Course experiment to determine an optimal time of exposure.

Before analyzing the mineral transporters expression, a time course experiment was performed to determine an optimal time of exposure to treatments. In order to analyze Caco-2 cells viability during the experiment, the "Alamar blue test" was performed. With this purpose, differentiated Caco-2 cells were exposed to the soluble fraction of Infant formula without thickener added (reference) (diluted 3:1 in SFM), and to SFM with different concentrations of $CaCl_2$ (5 mM), $FeSO_4$ (200 μM), and $ZnSO_4$ (50 μM) as positive controls. SFM was used as negative control. Five different times of exposition were analyzed (0 h, 2 h, 4 h, 8 h). Absorbance increment between 560 and 590 nm was determined. As can be seen in table 2.10, cell viability maintained stable during the first 4 hours of exposition, but it was halved reduced by 8h of exposition to reference. Results for 16h were discarded as cell viability was seriously compromised.

Table 2.10.- *Alamar blue test results (ΔAbsorbance$_{560-590nm}$). Assessment of Caco-2 cells viability during the time course experiment, for each time (0 h, 2 h, 4h, 8h) and treatment. Results are presented as mean ± SD*

	EXPOSITION TIME								
	2h			**4h**			**8h**		
	Mean	**±**	**S.D.**	**Mean**	**±**	**S.D.**	**Mean**	**±**	**S.D.**
SFM	0.41	±	0.02	0.44	±	0.03	0.39	±	0.01
$CaCl_2$	0.45	±	0.01	0.47	±	0.02	0.42	±	0.03
$FeSO_4$	0.39	±	0.01	0.45	±	0.01	0.40	±	0.01
$ZnSO_4$	0.40	±	0.02	0.43	±	0.01	0.48	±	0.01
Reference	0.43	±	0.03	0.47	±	0.01	0.23	±	0.01

“Alamar Blue test” was followed by the analysis of CalD9k, DMT1, FTH1, FTL and Zip-4 encoding genes expression (CNRQ). Differentiated Caco-2 cells were exposed to the previously defined treatments for 2, 4 and 8 h. In Figure 2.17, qPCR results (CNRQ) for CalD9K, DMT1, FTH1, FTL, and Zip-4 are shown. Only MT1M expression seemed to be affected by treatment and exposition time, increasing from 2h to 8h only when Zn control and reference were added.

According to these results and, in order to ensure a quantifiable gene expression with a minimum impact on cell availability, an exposure time of 6h was chosen.

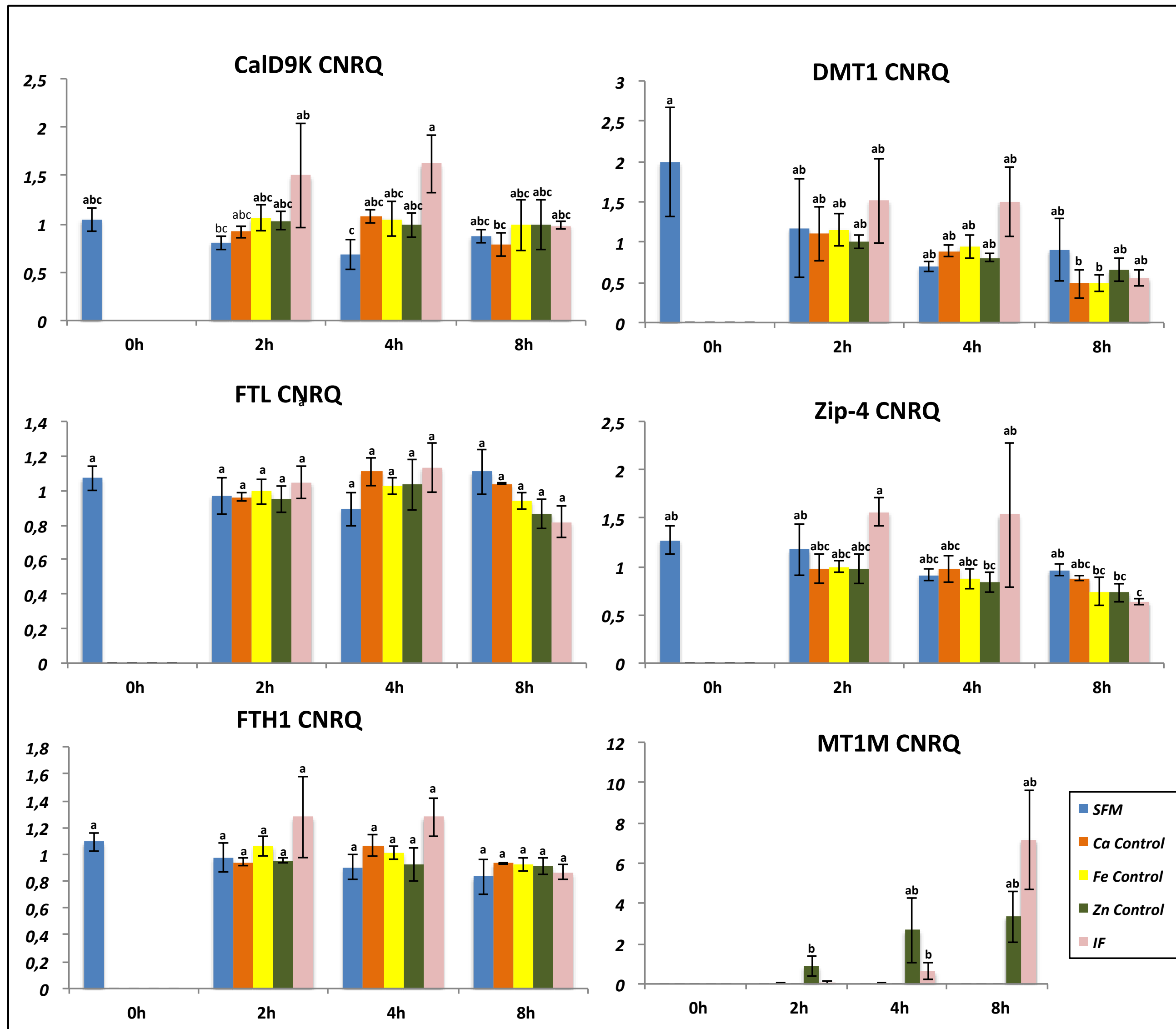


Figure 2.17.- *Calibrated and normalized relative gene expression (CNRQ) levels of mineral transporters: Calbindin-D9K (CalD9K), Divalent Metal Transporter-1 (DMT1), Ferritin Heavy chain-1 (FTH1), Ferritin Light chain (FTL), Metallothionein-1M (MT1M), and Zn Transporter Zip-4. Caco-2 cells were exposed to reference. As positive controls, different minerals concentrations in SFM solution were chosen [$CaCl_2$ (5 mM), $FeSO_4$ (200 μM), and $ZnSO_4$ (50 μM)]. As negative control, SFM was chosen. Different letters (a-c) indicate significant differences ($p<0.05$) between times of exposition (0 h, 2 h, 4 h and 8 h). Data are expressed as mean ± SD*

4.4.2.- Calibrated and Normalized Relative gene expression quantification (CNRQ).

After differentiation, Caco-2 cells were exposed to soluble fractions of infant formula supplemented with LBG, Mhdp and gRS. SFM and soluble fraction of infant formula without thickening agents added (reference) were respectively used as negative control and reference. As positive controls $CaCl_2$ (5 mM), $FeSO_4$ (200 µM), and $ZnSO_4$ (50 µM) were used. Soluble fractions were diluted 3:1 in SFM. The gene expression of Calbindin-D9K (CalD9k), Divalent Metal Transporter-1 (DMT1), Ferritin Heavy chain-1 (FTH1), Ferritin Light chain (FTL), Metallothionein-1M (MT1M), Zn Transporter Zip-4 (Zip-4) and Zn Transporter-1 (ZnT-1) were analyzed. With this purpose, a Calibrated and Normalized Relative gene expression quantification (CNRQ) was performed. For normalization, the following Housekeeping genes were used: B2M, SDH-A, YWHAZ and HPRT-1. In order to remove the possible effect of minerals contained in enzyme solutions added during the *in-vitro* digestion process, cells were also exposed to these solutions. Their respective CNRQ was subtracted from the results obtained for each sample and reference. In Figure 2.18, CNRQs for each transporter encoding gene and condition are shown.

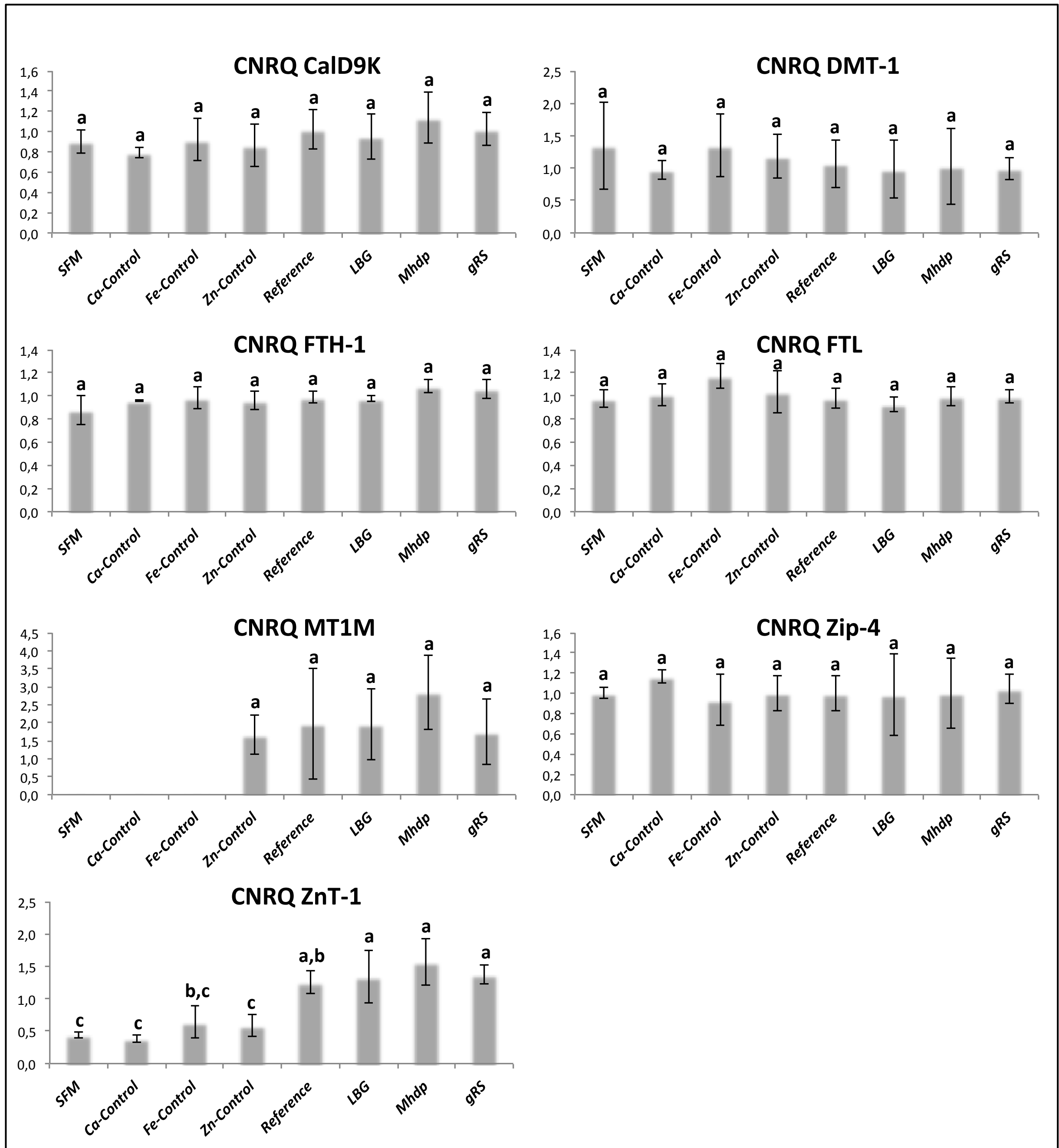


Figure 2.18.- *Influence of Infant formulas with thickeners added (LBG, Mhdp and gRS) on Calibrated and Normalized Relative Quantification for different mineral transporters encoding genes (CNRQ): Calbindin-D9K (CalD9K), Divalent Metal Transporter-1 (DMT1), Ferritin Heavy chain-1 (FTH1), Ferritin Light chain (FTL), Metallothionein-1M (MT1M), Zip-4 and Zn Transporter-1 (ZnT1). Infant formula without thickeners was considered as reference. As positive controls $CaCl_2$ (5 mM), $FeSO_4$ (200 µM), and $ZnSO_4$ were used. Serum Free Medium (SFM) was added as negative control. Different letters (a,b,c) denotes significant differences for each treatment and gene (<0.05). Data are expressed as mean ± SD*

4.4.3.- Immunoassay ELISA for cytoplasmic ferritin quantification

After the exposition, cells were maintained for 24h in SFM, and cells proteins extracted. Total protein concentration in each sample was assessed by Bradford assay. Per each treatment (Infant formula with LBG, Mhdp, gRS; Infant formula without thickeners or reference; $CaCl_2$, $FeSO_4$, and $ZnSO_4$ solutions; and SFM) cytoplasmic ferritin concentration (ng/mg of protein) was determined by immunoassay ELISA. In Figure 2.19, results can be seen.

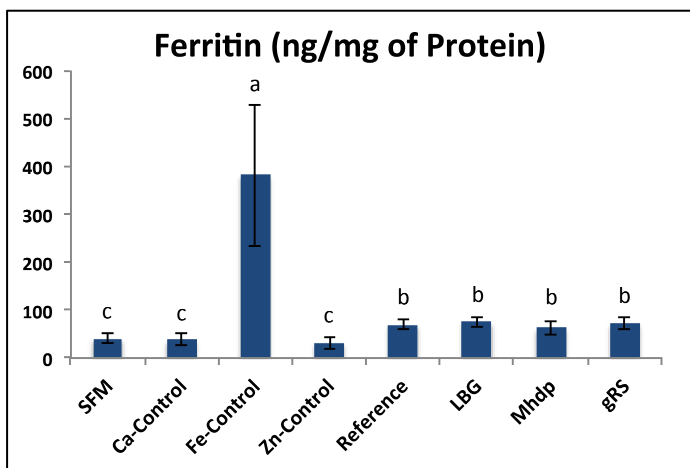


Figure 2.19.- *Cytoplasmic Ferritin quantification by Inmunoassay ELISA. The influence of different treatments (SFM; $CaCl_2$, $FeSO_4$, and $ZnSO_4$ solutions; Infant formula with LBG, Mhdp, gRS; and Infant formula without thickeners (reference) are evaluated. Different letters (a,b,c) denotes significant differences for each treatment and gene (<0.05). Data are expressed as mean ± SD*

4.5.- Discussion.

4.5.1.- Mineral Solubility and dialysability.

Regarding mineral solubility (Figure 2.12), Ca was the only mineral negatively affected by the three thickening agents when they were added in high concentrations (> 50 % of maximum legal limit) compared with the standard formula (Hero Baby®1 without thickening agents). This negative effect was significantly higher ($p<0.05$) for LBG than for modified starches at the same concentration (100 %). When Fe and Zn solubility were studied (Figure 2.12), a significant negative effect was observed after

adding LBG in high concentrations (> 50 % of maximum legal limit), whereas no negative effect occurred when modified starches were used as thickeners. Moreover, high concentration of modified starches (> 50 % of maximum legal limit) showed a mildly significant positive effect on Zn solubility with respect to the standard formula (Reference).

In contrast to modified starches, the negative effect on mineral solubility described for LBG could be explained by both: the possible degradation of starches across the *in-vitro* digestion process; and/or the content of the hexa (IP_6) and penta (IP_5) forms of IP detected in the thickening agents (Hurrell, 2004). It must be noted that, during the gastrointestinal process, optimal conditions for α-amylase were achieved (Frontela *et al*., 2009). Based on this, amylase could be responsible for breaking modified starches into oligosaccharides. Moreover, these conditions might favor endogenous phytase activity, which has an optimal temperature of around 55 ºC and which depends on the phytate content. Although the critical phytate (IP_5 + IP_6)/Fe molar ratio has not been well established, for an optimal mineral absorption, it should be reduced to below 0.4:1 (Hurrell, 2004). In the case of Ca, it should not be higher than 0.24 (Gibson *et al*., 2010) and in the case of Zn, ratios above 1.5:1 may inhibit Zn availability (Ma *et al*., 2007). As can be seen on Table 2.6, LBG showed the highest amount of phytate (IP_5 + IP_6) (109.02 mg/100 g) when compared to Mhdp and gRS (36.17 mg/100 g and 41.39 mg/100 g, respectively). However, as a result of the legal limit of addition for each thickening agent (European Parliament and Council, 1995; European Parliament and council, 2006), the final content of phytate in infant formula should not compromise the mineral availability, as they were under the critical ratio defined by different authors (Hurrell, 2004; Ma *et al*., 2007; Gibson *et al*., 2010).

Concerning mineral dialysability (Figure 2.13), Ca was affected by the addition of LBG and Mhdp/gRS at concentration levels of 15 % and 7.5 %, respectively. When Fe and Zn dialysability were analyzed, only formula added with a 50 % and 100 % concentration of LBG showed a significantly negative effect, which was more important for Fe (2.224 % and 1.857 % for LBG concentrations of 50 % and 100 %, respectively) than for Zn (7.656 % and 7.541 % for LBG concentrations of 50 % and 100 %, respectively). Meanwhile, Mhdp and gRS did not seem to affect Fe or Zn dialysability when compared with standard formula. These results are in agreement with those observed by Bosscher *et al*., (2003[b]), who studied dairy infant formulas with pregelatinised starches added. These authors reported that LBG, brought about reduced availability of both Fe and Zn. Meanwhile, pregelatinised starches used as thickeners increased mineral availability compared to non-thickened infant formulas.

To determine a possible linear relationship between the different concentrations of each thickening agent added to infant formula and Ca, Fe and Zn solubility and dialysability, a Pearson's correlation test was run (Table 2.8). A significant negative correlation was found between for Ca, Fe and Zn solubility (-0.909, -0.912 and -0.834, respectively) and dialysability (-0.953, -0.912 and -0.783, respectively) when LBG was used as a thickening agent. These results are in accordance with the negative effect of LBG on mineral availability observed in the present study. However, this highly significant negative effect was only found for Ca but not for Fe or Zn, when Mhdp and gRS were added to the standard infant formula. The explanation for these findings could be that, due to the presence of ionisable groups, LBG could bind Ca, Fe and Zn, forming unabsorbable complexes and decreasing mineral solubility and dialysability. In addition, LBG might decrease the availabilities of Ca, Fe and Zn as a consequence of its gel-forming

capacities. This will create a viscous environment in the small intestine, and thus impair the digestion of food components. Related to this, Bosscher *et al*. (2003[b]) suggested that LBG could form strong complexes with metal ions, rendering them unavailable for absorption.

Regarding modified or pre-gelatinized starches, Mhdp and gRS might be considered digestible carbohydrates. According to this possibility, during digestion process, bound minerals could be released to the intestinal lumen, increasing mineral availability in comparison with LBG. Nevertheless, the *in-vitro* availability of Ca seems to be decreased, which could be due to the formation of un-absorbable complexes (Agget *et al*., 2002[a]; Commission Directive, 2006). The results of similar published *in-vitro* studies also suggest that the bioavailability of Ca, Fe and Zn in infant formula may
be decreased by thickened formulas with non-digestible carbohydrates such as LBG, but not by those with modified starches added (Bosscher *et al*., 2003[a]; Bosscher *et al*., 2000; Commission Directive, 2006).

4.5.2.- Caco-2 cells. Uptake and transport:

Mineral Uptake and transport by Caco-2 cells was assessed by a bicameral study by exposing cells to the soluble fractions collected after an *in-vitro* digestion process. Results for Ca, Fe and Zn uptake and transport can be seen in Figures 2.14, 2.15 and 2.16. In addition, mineral transport efficiency (%) and uptake efficiency (%) with respect to mineral solubility during the digestion process have been presented in table 2.9. As reference, formula without thickeners was added.

According to these results, the addition of LBG as thickener, resulted in a Ca (1.37 ± 0.22 mg/mg of Protein), Fe (0.56 ± 0.07 µg/mg of Protein) and Zn (0.45 ± 0.10 µg/mg of Protein) cellular retention values significantly lower than the ones obtained for reference (Ca: 7.10 ± 0.65 mg/mg of Protein; Fe: 1.04 ± 0.08 µg/mg of Protein; Zn: 0.69 ± 0.15 µg/mg), Mhdp (Ca: 4.27 ± 0.65 mg/mg of Protein; Fe: 1.51 ± 0.14 µg/mg of Protein; Zn: 1.12 ± 0.27 µg/mg of protein) and gRS (Ca: 5.32 ± 1.73 mg/mg of Protein; Fe: 0.67 ± 0.03 µg/mg of Protein; Zn: 1.30 ± 0.28 µg/mg of protein). These values are in concordance with the effect of LBG, Mhdp and gRS on mineral solubility and dialysability (Figures 2.12 and 2.13). As it has been exposed in the previous section, the addition of LBG caused a decrease in Ca, Fe and Zn solubility and dialysabily. This negative effect was not described for Mhdp or gRS, except for Ca solubility and dialysability, which was also negatively affected by the addition of modified starches. However, in the case of Ca solubility the negative effect of Mhdp and gRS was significantly lower than the negative effect caused by LBG (Figure 2.12).

When mineral transport (%) to the basolateral chamber was analized, no concordance with mineral retention, or with *in-vitro* solubility and dialysability was observed. In this regard, despite the LBG addition resulted in a low mineral solubility, dialysability and retention, Ca and Fe transport (%) were surprisingly high. With regard to reference and starches (Mhdp and gRS), the transport (%) varied depending on the mineral, without a trend in their behavior. As transport (%) is determinant for uptake (%) and efficiency calculation, this lack of concordance can be also described in table 2.9. A possible explanation can be proposed if consider that transport (%) not only depends on interactions between minerals, and food components as Phytic acid, but also on different factors as paracellular transport or monolayer integrity.

Similar studies have been previously published. In this regard, Perales *et al*., (2005) analyzed Ca solubility, dialysability and transport/uptake by Caco-2 cells from milk based formulas. According to this authors, Ca solubility is not a good indicator of Ca bioavailability since, the highest values of Ca transport/retention/uptake corresponded to the lowest Ca solubility. These results are partially in concordance with the ones obtained in our study, as LBG resulted in the lower Ca solubility but the higher Ca transport (%) (Figure 2.14). On the contrary, a concordance between Ca solubility and dialysability, and Ca retention was defined in our study. According to Perales *et al*., (2005) the lack of concordance between Ca solubility and Ca transport/uptake could be explained by the interaction of Ca with food components, forming low-weight molecular aggregates that increases facilitates its absorption and transport. With regard to Fe, Perales *et al*., (2007) analyzed the possible correlation between *in-vitro* solubility and dialysability percentages versus transport/uptake by Caco-2 cells. A low correlation was described between these parameters, but a clear dependence between Fe uptake efficiency and the ascorbic acid content, as well as the ascorbic acid/Fe molar ratio was defined. The influence of ascorbic acid concentration and Caco-2 cells Fe uptake by Caco-2 cell have been also described by Glahn *et al*., (1998[b]). What is more, these authors also described a negative relationship between high concentrations of citrate and Fe uptake. With independence of these authors, it has to be considered that in our experiments, a standard infant formula was used as basal ingredient, so vitamin C/citrate content is stable and cannot explain differences in Fe uptake/transport. What is clear is that Fe solubility seems to be dependent of mineral solubility among other factors. With regard to Zn, no comparable studies have been found.

4.5.3.- Caco-2 cells. Mineral transporters encoding gene expression (CNRQ):

In Figure 2.17, time course experiment results can be seen. According to these data, mineral transporters encoding gene expressions (CNRQ) remained generally unvariable during the first 4 hours of exposition. In the case of CalD9K, DMT-1 and Zip-4, when infant formula without thickeners was added, CNRQ values started to decrease after 8 h of exposition.

With regard to MT1M expression across the time course experiment (Figure 2.17), with the exception of $ZnSO_4$ and infant formula (IF) treatments, gene expression maintained under detection limit, which denotes its specificity as intracellular Zn binding protein (Cousins, 2010; Kambe *et al*., 2015). In the case of $ZnSO_4$ treatment, after 2 h of exposition, MT1M expression increased to a stable CNRQ value of 3.01±0.48. With regard to infant formula treatment, MT1M expression (CNRQ) exceeded limit of detection after 2-4 h of exposition, being significantly lower (0.62±0.40) than CNRQ detected for $ZnSO_4$ supplementation. These significant differences in MT1M expression between treatments at 4 h of exposition could be related to Zn concentration, being higher for ZnSO4 treatment (1.44 mg/mL) than for IF treatment (0.02 mg/mL). However, after 8 h of exposition to IF, MT1M expression was increased to 7.17±2.47 without significant differences (<0.05) with respect to $ZnSO_4$ treatment. These findings are in concordance with those presented by Martin *et al*., (2013). These authors analyzed jejunal Zn transporters expression after exposition to different concentrations of dietary Zn supplementation in pigglets and jejunal cell line from porcine (Cell-J2). According to them, when Cell-J2 are exposed to a high dietary Zn supplementation level, mRNA expression is significantly higher than the one obtained without dietary Zn supplementation. What is more, this increment in MT1M expression was accompanied

to a downregulation in Zip-4 expression. According to the results presented in table 2.17, despite no significant differences in Zip-4 expression were described between 4 and 8 h of exposition to IF and $ZnSO_4$ treatment, the average CNRQ expression values for Zip-4 were slightly lower for 8 h ($ZnSO_4$: 0.73±0.03; IF: 0.64±0.08) than for 4 hours of exposition ($ZnSO_4$: 0.84±0.11; IF: 1.53±0.75). The absence of statistical significance could be attributed to the maximum time of exposition chosen (8 h). In this regard, after exposition to different concentration of dietary Zn, an adaptive process of 24 h has been defined for intestinal MT1M/Zip-4 expression regulation, (Martin *et al*., 2013).

Regarding time course experiments results for FTH-1 and FTL encoding genes expression (Figure 2.17), both genes were expressed at a similar level (CNRQ ≈ 1), maintaining a stable expression across the experiment. What is more, no significant differences (<0.05) were described between ingredients. The stable expression of FTH-1 and FTL can be explained assuming that response of ferritin to dietary Fe concentration is mainly regulated at a posttranscriptional level (Sammarco *et al*., 2008). This posttranscriptional regulation is due to the recruitment of mRNA previously stored at a cytoplasmic level in the presence of Fe abundance, being mediated by two different RNA-binding proteins, named Fe Regulatory Proteins (IRP-1 and IRP-2). In scarce of intracellular Fe, IRP-1 assumes an special conformational structure which allows its interactions with ferritin encoding RNAm, repressing its translation. With regard to IRP-2, it is destroyed under Fe-scarce conditions (Torti & Torti, 2002). Despite no statistical significance in FTH-1 and FTL expression (CNRQ) was found, when IF was added, the average CNRQs for both genes were higher after 4 h of exposition (FTH-1: 1.28±0.14; FTL: 1.23±0.14) than after 8 h (FTH-1: 0.86±0.06; FTL: 0.82±0.09).

Apart from an optimal gene expression, during the time course experiment, Caco-2 cells viability was assessed by the “Alamar blue test” or “Resazurin Reduction test”. Results (ΔAbsorbance $_{560\text{-}590\ nm}$) can be seen in table 2.10. According to these results, ΔAbsorbance $_{560\text{-}590\ nm}$ values obtained ranged between 0.47 and 0.39 across the time course experiments. However, the addition of IF resulted in a drastic absorbance reduction, from 0.47 to 0.23, after 8 h of exposition, which indicates cytotoxicity. Indeed, other authors have indicated a potential cytotoxicity associated to the digestate itself, which was not only related to the presence of proteolytic or lipolytic enzimes, but also to components in foods (Gangloff *et al*., 1996; Glahn *et al*., 1996). According to these results, and considering minerals transporters CNRQ across the time course experiment, an optimal exposition time of 6 h was chosen. This optimal time of exposition has been also proposed by Garrett *et al*., (1999), who reported that, after 4-6 hours exposition to diluted intestinal digestion, cells maintained their morphological and biochemical integrity.

Once the optimal exposition time was determined (6 h), Caco-2 cells were exposed to the different treatments (infant formulas with and without thickeners added) to determine Ca, Fe and Zn transporters CNRQ. Results are shown in Figure 2.18. According to them, after 6 h of exposition, no significant differences were found for CalD9k, FTH-1, FTL and DMT-1 expression between treatments.

With regard to CalD9K, it has been described that its gene expression is not determined by Ca availability, but by the steroid hormone 1, 25-Dihydroxyvitamin-D3 concentration (Wood *et al*., 2001; Fleet *et al*., 2002; Wasserman; 2004). According to these authors, the interaction between 1,25-dihydroxyvitamin-D3 and its specific

nuclear receptor stimulates the transcription of different genes involved in Ca transport, including CalD9K. As a result, 1,25-dihydroxyvitamin D3 has been proposed as the main hormonal regulator of active intestinal Ca transport. In this respect, the presence of vitamin D in infant formula (1.2 μg/100 mL according to manufacturer specifications) could explain the non-significant differences in CalD9k CNRQ values between treatments (infant formula with or without thickeners added). Despite the similar CalD9k gene expression between treatments, statistical differences in Ca transport and retention by Caco-2 cells (Figure 2.14) have been reported in section 4.5.2. This difference between CalD9k expression and Ca intestinal transport has been also reported by *in-vitro* and *in vivo* studies. According to the work published by Fleet *et al*., (2002), a high expression of CalD9k in Caco-2 cells do not ensure high rates of Ca transport. With regard to *in vivo* studies, Krisinger *et al*., (1991) reported that the administration of 1,25-dihydroxyvitamin D3 to rat, induce the expression of intestinal Cald9K encoding gene but no Ca intestinal transport. Similar results were reported by Spencer *et al*., (1979), according to whom the induction of intestinal CalD9k expression by 1,25-dihydroxyvitamin D3 administration in rachitic chicks was not correlated with a maintained high Ca absorption. These results lead to the idea that Ca transport by Caco-2 cells could not be only regulated by CalD9k expression, but by other transcellular mechanism and paracellular transport, as well as by the interaction of Ca with food matrix components.

In relation to the absence of statistical differences in ferritin encoding genes expression (FTH-1 and FTL) between treatments, a possible explanation has been previously exposed, being based on a regulation of FTH-1 and FTL at a posttranscriptional level (Torti & Torti, 2002; Sammarco *et al*., 2008). For this reason a

cytoplasmic ferritin quantification by immunoassay ELISA was performed. As can be seen in Figure 2.19, no significant differences were found between infant formulas with or without thickeners added. For these treatments, an average ferritin concentration of 69.91±1.91 ng/mg of protein was defined. On the contrary, when $FeSO_4$ was added, ferritin concentration was significantly higher (381.52±147.56 ng/mg of protein) than the results obtained for the rest of treatments. An explanation to these finding could be explained by the different concentration of Fe between treatment, which were approximately 100 times higher for $FeSO_4$ treatment (3.04 mg/mL) than for infant formulas (0.04 mg/mL). As it has been previously explained, a high exposition to Fe results in a high cytoplasmic ferritin content without modifying encoding gene expression (Torti & Torti, 2002; Sammarco *et al.*, 2008). This is an important homeostatic mechanism for minimizing free intracellular Fe and its reducing/oxidizing activity (Ponka, 2000; Collard, 2009; Brissot *et al.*, 2012). Despite no differences in ferritin content have been described, differences in Fe retention by Caco-2 cells have been found between infant formula with or without thickeners added (Figure 2.15). In this way, gRS and LBG resulted in the lower Fe retention (0.67±0.03 and 0.56±0.07 µg/mg of protein respectively), in comparison to formula without thickener added or Mhdp (1.04±0.08 and 1.51±0.14 µg/mg of protein respectively). The interaction between different food matrix components, as well as the presence of Fe bounded to mobilferrin as a shuttle protein between apical membrane and ferritin deposit (Smith, 1997) should be having into account.

With regard to Divalent Metal Transporter (DMT-1), it is being defined as the main responsible for Fe intestinal absorption, localized at the apical surface of Caco-2 cells (Sharp *et al.*, 2002; Johnson *et al.*, 2005). According to Johnson *et al.*, (2005), when

caco-2 cells were exposed to a non-heme Fe source, a relocation of DMT-1 protein between membrane and cytoplasm was found. However, no differences in DMT-1 encoding gene expression were observed within the first 24 h of exposition. After 24 h hours, DMT-1 expression was reported to be lower in non-heme Fe exposed cells against non exposed. Therefore, according to these authors, DMT-1 trafficking in Caco-2 cells was proposed as the main homeostatic mechanism for Fe intestinal absorption regulation after an acute (<24 h) non-heme Fe exposition. Similar explanation has been proposed by Sharp *et al*., (2002), who reported changes in DMT-1 expression after 72 h form Fe exposition. These results are in agreement with the ones presented in Figure 2.18, where no significant ($p<0.05$) differences in DMT-1 expression between treatments can be seen. The reason could be that time of exposure (6 h) was not enough for inducing changes in DMT-1 expression. At this respect, in order to evidence differences associated to Fe sources acute expositions, a direct quantification of DMT-1 transporter should be having into account.

In contrast to Ca and Fe transporters, Zn transporters expression seemed to be more influenced by Zn exposition. As can be seen in Figure 2.18, in the case of MT1M encoding genes, only $ZnSO_4$ and infant formulas with and without thickeners added, resulted in an expression (CNRQ) over the detection limit. However no differences were found between treatments. With regard to ZnT-1, the higher expression was obtained when infant formulas with and without thickeners added, showing an average CNRQ value of 1.38±0.13. On the contrary, the exposition to $ZnSO_4$ control resulted in a significantly lower ZnT-1 CNRQ values (0.58±0.17) when comparing to infant formulas exposition. When analyzing Zip-4 expression, no significant differences ($p<0.05$) were found between the different treatments, showing an average expression of 1.02±0.08.

According to the research performed by Martin *et al*., (2013), a high exposition of dietary Zn resulted in a cellular upregulation of ZnT-1 and MT1M, and downregulation of Zip-4 gene expression, when comparing with low Zn treatments. According to these authors, when intestinal cells are exposed to a Zn overload, this homeostatic mechanism promotes, an increment in Zn transference of intracellular Zn into extracellular compartments mediated by ZnT-1 and MT1M, while reduces Zn uptake from intestinal lumen by downregulation of Zip4 expression. The results presented in Figure 2.18, partially supports those presented by Martin *et al*., (2013), as MT1M gene expression was upregulated after the exposition to dietary Zn sources. On the contrary, no differences where found in Zip4 gene expression between treatments. In the same way, despite $ZnSO_4$ treatment presented a higher Zn concentration (1.44 mg/mL) than infant formulas (0.02 mg/mL), average ZnT1 CNRQ values were significantly lower for $ZnSO_4$ control (0.58±0.17) than for infant formulas with or without thickeners added (1.38±0.13). A possible explanation for the differences found between the results presented by Martin *et al*., (2013) and the one presented in Figure 2.18 could be the difference in exposures times, which were 24 h for the former and 6 h for our experiment. In this way, a longer exposition should be needed for stabilizing the regulation of Zn intestinal homeostasis by MT1M/Zip-4/Zip-1 gene expression. At this respect, it has to be taken into account that, whereas Martin *et al*., (2013) worked with dilutions of $ZnSO_4$ in cell culture medium, we worked with soluble fractions of intestinal digestion, which lead to a measurable toxic effect on Caco-2 cells after long time of exposition (>8 h) (Table 2.10).

In order to increase time of exposure without affecting cell viability, a modification of the method should be considered. In this way, Gandolff *et al*., (1996) and Glahn *et al*., (1996), proposed the introduction of a dialysis bag between apical chamber and cell monolayer, in order to retain high molecular weight toxic compounds without affecting mineral diffusion. Limitations to this modification could be that uptake of mineral-organic compounds complex will be impaired. Another more physiological modification has been proposed by Hilgendorf *et al*., (2000). According to these authors Caco-2/HT29-MTX cocultures offer a more flexible *in-vitro* model for studying nutrients availability. HT29-MTX differenciates into mature globets cells, producing mucus, which covers the Caco-2 cells monolayer. However, associated to this coculture, a lower expression of cellular carriers has been described. In this regard, Laparra *et al*., (2009), reported a lower dietary-Fe uptake in Caco-2/HT29-MTX co-cultures than in Caco-2 cultures.

Despite the reported effect of LBG, Mhdp, and gRS on Ca, Fe and Zn *in-vitro* solubility and dialysability, as well as in Caco-2 cells mineral uptake and transport, no physiological consequences have been described by *in vivo* studies. At this respect, Levtchenko *et al*., (1997) analyzed the effect of AR infant formula on anthropometric and serum parameters from 40 babies during the first 13 weeks of life. Among others, Fe, Fe binding capacity, Ca, and Zn content in serum were analyzed, but all the results were within normal ranges.

5.- CONCLUSIONS

According to the results obtained, it can be conclude that LBG negatively affects the *in-vitro* availability of Ca, Fe and Zn, decreasing mineral solubility and dialysability, whereas modified starches (Mhdp and gRS) only affect Ca solubility and dialysability. This negative effect on mineral solubility and dialysability is extended to mineral retention by Caco-2 cells. However, no relation between *in-vitro* solubility/dialysability, and mineral transport/uptake by Caco-2 cells is observed, assuming other factors such as interactions with food components, or monolayer integrity as modifiers of these parameters.

With regard to mineral transporter expression in caco-2 cells, only metallothionein (MT1M) and Zn transporter-1 (ZnT-1) encoding genes are specifically stimulated by Zn exposition. For the rest of mineral transporters, no differences between treatments can be defined. In the case of Ferritin encoding genes, they are regulated in a posttranscriptional level so ferritin quantification is a better indicator of Fe availability. However, no differences were found between treatments. With regard to exposure time, 6 hours are insufficient for analysing metal transporters gene expression but a longer exposure could results in cytotoxicity induced by soluble intestinal fraction components. In order to improve these analysis, a revision of the method should be needed with the aim of increasing time of exposition and maintaining cell viability.

6.- BIBLIOGRAPHY

Aggett P.J., Agostoni C., Goulet O., Hernell O., Koletzko B., Lafeber H.L., Michaelsen K.F., *et al.* (2002)[a]. Antireflux or Antiregurgitation Milk Products for infants and Young Children: A commentary by the ESPGHAN Committee on Nutrition. Journal of Pediatric Gastroenterology and Nutrition; 34(5): 496 – 498.

Aggett, P. J., Agostoni, C., Axelsson, I., Bresson, J. L., Goulet, O., Hernell, O., *et al.* (2002)[b]. Iron metabolism and requirements in early childhood: do we know enough?: a commentary by the ESPGHAN Committee on Nutrition. Journal of pediatric gastroenterology and nutrition; 34(4): 337 – 345.

Agostoni C. (2004). Antireflux or antiregurgitation milk products for infants and young children: a commentary by the ESPGHAN Committee on Nutrition. Acta Pædiatrica; 93 (4): 456.

Alarcon E., del Pozo M.D., Bartolomé B., Navarro B., Escudero R., Gonzalez I., *et al.* (2011). Urticaria and angioedema due to ingestion of carob gum: a case report. Journal of Investigion Allergology and Clinical Immunology; 21(1): 77 – 78.

Almeida A.A., Lopes M.P.V.C., Silva M.S.A., & Barrado E. (2008). Trace elements in human milk: Correlation with blood levels, inter-element correlations and changes in concentration during the first month of lactaction. Journal of Trace elements in Medicine and Biology; 22(3): 196 – 205.

Amaro M.A., & Camara F. (2004). Iron availability: An updated review. International Journal of Food Science and Nutrition; 55(8): 597 – 606.

Andreas N.J., Kampmann B., & Le-Doare K.M. (2015). Human breast milk: A review on its composition and bioactivity. Early human development; 91(11): 629 – 635.

Andrews N.C. (2000). Intestinal iron absorption: current concepts circa 2000. Digestive and Liver Disease; 32(1): 56 – 61.

Ballard O., Morrow A.L. (2013). Human milk composition nutrients and bioactive factors. Pediatric Clinics North America; 60(1): 49 – 74.

Bass J.K., & Chan G.M. (2006). Ca nutrition and metabolism during infancy. Nutrition; 22(10): 1057 – 1066.

Bauer J., & Gerss J. (2011). Longitudinal analysis of macronutrients and minerals in human milk produced by mothers of preterm infants. Clinical nutrition; 30(2): 215 – 220.

Birch, J.L., & Newell, S.J., (2009). Gastroesophageal reflux disease in preterm infants: current management and diagnostic dilemmas. Archives of Disease in Childhood-Fetal and Neonatal Edition; 94(5): 379 – 383.

Boato F., Wortley G.M., Liu R.H., & Glahn R.P. (2002). Red grape juice inhibits iron availability: application of an *in-vitro* digestion/Caco-2 cell model. Journal of Agricultural and Food Chemistry; 50(23): 6935 – 6938.

Bocca B., Alimonti A., Coni E., Di Pasquale M., Giglio L., Bocca A.P., & Caroli S. (2000). Determination of the total content and binding pattern of elements in human milk by high performance liquid chromatography-

inductively coupled plasma atomic emission spectrometry. Talanta; 53(2): 295 – 303.

Bosscher D., Van Caillie-Bertrand M., Van Dyck K., Robberecht H., Van Cauwenbergh R. & Deelstra H. (2000). Thickening infant formula with digestible and indigestible carbohydrate: availability of Ca, iron, and Zn *in-vitro*. Journal of Pediatric Gastroenterology and Nutrition; 30: 373 – 378.

Bosscher D., Van Caillie-Bertrand, M. & Deelstra H. (2003)[a]. Do thickening properties of locust bean gum affect the amount of Ca, iron and Zn available for absorption from infant formula? *In-vitro* studies. International Journal of Food Science and Nutrition; 54: 261 – 268.

Bosscher D., van Caillie-Bertrand M., van Cauwenbergh R., & Deelstra H. (2003)[b]. Availabilities of Ca, Iron and Zn from Infant Formulas is Affected by Soluble Dietary Fibers and Modified Starch Fractions. Nutrition; 19(7-8): 641 – 645.

Boundry G., David E.S., Douard V., Monteiro I.M., Le Huërou-Luron I., & Ferraris R.P. (2010). Role of intestinal transporters in neonatal nutrition: carbohydrates, proteins, lipids, minerals, and vitamins. Journal of Pediatric Gastroenterology and Nutrition; 51(4): 380 – 401.

Bradford M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry; 72(1-2): 248 – 254.

Brissot P., Ropert M., Le Lan C., & Loréal O. (2012). Non-transferrin bound iron: a key role in iron overload and iron toxicity. Biochemica et Biophysica Acta (BBA)-General Subjects; 1820(3): 403 – 410.

Bronner F. (2003). Mechanisms of intestinal Ca absorption. Journal of Cellular Biochemistry; 88(2): 387 – 393.

Bronner F., & Pansu D. (1999). Nutritional aspects of Ca absorption. The Journal of Nutrition; 129(1): 9 – 12.

Brown K.H., Wuehler S.E., & Peerson J.M. (2001). The importance of Zn in human nutrition and estimation of the global prevalence of Zn deficiency. Food and Nutrition Bulletin; 22(2): 113 – 125.

Carter R.C., Jacobson J.L., Burden M.J., Armony-Sivan R., Dodge N.C., Angelilli M.L., *et al.* (2010). Iron deficiency anemia and cognitive function in infancy. Pediatrics; 126(2): 427 – 434.

Chen, T. R. (1977). In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. Experimental Cell Research; 104(2): 255 – 262.

Christakos S., Dhawan P., Porta A., Mady L.J., & Seth, T. (2011). Vitamin D and intestinal Ca absorption. Molecular and Cellular Endocrinology; 347(1): 25 – 29.

Collard K.J. (2009). Iron homeostasis in the neonate. Pediatrics; 123(4): 1208 – 1216.

Commission Directive 2006/141/EC of 22 December 2006 on infant formulae and follow-on formulae and amending Directive 1999/21/EC. Available at: http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:02006L0141-20130918

Commission of the European Communities, Scientific Committee for Food. (1992). Opinion on certain additives for use in infant formula, follow-up on formula and weaning foods. 106th Meeting of the SCF on the 21 March 97. Available at: http://ec.europa.eu/food/fs/sc/oldcomm7/out06_en.html

Commission of the European Communities, Scientific Committee for Food. (1997). Opinion on certain additives to foods for infants and young children in good health and in foods for special medical purposes for infants and young children: Locust bean gum. Document XXIV/1270/97. Annex II. Available at: https://ec.europa.eu/food/sites/food/files/safety/docs/sci-com_scf_7_out06_en.pdf

de Barboza G.D., Guizzardi S., & de Talamoni N.T. (2015). Molecular aspects of intestinal Ca absorption. World Journal of Gastroenterology: 21(23): 7142 – 7154.

Dizdar E.A., Sari F.N., Degirmencioglu H., Canpolat F.E., Oguz S.S., Uras N., & Dilmen U. (2014). Effect of mode of delivery on macronutrient content of breast milk. The Journal of Maternal-Fetal & Neonatal Medicine; 27(11): 1099 – 1102.

Domellöf M., Braegger C., Campoy C., Colomb V., Decsi T., Fewtrell M., *et al.* (2014). Iron requirements of infants and toddlers. Journal of Pediatric Gastroenterology and Nutrition; 58(1): 119 – 129.

Domellöf M., Lönnerdal B., Dewey K.G., Cohen R.J., & Hernell O. (2004). Iron, Zn, and copper concentrations in breast milk are independent of maternal mineral status. The American Journal of Clinical Nutrition; 79(1): 111 – 115.

Dorea J.G. (2000). Zn in human milk. Nutrition Research; 20(11): 1645 – 1687.

Etcheverry P., Grusak M.A., & Fleige L.E. (2012). Application of *in-vitro* bioaccessibility and bioavailability methods for Ca, carotenoids, folate, iron, magnesium, polyphenols, Zn, and vitamins B6, B12, D, and E. Frontiers in physiology; 3: 1 – 22.

European Parliament and Council Directive 95/2/EC of 20 February 1995 on food additives other than colours and sweeteners. Available at: http://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=LEGISSUM:l21070a&from=ES

Fairweather-Tait, S., Lynch, S., Hotz, C., Hurrell, R., Abrahamse, L., Beebe, S., *et al.* (2005). The usefulness of *in-vitro* models to predict the bioavailability of iron and Zn: a consensus statement from the HarvestPlus expert consultation. International Journal for Vitamin and Nutrition Research; 75(6): 371 – 374.

Ferrer-Lorente B., Vitoria-Miñana I., & Dalmau-Serra J. (2009). Indicaciones para fórmulas lácteas especiales: fórmulas para problemas "menores", fórmulas sin lactosa y fórmulas de proteína de soja. Acta Pediatrica Española; 67(7): 333 – 337.

Fleet J.C., Eksir F., Hance K.W., & Wood R.J. (2002). Vitamin D-inducible Ca transport and gene expression in three Caco-2 cell lines. American Journal of Physiology-Gastrointestinal and Liver Physiology; 283(3): 618 – 625.

Fomon S.J. (2001). Infant feeding in the 20th century: formula and beikost. The Journal of Nutrition; 131(2): 409 – 420.

Forrellat-Barrios M., Fernández-Delgado N., & Hernández-Ramírez P. (2012). Regulación de la hepcidina y homeostasis del hierro: avances y perspectivas. Revista Cubana de Hematología, Inmunología y Hemoterapia; 28(4): 347 – 356.

Frontela C., Haro J.F., Ros G. & Martinez C. (2008). Effect of Dephytinization and Follow-on Formula Addition on *in-vitro* Iron, Ca, and Zn Availability from Infant Cereals. Journal of Agricultural and Food Chemistry; 56: 3805 – 3811.

Frontela C., Ros G. & Martinez C. (2009). Iron and Ca availability from digestion of infant cereals by Caco-2 cells. European Food Research and Technology; 228(5): 789 – 797.

Fuqua B.K., Vulpe C.D., & Anderson G.J. (2012). Intestinal iron absorption. Journal of Trace Elements in Medicine and Biology; 26(2): 115 – 119.

Gage H., Morgan J., Williams P., Schmid M., Laitenen K., Von Rosen J., *et al.* (2010). Infant feeding intentions of new mothers in five European countries. Proceedings of the Nutrition Society; 69(OCE1): 69.

Gangloff M.B., Glahn R.P., Miller D.D., & Van Campen D.R. (1996). Assessment of iron availability using combined *in-vitro* digestion and Caco-2 cell culture. Nutrition Research; 16(3): 479 – 487.

Ganz, T. (2013). Systemic iron homeostasis. Physiological reviews; 93(4): 1721 – 1741.

García-López R. (2011). Composición e inmunología de la leche humana. Acta pediátrica de México; 32(4): 223-230.

Garrett D.A., Failla M.L., & Sarama R.J. (1999). Development of an *in-vitro* digestion method to assess carotenoid bioavailability from meals. Journal of Agricultural and Food Chemistry; 47(10): 4301 – 4309.

Gibson R.S., Bailey K.B., Gibbs M., & Ferguson E.L. (2010). A review of phytate, iron, Zn, and Ca concentrations in plant-based complementary foods used in low-income countries and implications for bioavailability. Food and Nutrition Bulletin; 31(2): 134 – 146.

Gidrewicz, D. A., & Fenton, T. R. (2014). A systematic review and meta-analysis of the nutrient content of preterm and term breast milk. BMC Pediatrics; 14(1): 1 – 14.

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

Glahn R.P., Lee O.A., Yeung A., Goldman M.I., & Miller D.D. (1998)[a]. Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an *in-vitro* digestion/Caco-2 cell culture model. The Journal of Nutrition; 128(9): 1555 – 1561.

Glahn R.P., Wien E.M., Van Campen D.R., & Miller D.D. (1996). Caco-2 cell iron uptake from meat and casein digests parallels in vivo studies: use of a novel *in-vitro* method for rapid estimation of iron bioavailability. The Journal of nutrition; 126(1): 332 – 339.

Gopalsamy, G. L., Alpers, D. H., Binder, H. J., Tran, C. D., Ramakrishna, B. S., Brown, I., *et al.* (2015). The relevance of the colon to Zn nutrition. Nutrients; 7(1): 572 – 583.

Greer F.R., & Krebs N.F. (2006). Optimizing bone health and Ca intakes of infants, children, and adolescents. Pediatrics; 117(2): 578 – 585.

Greer F.R., Tsang R.C., Levin R.S., Searcy J.E., Wu R., & Steichen J.J. (1982). Increasing serum Ca and magnesium concentrations in breast-fed infants: Longitudinal studies of minerals in human milk and in sera of nursing mothers and their infants. The Journal of pediatrics; 100(1): 59 – 64.

Guo, M. (2014). Human Milk Biochemistry and Infant Formula. In Manufacturing Technology; Elsevier: Cambrdige, UK.

Hallberg L. & Hulthen L. (2007). Prediction of dietary iron absorption: an algorithm for calculating absorption and bioavailability of dietary iron. American Journal of. Clinical. Nutrition; 2(71): 1147 – 1160.

Hernández A.G., Dagach R.U., & Serra J.D. (2006). Bases para una alimentación complementaria adecuada de los lactantes y los niños de corta edad. En Anales de Pediatría Barcelona; 65(5): 481 – 495.

Hegar B., Rantos R., Firmansyah A., de Schepper J., & Vandenplas Y. (2008). Natural evolution of infantile regurgitation versus the efficacy of thickened formula. Journal of Pediatric Gastroenterology and Nutrition; 47(1): 26 – 30.

Hellemans J., & Vandesompele J. (2011). Quantitative PCR data analysis–unlocking the secret to successful results. In PCR troubleshooting and optimization: the essential guide. Caister Academic Press Norwich, UK. Available at: https://www.qbaseplus.com/sites/default/files/publication/hellemans_vandesompele_2011.pdf

Hernandez-Aguilar M.T., & Aguayo-Maldonado J. (2005). La lactancia materna. Cómo promover y apoyar la lactancia materna en la práctica pediátrica. Recomendaciones del Comité de Lactancia de la AEP. Anales de Pediatria (Barc); 63(4): 340 – 356.

Hilgendorf C., Spahn-Langguth H., Regårdh C.G., Lipka E., Amidon G.L., & Langguth P. (2000). Caco-2 versus Caco-2/HT29 - MTX co-cultured cell lines: Permeabilities via diffusion, inside and outside directed carrier-mediated transport. Journal of Pharmaceutical Sciences; 89(1): 63 – 75.

Horvath A., Dziechciarz P., & Szajewska H. (2008). The Effect of Thickened-Feed Interventions on Gastroesophageal Reflux in Infants: Systematic Review and Meta-analysis of Randomized, Controlled Trials. Pediatrics; 122(6): 1268 – 1277

Hoenderop J.G., Nilius B., & Bindels R.J. (2005). Ca absorption across epithelia. Physiological reviews; 85(1): 373 – 422.

Huang Z.L., Dufner-Beattie J., Andrews G.K.. (2006). Expression and regulation of SLC39A family Zn transporters in the developing mouse intestine. Development Biology; 295: 571 – 579.

Hurrell R. (2004). Phytic acid degradation as a means of improving

iron absorption. International Journal for Vitamin and Nutrition Research; 74(6): 445 – 452.

Infante-Pina D., Lara-Villoslada F., López-Ginés G., Morales-Hernández M.E. (2010). Estudio del comportamiento reológico *in-vitro* de las fórmulas antirregurgitación. Anales de Pediatría (Barc); 75(5): 302 – 308.

Jarjou L.M., Prentice A., Sawo Y., Laskey M.A., Bennett J., Goldberg G.R., & Cole T.J. (2006). Randomized, placebo-controlled, Ca supplementation study in pregnant Gambian women: effects on breast-milk Ca concentrations and infant birth weight, growth, and bone mineral accretion in the first year of life. The American Journal of Clinical Nutrition; 83(3): 657 – 666.

Johnson D.M., Yamaji S., Tennant J., Srai S.K., & Sharp P.A. (2005). Regulation of divalent metal transporter expression in human intestinal epithelial cells following exposure to non - haem iron. FEBS Letters; 579(9): 1923 – 1929.

Jou M.Y., Philipps A.F., Kelleher S.L., & Lönnerdal B. (2010). Effects of Zn exposure on Zn transporter expression in human intestinal cells of varying maturity. Journal of Pediatric Gastroenterology and Nutrition; 50(6): 587 – 595.

Kambe T., Tsuji T., Hashimoto A., & Itsumura N. (2015). The physiological, biochemical, and molecular roles of Zn transporters in Zn homeostasis and metabolism. Physiological Reviews; 95(3): 749 – 784.

Kanyshkova T. G., Buneva V. N., & Nevinsky G. A. (2001). Lactoferrin and its biological functions. Biochemistry (Moscow); 66(1): 1 – 7.

Khodayar-Pardo P., Mira-Pascual L., Collado M.C., & Martinez-Costa C. (2014). Impact of lactation stage, gestational age and mode of delivery on breast milk microbiota. Journal of Perinatology; 34(8): 599 – 605.

Kiela P.R., & Ghishan F.K. (2016). Physiology of intestinal absorption and secretion. Best Practice & Research Clinical Gastroenterology; 30(2): 145 – 159.

King J.C., Shames D.M., & Woodhouse L.R. (2000). Zn homeostasis in humans. The Journal of Nutrition; 130(5): 1360 – 1366.

Kirmemis O. (2017). Regurgitation in Infants. In: Esophageal and Gastric Disorders in Infancy and Childhood (pp. 1141-1147). Springer. Berlin, Heidelberg.

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(5): 584 – 599.

Krebs N.F. (1998). Zn supplementation during lactation. American Journal of Clinical Nutrition; 68(2): 509 – 512.

Krisinger J., Strom M., Darwish H. D., Perlman K., Smith C., & DeLuca H.F. (1991). Induction of calbindin-D 9k mRNA but not Ca transport in rat intestine by 1, 25-dihydroxyvitamin D3 24-homologs. Journal of Biological Chemistry; 266(3): 1910 – 1913.

Kunz C., & Lönnerdal B. (1992). Re-evaluation of the whey protein/casein

ratio of human milk. Acta Paediatrica; 81(2): 107 – 112.

Lara-Villoslada F., Olivares M., & Xaus J. (2005). The balance between caseins and whey proteins in cow's milk determines its allergenicity. Journal of Dairy Science; 88(5): 1654 – 1660.

Larsson D., & Nemere I. (2002). Vectorial transcellular Ca transport in intestine: integration of current models. BioMedical Research International; 2(3): 117 – 119.

Leong W., Bowlus C.L., Tallkvist J., & Lönnerdal B. (2003). DMT1 and FPN1 expression during infancy: developmental regulation of iron absorption. American Journal of Physiology Gastrointestinal and Liver Physiology; 285: 1153 – 1161.

Levtchenko E., Hauser B., & Vandenplas Y. (1997). Nutritional value of an" anti-regurgitation" formula. Acta Gastro-enterologica Belgica; 61(3): 285 – 287.

Liu X., Villalta P.W., & Sturla S.J. (2009). Simultaneous determination of inositol and inositol phosphates in complex biological matrices: quantitative ion-exchange chromatography/tandem mass spectrometry. Rapid Communications in Mass Spectrometry; 23(5): 705 – 712.

Liuzzi J.P., Bobo J.A., Cui L., McMahon R.J., & Cousins R.J. (2003). Zn transporters 1, 2 and 4 are differentially expressed and localized in rats during pregnancy and lactation. The Journal of Nutrition; 133(2): 342 – 351.

Liuzzi, J.P., & Cousins, R.J. (2004). Mammalian Zn transporters. Annual Revisions on Nutrition; 24: 151 – 172.

Lopez A., Cacoub P., Macdougall I.C., & Peyrin-Biroulet L. (2016). Iron deficiency anaemia. The Lancet; 387(10021): 907 – 916.

Ma G., Li Y., Jin Y., Zhai F., Kok F.J., & Yang X. (2007). Phytate intake and molar ratios of phytate to Zn, iron and Ca in the diets of people in China. European Journal of Clinical Nutrition; 61(3): 368 – 374.

Malacarne, M., Martuzzi, F., Summer, A., & Mariani, P. (2002). Protein and fat composition of mare's milk: some nutritional remarks with reference to human and cow's milk. International Dairy Journal; 12(11): 869 – 877.

Martin C.R., Ling P.R., & Blackburn G.L. (2016). Review of infant feeding: key features of breast milk and infant formula. Nutrients; 8(5): 1 – 11.

Martin L., Lodemann U., Bondzio A., Gefeller E.M., Vahjen W., Aschenbach J. R., *et al.* (2013). A high amount of dietary Zn changes the expression of Zn transporters and metallothionein in jejunal epithelial cells *in-vitro* and in vivo but does not prevent Zn accumulation in jejunal tissue of piglets. The Journal of Nutrition; 143(8): 1205 – 1210.

Meunier L., Garthoff J.A., Schaafsma A., Krul L., Schrijver J., van Goudoever, J. B., *et al.* (2014). Locust bean gum safety in neonates and young infants: an integrated review of the toxicological database and clinical evidence. Regulatory Toxicology and Pharmacology; 70(1): 155 – 169.

Miller D.D., Schricker B.R., Rasmussen R.R., & Van Campen D. (1981). An *in-vitro* method for estimation of iron availability from meals. The American

Journal of Clinical Nutrition; 34(10): 2248 – 2256.

Minekus M., Alminger M., Alvito P., Ballance S., Bohn T., Bourlieu C., *et al.* (2014). A standardised static *in-vitro* digestion method suitable for food–an international consensus. Food & Function; 5(6): 1113 – 1124.

Miller G.D., Jarvis J.K., Mcbean L.D. (2001). The importance of meeting Ca needs with food. Journal of American College of Nutrition; 20(2): 168 – 185.

Milman N., Ibsen K.K., & Christensen J.M. (1987). Serum ferritin and iron status in mothers and newborn infants. Acta Obstetricia et Gynecologica Scandinavica; 66(3): 205 – 211.

Mitoulas L.R., Kent J.C., Cox D.B., Owens R.A., Sherriff J.L., & Hartmann P.E. (2002). Variation in fat, lactose and protein in human milk over 24 h and throughout the first year of lactation. British Journal of Nutrition; 88(01): 29 – 37.

Molska A., Gutowska I., Baranowska-Bosiacka I., Noceń I., & Chlubek D. (2014). The content of elements in infant formulas and drinks against mineral requirements of children. Biological Trace Element Research; 158(3): 422 – 427.

Muñoz-Hoyos A., & Molina-Carballo A. (2010). Hierro. In: Gil-Hernandez A., ed., Tomo 1. Bases Fisiologicas y Bioquimicas de la nutricion, 2nd Ed. Sevilla: Grupo Accion Medica: 927-972.

Neville M.C., Allen J.C., Archer P.C., Casey C.E., Seacat J., Keller R.P., *et al.* (1991). Studies in human lactation: milk volume and nutrient composition during weaning and lactogenesis. The American Journal of Clinical Nutrition; 54(1): 81 - 92.

NIH (National Institutes of Health). (2017). Nutrient recommendations: Dietary Reference Intakes (DRI). U.S. Department of Health & Human Services [Internet]. (Cited 2017 April 20). Available at: https://ods.od.nih.gov/Health_Information/Dietary_Reference_Intakes.aspx

Nommsen L.A., Lovelady C.A., Heinig M.J., Lonnerdal B., & Dewey K.G. (1991). Determinants of energy, protein, lipid, and lactose concentrations in human milk during the first 12 mo of lactation: the DARLING Study. The American Journal of Clinical Nutrition; 53(2): 457 – 465.

O'brien J., Wilson I., Orton T., & Pognan F. (2000). Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. European Journal of Biochemistry; 267(17): 5421 – 5426.

Pang W.W., & Hartmann P.E. (2007). Initiation of human lactation: secretory differentiation and secretory activation. Journal of Mammary Gland Biology and Neoplasia; 12(4): 211 – 221.

Perales S., Barberá R., Lagarda M.J., & Farré R. (2005). Bioavailability of Ca from milk-based formulas and fruit juices containing milk and cereals estimated by *in-vitro* methods (solubility, dialyzability, and uptake and transport by Caco-2 cells). Journal of Agricultural and Food Chemistry; 53(9): 3721 – 3726.

Pérez G., Vittori D., Pregi N., Garbossa G., & Nesse A. (2005). Homeostasis del hierro.: Mecanismos de absorción, captación celular y regulación. Acta

Bioquímica Clínica Latinoamericana; 39(3): 301 – 314.

Piana C., Wirth M., Gerbes S., Viernstein H., Gabor F., & Toegel S. (2008). Validation of reference genes for qPCR studies on Caco-2 cell differentiation. European Journal of Pharmaceutics and Biopharmaceutics; 69(3): 1187 – 1192.

Picciano, M. F. (2001). Nutrient composition of human milk. Pediatric Clinics of North America; 48(1): 53 – 67.

Ponka, P. (2000). Iron metabolism: physiology and pathophysiology. The Journal of Trace Elements in Experimental Medicine; 13(1): 73 – 83.

Pozzi C.M.C., Braga C.P., Vieira J.C.S., Cavecci B., de Queiroz J.V., de Souza Barbosa H., *et al*. (2015). Metal ions bound to the human milk immunoglobulin A: Metalloproteomic approach. Food Chemistry; 166: 492-497.

Prado E.L., & Dewey K.G. (2014). Nutrition and brain development in early life. Nutrition Reviews; 72(4): 267 – 284.

Pronczuk J., Moy G., Vallenas C. (2004). Breast milk: an optimal food. Enviroment Health Perspectives; 112(12): 722 – 723.

Reddy M.B., Hurrel R.F., Cook J.D. (2006). Meat consumption in a varied diet marginally influences nonheme iron absorption in normal individuals. Journal of Nutrition; 136: 576 – 581.

Reilly C. (2007). Transition Metals: Chromium, Manganese, Iron, Cobalt, Nickel, Copper and Molybdenum, In: Metal Contamination of Food: Its Significance for Food Quality and Human Health, Third Edition, Blackwell Science Ltd, Oxford, UK.

Reuss, L. (2001). Tight junction permeability to ions and water. Tight Junctions; 2: 61 – 88.

Rivera-Tapia J.A., Castillo-Viveros L.V., & Sánchez-Hernández J.A. (2010). Detección de micoplasmas en cultivos celulares. Revista Ciencias Biomédicas; 1(2): 185 – 189.

Sandberg A.S. (2005). Methods and options for *in-vitro* dialyzability; benefits and limitations. International Journal for Vitamin and Nutrition Research; 75(6): 395 – 404.

Sandstead H.H., Frederickson C.J., & Penland J.G. (2000). History of Zn as related to brain function. The Journal of Nutrition; 130(2): 496 – 502.

Sammarco M.C., Ditch S., Banerjee A., & Grabczyk E. (2008). Ferritin L and H subunits are differentially regulated on a post-transcriptional level. Journal of Biological Chemistry; 283(8): 4578 – 4587.

Savino F., Muratore M.C., Silvestro L., Oggero R., & Mostert M. (1999). Allergy to carob gum in an infant. Journal of Pediatrics Gastroenterology and Nutrition; 29(4): 475 – 476.

Sharp P., Tandy S., Yamaji S., Tennant J., Williams M., & Srai S.K.S. (2002). Rapid regulation of divalent metal transporter (DMT1) protein but not mRNA expression by non-haem iron in human intestinal Caco-2 cells. FEBS Letters; 510(1): 71 – 76.

Siddappa A.M., Rao R., Long J.D., Widness J.A., & Georgieff M.K. (2007). The assessment of newborn iron stores at birth: a review of the literature and

standards for ferritin concentrations. Neonatology; 92(2): 73 – 82.

Smith, J. E. (1997). Iron metabolism and its disorders. Clinical Biochemistry of Domestic Animals; 5: 223 – 239.

Spencer R., Charman M., Wilson P., Lawson E. (1979). Vitamin D-stimulated intestinal Ca absorption may not involve Ca-binding protein directly. Nature; 263: 161 – 163.

Torti F.M., & Torti S.V. (2002). Regulation of ferritin genes and protein. Blood; 99(10): 3505 – 3516.

UNICEF (2010). Malnutrition in Namibia: Te time to act is now!. Available at: https://www.unicef.org/namibia/na.Malnutrition_final.pdf

UNICEF/WHO (1990). Innocenti Declaration. Florence. Available at: https://www.unicef.org/programme/breastfeeding/innocenti.htm

Van Itallie C.M., & Anderson J.M. (2006). Claudins and epithelial paracellular transport. Annual Review of Physiology 68: 403 – 429.

Vandenplas, Y. (2009)[a]. Thickened infant formula does what it has to do: decrease regurgitation. Pediatrics; 123: 549 – 550.

Vandenplas Y., Hauser B., Devreker T., Mahler T., Degreef E. & Veereman-Wauters G. (2013). Gastro-esophageal reflux in children: Symptoms, diagnosis and treatment. Journal of Pediatric Sciences; 3: 1 – 20.

Vandeplas, Y., Rudolph C.D., Di Lorenzo C., Hassall E., Liptak G., Mazur L., *et al.*, (2009)[b]. Pediatric Gastroesophageal Reflux Clinical Practice Guidelines: Joint Recommendations of the North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition (NASPGHAN) and the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN). Journal of Pediatric Gastroenterology and Nutrition; 49(4): 498 - 547.

Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A., & Speleman F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology; 3(7): Research0034-1.

van Goor M.K., Hoenderop J.G., & van der Wijst J. (2016). TRP channels in Ca homeostasis from hormonal control to structure-function relationship of TRPV5 and TRPV6. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research; 1 – 11.

Victora C.G., Bahl R., Barros A.J., França G.V., Horton S., Krasevec J., *et al.* (2016). Breastfeeding in the 21st century: epidemiology, mechanisms, and lifelong effect. The Lancet; 387(10017): 475 - 490.

Wang L., Klopot A., Freund J.N., Dowling L.N., Krasinski S.D., & Fleet J.C. (2004). Control of differentiation-induced calbindin-D9k gene expression in Caco-2 cells by cdx-2 and HNF-1α. American Journal of Physiology-Gastrointestinal and Liver Physiology; 287(5): 943 – 953.

Wasserman, R. H. (2004). Vitamin D and the dual processes of intestinal Ca absorption. The Journal of Nutrition; 134(11): 3137 – 3139.

Wasserman R.H., Chandler J.S., Meyer S.A., Smith C.A., Brindak M.E., Fullmer C.S., *et al.* (1992). Intestinal Ca Transport and Ca Extrusion Processes at the Basolateral Membrane1, 2. The Journal of Nutrition, 122(3S): 662 – 671.

WHO (World Health Organization). (2009) Infant and young child feeding: Model chapter for textbooks for medical students and allied health professionals. World Health Organization. Geneva. Available at: http://www.who.int/maternal_child_adolescent/documents/9789241597494/en/

WHO/FAO. (2011). Codex Alimentarius: standard for infant formula and formulas for special medical purposes intended for infants. Available from: http://www.codexalimentarius.org/download/standards/288/CXS_072e.pdf

Wood R.J., Tchack L., & Taparia S. (2001). 1,25-Dihydroxyvitamin D3 increases the expression of the CaT1 epithelial Ca channel in the Caco-2 human intestinal cell line. BMC Physiology; 1(1): 1 - 11.

Yee S. (1997). *In-vitro* permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man—fact or myth. Pharmaceutical Research; 14(6): 763 – 766.

Yamawaki N., Yamada M., Kan-no T., Kojima T., Kaneko T., & Yonekubo A. (2005). Macronutrient, mineral and trace element composition of breast milk from Japanese women. Journal of Trace Elements in Medicine and Biology; 19(2): 171 – 181.

Yasuda H., Yoshida K., Yasuda Y., & Tsutsui T. (2011). Infantile Zn deficiency: association with autism spectrum disorders. Nature Scientific Reports; 1(129): 1 – 5.

Zödl B., Zeiner M., Sargazi M., Roberts N.B., Marktl W., Steffan I., & Ekmekcioglu C. (2003). Toxic and biochemical effects of Zn in Caco-2 cells. Journal of Inorganic Biochemistry; 97(4): 324 – 330.

Effect of different thickening agents on infant gut microbiota. CHAPTER 3

1.- INTRODUCTION

Microorganisms colonize every exposed surface of the human body, including the skin surface as well as the genitourinary, gastrointestinal and respiratory tract. Between these organs, the gastrointestinal tract is the most heavily colonized, containing over 70% of all the microorganisms in the human body (Sekirov *et al.*, 2010). These microorganisms, that reside in and on the human body, constituted the human microbiota (Clemente *et al.,* 2012).

Gut microbiota refers to the entire population of microorganisms that colonizes the human gastrointestinal tract. Under this term, not only bacteria, but also fungi, archaea, viruses and unicellular eukaryotes are included (Sekirov *et al.*, 2010; Jandhyala *et al.*, 2015). It has been estimated that the collective human gut microbiota is inhabited by 1 x 10^{13} to 1 x 10^{14} microorganisms, which contains over 35000 bacterial species (Frank *et al.*, 2007; Cryan & Dinan, 2012; Jandhyala *et al.*, 2015). In parallel, the group of genes encoded by the human microbiota is known as gut microbiome (Clemente *et al.*, 2012). According to a metagenomic study, it has been estimated that there are 3.3 million of non-redundant genes in human gut, which is 150 times more genes than human genome (Qin *et al.*, 2010).

All the microorganisms contained in gut microbiota interact one with another and with the host (Clemente *et al.*, 2012). There has recently been an increasing interest in these interactions and their consequences on human health. In this way, gut microbiota plays an important role in the functionality of immune response, as well as in the regulation of gastrointestinal motility, intestinal barrier homeostasis and

development, nutrient metabolism or fat distribution (Cryan & Dinan, 2012). Different diseases as inflammatory bowel disease, metabolic diseases (i.e. obesity and diabetes) allergic disease or neurodevelopmental diseases have been associated with an imbalance of human gut microbiota composition (Sekirov *et al*., 2010; Cryan & Dinan 2012; Jandhyala *et al*., 2015).

Despite being so relevant, the composition of human gut microbiota cannot be considered as a non-dynamic or a passive equilibrium. On the contrary, its composition is going to be influenced by different factors, as the host age, diet, environmental exposures and antimicrobial treatments. What is more, its composition is going to be different across the length of the intestinal tract, as well as across its section. In other words, differences can be find between the intestinal lumen and the epithelial surface (Palmer *et al*., 2007; Sekirov *et al*., 2010; Putignani *et al*., 2014; Conlon & Bird, 2015; Jandhyala *et al*., 2015).

1.1.- Gut microbiota in healthy adults

1.1.1.- Constitution of healthy gut microbiota.

The intestinal microbiota comprises mostly anaerobic, bacterial species which dominates the facultative anaerobes and aerobes groups (Sekirov *et al*., 2010; Arboleya *et al*., 2012[c]). During the last years, the combination of amplified 16S rRNA analyses and metagenomic surveys have permitted the definition of the most abundant phylotypes within the human faecal microbiota (Eckburg *et al*., 2005; Qin *et al*., 2010; Walker *et al*., 2011). According to these studies, although gut microbiota contains over 395 bacteria

phylotypes, it is dominated by two phyla, Bacteroidetes and Firmicutes, followed by the phylum Actinobacteria, Proteobacteria, Verrucomicrobia, Fusobacteria, and Cyanobacteria in lower proportions.

Composition of individual gut microbiota is not homogeneous neither, across the gastrointestinal tract nor across the life stages. In this way, different factors will influence it. Among this factors, the gastrointestinal track section analyzed, age, diet or lifestyle have been defined as decisive for its composition (Eck burg *et al*., 2005; Sekirov *et al*., 2010; Ottman *et al*., 2012; Conlon & Bird, 2015; Jandhyala *et al*., 2015).

1.1.2.- Longitudinal variations of gut microbiota composition across the gastrointestinal track.

The composition of gut microbiota across the gastrointestinal track is not homogeneous distributed. As it has been represented in figure 3.1, a marked difference in diversity and concentration of bacteria from the oesophagus to the rectum has been defined (O'Hara & Shanahan, 2006; Sekirov 2010; Jandhyala *et al*., 2015). Regarding the bacterial density, it will be progressively increasing its number and diversity as we move distally across the digestive system. The number of bacterial cells in distal esophagus and stomach varies from 10^1 to 10^3 bacteria per gram of content (O'Hara & Shanahan, 2006).

Regarding the bacterial flora in oral cavity, multispecies biofilms attached to different surfaces as cheek, tongue or teeth have been found. *Gemella*, *Granulicatella*, *Streptococcus*, and *Veillonella* have been defined as the predominant genus (Aas *et al*.,

2005). However, some studies indicate differences in bacterial diversity depending on the oral surface (Bik *et al*., 2010; Simón Soro *et al*., 2013). For instance, and according to Simón-Soro *et al*., (2013), Streptococcus is more abundant at anterior surface of teeth than at lingual surface.

Eight genera (*Streptococcus spp., Prevotella spp., Actinomyces spp., Gemella spp., Rothia spp., Granulicatella spp., Haemophilus spp. and Veillonella spp*.) has been identified in throat and distal esophagus, being *Streptococcus spp*., followed by *Prevotella spp*., the dominant genus (Pei *et al*., 2004; Andersson *et al*., 2008).

Once in stomach, its acidity kills many bacteria ingested form the upper digestive track determining the gastric microbial community. *Helicobacter pylori* seems to be the dominant phylotipe, and its presence could be decisive for the entire gastric microbiota establishment. In this way, when *H. pylori* is absent or appears in low concentration as a commensal, the gastric microbiota will be diverse, including genus as *Streptococcus*, *Prevotella, Veillonella* or *Rothia*. Furthermore, a high variability among subjects has been described (Bik *et. al*., 2006; Andersson *et al*., 2008; Jandhyala *et al*., 2015).

The number of bacterial cells varies from approximately 10^4 to 10^7 as we move from the duodenum to the ileum (Sekirov *et al*., 2010). Unlike fecal microbiota, its composition in small intestine shows a high variability in the same individual and in the same day. This high variability has been related to dietary variation (Booijink *et al*., 2010). Despite this intra-individual variation, there is an increasing trend in anaerobic/facultative anaerobic groups as *Bacteroides*, *Clostridium*, *Enterococcus*, γ-*Proteobacteria* or *Lactobacillus* as we move from the duodenum to the ileum. Among

these microorganisms, Streptococcus and Veillonella has been isolated in a high abundance (Hayashi *et al*., 2005; Booijink *et al*., 2010; El Aidy *et al*., 2015; Jandhyala *et al*., 2015)

The large intestine contains around 10^{11} – 10^{12} bacteria per gram of content, which represent over 70% of all microorganisms contained in the human body (Sekirov *et al*., 2010; Jandhyala *et al*., 2015). Colonic and cecal microbiota is more complex but more stable than the small intestine microbiota (Booijink *et al*., 2010). In this way, Eckburg *et al*., (2005) described higher differences between individuals than intra-subject. Regarding to the results provided by different authors (Eckburg *et al*., 2005; Hayashi *et al*., 2005; Lay *et al*., 2005; Arumugam *et al*., 2011; Jadhyala *et al*., 2015), most of the microorganism contained in the large intestine are included in *Firmicutes* and *Bacteroidetes* phyla. Regarding *Firmicutes* phylum, a high percentage of the bacteria detected belongs to the *Clostridia class*, being *Clostridium coccoides* and *Clostridium leptum* the dominant groups. On the contrary, *Bacteroidetes* phylotipes (eg. genus *Bacteroides* or *Prevotella*) are more variable between individuals, being *Bacteroides* the most abundant and most variable genus. Apart from these groups, *Bifidobacterium*, *Streptococcus* and *Lactobacillus* genus, are also present in colonic microbiota. Less abundant are *Proteobacteria* (including *E. coli*), *Ctinobacteria, Fusobacteria* or *Verrucomicrobia* phyla, which represent about 0.1% of colonic bacteria.

Hayashi *et al.,* (2005) reported differences between colonic and recto-sigmoidal colonic microbiota. *Clostridium coccoides*, *Clostridium leptum* and the *Bacteroides* group maintained as the predominant species, but Proteobacteria increased their relevance.

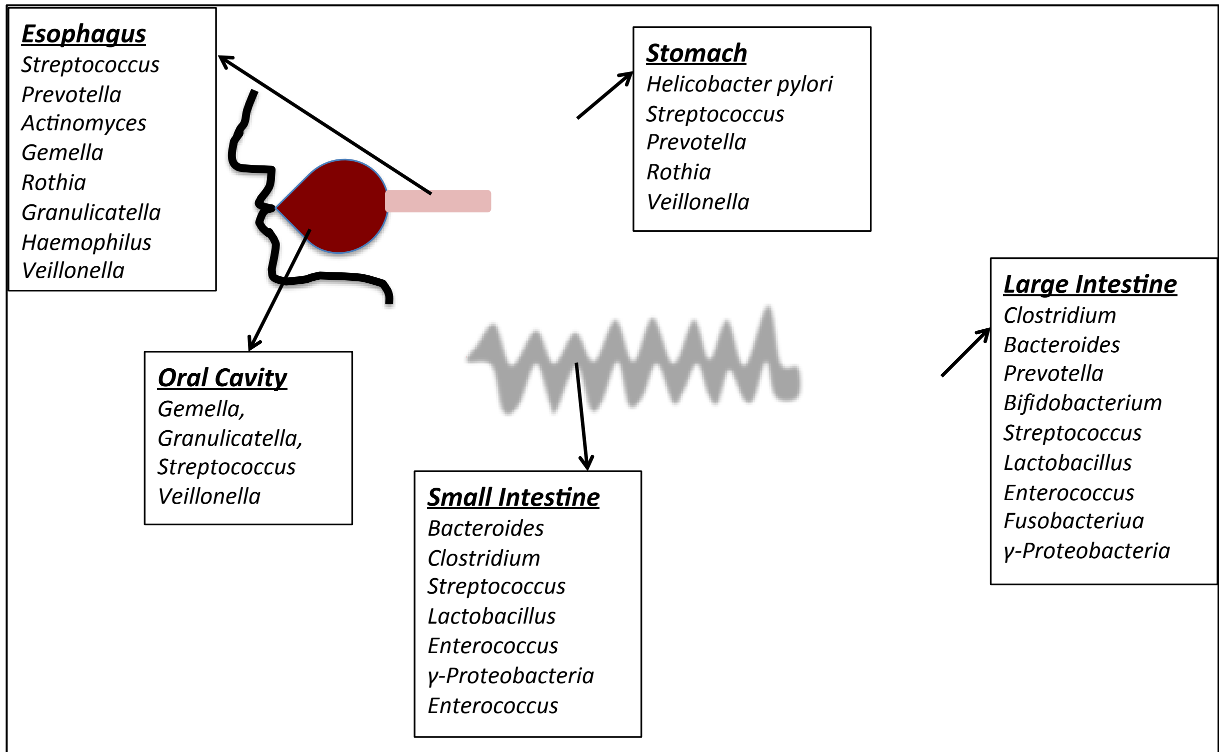


Figure 3.1.- *Variations on microbiota composition across the gastrointestinal tract*

Recently, Arumugam *et al*., (2011), proposed a classification of gut microbiota based on the identification of different clusters of well-balanced host-microbial symbiotic states, that are not geographical dependent but can respond differently to diet and/or drugs. In this work, three different clusters, referred as enterotypes, have been proposed:

- **Enterotype 1**: With a high abundance of *Bacteroides*. This enterotype presents a very broad saccharolytic potential, containing genes that encodes for proteases, hexoaminidases and galactosidases. These enzymes enable the bacteria to obtain energy from carbohydrates and proteins. The predominant bacteria included in this enterotype present a more active synthesis of biotin, riboflavin, pantothenate and ascorbate than the other enterotypes.

- **Enterotype 2**: Enriched in *Prevotella* and *Desulfovibrio*, which act together to degrade mucin glycoprotein presented in the mucosa layer. Regarding vitamin synthesis, this enterotype is enriched in thiamine and folate synthesis.
- **Enterotype 3**: Is the most frequent Enterotype, with abundance in *Ruminococcus* and *Akkermansia*. Both genus comprise species with enzymes that degrade mucin, as well as membrane transporters for simple sugars.

Arumugam *et al*., (2011) proposed that these enterotypes are not only driven by nutritional habits or physiological condition of the host (e.g. age or BMI), but also by functional markers as genes.

1.1.3.- Transversal or Axial variations of gut microbiota in the intestine.

It has been published that, in addition to the longitudinal variation across the digestive tract, there are differences between luminal and epithelial microbiota composition (Palestrant *et al.*, 2004; Eckburg *et al*., 2005; Swidsinsky *et al*., 2005). According to Swidsinski *et al.*, (2007), these differences could be explained by the viscosity gradient within the mucus layer. The enterocyte layer in intestine is lined by thick mucus coverage. Together with the enterocyte layer, this mucus provides a barrier to pathogens invasion and, at the same time, allows an efficient exchange of nutrients. Palestrant *et al.,* (2005) determined the presence of adherent mucus areas across the intestinal epithelium. These areas were more common in cecum, ascending colon and transverse colon, whereas they where absent in the small intestine. These authors identified bacterial colonies associated to these areas, making biofilms. These colonies of bacteria followed the contours of intestinal epithelium, and were oriented in parallel to

the direction of content flow. In these biofilms, bacteria would grow in distinct layers, explaining the differences between the bacteria on the top from those on the inner layers. Swidsinsky *et al.,* 2005 found that only *Clostridium*, *Lactobacillus* and *Enterococcus* were able to penetrate the inner mucus layer, whereas *Bacteroides*, *Bifidobacterium*, *Streptococcus*, *Ruminococcus* and members of *Enterobacteriacea*, together with the previously mentioned genus, remained closer to the intestinal lumen. These differences have been represented in figure 3.2.

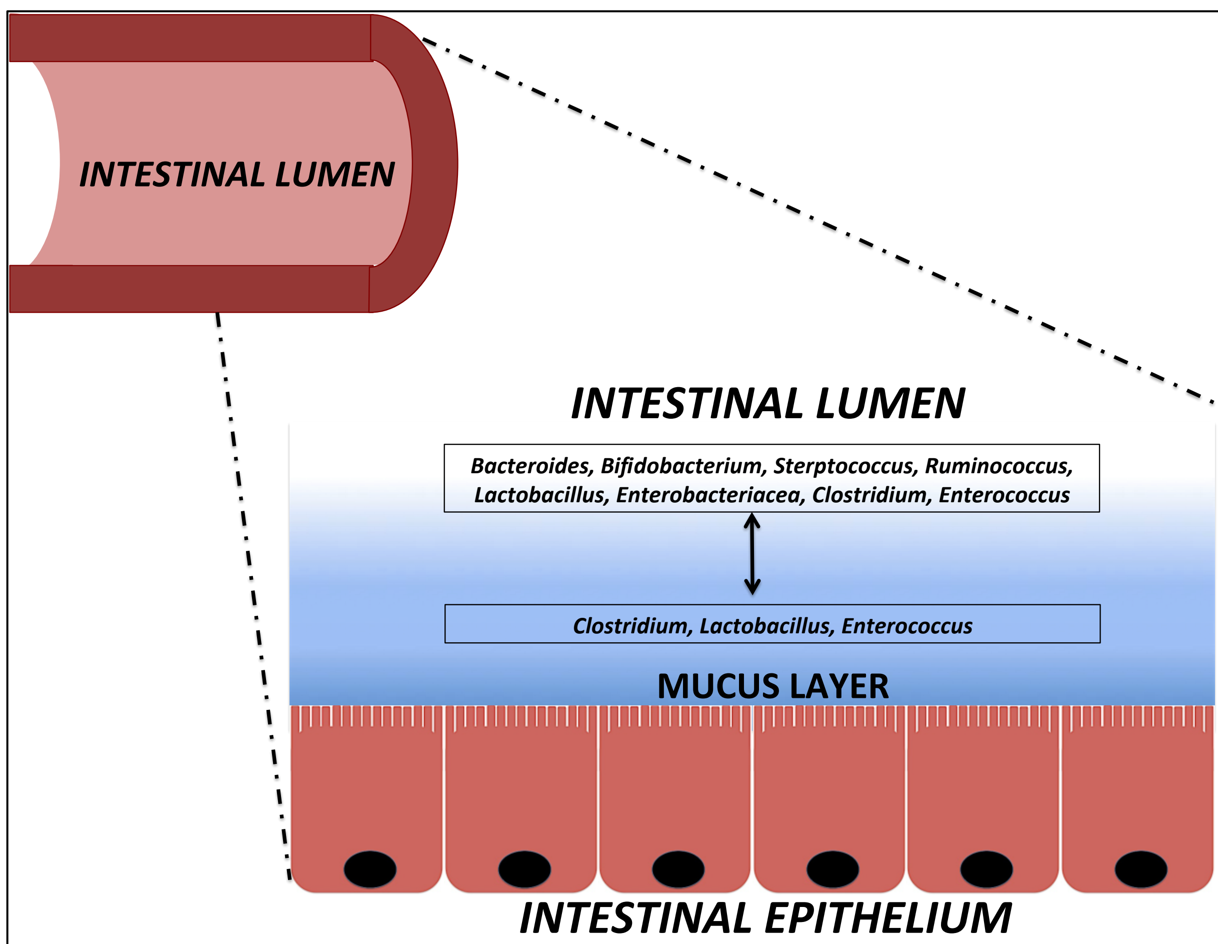


Figure 3.2.- *Variations on microbiota composition between the intestinal epithelium and the intestinal lumen.*

These differences between luminal and mucosal microbiota could explain the differences founded by different authors between fecal microbiota and the bacterial presented in the intestinal mucosa (Eckburg *et al*., 2005; Palestrant *et al*., 2005; Swidsinsky *et al*., 2005).

1.2.- Physiological relevance of gut microbiota.

As it has been mentioned in the previous section, human gastrointestinal tract is inhabited by, approximately, 10^{13} to 10^{14} microorganisms. Composition and distribution of gut microbiota is a result of a co-evolution between both, the microorganism and the host, from birth until adult age. As a result, a symbiosis-like relationship has been established among them. The coevolution process is affected by the host genome and different external factors, as diet or the environment (O'Hara & Shanahan, 2006; Nicholson *et al*., 2012).

The understanding of human and gut microbiota symbiosis, as well as its effect on human health, have gained a growing interest during the last years. Nicholson *et al*., (2012), define this symbiosis as "*immune-mediated signaling processes together with a direct chemical interaction*". According to this definition, gut microbiota plays an essential role on the host's immune system, as well as on the gut physiology and metabolism. What is more, microbiota affects not only gastrointestinal tract, but also other organs and systems such as liver, nervous system, endocrine system or systemic immune response (O'Hara & Shanahan, 2006; Sekirov *et al*., 2010; Clemente *et al*., 2012; Cryan & Dinan, 2012; Nicholson *et al*., 2012; Ottman *et al*., 2012).

1.2.1.- Gut microbiota and immune system.

The immune system plays an essential role for the human-microbiota homeostasis maintenance. Not only the immune system determines the composition of gut microbiota but also, microbiota have an impact in its development and maturation (Hooper *et al*., 2012; Nicolson *et al*., 2012).

Gastro-Intestinal microbiota as a whole, can be considered a great source of antigens which are in contact with the immune cells and receptors presented in the gastro-intestinal mucosa (Sekirov *et al*., 2010). The intestinal immune system has been named as Gut Associated Lymphoid Tissue (GALT), being formed by different components (Sekirov *et al*., 2010; Jandhyala *et al*., 2015): which, in brief, are

- Peyer's patches
- Small intestine lymphoid tissue
- Lymphoid aggregates presented in the large intestine
- Immune cells spreaded in the lamina propia.

Cell population is composed by effectors and regulatory T cells, immunoglobulin (Ig) producer B cells, macrophages and dendritic cells.

The presence of gut microbiota is necessary for an adequate development of the GALT. In this process, not only the presence of microorganism and their structural antigens (for instance, lipopolysaccharide, peptidoglycan, or flagellin), but also the production of different metabolites as short chain fatty acid or aldehydes are necessary. In their works, Jandhyala *et al*., (2015) and Rooks & Garret (2016) gather some specific pathways of the immunomodulatory mechanism in which gut microbiota is involved:

- Induction of IgA production by B cells through Dendritic cells activation. This activation seems to be mediated by the production of cytokines such as TGF-β and My-D88 protein. IgA contribute to the maintenance of mucosal barrier integrity.
- Maintenance of an adequate balance between T helper cells, Th1 and Th2.
- Optimal production of immunomodulatory cytokines as IL-10 or IL-1β by T cells, macrophages and dendritic cells.

Different studies performed with axenic or germ-free animals have demonstrated the relevance of gut microbiota for an adequate development of the intestinal and the systemic immune system (Macpherson & Harris, 2004; Mazmanian *et al*., 2005; Pabst 2012; Jandhyala *et al*., 2015; Rooks & Garret, 2016). According to these studies, germ-free animals show several immune alterations which lead to a higher infection susceptibility and to an ineffective immune response, for instance:

- Axenic mice presented a reduced number of IgA producing B cells and a lower secretion of mucosal immunoglobulins.
- Underdevelopment of GALT, including Peyer's patch hypoplasia and fluidification of mucus layer.
- Germ-free mice presented an unbalanced relationship between Th1 and Th2 cells. In this way, an excess of Th2 against a lower amount of Th1 cells has been described, being related to a higher susceptibility to allergic disorders.

According to these works, gut microbiota not only contributes to an adequate development of gastro-intestinal immune system, but also contributes to the normal development and function of systemic immune response.

As it has been presented in figures 3.1 and 3.2, a variation in the distribution of gut microbiota across the digestive tract has been described. This distribution is, in part, determined by the host's immune system with the objective of controlling their overgrowth, protecting against pathogens or avoiding a harmful inflammatory response. In this regulatory mechanism, the secretion of different products by the mucosa cells seems to be involved. One of these products is the IgA secreted by B cells (plasma cells) located in the intestinal mucosa (Tsuji *et al*., 2008; Mirpuri *et al*., 2014; Planer *et al*., 2016). For its active secretion, the presence of bacteria, lipopolysaccharide and flagellin seems to be necessary (Tsuji et al., 2008). Secreted IgA plays an important role in preventing bacterial overgrowth. In this regard, Mirpuri *et al*., (2014) determined that, a deficiency of IgA in newborn mice, was associated to an abnormal development and composition of gut microbiota in adult mice, existing a relationship between this microbial dysbiosis and a predisposition for intestinal inflammation. These findings have been also described by Planer *et al*., (2016), who related an abnormal IgA response with an abnormal microbial colonization and inflammatory/immune diseases. In humans, an IgA deficiency has been associated to autoimmune disorders, allergic disorders, or gastrointestinal and pulmonary disorders/infections (Yel, 2010). As can be deduced, IgA not only affects microbiota development and composition, but also is necessary for preventing pro-inflammatory responses against gut microbiota and its antigens. In this way, Fernandez *et al*., (2003) described that IgA plays an anti-

inflammatory role as is involved in the neutralization of bacterial lipopolysaccharide in the intestinal epithelial cells.

Apart from secreted IgA, it has been described a secretion of antimicrobial peptides by the host's enteric cells. These peptides would be involved in controlling microbial growth. Among these enteric antimicrobial products, lysozyme secreted by Paneth cells, microbe bindings lectins corticostatin/defensin precursors or phospholipase A2 are included (Harwig *et al*., 1995; Cash *et al*., 2006; Meyer-Hoffert *et al*., 2008). Meyer-Hoffert *et al.,* (2008) determined that, the secretion of these antimicrobial substances occurs in the intestinal crypts, diffusing actively through the mucus layer. In this way, a concentration gradient of these substances was described by these authors, being more abundant in close proximity to the epithelial barrier and absent in the intestinal lumen. The distribution of antimicrobial peptides could explain the variations in microbiota composition between the intestinal epithelium and the lumen described in figure 3.2.

1.2.2.- Gut microbiota and the protection against income potentially pathogens.

Apart from stimulating an optimal development of the GALT, gut microbiota offers protective functions against incoming pathogens. This function has been classically termed "colonization resistance" (Van der Waaij *et al*., 1971; Buffie & Pamer, 2013) being a complex mechanism which results from the combination of different factors (O'Hara & Shanahan, 2006; Stecher & Hardt, 2008; Sekirov *et al*., 2010; Buffie & Pamer, 2013):

- Production of antimicrobial factors.
- Nutrient competition.
- Modulation of immune response.
- Receptor competition and adhesion inhibition.

For instance, *Bifidobacterium spp*., between other described functions, secrete antimicrobial substances like organic acids and peptides derived from their metabolism, inhibit the adhesion of pathogens such as *Clostridium difficile* or enterohaemorragic *E. coli*, and modulates the immune response and cell proliferation through gene expression enhancement (Gagnon *et al*., 2004; Trejo *et al*., 2006; Nicholson *et al*., 2009; Allen *et al*., 2013; Buffic & Pamer, 2013).

In the same way, different protective function has been attributed to *Lactobacillus spp*. The production of lactic acid by these bacteria not only decreases the intestinal pH, but also enhance the lysozyme activity and facilitates the disruption of the gram negative external membrane (Alakomi *et al*., 2000). Other studies have demonstrated that some *Lactobacillus* species are able to produce antimicrobial compounds against different microorganisms as *E. coli*, *Staphylococcus aureus* or *C. difficile* (Allen *et al*., 2013; Sambanthamoorthy *et al*., 2014). As it has been mentioned, *Bifidobacterium spp*., *Lactobacillus spp* are also able to inhibit the adhesion of microorganisms to the intestinal cells, displacing pathogen and avoiding their colonization (Buffie & Pamer, 2013).

1.2.3.- Metabolic function of gut microbiota.

It has been demonstrated that there is an important interaction between the host and microbial metabolism. During the digestion process, the microbiota is able to interact with different metabolites resulting from the ingestion and transformation of different foods and components by the host. As a result of this interaction, there is an improvement of nutrient transformation/absorption. In the same way, microbiota is able to produce essential nutrients such as vitamins that otherwise would not be available for satisfying human needs. Apart from these functions, gut microbiota also contributes to the intestinal detoxification of harmful substances and metabolites, and to the regulation of the host metabolism (Nicholson *et al*., 2012; Jandhyala *et al*., 2015).

- *Production and relevance of Short Chain Fatty Acids (SCFAs)*.

Complex carbohydrates, classified as dietary fiber, cannot be digested by the host enzymes. However, colonic microorganisms are able to metabolize these indigestible oligosaccharides as well as other carbohydrates and proteins presented in the colonic lumen during the digestion process. As a result of their colonic fermentation, Short-Chain Fatty Acids (SCFAs) such as acetate, propionate and butyrate, are obtained. These SCFAs has been associated to multiple biological activities, being the most interesting metabolites produced by gut microbiota (Puertollano *et al*., 2014; Jandhyala *et al*., 2015). In table 3.1 a compilation of SCFAs production and their most relevant metabolic functions are shown. During colonic carbohydrate fermentation, acetate, propionate and butyrate are respectively produced in a 3:1:1 ratio (Puertollano *et al*., 2014; Raman *et al*., 2016). SCFAs are mainly produced in the proximal colon in high concentrations (70-140 mM), being transported through the distal colon by the intestinal flow. However,

due to the fact that SCFAs are absorbed and mainly used by the colonocytes for their metabolic maintenance (butyrate provides around 60 – 70% of colonocytes energy requirements), their concentration in distal colon are lower (20 – 40 mM) (Wong *et al.*, 2006; Suzuki *et al.*, 2008; Raman *et al.*, 2016).

Table 3.1.- *SCFAs production and their most relevant metabolic functions*

<table>
<tr><th>SCFAs</th><th>Main Colonic Producers</th><th>Metabolic Function</th></tr>
<tr><td>Butyrate</td><td>Clostridium, Faecalibacterium, Fusobacterium, Eubacterium, Roseburia.</td><td>• Regulation of energy homeostasis.
➢ Stimulation of leptin production in adipocytes.
➢ Induction of glucogen-like peptides by enteroendocrine cells.
• Nutrient Source for colonocytes. Butyrate provides arround 60 – 70% of their energy requirements, being also a substrate for ketone bodies and CO_2 production.
• Regulation of cell growth and differentiation. Butyrate is a stronger inhibitor of hystone deacetilases, inducing gene transcription/expression.
• Anti-inflamatory activity. Butyrate repress the production pro-inflamatory molecules as TNFα, or Nitric Oxide.</td></tr>
<tr><td>Acetate</td><td>Bacteroides, Bifidobacterium, Clostridium, Lactobacillus, Atopobium, Fusobacterium, Rhuminococcus, Enterobacterium, Eubacterium.</td><td rowspan="2">• Regulation of inflamatory response and anti-inflamatory activity. Acetate regulates chemotaxis of inflamatory cells as neutrophils.
• Metabolic substrates for different tissues (mainly hepatocytes). Acetate and particularly propionate, are used as metabolic substrates for lipid synthesis, energy/metabolism/gluconeogenesis and oxidation.
• Regulation of cell growth and differentiation. As butyrate, acetate and propionate seems to be mild inhibitors of hystone deacetilases.</td></tr>
<tr><td>Propionate</td><td>Bacteroides, Clostridium, Prevotella</td></tr>
<tr><td colspan="3">References: Nicholson et al., 2012; den Besten et al., 2013; Puertollano et al., 2014; Tan et al., 2014; Jahdhyala et al., 2015;</td></tr>
</table>

Apart from the metabolic functions presented in table 3.1, an antimicrobial function has been also associated to SCFAs. In this way, together with other fermentation products as lactate, SCFAs decrease colonic pH and inhibit the growth of some potentially pathogens. Apart form this effect on pH, it has been described that

acetate promotes the release of Reactive Oxygen Species (ROS), which are efficient bactericidal factors (Nicholson *et al*., 2012; Tan *et al*., 2014)

The effect of SCFAs on gastrointestinal motility has been also analyzed by different authors. Grider & Piland (2007) developed an *in-vitro* study with rats and guinea pigs colon, finding that physiological concentrations of SCFAs were able to stimulate the peristaltic reflex (ascending contractions and descending relaxation). In this stimulation, the release of serotonin and calcitonin-gene related peptide, as well as the activation of enteric reflex pathways are involved. According to Hurst *et al*., (2014) acetate, propionate and butyrate could present a different effect on the regulation of gastrointestinal motility. These authors worked *in-vitro* with colonic segment obtained from guinea pigs. After adding different solutions of each SCFA in a concentration range of 10 – 100mM, the effect on colonic motility was analyzed. According to their results, butyrate would increase full-length peristaltic propagations and decreased short propagations. On the contrary, propionate and acetate would decrease total and short propagations.

- *Synthesis of vitamins*.

Vitamins play an important role in different metabolic pathways, acting for instance, as cofactors of different enzymes. As essential nutrients, the human organism is unable to synthetize them. Apart from the diet, gut microbiota is an endogenous source for some of these vitamins. In this regard, different *Bifidobacterium* strains have been catalogued as vitamin K and different soluble vitamins from B group producers, such as B12, biotin, folates, thiamine, riboflavin or pyridoxine (Nicholson *et al*., 2012;

LeBlanc *et al*., 2013; Patterson *et al*., 2014). For instance, in the case of folates, *Bifidobacterium* strains have been classified as high level folates producer (*Bifidobacterium bifidum*, *Bifidobacterium longum subsp infantis*) and low level folates producers (*Bifidobacterium breve*, *Bifidobacterium longum subsp longum*, *Bifidobacterium adolescentis*) (Pompei *et al*., 2007; LeBlanc *et al*., 2012).

Apart from *Bifidobacterium*, other groups seem to be able to partially produce vitamins or to produce vitamin-like compounds. This is the case of *Lactobacillus*. In the case of folates, the majority of Lactobacillus strains, are unable to synthetize folates *de novo* without the addition of para-amino benzoic acid as precursor. With regard to cobalamin (B12 vitamin), some *Lactobacillus* strains as *L. reuteri* seems to be able to produce cobalamin-like products. Regarding other vitamins from B group, *Lactobacillus helveticus* is able to produce thiamine and pyridoxine (LeBlanc *et al*., 2012).

- *Transformation of food components and synthesis of bioactive compounds*.

Some foods are rich in potentially bioactive compounds. However, in some cases they are in an inactive form, linked to different food components. In these regards, polyphenols are presented in different vegetables as tea, cocoa seeds, pomegranate, or wine, but they are usually glycosylated (linked to carbohydrates such as glucose, rhamnose, galactose or ribulose), or bounded to organic acids and lipids. In this form, polyphenols cannot be absorbed or are biologically inactive (Marín *et al*., 2015). Some of these bioactive compound (5-10% of total polyphenol intake) can be digested by human digestive enzymes, as for instance β-glucosidase, that hydrolyzes glucose-linked glycosylated flavonoids, and absorbed in the small intestine. On the contrary, the

majority of ingested polyphenols (90 – 95%) are linked to complex sugars as rhamnose or esterified to organic acids and lipids, needing to be processed by the colonic bacterial enzymes, like α-rhamnosidases (Cardona *et al*., 2013; Marín *et al*., 2015). After being absorbed, polyphenols are modified in different tissues, mainly in enterocytes and hepatocytes. The main modification routes are conjugations (methylation, sulfation, or glucuronidation) resulting in different water soluble metabolites that are distributed by the blood stream and excreted in the urine (Manach *et al*., 2004; Cardona *et al*., 2013). Different beneficial effects against chronic diseases have been attributed to polyphenols, such as cardiovascular, inflammatory or immune diseases. However, possible negative molecular interactions and toxicity have been also associated to their intake (Skibola & Smith, 2000; Fraga *et al*., 2010; Khurana *et al*., 2013; Murakami, 2014). Apart from their physiological effects, an effect on the composition and activity of microbiota has been attributed to polyphenols. Parkar *et al*., (2013) attributed this effect not to the original compounds presented in plants, but to the product resulting from their fermentation. In this regard, these authors concluded that the *in-vitro* growth of *Bifidobacterium, Bacteroidetes* and *Firmicutes* was stimulated by the presence of these compounds. Other studies also reported a positive effect on *Lactobacillus* population (Tzounis *et al*., 2011; Viveros *et al*., 2011). In parallel, an inhibitory effect on potentially pathogen microorganisms, such as *Staphylococcus aureus, Listeria monocytogenes*, Candida or Pseudomonas, has been attributed to the intake and transformation of polyphenols (Marín *et al*., 2015).

Another example is rutin, a flavonoid presented in plants, which is metabolized by human microbiota resulting in different compounds as quercetin. Quercetin presents higher anti-apoptotic and antioxidant activity than rutin (Chow *et al*., 2005; Yang *et al*., 2012).

Gut microbiota also plays an important role in bile salt metabolism and their enterohepatic cycle. Some groups as *Bacteroides, Eubacterium, Clostridium, Lactobacillus* or *Escherichia*, synthetize bile salt hydrolase enzymes, which form unconjugated free bile acids as deoxycholic acid and lithocholic acid. These products will be reabsorbed and transported to the liver for being recycled (Nicholson *et al*., 2012).

- *Effect on mineral absorption*.

Due to carbohydrate fermentation, gut microbiota produces different compounds as lactate or short chain fatty acids. The presence of these compounds on intestinal lumen decrease pH, stimulating the absorption of divalent cations such as Ca^{+2} or Fe^{+2} (Ramakrishna, 2013). Regarding calcium absorption, it seems that the positive effect depends on the age, being microbiota interventions more effective on the adolescence (Whisner & Weaver, 2013). Apart from the effect on intestinal pH, it seems that microbiota could affect the expression of mineral transporters in enterocytes membrane. In this way, Deschemin *et al*., (2016) have found that germ free mice present higher expression of duodenal cytochrome b and divalent metal transporter but lower expression of ferroportin in comparison with colonize mice. These could induce iron depletion in germ free mice's intestinal cells. Taking into account that different complex

mechanisms beyond the effect on intestinal pH could exist, the impact of gut microbiota on intestinal mineral absorption is still unclear,

- *Drug metabolism by gut microbiota.*

Drugs, especially when they are orally administered, can be a subtract for gut microbiota metabolism. As a result, a modification in their availability, activity or even an increase in their toxicity has been described (Sousa *et al*., 2008). For instance, metronidazole is an antimicrobial agent used, among others, for anaerobic bacterial infection, recurrent ulcerative colitis ulcerative or inflammatory bowel disease treatment. For being effective, metronidazole-N group need to be reduced by bacterial nitro-reductases (Rafii *et al*., 2003). Another example could be Sulfinpyrazone, used for treatment of inflammatory arthritis caused by uric acid or gout. It is composed by sulfoxide, which is reduced to sulfide by gut microbiota, increasing its platelet anti-aggregant activity or its cyclo-oxigenase-inhibitory effect (Kang *et al*., 2013).

An increase in pharmacological toxicity has been also attributed to microbiota metabolism. 1-β-D-arabinofuranosyl-5-(E)-2-bromovinyl uracil, is used against Herpes-virus type-1 and varicella-zoster virus. However, serious negative secondary effects have been declared when this drug is used in combination with the anti-tumoral 5-fluorouracil. The metabolites resulting from intestinal bacterial degradation of 1-β-D-arabinofuranosyl-5-(E)-2-bromovinyl uracil, interfere 5-fluorouracil degradation. As a result, 5-fluorouracil accumulates in blood and increase its toxicity (Li & Jia, 2013).

1.2.4.- Gut microbiota and disease.

As it has been mentioned above, gut microbiota plays an important role in different physiological functions of the host. Therefore, it is necessary to maintain an optimal relationship between gut microbiota composition and metabolism and the host's organism. In this way, during the last year, different studies have tried to clarify a possible relationship between intestinal microbial dysbiosis or imbalance, and several diseases, not only at a gastrointestinal level, but also metabolic diseases (Sekirov *et al*., 2010). In table 3.2., a compilation of different research works about microbiota and related diseases can be found.

In recent years, the studies on a possible relationship between microbiota and nervous system development and function have gained relevance. In this regard, a Gut-Brain Axis has been defined to describe any interaction between the gastrointestinal tract and central nervous system (Bercik *et al*., 2012). In principle, this interaction was related to hormone or different nutrients production, as different peptides, carbohydrates or fatty acids. Among these products, microbial bioactive metabolites are included (Banks, 1980; Stilling *et al*., 2014). Apart from this biochemical interaction, germ-free animal's studies have revealed a neural-microbiota communication through vagus and spinal cord afferent nerves (Gaykema *et al*., 2004; Lyte *et al*., 2006). Related to these studies, evidences of the impact of gut microbiota on nervous system health and diseases have begun to come to light. For instance, Finegold *et al*., (2010) described an altered intestinal microbiota composition in children with autism, with the highest differences between *Bacteroidetes* and *Firmicutes* phylum. *Bacteroidetes* were isolated at high levels in severely affected autistic children, whereas *Firmicutes* were more abundant in non-autistic children. *Actinobacterium* and *Proteobacterium* phylum also

show differences, being the former less abundant and the latter more abundant than in healthy children. In this work, severely affected autistic children also show a reduced *Bifidobacterium* population.

Table 3.2.- *Relationship between microbiota and gut/metabolic diseases.*

Gastro-Intestinal Tract Disorders	
• ***Gastro-duodenal peptic ulcera and gastric adenocarcinoma***.	
A predominant *Helicobacter pylori* gastric microbial profile have been strongly correlated to gastro-duodenal lesions and gastric cancer.	(Watari *et al.*, 2014)
• ***Cholelithiasis***.	
A relationship between gut micorbiota dysbiosis and a predisposition to cholestrol gall-blade stones formation has been proposed. An increase of gut bacterial phylum *Proteibacterium* and a decrease of *Faecalibacterium*, *Lachnospira* and *Roseburia* have been detected in gallstone patients.	(Wu *et al.*, 2013)
• ***Celiac Disease***	
Differences in microbiota composition have been found in patients with celiac disease respect to control. In this way, a reduced number of Lactobacillus and *Bifidobacterium* and a higher number of *Bacteroides, Staphylococcus* and *Escherichia coli* have been observed. The high presence of *Proteobacteria* have been also associated with gastro-intestinal symptomatology.	(Wacklin *et al.*, 2013; Cenit *et al.*, 2015)
Metabolic Disorders	
• ***Obesity***.	
Microbiota from obese individuals seems to show an imbalance in the *Firmicutes*:*Bacteroidetes ratio*. A high level of *Lactobacillus ruteri* and low level of *L. casei, L.paracasei* and *L.plantarum*, together with low levels of *Bifidobacterium* have been also asociated to obesity.	(Clemente *et al.*, 2012; Million *et al.*, 2013)
• ***Diabetes***.	
Both, reduction of highly butyrate-producing bacteria (as *Roseburia* and *Faecalibacterium*), and a functional dysbiosis towards a increased gut oxidative stress, have been associated with Type 2 Diabetes. Imbalances in the amount of different *Clostridium* species, as well as in *Bifidobacterium* and *Lactobacillus*, together with a reduced Firmicutes:Bacteroidetes ratio have been described in children with type 1 diabetes.	(Qin *et al.*, 2012; Karlsson *et al.*, 2013; Murri *et al.*, 2013; de Goffau *et al.*, 2014)

1.3.- Establishment of the infant gut microbiota. Involved factors.

During most of adult's life, microbiota will remain relatively stable (Ottman *et al*., 2012). In this way, differences between individuals are larger than Intra-individuals, for periods of a year or more (Eckburg *et al*., 2005; Palmer *et a.,*. 2007). On the contrary, during the first year of life, infant gastro-intestinal microbiota has to progress from an almost sterile to a complex and varied community, being its composition and structure subject to a greater variability than during the adulthood. After 2 – 3 years, infant microbiota will converge towards an adult-like pattern (Palmer *et al*., 2007; Koening *et al*., 2011, Rodriguez *et al*., 2015). During this period, a succession of different species will occur, influenced not only by the special conditions of the immature infant gastrointestinal tract, but also by the interactions between the different microorganisms. This process involves species that maintain during the adult life, but also specific species from early infancy (Matamoros *et al*., 2013).

Different authors have described the patterns of infant gut microbiota establishment (Adleberth, 2008; Vael & Desager 2009; Matamoros *et al*., 2013; Rodríguez *et al*., 2015). According to these authors, the first stages of infant microbiota development are dominated by *Proteobacteria* and *Actinobacteria* phylum. As the gastrointestinal tract develops, the microbial community becomes more complex than in the previous stages, being dominated by the *Firmicutes* and *Bacteroidetes* phylum. This first evolution pattern is being related to the modifications of the gastrointestinal tract environment. After birth, neonatal gut is mainly colonized by facultative anaerobe bacteria, belonging to the Enterobacteriaceae family, as *Escherichia*. Apart from them,

other bacteria belonging to the *Firmicutes* phyla, such as *Enterococcus* or *Streptococcus* have been isolated in less proportions. As these species develop and multiply, oxygen supplies become depleted, leading to a reduced environment in which anaerobic bacteria, as *Bifidobacterium*, *Bacteroides* or *Clostridium*, can survive and proliferate.

However, colonization and infant microbiome development is a much more complex process than the previously explained. In this regard, it is affected by different factors as intrauterine ambience, gestational age, mode of delivery, genetics, diet, or even, environment and geographical location (Bergström *et al*., 2014; Rodriguez *et al*., 2015; Echarri *et al*., 2011). Among them, Bergström *et al*., (2014) define breastfeeding and introduction of complementary feeding (9 – 18 months) as the most determinants factors affecting on the microbiota composition.

As it has been explained in previous sections, gut microbiota is involved in many physiological functions, being its imbalance related to different diseases and metabolic disorders. As gut microbiota development mainly occurs during the first years of life, many authors considers this period as crucial and susceptible to different intervention to ensure an adequate establishment and to reduce the risk of diseases during life (Adleberth, 2008; Vael & Desager, 2009; Bergström *et al*., 2014; Rodríguez *et al*., 2015). Therefore, research about the way in which these factors interact with microbiota establishment and composition are crucial.

1.3.1.- Prenatal Colonization of Fetus' Gastrointestinal Tract

During uterine development, it has been accepted that the fetus floats in the amniotic fluid, which is defined as a sterile environment (Putignani *et al*., 2014). In fact, the presence of microbes in the amniotic cavity has been related to the rupture of fetal membranes. Process as chorioamnionitis and intra-amniotic inflammation, as well as preterm delivery are associated with the microbial invasion of amniotic fluid (Di Giulio *et al*., 2008; Di Giulio *et al*., 2010). As a consequence, baby's gastrointestinal tract has been considered as mostly sterile at birth, being colonized during and/or after the labour (Mackie *et al*., 1999).

Despite this assumption, recent studies have demonstrated the presence of a microbial community in the meconium and fecal samples obtained from preterm babies during their first months of life (Jimenez *et al*., 2008; Moles *et al*., 2013; Ardissone *et al*., 2014). According to Moles *et al*. (2013), *Firmicutes* was the main bacterial group detected in meconium, whereas fecal samples show abundance in *Enterococcus*, *E. coli*, *Klebsiella* or *Serratia*. However, the amniotic colonization route for these microorganism is not clear.

On one hand, the ascending route seems to be accepted for the microorganism presented in mother's vagina (Vinturache *et al*., 2016). According to the work published by Hansen *et al*., (2014), some bacteria could be able to pass through the cervical mucus plug, being thus possible this colonization route.

On the other hand, recent studies have demonstrated other possible colonization routes. In this way, Stout *et al*., (2013) suggested that bacteria located in the endometrium mucosa could be incorporated to the placenta during its uterine implantation. In addition, Jimenez et al., (2008), performed an experiment which consisted in the oral administration of a labeled *Enterococcus faecium* strain to pregnant mice. Subsequently, this labeled strain was isolated from the meconium and amniotic fluid obtained from preterm fetuses. With this experiment, a possible colonization route between the mother's gastrointestinal tract and the fetus though the bloodstream could be considered.

The presence of bacteria in the amniotic cavity has been associated with different inflammatory process (Di Giulio *et al*., 2010). However, the presence of genus as *Lactobacillus*, seems to reduce this inflammatory response (Fichorova *et al*., 2011). In fact, Rautava *et al*., (2012) performed an experiment in which prebiotics, consisted in *Bifidobacterium lactis* and *Lactobacillus rhamnosus,* were supplied to pregnant woman. Their posterior presence in amniotic fluid and placenta was associated with a modulation of the Toll-Like –Receptors genes expression in the fetal intestine respect the samples obtained from mothers that didn't receive the prebiotics. In a recent study performed by Gunjan *et al*. (2016), a pessary containing Lactobacillus and antibiotics was applied to mothers with abnormal vaginal flora in early pregnancy. As a result, a decrease in the rate of preterm delivery and low birth weight was obtained. This relationship between the presence of Lactobacillus, and a later date of delivery/higher birth rate, has been also mentioned by Dasanayake *et al*., (2005).

1.3.2.- Gestation time and infant microbiota.

Gestation time seems to have a great influence in the infant microbiota establishment. According to Arboleya *et al.*, (2012[b]), microbiota composition in preterm neonates differs significantly from that of full-term neonates. These authors observed reduced variability and microbial diversity in pre-term neonates, with high levels of the facultative anaerobes microorganisms *Enterobacteriaceae*, *Enterococaceae* and *Lactobacillus* group, and low levels of the anaerobes *Bifidobacterium*, *Bacteroides* and *Atopobium*. In concordance with these findings, low concentrations of SCFAs and a proliferation of potentially pathogenic microorganisms such as *Klebsiella pneumonia* or *Clostridium difficile* were reported by Arboleya *et al.*, (2012[b]) in pre-term neonates. In this study, *C. difficile* was absent in full-term neonates. The presence of this microorganism has been related to an imbalanced gut-microbiota and low levels of *Bifidobacterium*, attributing colonization resistance to the latter (Rousseau *et al*, 2010).

With regard to full-term infants, it has been described a higher microbial diversity and abundance in *Bifidobacterium*, *Streptococcus* and *Lactobacillus* respect the pre-term infants (Arboleya *et al.*, 2012[a]; Arboleya *et al.*, 2012[b]). Despite microbiota differences between pre-term and full-term neonates, as babies grow, they were attenuated, ceasing to be significant at 3 months of age (Arboleya *et al.*, 2012[b]).

1.3.3.- Mode of delivery and its influence on the newborn's microbiota.

Mode of delivery is an important factor affecting neonate's gut colonization. Depending on mode of delivery (vaginal delivery or cesarean section), neonates will be exposed to different microorganisms. In contrast with cesarean section, vaginally delivered neonates are highly exposed to maternal intestinal and vaginal microbiota,

being considered as one of the most important maternal contribution to the early neonate's microbiota acquisition (Adleberth, 2008; Khodayar-Pardo *et al*, 2014).

Regarding vaginally delivered neonates, different studies show that their intestines are colonized by mother's vaginal and intestinal microbiota, which includes species as *Lactobacillus, Bifidobacterium*, and *Bacteroides*, together with facultative anaerobic *Enterobacteraciae* as *Escherichia coli* (Adlerberth *et al*., 2006; Dominguez-Bello *et al.*, 2010; Khodayar-Pardo *et al*., 2014).

In the case of neonates delivered by cesarean section, the exposure to maternal vaginal and fecal microbiota is negligible, showing a lower microbiota diversity than vaginally delivered neonates. As a result, microbes from the maternal skin and the perinatal environment colonize the former (Rodríguez *et al*., 2015). Domínguez-Bello *et al*., (2010) found that cesarean neonates presented an intestinal microbiota very similar to that isolated form maternal skin, being dominated by *Staphylococcus*, *Corynebacterium*, and *Propionibacterium spp*. What is more, these neonates show a delayed colonization by *Escherichia coli* or *Bacteroides* against other *Enterobacteriaceae* species as *Klebsiella* or *Enterobacter*. Apart from these findings, the potentially pathogens *Clostridium perfringens* and *C. difficile* have been also isolated form neonates delivered by cesarean section in higher proportion than in vaginally delivered ones (Adlerberth, 2008).

Due to the low exposition to maternal intestinal and vaginal microbiota, colonization by *E.coli* and *Bacteroides* is delayed in babies delivered by cesarean section respect the vaginally delivered ones. This disturbance of infant microbiota seems to

maintain during the first years of life (Adlerberth *et al*., 2006; Adlerberth, 2008; Rodríguez *et al*., 2015).

1.3.4.- Other perinatal factors involved in the microbiota establishment.

Apart from prenatal environment, gestation time, and delivery mode, there have been identified other influencing factors involved in the development of neonate's intestinal microbiota. Among them, the use of antibiotic prophylaxis protocols, and the environmental hygienic conditions, have been identified as relevant influential factors (Fanaro *et al*., 2003; Rodríguez *et al*., 2015)

Antibiotherapy is a common procedure included in preterm infants and neonatal intensive care units. The use of antibiotic protocols for neonates are aimed to prevent central line associated bloodstream infection and nosocomial infections (Schulman *et al*., 2015). However, their use has been associated with disturbances in the enteric microbiota establishment. In fact, different studies have indicated a low microbiota diversity, as well as a reduced number of *Bifidobacterium* in neonates exposed to antibiotics (Kumar *et al*., 2015). On the contrary, coagulase-negative staphylococci, enterobacterias, enterococci or proteobacteria have been isolated in higher proportions in these children (Adlerberth, 2008; Matamoros *et al*., 2012; Rodríguez *et al*., 2015)

The delayed exposition to some bacterial groups, together with a lower microbial diversity could affect the physiological development of babies' gastrointestinal tract and its associated immune system (Fanaro *et al*., 2003). Indeed, Abrahamsson *et al*., (2014) found a correlation between the low gut microbiota diversity at 1 week of age, and the development of asthma in children at seven years of age. Nevertheless, these authors did

not find any relationship between microbiota composition at 12 months, and asthma development This correlation has also been observed by Russell *et al*., (2013) in murine models treated with the antibiotic vancomycin during their first days of life. This treatment resulted in a disturbance of gut microbiota and an increased allergic asthma susceptibility. However, this effect was not observed when the treatment was administered in adult mice. According to these studies, the administration of antibiotics and related disturbances of infant microbiota during the first days of life, could have a great impact on the adequate development of the intestinal associated immune system.

Environmental hygienic conditions have been defined as another relevant factor involved in microbiota establishment. With the aim of reducing the potentially pathogenic microbial presence on maternal and neonate care unit, strict hygienic procedures have been implemented (Boyce & Pittet, 2002). As a result, a modification in the colonization pattern of neonates, with a low presence of enterobacteria, has been attributed to the hygienic procedures in hospitals from Sweden. On the contrary, this routine is not performed in developing countries, resulting thus in a high bacterial exposition from birth (Fanaro *et al*., 2003).

During the last years, there has been theorized a direct relationship between the increasing prevalence of immune diseases in developed countries, and the lower microbial exposure during the early infancy. This theory was initially proposed by Strachan (1989), according to whom "*higher standards of personal cleanliness have reduced the opportunity for cross infection in young families. This may have resulted in more widespread clinical expression of atopic disease, emerging earlier in wealthier people*". In this work, Strachan (1989) attributes a protective role against allergic

diseases to infections in early childhood. After that, different epidemiological and experimental studies have been performed, resulting in the postulation of the "hygiene hypothesis" (Garn & Renz, 2007). According to this hypothesis, the strict hygiene practices, together with the increasing "sterilization" of the environment in developed countries, would reduce the exposure to different microorganism and related microbial factors since the early life stages. As a result, their immunizing potential exposure could not be enough to guarantee an adequate development of immune system, leading to a predisposition to immune disorders as allergic diseases.

1.3.5.- Relevance of infant nutrition on gut microbiota establishment and modulation.

Apart from the perinatal factors mentioned, diet is being defined as an important factor, which can modify the composition and metabolism of gut microbiota (Scott *et al*., 2013). Regarding to the importance of diet in early infancy, it seems that, the breastfeeding to weaning/solid foods shipment (9 – 36 months of age) is a critical point, determining the transition between the infant and adult microbiota (Bergström *et al*., 2014). Whereas Bifidobacteria, lactobacilli and Enterobacteriaceae dominate preweaned infant microbiota, an increment on butyrate producers, mainly *Bacteroides* and some *Clostridium* species, are associated with the introduction of weaning and solid foods (Salminen & Isolauri, 2006; Bergström *et al*., 2014; Rodríguez et al., 2015). Despite this global overview, more specific infant microbiota differences have been attributed, among other factors, to infant diet. In this way, the influence of breastfeeding versus bottle-feeding, on the development of neonate microbiota, has been widely studied. In table 3.3, published differences between breastfed and formula-fed infants in bacterial prevalence, have been presented.

Table 3.3.- *Published differences between breastfed and bottle-fed infants.*

Bacterial prevalence differences	
Breastfed infants	***Bottle-fed infants***
↓ *Bacteroides*	↑ Bacteroides
↓ *Clostridium*	↑ *Clostridium* (*C. difficile*)
↓ *Enterococcus*	↓ *Staphylococcus*
↓ Enterobacteria	↑ *Enterococcus*
↑ *Staphylococcus*	↑ Enterobacteria (*Klebsiella)*
↑ *Streptococcus*	
References: Adleberth *et al*., (2008); Adleberth & Wold, (2009); Vael & Desager, (2009); Matamoros *et al*., (2013).	

As can be seen, the presence of Staphylococcus and Streptococcus has been associated with breastfeeding. This finding has been related to the microbiota presented in breast-milk (Matamoros *et al*., 2013; Rodríguez *et al*., 2015). Despite being classically considered sterile, different studies have analyzed the presence of different microorganisms on breast milk, concluding that *Staphylococcus* and *Streptococcus*, together with other bacteria such as *Bifidobacterium* or *Lactobacillum*, are the predominant groups (Collado *et al*., 2009; Hunt *et al*., 2011; Fernandez *et al*., 2013). Regarding to the origin of these microorganisms, two different pathways have been described. On one hand, microorganisms would arrive into the mammary ducts from the mother skin, which will be favored during suckling. On the other hand, another possible pathway has been recently described. Non-pathogenic bacteria would be translocated to the mammary gland from the intestinal epithelium driven by dendritic cells (Fernandez *et al*., 2013).

Different authors have also discussed the differences reported in *Bacteroides* prevalence. There is a controversy with regard to *Bacteroides* early gut colonization and the ulterior susceptibility to allergies and chronic inflammation. In this regard, Marques *et al*., (2010), suggested that early presence of this group could exert a positive effect on the development of mucosal associated-immune system. This suggestion is in agreement with Jakobsson *et al*., (2014), who determined that a delayed *Bacteroides* colonization leads to a reduced maturation of intestinal immune system and a high predisposition to immune disorders. Concerning to the effect of diet on early *Bacteroides* colonization, it seems that it is one predominant group in formula-fed infant, whereas its presence is much more lower in breastfed infants (Adleberth & Wold, 2009; Bergström *et al*., 2014). However, in the study performed by Jost *et al*., (2012), *Bacteroides* was highly in the feces of 4 of the 7 analyzed children. In this study, subjects were vaginally-delivered and breastfed. Furthermore, the presence of *Bacteroides* was inversely correlated with the presence of *Bifidobacterium*. These authors related the presence of *Bacteroides* to a vaginal-fecal maternal transmission during delivery, as the same *Bacteroides* species were isolated from both, neonates and maternal feces, displacing the proliferation of other species as *Bifidobacterium*. The reported *Bifidobacterium*/*Bacteroides* ratio displacement could support the increase in allergy and chronic inflammation susceptibility, which is related to early *Bacteroides* colonization. What is more, according to this study, *Bacteroides* early gut colonization could be related, not only to the mode of feeding, but also to other factors as maternal transmission during the delivery. In this regard, Fallani *et al*., (2010), also reported that geographical origin was another strong factor for *Bacteroides* early colonization. In this study, a “north-south” geographical gradient was described, with a high proportion of *Bifidobacterium,* and other species as *Clostridium difficile* or *C. perfringens,* in north European countries. On the contrary, south

European countries showed higher proportions of *Bacteroides*, *Enterobacteria* and *Lactobacillus*. With regard to Spain, Echarri *et al*., (2011) also described geographical differences in infant microbiota between two different locations, Asturias and Spain.

As can be observed, *Bifidobacterium* and *Lactobacillus* genus have not been included in table 3.3. This fact meets the controversial reported effect of infant diet (breastfeeding or formula-feeding) on both bacterial populations. Breast-milk is composed, among other ingredients, by oligosaccharides, acting as bifidogenical substrates (Salminen & Isolauri, 2006). Classically, a higher proportion of *Bifidobacterium* has been attributed to breastfed infants than to bottle-fed ones (Yoshioka *et al*., 1983; Balmer & Wharton, 1989; Harmsen *et al*., 2000; Rinne *et al*., 2005; Matamoros *et al*., 2013). However, during the last years, this statement has been discarded by different authors, as no differences in *Bifidobacterium* counts have been reported between both, breastfed and bottle-fed infants (Penders *et al*., 2005; Adlerberth, 2008; Adlerberth & Wold, 2009). An explanation to these findings could be the improvement of infant formula composition during the last decade with the aim of make them more like breast-milk (Adlerberth & Wold, 2009; Salminem & Isolauri, 2010). Despite no differences in bifidobacterial counts have been reported, some authors indicate that *Bifidobacterium* species are different between two types of feeding (for instance, *B. breve* was more commonly isolated among breastfed infants), being a more complex bacterial community in breastfed infants (Salminem & Isolauri, 2006; Roger *et al*., 2010).

As in the case of *Bifidobacterium* genus, no differences in *Lactobacillus* counts have been found between breastfed and bottle-fed infants (Adleberth & Wold, 2009;

Salminem & Isolauri, 2010). According to these authors, despite these similarities, the presence of specific lactobacilli species, as *L. rhamnosus*, could be favored by breastfeeding. This statement is in contrast with the higher presence of lactobacillus attributed by other authors to breastfed infants (Rinne *et al*., 2005; Matamoros *et al*., 2012).

1.4.- Non-digestible carbohydrates as ingredients in infant formulas. Its role on gut microbiota metabolism and modulation.

Colonic microbial metabolism is based in both, endogenous substrates as mucin or sloughed intestinal cells, as well as food components arriving into the colon. Among them, carbohydrates are the main bacterial substrates, especially those resisting gastrointestinal digestion and absorption (Conlon & Bird, 2015). At this respect, human gastrointestinal tract can not synthesize the enzymes needed for degrading structural polysaccharides presented in plants, which includes resistant starches, non-starch polysaccharides, oligonucleotides or lignin. These compounds are generally known as dietary fiber. However, these ingredients can be degraded by the colonic microbiota, being incorporated into specific fermentative pathways (Tungland & Meyer, 2002; Scott *et al.,* 2013, Conlon & Bird, 2015). Due to the bacterial metabolism of these food components, a complementary energy supply, together with the synthesis of functional metabolites such as SCFAs, lactic acid or vitamins, are possible (Conlon & Bird, 2015). Dietary fiber is being recognized as having health benefits, not only for an adequate gastrointestinal functionality, but also for maintaining a balanced intestinal microbiota.

Is for that reason that a 30 g daily intake of dietary fiber has been recommended (Tungland & Meyer, 2002). An inadequate intake of dietary fiber in adults has been related with different gastrointestinal disorders, such as chronic inflammatory syndromes, constipation or gastrointestinal cancer (Aggett *et al*., 2003).

Apart from resistant-carbohydrates, other macronutrients as fats or proteins can be fermented by colonic microbiota. However, in contrast with carbohydrates, fat and protein fermentation is being associated with the production of non-desirable metabolites. In the case of proteins, the production of nitrosamines, ammonia, phenols, indols, thiols or sulphides are associated to their colonic degradation (Macfarlane *et al*., 2006; Scott *et al*., 2012).

1.4.1.- Prebiotics in infant nutrition

Gibson *et al*. (2004) defined prebiotic as "*a selectively fermented ingredient that allows specific changes, both in the composition and/or activity of gastrointestinal microbiota that confers benefits upon host well-being and health*". According to this definition, there are three key-points for a food component to be considered as prebiotic:

- To resist the enzymatic hydrolysis and absorptive process during the gastrointestinal digestion.
- To be fermented by colonic microbiota.
- To have a positive selective effect on the growth and/or activity of health and wellbeing associated microbiota.

Concerning to the positive selective effect on specific microbiota populations, the concept of "healthy microbiota" needs to be introduced. Abundance in *Bifidobacterium* and *Lactobacillus* genera has been considered as a healthy and well balanced microbiota (Macfarlane *et al*., 2006). This association is being established because these genera do not include pathogenic species, as well as their higher presence was originally associated to breastfeeding and its content in oligosaccharides. Furthermore, there are carbohydrate fermenters, and their metabolites (SCFAs or lactic acid) which have been associated with good health maintenance. On the contrary, a positive effect on the activity of other proteolytic and aminoacid fermenter groups, such as *Bacteroides* or *Clostridium*, may have negative feature on infant development and health (Meyer & Stasse-Wolthuis, 2009). However, besides bifidobacteria and lactobacilli, other species such as *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* are nowadays considered as beneficial (Venema, 2016). For this reason, different authors claims that this concept should not be limited to the presence of a certain number of species, but needs to be extended to a new one: "healthy microbiome" (Bäckhed *et al*., 2012, Alonso & Guarner, 2013). According to these authors, this new concept includes an individualized and stable microbial distribution that makes the host less susceptible to certain kind of dysbiosis-associated diseases, such as immunologic disorders, diabetes, chronic inflammatory syndromes, cancer or obesity. When referring to prebiotics, they should include different ingredients with the potential of changing the host microbiota into a more beneficial one, promoting the development and function of its beneficial members (Bäckhed *et al*., 2012). Unfortunately, beyond this ideal definition, there are not enough results and evidences for establishing the characteristic of a "healthy gut microbiota" in humans (Alonso & Guarner; 2013). At this respect, published experimental data suggest that the unbalanced presence of some specific bacterial

groups, is related with specific disorders, as was explained in table 3.2. For instance, the increment of clostridia counts is related to an increment in SCFA butyrate production. This butyrate overproduction during infancy could lead to an obesity predisposition later in life (Bergström *et al*., 2014). According to these concepts, prebiotics not only should increase the growth and/or activity of desirable microbiota, but also limit the presence of potentially harmful microorganisms.

In principle, fructo-oligosaccharides (FOS), gluco/galacto-oligosaccharides (GOS) and lactulose are the food components that best fit the prebiotic definition (Gibson *et al*., 2004). Among them, fructans as inulin and related FOS compounds, as well as GOS are being widely used as prebiotic ingredients, because of their low production cost, and the improvement of organoleptic properties associated to their addition (Macfarlane *et al*., 2006). Regarding to infant nutrition, human-milk oligosaccharides are considered as the prebiotic reference, since they promote the increment in bifidobacteria/lactobacilli populations and activity, in breastfed infants (Quigley, 2010).

As it has been indicated in section 1.3.5, available infant formula composition has been improved during the last decade, in order to make them and breast-milk more alike (Adlerberth & Wold, 2009; Salminem & Isolauri, 2010). Claiming to their prebiotic potential, the composition of many of these formulas has been improved through GOS, FOS and GOS/FOS addition (Scott *et al*., 2013; Sabater *et al*., 2016).

GOS supplementation leads to an increment of *Bifidobacterium*, decreasing the classically described differences between breastfed and bottle-fed neonates (Adlerberth & Wold, 2009). Recently, Simeoni *et al*., (2016) published one study with 115 infants

randomly fed with different types of foods: Breast-milk, a control formula without prebiotic added, and a test formula with bovine-milk derived GOS added. In this study, total bacteria counts were similar for the three treatments during the experimental phase (2, 6 and 12 weeks of age), but infants fed with the control formula showed a high diversity of fecal microbiota. On the contrary, breastfeeding and supplemented-formula feeding led to a shift towards a *Bifidobacterium*-dominated gut microbiota after 6 weeks of intervention. Regarding other groups, Simeoni *et al*., (2016) described a higher presence of *Bacteroides* and *Ruminococcus* in non-supplemented formula infant fed, than in those fed with breast-milk or supplemented infant formula. In the same study, physicochemical differences of stools were also attributed to the different treatment analyzed. Infant fed with the supplemented formula show a stool consistency and color similar to the observed for breastfed infants. Differences were also found in pH, being significantly lower for supplemented formula and breast-milk treatments than for the non-supplemented formula. These findings were attributed to a higher production of lactic acid and SCFAs in the formers than in the latter. Despite this bifidogenic effect, Klaassens *et al*., (2009) reported that, *Bifidobacterium* species detected in infants fed with GOS-supplemented infant formula, differ from those detected in breastfed. In this regard, *B. longum subsp. infantis* was reported as the major bifidobacteria specie detected in children fed with both, breast-milk and supplemented infant formula. *B. bifidum*, and *B. longum* were also detected in all infants but in less amount. Nevertheless, *B. adolescentis* was only detected in supplemented formula-fed infants. The association between type of diet and differences in the *Bifidobacterium* species distribution has been also reported by Simeoni *et al*., (2016). In this study, *B. longum* and *B. bifidum* dominated gut microbiota in infants fed with supplemented infant formula, whereas *B. bifidum* and *B. breve* dominated gut microbiota in breastfed infants.

Apart from GOS, inulin and related FOS are also used as prebiotic ingredients in infant formula. According to Meyer & Stasse-Wolthuis (2009), inulin/FOS supplementation leads to a bifidogenic effect on gut microbiota, which basically depends on the initial number of *Bifidobacterium* before the supplementation. According to Closa-Monasterolo *et al*., (2013), apart from this bifidogenic effect, supplementation with inulin/FOS has also effects on other microbial populations, decreasing the number of *Bacteroides* and *Enterobacteriaceae* like the one detected in breastfed infants. Furthermore, inulin/FOS supplementation could modify the colonic enzymatic metabolism, reducing the activity of different enzymes such as protease, nitroreductase or glycocholic acid hydroxylase, which are suggested to be involved in the production of carcinogens (Meyer & Stasse-Wolthuis, 2009). Regarding to the effect of these ingredients on stool characteristics, Closa-Monasterolo *et al*., (2013) reported that formula supplementation resulted in a higher frequency of deposition and softer consistence than the observed in non-supplemented formula fed infants, being similar to those obtained with breastfeeding.

With regard to the prebiotic safety and tolerance as ingredients in infant nutrition, the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) Committee has indicated that, there are no scientific evidences of adverse effects on growth and health status associated to the use of available prebiotic-supplemented infant formula (Braegger *et al*., 2011). In the study performed by Closa-Monasterolo *et al*., (2013), the prevalence of gastrointestinal symptoms associated to a fermentation process and dysbiosis, such as diarrhea, vomiting, regurgitation or

flatulence, was similar to that described in breastfed and non-supplemented formula fed infants. In the same way, growth pattern was similar in the three groups of children.

Despite no negative evidences have been reported, the ESPGHAN Committee (Braegger *et al*., 2011) concludes that, there should be performed more studies using "*validated clinical outcome measures to assess the effects of prebiotic supplementation of formulae*". Another request is that their "*optimal dose and intake duration*" should be defined. In the case of Spain, the current legislation establishes the legal limit for their addition as ingredient. Regarding to infant formulas, they could be added up to a maximum legal limit of 0.8/100 mL in a combination of 90% GOS and 10% FOS (BOE, 2008). In this regard, Sabater *et al*., (2016) performed a recent study with the aim of quantify the concentration of these ingredients in commercial infant formulas. According to it, the addition of these ingredients are in a range of 1.6 – 5.0 g/100g for FOS, 1.7 – 3.2 g/100 g for GOS and 0.08 – 0.25/2.3 – 3.8 g/100g for GOS/FOS (Concentrations are related to reconstituted infant formula).

1.4.2.- Other non-digestible carbohydrates used in infant nutrition: Locust bean gum and resistant starches.

Apart from prebiotics, other non-digestible carbohydrates are being added as ingredients to infant formula. Among them, galactomannans as locust bean gum (LBG), guar gum, pectin or starches are widely used as texturizers and thickeners (Agget *et al*., 2003). Concerning infant formulas, these ingredients are being widely used as thickeners, resulting in an especial type of formulas known as "Anti-Regurgitation" (AR) infant formula. Pediatric guidelines on the management of gastroesophageal reflux and regurgitation in infants, recommend the use of these special formulae with the aim of

reducing the frequency of regurgitation and vomiting. Their use should be limited to healthy term infants with uncomplicated gastroesophageal reflux (Vandenplas *et al*., 2009).

According to Tungland & Meyer (2002) and Aggett *et al*., (2003), the addition of non digestible carbohydrates could have different consequences on gut functionality and metabolism. In this way, these ingredients can be fermented by gut microbiota, generating an extra energy source. Apart from that, fermentation includes SCFAs formation, which lead to the modulation of water co-transport, epithelial growth and intestinal motility. Is for that reason that the addition of these ingredients to infant formula could have a positive effect on the management of constipation, increasing stool weight, softness and frequency. In parallel, non digestible carbohydrates reduces energy intake and the digestion of fat and carbohydrates, providing satiety effects and improving postprandial glycemic index. These aspects could be positive for the management of obesity and *Diabetes mellitus.*

This generally accepted beneficial role of non digestible carbohydrates have been also criticized. Goodlad & Englyst (2001), advised about the possible negative effect associated to the ingestion of non digestible carbohydrates. According to them, SCFAs derived from their fermentation, could stimulate cell proliferation and increase polyps formation. With regard to the reduction of caloric intake and carbohydrates-fats digestibility, Aggett *et al*., (2003) proposed another possible negative effect. In this regard, if non digestible carbohydrates are added in high amount, will increase fecal loss of energy, contributing thus to malnutrition. Other possible related negatives effects

could be pain, discomfort or diarrhea due to an excessive colonic fermentation and content acidification, especially at high or excessive intakes (Grabitske & Slavin, 2009).

LBG is commonly used as thickener in infant nutrition. Despite this fact, only a few reports have been found about their impact on infant gut microbiota. Penders *et al*., (2006) analyzed the effect of LBG against GOS supplementation, on bottle fed infants microbiota. Infants who received GOS supplementation show higher fecal *Bifidobacterium* and *Lactobacillus* counts than those supplemented with LBG. When compared with exclusively breastfed infants, all of them presented higher counts of *Escherichia coli*, *Clostridium difficile*, *Bacillus fragilis-group*, and *Lactobacillus*. In the study performed by Crociani *et al*., (1994), the *Bifidobacterium* group show a low LBG fermentation capacity, being exclusively degraded by *B. dentium*. Concerning to its safety, Meunier *et al*., (2014) performed a systematic review about the addition of locust bean gum to infant foods. In this study, no negative effects have been attributed to their use at a "therapeutic" concentration, which has been defined as 0.5 g/100 mL. However, these authors recommend the use of these formulae under medical supervision, and only for the treatment of frequent regurgitation.

Regarding to resistant starches, different microbial groups are able to ferment these compounds as *Eschericia, Bacteroides, Bifidobacterium* or *Eubacterium*. Is for that reason that a potential for resistant starches to modify microbiota composition has been attributed (Bird *et a*l., 2000). However, the scientific evidences about their prebiotic potential are unclear, being mainly based on *in-vitro* and animal studies (Topping *et al*., 2003). Animal studies were respectively performed in piglets and mice by Brown *et al*., (1997) and Brown *et al*., (1998). In both studies, an increment in fecal counts of

Bifidobacterium and *Lactobacillus* were described. In a recent study performed by Tachon *et al*., (2013), 20-months mice were fed with a diet containing different concentration of resistant starch. Animals that received starch supplementation showed higher levels of *Bacteroidetes* and *Bifidobacterium*, *Akkermansia*, and *Allobaculum* species, than those who did not receive supplementation. Furthermore, this prebiotic effect depended on the starch concentration. Other studies have demonstrated that the bacterial fermentation of resistant starches result in SCFAs butyrate, propionate and acetate production in rats (Charrier *et al*., 2013; Kalmokoff *et al*., 2013).

According to the ESPGHAN Committee (Aggett *et al*, 2003), due to the lack of scientific consensus and professional guidelines, the establishment of quantitative and qualitative recommendations about the addition of non-digestible carbohydrates to infant foods, cannot be set. In this way, different research topics have been proposed, among them:

- Metabolic-effects and dose-response of individual components of non digestible carbohydrates in early life.
- Influence of non digestible carbohydrates on the early acquisition and modulation of colonic microbiota.
- Interaction of colonic microbiota with these compounds and its health consequences.

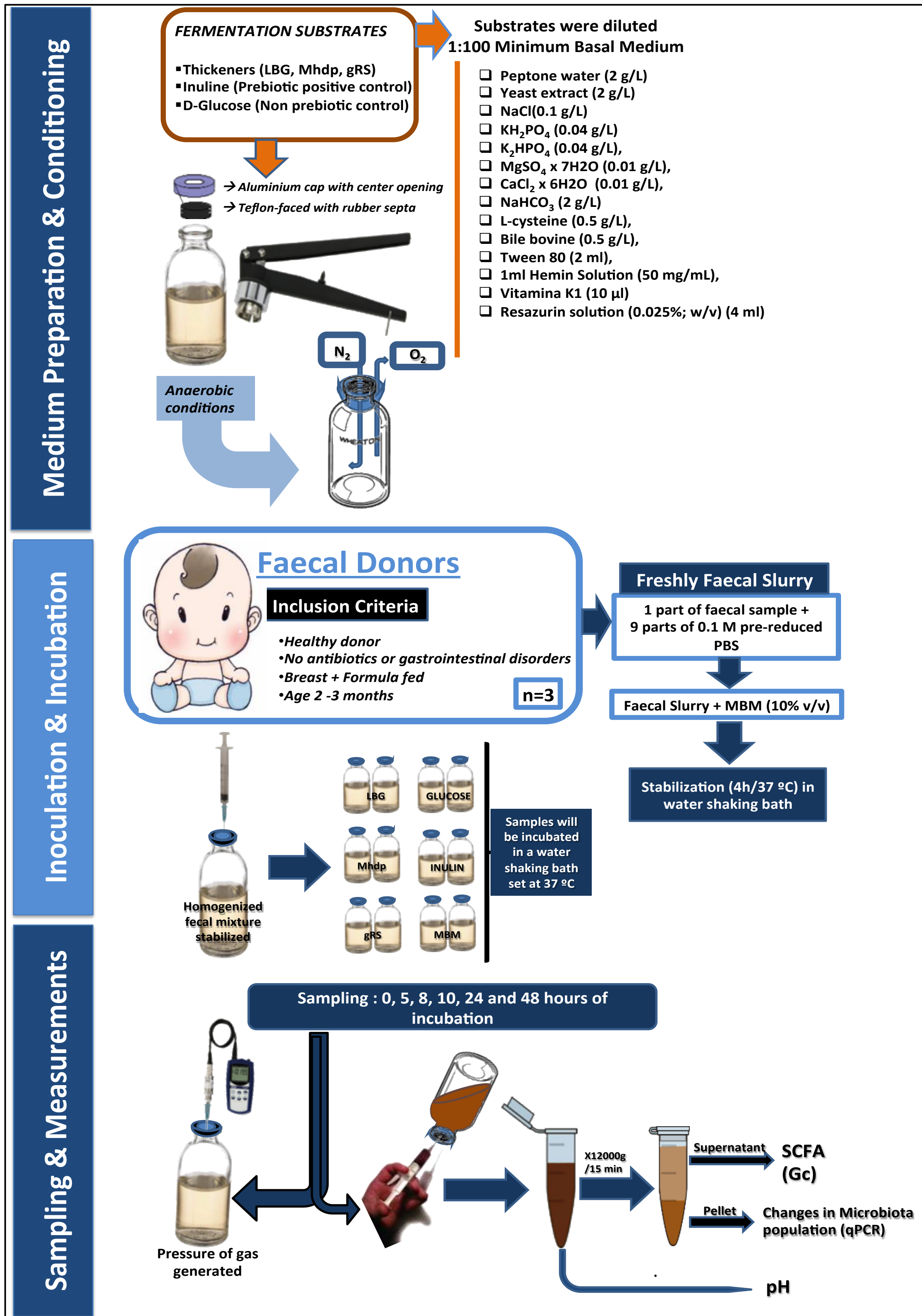
2.- AIM OF THE STUDY

As it has been explained in the previous section, gut microbiota is essential for an adequate development and health. It will be established during the first years of life, being influenced by different factors. Among these factors, infant nutrition is considered as one of the most relevant. In fact, the ability to modulate the microbiota has been attributed to food ingredients and components. Regarding infant nutrition, resistant starches and gums are nowadays being used as thickeners and texturizers. However, ESPAGHAN committee has stated the need to investigate some unclear aspect about their use and effect on infant microbiota and health.

According to this suggestion, the aim of this study was to determine the *in-vitro* gut fermentability and the effect on infant microbiota, of three thickening ingredients, locust bean gum (LBG), maize distarch phosphate (Mhdp) and pre-gelatinized rice starch (gRS), using a stirred and non-pH controlled faecal batch culture method.

3.- MATERIAL & METHODS

3.1.- Experimental design



3.2.- Faecal donors selection and samples collection.

After informed consent, three fecal donors were included in the study. The inclusion criteria considered, are listed below:

- To be in an age range between 2 and 3 months
- No use of antibiotics or any history of gastrointestinal disorder
- Mixed-fed by the combination of breast-milk and infant formula.

Mothers took freshly fecal samples, after receiving detailed instructions. Faeces were stored in a sterile sample container and immediately placed in an anaerobe jar. Stool samples were maintained under anaerobic atmosphere until their reception in the lab and processing. Stool samples from each donor were collected and processed independently.

3.3.- Minimum basal medium. Preparation and pre-reduction.

A minimum basal medium (MBM) for cultivation of infant feces was prepared according to the method described by Olano-Martín *et al*., (2002) and Al-Tamimi *et al*., (2006). MBM contained per liter: peptone water (2 g), yeast extract (2 g), NaCl (0.1 g), KH_2PO_4 (0.04 g), K_2HPO_4 (0.04g), $MgSO_4$ x $7H_2O$ (0.01 g), $CaCl_2$ x $6H_2O$ (0.01 g), $NaHCO_3$ (2 g), L-cysteine (0.5 g), bile bovine (0.5 g), Tween 80 (2 ml), and 4 mL of resazurin solution (0.025%; w/v). Resazurin was added as anaerobiosis/redox potential indicator (Rechner *et al*., 2004).

All the ingredients were dissolved in bi-distilled water and sterilized (121º C, 15 min). After sterilization, vitamin K1 (10 µl) and 1ml of hemin solution (50 mg/ml) were added. In order to avoid precipitation, hemin was dissolved in NaOH 0.01M.

After preparation, the medium was dispensed in 100 ml Wheaton serum bottles (Wheaton Science Products, Millville, NJ) previously autoclaved. Each bottle contained different fermentation substrates (1% w/v):

- Thickeners: Locust bean gum, LBG (Grinsted LBG 860, Danisco, Portugal), maize hidroxipropilated distarch phosphate, Mhdp (Multi-Thick®104, Abbott Nutrition, Spain) and pre-gelatinized rice starch, gRS (Beneo-Remy Industries, Belgium).
- As Prebiotic positive and negative control, inulin (Alfa AESAR, Ward Hill, MA) (Beards et al., 2010) and D-glucose (Sigma-Aldrich, St Louis, USA) (Olano-Martin *et al*., 2000; Beards *et al*., 2010), were respectively used.
- MBM without any substrate added, was considered as reference. For each ingredient and donor, a technical duplicate was done.

Serum bottles were sealed with 20MM silicone/teflon septums and aluminum seals, using a hand operated crimper W225303 (Wheaton Science Products, Millville, NJ). Each serum bottle was sparged with oxygen-free N_2 until achieving anaerobic conditions. With this purpose two sterile needles were placed in the septums for sparging the N_2 and displacing the O_2. Bottles were maintained at 37º C until use.

3.4.- Faeces processing and medium inoculation.

Freshly faeces were immediately processed after being received. 1 part of stool sample and 9 parts of 0.1 M pre-reduced Phosphate buffered saline (PBS) solution (catalog number P3813-10PAK, Sigma-Aldrich, St Louis, USA) were homogenized for 60s in a stomacher. A 10% v/v dilution was done by mixing the homogenized fecal solution with pre-reduced MBM. After dilution, samples were maintained for 4h in a water shaking bath set at 37ºC, until microbiota stabilization (Arboleya *et al*., 2013).

Once stabilized, a 10% v/v inoculum was done, by injecting the fecal dilution in the sterile Wheaton serum bottles previously prepared (see section 3.3). After the inoculation, bottles were maintained in a water shaking bath set at 37ºC. Sampling times were set at 0, 5, 8, 10, 24 and 48h (Arboleya *et al*., 2013; Beards *et al*., 2010; Manderson *et al*., 2005).

3.5.- Total gas production measurement.

The gas pressure generated by fecal inoculum in each bottle was measured at 5, 8, 10, 24 and 48 h. With this purpose, a sterile needle (25G x 2”) was inserted into the septum, previously attached to a pressure transmitter CPT6200 and connected to a digital hand held pressure indicator (WIKA Instruments, S.A.U., Barcelona, Spain) (Sarbini *et al*., 2011). Per each sampling time and bottle, gas pressure was measured twice, before (P_0) and after aliquots extraction (P_1). Total gas production (P_t) (kPa), was calculated as follows:

$$P_t = \sum_{n=0}^{48h} (P_{0_{t_n}} - P_{1_{t_{n-1}}})$$

Pressure at 0 h was considered as 0 kPa.

3.6.- Aliquots extraction and pH determination.

After the first gas measurement for each sampling time, 4 mL aliquots from each serum bottle were collected using sterile needles. From each aliquot, a volume of 1.5 ml was centrifuged (12000 g, 20 min). Cell free supernatants were used for short chain fatty acid (SCFA) analysis by gas chromatography, whereas resulting pellets were used for microbial population quantification by qPCR. The rest of each aliquot (2.5 mL) was used for pH measurement by a Crison MicropH 2001 pH-meter (Crison, Germany). Work-flow scheme has been presented in figure 3.3.

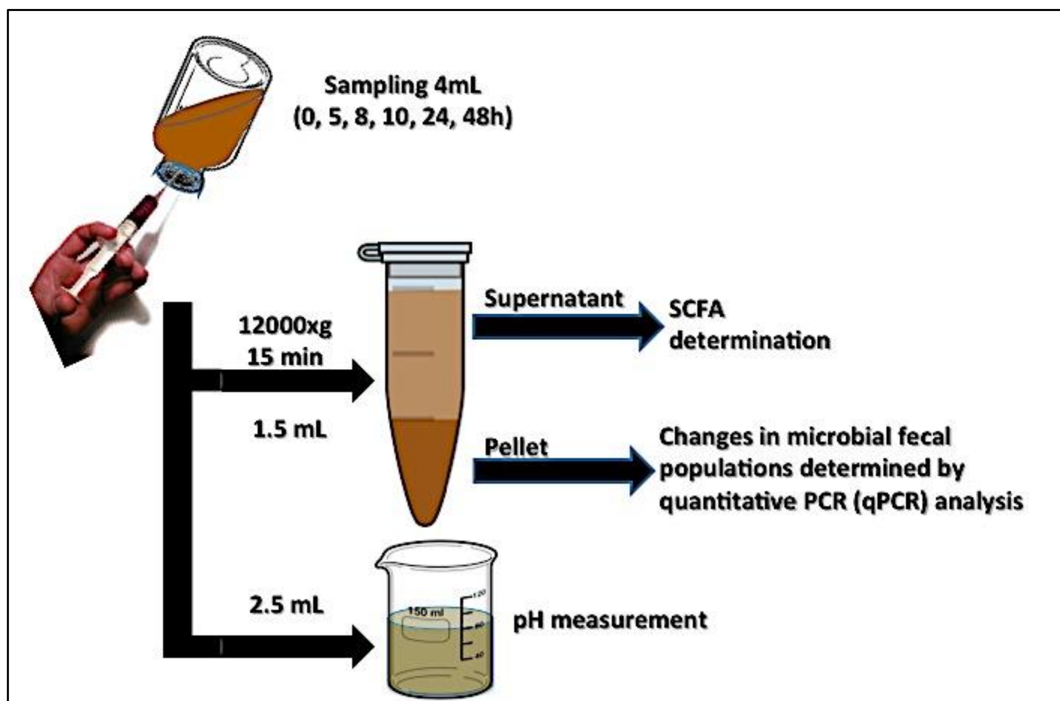


***Figure 3.3**.- Sampling work-flow scheme. Aliquots extraction and pH measurement.*

3.7.- SCFAs quantification.

Cell free supernatants were used for SCFAs quantification by gas chromatography. Before analysis, samples were conditioned as follow: 100 µL of cell free supernatant were added to 650 µL of a mixture of formic acid (20%), methanol and 2-ethyl butyric acid (internal standard, 2 mg/ml in methanol) at a ratio of 1:4.5:1. The mixtures were homogenized by vortexing and filtered through a 13 mm (diameter), 0.22 µm (pore size) PTFE filter (Salazar *et al.*, 2008). Samples were analyzed using a GC (Agilent 7890A) equipped with a flame ionization detector and a Nukol™ GC-column (30 m x 0,25 x 0,25 µm). The chromatographic conditions are represented in table 3.4. SCFAs peaks were integrated and quantified using Agilent Chem Station software.

Table 3.4.- *Chromatographic conditions*

Equipment Parameters	Conditions
Elution Method	Constant pressure injection
Gas flux	He_2 25 ml/min (carrier) Air 400 ml/min H_2 30 ml/min
Injection method	Splitless
Volume of injection	2 µl
Oven temperature	80ºC
Temperature ramp	80ºC for 5 min 5ºC/min until 185ºC
Injector temperature	220ºC
Detector temperature	220ºC
Injector Preassure	58.99 kPa

Each SCFAs concentration (mM) was calculated using linear regression (R ≥ 0.99) from a six points standard curve, subtracting their corresponding results at 0 h. Standard curves were obtained by serial dilutions of a volatile acid standard mix (Supelco, Bellefonte, PA, USA). Diluted concentrations are presented in table 3.5. Calibration curves for acetic, propionic and butyric acids can be observed in figure 3.4.

Table 3.5.- *Concentrations of volatile acid standard mix used in SCFAs standard curve*

	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6
Standard Mix	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

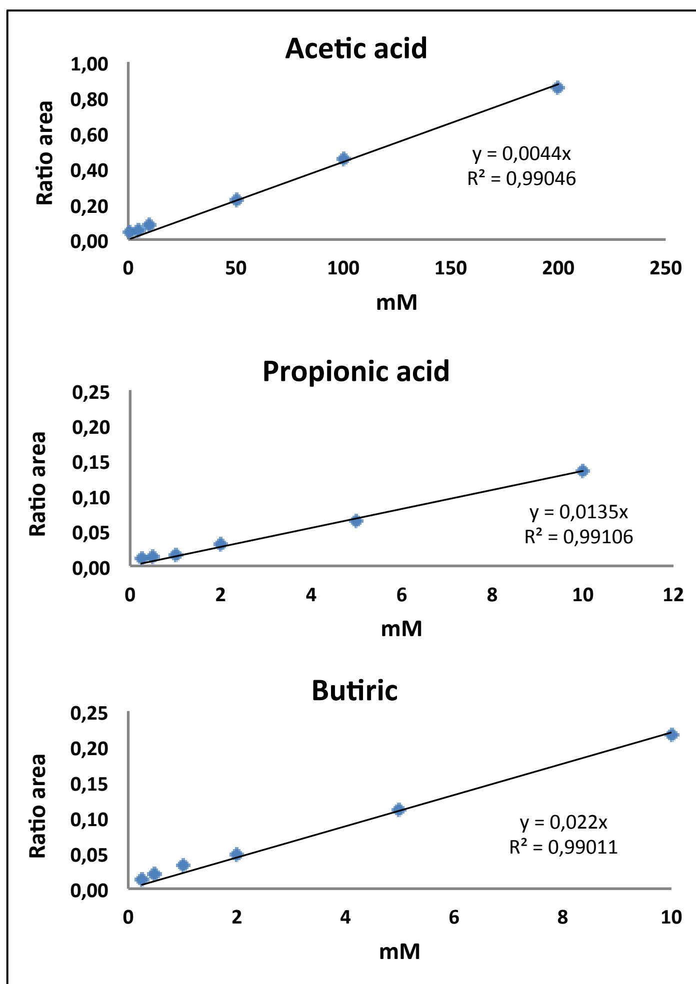


Figure 3.4.- *Acetic, propionic and butyric acid calibration curves.*

3.8.- Analysis of faecal microbial populations by qPCR

Pellets resulting from the previous step (see section 3.6.) were used for faecal microbial population quantification. Bacterial DNA from each sample was extracted using the QIAamp DNA Stool Minikit (Qiagen, Germany) according to the manufacturer's instructions. After extraction, DNA was stored at -80ºC until use.

3.8.1.- Primers and qPCR conditions

Primers against 16s ribosomal gen were used for faecal microbiota characterization (table 3.6). With this purpose, the following groups were included: *Atopobium* cluster (including *Atopobium* and *Collinsella*), *Bacteroides* (*Bacteriodes-Prevotella-Porphiromonas*), *Enterobacteriaceae*, *Enterococaceae*, *Lactobacillus*, *Bifidobacterium*, Clostridia IX (*Clostridium leptum - Faecalibacterium praustnitzii*) and Clostridia XIVa (*Clostridium Coccoides – Eubacterium rectale*). All the Primers were purchased from Sigma-Aldrich (Barcelona, Spain).

The qPCR protocol was performed in a 96-well CFX96 Real-Time PCR thermocycler and detection system (Bio-Rad, Madrid, Spain). The temperature cycling protocol has been detailed in figure 3.5., which included:

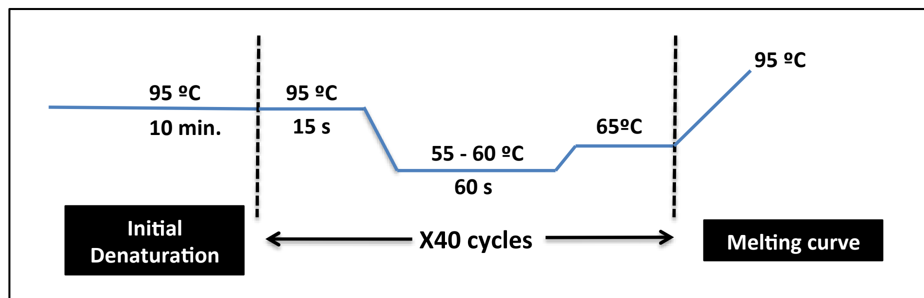
- an initial denaturation step at 95 °C for 10 min,
- followed by 40 cycles of denaturation at 95 °C for 15 s,
- annealing at 55º/60º depending on the group (see table 3.6) for 60 s,
- and extension at 72 °C
- After the completion of 40 cycles, a Melting-curve analysis was performed for one single amplification product verification.

Reactions were conducted in a volume of 25 µL per reaction, containing: 1 µL cDNA, 12.5 µL SensiMix™ SYBR No-ROX (1x) (Bioline, London, UK), 0.5 µL of both primer (0.2 mM), and 10.5 µL nuclease-free water (AppliChem, Darmstadt, Germany).

Table 3.6.- *Specific primers used in the study to target different bacterial groups*

Bacterial Group	Amplicon size (NCBI)	Primer Sequence (5' to 3')	Annealing Tº	Reference
***Atopobium* cluster**	**190 pb**	**F: GGGTTGAGAGACCGACC** **R: CGGRGCTTCTTCTGCAGG**	**55 ºC**	**Matsuki *et al.*, 2004**
Bacteroides-Prevotella	**108 pb**	**F: GAGAGGAAGGTCCCCCAC** **R: CGCKACTTGGCTGGTTCAG**	**60 ºC**	**Ramirez-Farias *et al.*, 2009**
Enterobacteriaceae	**428 pb**	**F: TGCCGTAACTTCGGGAGAAGGCA** **R: TCAAGGACCAGTGTTCAGTGTC**	**60 ºC**	**Matsuda *et al.*, 2007**
Enterococaceae	**115 pb**	**F: CCCATCAGAAGGGGATAACACTT** **R: ACCGCGGGTCCATCCATC**	**60 ºC**	**Matsuda *et al.*, 2007**
Lactobacillus	**331 pb**	**F: AGCAGTAGGGAATCTTCCA** **R: CATGGAGTTCCACTGTCCTC**	**60 ºC**	**Rinttila *et al.*, 2004**
Bifidobacterium	**236 pb**	**F: GATTCTGGCTCAGGATGAACGC** **R: CTGATAGGACGCGACCCCAT**	**60 ºC**	**Gueimonde *et al.*, 2004**
***Clostridia* IV**	**314 pb**	**F: TTAACACAATAAGTWATCCACCTGG** **R: ACCTTCCTCCGTTTTGTCAAC**	**60 ºC**	**Ramirez-Farias *et al.*, 2009**
***Clostridia* XIVa**	**429 pb**	**F: CGGTACCTGACTAAGAAGC** **R: AGTTTYATTCTTGCGAACG**	**55 ºC**	**Rinttila *et al.*, 2004**

Figure 3.5.- *qPCR. Temperature cycling protocol*



3.8.2.- Quantification of DNA concentration. Standard curves.

For each target group (table 3.6), pure bacterial strains were obtained from, the Spanish Type Culture Collection (CECT) or the German Collection of microorganism and cell cultures (DSMZ). Microorganisms were reconstituted and grown according to the provided instruction. Reference numbers are provided in table 3.7.

Table 3.7.- *Pure bacterial strain used for DNA quantification. Spanish Type Culture Collection (CECT) and from the German Collection of microorganism and cell cultures (DSMZ)*

Strain	Reference number (CECT/DSMZ)
Lactobacillus gasseri	DSMZ 20077
Bifidobacterium longum	CECT 4503
Clostridium coccoides	DSMZ 7935
Clostridium leptum	DSMZ 753
Bacteroides tetaiotaomicron	DSMZ 2079
Collinsella intestinalis	DSMZ13280
Enterococcus faecalis	DSMZ 2478

E. coli DNA was used for setting the *Enterobacteriaceae* standard curve. Pure strains were isolated from food samples using the Rapid *E. coli* medium (Bio-Rad Laboratories, Madrid, Spain), a specific chromogenic agar medium for its detection and isolation. The medium was incubated at 45°C for 24 h. Positive colonies were confirmed by the biochemical characterization mini-kit API 20E (Biomerieux, Madrid, España).

From each pure culture, DNA was extracted using the QIAamp DNA Stool Minikit. After extraction, DNA was amplified according to the previously described PCR protocol (see section 3.8.1.). After amplification, DNA was cleaned up using the QIAquick PCR Purification Kit (Qiagen, Germany), to remove any remaining enzyme and nucleotides after the amplification. Immediately after cleaning, DNA concentration and integrity was analysed. Concentration was measured at 260/280 nm ratio, using a spectrophotometer (NanoDrop-1000, Thermo Scientific, Villebonsur Yvette, France). DNA integrity was measured on the Agilent 2100 Bioanalyzer with the DNA 1000 Nano LabChip kit (Agilent, Diegem, Belgium). Samples were maintained at – 80 °C until use.

Bacterial DNA concentration per sample, expressed as logarithm of genome equivalent/mL, was calculated using linear regression (R ≥ 0.99) from a seven points (Ct values) standard curve. Curves were established from a 10-dilution (10^8 - 10^2 logarithm of genome equivalents/mL) of DNA extracted and purified from pure bacterial cultures. The theoretical genome equivalents were calculated assuming (Collado *et al.*, 2010; Morpeth *et al.*, 2014):

- Similarities between the amplicon size and the 16S ribosomal RNA gene copy number for each microbial group.
- The average weight of a single DNA base pair (bp) is 650 g/mol.

$$Genome\ equivalents = \frac{Amplicon\ size\ (bp)\ x\ 1\ mol}{(6.023\ x\ 10^{23}\ molecules)x\ (650\ {}^{g}/_{mol})}$$

For each target gene, Amplicon length/size (pb) was obtained combining the Bioanalyzer results, with the data provided by the Primer-Blast tool from the National Center for Biotechnology Information (NCBI). (Available: http://www.ncbi.nlm.nih.gov).

For each bacterial group included in the study, standard curves have been presented in figure 3.6.

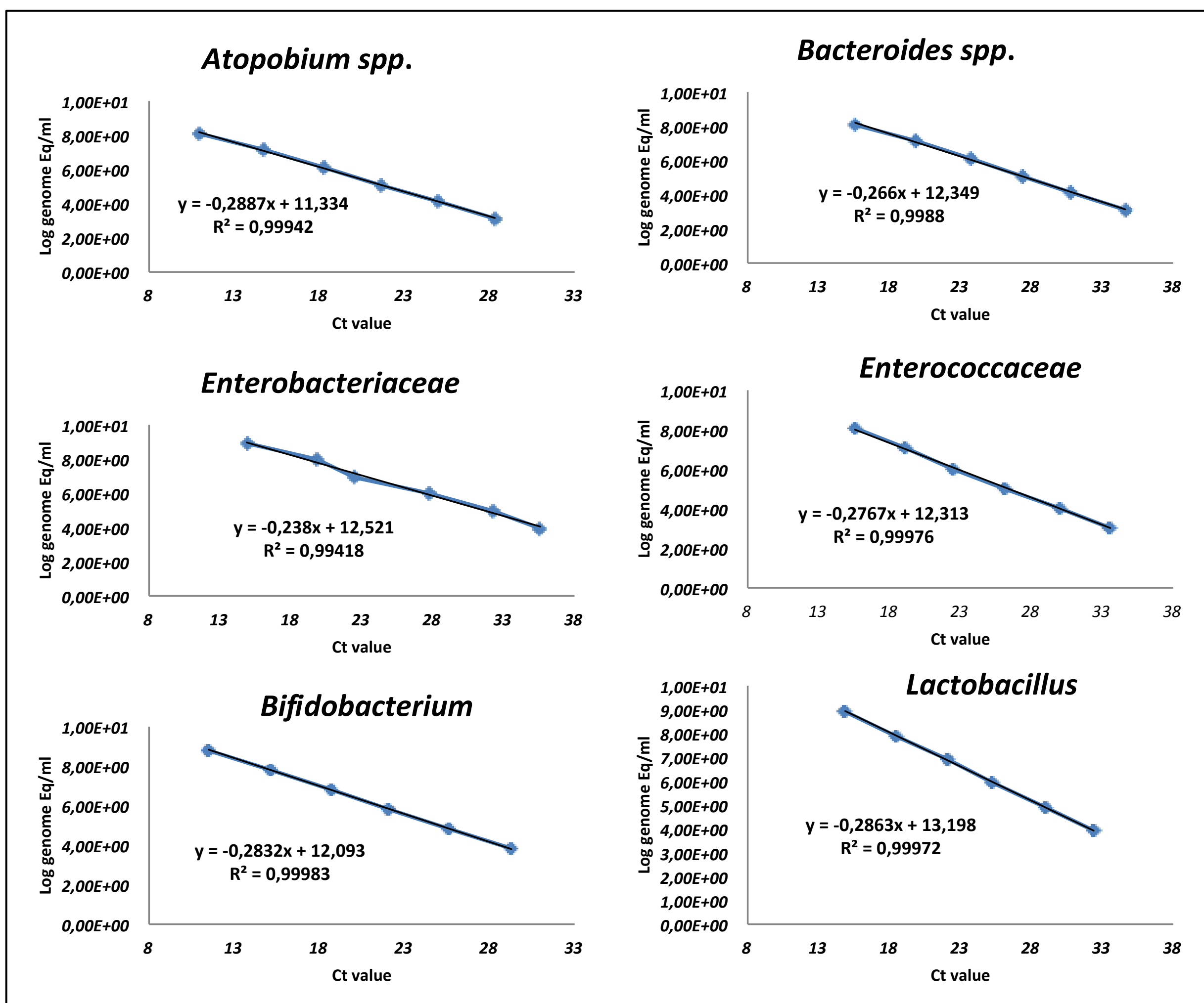


Figure 3.6.- *qPCR. Standard curves for bacterial DNA concentration quantification.*

Despite being included in the study, the quantification of *Clostridia IV* and *Clostridia XIVa*, are not shown as the culture of pure strains was not successful.

3.9.- Statistical analyses

Statistical analyses were performed using the IBM Statistical Package for the Social Sciences (IBM-SPSS) v.19.0 Inc., (Chicago, IL, USA). Data normality and homoscedasticity were confirmed by the Shapiro-Wilk/Kolmogorov-Smirnov test and Levene test, respectively, (significance level $p < 0.05$).

Per each ingredient and donor, differences in total gas production, pH, SCFAs quantification, and evolution of increments of bacterial genome equivalents logarithm per mL, were analyzed by ANOVA followed by Tuckey test for multiple comparisons ($p<0.05$). In order to analyse the relation between time of fermentation, and pH and increments of bacterial genome equivalents logarithm per mL, Pearson's correlation coefficients were determined ($p<0.05$).

In addition to each variable individual analysis, a Principal Component Analysis (PCA) followed by Varimax rotation, was performed as a means to provide an integrative overview of the effect of thickeners addition on the analysed variables (pH, SCFAs production and bacterial quantification).

4.- RESULTS & DISCUSSION

In order to characterize *in-vitro* gut fermentability and the effect on infant fecal microbiota, of locust bean gum (LBG), maize distarch phosphate (Mhdp) and pre-gelatinized rice starch (gRS), a stirred and non-pH controlled faecal batch culture method was designed. With this aim, gas production, pH evolution and faecal microbiota DNA were quantified at 0, 5, 8, 10, 24 and 48h of incubation.

4.1.- Total gas production.

Total gas production summations (Kpa; mean ± S.D.), per ingredient and donor, have been presented in figure 3.7. As can be seen, all the ingredients analysed showed a similar behaviour within each donor. In this regard, Mhdp, gRS and D-glucose (prebiotic negative control) resulted in a significant ($p<0.05$) higher total gas production than the one obtained for LBG and inulin, with an approximate increment of 25 – 35 KPa for the former respect to the latter. When total gas production for Mhdp, gRS and D-glucose were analysed, no significant differences were found but for donor 3, in which the addition of Mhdp resulted in a significantly lower total gas production (67.4±8.2 KPa) than the one obtained with D-glucose (87.7±2.9 KPa). In the same way, no significant differences were described when LBG and inulin were compared within the same donor. The lowest total gas production values (1.0±1.0; 7.7±4.0; and 8.7±1.8 KPa for donor 1, 2 and 3, respectively), were obtained from the reference (MBM without any fermentation substrate added) in comparison with the rest of ingredients. For donor 2 and 3, no significant differences were found between LBG (26.2±4.9 and 17.9±3.0 KPa) and MBM.

In general, when total gas production per ingredient was compared between donors, donor 1 resulted in significantly higher values than donor 2 or 3 for all the ingredients but for the reference MBM, which was significantly lower (1.0±1.0 KPa) than donor 2 and 3 (7.7±4.0, and 8.7±1.8 KPa respectively). In the case of gRS and D-glucose, no significant differences were found between donor 1 and 3.

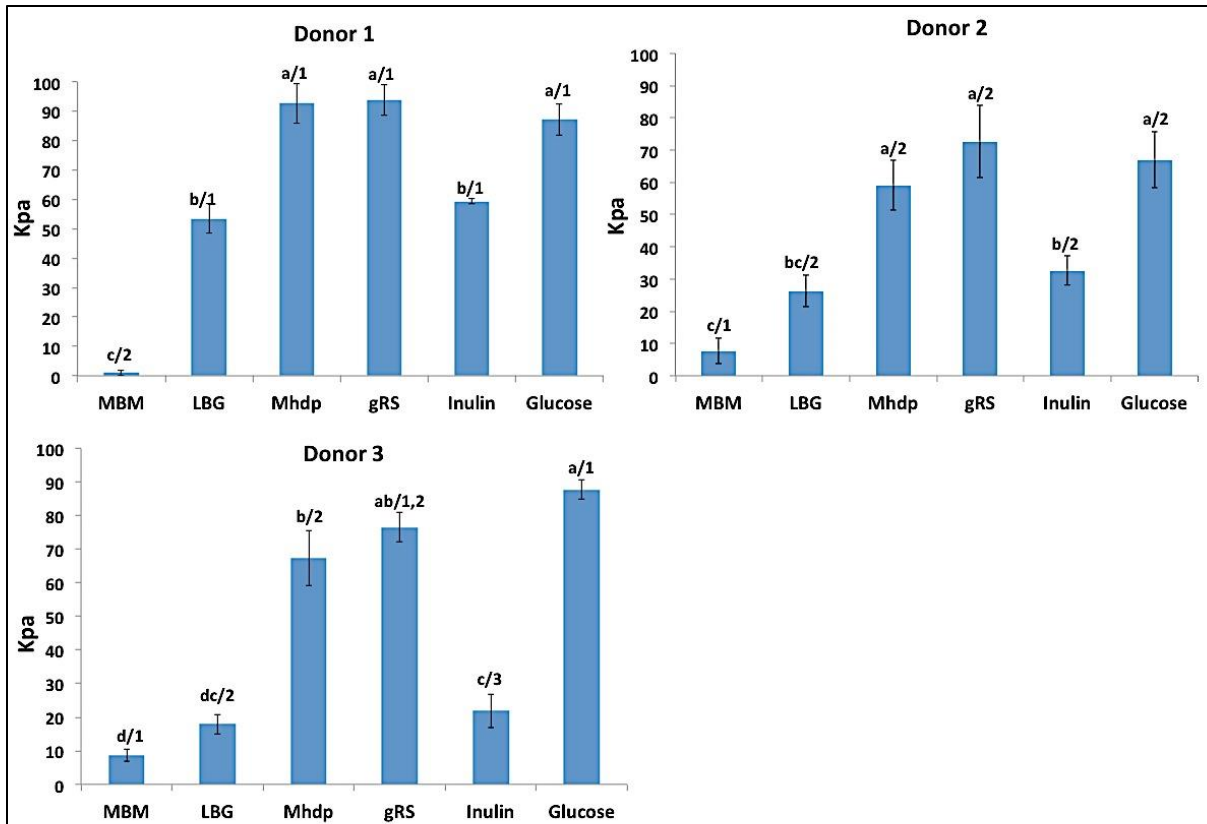


Figure 3.7.- *Total gas production (KPa) per ingredient and donor. Results have been presented as mean ± S.D. Different letters (a,b,c) denote significant differences between different ingredients within the same donor. For the same ingredient, different numbers (1,2,3) denote significant differences between different donors. Significance level p<0.05.*

4.2.- pH evolution.

In figure 3.8, pH evolution (from 0 to 48 h) for each ingredient and donor can be seen. Per each aliquot, three pH measurements were done, presenting values as mean±SD. For each donor and ingredient, Pearson's correlation coefficient for relation between time and pH are presented in table 3.8. The fixed level of significance was $p<0.05$

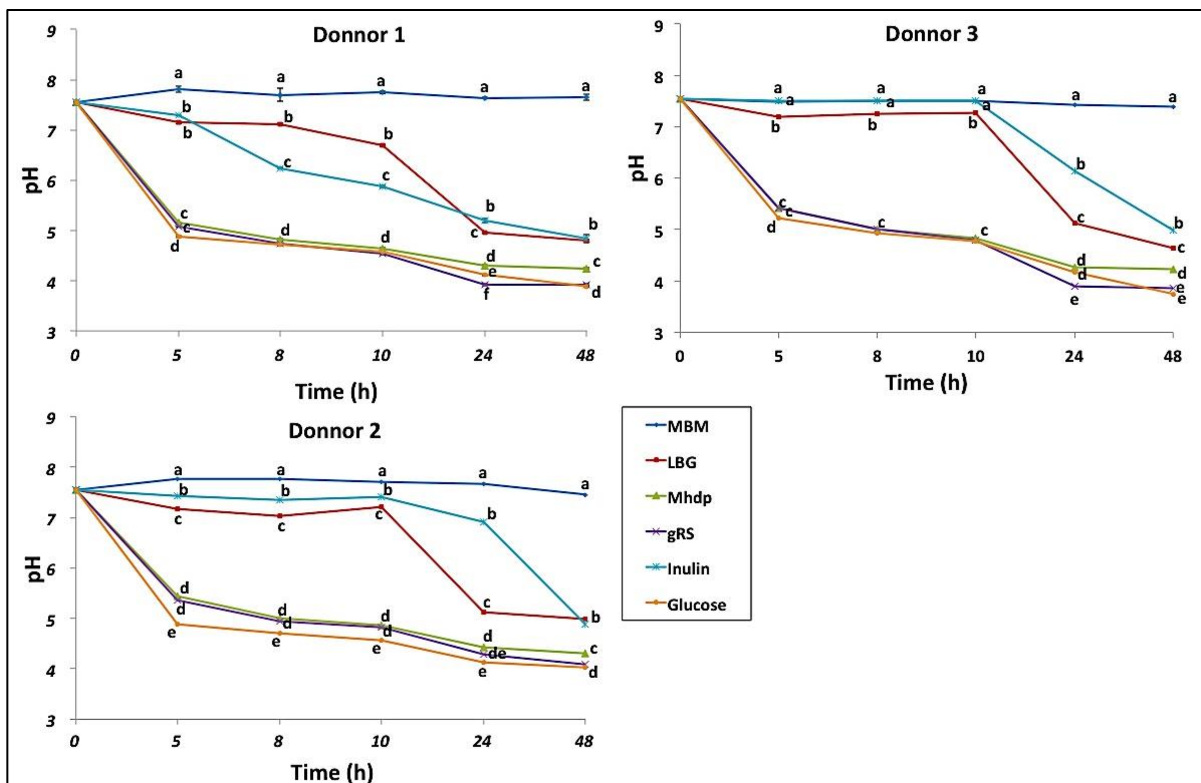


***Figure 3.8**.- pH evolution per ingredient and donor. Results have been presented as mean ± S.D. Different letters (a,b,c) denote significant differences between different ingredients within the same sampling time and donor. Significance level p<0.05.*

According to these results, when no ingredients were added (MBM), pH maintained stable across the experiment, with values ranging from 7.81±0.06 to 7.55±0.01 for donor 1, from 7.71±0.02 to 7.46±0.01 for donor 2, and from 7.55±0.03 to 7.39±0.04 for donor 3. On the contrary, when the different fermentation substrates were evaluated, a pH decrease was detected. As can be seen in table 3.8, the addition of all the ingredients resulted in a significant negative correlation ($p<0.01$) between time and pH. On the contrary, no significant correlations were detected between pH and time of fermentation when no ingredients were added (MBM).

Table 3.8.- Pearson's correlation coefficient for relation between time and pH per faecal donor and ingredient.

	Pearson's correlation coefficients		
Ingredients	**Donor 1**	**Donor 2**	**Donor 3**
MBM	-0.500	-0.245	-0.353
LBG	-0.928*	-0.869*	-0.880*
Mhdp	-0.984*	-0.985*	-0.977*
gRS	-0.972*	-0.986*	-0.967*
inulin	-0.982*	-0.802*	-0.889*
D-glucose	-0.979*	-0.977*	-0.979*

*$p<0.05$

Despite significant correlations between time and pH, pH decrease was more or less pronounced depending on the ingredient added. In the case of Mhdp, gRS and D-glucose, pH rapidly decreased to values ≈5 after 5h of incubation, and then continued to decrease though less sharply, reaching a minimum at 24-48h. This minimum ranged between 3.75±0.03 and 4.02±0.05 for D-glucose, 4.24±0.02 and 4.30±0.03 for Mhdp, and 3.86±0.01 and 4.08±0.04 for gRS. For the three donors, at 5h of fermentation the pH

range registered for D-glucose (4.88±0.02 – 4.92±0.03) was significantly lower ($p<0.001$) than for Mhdp (5.44±0.05 – 5.15±0.03) and gRS (5.41±0.01 – 5.47±0.02). However, at the end of the experiment (48h), the addition of gRS and D-glucosa resulted in a significantly lower pH range (4.08±0.02 – 3.75±0.06) than the one obtained with the addition of Mhdp (4.30±0.04 – 4.23±0.02).

Regarding LBG and inulin, both ingredients had a similar effect on pH, being different from that observed for Mhdp, gRS and D-glucose. At this respect, when LBG was added as ingredient, pH values maintained within the range 7.55±0.05 - 6.69±0.02 until 10h of fermentation. After this point, pH drastically decreased to the range 5.12±0.06 – 4.64±0.02 at 24 – 48h of fermentation. Regarding inulin, for donor 2 and 3, pH maintained in the range 7.55±0.05 – 7.32±0.06 until 10h of fermentation, decreasing progressively to a minimum ranged between 4.98±0.01 and 4.89±0.04 at 48h. For donor 2 and 3, pH values were significantly higher ($p<0.01$) for inulin than for LBG. Nevertheless, at 48h of fermentation, no significant differences were found between inulin and LBG for donor 2. When the effect of inulin in donor 1 was studied, a progressive pH decrease was observed, dropping from 7.55±0.04 at 0h of fermentation, to 4.84±0.08 at 48h. For times 8h and 10h, pH values for inulin and donor 1, were significantly lower than those reported for LBG. In the same way as described for donor 2, no significant differences were found between inulin and LBG at 48h.

For donors 1, 2 and 3, as well as for sampling time 5, 8, 10, 24 and 48h, pH values obtained for Mhdp, gRS and D-glucose were significantly lower ($p<0.01$) than for LBG and inulin.

4.3.- SCFAs quantification.

SCFAs results are shown in table 3.9 per each ingredient and fermentation time, (donor 1), table 3.10 (Donor 2) and table 3.11 (donor 3). These are expressed as molar proportions (%) with respect to the total molar concentration (mM), which was calculated as the sum of previously calculated acetate, propionate and butyrate concentrations (mM) for the same time and ingredient. In addition, the ratio acetate:propionate (A:P) has been included.

When SCFAs total molar concentration was analyzed, the non-addition of fermentable ingredients (MBM), resulted in a stable concentration evolution between the different times considered in the study (5, 8, 10, 24 and 48h). In this regard, total molar concentration values ranged from 13.83±3.72 to 20.93±5.60 mM for donor 1, from 13.55±1.07 to 17.06±0.25 mM for donor 2, and from 6.54±3.72 to 12.34±3.27 mM for donor 3. On the contrary, when MBM results were compared with those obtained with the addition of different fermentable substrates (LBG, Mhdp, gRS, inulin, and D-glucose), a time-dependent effect was observed. In this way, at 5 and 8h, no significant differences ($p<0.05$) were found between the SCFAs total molar concentration values obtained with MBM and the fermentable ingredients, for donor 2 and 3. In the case of donor 1, the initial molar concentrations obtained with Mhdp (22.18±0.13 mM), gRS (25.78±1.40 mM) and D-glucose (21.87±0.29 mM) were significantly higher than the ones observed for inulin (13.00±0.98 mM), LBG (13.07±1.62 mM) and MBM (13.83±3.72 mM), but at 8h only the addition of gRS (24.37±2.66 mM) resulted in significant differences with regard to MBM (15.97±1.14 mM). For Donor 3, at 10h, the highest molar concentration was obtained for Mhdp (12.04±0.90 mM), gRS (12.73±0.23 mM)

and D-glucose (12.59±1.25 mM), in comparison with the significantly lower values obtained for MBM (8.12±1.28 mM), LBG (8.00±0.74 mM) and inulin (9.12±0.55 mM). For donor 1 and 2, the significantly lowest molar concentration values were obtained for LBG (12.65±0.72 mM) and inulin (9.25±0.86 mM) respectively. However, for the three donors, after 24 and 48h of fermentation, the total molar concentration obtained for LBG and inulin were approximately 4-6 times higher than the initial concentration (5h). With regard to Mhdp, gRS and D-glucose, the molar concentrations obtained after 24-48h of fermentation, were approximately 1 or 3 times higher than the initial concentration (5h). In this way, after 24-48h of fermentation, SCFAs total molar concentrations for LBG and inulin were significantly higher ($p<0.05$) than for MBM, Mhdp, gRS and D-glucose.

In relation to acetate, propionate and butyrate, acetate was the most relevant contributor to the previously described SCFAs total molar concentration. Nevertheless, this contribution was different depending on the ingredient evaluated and time of fermentation. In this regard, the addition of Mhdp, gRS and D-glucose resulted in a stable acetate molar proportion during the experiment, maintained within a range from about 85% to about 97%. For these ingredients, propionate molar proportions ranged from about 5% to about 13%. In contrast with the previous ingredients, the addition of LBG and inulin resulted in a time-dependent significant decrease of acetate molar proportion against a significant increase of propionate. In this way, A:P ratio decreased from values about 10 at 5 h, to values about 2 at 48 h. This significant variation of acetate and propionate described for LBG and inulin, was more pronounced from 10-24h of fermentation, which coincides with the previously reported significant increment of SCFAs total molar concentration.

__Table 3.9__.- Short chain fatty acids (acetate, propionate and butyrate) quantification at 5, 8, 10, 24 and 48h, in faecal donor 1. Different letters (a,b,c,d) denote significant differences (p<0.05) between different times (h) within the same ingredient and measurement (Molar proportions, ratio A:P, and total molar concentration). Different numbers (1,2,3) denote significant differences (p<0.05) in total molar concentration, between different ingredients, within the same time.

		Donor 1														
		Time (h)														
		5			8			10			24			48		
Ingredient	**Molar Proportions (%)**	Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD	
MBM	Acetate	90,11	± 0,77	a	89,39	± 1,35	a	91,41	± 3,26	a	92,75	± 3,96	a	93,16	± 3,70	a
	Propionate	8,53	± 1,20	a	9,97	± 1,17	a	7,46	± 3,69	a	6,09	± 3,62	a	4,48	± 1,61	a
	Butyrate	1,37	± 0,43	a	0,66	± 0,18	a	1,13	± 0,43	a	1,16	± 0,34	a	2,36	± 2,09	a
	Ratio A:P	10,68	± 1,59	a	9,04	± 0,30	a	14,08	± 1,20	a	18,73	± 1,44	a	22,39	± 0,90	a
	Total Molar Concentration (mM)	13,83	± 3,72	a/1	15,97	± 1,14	a/23	19,48	± 2,26	a/2	17,72	± 2,52	a/3	20,93	± 5,60	a/2
LBG	**Molar Proportions (%)**															
	Acetate	88,10	± 0,05	a	87,11	± 2,32	a	74,55	± 1,61	b	67,35	± 1,08	c	67,90	± 0,55	c
	Propionate	11,04	± 0,19	c	11,95	± 2,14	c	25,12	± 1,67	b	32,44	± 1,06	a	31,86	± 0,52	a
	Butyrate	0,87	± 0,24	ab	0,94	± 0,18	a	0,33	± 0,06	bc	0,21	± 0,02	c	0,24	± 0,04	c
	Ratio A:P	7,98	± 0,13	a	7,42	± 1,53	a	2,98	± 0,26	b	2,08	± 0,10	b	2,13	± 0,05	b
	Total Molar Concentration (mM)	13,07	± 1,62	b/2	12,65	± 0,72	b/3	25,06	± 1,94	b/12	55,94	± 1,85	a/1	61,28	± 2,27	a/1
Mhdp	**Molar Proportions (%)**															
	Acetate	92,59	± 1,49	a	93,70	± 0,15	a	94,66	± 0,83	a	92,92	± 0,77	a	91,96	± 1,27	a
	Propionate	6,27	± 0,86	a	5,96	± 0,08	a	5,01	± 0,76	a	6,86	± 0,74	a	7,86	± 1,26	a
	Butyrate	1,14	± 0,62	a	0,34	± 0,07	a	0,34	± 0,07	a	0,22	± 0,04	a	0,17	± 0,01	a
	Ratio A:P	14,93	± 2,29	a	15,72	± 0,23	a	19,15	± 3,08	a	13,63	± 1,58	a	11,86	± 2,06	a
	Total Molar Concentration (mM)	22,18	± 0,13	c/1	23,25	± 2,42	c/12	30,39	± 1,95	b/12	39,52	± 1,64	a/2	40,87	± 1,06	a/12
gRS	**Molar Proportions (%)**															
	Acetate	94,51	± 0,35	b	94,78	± 0,34	ab	96,63	± 0,89	a	96,53	± 0,22	ab	96,41	± 0,54	ab
	Propionate	4,77	± 0,29	a	4,93	± 0,32	a	3,17	± 0,88	a	3,35	± 0,24	a	3,45	± 0,52	a
	Butyrate	0,73	± 0,07	a	0,29	± 0,02	b	0,20	± 0,01	bc	0,12	± 0,02	bc	0,14	± 0,02	bc
	Ratio A:P	19,87	± 1,27	a	19,25	± 1,33	a	31,70	± 9,03	a	28,88	± 2,11	a	28,29	± 4,44	a
	Total Molar Concentration (mM)	25,78	± 1,40	b/1	24,37	± 2,66	b/1	34,49	± 5,69	ab/1	41,63	± 1,67	a/2	44,29	± 5,01	a/12
Inulin	**Molar Proportions (%)**															
	Acetate	87,40	± 0,23	a	85,23	± 0,68	a	78,80	± 2,25	b	68,17	± 1,92	c	63,87	± 0,24	c
	Propionate	11,87	± 0,34	c	14,30	± 0,80	c	20,95	± 2,19	b	31,21	± 2,16	a	35,65	± 0,22	a
	Butyrate	0,74	± 0,57	a	0,48	± 0,12	a	0,25	± 0,05	a	0,62	± 0,24	a	0,48	± 0,02	a
	Ratio A:P	7,37	± 0,19	a	5,97	± 0,38	b	3,79	± 0,50	c	2,19	± 0,21	d	1,79	± 0,02	d
	Total Molar Concentration (mM)	13,00	± 0,98	b/2	18,89	± 1,80	b/123	23,32	± 0,11	b/12	50,57	± 3,03	a/1	51,98	± 7,59	a/1
D-glucose	**Molar Proportions (%)**															
	Acetate	95,30	± 0,55	a	95,13	± 0,38	a	95,85	± 0,38	a	94,98	± 0,44	a	93,74	± 1,57	a
	Propionate	4,05	± 0,46	a	4,43	± 0,30	a	3,84	± 0,30	a	4,77	± 0,43	a	4,75	± 0,92	a
	Butyrate	0,64	± 0,09	ab	0,44	± 0,08	ab	0,32	± 0,08	ab	0,24	± 0,01	b	1,51	± 0,65	a
	Ratio A:P	23,66	± 2,80	a	21,51	± 1,54	a	25,46	± 1,54	a	19,98	± 1,88	a	20,14	± 4,22	a
	Total Molar Concentration (mM)	21,87	± 0,29	c/1	22,22	± 1,98	c/12	27,41	± 1,25	bc/12	38,80	± 2,00	ab/2	41,76	± 6,35	a/12

Ingredient	Molar Proportions (%)	5 Mean	SD		8 Mean	SD		10 Mean	SD		24 Mean	SD		48 Mean	SD	
Donor 2																
		Time (h)														
MBM	Acetate	93,41	± 0,17	a	89,30	± 0,67	a	87,13	± 5,77	a	91,40	± 5,78	a	89,50	± 2,51	a
	Propionate	5,15	± 0,26	a	8,52	± 0,59	a	9,67	± 4,21	a	7,05	± 6,27	a	8,40	± 4,03	a
	Butyrate	1,44	± 0,10	a	2,17	± 0,08	a	3,20	± 1,56	a	1,55	± 0,48	a	2,10	± 1,52	a
	Ratio A:P	18,15	± 0,95	a	10,51	± 0,80	a	10,10	± 1,16	a	22,03	± 2,24	a	12,12	± 1,11	a
	Total Molar Concentration (mM)	16,98	± 3,72	a/1	13,55	± 1,07	a/2	16,42	± 0,91	a/2	15,85	± 2,52	a/3	17,06	± 0,25	a/5
LBG	**Molar Proportions (%)**															
	Acetate	91,58	± 0,31	a	84,15	± 0,97	b	75,13	± 3,58	c	76,42	± 1,61	c	70,86	± 0,75	c
	Propionate	6,86	± 0,47	b	14,43	± 1,10	b	23,44	± 3,71	a	22,89	± 1,62	a	28,33	± 0,93	a
	Butyrate	1,57	± 0,16	a	1,42	± 0,13	a	1,44	± 0,13	a	0,69	± 0,01	b	0,81	± 0,18	b
	Ratio A:P	13,39	± 0,97	a	5,85	± 0,51	b	3,26	± 0,67	c	3,35	± 0,31	c	2,50	± 0,11	c
	Total Molar Concentration (mM)	17,96	± 3,56	c/1	15,39	± 0,24	c/12	14,45	± 1,20	c/2	36,11	± 1,85	b/1	48,82	± 2,27	a/1
Mhdp	**Molar Proportions (%)**															
	Acetate	91,22	± 0,03	a	87,40	± 0,88	bc	91,32	± 0,84	a	87,01	± 0,51	c	89,49	± 0,34	ab
	Propionate	6,71	± 0,08	c	10,99	± 0,53	ab	7,55	± 0,61	c	12,45	± 0,57	a	10,20	± 0,35	b
	Butyrate	2,07	± 0,05	a	1,61	± 0,35	ab	1,14	± 0,23	bc	0,54	± 0,06	cd	0,32	± 0,02	d
	Ratio A:P	13,59	± 0,16	a	7,97	± 0,46	a	12,14	± 0,21	b	7,00	± 0,36	b	8,78	± 0,34	b
	Total Molar Concentration (mM)	21,84	± 2,24	a/1	14,40	± 0,27	b/12	14,47	± 0,53	b/2	22,38	± 0,71	a/2	24,95	± 1,53	a/4
gRS	**Molar Proportions (%)**															
	Acetate	92,39	± 1,51	a	86,43	± 1,13	ab	86,35	± 4,65	a	85,25	± 0,01	ab	88,92	± 0,36	ab
	Propionate	6,11	± 1,20	a	12,45	± 0,78	a	12,79	± 4,73	a	14,31	± 0,01	a	10,80	± 0,38	a
	Butyrate	1,50	± 0,30	a	1,12	± 0,36	b	0,88	± 0,09	bc	0,44	± 0,01	c	0,28	± 0,01	bc
	Ratio A:P	15,45	± 3,29	a	6,96	± 0,53	a	7,32	± 0,26	a	5,96	± 0,00	a	8,24	± 0,32	a
	Total Molar Concentration (mM)	18,20	± 3,97	b/1	13,94	± 1,49	b/2	14,73	± 0,17	ab/2	20,97	± 0,43	a/23	31,81	± 2,10	a/3
Inulin	**Molar Proportions (%)**															
	Acetate	90,86	± 0,44	a	88,62	± 0,25	a	69,44	± 0,63	b	70,79	± 0,74	b	65,19	± 1,10	c
	Propionate	7,62	± 0,19	c	9,98	± 0,04	c	29,50	± 0,60	b	28,64	± 0,80	b	34,55	± 1,04	a
	Butyrate	1,52	± 0,25	a	1,41	± 0,21	a	1,06	± 0,03	ab	0,57	± 0,06	bc	0,26	± 0,06	c
	Ratio A:P	11,93	± 0,35	a	8,88	± 0,06	b	2,35	± 0,07	c	2,47	± 0,10	c	1,89	± 0,09	c
	Total Molar Concentration (mM)	9,67	± 0,08	c/1	9,25	± 0,86	c/3	10,79	± 0,03	c/3	33,93	± 1,22	b/1	43,26	± 1,11	a/12
D-glucose	**Molar Proportions (%)**															
	Acetate	94,14	± 2,61	a	96,11	± 0,27	a	93,61	± 0,27	a	95,79	± 0,45	a	95,11	± 1,00	a
	Propionate	4,97	± 2,44	a	3,41	± 0,29	a	6,16	± 0,29	a	3,94	± 0,42	a	3,98	± 0,65	a
	Butyrate	0,89	± 0,17	a	0,48	± 0,01	a	0,23	± 0,01	a	0,27	± 0,03	a	0,91	± 0,35	a
	Ratio A:P	21,66	± 1,69	a	28,25	± 3,11	a	16,17	± 3,11	a	24,46	± 3,11	a	24,21	± 3,95	a
	Total Molar Concentration (mM)	13,32	± 3,97	b/1	17,98	± 1,23	b/1	19,96	± 1,25	b/1	21,88	± 0,34	b/2	36,49	± 2,07	a/23

***Table 3.10**.- Short chain fatty acids (acetate, propionate and butyrate) quantification at 5, 8, 10, 24 and 48h, in faecal donor 2. Different letters (a,b,c,d) denote significant differences (p<0.05) between different times (h) within the same ingredient and measurement (Molar proportions, ratio A:P, and total molar concentration). Different numbers (1,2,3) denote significant differences (p<0.05) in total molar concentration, between different ingredients, within the same time.*

		Donor 3									
		Time (h)									
		5		8		10		24		48	
Ingredient	**Molar Proportions (%)**	Mean ± SD		Mean ± SD		Mean ± SD		Mean ± SD		Mean ± SD	
MBM	Acetate	90,79 ± 0,55	a	87,97 ± 4,73	a	88,08 ± 6,64	a	89,74 ± 1,15	a	85,22 ± 6,10	a
	Propionate	8,23 ± 0,36	a	9,37 ± 2,90	a	6,34 ± 6,58	a	7,95 ± 1,16	a	14,16 ± 6,48	a
	Butyrate	0,98 ± 0,18	a	2,66 ± 1,83	a	0,59 ± 0,06	a	2,31 ± 0,00	a	0,62 ± 0,38	a
	Ratio A:P	11,05 ± 0,56	a	9,95 ± 3,59	a	13,90 ± 1,51	a	11,42 ± 0,35	a	6,83 ± 3,55	a
	Total Molar Concentration (mM)	6,54 ± 3,72	a/12	10,28 ± 0,28	a/123	8,12 ± 1,28	a/3	6,68 ± 2,52	a/3	12,34 ± 3,27	a/2
LBG	**Molar Proportions (%)**										
	Acetate	91,88 ± 0,75	a	89,47 ± 0,22	a	72,97 ± 0,71	b	76,85 ± 3,37	b	74,42 ± 0,13	b
	Propionate	7,22 ± 0,68	b	9,72 ± 0,40	b	26,52 ± 0,65	a	22,43 ± 3,48	a	25,22 ± 0,11	a
	Butyrate	0,90 ± 0,07	a	0,82 ± 0,18	a	0,51 ± 0,06	ab	0,72 ± 0,11	ab	0,37 ± 0,02	b
	Ratio A:P	12,79 ± 1,32	a	9,22 ± 0,40	b	2,75 ± 0,09	c	3,48 ± 0,69	c	2,95 ± 0,02	c
	Total Molar Concentration (mM)	7,23 ± 0,77	c/12	9,05 ± 1,92	c/23	8,00 ± 0,74	c/3	36,17 ± 1,85	b/1	53,52 ± 2,27	a/1
Mhdp	**Molar Proportions (%)**										
	Acetate	94,90 ± 1,00	a	94,67 ± 0,72	a	94,59 ± 0,44	a	95,13 ± 0,54	a	95,25 ± 1,36	a
	Propionate	4,31 ± 0,80	a	4,69 ± 0,73	a	4,78 ± 0,36	a	4,49 ± 0,52	a	4,51 ± 1,16	a
	Butyrate	0,78 ± 0,20	a	0,63 ± 0,01	ab	0,64 ± 0,08	ab	0,38 ± 0,02	ab	0,24 ± 0,20	b
	Ratio A:P	22,41 ± 4,39	a	20,43 ± 3,35	a	19,87 ± 0,32	a	21,35 ± 2,61	a	21,87 ± 5,91	a
	Total Molar Concentration (mM)	8,52 ± 0,25	b/12	12,03 ± 0,60	b/12	12,04 ± 0,90	b/12	26,50 ± 2,85	a/2	24,40 ± 4,30	a/2
gRS	**Molar Proportions (%)**										
	Acetate	95,65 ± 0,48	b	95,42 ± 0,04	b	96,28 ± 0,26	b	97,44 ± 0,03	a	97,76 ± 0,20	a
	Propionate	3,74 ± 0,32	a	4,11 ± 0,10	a	3,40 ± 0,17	a	2,36 ± 0,04	b	2,10 ± 0,17	b
	Butyrate	0,61 ± 0,16	a	0,46 ± 0,05	ab	0,33 ± 0,09	ab	0,20 ± 0,02	b	0,14 ± 0,04	b
	Ratio A:P	25,68 ± 2,31	b	23,21 ± 0,56	b	28,35 ± 0,25	b	41,31 ± 0,76	a	46,71 ± 3,78	a
	Total Molar Concentration (mM)	8,69 ± 0,88	b/12	12,45 ± 1,10	ab/12	12,73 ± 0,23	ab/1	21,74 ± 2,36	ab/2	24,65 ± 7,52	a/2
Inulin	**Molar Proportions (%)**										
	Acetate	92,55 ± 0,45	a	92,11 ± 1,02	ab	76,32 ± 5,56	c	80,11 ± 1,37	bc	80,86 ± 3,73	bc
	Propionate	6,65 ± 0,41	c	7,31 ± 0,91	bc	23,34 ± 5,52	a	19,51 ± 1,46	ab	18,85 ± 3,69	ab
	Butyrate	0,80 ± 0,05	a	0,58 ± 0,11	ab	0,34 ± 0,05	bc	0,38 ± 0,09	bc	0,29 ± 0,04	c
	Ratio A:P	13,94 ± 0,92	a	12,71 ± 1,72	b	3,39 ± 1,04	c	4,12 ± 0,38	c	4,39 ± 1,06	c
	Total Molar Concentration (mM)	5,81 ± 0,24	c/2	7,20 ± 0,30	c/3	9,12 ± 0,55	c/23	36,16 ± 0,60	b/1	45,01 ± 2,20	a/1
D-glucose	**Molar Proportions (%)**										
	Acetate	97,20 ± 0,21	a	97,08 ± 0,17	a	95,82 ± 0,17	a	96,60 ± 0,47	a	96,47 ± 0,43	a
	Propionate	1,94 ± 0,25	b	2,39 ± 0,27	ab	3,84 ± 0,27	a	3,11 ± 0,42	ab	3,22 ± 0,38	ab
	Butyrate	0,86 ± 0,04	a	0,52 ± 0,11	b	0,35 ± 0,11	b	0,29 ± 0,05	b	0,31 ± 0,05	b
	Ratio A:P	50,45 ± 6,64	a	40,81 ± 4,69	ab	25,36 ± 4,69	b	31,39 ± 4,35	ab	30,20 ± 3,68	ab
	Total Molar Concentration (mM)	9,65 ± 1,32	b/1	13,73 ± 0,48	b/1	12,59 ± 1,25	b/1	24,96 ± 1,31	a/2	21,21 ± 2,22	a/2

Table 3.11.- *Short chain fatty acids (acetate, propionate and butyrate) quantification at 5, 8, 10, 24 and 48h, in faecal donor 3. Different letters (a,b,c,d) denote significant differences ($p<0.05$) between different times (h) within the same ingredient and measurement (Molar proportions, ratio A:P, and total molar concentration). Different numbers (1,2,3) denote significant differences ($p<0.05$) in total molar concentration, between different ingredients, within the same time.*

When no fermentable ingredients were added (MBM), no significant differences for total molar proportions between fermentation times were described.

With regard to butyrate molar proportion, it remained stable for MBM. On the contrary, when fermentable ingredients were added, its molar proportions significantly decreased across the experiment, from 5h to 48h of fermentation.

4.4.- Analysis of faecal microbial populations by qPCR results

For each bacterial group analyzed and donor, the increments across the experiment of genome equivalents logarithm per mL respect initial time (0h), were calculated. In parallel, per each ingredient and donor, the Pearson's correlation coefficients between time of fermentation, and increment of genome equivalents logarithm per milliliter, within the same bacterial group, were also calculated. These results have been presented in figures 3.9, 3.10, 3.11 and 3.12.

In figure 3.9, the results for *Lactobacillus* have been presented. According to the Pearson's correlation coefficients, all the ingredients showed a significant ($p<0.05$) positive correlation between time of fermentation and increments of quantified DNA (log genome equivalents/mL). On the contrary, for donor 1 ($r=0.342$) and 3 ($r=-0.522$), the non-addition of fermentable ingredients (MBM) resulted in a non-significant correlation between both variables, being significantly negative for donor 2 ($r=-0.806$). From these results, it can be deduced that the addition of LBG, Mhdp, gRS, Inuline and D-glucose results in a positive effect on *Lactobacillus* population.

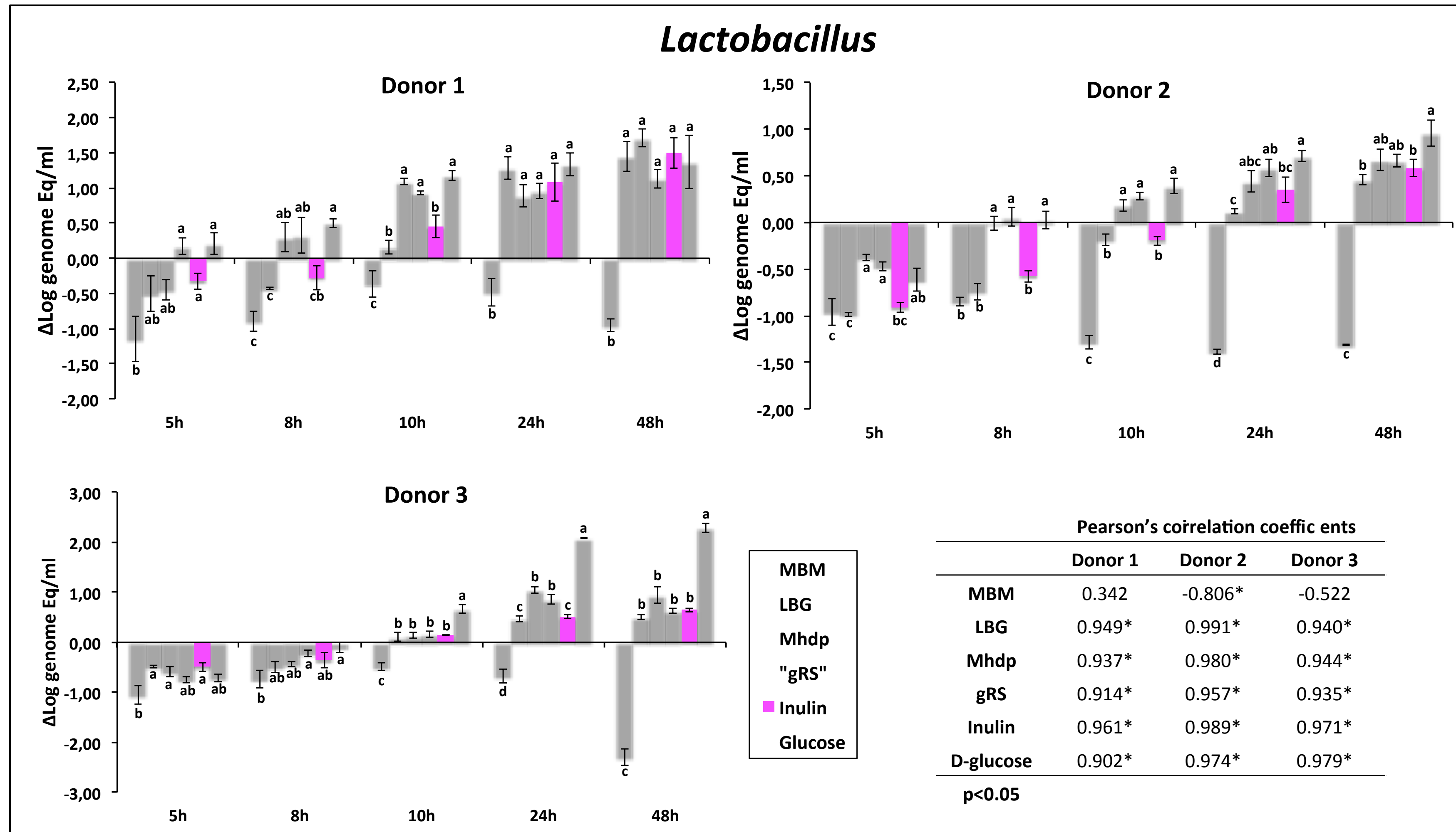
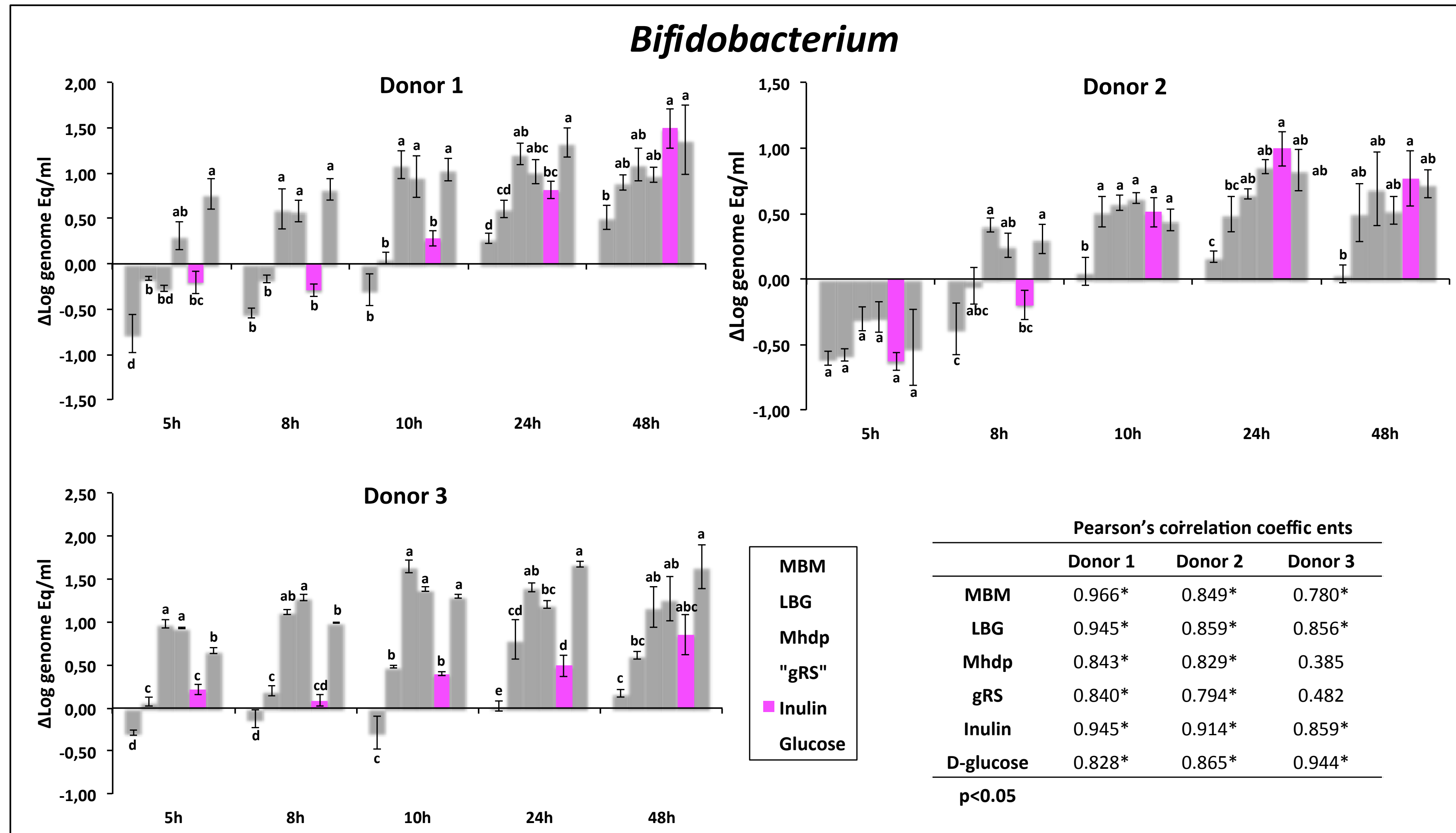


***Figure 3.9**.- Evolution of increments of Lactobacillus genome equivalents logarithm per mL with time of fermentation, per ingredient and donor. Results have been presented as mean ± S.D. For each donor, different letters (a,b,c,d) denote significant differences between ingredients within the same time. Per each donor and ingredient, Pearson's correlation coefficients for the variation of bacterial DNA quantification in relation to time, are presented in annexed table. Significance level p<0.05.*

When analyzing the relationship between the different ingredients within the same fermentation time and donor (figure 3.9), during the first hours (5h and 8h), *Lactobacillus* presented a negative growth with respect to the initial conditions (0h), unless for Mhdp, gRS and D-glucose, which resulted in a positive *Lactobacillus* increment after 8 h of fermentation, for donor 1 (0.30±0.21, 0.32±0.25 and 0.50±0.07 Δlog genome eq/mL for Mhdp, gRS and D-glucose respectively) and donor 2 (-0.01±0.08, 0.06±0.10 and 0.03±0.09 Δlog genome eq/mL for Mhdp, gRS and D-glucose respectively). This positive effect on *Lactobacillus* growth was extended for all the ingredients evaluated after 10h of fermentation, in all 3 donors. In donor 1 and 2, this effect was significantly higher for Mhdp (1.09±0.04 and 0.19±0.06 Δlog genome eq/mL for donor 1 and 2), gRS (0.93±0.03 and 0.28±0.04 Δlog genome eq/mL for donor 1 and 2) and D-glucose (1.18±0.07 and 0.39±0.08 Δlog genome eq/mL for donor 1 and 2), than for LBG (0.16±0.10 and -0.19±0.06 Δlog genome eq/mL for donor 1 and 2) and inulin (0.45±0.16 and -0.20±0.05 Δlog genome eq/mL for donor 1 and 2). In the case of donor 3, the increments of *Lactobacillus* amount of DNA respect time 0h for LBG (0.11±0.10 Δlog genome eq/mL), Mhdp (0.14±0.06 Δlog genome eq/mL), gRS (0.17±0.06 Δlog genome eq/mL) and inulin (0.15±0.01 Δlog genome eq/mL), where significantly lower ($p<0.05$) than the one obtained with D-glucose (0.66±0.08 Δlog genome eq/mL) after 10h of fermentation. Differences in the *Lactobacillus* DNA amount increments respect to time 0h, between different ingredients, were minimized after 24-48h hours of fermentation. In this way, for donor 1, no statistical differences between LBG, Mhdp, gRS, inulin and D-glucose were found at 24 and 48h, with values ranged from 0.89±0.16 to 1.45±0.22 Δlog genome eq/mL.



Pearson’s correlation coeffic ents

	Donor 1	Donor 2	Donor 3
MBM	0.966*	0.849*	0.780*
LBG	0.945*	0.859*	0.856*
Mhdp	0.843*	0.829*	0.385
gRS	0.840*	0.794*	0.482
Inulin	0.945*	0.914*	0.859*
D-glucose	0.828*	0.865*	0.944*

p<0.05

***Figure 3.10**.- Evolution of increments of Bifidobacterium genome equivalents logarithm per mL with time of fermentation, per ingredient and donor. Results have been presented as mean ± S.D. For each donor, different letters (a,b,c,d) denote significant differences between ingredients within the same time. Per each donor and ingredient, Pearson’s correlation coefficients for the variation of bacterial DNA quantification in relation to time, are presented in annexed table. Significance level p<0.05.*

For donor 2 and 3, LBG, Mhdp, gRS and inulin resulted in positive but significantly lower increments of *Lactobacillus* amount of DNA (ranged from 0.46 to 0.90 Δlog genome eq/mL at 48h) with respect to the ones obtained for D-glucose (ranged between 1 and 2 Δlog genome eq/mL at 48h).

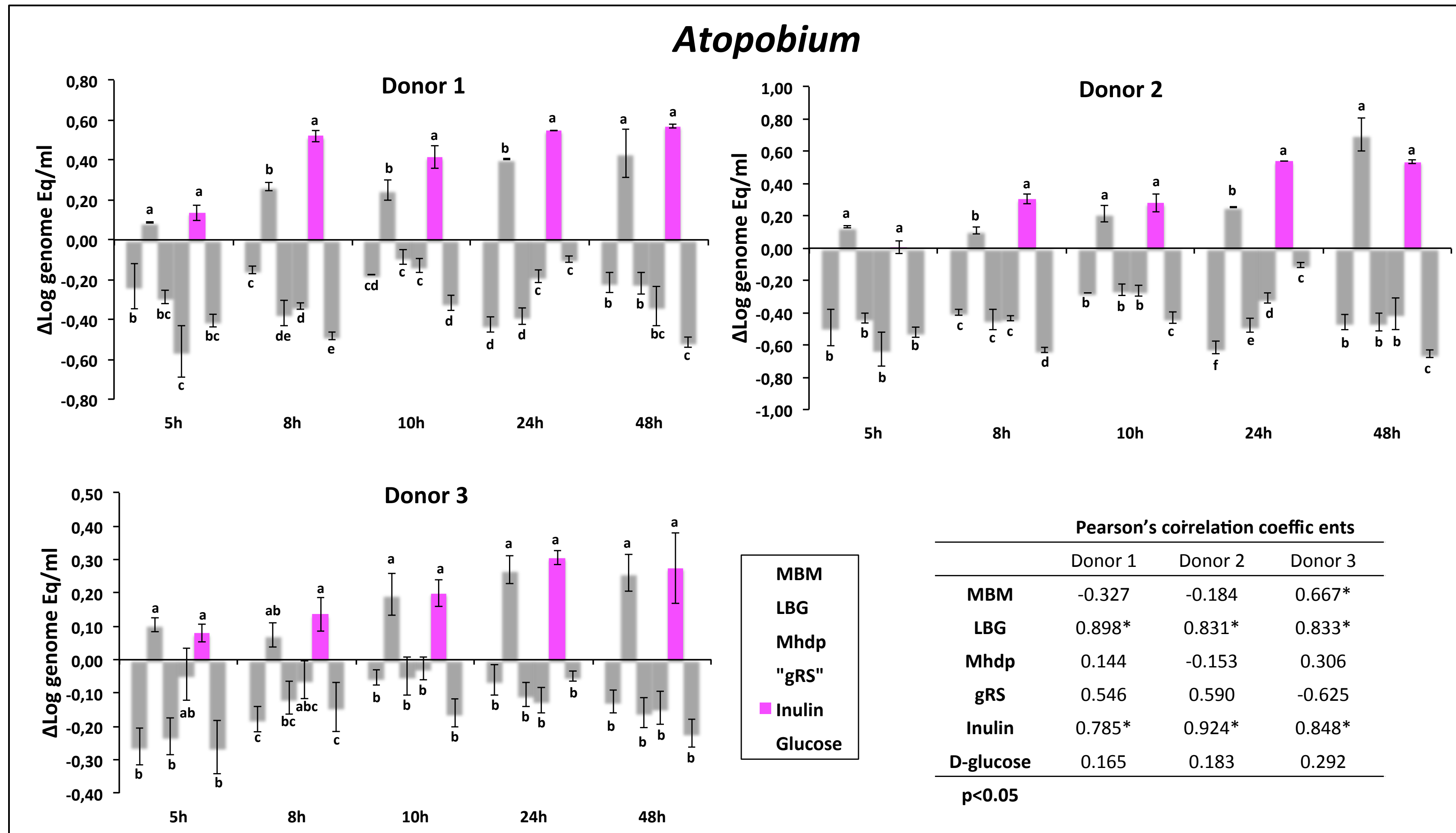
With regard to *Bifidobacterium* (figure 3.10), its behavior for the different ingredients was similar to that of *Lactobacillus*. In this way, all the ingredients evaluated, showed a significant ($p<0.05$) positive correlation between the *Bifidobacterium* DNA amount increments, and time of fermentation. For donor 3, this positive correlation was described for MBM, LBG, inulin and D-glucose, but not for Mhdp and gRS.

When the relationship between the different ingredients, within the same fermentation time and donor was analyzed for *Bifidobacterium* (figure 3.10), during the first hours (from 5h to 10h), the addition of Mhdp, gRS and D-glucose resulted in a significantly higher growth increment than the ones described for LBG and inulin, for donor 1 and 3. For donor 2, this significant effect was maintained until 8h of fermentation. In this regard, after 10 h of fermentation, the *Bifidobacterium* DNA amount increments respect time 0h, registered for Mhdp, gRS and D-glucose ranged from 0.96±0.23 to 1.09±0.15, and from 1.30±0.02 to 1.65±0.07 Δlog genome eq/mL for donor 1 and 3 respectively, with no significant differences between them. This results were significantly higher ($p<0.05$) than these obtained for LBG and inulin, which ranged from 0.07±0.07 to 0.28±0.08, and from 0.40±0.03 to 0.48±0.01 Δlog genome eq/mL for Donor 1 and 3 respectively. In the case of donor 2, no differences were found between ingredients at 10h of fermentation, with values between 0.45±0.08 and 0.62±0.04 Δlog genome eq/mL. As increasing fermentation time, the previously reported differences

among ingredients were minimizing. As a result, no significant differences between the addition of LBG, Mhdp, gRS, inulin and D-glucose were found after 48h of fermentation, except for donor 3, in which LBG resulted in a significantly lower *Bifidobacterium* DNA amount increments (0.61±0.04 Δlog genome eq/mL) than the obtained with D-glucose (1.64±0.25 Δlog genome eq/mL).

With regard to the non-addition of fermentable ingredients (MBM), a significant ($p<0.05$) positive correlation between the *Bifidobacterium* DNA amount increments and time of fermentation was described. As can be seen in figure 3.10, from 10 - 24h onwards, growth increments of *Bifidobacterium* respect to 0h, were positive.

In relation to *Atopobium* and *Bacteroides* DNA amount increments respect to 0h, both bacterial groups showed a similar behavior for each ingredient, in all three donors. As can be seen in figures 3.11 and 3.12, the addition of Mhdp, gRS and D-glucose resulted in a negative *Atopobium* and *Bacteroides* DNA amount increments across the experiment (from 0 to 48h). These results were similar to the ones obtained with the non-addition of any fermentable ingredient (MBM). On the contrary, a positive of both bacterial groups DNA amount increments were obtained for the addition of LBG and inulin as fermentable ingredients. What is more, a significant ($p<0.05$) and positive ($p<0.05$) correlation between increments of *Atopobium* and *Bacteroides* genome equivalents logarithm per mL and time of fermentation were obtained for LBG and inulin (figures 3.11 and 3.12).



Pearson's correlation coefficients	Donor 1	Donor 2	Donor 3
MBM	-0.327	-0.184	0.667*
LBG	0.898*	0.831*	0.833*
Mhdp	0.144	-0.153	0.306
gRS	0.546	0.590	-0.625
Inulin	0.785*	0.924*	0.848*
D-glucose	0.165	0.183	0.292

p<0.05

***Figure 3.11**.- Evolution of increments of Atopobium genome equivalents logarithm per mL with time of fermentation, per ingredient and donor. Results have been presented as mean ± S.D. For each donor, different letters (a,b,c,d) denote significant differences between ingredients within the same time. Per each donor and ingredient, Pearson's correlation coefficients for the variation of bacterial DNA quantification in relation to time, are presented in annexed table. Significance level p<0.05.*

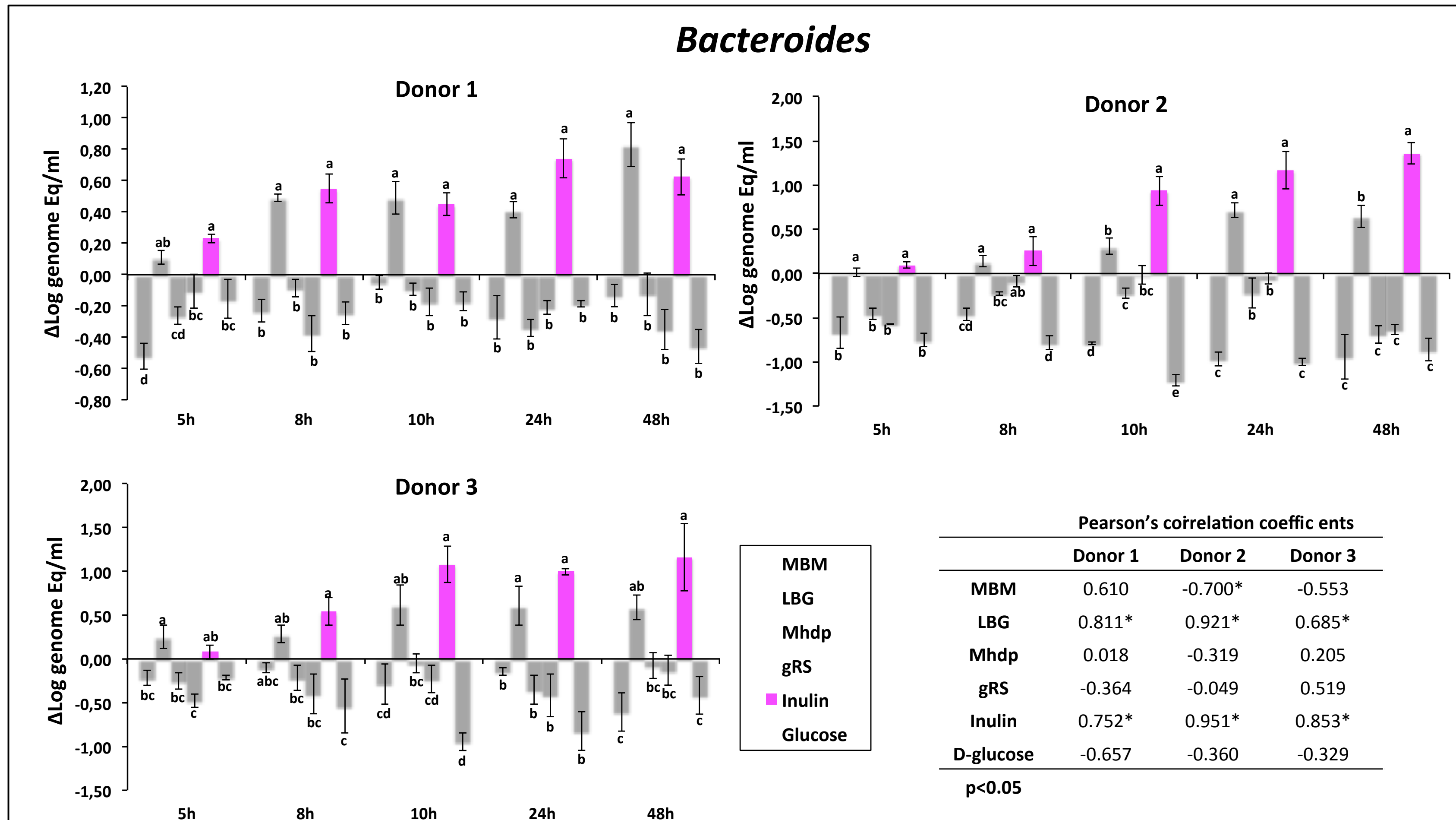


***Figure 3.12**.- Evolution of increments of Bacteroides genome equivalents logarithm per mL with time of fermentation, per ingredient and donor. Results have been presented as mean ± S.D. For each donor, different letters (a,b,c,d) denote significant differences between ingredients within the same time. Per each donor and ingredient, Pearson's correlation coefficients for the variation of bacterial DNA quantification in relation to time, are presented in annexed table. Significance level p<0.05.*

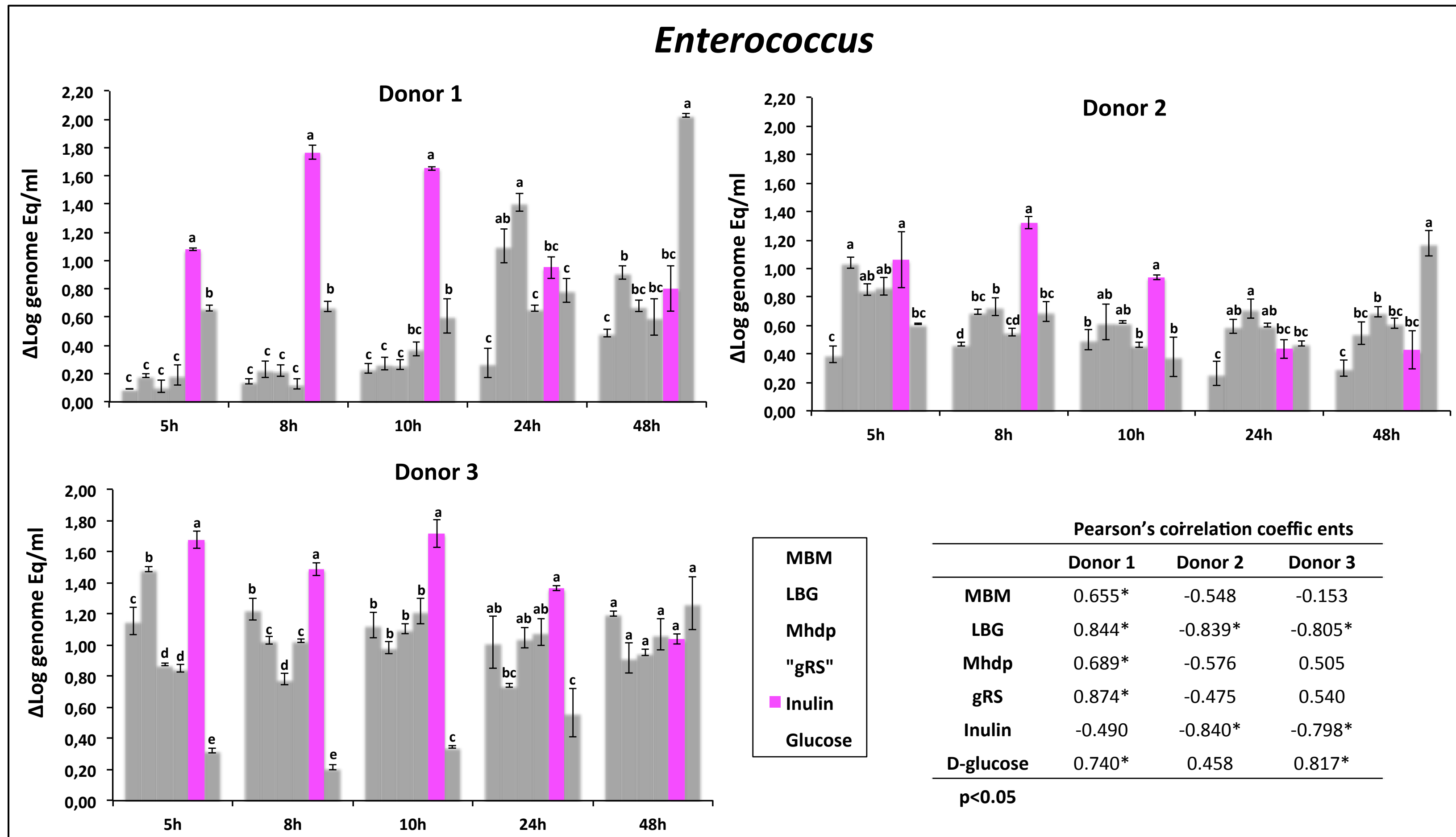
According to the results presented in figure 3.11, when LBG and inulin where added, *Atopobium* DNA amounts increments varied from a value of 0.09±0.01 (donor 1), 0.13±0.02 (donor 2) and 0.11±0.02 (donor 3) Δlog genome eq/mL at 5h of fermentation, to values of 0.43±0.12 (donor 1), 0.70±0.10 (donor 2) and 0.26±0.06 (donor 3) Δlog genome eq/mL at 48h of fermentation. In the same way, inulin resulted in a variation of *Atopobium* DNA amount increments from 0.14±0.04 (donor 1), 0.01±0.04 (donor 2) and 0.08±0.03 (donor 3) Δlog genome eq/mL at 5h of fermentation, to 0.57±0.01 (donor 1), 0.53±0.01 (donor 2) and 0.28±0.11 (donor 3) Δlog genome eq/mL at 48h

With regard to *Bacteroides* DNA amount increments (figure 3.12), the addition of LBG resulted in a variation from 0.11±0.04 (donor 1), 0.02±0.05 (donor 2) and 0.26±0.13 (donor 3) Δlog genome eq/mL at 5h, to 0.83±0.14 (donor 1), 0.65±0.13 (donor 2), and 0.59±0.14 (donor 3) Δlog genome eq/mL at 48h. The values obtained with the addition of inulin varied from 0.23±0.03 (donor 1), 0.10±0.04 (donor 2) and 0.08±0.08 (donor 3) Δlog genome eq/mL at 5h, to 0.62±0.11 (donor 1), 1.36±0.13 (donor 2), and 1.16±0.38 (donor 3) Δlog genome eq/mL at 48h.

When the *Enterococcus* results were analysed (figure 3.13), Δlog genome eq/mL across the experiment varied depending on the ingredient and the donor. In this way, when LBG and inulin where added, a significant ($p<0.05$) negative correlation between the *Enterococcus* DNA amount increments and time of fermentation was described for donor 2 (-0.839 for LBG and -0.840 for inulin) and donor 3 (-0.805 for LBG and -0.798 for inulin), but not for donor 1. Indeed, for donor 1, the addition of LBG, resulted in a significant and positive correlation. As can be seen in figure 3.13, when LBG was added, *Enterococcus* DNA amount increments varied from 1.04±0.04 (5h) to 0.55±0.08 Δlog

genome eq/mL (48h) for donor 2, and from 1.49±0.02 (5h) to 0.92±0.10 (48h) Δlog genome eq/mL for donor donor 3. On the contrary, the addition of LBG in donor 1 resulted in an increase of *Enterococcus* DNA amount increments with time, varying from 0.19±0.01 (5h) to 0.91±0.05 (48h) Δlog genome eq/mL. For donor 1, when the relationship between *Enterococcus* DNA amount increments and time of fermentation where analyzed, a non significant correlation was obtained (-0.490). As it has been shown in figure 3.13, from 5h to 10 h of fermentation, *Enterococcus* DNA amount increments increased from 1.08±0.01 to 1.65±0.01 Δlog genome eq/mL. After this time, its values decreased, reaching a minimum of 0.81±0.16 Δlog genome eq/mL after 48h of fermentation. With regard to the rest of ingredients, a significant and positive correlation between the *Enterococcus* DNA amount increments and time of fermentation, was defined for donor 1 when Mhdp (0.689), gRS (0.874) and D-glucose (0.740) were added. However, for the rest of donors, a significant and positive correlation was observed only for donor 3 and D-glucose (0.817). According to the data presented in figure 3.13, when results for donors 1 and 2 where analyzed, after 48h of fermentation, glucose resulted in the highest *Enterococcus* DNA amount increment, whose values were 2.03±0.01 and 1.27±0.27 Δlog genome eq/mL respectively. On the contrary, no significant differences where found between LBG, Mhdp, gRS and inulin, with a value range between 0.60±0.13 and 0.91±0.05 Δlog genome eq/mL for donor 1, and between 0.43±0.13 and 0.69±0.04 Δlog genome eq/mL for donor 2. These values where significantly lower than the ones defined for D-glucose. In contrast to the results explained for donor 1 and 2, after 48h of fermentation, no significant differences where described between LBG (0.92±10 Δlog genome eq/mL), Mhdp (0.95±0.02 Δlog genome eq/mL), gRS (1.07±0.10 Δlog genome eq/mL), inulin (1.04±0.03 Δlog genome eq/mL) and D-glucose (1.27±0.17 Δlog genome eq/mL) for donor 3.

In relation to the *Enterobacteriaceae* DNA amount increments respect to 0h, varied depending on the donor. As it has been shown in figure 3.14, Pearson's correlation coefficients were significant (<0.05) only for LBG (0.659) and Mhdp (-0.739). In this regard, the addition of LBG resulted in a DNA amount increment variation from 0.66±0.08 Δlog genome eq/mL at 5h of fermentation, to 1.10±0.06 Δlog genome eq/mL at 10h. From this point, DNA amount increment variations maintained stable, with values ranged from 1.03±0.06 to 0.99±0.04 Δlog genome eq/mL. On the contrary, when Mhdp was added, *Enterobacteriaceae* DNA amount increments respect to 0h, decreased from 1.26±0.04 at 5h, to 0.93±0.01 Δlog genome eq/mL at 48h. In the case of D-glucose, values maintained in a range of 1.20 – 1.50 Δlog genome eq/mL. For the rest of ingredients, values increased and decreased during the experiment without a defined pattern. For donor 2, no significant differences where found among all the ingredients analysed during the fermentantion assay (from 5h to 48h). In this donor, only D-glucose showed a significant and positive correlation between the *Enterobacteriaceae* DNA amounts increment and time of fermentation, with values that varied from 0.45±0.08 Δlog genome eq/mL at 5h of fermentation, to 0.62±0.09 Δlog genome eq/mL at 48h. When data from donor 3 where analysed, a non-significant correlation between the *Enterobacteriaceae* DNA amount increments and time of fermentation, was obtained for all the ingredients. In this way, no statistical differences where defined between LBG, Mhdp, gRS, inulin and D-glucose until 48h of fermentation. At 48h of fermentation, gRS (0.12±0.08 Δlog genome eq/mL) resulted in a significantly lower DNA amount increment than the one registered for LBG (values ranged between 0.17±0.08 and 0.28±0.07 Δlog genome eq/mL). The values for the rest of ingredient did not differ significatively with respect to LBG or gRS.

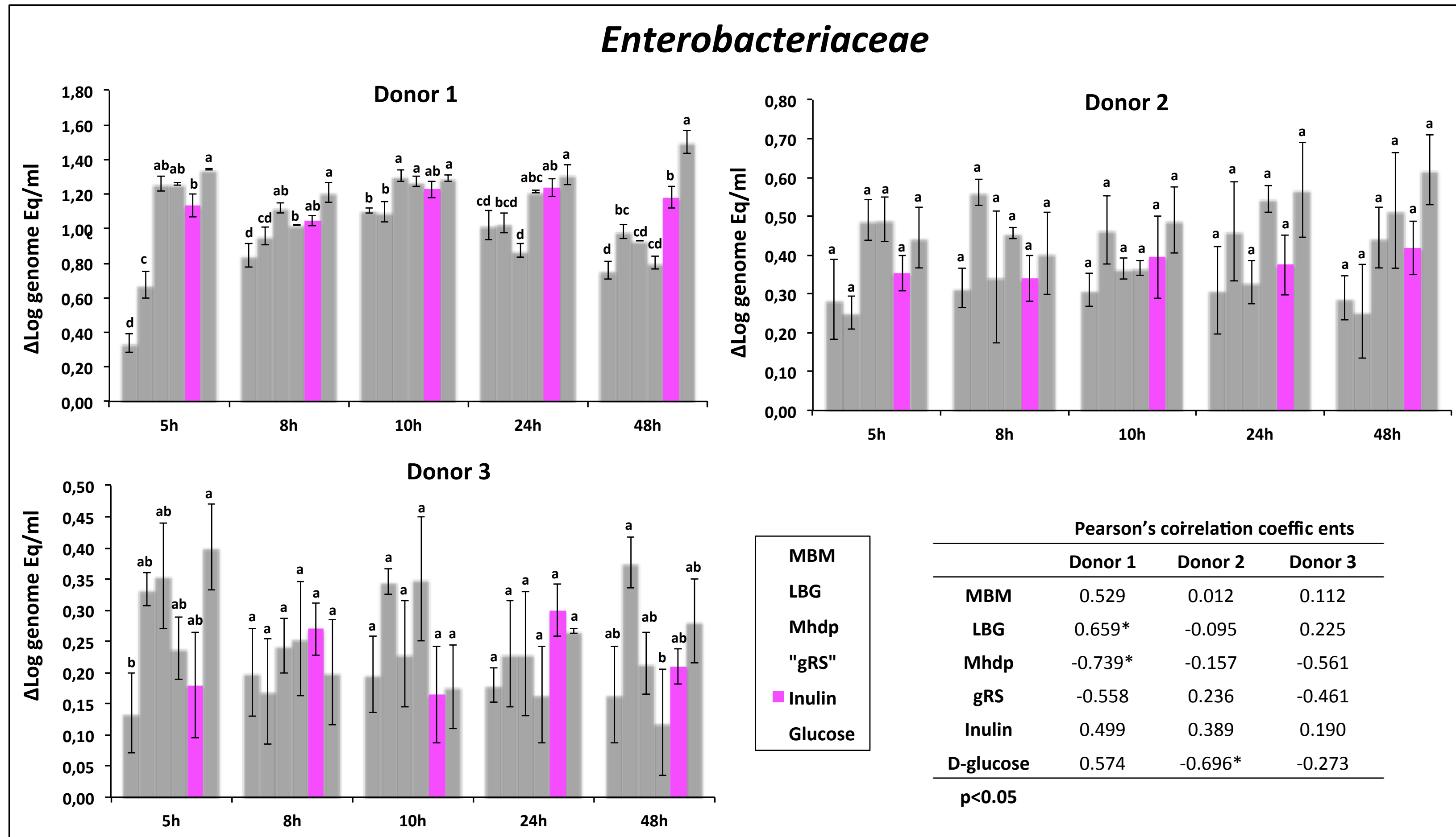


Pearson's correlation coefficients

	Donor 1	Donor 2	Donor 3
MBM	0.655*	-0.548	-0.153
LBG	0.844*	-0.839*	-0.805*
Mhdp	0.689*	-0.576	0.505
gRS	0.874*	-0.475	0.540
Inulin	-0.490	-0.840*	-0.798*
D-glucose	0.740*	0.458	0.817*

p<0.05

***Figure 3.13**.- Evolution of increments of Enterococcus genome equivalents logarithm per mL with time of fermentation, per ingredient and donor. Results have been presented as mean ± S.D. For each donor, different letters (a,b,c,d) denote significant differences between ingredients within the same time. Per each donor and ingredient, Pearson's correlation coefficients for the variation of bacterial DNA quantification in relation to time, are presented in annexed table. Significance level p<0.05.*



	Pearson’s correlation coefficients		
	Donor 1	Donor 2	Donor 3
MBM	0.529	0.012	0.112
LBG	0.659*	-0.095	0.225
Mhdp	-0.739*	-0.157	-0.561
gRS	-0.558	0.236	-0.461
Inulin	0.499	0.389	0.190
D-glucose	0.574	-0.696*	-0.273

p<0.05

***Figure 3.14**.- Evolution of increments of Enterobacteriaceae genome equivalents logarithm per mL with time of fermentation, per ingredient and donor. Results have been presented as mean ± S.D. For each donor, different letters (a,b,c,d) denote significant differences between ingredients within the same time. Per each donor and ingredient, Pearson’s correlation coefficients for the variation of bacterial DNA quantification in relation to time, are presented in annexed table. Significance level p<0.05.*

4.5.- Principal Components – Varimax Rotation Analysis

Principal components analysis (PCA) was applied to describe the possible relationship among SCFAs, different bacterial groups and the different ingredients evaluated (MBM, LBG, Mhdp, gRS, inulin and D-glucose), in all three donors. According to the previous results and, in order to evidence the relationship between these variables, fermentation time was set at 24h for the analysis. A three components solution, with a total variance explained of 67.87% was chosen, showing the Varimax-rotated components distribution in Figure 3.15. Table 3.12 shows the matrix of rotated components.

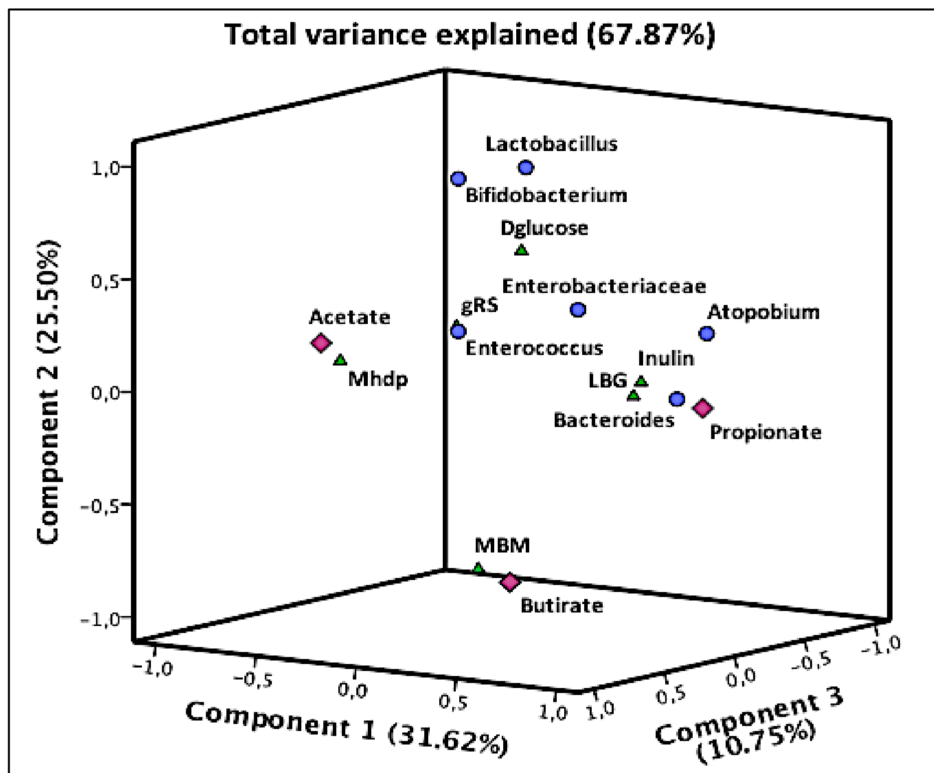


***Figure 3.15.**.- Varimax-rotated components distribution. With blue circles, purple diamonds and green triangles, the analysed bacterial groups, SCFAs (acetate propionate and butyrate) and, the different fermentation substrates, have been respectively represented.*

***Table 3.12.**.- Matrix of rotated components.*

	components		
Variables	**C.1**	**C.2**	**C.3**
MBM	-0.27	-0.89	-0.15
LBG	0.55	-0.02	-0.08
Mhdp	-0.30	0.17	0.79
gRS	-0.27	0.22	0.02
Inulin	0.68	0.07	0.04
Dglucose	-0.38	0.45	-0.61
Bifidobacterium	-0.25	0.88	0.03
Lactobacillus	0.05	0.95	-0.03
Acetate	-0.96	0.07	-0.01
Propionate	0.96	-0.02	0.01
Butirate	-0.04	-0.90	-0.05
Bacteroides	0.92	0.03	0.13
Enterococcus	0.20	0.34	0.67
Atopobium	0.88	0.28	-0.14
Enterobacteriaceae	0.10	0.28	-0.33

According to these results, and together with the previously presented total gas production results (section 4.1) and pH evolution (section 4.2), Component 1 has been defined as "*slow fermented ingredients*", which correlated positively with LBG (0.55) and inulin (0.68). In fact, both ingredients resulted in a significantly lower total gas production than that obtained for Mhdp, gRS or D-glucose (figure 3.7). What is more, LBG and inulin provided a delayed drop of pH (24h) in comparison with the rest of ingredients (5h) (see figure 3.8). Regarding to SCFAs production, while propionate (0.96) was positively marked by Component 1, acetate (-0.96) was inversely correlated to it (table 3.12). These findings are in concordance with previously analyzed tables 3.8, 3.9 and 3.10, in which the addition of LBG and inulin, resulted in a significant increment of propionate and a decrease of acetate, which was more evident from 10h onwards, associated to a significantly higher SCFAs total molar concentration than that obtained for the rest of ingredients evaluated. Component 1 is also positively correlated with *Atopobium* (0.88) and *Bacteroides* (0.92). As it has been shown in figures 3.11 and 3.12 respectively, these bacterial groups only presented a positive correlation between time

of fermentation and growth, when LBG and inulin were added as ingredients for the three donors.

Following the same deductive approach, Component 2 has been defined as "*rapidly fermented ingredients*", being positively correlated with *Bifidobacterium* (0.88) and *Lactobacillus* (0.95), and negatively correlated (-0.89) with the absence of fermentable ingredients (MBM) and the production of butyrate (-0.90). With regard to D-glucose and gRS, no marked correlations have been found with any of the three components included in the analysis (table 3.12). However, D-glucose and gRS show a slight positive correlation with this second component (0.45 and 0.22 respectively). Indeed, according to figures 3.9 and 3.10, *Lactobacillus* and *Bifidobacterium* populations rapidly increased when both ingredients where added, whereas a null or minor increment was described with MBM. In relation with the previously explained first component ("*slow fermented ingredients*"), D-glucose (-0.38) and gRS (-0.27) show a slight negative correlation.

Third Component is positively related to the variable Mhdp (0.79). Indeed, when Mhdp was added, total gas production (figure 3.7) in donor 1 and 2, were slightly lower than the one obtained for D-glucose, but was significant ($P<0.05$) in donor 3. In parallel, pH ranges at 48h (section 4.2), were significantly lower ($p<0.01$) for D-glucose and gRS (4.08±0.02 – 3.75±0.06) than for Mhdp (4.30±0.04 – 4.23±0.02). In any way, total gas production and pH values were, respectively, significantly higher and lower than the ones obtained with the addition of LBG and inulin. Under these premises, third component has been defined as "*moderate fermented ingredients*". With regard to the other variables, only *Enterococcus* is positively correlated with third component (0.67),

although it is also positively correlated, in a less marked way, with Component 1 (0.20) and Component 2 (0.34), denoting its variability with different ingredients, donors and fermentation time. In this respect, when analysing *Enterococcus* population increment (figure 3.13), it was not possible to establish a pattern between its behaviour and the different ingredients and fermentation time among donors.

With regard to *Enterobacteriaceae* group, no correlations were clearly defined with any of the components. At this respect, it was slightly positively correlated with Component 1 (0.10) and 2 (0.28), but negatively correlated with Component 3 (-0.33). These findings are in concordance with figure 3.14, in which no clear effect could be established about ingredients and fermentation time on the *Enterobacteriaceae* population.

4.6.- Discussion

The relevance of resistant carbohydrates as gut microbial fermentation substrates, and determination of their final fermentation products as a way of monitoring *in vivo* and *in-vitro* gut microbiota activity, are widely described in the published bibliography (Cummings *et al*., 1987; Glenn *et al*., 1995; Rycroft *et al*., 2001; Flint *et al*., 2014). Basically, as a result of this fermentation activity, there is an increase of bacterial population, as well as gas and SCFA production, together with a faecal pH acidification (Raninen *et al*., 2011). However, the final fermentation product proportions are different depending on the type of fermented substrate and the composition of intestinal microbiota. With regard to resistant carbohydrates, their fermentability is

mainly related to their degree of polymerization and degree of branching (Mussatto & Mancilha, 2007). As it has been explained during the reported results (section 4.5), the different ingredients evaluated in this chapter, has been classified as "*rapidly fermented ingredients*", "*moderate fermented ingredients*" and "*slow fermented ingredients*". This classification attends to the gas production, pH evolution and SCFAs concentration registered during the *in-vitro* fermentation process.

According to the results obtained, modified starches (Mhdp and gRS), together with D-glucose, showed a similar behavior, being classified as moderately or rapidly fermentable ingredients. In fact, these ingredients resulted in the highest total gas production, and a pronounced drop in pH after 5h of fermentation (3.75±0.03 - 4.30±0.03). On the contrary, when slowly fermented ingredients (inulin and LBG) were analyzed, the decrease in pH was delayed until 10h of fermentation, being the total gas production significantly lower than for the previous ingredients. These results are in concordance with the ones published by Beards *et al*., (2010), in which, after a 24h faecal batch fermentation procedure, glucose and modified starch resulted in a higher gas production (≈27 psi/mL) than inulin (≈17 psi/mL) or galactomannans (≈23 psi/mL). In the same way, Kaur *et al.,* (2011), during an *in-vitro* fecal fermentation assay, described that modified starch lead to a rapidly pH-increase after 5-8h of fermentation (≈8-12 ml/50 mg carbohydrate). On the contrary gas production was lower for inulin (≈6 ml/50 mg carbohydrate after 8h of fermentation). With regard to the pH evolution, according to Gielt *et al*., (2012) the medium acidification during the fermentation experiment is related to the accumulation of organic acid production. These authors described a good correlation between the production of organic acids and the recovery of water soluble sugars during complex carbohydrates fermentation, establishing that

the rate of production of organic acids is a good reflection of the rate of fermentation. Under this basis, and together with the total gas production, analysis of pH evolution during the batch experiment could be a good indicator of the rate of fermentation, being higher for glucose, gRS and Mhdp (moderately or rapidly fermentable ingredients) than for LBG and inulin (slowly fermented ingredients). In their article, Gielt *et al*., (2012) analyzed different factors that could affect *in-vitro* fermentability of different carbohydrates, establishing the degree of substitution as the most influential factor, followed by the composition of monomer and linkage type. The chain length was the less influential factor. This could explain why LBG (highly polydisperse polysaccharide, consisting of a linear chain of (1→4)-linked β-D-mannopyranosyl units with randomly distributed (1→6)-linked-d-galactopyranosyl residues as side chains and an average degree of branching of 25%) as well as inulin (a polydisperse β(1-2) fructose polymer with an α(1-2) glucose end unit linked) were more slowly fermented than modified starches or glucose. (Niness, 1999; Sittikijyothinet *et al*., 2005). When evaluating the use of these ingredients as infant formulas thickeners, rate of substrate fermentation should be considered, as it could be related with gastrointestinal disorders. In this way, rapid fermentation can result in abdominal pain, discomfort, bloating and flatulence (Cummings *et al*., 2001).

With regard to SCFAs (acetate, propionate, butyrate) total molar concentration, a time-dependent increment was observed for all the ingredients evaluated. This increment at 24-48h respect to the initial concentration (5h), was higher for inulin and LBG (4-6 times higher) than for glucose, Mhdp and gRS (1-3 times higher). Kaur *et al*., (2011) also reported differences regarding to SCFAs total molar concentration depending on the ingredient evaluated. In this way, inulin or β-glucan resulted in an

increment of 3.5 at 48h respect 0h, whereas the increment for modified starch was lower (2.1 times higher).

Acetate was the most relevant contributor to the SCFAs total molar concentration. However, whereas for glucose, Mhdp and gRS, acetate proportions remained stable (85-97%) across the *in-vitro* fermentation process, when LBG and inulin were analyzed, a decrease in acetate towards an increase in propionate proportions were detected, decreasing the acetate:propionate ratio from 10 at 5h to 2 at 48h. Glucose, Mhdp and gRS resulted in stable propionate proportion (5-13%). Butyrate molar proportion decreased from 5h to 48h of fermentation for all the fermentable ingredients evaluated. These results are mainly in agreement with the ones published by Beards *et al*., (2010), according to which, after 24h of fermentation, glactomannan and inulin resulted in a significant increase in acetate (from 1.5 to 17.1 mMol for inulin, and from 1.6 to 7.3 mMol for galactomannans), and propionate (from 0.5 to 4.7 mMol for inulin, and from 0.7 to 4.4 mMol for galactomannans). No significant results were described for modified starches and glucose.

The increment of propionate molar proportions registered for LBG and inulin, as well as the decrease in butyrate molars proportion registered for all the ingredients could be explained by metabolic cross-feeding between butyrate, propionate and acetate producers, as well as by the effect of the different ingredients on the microbial populations. At this respect, *Bifidobacterium* and *Lactobacillus* have been defined as lactate and acetate producers (Beards *et al*., 2010; Nicholson *et al*., 2012; Jahdhyala *et al*., 2015). According to the results presented in this chapter, all the ingredient evaluated resulted in a fermentation time-dependent positive increment of both bacterial

populations. However, this positive increment was registered earlier for Mhdp, gRS and D-glucose (5 - 8h of fermentation) than for LBG and inulin, in which the positive increment was registered from 10h onwards. Differences in the increments of *Lactobacillus* and *Bifidobacterium* populations respect to time 0h, between different ingredients, were minimized after 24-48h hours of fermentation. Despite the production of lactate was not analyzed, these results together with the previously explained pH evolution, are in agreement with the proposal of *Lactobacillus* and *Bifidobacterium* as lactate and acetate producers (Beards *et al*., 2010; Nicholson *et al*., 2012; Jahdhyala *et al*., 2015). Indeed, the addition of glucose, Mhdp and gRS resulted in a pronounced drop in pH after 5h, which is in concordance with the positive increment in *Lactobacillus* and *Bifidobacterium* population. With regard to inulin and LBG, the positive increment in both bacterial populations, as well as the drop in pH was detected from 10h on wards. Whereas *in vivo* organic acid/SCFAs production is compensated by the colonic mucosa absorption, during a static faecal batch fermentation assay, these acids accumulate, decreasing the pH and explaining the previous findings (Gielt et al., 2012). In comparison with slowly fermentable ingredients (LBG and inulin), the earlier acidification of culture medium detected for moderate or rapidly fermentable ingredients (glucose, Mhdp and gRS), could explain the differences in other bacterial groups as a drastically drop in pH could inhibit the growth of some microorganism (Gietl *et al*., 2012).

According to the results presented in this chapter, *Bacteroides* group showed a different behavior depending on the ingredient added. In this way, when glucose, Mhdp and gRS were evaluated, both groups showed a negative increment at 24-48h respect 5h. The contrary effect was observed for LBG and inulin, *Bacteroides* presented a positive

increment with time of fermentation. This behavior can be explained by the previously published literature. Walker *et al*., (2005) analyzed the effect of pH on *Bacteroides* spp. growth, explaining that a pH of 5.5 or lower determines a poorly growth of this bacterial group. This bacterial group is also being related to a high production of propionate associated to metabolic cross-feeding. According to Walker *et al*., (2005) and Hosseini *et al*., (2011), *Bacteroides* is able to produce propionate using lactate and other fermentation products as substrate. This could explain why, a rapidly drop in pH associated to glucose, Mhdp and gRS fermentation is accompanied by a negative increment in *Bacteroides* population and a non significant propionate molar proportion increment. On the contrary, LBG and inulin fermentation resulted in a delayed pH decrease, which can be associated with a positive increment of *Bacteroides* population and propionate molar proportions. The presence and growth of *Bacteroides* population should be considered as a desirable response to diet, as it has been related to a broad saccharolytic and proteolitic potential, as well as to an active synthesis of different vitamins as biotin, riboflavin, pantothenate and ascorbate (Arumugam *et al*., 2011). What is more, the early presence of *Bacteroides* in infant gut microbiota could exert a positive effect on the development and maturation of mucosal associated-inmune system, reducing the predisposition to immune disorders (Marques *et al*., 2010; Jakobsson *et al*., 2014). On its behalf, propionate production has been associated with a decrease in the intestinal motility, and a mild modulator of cell growth and differentiation through the inhibition of histone de-acetilase. Together with this functions, propionate can be used as a metabolic substrate for lipid synthesis, energy metabolism, gluconeogenesis and oxidation (Nicholson *et al*., 2012; Hurst *et al.,* 2014; Puertollano *et al*., 2014; Tan *et al*., 2014; Jahdhyla *et al*., 2015).

Regarding butyrate, a similar metabolic cross-finding has been explained between *Bifidobacterium spp* and butyrate producers bacteria. At this respect, different authors (Belenguer *et al*., 2006; Falony *et al*., 2006) described two metabolic pathways using lactate/acetate for butyrate production and vice verse. The growth of *Bifidobacterium* as well as other butyrate consumer species through metabolic cross-feeding, could explain the decrease in butyrate molar proportion registered for all the ingredients evaluated.

In contrast with glucose, Mhdp and gRS, the fermentation of inulin and LBG resulted in a positive increment of *Atopobium* cluster. According to Collado *et al*., (2007), the presence of *Atopobium* cluster could be related to the type of feeding and infant age. In this regard, Rajilić-Stojanović *et al*., (2009) described an elderly:young ratio with a value of 2, which denotes a slight increment of *Atopobium* population associated to age. With regard to the type of feeding, the presence of *Atopobium* in feces has been associated to bottle-feeding (Vael & Desager, 2009). However, based on the consulted bibliography, the relevance of this group in infant gut microbiota seems to be unclear.

About this study, it has to be taken into account that due to its *in-vitro* performance, the physiological process that interacts with the microbiota and its metabolites have been omitted. Another factor to take into account is that, despite being considered during the methodological design, the quantification of Clostridia IX (*Clostridium leptum* - Faecalibacterium praustnitzii) and Clostridia XIVa (*Clostridium Coccoides – Eubacterium rectale*) was not possible.

Considering these factors, and the results obtained, a possible future investigation should consider the *in vivo* and/or *in-vitro* evaluation of the impact of Mhdp, gRS and LBG on infant faecal microbiota, using a dynamic simulator of gastrointestinal fermentation.

5.- CONCLUSIONS

Attending to its fermentability, LBG can be defined as a "*slowly fermented ingredient"*, resulting in a moderate gas production and a slow drop of pH as well as in a decreasing acetate:propionate ratio. These properties are related to the development of a varied fecal microbiota, increasing *Atopobium* and *Bacteroides* populations. On the contrary, modified starches, Mhdp and gRS are classified as "*rapidly fermented ingredients*", resulting in a high gas production, a sudden drop of pH and a majority production of Acetate. These properties promote the development of a less varied fecal microbiota, with absence or minimal development of *Atopobium* and *Bacteroides* groups.

According to these conclusions it would be needed to clarify if a high gas production associated to "rapidly fermentes ingredients", as well as a high propionate and a more varied fecal microbiota associated to "slowly fermented ingredients", could have any impact on infant health and development. In this way, *in-vivo* studies should be considered.

6.- BIBLIOGRAPHY

Aas, J.A., Paster, B. J., Stokes, L. N., Olsen, I., & Dewhirst, F. E. (2005). Defining the normal bacterial floraof the oral cavity. Journal of Clinical Microbiology; 43(11): 5721 – 5732.

Abrahamsson, T. R., Jakobsson, H. E., Andersson, A. F., Björkstén, B., Engstrand, L., & Jenmalm, M. C. (2014). Low gut microbiota diversity in early infancy precedes asthma at school age. Clinical & Experimental Allergy; 44(6): 842 – 850.

Adlerberth I. (2009). Factors influencing the establishment of the intestinal microbiota in infancy. Personalized Nutrition for the Diverse Needs of Infants and Children; 62: 13 – 33.

Adlerberth, I., Lindberg, E., Åberg, N., Hesselmar, B., Saalman, R., Strannegård, I. L., & Wold, A. E. (2006). Reduced enterobacterial and increased staphylococcal colonization of the infantile bowel: an effect of hygienic lifestyle?. Pediatric research; 59(1): 96 – 101.

Adlerberth, I., & Wold, A. E. (2009). Establishment of the gut microbiota in Western infants. Acta paediatrica; 98(2): 229 – 238.

Aggett, P. J., Agostoni, C., Axelsson, I., Edwards, C. A., Goulet, O., Hernell, O., *et al.* (2003). Nondigestible carbohydrates in the diets of infants and young children: a commentary by the ESPGHAN Committee on Nutrition. Journal of pediatric gastroenterology and nutrition; 36(3): 329 – 337.

Alakomi, H. L., Skyttä, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., & Helander, I. M. (2000). Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. Applied and environmental microbiology; 66(5): 2001 – 2005.

Allen, S. J., Wareham, K., Wang, D., Bradley, C., Hutchings, H., Harris, W., ... & Mack, D. (2013). Lactobacilli and bifidobacteria in the prevention of antibiotic-associated diarrhoea and Clostridium difficile diarrhoea in older inpatients (PLACIDE): a randomised, double-blind, placebo-controlled, multicentre trial. The Lancet; 382(9900): 1249 – 1257.

Alonso, V. R., & Guarner, F. (2013). Linking the gut microbiota to human health. British Journal of Nutrition; 109(2): 21 – 26.

Al-Tamimi M.A.H.M., Palframan R.J., Cooper J.M., Gibson G.R., Rastall R.A. (2006). In-vitrofermentation of sugar beet arabinan and arabino-oligosaccharides by the human gut microflora. Journal of Applied Microbiology; 100: 407 - 414.

Andersson, A. F., Lindberg, M., Jakobsson, H., Backhed, F., Nyrén, P., & Engstrand, L. (2008). Comparative analysis of human gut microbiota by barcoded pyrosequencing. PloS one; 3(7): 2836 – 2836.

Arboleya, S., Ang, L., Margolles, A., Yiyuan, L., Dongya, Z., Liang, X., *et al.* (2012[a]). Deep 16S rRNA metagenomics and quantitative PCR analyses of the premature infant fecal microbiota. Anaerobe; 18(3): 378 – 380.

Arboleya, S., Binetti, A., Salazar, N., Fernández, N., Solís, G., Hernández-Barranco, A., *et al.* (2012[b]). Establishment and development of intestinal microbiota in preterm neonates. FEMS microbiology ecology; 79(3): 763 – 772.

Arboleya S., Salazar N., Solís G., Fernández N., Hernández-Barrancosa A.M., Cuesta I., *et al.* (2013). Assessment of intestinal microbiota modulation ability of Bifidobacterium strains in in-vitro fecal batch cultures from preterm neonates. Anaerobe; 19: 9 – 16.

Arboleya, S., Solís, G., Fernández, N., de los Reyes-Gavilán, C. G., & Gueimonde, M. (2012[c]). Facultative to strict anaerobes ratio in the preterm infant microbiota: a target for intervention?. Gut microbes; 3(6): 583 – 588.

Ardissone, A. N., Diomel, M., Davis-Richardson, A. G., Rechcigl, K. T., Li, N., Drew, J. C., *et al.* (2014). Meconium microbiome analysis identifies bacteria correlated with premature birth. PLoS One; 9(3): 1 – 8.

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., *et al.*, (2011). Enterotypes of the human gut microbiome. Nature; 473(7346): 174 – 180.

Bäckhed, F., Fraser, C. M., Ringel, Y., Sanders, M. E., Sartor, R. B., Sherman, P. M., *et al.* (2012). Defining a healthy human gut microbiome: current concepts, future directions, and clinical applications. Cell host & microbe; 12(5): 611 – 622.

Balmer, S. E., & Wharton, B. A. (1989). Diet and faecal flora in the newborn: breast milk and infant formula. Archives of Disease in Childhood; 64(12): 1672 – 1677.

Banks, W. A. (1980). Evidence for a cholecystokinin gut-brain axis with modulation by bombesin. Peptides; 1(4): 347 – 351.

Beards E., Tuohy K., Gibson G. (2010). Bacterial, SCFA and gas profiles of a range of food ingredients following in-vitro fermentation by human colonic microbiota. Anaerobe; 16(4): 420 – 425.

Belenguer, A., Duncan, S. H., Calder, A. G., Holtrop, G., Louis, P., Lobley, G. E., & Flint, H. J. (2006). Two routes of metabolic cross-feeding between Bifidobacterium adolescentis and butyrate-producing anaerobes from the human gut. Applied and environmental microbiology; 72(5): 3593-3599.

Bercik, P., Collins, S. M., & Verdu, E. F. (2012). Microbes and the gut-brain axis. Neurogastroenterology & Motility; 24(5): 405 – 413.

Bergström, A., Skov, T. H., Bahl, M. I., Roager, H. M., Christensen, L. B., Ejlerskov, K. T., *et al.* (2014). Establishment of intestinal microbiota during early life: a longitudinal, explorative study of a large cohort of Danish infants. Applied and environmental microbiology; 80(9): 2889 – 2900.

Bik, E.M., Eckburg, P.B., Gill, S.R., Nelson, K.E., Purdom, E.A., Francois, F., *et al.*, (2006). Molecular analysis of the bacterial microbiota in the human stomach. Proceedings of the National Academy of Sciences of the United States of America; 103(3): 732 – 737.

Bik, E.M., Long C.D., Armitage G.C., Loomer P., Emerson J., Mongodin E.F., *et al.* (2010). Bacterial diversity in the oral cavity of 10 healthy individuals. The ISME Journal; 4(8): 962 – 974.

BOE. Boletin Oficial del Estado (2008). Real Decreto 867/2008, de 23 de mayo, por el que se aprueba la reglamentación técnico-sanitaria específica de los preparados para lactantes y de los preparados de continuación. Disponible en: https://www.boe.es/boe/dias/2008/05/30/pdfs/A25121-25137.pdf

Booijink, C. C., El-Aidy, S., Rajilić-Stojanović, M., Heilig, H. G., Troost, F. J., Smidt, H., *et al.* (2010). High temporal and inter-individual variation detected in the human ileal microbiota. Environmental microbiology; 12(12): 3213 – 3227.

Boyce, J. M., & Pittet, D. (2002). Guideline for hand hygiene in health-care settings: recommendations of the Healthcare Infection Control Practices Advisory Committee and the HICPAC/SHEA/APIC/IDSA Hand Hygiene Task Force. American journal of infection control; 30(8): 1 – 46.

Braegger, C., Chmielewska, A., Decsi, T., Kolacek, S., Mihatsch, W., Moreno, L., *et*

***al.* (2011).** Supplementation of infant formula with probiotics and/or prebiotics: a systematic review and comment by the ESPGHAN committee on nutrition. Journal of pediatric gastroenterology and nutrition; 52(2): 238 – 250.

Brown I, Warhurst M, Arcot J, Playne M, Illman RJ & Topping DL. (1997). Faecal numbers of Bifidobacteria are higher in pigs fed Bifidobacterium longum with a high amylose cornstarch than with a low amylose cornstarch. Journal of Nutrition; 127: 1822 – 1827.

Brown IL, Wang X, Topping DL, Playne MJ & Conway PL (1998). High amylose maize starch as a versatile prebiotic for use with probiotic bacteria. Food Australia; 50: 602 – 609.

Buffie, C. G., & Pamer, E. G. (2013). Microbiota-mediated colonization resistance against intestinal pathogens. Nature Reviews Immunology; 13(11): 790 – 801.

Cardona, F., Andrés-Lacueva, C., Tulipani, S., Tinahones, F. J., & Queipo-Ortuño, M. I. (2013). Benefits of polyphenols on gut microbiota and implications in human health. The Journal of nutritional biochemistry; 24(8): 1415 – 1422.

Cash, H. L., Whitham, C. V., Behrendt, C. L., & Hooper, L. V. (2006). Symbiotic bacteria direct expression of an intestinal bactericidal lectin. Science; 313(5790): 1126 – 1130.

Cenit, M. C., Olivares, M., Codoñer-Franch, P., & Sanz, Y. (2015). Intestinal microbiota and celiac disease: cause, consequence or co-evolution?. Nutrients; 7(8): 6900 – 6923.

Charrier, J. A., Martin, R. J., McCutcheon, K. L., Raggio, A. M., Goldsmith, F., Goita, M. F., *et al.* (2013). High fat diet partially attenuates fermentation responses in rats fed resistant starch from high-amylose maize. Obesity; 21(11): 2350 – 2355.

Chow, J. M., Shen, S. C., Huan, S. K., Lin, H. Y., & Chen, Y. C. (2005). Quercetin, but not rutin and quercitrin, prevention of H 2 O 2-induced apoptosis via anti-oxidant activity and heme oxygenase 1 gene expression in macrophages. Biochemical pharmacology; 69(12): 1839 – 1851.

Clemente, J. C., Ursell, L. K., Parfrey, L. W., & Knight, R. (2012). The impact of the gut microbiota on human health: an integrative view. Cell; 148(6): 1258 – 1270.

Collado, M. C., Calabuig, M., & Sanz, Y. (2007). Differences between the fecal microbiota of coeliac infants and healthy controls. Current issues in intestinal microbiology; 8(1): 9-14.

Collado, M. C., Delgado, S., Maldonado, A., & Rodríguez, J. M. (2009). Assessment of the bacterial diversity of breast milk of healthy women by quantitative real-time PCR. Letters in Applied Microbiology; 48(5): 523 – 528.

Collado, M. C., Isolauri, E., Laitinen, K., & Salminen, S. (2010). Effect of mother's weight on infant's microbiota acquisition, composition, and activity during early infancy: a prospective follow-up study initiated in early pregnancy. The American journal of clinical nutrition; 92(5): 1023 – 1030.

Conlon, M. A., & Bird, A. R. (2015). The impact of diet and lifestyle on gut microbiota and human health. Nutrients; 7(1): 17 – 44.

Crociani, F., Alessandrini, A., Mucci, M. M., & Biavati, B. (1994). Degradation of complex carbohydrates by Bifidobacterium spp. International journal of food microbiology; 24(1-2): 199 – 210.

Cryan, J. F., & Dinan, T. G. (2012). Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nature reviews neuroscience; 13(10): 701 – 712.

Cummings, J. H., Macfarlane, G. T., & Englyst, H. N. (2001). Prebiotic digestion and fermentation. The American journal of clinical nutrition; 73(2):415-420.

Cummings, J. H., Pomare, E. W., Branch, W. J., Naylor, C. P., & Macfarlane, G. T. (1987). Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut; 28(10): 1221 – 1227.

Dasanayake, A. P., Li, Y., Wiener, H., Ruby, J. D., & Lee, M. J. (2005). Salivary Actinomyces naeslundii genospecies 2 and Lactobacillus casei levels predict pregnancy outcomes. Journal of periodontology; 76(2): 171 – 177.

de Goffau, M. C., Fuentes, S., van den Bogert, B., Honkanen, H., de Vos, W. M., Welling, G. W., *et al.* (2014). Aberrant gut microbiota composition at the onset of type 1 diabetes in young children. Diabetologia; 57(8): 1569 – 1577.

den Besten, G., van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D. J., & Bakker, B. M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. Journal of lipid research, 54(9): 2325 – 2340.

Deschemin, J. C., Noordine, M. L., Remot, A., Willemetz, A., Afif, C., Canonne-Hergaux, F., *et al.* (2016). The microbiota shifts the iron sensing of intestinal cells. The FASEB Journal; 30(1): 252 – 261.

Di Giulio, D. B., Romero, R., Amogan, H. P., Kusanovic, J. P., Bik, E. M., Gotsch, F., *et al.* (2008). Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. PloS one; 3(8): 1-10.

Di Giulio, D. B., Romero, R., Kusanovic, J. P., Gómez, R., Kim, C. J., Seok, K. S., *et al.* (2010). Prevalence and diversity of microbes in the amniotic fluid, the fetal inflammatory response, and pregnancy outcome in women with preterm pre-labor rupture of membranes. American journal of reproductive immunology; 64(1): 38 – 57.

Dominguez-Bello, M. G., Costello, E. K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., & Knight, R. (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proceedings of the National Academy of Sciences; 107(26): 11971 – 11975.

Doré, J., & Blottiere, H. (2015). The influence of diet on the gut microbiota and its consequences for health. Current opinion in biotechnology; 32: 195 – 199.

Echarri, P. P., Graciá, C. M., Berruezo, G. R., Vives, I., Ballesta, M., Solís, G., *et al.* (2011). Assessment of intestinal microbiota of full-term breast-fed infants from two different geographical locations. Early human development; 87(7): 511 – 513.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., *et al.* (2005). Diversity of the human intestinal microbial flora. Science; 308(5728): 1635 – 1638.

El Aidy, S., van den Bogert, B., & Kleerebezem, M. (2015). The small intestine microbiota, nutritional modulation and relevance for health. Current opinion in biotechnology; 32: 14 – 20.

Fallani, M., Young, D., Scott, J., Norin, E., Amarri, S., Adam, R., *et al.* (2010). Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. Journal of pediatric gastroenterology and nutrition; 51(1): 77 – 84.

Falony, G., Vlachou, A., Verbrugghe, K., & De Vuyst, L. (2006). Cross-feeding between Bifidobacterium longum BB536 and acetate-converting, butyrate-producing colon bacteria during growth on oligofructose. Applied and environmental microbiology; 72(12): 7835-7841.

Fanaro, S., Chierici, R., Guerrini, P., & Vigi, V. (2003). Intestinal microflora in early infancy: composition and development. Acta paediatrica; 92(441): 48 – 55.

Fernandez, L., Langa, S., Martín, V., Maldonado, A., Jiménez, E., Martín, R., & Rodríguez, J. M. (2013). The human milk microbiota: origin and potential roles in health and disease. Pharmacological Research; 69(1): 1 – 10.

Fernandez, M. I., Pedron, T., Tournebize, R., Olivo-Marin, J. C., Sansonetti, P. J., & Phalipon, A. (2003). Anti-inflammatory role for intracellular dimeric immunoglobulin a by neutralization of lipopolysaccharide in epithelial cells. Immunity; 18(6): 739 – 749.

Fichorova, R. N., Onderdonk, A. B., Yamamoto, H., Delaney, M. L., DuBois, A. M., Allred, E., & Leviton, A. (2011). Maternal microbe-specific modulation of inflammatory response in extremely low-gestational-age newborns. MBio; 2(1): 1 – 10.

Finegold, S. M., Dowd, S. E., Gontcharova, V., Liu, C., Henley, K. E., Wolcott, R. D., *et al.* (2010). Pyrosequencing study of fecal microflora of autistic and control children. Anaerobe; 16(4): 444 – 453.

Fraga, C. G., Galleano, M., Verstraeten, S. V., & Oteiza, P. I. (2010). Basic biochemical mechanisms behind the health benefits of polyphenols. Molecular aspects of medicine: 31(6): 435 – 445.

Frank, D. N., Amand, A. L. S., Feldman, R. A., Boedeker, E. C., Harpaz, N., & Pace, N. R. (2007). Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proceedings of the National Academy of Sciences; 104(34): 13780 – 13785.

Gagnon, M., Kheadr, E. E., & Le Blay, G. (2004). In-vitro inhibition of Escherichia coli O157: H7 by bifidobacterial strains of human origin. International journal of food microbiology; 92(1): 69 – 78.

Garn, H., & Renz, H. (2007). Epidemiological and immunological evidence for the hygiene hypothesis. Immunobiology; 212(6): 441 – 452.

Gaykema, R. P., Goehler, L. E., & Lyte, M. (2004). Brain response to cecal infection with Campylobacter jejuni: analysis with Fos immunohistochemistry. Brain, behavior, and immunity; 18(3): 238 – 245.

Gibson, G. R., Probert, H. M., Van Loo, J., Rastall, R. A., & Roberfroid, M. B. (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutrition research reviews, 17(02), 259-275.

Gietl, E., Mengerink, W., de Slegte, J., Gibson, G., Rastall, R., & van den Heuvel, E. (2012). Factors involved in the in-vitro fermentability of short carbohydrates in static faecal batch cultures. International Journal of Carbohydrate Chemistry. (2012), Article ID 197809, 10 pages. Available in: https://www.hindawi.com/journals/ijcc/2012/197809/abs/

Glenn, G. R., & Roberfroid, M. B. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. Journal of nutrition; 125: 1401 – 1412.

Goodlad, R. A., & Englyst, H. N. (2001). Redefining dietary fibre: potentially a recipe for disaster. The Lancet; 358(9296): 1833 – 1834.

Grabitske, H. A., & Slavin, J. L. (2009). Gastrointestinal effects of low-digestible carbohydrates. Critical reviews in food science and nutrition; 49(4): 327 – 360.

Grider, J. R., & Piland, B. E. (2007). The peristaltic reflex induced by short-chain fatty acids is mediated by sequential release of 5-HT and neuronal CGRP but not BDNF. American Journal of Physiology-

Gastrointestinal and Liver Physiology; 292(1): 429 – 437.

Gueimonde, M., Tölkkö, S., Korpimäki, T., & Salminen, S. (2004). New real-time quantitative PCR procedure for quantification of bifidobacteria in human fecal samples. Applied and Environmental Microbiology; 70(7): 4165-4169.

Gunjan, K., Anupam, K. S., & Arvind, K. (2016). Intravaginal Pessary Containing lactobacillus, Clindamycin, Clotrimazole decreases the Rate of preterm delivery and Incidence of Low Birth Weight in Asymptomatic Mothers with Abnormal Vaginal Flora in Early Pregnancy. International Journal of Health Sciences and Research; 6(4): 72 – 79.

Hansen, L. K., Becher, N., Bastholm, S., Glavind, J., Ramsing, M., Kim, C. J., *et al.* (2014). The cervical mucus plug inhibits, but does not block, the passage of ascending bacteria from the vagina during pregnancy. Acta obstetricia et gynecologica Scandinavica; 93(1): 102 – 108.

Harmsen, H. J., Wildeboer–Veloo, A. C., Raangs, G. C., Wagendorp, A. A., Klijn, N., Bindels, J. G., & Welling, G. W. (2000). Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. Journal of pediatric gastroenterology and nutrition: 30(1); 61 – 67.

Harwig, S. S., Tan, L., Qu, X. D., Cho, Y. O. O. N., Eisenhauer, P. B., & Lehrer, R. I. (1995). Bactericidal properties of murine intestinal phospholipase A2. Journal of Clinical Investigation; 95(2): 603.

Hayashi, H., Takahashi, R., Nishi, T., Sakamoto, M., & Benno, Y. (2005). Molecular analysis of jejunal, ileal, caecal and recto-sigmoidal human colonic microbiota using 16S rRNA gene libraries and terminal restriction fragment length polymorphism. Journal of medical microbiology; 54(11): 1093 – 1101.

Hitti, J. (2001) Vaginal indicators of amniotic fluid infection in preterm labor. Obstetric and Gynecoly; 97: 211 – 219.

Hooper, L. V., Littman, D. R., & Macpherson, A. J. (2012). Interactions between the microbiota and the immune system. Science; 336(6086): 1268 – 1273.

Hosseini, E., Grootaert, C., Verstraete, W., & Van de Wiele, T. (2011). Propionate as a health-promoting microbial metabolite in the human gut. Nutrition Reviews; 69(5): 245-258.

Hunt, K. M., Foster, J. A., Forney, L. J., Schütte, U. M., Beck, D. L., Abdo, Z., *et al.* (2011). Characterization of the diversity and temporal stability of bacterial communities in human milk. PloS one; 6(6): 1 – 8.

Hurst, N. R., Kendig, D. M., Murthy, K. S., & Grider, J. R. (2014). The short chain fatty acids, butyrate and propionate, have differential effects on the motility of the guinea pig colon. Neurogastroenterology & Motility; 26(11): 1586 – 1596.

Jakobsson, H. E., Abrahamsson, T. R., Jenmalm, M. C., Harris, K., Quince, C., Jernberg, C., ... & Andersson, A. F. (2014). Decreased gut microbiota diversity, delayed Bacteroidetes colonisation and reduced Th1 responses in infants delivered by caesarean section. Gut; 63(4): 559 – 566.

Jandhyala, S. M., Talukdar, R., Subramanyam, C., Vuyyuru, H., Sasikala, M., & Reddy, D. N. (2015). Role of the normal gut microbiota. World journal of gastroenterology – WJG; 21(29): 8787 – 8803.

Jiménez, E., Marín, M. L., Martín, R., Odriozola, J. M., Olivares, M., Xaus, J., *et al.* (2008). Is meconium from healthy newborns actually sterile?. Research in microbiology; 159(3): 187 – 193.

Jost, T., Lacroix, C., Braegger, C. P., & Chassard, C. (2012). New insights in gut

microbiota establishment in healthy breast fed neonates. PloS one; 7(8): 1 – 9.

Kalmokoff, M., Zwicker, B., O'hara, M., Matias, F., Green, J., Shastri, P., ***et al.*** **(2013).** Temporal change in the gut community of rats fed high amylose cornstarch is driven by endogenous urea rather than strictly on carbohydrate availability. Journal of applied microbiology; 114(5): 1516 – 1528.

Kaur, A., Rose, D. J., Rumpagaporn, P., Patterson, J. A., & Hamaker, B. R. (2011). In-vitro Batch Faecal Fermentation Comparison of Gas and Short-Chain Fatty Acid Production Using "Slowly Fermentable" Dietary Fibers. Journal of food science, 76(5): 137 - 142.

Khodayar-Pardo, P., Mira-Pascual, L., Collado, M. C., & Martinez-Costa, C. (2014). Impact of lactation stage, gestational age and mode of delivery on breast milk microbiota. Journal of Perinatology; 34(8): 599 – 605.

Kumar, H., Rautava, S., Collado, M., Borzykh, N., Löyttyniemi, E., Isolauri, E., & Salminen, S. (2015). Neonatal Antibiotic Exposure Alters Compositional Gut Microbiota Development During the First 6 Months of Life. The FASEB Journal; 29(1): 575 – 583.

Kang, M. J., Kim, H. G., Kim, J. S., Oh, D. G., Um, Y. J., Seo, C. S., ... & Jeong, H. G. (2013). The effect of gut microbiota on drug metabolism. Expert opinion on drug metabolism & toxicology; 9(10): 1295 – 1308.

Karlsson, F. H., Tremaroli, V., Nookaew, I., Bergström, G., Behre, C. J., Fagerberg, B., ***et al.*** **(2013).** Gut metagenome in European women with normal, impaired and diabetic glucose control. Nature; 498(7452): 99 – 103.

Khurana, S., Venkataraman, K., Hollingsworth, A., Piche, M., & Tai, T. C. (2013). Polyphenols: benefits to the cardiovascular system in health and in aging. Nutrients, 5(10): 3779 – 3827.

Koenig, J. E., Spor, A., Scalfone, N., Fricker, A. D., Stombaugh, J., Knight, R., ***et al.*** **(2011).** Succession of microbial consortia in the developing infant gut microbiome. Proceedings of the National Academy of Sciences; 108(1): 4578 – 4585.

Lay, C., Rigottier-Gois, L., Holmstrøm, K., Rajilic, M., Vaughan, E. E., de Vos, W. M., ***et al.*** **(2005).** Colonic microbiota signatures across five northern European countries. Applied and Environmental Microbiology; 71(7): 4153 – 4155.

Lyte, M., Li, W., Opitz, N., Gaykema, R. P., & Goehler, L. E. (2006). Induction of anxiety-like behavior in mice during the initial stages of infection with the agent of murine colonic hyperplasia Citrobacter rodentium. Physiology & behavior; 89(3): 350 – 357.

Macfarlane, S. M. G. T., Macfarlane, G. T., & Cummings, J. T. (2006). Review article: prebiotics in the gastrointestinal tract. Alimentary pharmacology & therapeutics, 24(5), 701-714.

Macpherson, A. J., & Harris, N. L. (2004). Interactions between commensal intestinal bacteria and the immune system. Nature Reviews Immunology; 4(6): 478 – 485.

Mackie, R.I., Sghir, A., Gaskins, H.R. (1999). Developmental microbial ecology of the neonatal gastrointestinal tract. Americal Journal of Clinical Nutrition; 69(5): 1035 – 1045.

Manderson K., Pinart M., Tuohy K.M., Grace W.E., Hotchkiss A.T., Widmer W., ***et al.*** **(2005).** In-vitro determination of prebiotic properties of oligosaccharides derived from an orange juice manufacturing by product stream. Applied and Environmental Microbiology; 71(12): 8383 – 8389.

Marín, L., Miguélez, E. M., Villar, C. J., & Lombó, F. (2015). Bioavailability of dietary

polyphenols and gut microbiota metabolism: antimicrobial properties. BioMed research international; 2015: 1 – 18.

Marques, T. M., Wall, R., Ross, R. P., Fitzgerald, G. F., Ryan, C. A., & Stanton, C. (2010). Programming infant gut microbiota: influence of dietary and environmental factors. Current opinion in biotechnology; 21(2): 149 – 156.

Matamoros, S., Gras-Leguen, C., Le Vacon, F., Potel, G., & de La Cochetiere, M. F. (2013). Development of intestinal microbiota in infants and its impact on health. Trends in microbiology; 21(4): 167 – 173.

Matsuda, K., Tsuji, H., Asahara, T., Kado, Y., & Nomoto, K. (2007). Sensitive quantitative detection of commensal bacteria by rRNA-targeted reverse transcription-PCR. Applied and environmental microbiology; 73(1): 32 – 39.

Matsuki, T., Watanabe, K., Fujimoto, J., Takada, T., & Tanaka, R. (2004). Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. Applied and Environmental Microbiology; 70(12): 7220-7228.

Mazmanian, S. K., Liu, C. H., Tzianabos, A. O., & Kasper, D. L. (2005). An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell; 122(1): 107 – 118.

Meunier, L., Garthoff, J. A., Schaafsma, A., Krul, L., Schrijver, J., van Goudoever, J. B., *et al*. (2014). Locust bean gum safety in neonates and young infants: an integrated review of the toxicological database and clinical evidence. Regulatory Toxicology and Pharmacology; 70(1): 155 - 169.

Meyer, D., & Stasse-Wolthuis, M. (2009). The bifidogenic effect of inulin and oligofructose and its consequences for gut health. European journal of clinical nutrition; 63(11): 1277 – 1289.

Million, M., Lagier, J. C., Yahav, D., & Paul, M. (2013). Gut bacterial microbiota and obesity. Clinical Microbiology and Infection; 19(4): 305-313.

Mirpuri, J., Raetz, M., Sturge, C. R., Wilhelm, C. L., Benson, A., Savani, R. C., *et al*. (2014). Proteobacteria-specific IgA regulates maturation of the intestinal microbiota. Gut microbes; 5(1): 28 – 39.

Moles, L., Gomez, M., Heilig, H., Bustos, G., Fuentes, S., de Vos, W., *et al*. (2013). Bacterial diversity in meconium of preterm neonates and evolution of their fecal microbiota during the first month of life. PloS one; 8(6): 1 – 13.

Morpeth, S. C., Huggett, J. F., Murdoch, D. R., & Scott, J. A. G. (2014). Making standards for quantitative real-time pneumococcal PCR. Biomolecular Detection and Quantification; 2: 1 – 3.

Murakami, A. (2014). Dose-dependent functionality and toxicity of green tea polyphenols in experimental rodents. Archives of biochemistry and biophysics, 557, 3-10.

Murri, M., Leiva, I., Gomez-Zumaquero, J. M., Tinahones, F. J., Cardona, F., Soriguer, F., & Queipo-Ortuño, M. I. (2013). Gut microbiota in children with type 1 diabetes differs from that in healthy children: a case-control study. BMC medicine; 11(1): 1 – 12.

Mussatto, S. I., & Mancilha, I. M. (2007). Non-digestible oligosaccharides: a review. Carbohydrate polymers; 68(3): 587 – 597.

Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., & Pettersson, S. (2012). Host-gut microbiota metabolic interactions. Science; 336(6086): 1262 – 1267.

Niness, K. R. (1999). inulin and oligofructose: what are they?. The Journal of nutrition; 129(7): 1402 - 1406.

O'Hara, A. M., & Shanahan, F. (2006). The gut flora as a forgotten organ. EMBO reports; 7(7): 688-693.

Olano-Martin E., Mountzouris K.C., Gibson G.R., Rastall R.A. (2000). In-vitro fermentability of dextran, oligodextran and maltodextrin by human gut bacteria. British Journal of Nutrition; 83(3): 247 – 255.

Olano-Martín E., Gibson G.R., Rastall R.A. (2002). Comparison of the in-vitro bifidogenic properties of pectins and pectic-oligosaccharides. Journal of Applied microbiology; 95(3): 505 – 511.

Ottman, N., Smidt, H., De Vos, W. M., & Belzer, C. (2012). The function of our microbiota: who is out there and what do they do?. Frontiers in cellular and infection microbiology; 2: 1 – 11.

Pabst, O. (2012). New concepts in the generation and functions of IgA. Nature Reviews Immunology; 12(12): 821 – 832.

Palestrant, D., Holzknecht, Z. E., Collins, B. H., Parker, W., Miller, S. E., & Bollinger, R. R. (2004). Microbial biofilms in the gut: visualization by electron microscopy and by acridine orange staining. Ultrastructural pathology; 28(1): 23 – 27.

Palmer, C., Bik, E. M., DiGiulio, D. B., Relman, D. A., & Brown, P. O. (2007). Development of the human infant intestinal microbiota. PLoS Biology; 5(7): 1556 – 1573.

Parkar, S. G., Trower, T. M., & Stevenson, D. E. (2013). Faecal microbial metabolism of polyphenols and its effects on human gut microbiota. Anaerobe, 23, 12-19.

Patterson, E., Cryan, J. F., Fitzgerald, G. F., Ross, R. P., Dinan, T. G., & Stanton, C. (2014). Gut microbiota, the pharmabiotics they produce and host health. Proceedings of the Nutrition Society; 73(04): 477 – 489.

Pei Z, Bini EJ, Yang L, Zhou M, Francois F, et al. (2004). Bacterial biota in the human distal esophagus. Proc Natl Acad Sci; 101: 4250 – 4255.

Penders, J., Thijs, C., Vink, C., Stelma, F. F., Snijders, B., Kummeling, I., *et al.* (2006). Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics; 118(2): 511 – 521.

Penders, J., Vink, C., Driessen, C., London, N., Thijs, C., & Stobberingh, E. E. (2005). Quantification of Bifidobacterium spp., Escherichia coli and Clostridium difficile in faecal samples of breast-fed and formula-fed infants by real-time PCR. FEMS microbiology letters; 243(1): 141 – 147.

Planer, J. D., Peng, Y., Kau, A. L., Blanton, L. V., Ndao, I. M., Tarr, P. I., *et al.* (2016). Development of the gut microbiota and mucosal IgA responses in twins and gnotobiotic mice. Nature; 534(7606): 263 – 266.

Pompei, A., Cordisco, L., Amaretti, A., Zanoni, S., Matteuzzi, D., & Rossi, M. (2007). Folate production by bifidobacteria as a potential probiotic property. Applied and environmental microbiology; 73(1): 179 – 185.

Puertollano, E., Kolida, S., & Yaqoob, P. (2014). Biological significance of short-chain fatty acid metabolism by the intestinal microbiome. Current Opinion in Clinical Nutrition & Metabolic Care; 17(2): 139 – 144.

Putignani, L., Del Chierico, F., Petrucca, A., Vernocchi, P., & Dallapiccola, B. (2014). The human gut microbiota: a dynamic interplay with the host from birth to senescence settled during childhood. Pediatric research; 76(1): 2-10.

Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., *et al.* (2012). A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature; 490(7418): 55 – 60.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., *et al.* (2010). A human gut microbial gene

catalogue established by metagenomic sequencing. Nature; 464(7285): 59 – 65.

Quigley, E. M. (2010). Prebiotics and probiotics; modifying and mining the microbiota. Pharmacological research; 61(3): 213 – 218.

Rafii, F., Wynne, R., Heinze, T. M., & Paine, D. D. (2003). Mechanism of metronidazole-resistance by isolates of nitroreductase-producing Enterococcus gallinarum and Enterococcus casseliflavus from the human intestinal tract. FEMS microbiology letters, 225(2), 195-200.

Rajilić-Stojanović, M., Heilig, H. G., Molenaar, D., Kajander, K., Surakka, A., Smidt, H., *et al.* (2009). Development and application of the human intestinal tract chip, a phylogenetic microarray: analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. Environmental microbiology; 11(7): 1736-1751.

Ramakrishna, B. S. (2013). Role of the gut microbiota in human nutrition and metabolism. Journal of gastroenterology and Hepatology; 28(4): 9 – 17.

Raman, M., Ambalam, P., & Doble, M. (2016). Short-chain fatty acids. In Probiotics and Bioactive Carbohydrates. In "Colon Cancer Management": 97 – 115. Springer. India.

Ramirez-Farias, C., Slezak, K., Fuller, Z., Duncan, A., Holtrop, G., & Louis, P. (2009). Effect of inulin on the human gut microbiota: stimulation of Bifidobacterium adolescentis and Faecalibacterium prausnitzii. British Journal of Nutrition; 101(04): 541 – 550.

Raninen, K., Lappi, J., Mykkänen, H., & Poutanen, K. (2011). Dietary fiber type reflects physiological functionality: comparison of grain fiber, inulin, and polydextrose. Nutrition reviews; 69(1): 9 – 21.

Rechner, A. R., Smith, M. A., Kuhnle, G., Gibson, G. R., Debnam, E. S., Srai, S. K. S., *et al*. (2004). Colonic metabolism of dietary polyphenols: influence of structure on microbial fermentation products. Free Radical Biology and Medicine; 36(2): 212 – 225.

Rinne, M., Kalliomaki, M., Arvilommi, H., Salminen, S., & Isolauri, E. (2005). Effect of probiotics and breastfeeding on the bifidobacterium and lactobacillus/enterococcus microbiota and humoral immune responses. The Journal of pediatrics; 147(2): 186 – 191.

Rinttilä, T., Kassinen, A., Malinen, E., Krogius, L., & Palva, A. (2004). Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. Journal of applied microbiology; 97(6): 1166 – 1177.

Rodríguez, J. M., Murphy, K., Stanton, C., Ross, R. P., Kober, O. I., Juge, N., *et al.* (2015). The composition of the gut microbiota throughout life, with an emphasis on early life. Microbial ecology in health and disease; 26(2015): 1 – 17.

Rooks, M. G., & Garrett, W. S. (2016). Gut microbiota, metabolites and host immunity. Nature Reviews Immunology; 16(6): 341 – 352.

Rousseau, C., Levenez, F., Fouqueray, C., Doré, J., Collignon, A., & Lepage, P. (2011). *Clostridium difficile* colonization in early infancy is accompanied by changes in intestinal microbiota composition. Journal of clinical microbiology; 49(3): 858 – 865.

Russell, S. L., Gold, M. J., Willing, B. P., Thorson, L., McNagny, K. M., & Finlay, B. B. (2013). Perinatal antibiotic treatment affects murine microbiota, immune responses and allergic asthma. Gut Microbes; 4(2): 158 – 164.

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. Journal of Applied Microbiology; 91(5): 878 – 887.

Sabater, C., Prodanov, M., Olano, A., Corzo, N., & Montilla, A. (2016). Quantification of prebiotics in commercial infant formulas. Food chemistry; 194: 6-11.

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. Applied Environmental Microbiology; 74(15): 4737 – 4745.

Salminen, S., & Isolauri, E. (2006). Intestinal colonization, microbiota, and probiotics. The Journal of pediatrics; 149(5): 115 – 120.

Sambanthamoorthy, K., Feng, X., Patel, R., Patel, S., & Paranavitana, C. (2014). Antimicrobial and antibiofilm potential of biosurfactants isolated from lactobacilli against multi-drug-resistant pathogens. BMC microbiology; 14(1): 1 – 9.

Sarbini R.S., Kolida S., Naeye T., Einerhand A., Brison Y., Remaud-Simeon M., *et al.* (2011). In-vitro fermentation of linear and α-1,2 branched dextrans by the human faecal microbiota. Applied and Environmental Microbiology; 77: 5307 – 5315.

Schulman, J., Dimand, R. J., Lee, H. C., Duenas, G. V., Bennett, M. V., & Gould, J. B. (2015). Neonatal intensive care unit antibiotic use. Pediatrics; 135(5): 826 – 833.

Scott, K. P., Gratz, S. W., Sheridan, P. O., Flint, H. J., & Duncan, S. H. (2013). The influence of diet on the gut microbiota. Pharmacological research; 69(1): 52 – 60.

Sekirov, I., Russell, S.L., Antunes, L.C.M., & Finlay, B.B. (2010). Gut microbiota in health and disease. Physiological reviews; 90(3): 859 – 904.

Simeoni, U., Berger, B., Junick, J., Blaut, M., Pecquet, S., Rezzonico, E., *et al.* (2016). Gut microbiota analysis reveals a marked shift to bifidobacteria by a starter infant formula containing a synbiotic of bovine milk-derived oligosaccharides and Bifidobacterium animalis subsp. lactis CNCM I-3446. Environmental Microbiology; 18 (7): 2185 – 2195.

Simón-Soro, A., Tomás, I., Cabrera-Rubio, R., Catalan, M. D., Nyvad, B., & Mira, A. (2013). Microbial geography of the oral cavity. Journal of dental research; 92(7): 616 – 621.

Sittikijyothin, W., Torres, D. & Gonçalves, M.P. (2005). Modelling the rheological behavior of galctomannan aqueous solutions. Carbohydrate Polymers; 59: 339 – 350.

Skibola CF, & Smith MT. (2000). Potential health impacts of excessive flavonoid intake. Free Radical Biology and Mededicine; 29(3-4): 375 – 383.

Sousa, T., Paterson, R., Moore, V., Carlsson, A., Abrahamsson, B., & Basit, A. W. (2008). The gastrointestinal microbiota as a site for the biotransformation of drugs. International journal of pharmaceutics; 363(1): 1 – 25.

Stecher, B., & Hardt, W. D. (2008). The role of microbiota in infectious disease. Trends in microbiology; 16(3): 107 – 114.

Stilling, R. M., Dinan, T. G., & Cryan, J. F. (2014). Microbial genes, brain & behaviour–epigenetic regulation of the gut–brain axis. Genes, Brain and Behavior; 13(1): 69 - 86.

Stout, M. J., Conlon, B., Landeau, M., Lee, I., Bower, C., Zhao, Q., *et al.* (2013). Identification of intracellular bacteria in the basal plate of the human placenta in term and preterm gestations. American Journal of Obstetrics and Gynecology; 208(3): 1 – 14.

Strachan, D. P. (1989). Hay fever, hygiene, and household size. BMJ: British Medical Journal; 299(6710): 1259 – 1260.

Suzuki, T., Yoshida, S., & Hara, H. (2008). Physiological concentrations of short-chain fatty acids immediately suppress colonic epithelial permeability. British journal of nutrition; 100(02): 297 – 305.

Swidsinski, A., Loening-Baucke, V., Lochs, H., & Hale, L.P. (2005). Spatial organization of bacterial flora in normal and inflamed intestine: a fluorescence in situ hybridization study in mice. World J Gastroenterol; 11(8): 1131 – 1140.

Swidsinski, A., Sydora, B. C., Doerffel, Y., Loenin-Baucke, V., Vaneechoutte, M., Lupicki, M., *et al*. (2007). Viscosity gradient within the mucus layer determines the mucosal barrier function and the spatial organization of the intestinal microbiota. Inflammatory bowel diseases; 13(8): 963 – 970.

Tan, J., McKenzie, C., Potamitis, M., Thorburn, A. N., Mackay, C. R., & Macia, L. (2014). The role of short-chain fatty acids in health and disease. Adv Immunol; 121(9): 92 – 112.

Topping, D. L., Fukushima, M., & Bird, A. R. (2003). Resistant starch as a prebiotic and synbiotic: state of the art. Proceedings-nutrition society of London; 62(1): 171 – 176.

Trejo, F. M., Minnaard, J., Perez, P. F., & De Antoni, G. L. (2006). Inhibition of Clostridium difficile growth and adhesion to enterocytes by Bifidobacterium supernatants. Anaerobe; 12(4): 186 – 193.

Tsuji, M., Suzuki, K., Kinoshita, K., & Fagarasan, S. (2008). Dynamic interactions between bacteria and immune cells leading to intestinal IgA synthesis. Seminars in immunology; 20(1): 59 – 66.

Tungland, B. C., & Meyer, D. (2002). Nondigestible oligo-and polysaccharides (Dietary Fiber): their physiology and role in human health and food. Comprehensive reviews in food science and food safety; 1(3): 90 – 109.

Tzounis, X., Rodriguez-Mateos, A., Vulevic, J., Gibson, G. R., Kwik-Uribe, C., & Spencer, J. P. (2011). Prebiotic evaluation of cocoa-derived flavanols in healthy humans by using a randomized, controlled, double-blind, crossover intervention study. The American journal of clinical nutrition; 93(1): 62 – 72.

Vael, C., & Desager, K. (2009). The importance of the development of the intestinal microbiota in infancy. Current opinion in pediatrics; 21(6): 794 – 800.

Vandenplas, Y., Rudolph, C. D., Di Lorenzo, C., Hassall, E., Liptak, G., Mazur, L., *et al*. (2009). Pediatric gastroesophageal reflux clinical practice guidelines: joint recommendations of the North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition (NASPGHAN) and the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN). Journal of pediatric gastroenterology and nutrition; 49(4): 498 – 547.

Van der Waaij, D., Berghuis-de Vries, J. M., & Lekkerkerk-Van der Wees, J. E. C. (1971). Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. Journal of Hygiene; 69(03): 405 – 411.

Venema, K. (2016). Infant gut microbiota, human milk oligosaccharides and prebiotics–what's new?. Journal of Pediatric Gastroenterology and Nutrition; 63(1): 44.

Vinturache, A. E., Gyamfi-Bannerman, C., Hwang, J., Mysorekar, I. U., Jacobsson, B., & Collaborative, T. P. B. I. (2016). Maternal microbiome–A pathway to preterm birth. Seminars in Fetal and Neonatal Medicine; 21(2): 94 – 99.

Viveros, A., Chamorro, S., Pizarro, M., Arija, I., Centeno, C., & Brenes, A. (2011). Effects of dietary polyphenol-rich grape

products on intestinal microflora and gut morphology in broiler chicks. Poultry science; 90(3): 566 – 578.

Wacklin, P., Kaukinen, K., Tuovinen, E., Collin, P., Lindfors, K., Partanen, J., ... & Mättö, J. (2013). The duodenal microbiota composition of adult celiac disease patients is associated with the clinical manifestation of the disease. Inflammatory bowel diseases; 19(5): 934 – 941.

Walker, A. W., Ince, J., Duncan, S. H., Webster, L. M., Holtrop, G., Ze, X., *et al*. (2011). Dominant and diet-responsive groups of bacteria within the human colonic microbiota. The ISME journal; 5(2): 220 – 230.

Watari, J., Chen, N., Amenta, P. S., Fukui, H., Oshima, T., Tomita, T., *et al.* (2014). Helicobacter pylori associated chronic gastritis, clinical syndromes, precancerous lesions, and pathogenesis of gastric cancer development. World Journal of Gastroenterology: WJG, 20(18), 5461.

Whisner, C. M., & Weaver, C. M. (2013). Galacto-oligosaccharides: prebiotic effects on calcium absorption and bone health. In Nutritional Influences on Bone Health (pp. 315-323). Springer London.

Wong, J. M., De Souza, R., Kendall, C. W., Emam, A., & Jenkins, D. J. (2006). Colonic health: fermentation and short chain fatty acids. Journal of clinical gastroenterology; 40(3): 235 – 243.

Wu, T., Zhang, Z., Liu, B., Hou, D., Liang, Y., Zhang, J., & Shi, P. (2013). Gut microbiota dysbiosis and bacterial community assembly associated with cholesterol gallstones in large-scale study. BMC genomics; 14(1): 1 – 11.

Yang, J., Qian, D., Jiang, S., Shang, E. X., Guo, J., & Duan, J. A. (2012). Identification of rutin deglycosylated metabolites produced by human intestinal bacteria using UPLC–Q-TOF/MS. Journal of Chromatography B; 898: 95 – 100.

Yel, L. (2010). Selective IgA deficiency. Journal of clinical immunology; 30(1): 10 – 16.

Yoshioka, H., Iseki, K. I., & Fujita, K. (1983). Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. Pediatrics; 72(3): 317 – 321.

GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

Based on the previously exposed results and considering the experimental conditions of the studies carried out, it can be concluded that:

- The addition of LBG as a thickening ingredient provides greater resistance to enzymatic degradation during the in-vitro digestive process, as well as a greater thickening effect compared to modified starches.
- Mhdp and gRS are completely degraded during the intestinal digestion process, thus providing an increase in viscosity, which is limited to the gastric environment.
- LBG addition has a negative effect on the availability and dialyzability of Ca, Fe, Zn, whereas modified starches (Mhdp and gRS) only negatively affect the solubility and dialyzability of Ca.
- This effect on in-vitro mineral availability is extensible to the mineral retention by Caco-2 cell cultures, but is not related to transport or uptake efficiency, or to the expression of molecules (Calbindin D-9k, DMT-1, Ferritin, MT1M, Zip-4 and ZnT1) involved in the cellular storage and transport of Ca, Fe and Zn.
- LBG has an in-vitro fermentation profile similar to that presented by inulin (probiotic positive control), causing a moderate total gas production and a slow decrease in pH, as well as a progresive decrease in the acetate: propionate ratio. This is related to the development of a varied fecal microbiota, enhancing the growth of *Atopobium* and *Bacteroides* groups.
- Mhdp and gRS are rapidly fermentable ingredients, with a behavior similar to that of D-glucose (probiotic negative control). Thus, the addition of Mhdp and gRS is related to a high total gas production, a sudden drop in pH and a majority production of acetate. On the contrary, its addition promotes the development of a less varied fecal microbiota, with absence or minimal development of *Atopobium* and *Bacteroides* groups.

ANNEXES

1.- FUNDINGS

This thesis was supported by the Seneca Foundation and Science and Technology Agency (Murcia Region, Spain) through the Project 11978/PI/09. Carlos A. González Bermúdez was supported by a predoctoral grant from the University of Murcia.

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3.- SCIENTIFIC PRODUCTION DERIVED FROM THIS THESIS

3.1.- Scientific Articles

González-Bermúdez C.A., Frontela Saseta C., Peso Echarri P., López Nicolás R., Martínez Graciá C. (2011). Use of Anti-Regurgitant Infant Formulas in Unweaned Babies. Effect on Mineral Availability. Revista Chilena de Nutrición; 38(4): 482 – 490. Available at: http://www.scielo.cl/scielo.php?script=sci_arttext&pid=S0717-75182011000400012

Gonzalez-Bermudez C.A., Frontela-Saseta C., López-Nicolás R., Ros-Berruezo G., Martínez-Graciá C. (2014). Effect of adding different thickening agents on the viscosity properties and in-vitro mineral availability of infant formula. Food Chemistry; 159: 5 – 11. Available at: http://ssu.ac.ir/cms/fileadmin/user_upload/Mtahghighat/tfood/asil-article/a-f2/Effect-of-adding-different-thickening-agents-on-the-viscosity-properties-and-in-vitro-mineral-availability-of-infant-formula_2014_Food-Chemistry.pdf

Gonzalez-Bermudez C.A., Castro A., Perez-Rea D., Frontela-Saseta C., Martínez-Graciá C., Nilsson L. (2015). Physicochemical properties of different thickeners used in infants foods and their relationship with mineral availability during in-vitro digestión process. Food Research International; 78: 62 – 70. Available at: http://www.sciencedirect.com/science/article/pii/S0963996915302465

Currently two more articles are under review for being published. On one hand, these articles will include the results about Caco-2 cells assays and mineral transporters gene encoding expression (Chapter 2). On the other hand, they will include the results about the effect of thickening agents (LBG, Mhdp and gRS) on infant gut microbiota (Chapter 3).

3.2.- Scientific Communications

3.2.1.- Oral Communications

González Bermúdez C.A., Frontela Saseta C., Peso Echarri P., López Nicolás R., Ros Berruezo G., Bernal Cava M.J., Martínez Graciá C. (2011). "Iron, Calcium and Zinc in-vitro solubility and availability from different comercial infant formulas with thickening agents." TRACELL 2011- 3rd International Symposium on Trace Elements & Health. Murcia. (Spain).

González Bermúdez C.A., López Nicolás R., Frontela Saseta C., Peso Echarri P., Bernal Cava M.J., Martínez Graciá C. (2011). "Influence of the Addition of different concentrations of Thickening Agents on in-vitro Mineral Availability in Infant Formula." COST Action FA0905. Second Annual conference. Venice (Italy).

González Bermúdez C.A. (2012). "Empleo de espesantes como ingredientes en fórmulas infantiles anti-regurgitación. Hacia una optimización del producto". Mediterranean Know How. Murcia (Spain).

González Bermúdez C.A., Miranda Miranda L., Legay S., Corvisy A., Klein S., Frontela Saseta C., Martínez Graciá C., Evers D. (2013). "Anti-Regurgitation (AR) Infant Fórmulas. Expression of mineral transporters and Storage Proteins in Caco-2 Cells after in-vitro gastrointestinal digestion". 4th Annual Conference in COST Action FA-0905. Oslo (Norway).

3.2.2.- Poster Communications

Gonzalez Bermudez C.A., Frontela Saseta C., Vidal Guevara M.L., Martínez Graciá C. (2009). "Bioaccesibilidad del calcio, hierro y zinc en fórmulas anti-regurgitación." V Congreso Nacional de Ciencia y Tecnología de los Alimentos. Murcia (Spain).

González Bermúdez C.A., Frontela Saseta C., Peso Echarri P., López Nicolás R., Bernal Cava M.J., Martínez Graciá C. (2012). "Effect of different thickening agents on viscosity of infant formula during in-vitro gastric digestion." COST Action FA-1005. 1st International Conference on Food Digestion. Cesena (Italy).

González Bermúdez C.A., Frontela Saseta C., López Nicolás R., Gómez Gómez V.P., Miranda Miranda L., Legay S., Corvisy A., Klein S., Evers D., Rincón León F., Martínez Graciá C. (2013). "The use of thickeners as ingredients. Evaluation of their effect on mineral availability using an in-vitro digestion model". 4th Annual Conference in COST Action FA-0905. Oslo (Norway)

Gonzalez Bermudez C.A., Castro A., Perez-Rea D., Frontela-Saseta C., Martínez-Graciá C., Nilsson L. (2014). "Effect of *in-vitro* digestión on physicoquemical (solubility and shear viscosity) and molecular properties of different thickening agents used in infant nutrition". COST Action FA-1005, INFOGEST. 3rd International Conference on Food Digestion. Wageningen, The Netherlands.

Gonzalez Bermudez C.A., Frontela Saseta C., Gomez Gomez V.P., Peso Echarri P., Lopez Nicolas R., Santaella Pascual M., Ros Berruezo G., Martinez Gracia M.C. (2015) "In-vitro fermentability of locust bean gum and modified rice and corn starches by infant gut microbiota". Cost Action FA-1005. Infogest. Improving health properties of food by sharing our knowledge on the digestive process. Naples (Italy).

González Bermúdez C.A., Frontela Saseta C., Gómez-Gómez V.P., Peso Echarri P., Santaella Pascual M., Martínez Graciá M. (2017). "¿Afectan los ingredientes espesantes a la microbiota fecal del lactante? Ensayo de fermentación in-vitro". Sociedad Española de Probióticos y Prebióticos, SEPyP. VIII Work-Shop. Santiago de Compostela (Spain).

3.3.- Awards

IV Edición del Premio Hero de Investigación Infantil. **2011.**“Reflujo Gastroesofágico en Lactantes y Fórmulas Anti-regurgitación. Eficacia y Posibles Efectos Adversos”.

4.- WHAT’S NEXT? THICKENERS COMBINATIONS.

As it has been described in this thesis, the addition of LBG, Mhdp and gRS as thickeners affects, almost *in-vitro*, to the mineral availability and intestinal microbiota development. On one hand, LBG resists the in vitro digestion process, remaining stable and providing a high viscosity to the infant formula. Due to this property, LBG negatively affects mineral availability and provides a slowly fermentable substrate to faecal microbiota. On the other hand, Mhdp and gRS are degraded during the *in vitro* intestinal digestion, providing a viscosity lower than the one registered for LBG, which is restricted to the gastric environment. Therefore, Mhdp and gRS don not affect *in vitro* mineral availability. Whith regard to faecal microbiota, starches are rapidly fermented, resulting in a higher gas production and a less varied microbiota than LBG.

In order to minimize the negative effect associated to these ingredients, one possible solution could be to optimize a combination of LBG, Mhdp and gRS. Whith this pourpose, and in collaboration with Dr. Francisco Rincón León, from the department of Food Science, Technology and Bromatology, University of Cordoba (Spain), a simplex-centroid augmented with interior points and centroid for three thickeners (LBG, MCS and MRS) was designed. The aim of this study was to determine a degree 2 polynomial model for LBG, Mhdp and gRS combination, minimizing the effect on mineral availability and improving the impact on faecal microbiota. Derived from this experimental design, different previous results have been obtained.

With regard to mineral availability a poster communication was presented in the COST Action FA-0905 conference, which was held in Oslo (Norway) in June (2013). Poster has been added in the next page.

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THE USE OF THICKENERS AS INGREDIENTS. EVALUATION OF THEIR EFFECT ON MINERAL AVAILABILITY USING AN *IN VITRO* DIGESTION MODEL

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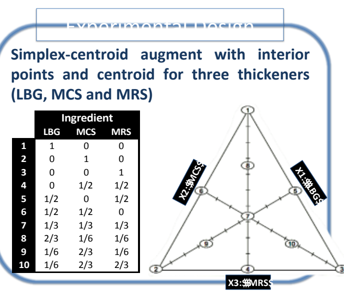
INTRODUCTION

Thickeners, as locust bean gum (LBG) or corn and rice modified starches (CMS and RMS), are being used as ingredients in many common products. Considered as additives, their use is allowed by the European Legislation. One of these products could be the anti-regurgitation infant formulas (AR-IF), recommended for the non complicated gastoresophageal reflux regurgitations nutritional management. Nevertheless, some studies has indicated that, the addition of this ingredients to the infant formulas could affect mineral bioavailavility[1,2]. The Objetive of the study was to clarify the effect of different combinations of MCS, MRS and LBG on Ca, Fe and Zn availability after *in vitro* digestion, when they are used alone, or combined with an infant formula (AR-IF).

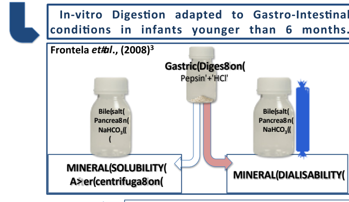
MATERIALS & METHODS



	Ingredient		
	LBG	MCS	MRS
1	1	0	0
2	0	1	0
3	0	0	1
4	0	1/2	1/2
5	1/2	0	1/2
6	1/2	1/2	0
7	1/3	1/3	1/3
8	2/3	1/6	1/6
9	1/6	2/3	1/6
10	1/6	2/3	2/3

- Ingredients were added up to a maximum concentration of 3.5 g/100 g of sample (with and without formula). When no formula was added, $CaCL_2$, $FeSO_4$ and $ZnSO_4$ where used as mineral sources.

In-vitro Digestion adapted to Gastro-Intestinal conditions in infants younger than 6 months.



Mineral content (AAS)

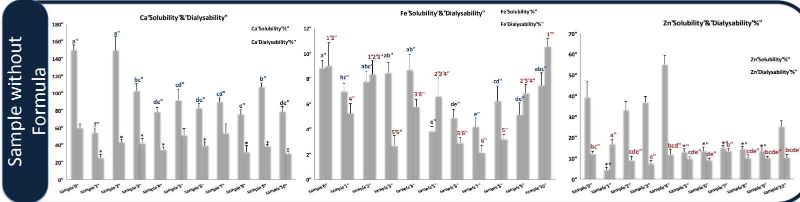
Solubility and Dialysability (%) respect the sample without thickeners added

Relationship between ingredient combinations and mineral solubility and dialysability through a degree 2 Polinomial model:

$$Y=b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3$$

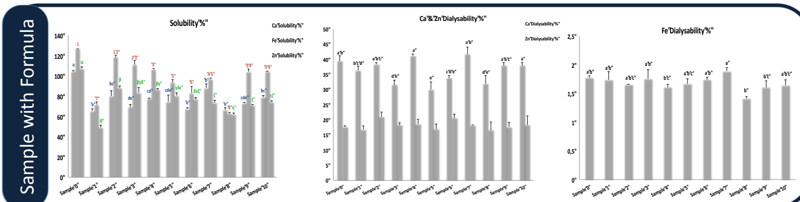
RESULTS & CONCLUSIONS

Sample without Formula

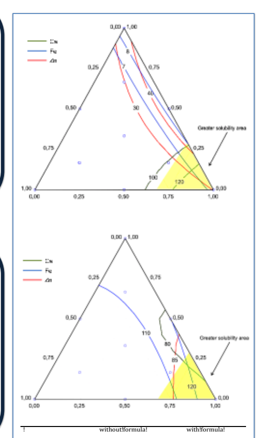


Different superindex denotes significant differences between samples, (p<0.05, **one-way ANOVA**, **Tukey** post-hoc) within the same mineral.
✕Denotes a significant difference between the control (without thickeners) and the formula (p<0.05, **one-way ANOVA**, **T3 Dunnett's Test**)

Sample with Formula



Different superindex denotes a significant difference between samples, (p<0.05, **one-way ANOVA**, **Tukey** post-hoc) within the same mineral.



Effect	without formula minerals Ca	Fe	Zn	with formula minerals Ca	Fe	Zn
Locust bean gum (A)	54.06	7.59	9.41	63.47	67.41	50.23
Corn starch (B)	138.85	6.92	31.53	78.40	120.10	87.67
Rice Starch (C)	91.94	8.81	34.99	69.54	112.46	83.92
AB		-11.90	-50.92		-51.88	
AC	86.90	-15.44	-46.60	43.51		31.35
BC	-157.00		49.18			
R^2	0.86	0.66	0.75	0.52	0.88	0.79
R^2_{adj}	0.83	0.59	0.70	0.42	0.85	0.74

Model reliable enough for predictions, R^2 and adjusted-R^2 Should be ≥ 75

- LBG used as ingredient without formula, seemed to have a significant negative effect on Ca solubility and dialyzability, and on Zn solubility. Nevertheless, no relationship was observed between different ingredients combinations and Fe solubility and dialyzability.
- When combining with formula, Ca solubility and dialysability, and Fe and Zn solubility were significantly lower for the samples with a high concentration of LBG (1,8). When Zn and Fe dialysability were studied, no significant differences were observed regarding the control (without thickeners added).
- A 2 degree polynomial model was obtained for Ca, Fe and Zn solubility. According to it, a higher solubility would be obtained for those combination in which LBG is used in a very low concentration regarding the others ingredients (MCS and MRS).

BIBLIOGRAPHY
[1]Boscher *et al.*, (2000). J Pediatr Gast Nutr; 30(4): 373 – 378.
[2]Bosscher *et al.*, (2003). Nutrition; 19: 641 - 645.
[3]Frontela et al., (2008). . J. Agric Food Chem; 56: 3805-3811

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