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Polyamine Functionality on Immune System Development and Intestinal Microbiota During Lactation. Influence of the Infant Formula Manufacturing on Polyamine Content and Bioactive Peptide Releasing During Digestion. Assay of Functionality in a Lactating BALB/cOlaHsd Mice Model

Funcionalidad de las Poliaminas sobre el Desarrollo del Sistema Inmune y la Microbiota Intestinal Durante el Periodo de Lactancia. Influencia del Procesado de las Fórmulas Infantiles en el Contenido en Poliaminas y en la Liberación de Péptidos Durante la Digestión. Ensayo de Funcionalidad Empleando Como Modelo Ratones BALB/cOlaHsd Lactantes

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AUTORIZAN:

La presentación de la Tesis Doctoral como compendio de publicaciones titulada "**Funcionalidad de las poliaminas sobre el desarrollo del sistema inmune y la microbiota intestinal durante el periodo de lactancia. Influencia del procesado de las fórmulas infantiles en el contenido en poliaminas y en la liberación de péptidos durante la digestión. Ensayo de funcionalidad empleando como modelo ratones BALB/cOlaHsd lactantes**", realizada por D. Carlos Gómez Gallego, bajo nuestra dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

En Murcia a, 23 de Junio de 2014

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REVIEW OF THE THESIS:

Polyamine Functionality on Immune System Development and Intestinal Microbiota During Lactation **by D. Carlos Gómez Gallego.**

This thesis discusses the influence of certain polyamines present in breast milk on the development of allergies and the role of the intestinal microflora. This is an interesting new theory, as previously most research has been focussed on carbohydrates and peptides and not on polyamines.

The thesis first focusses on a literature study, followed by several studies in a mouse model. The set-up of these studies follows a logical pattern. All studies have been published (or have been submitted), three in well-known English peer-reviewed journals.

The first study determines the activity of the polyamine oxidase enzyme in commercial samples. The study also determines the formation of different, possibly bioactive, protein fragments in commercial samples. This study is well performed and reaches interesting results.

The second study describes the effect of supplementation of polyamines to formula in a mouse model. The supplementation has interesting effects on the microflora, even on species which are known to be saccharolytic (bifidobacteria) and would thus not benefit directly from the supplementation. Their increase indicates a general improved health status in the mouse model. The results also show a flora similar to a flora obtained by normal lactation. The study is well designed and well presented.

The third study follows up on the previous study and shows similar interesting results.

The fourth study adds a new dimension by studying the influence on the immune system rather as on the flora. Similarly as in the previous studies a positive effect was found on supplementation of infant formula, resembling the normal lactation state. As in the previous studies, the results are shown in a clear way and the study is well designed.

The total study provides a number of insights on the effect of polyamines on the microflora and immune system in a mouse model. The result so far are very interesting, but still in an initial stage. The questions remain what effect these changes in flora have on the total development of the organism, and whether the observed immunological changes indeed have an effect on allergies in a later stage of life. And, obviously, it remains to be seen whether these effects also are valid in humans.

As a whole, the thesis provides a number of new insights, is well carried out, of high level, well written and is scientifically sound. It may have opened a whole new field in intestinal research which may have profound effects on research on infant formula.

With kind regards,

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A quien pueda interesar,

En la literatura científica existen datos controvertidos en relación a la presencia de poliaminas en la dieta y en las formulas infantiles, especialmente en relación al uso de formulas enterales en el tratamiento de niños con cáncer. Un estudio llevado a cabo por Medina y col. (2003) en relación a la administración de fórmula que contienen L-arginina, un aminoácido que forma parte de la vía secundaria de biosíntesis de poliaminas en animales, muestra concentraciones varias veces superiores a las recomendadas. Además, un gran número de fórmulas disponibles en el mercado alcanzan concentraciones en relación en la ingestión de alimentos con altos niveles de poliaminas por los niños, debido a cambios en la composición, características naturas y proporción de macronutrientes.

Sin embargo, la presente tesis aporta información de gran importancia para resolver las dudas y controversias en relación a la presencia de poliaminas en fórmulas infantiles y comparando dichas fórmulas con la composición de la leche materna. Además, este trabajo aporta valiosa información, reduciendo los datos contradictorios presentes actualmente en la literatura.

El principal objetivo de la presente tesis fue evaluar la influencia del procesado de fórmulas infantiles en el perfil del poliaminas y péptidos bioactivos, y evaluar si la adición de poliaminas después del procesado podía mejorar el desarrollo del sistema inmune y el patrón de colonización microbiana de forma similar a lo que lo hace la leche materna.

Los resultados de este trabajo son muy positivos, produciendo varias publicaciones originales en el periodo entre 2008-2014. Se ha desarrollado una metodología y las herramientas para recoger leche de ratones, lo que resulta en una importante contribución a los investigadores que utilizan modelos animales para investigar y extrapolar resultados del periodo de lactancia en humanos. Los resultados han sido publicados en revistas con un alto

índice de impacto, reflejándose la madurez y la calidad del trabajo realizado por los autores implicados, especialmente del estudiante de doctorado evaluado aquí.

La información en relación a la calidad proteica de las fórmulas infantiles, la digestibilidad de los péptidos encontrados y la aplicación de poliaminas como suplemento para mejorar el patrón de colonización microbiana, lo que contribuiría a mejorar el sistema inmune, la maduración y el desarrollo de tracto gastrointestinal de los niños, son algunas de las importantes contribuciones demostradas en el estudio. Además, la información en relación a la expresión de genes implicados en la proliferación y diferenciación de linfocitos T y B, marcadores de membrana, proteínas que regulan la trascripción y la señalización celular, nos lleva a pensar en la creciente importancia y en la aplicación de las fórmulas infantiles, y aporta datos sobre lo que sucede cuando las utilizamos.

Quiero expresar mi admiración y gran respeto por el grupo de gente implicada en este trabajo. Quiero dar la enhorabuena a todo el grupo en especial a Carlos Gómez Gallego, por su brillante trabajo, por su dedicación a las ciencias de la salud y especialmente por el conocimiento aportado y las cuestiones que se han respondido, haciéndonos optimistas en relación a las acciones adoptadas por los profesionales de la salud durante el seguimiento de madres y niños. Por todo ello, considero esta tesis suficiente para acceder al grado de doctor.

Agradezco la oportunidad de poder evaluar esta excelente tesis y me pongo a su disposición en el futuro.

Atentamente,

Prof. Dr. Maria do Carmo Gouveia Peluzio

A mis padres y hermano

“I was taught that the way of progress is neither swift nor easy”
Marie Curie

LIST OF ORIGINAL PUBLICATIONS

I. **Gómez-Gallego, C**., Collado, M. C., Ilo, T., Jaakkola, U. M., Bernal, M. J., Periago, M. J., Salminen, S., Ros, G., Frias, R. Infant formula supplemented with polyamines alters the intestinal microbiota in neonatal BALB/cOlaHsd mice. Journal of Nutritional Biochemistry. 2012; 23(11):1508-13.

II. **Gómez-Gallego, C**., Collado, M. C., Pérez, G., Ilo, T., Jaakkola, U.M., Bernal, M. J., Periago, M. J., Frias, R., Ros, G., Salminen, S. Resembling breast milk: influence of polyamine-supplemented formula on neonatal BALB/cOlaHsd mouse microbiota. British Journal of Nutrition. 2014; 111: 1050-1058.

III. **Gómez-Gallego, C**., Frias, R., Pérez-Martínez, G., Bernal, M. J., Periago, M. J., Salminen, S., Ros, G., Collado, M. C. Polyamine supplementation in infant formula: Influence on lymphocyte populations and immune system-related gene expression in a Balb/cOlaHsd mouse model. Food Research International. 2014; 59: 8-15.

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CONTENTS

1. GENERAL INTRODUCTION

Infant formula composition and health

As has been recommended by a large number of health or breastfeeding organizations, infants should be fully breastfed for at least six months whenever feasible and should continue breastfeeding (along with being giving appropriate complementary foods) up to two years of age or beyond (World Health Organization, 2013). If full breastfeeding is not possible, safe and suitable infant formula should be used. Although human milk is considered the best dietary option for children, more than 70 percent of children in Western Europe are being fed with manufactured formula from the twelfth week of life onward (Bosscher *et al*., 2002). During recent decades, infant formula has been nutritionally improved, but human milk is more complex and difficult to mimic. Considering such nutritional compounds as fat, carbohydrates, minerals and vitamin contents, infant formula is similar to mature human milk, though it is not able to reproduce the same composition of functional compounds, protein and amino acids as the profile of human milk. In addition, it is important to take into consideration that nutritional exposures in early life are significant determinants of the development and future health of all organ systems, so changes in exposure to dietary components during lactation might have implications for later health status.

It is known that postnatal nutritional exposure is critical for the ongoing developmental maturation of many organ systems and for optimal physiological functions. Early Programming Theory is based in the fact that environmental exposure, including nutritional exposure during this period of life, can result in permanent changes in many physiological processes. The effects of early programming extend to adult health and are linked to the risk of some diseases even many decades later (Amarasekera *et al*., 2013).

The differences between formula-fed children and breastfed children with respect to intestinal inflammation (Goldman, 2000), necrotizing enterocolitis (Lucas and Cole, 1990), overweight and obesity (Horta *et al*., 2007), childhood type 1-diabetes (Norris and Scott, 1996), Crohn's disease (Rigas *et al*., 1993), rheumatoid arthritis (Mason *et al.*, 1995) and lymphomas (Davis, 1988) may indicate the specific vulnerability of the immune system to environmental changes at this early age (Amarasekera *et al*., 2013). Infants benefit from breastfeeding not only via immediate protection against gastrointestinal and respiratory infections, but also via a lower risk of obesity and diabetes in adult life (Robinson and Fall, 2012). The immune system can be seen as an integral part of the pathogenesis of these diseases, and it may mediate some of the effects of nutritional changes. Moreover, changes in diet have been associated with changes in the gut microbiome, metabolic responses and immune function (Le Hutoërou-Luron, *et al*., 2010). The microbiome is known to play an essential role in the maturation of the immune system and the establishment of the gut barrier (Artis, 2008). The human gastrointestinal tract is inhabited by a complex and dynamic population of around 500–1000 different microbial species. At birth, the neonate is exposed to microbes, and the

infant gut begins to be gradually colonised by a rapidly diversifying microbiota (Walker, 2013). After birth, the impact of the infant diet on the microbial composition and the changes in microbiota composition during weaning are more drastic in breastfed than in formula-fed infants. After weaning, the gut microbiota continues to develop towards the adult-like pattern (Collado *et al*., 2012).

Indeed, some bacterial strains in the intestinal tract are likely to create beneficial effects on intestinal epithelial cells and the immune system through mechanisms that include, among others, 1) the competitive exclusion and release of compounds which can inhibit pathogens; 2) the enhancement of barrier function; 3) the modulation of immune cell response and 4) the reduction of inflammation by the inhibition of NK-κB activation (for a review, see Rivera Guzman *et al*., 2013). In humans, there is also evidence that infants who go on to develop allergic diseases have an altered pattern of gut microbiota in early life (Walker, 2013), indicating the importance of a balanced microbial colonization pattern in early programming.

The abovementioned observed differences in health status between breastfed and formula-fed children could be dependent on compounds that are part of the peptidic and non-protein nitrogen fraction of human milk, which includes bioactive peptides and polyamines.

The fact that the changes in health between breastfed and formula-fed children extend through adult life suggests that early exposures are associated with epigenetic changes. Epigenetic mechanisms include DNA methylation, post-translational modification to histone tails and regulation through non-coding RNAs, which regulate the expression of genes to produce changes in cellular function without changes in the underlying DNA sequence (Waterland and Michels, 2007). Modulation of gene expression through epigenetic changes is one important mechanism by which dietary exposures can lead to changes in immune development through changes in the expression of immune genes (Amarasekera *et al.*, 2013).

Bioactive peptides

Enzymatic digestion of proteins results in protein fragments, some of which could show biological properties different to the ones exhibited by the intact precursor molecule. These fragments, called bioactive peptides, consist of 3–20 amino acids, and their biological activities are dependent on their sequence (Raikos and Dassios, 2014).

Human milk is obviously the reference standard for manufactured infant formulas, but protein compositional differences exist between them. For instance, infant formula contains three to four times the protein content and more caseins, compared with human milk (Raikos and Dassios, 2014). The serum protein predominant in human milk is α-lactalbumin, which is absent or included in lower amounts in the majority of commercial formulas (Jackson *et al*., 2004). This protein is the main font of essential amino acids, it has prebiotic activity on *Bifidobacterium spp.* (Maase and Stejins, 2002) and it releases antimicrobial (Pellegrini *et al*., 1999), immunomodulatory (Baró *et al*., 2001) and opioid

peptides (Chatterton *et al*., 2006). Furthermore, human milk does not contain the β-lactoglobulin or α_{s2}-casein that is present in cow milk-based formulas (Raikos and Dassios, 2014). Therefore, there are differences in exposure to dietary components both with respect to the level of quality and the amount of proteins between breast- and formula-fed children. Moreover, the protein content of human milk changes throughout the lactation period, decreasing rapidly during the first months of lactation (14–16 g/l during early lactation, 8–10 g/l at 3–4 months and 7–8 g/l at 6 months and later) (Le Huërou-Luron, *et al*., 2010). This decrease is mainly related to a change in the ratio of whey:casein proteins from 80:20 during the first days of lactation to 60:40 at 2–3 months (Le Huërou-Luron, *et al*., 2010). Therefore, the amino acid content of human milk and the profile of majority peptides erased after gastrointestinal digestion also vary during lactation. Milk proteins are an important source of bioactive peptides which could act positively on infant health, having opiate, antithrombotic, antihypertensive, antimicrobial, antioxidant, immunomodulating or mineral absorption properties (Korhonen *et al*., 1998; Raikos and Dassios, 2014). Some of them are also able to influence insulin secretion or intestinal motility and secretion (Korhonen *et al*., 1998).

During the lactating period, the intestinal permeability is higher than that in adults; at the same time, the resistance of these peptides to proteolytic action is higher due to the soft intestinal conditions (Baró *et al*., 2001). Therefore, the number of peptides which could raise the systemic circulation and exert an influence beyond just the gastrointestinal tract is higher in newborns.

Of special interest for infant development are opioid and immunomodulating peptides. Milk opioid peptides derived from caseins and serum proteins (mainly α-lactalbumin, β-casein, κ-casein and lactoferrin) have a local effect without the necessity of systemic absorption, by reducing and modulating gastrointestinal motility, intestinal permeability and intestinal hormone secretion (Baró *et al*., 2001). These changes, together with the presence of immunomodulatory and antimicrobial peptides, mainly derived from α-lactalbumin and β- and κ-casein (Raikos and Dassios, 2014), could modulate the environment of the intestinal lumen and lead to a microbial colonization pattern which could have an effect on health and immune system development.

Due to the protein content, both human milk and infant formulas based on cow's milk are potential sources of bioactive peptides, but there are differences in the bioactive peptides released, owing to their protein composition. These differences might be responsible, at least in part, for differences in health status between breastfed and formula-fed children.

Polyamines

Polyamines are organic polycations that are present in all mammalian cells. They have significant interest due to their reported biological roles in eukaryotic cells, being essential to cell proliferation and differentiation. Putrescine, spermidine and spermine are the most representative molecules of this group (Figure 1.1). Their essential role is reflected in the fact that their structure and metabolism has been rigorously conserved along evolution (Löser, 2000). Their metabolism is strictly regulated

through a wide number of compensatory mechanisms, with the aim of regulating intracellular homeostasis through unique biosynthetic and catabolic processes (Löser, 2000; Wallace *et al*., 2003).

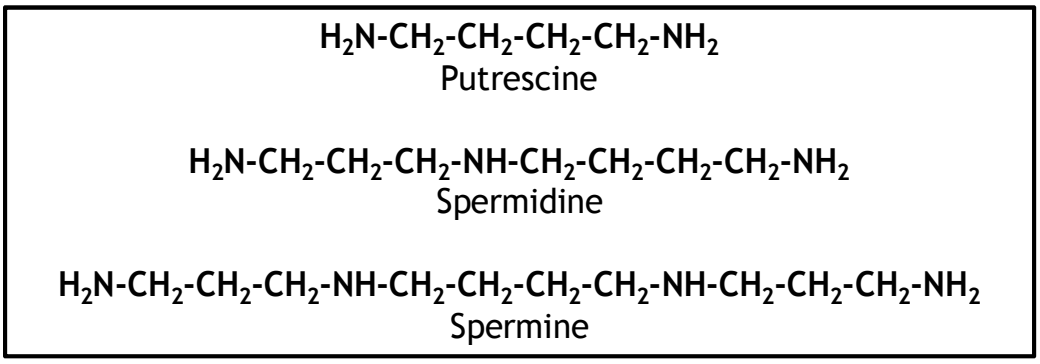

Figure 1.1. Structure of main polyamines present in human milk. Their activity is dependent of their posible positive charges at physiological pH being spermine the most active and putrescine the least active in some biological processes (Larqué *et al*., 2007).

The presence of polyamines has been demonstrated in human milk, the most abundant polyamines being spermidine and spermine (Pollack *et al*., 1992; Romain *et al*., 1992; Buts *et al*., 1995). A large individual variation in polyamine levels in human milk was recorded by other authors (Table 1.1), but in general, the average daily intake of polyamines during lactation was estimated to be 3.5 µmol/day (Buts *et al*., 1995). The variations in polyamine concentration in milk could be caused by different factors such as genetic factors, time of lactation, volume of milk in the mammary gland, diet and nutritional status, the age of the mother, ethnic origin, etc. (Löser, 2000; Baró *et al*., 2001). Nonfermented dairy products and cow's milk usually have low amounts of polyamines due to the degradation associated with high diamino oxidase activity and polyamine oxidase activity (Löser, 2000) present in these products. Hence, the average polyamine content in infant formulas is around ten times less than in human milk (Buts *et al*. 1995), and children who are formula-fed are going to consume fewer dietary polyamines than those who are breastfed.

Table 1.1. Polyamine concentration (ppm) in human milk.

	Putrescine	**Spermidine**	**Spermine**
Pollack *et al*. 1992	0.026 ± 0.020	0.282 ± 0.151	0.455 ± 0.300
Romain *et al*. 1992	0.114 ± 0.019	1.033 ± 0.158	1.341 ± 0.275
Buts *et al*. 1995	0.021 ± 0.003	0.320 ± 0.029	0.633 ± 0.032

Data are expressed as mean ± SD.

Although the majority of the cells of a vertebrate organism can synthesize polyamines, the requirements of polyamines for cellular growth and differentiation, depending of the cell type and the physiological status, determine in what proportion the uptake from external sources (dietary polyamines or microbiota-produced polyamines) are necessary (Seiler and Raul, 2007). Due to the high proliferation rate of the intestinal mucosa, it seems to have a high demand for polyamines (Löser, 2000), something that may be particularly important during lactation. Studies carried out with newborn pigs suggest that enterocytes appear to depend entirely on polyamine uptake from external sources (Blachier *et al*., 1992, Sabater-Molina *et al*., 2009).

In parallel to gastrointestinal development, polyamines stimulate maturation of associated organs such as the pancreas, and they initiate differentiation of the liver (for a review, see Dandrifosse *et al*. 1999). Oral spermidine and spermine administration during lactation also seems to have a positive effect on cell differentiation and development, not only of intraepithelial lymphocytes in the small intestine but also of other lymphoid organs such as the spleen (Steege *et al*., 1997; Jolois *et al*., 2002; Pérez-Cano *et al*., 2010). This demonstrates how oral spermidine and spermine administration plays a significant role in the growth and development of the digestive system of neonates.

Dandrifosse *et al*. (2000) postulated that an insufficient polyamine intake could play an important role in the induction of sensitization to dietary allergens, based on the following: Concentrations of polyamines are generally lower in infant formulas than in human breast milk, the spermine and spermidine intake of breastfed babies is higher than that of formula-fed babies, and the probability of developing an allergy can reach 80% during lactation, when the mean concentration of spermine in milk or formula is lower than 0.4 ppm milk. This probability is almost abolished at concentrations higher than 2.63 ppm in milk (Dandrifosse *et al.*, 2000). Therefore, the possible protective effect of breast milk against allergies could be explained, at least partially, by its polyamine levels and their influence on immune system development.

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2. OBJECTIVES

The main objective of the present thesis was to evaluate if the influence of the addition of polyamines after processing could improve immune system development and microbial colonization patterns in a way similar to breastfeeding.

Consistent with the main objective, the specific objectives of this research were as follows:

1. To estimate the differences in intestinal microbiota, immune system cell populations and immune system-related gene expression between breastfeeding and commercial infant formula feeding in a mouse model.

2. To evaluate the effect of polyamine supplementation of a commercial infant formula on intestinal microbiota, immune system cell populations and immune system-related gene expression in a mouse model.

3. DESIGN AND STRUCTURE

Experimental design

The present thesis is part of a project structured in two studies which results have been published or which are in the process of being revised for scientific journals. The experimental stage has two fundamental steps:

1) Characterization of infant formulas through the manufacturing process and their influence in polyamine content, protein digestibility and bioactive peptide release after simulation of gastrointestinal digestion *in vitro*.

2) Evaluation of the effect of polyamine addition to an infant formula on immune system development and microbial colonization patterns *in vivo*, employing an animal model (object of the thesis).

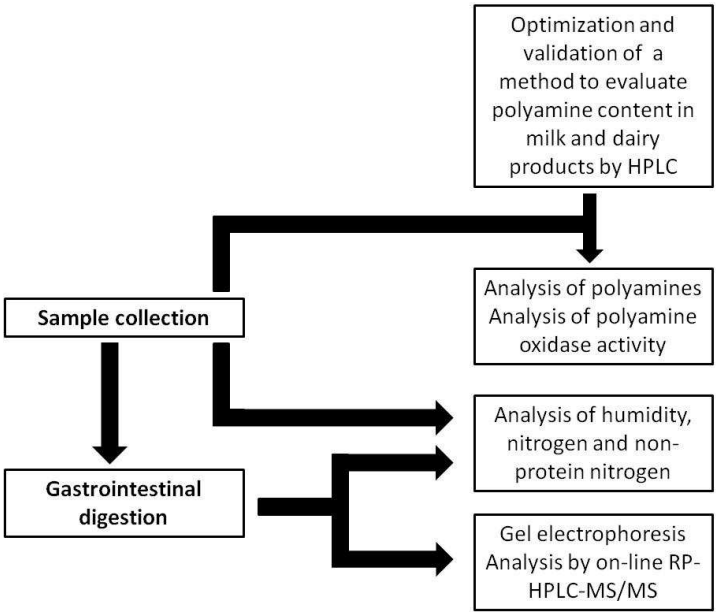

Figure 3.1. Diagram of the experience to characterise infant formulas.

For the previous characterization of infant formula (Figure 3.1), as described in annex 9.1, infant formula samples were supplied by Hero España S.A. (Alcantarilla, Spain) at different representative stages of the manufacturing process. Analyses of the samples were performed at the Department of Food Science and Technology of the University of Murcia, the Department of Research and Development of the Global Technology Centre for Infant Nutrition from the Hero Group and at the Institute of Industrial Fermentations (IFI) from the Spanish National Research Council. Data about

infant formula composition are included in annex 9.1 because it was the infant formula employed for the main study of this thesis.

The study with animals (Figure 3.2) was carried out in the Central Animal Laboratory of the University of Turku (see chapters 4.1–4.3). Analyses of the samples from this study were performed at the Functional Foods Forum of the University of Turku, Department of Biotechnology of the Institute of Agrochemistry and Food Technology (IATA) from the Spanish National Research Council (CSIC) and also in the Department of Food Science and Technology of the University of Murcia.

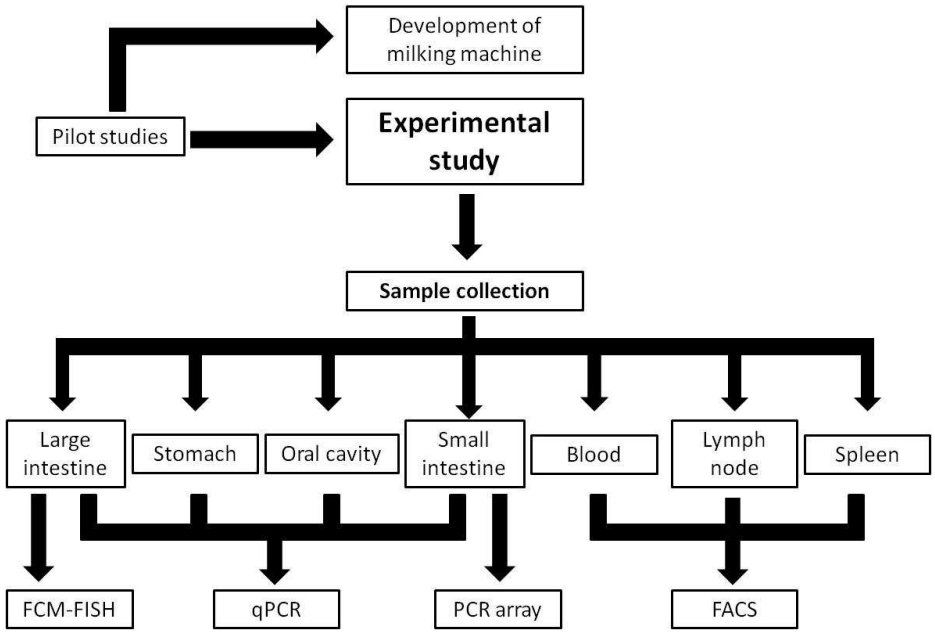

Figure 3.2. Diagram of the in vivo study. The development of milking machine as part of the pilot studies is included as annex 9.2.

4. CHAPTERS

4.1

Gómez-Gallego, C., Collado, M. C., Ilo, T., Jaakkola, U. M., Bernal, M. J., Periago, M. J., Salminen, S., Ros, G., Frias, R.

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Infant formula supplemented with polyamines alters the intestinal microbiota in neonatal BALB/cOlaHsd mice☆,☆☆,★

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Abstract

Polyamines play a critical role in the development of intestinal and immune systems during the infant breastfeeding period, but the effect of polyamines on the microbiota has not been reported. The aim of our study was to characterize the impact on the colonization pattern in neonatal BALB/cOlaHsd mice after supplementing an infant formula (IF) with a mixture of putrescine (PUT), spermidine (SPD) and spermine (SPM). A total of 48 pups (14 days old) were randomly assigned to 4-day intervention groups as follows: breast-fed (unweaned) pups (n=12); weaned pups (n=12) fed an infant formula (IF); weaned pups (n=12) fed an IF enriched with a low concentration of PUT, SPD and SPM (2.10, 22.05 and 38.00 μg/day, respectively); and weaned pups (n=12) fed with IF enriched with a high concentration of PUT, SPD and SPM (8.40, 88.20 and 152.00 μg/day, respectively) of polyamines in accordance with normal proportions found in human milk. Microbiota composition was analyzed by fluorescent in situ hybridization (FISH) with flow cytometry detection. Microbiota changes in formula-fed mice were significantly greater following supplementation with polyamines (P<.01). *Bifidobacterium* group bacteria, *Akkermansia*-like bacteria and *Lactobacillus–Enterococcus* group levels were higher in the groups fed infant formula supplemented with polyamines, resulting in even higher numbers of bacteria than in the breastfed pups. Our findings indicate that infant formulas enriched with polyamines may interact with gut microbiota, suggesting that further studies in human infants are required to assess the impact of polyamines on both growth and microbiota levels.

Keywords: Polyamines; Putrescine; Spermidine; Spermine; Microbiota; BALB/c; Infant formula; Breastfeeding

1. Introduction

In all mammals, maternal milk is the key source of nutrition during the early developmental phase. Human milk is a complex composition of nutrients and bioactive components, such as nucleotides, hormones, growth factors and anti-inflammatory and immunomodulatory agents [1,2], designed to fulfil the needs of the growing young infant. Protective compounds, such as cytokines, oligosaccharides and even microbes, in breast milk provide the newborn with the means to adapt to the environment [3,4]. Among other compounds detected in human milk, polyamines, such as spermidine, spermine and putrescine, are of great interest due to their reported biological roles in eukaryotic cells, stimulating cellular proliferation and differentiation [5]. Moreover, there is evidence that polyamines participate in several processes related to the immune system, including immune system development [6,7], inflammatory response modulation [8,9] and normal function of the immune system [10]. Polyamines may also have a potential role in the growth and development of the digestive tract wall and colonic mucosa in neonatal mammals [11]. Other reports suggest that they participate in the maintenance of intestinal mucosal integrity by regulating epithelial barrier functions through expression of E-cadherin [12,13]. High levels of spermine and spermidine have been shown to be immunoprotective by decreasing the permeability of the intestinal mucosa [14]. These factors suggest that such components may have an important role in the development of gut microbiota and the immune system.

There is increasing evidence that breastfeeding has both short- and long-term beneficial effects on the infant and provides an optimal

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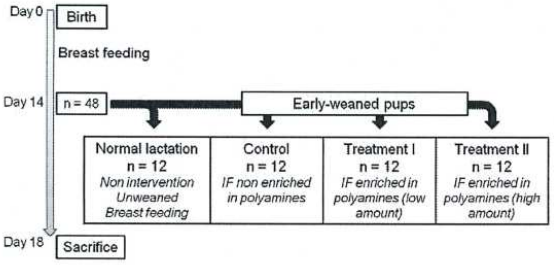

Fig. 1. Experimental study design.

source of nutrition. The most important effects are improved cognitive development, reduced incidence of immune-related diseases (e.g., allergies, type 1 diabetes and inflammatory bowel disease) and protection from infections [15]. Furthermore, polyamines have also demonstrated that they affect the allergy response, thereby increasing the protective effect of breast milk [14,16]. Due to the benefits it provides, exclusive breastfeeding during the first months is recommended by the World Health Organization [17]. When breastfeeding is not possible, infant formula may be the only option for mothers.

The mean polyamine concentrations of 0.021, 0.320 and 0.633 ppm have been reported in human milk for PUT, SPD and SPM, respectively. The concentrations may vary depending on factors such as ethnic origin, diet and age of the mother or lactation stage [18-20]. However, the content of polyamines in current infant formulas (IF) is low, about 10 times less than in breast milk [18]. This may be partly due to the high polyamine oxidase activity and diamine oxidase activity in IF [5], which resist thermic treatments during processing. Thus, supplementation with polyamines may ensure better nutrition of infants and ensure the inclusion of bioactive factors present in breast milk making IF more similar to human milk.

We hypothesised that polyamines, considered as one bioactive factor that acts on infant health impacting gastrointestinal tract development and maturation and the immune system, could modulate microbial colonization patterns. To test this hypothesis, the aim of this study was to assess the impact of supplementation of an IF with a mixture of different polyamines (PUT, SPD and SPM) in concentrations present in breast milk on the neonatal microbiota compositions in BALB/cOlaHsd mice.

2. Material and methods

2.1. Animals

A total of 48 pups, derived from a breeding colony of BALB/cOlaHsd mice supplied by Harlan Laboratories (Horst, Netherlands), were used in this study. The progenitor mice were 8 weeks of age and were acclimatized for 30 days prior to breeding. All the mice were determined to be healthy on the basis of individual physical examinations and pathogen free based on results of the routine microbiological screening performed in the colony in accordance with European recommendations [21].

All the mice were maintained in stainless steel Eurostandard Type II cages (36.5×20.7×14.0 cm) protected with filter tops. The cages had solid bottoms, were covered with Aspen chip bedding (Tapvei, Kaavi, Finland) and were provided with some nesting material. Cage changes for the adult mice were undertaken twice a week, but never during the study period using the pups. The environment in the room consisted of a temperature range of 23°C (±3°C), a relative humidity of 55 ±15% and an artificial illumination of a 12-h light/dark cycle (lights on at 0600). They were maintained on a standard laboratory diet [RM3 (E) SOYA-FREE, product code 801710, Special Diets Services, Witham, Essex, UK] and were allowed free access to water.

Day of birth was referred to as Day 0 of neonatal life. At the commencement of the study, all the pups were 14 days of age, and their mean body weight was 7.94±1.01 g. Throughout the study period, all the pups in the breastfed group had free access to the dam's nipples, and all the pups in the other groups had free access to a standard moist diet consisting of a porridge made by adding warm water to an infant formula at a final proportion of 65 g of infant formula/15 ml of water.

All the adult mice were fed a standard mouse chow [RM3 (E) SOYA-FREE] *ad libitum*. Tap water was provided without restrictions to both adult and infant mice in polycarbonate bottles.

The animals were weighed daily during the intervention study, and handling was done in the same time range to avoid the influence of biological rhythms.

This study was performed at the Central Animal Laboratory, University of Turku, Finland. Pilot experiments were performed to optimize all the experimental procedures including handling and treatments.

The experimental protocol was approved by the National Ethics Committee for Animal Experiments in Finland. The mice were handled in accordance with Finnish legislation and the Council of European Convention ETS 123 on the use of vertebrate animals for scientific purposes.

2.2. Study design

The animals were randomly assigned to dietary intervention groups and treated as described in Fig. 1. Intervention was given for 4 days. The study groups were breastfed (unweaned) pups (normal lactation, $n=12$); weaned pups fed on IF (control, $n=12$); and weaned pups fed on IF enriched with low (Treatment I, $n=12$) and high (Treatment II, $n=12$) concentrations of polyamines.

Table 1
Nutritional composition of infant formula

Energetic value	kJ	2158	Vitamin A	μg	523.0
	kcal	516	Vitamin D	μg	7.8
Proteins	g	10.2	Vitamin E	mg	7.8
Casein	g	5.1	Vitamin K	μg	52.0
Serum	g	5.1	Vitamin C	mg	52.0
Carbohydrates	g	56.9	Vitamin B_1	μg	523.0
Fats	g	27.5	Vitamin B_2	μg	785.0
Linoleic acid	mg	4129.5	Niacin	mg	5.2
Linolenic acid	mg	311.8	Vitamin B_6	μg	523.0
Na	mg	154.0	Folic acid	μg	78.0
K	mg	495.0	Vitamin B_{12}	μg	2.1
Cl	mg	326.0	Biotin	μg	16.0
Ca	mg	387.0	Pantothenic acid	μg	2353.0
P	mg	246.0	L-Carnitine	mg	7.8
Ca/P balance		1.6	Taurine	mg	42.0
Mg	mg	46.0	Inositol	mg	26.1
Fe	mg	6.6	Choline	mg	63,0
Zn	mg	4.2	Adenosine 5′-monophosphate	mg	3,6
Cu	μg	314.0	Cytosine 5′-monophosphate	mg	12,4
I	μg	78.0	Guanosine 5′-monophosphate	mg	2,1
Se	μg	7.0	Uridine 5′-monophosphate	mg	6,5

Values are expressed as per 100 g of commercial dry product.

Table 2
Sequences of the probes used in FISH

Probe	Target	Sequence from 5′ to 3′	Hybridisation temperature (°C)	Reference
EUB 338	Total bacteria	GCT GCC TCC CGT AGG AGT	50	Amann et al., 1990 [28]
Bif 164	*Bifidobacterium*	CAT CCG GCA TTA CCA CCC	50	Langendijk et al., 1995 [29]
Bac 303	*Bacteroides–Prevotella*	CCA ATG TGG GGG ACC TT	45	Manz et al., 1996 [30]
Chis 150	*Clostridia* subgrp. *perfringens/Histolyticum*	TTA TGC GGT ATT AAT CTY CCT TT	50	Franks et al., 1998 [31]
Lab 158	*Lactobacillus–Enterococcus*	GGT ATT AGC AYC TGT TTC CA	45	Harmsen et al., 1999 [32]
Muc 1437	*Akkermansia*-like bacteria	CCT TGC GGT TGG CTT CAG AT	50	Collado et al., 2007 [22]

Y represents a (C/T) wobble nucleotide.

The unweaned group was caged during the study in pairs (one male and one female) with a mother. In the weaned groups, the pups were caged in pairs (one male and one female) with a 28–32-day-old female mouse who acted as trainer to teach them how to eat and drink. Infant formula, both nonenriched and enriched with polyamines, was administered to the control and treatment groups, respectively, by oral gavage twice daily. Handling was done in the same time range to avoid the influence of biological rhythms.

After the 4-day diet intervention, the animals were anesthetized with isoflurane and samples were collected. Immediately after euthanasia by cervical dislocation, the entire intestinal tract was removed from the pups on Day 18. The full contents of the large intestines were emptied and diluted [1:10 (w/v)] in sterile phosphate buffered saline (PBS). Then, one volume was transferred into three volumes of 4% paraformaldehyde and fixed at 4°C overnight. After fixation, the intestinal contents were centrifuged (12,000×*g*, 3 min, 4°C) and washed with PBS three times [22]. Then, bacterial pellets were stored in PBS–ethanol (1:1) at −20°C until analyzed.

2.3. Formula and formula supplemented with polyamines

Infant formula was supplied by Hero España (Alcantarilla, Spain). The basic formula was designed according to the European Infant Formula directive and the ESPGHAN recommendations [23,24]. It was a commercial IF targeted for babies during the first 6 months, fortified with nucleotides, α-lactalbumin, and ω-3 and ω-6 fatty acids (Table 1).

Nonenriched formula and formula with polyamines (100 μl) were made with warm water following the manufacturer's instructions and given to the pups twice daily by oral gavage. The polyamines tested in the study were putrescine (D13208, Aldrich, Steinheim, Germany), spermidine (S2626, Sigma, Steinheim, Germany) and spermine (85590, Fluka, Steinheim, Germany). The concentration levels tested were 2.10 μg/day PUT, 22.05 μg/day SPD and 38.00 μg/day SPM for the low concentration group, and for the high concentration group the levels were 8.40 μg/day PUT, 88.20 μg/day SPD and 152.00 μg/day SPM — Treatment II was four times higher than Treatment I. The polyamines were prepared in water solution and kept refrigerated at 4°C until addition to the IF. The amount of polyamines for each group was added to the IF immediately before administering it to the mice to avoid degradation by polyamine oxidase. The proportion between the different polyamines (3.38% for PUT, 35.48% for SPD and 61.14% for SPM) was based on the proportion of these polyamines found in human milk [18-20], and daily intake was lower than nonobserved adverse effect levels [25].

Table 3
Bacterial counts as percentage hybridised with each group-specific probe relative to total bacteria hybridised with the EUB 338 probe

	Treatment			
	Normal lactation	Control	Treatment I	Treatment II
Bacteroides/Prevotella	57 ± 14^{a}	20 ± 8^{c}	35 ± 8^{b}	49 ± 12^{a}
Bifidobacterium	21 ± 5^{b}	10 ± 7^{c}	27 ± 8^{ab}	34 ± 13^{ab}
Clostridia subgrp. *perfringens/Histolyticum*	11 ± 5^{ab}	5 ± 3^{c}	12 ± 4^{ab}	17 ± 11^{ab}
Lactobacillus/Enterococcus	9 ± 3^{bc}	6 ± 3^{c}	13 ± 3^{ab}	16 ± 4^{a}
Akkermansia-like bacteria	15 ± 2^{b}	11 ± 2^{c}	20 ± 6^{ab}	27 ± 8^{a}

Data are expressed as mean±S.D. (n=12). Significant differences among microbial counts are shown with letters; groups with different letters (a, b, c) in the same row have statistically significant differences in bacterial group population at a level of P<.01.

2.4. Microbiota composition

The culture-independent analysis of the microbiota was carried out by fluorescent in situ hybridization (FISH) combined with flow cytometry (FCM-FISH) using 16S rRNA oligonucleotide probes on 96-well plates. FCM-FISH was performed as described previously [22,26]. Determination of specific bacteria was performed by combining each of the group-specific Cy3 probes with the EUB 338-FITC probe and counting double-positive cells [27]. Specific bacterial group probe sequences are presented in Table 2. All oligonucleotides were purchased from Thermo Electron Corporation (Bioscience Technologies Division, Ulm, Germany).

Data acquisition was performed with an LSR II flow cytometer equipped with an HTS 96-well plate reader (Becton Dickinson, San Jose, CA, USA). A 15-mV argon ion laser (488 nm) was used to measure forward and side scatter (488±10 nm), green fluorescent for FITC (530±30 nm) and red fluorescent for Cy3 (575 ±26 nm). Forty microliters of the samples was collected in duplicate for each sample. Data were analysed with BD FACSDiva software (Becton Dickinson). Results were expressed as cells hybridising with the group-specific Cy3 probes as a proportion of the total bacteria hybridising with the EUB 338-FITC bacteria domain probe.

2.5. Statistical analysis

Statistical analysis was performed using SPSS software version 15.0. The analysis for normal distribution of the data was performed by a Kolmogorov–Smirnov test. One-way ANOVA and Tukey's and Games–Howell post hoc tests were used. Significant differences were considered at $P\leq.05$.

3. Results

The proportions of cells hybridised with the group-specific probes among the bacteria detected with the EUB 338 probe are presented in Table 3. The box-and-whisker diagrams of the bacterial group populations are presented in Figs. 2 and 3.

The proportions of the bacterial groups characterised were increased by polyamine enrichment of the IF to similar or higher levels as those found in the breastfed group and to higher levels than in the control group.

Bifidobacterium, *Bacteroides-Prevotella* and *Clostridium* groups were the most predominant groups in the large intestines of the mice. They were significantly higher in the treatment and normal lactation groups than in the formula-fed pups (P<.01), as shown in Figs. 2 and 3.

Lactobacillus–Enterococcus group levels were also higher in the breastfed group than in the control group (P<.001). The microbial levels detected in the formula-fed group increased with the supplementation of polyamines, and the highest cell counts were detected in the high polyamine concentration group (Fig. 3). However, *Bifidobacterium* group and *Lactobacillus* group cell counts were higher in the infant formula supplemented with polyamine groups, differing from the levels observed in the breastfed pups.

Akkermansia-like bacteria populations in the different groups displayed the same behaviour as *Bifidobacterium*, which could be related to the healthy status of the intestinal tracts.

Microbial levels between the breastfed group and the infant formula supplemented with higher concentration of polyamines were

4. Discussion

To our knowledge, this is the first study demonstrating that supplementation of infant formula with polyamines has a significant impact on the gut microbiota composition and activity in neonatal BALB/cOlaHsd mice. The results demonstrated that polyamines in infant formula interact with microbiota development, and microbiota composition in the supplemented formula groups resembled closely that of the breastfed groups.

Although intake of dietary polyamines has been known to have a direct impact on health, limited data are available on polyamine content in foods and their effects. Polyamines are common components present in human breast milk, the preferred and recommended source of nutrition for infants. With the early core microbiota formation being dependent on exposure to the microbes that first colonise the gastrointestinal tract, the establishment of a 'healthy' microbiota in early life is likely to be critical for normal development. In the present study, we assessed the effect of infant formula supplementation with polyamines on the intestinal microbiota composition. In this study, the most predominant groups found in mouse large intestinal contents are similar to those found in human infants [33,34]. These bacteria belong to groups which may be associated with mucin, and mucin production could be correlated with an increase of these microorganisms.

The high *Bifidobacterium* levels found in the large intestines of the pups fed with IF enriched with polyamines could be a biological index of the health status of the intestinal tract [35]. These results suggest that polyamines increase the number of *Bifidobacterium* species in the intestine and promote a healthy mucosal status. Bifidobacteria are the predominant microbiota of healthy breast-fed infants and are considered to be a hallmark of a healthy breastfed infant. Bifidobacteria have a biological role in mucosal host-microbe crosstalk, immune regulation and inflammation control [15,36]. Compared with formula, human milk is of a more complex composition, providing both optimal nutrition for the newborn and protective nutrients that contribute to the development of mucosal defences.

A recent study reported that high numbers of bifidobacteria may correlate positively with the normalization of inflammatory status and improved glucose tolerance and glucose-induced insulin secretion [37]. Therefore, infant formula supplemented with polyamines might ensure improved nutrition for infants, mimicking the effects of breastfeeding and ensuring the inclusion of bioactive factors present in breast milk. The enrichment of a normal infant formula with polyamines in the same proportion as human milk may increase the relative populations of bifidobacteria in the large intestine to a comparable level as healthy breastfed infants. There are three possible explanations for this: (1) polyamines, which are reported to be cellular growth modulators [10,38], increase specifically the proliferation of these bacterial groups; (2) polyamines have an inhibitory effect against other bacterial groups, made possible by a higher proliferation of this bacterial group; and/or (3) the stimulation of the immune system exerted by polyamines allows a greater spread of these beneficial host microbes. Thus, strategies targeting gut microbiota modulation in favour of the bifidobacteria group could be a useful tool for improving health in early as well as in later life. Moreover, *Bifidobacterium* species have been suggested as the key biological markers of a healthy breastfed infant gut; thus, it is important to analyze further bifidobacteria species composition. Differences in *Bifidobacterium* species composition have been related to inflammatory diseases such as allergy, metabolic disorders and obesity [39,40], and different immunomodulatory properties have been attributed to different *Bifidobacterium* species.

We found lower levels of the *Lactobacillus–Enterococcus* group in formula-fed neonatal BALB/cOlaHsd pups than in those that were breastfed or were fed polyamine-supplemented formulas. Together with bifidobacteria, the *Lactobacillus* group is one of the target groups in the intestinal microbiota of breast-fed infants, whereas infants who receive cow's milk-based infant formulas, which naturally contain low levels of oligosaccharides, often have higher concentrations of potentially pathogenic bacteria, such as *Enterobacteriaceae* and *Clostridia*, in their intestinal microbiota [41]. However, scarce information about the dietary impact on *Lactobacillus* group levels is available.

Our results showed that pups fed IF enriched with polyamines and breastfed pups had higher levels of *Akkermansia*-like bacteria in their large intestines. *Akkermansia muciniphila* has been shown to be a common member of the gut microbiota of human and animals and has the capability to utilise intestinal mucus as a source of nutrients at the same time promoting the development of innate and adaptive immune responses [41]. The high prevalence of *Akkermansia*-type bacteria in diverse gut ecosystems also supports its nonpathogenic nature, and its presence is probably important for equilibrium of the microbial ecosystem. *A. muciniphila* has so far not been correlated with any disease or sign of pathogenicity [42]. A recent study [43] reported that it was negatively correlated with acute appendicitis, together with *Faecalibacterium prausnitzii, Eubacterium rectale* and *Bacteroides* spp, inhabitants of the intestinal ecosystem. Although several studies have reported the potential involvement of mucin-degrading bacteria, such as *A. muciniphila*, in the pathogenesis of inflammatory diseases [44,45] and in animal models, higher levels of *Akkermansia* have also been associated with allergic diarrhoea, while lower levels were associated with diets with lower allergic symptoms and fasting in mice [46,47]. Further studies are necessary to clarify the roles of and variations in *Akkermansia*-like bacteria populations in health-disease status.

Our results demonstrated that polyamine supplementation of infant formula had an effect on all bacterial groups analyzed in similar amounts to what we reported for normal lactation. Differences between Treatment Groups I and II including an increase in *Bacteroides–Prevotella* group with increasing polyamine concentration suggest that polyamines may be used by these microorganisms as growth factors. Polyamines may be the preferred substrate or they may stimulate proliferation and differentiation of mucosa cells in specific environments which are beneficial for these bacterial groups.

5. Conclusions

Changes in lifestyle during recent decades, including nutritional habits of nursing mothers, may influence breast milk composition, duration of breastfeeding and the physiological properties of some bioactive components present in breast milk. Surprisingly, little attention has been paid to the role of breastfeeding vs. formula feeding on the functional development of the digestive tract. Our results demonstrate the potential effect of polyamines on intestinal microbial composition with a resulting impact on health; such an effect should be further studied in human infants.

Taken together, enrichment of IF with polyamines influences microbial colonization patterns in mice, promoting similar microbiota to that found in mice with normal lactation. We suggest that the phenomenon could be related to that seen in healthy breastfed human infants [33]. Further studies are warranted to verify this phenomenon and the correlation with other factors associated with health status such as mucosal permeability to macromolecules or short-chain fatty acid production.

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Resembling breast milk: influence of polyamine-supplemented formula on neonatal BALB/cOlaHsd mouse microbiota

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Abstract

Infant microbiota is influenced by numerous factors, such as delivery mode, environment, prematurity and diet (breast milk or formula). In addition to its nutritional value, breast milk contains bioactive substances that drive microbial colonisation and support immune system development, which are usually not present in infant formulas. Among these substances, polyamines have been described to be essential for intestinal and immune functions in newborns. However, their effect on the establishment of microbiota remains unclear. Therefore, the aim of the present study was to ascertain whether an infant formula supplemented with polyamines has an impact on microbial colonisation by modifying it to resemble that in breast-fed neonatal BALB/c mice. In a 4 d intervention, a total of sixty pups (14 d old) were randomly assigned to the following groups: (1) breast-fed group; (2) non-enriched infant formula-fed group; (3) three different groups fed an infant formula enriched with increasing concentrations of polyamines (mixture of putrescine, spermidine and spermine), following the proportions found in human milk. Microbial composition in the contents of the oral cavity, stomach and small and large intestines was analysed by quantitative PCR targeted at fourteen bacterial genera and species. Significantly different ($P<0·05$) microbial colonisation patterns were observed in the entire gastrointestinal tract of the breast-fed and formula-fed mice. In addition, our findings demonstrate that supplementation of polyamines regulates the amounts of total bacteria, *Akkermansia muciniphila*, *Lactobacillus*, *Bifidobacterium*, *Bacteroides–Prevotella* and *Clostridium* groups to levels found in the breast-fed group. Such an effect requires further investigation in human infants, as supplementation of an infant formula with polyamines might contribute to healthy gastrointestinal tract development.

Key words: Polyamines: Microbiota: Infant formulas: Breast-feeding

Microbiota is known to play an important role in the maturation of the immune system and the establishment of the gut barrier. It has been well established that early microbial colonisation provides the neonate with vital stimuli that guide the maturation of the immune system. It is also well known that disturbances in this process can result in the development of immune disorders, which are regarded as a failure in the development of a balanced immune response[1,2], as well as a predisposition to diseases later in life[3]. The development of the human microbiome is a complex process that might begin during the perinatal period, when the infant is exposed to the mother's microbiota, and continues to develop over the individual's lifetime. The first microbes and the succession of microbiota provide important stimuli for the maturation of the intestinal immune system[4]. Thus, the establishment of healthy gut microbiota in early life is likely to be critical for normal development, acting a key step in the development of long-term well-being. Aberrancies in early microbial colonisation have been reported to be associated with a higher risk of a variety of diseases, including allergies, gut inflammatory conditions, and, more recently, obesity and diabetes[5]. Breast milk delivers numerous growth factors to the infant's gut, influencing the colonisation and maturation of bacteria in the intestinal mucosa, as well as antibacterial factors that influence the colonisation process. The intestinal microbiota of healthy breast-fed infants is mainly composed of bifidobacteria[6,7], the amounts of which can reach up to 60–90% of the total faecal microbiota[8].

Abbreviations: IF, infant formula; PUT, putrescine; SPD, spermidine; SPM, spermine.

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The microbial profile of formula-fed infants is more complex than, but similar to, that of adults, with predominant facultative anaerobes, such as *Bacteroides* and *Clostridium*, followed by *Staphylococcus*, *Streptococcus* and bacteria of the Enterobacteriaceae family. The colonisation of bifidobacteria is delayed[9–13]. The microbial colonisation pattern is characterised by changes in the main bacterial groups: the *Bacteroides–Prevotella* group; *Bifidobacterium*, *Clostridium*, *Lactobacillus and Staphylococcus* genera; Enterococcaceae and Enterobacteriaceae families[1,14]. Factors such as delivery mode, birth environment, prematurity, hygiene measures, maternal vaginal and cutaneous microbiota and infant feeding type (breast milk or formula) influence the establishment of microbiota[1,6,15]. It has been demonstrated that breast-fed infants are colonised with less number of bacteria of the Enterobacteriaceae family and *Clostridium* group compared with the formula-fed infants[1,16]. Other studies have also demonstrated that human infants given an infant formula are more affected by gastrointestinal disorders than the breast-fed infants. These differences are associated with an increase in local inflammation and, thus, different microbial populations[17,18].

Breast milk is known to have a complex composition of nutrients and bioactive components that are designed to fulfil the needs of the young growing infant. Protective nutrients, such as cytokines, oligosaccharides and even microbes, in breast milk provide the newborn with the means to adapt to his or her particular environment[19,20]. Breast milk contains polyamines such as spermidine (SPD), spermine (SPM) and putrescine (PUT)[21,22], which are gaining relevance due to their reported biological roles in eukaryotic cells[23]. Polyamines are involved in the growth and development of the digestive tract wall and colonic mucosa in neonatal mammals[24] by helping with the maintenance of intestinal mucosal integrity[25,26] and intestinal permeability[27]. Moreover, there is evidence that polyamines participate in several processes related to the immune system, including its development and maturation[28,29], inflammatory response[30–32] and normal functioning[33]. The concentration and effect of these compounds in infant formulas compared with those in human milk are of special interest, because their concentrations are lower than those in human milk[34].

In a previous study[35], a fluorescence *in situ* hybridisation analysis of samples of intestinal content has shown that an infant formula enriched with polyamines influences microbial colonisation patterns with a higher number of beneficial bacteria, such as *Bacteroides–Prevotella*, *Bifidobacterium* and *Akkermansia*-like bacteria, as well as the *Clostridium* subgroup. The *perfringens/histolyticum* and *Lactobacillus–Enterococcus* groups have been observed by fluorescence *in situ* hybridisation in samples of the large intestine from mouse pups fed breast milk, an infant formula and an infant formula enriched with polyamines. These previously reported animal model results are in accordance with the fact that the *Bifidobacterium*-dominated microbiota and *Lactobacillus* spp. are more frequently present in breast-fed human infants than in the formula-fed infants[1], suggesting that polyamines may interact with microbiota, driving the microbiota to a breast-feeding standard. However, further studies targeting more bacterial groups and gastrointestinal sites are needed to determine the effect of polyamines on microbiota, mainly during early life. Thus, to expand our previous knowledge, in the present study, we aimed to compare the differences in the development of microbiota in the entire gastrointestinal tract of infants fed breast milk and a manufactured formula and to ascertain whether an infant formula enriched with different concentrations of polyamines influences early microbial colonisation in the neonatal BALB/cOlaHsd mouse model.

Materials and methods

Animals and study design

A total of sixty pups, derived from a breeding colony of BALB/cOlaHsd mice supplied by Harlan Laboratories®, were used in the present study. The progenitor mice were 8 weeks old and were allowed to acclimatise for 30 d before breeding. All the mice were determined to be healthy on the basis of individual physical examinations and to be pathogen free based on the results of routine microbiological screening carried out in the colony in accordance with European recommendations[36].

On day 14 after birth, same-day-born litters were mixed and individually identified. Individual pups were randomly assigned to one of the four dietary groups according to dietary treatment. The study groups were breast-fed (unweaned) pups (n 12), early-weaned pups fed an infant formula (IF) (n 12) and early-weaned pups fed an IF enriched with low (n 12), intermediate (n 12) and high (n 12) concentrations of polyamines. The non-enriched formula and formula enriched with polyamines were prepared with warm water, following the manufacturer's instructions, and given to the pups twice daily (100 μl each time) by oral administration. During the study, the unweaned pups were caged in pairs (one male and one female) with a mother. The weaned pups were caged in pairs (one male and one female) with a 28–32-d-old female mouse acting as a trainer to teach them how to eat and drink. Infant formulas, both non-enriched and enriched with polyamines, were orally administered to the control and treatment groups, respectively, twice daily. Handling was done at the same time range to avoid the influence of biological rhythms. The early-weaned pups were fed a porridge made with the IF not enriched with polyamines.

The study was carried out at the Central Animal Laboratory, University of Turku, Finland. Pilot experiments were carried out to optimise handling and treatment. The experimental protocol was approved by the National Ethics Committee for Animal Experiments in Finland (ESLH-2009-04845/Ym-23). The mice were handled in accordance with Finnish legislation and the Council of European Convention ETS 123 on the use of vertebrate animals for scientific purposes.

Formulas and polyamines

PUT (D13208; Aldrich), SPD (2626; Sigma) and SPM (85590, Fluka) were added to the IF (3·38% PUT, 35·48% SPD and 61·14% SPM) based on the proportions found in human

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milk[21,22,34]. The concentrations tested in the three polyamine groups were as follows: (1) low: 2·10 μg/d PUT, 22·05 μg/d SPD and 38·00 μg/d SPM; (2) intermediate: 4·20 μg/d PUT, 44·10 μg/d SPD and 76·00 μg/d SPM; (3) high: 8·40 μg/d PUT, 88·20 μg/d SPD and 152·00 μg/d SPM. The polyamines were prepared in water and kept refrigerated at 4°C until their addition to the IF. The polyamines were added to the IF immediately before feeding it to the mice to avoid degradation by polyamine oxidase.

The manufactured formula used in the present study was a commercial IF used for babies up to 6 months of age and fortified with nucleotides, α-lactalbumin, and *n*-3 and *n*-6 fatty acids, supplied by HERO España S.A. The commercial formula that was chosen contained no oligosaccharides or pre- or probiotics that could influence the microbial colonisation patterns. The non-enriched formula and formula enriched with polyamines (100 μl) were prepared with warm water, following the manufacturer's instructions, and given to the pups twice daily by oral administration.

Sample collection and DNA extraction

After the 4 d dietary intervention, the pups were anaesthetised with isoflurane and killed by cervical dislocation, following which the entire intestinal tract was removed. Samples of oral mucosa were collected using a sterile swab, and the contents of the stomach, small intestine and large intestine, including the caecum, were collected for further analysis. DNA was extracted using a modified QIAGEN stool DNA extraction kit (QIAGEN) with a previous bead-beating step.

Microbial composition analysis by quantitative PCR

PCR primers used for the characterisation of microbiota in the present study included those specific for total bacteria; *Bifidobacterium* genus and species, including *B. longum*, *B. breve*, *B. bifidum*, *B. animalis–lactis* and *B. catenulatum*; *Bacteroides–Prevotella* group; *Clostridium coccoides*; *Clostridium leptum* subgroup; *Akkermansia muciniphila*; *Lactobacillus* group; Enterobacteriaceae family; *Enterococcus* group; and *Staphylococcus* and *Streptococcus* group (Table 1). These oligonucleotides were purchased from Isogen (Isogen Life Science). Quantitative PCR were carried out as described previously[49]. Quantitative PCR amplification and detection were carried out using the LightCycler® 480 Real-Time PCR System (Roche). Each 10 μl reaction mixture contained SYBR® Green PCR Master Mix (Roche), 0·5 μl of each of the specific primers, at a concentration of 0·25 μM, and 1 μl of template DNA. The fluorescent products were detected in the last step of each cycle. A melting curve analysis was carried out after amplification to distinguish the targeted PCR products from the non-targeted PCR products. The concentration of bacteria in each sample was calculated by comparing the C_t values obtained from the standard curves. These were constructed using serial 10-fold dilutions of pure culture-specific DNA fragments corresponding to 10^2–10^8 gene copies/ml.

Statistical analysis

The SPSS 15.0 software (IBM) was used for statistical analysis. Due to non-normal distribution, a non-parametric test was used, and microbial data are expressed as medians with interquartile ranges. Comparisons among the data of more than two groups of pups were made by applying the Kruskal–Wallis test, and comparisons between data of two groups were made by applying the Mann–Whitney *U* test. The Bonferroni adjustment test was also used to correct the significance of multiple test comparisons among the three groups, which has the advantage of reducing type I errors and the disadvantage of increasing type II errors. The χ^2 test was used to establish differences in bacterial prevalence among the studied groups. The possible correlation between variables was studied by applying Spearman's rank correlation coefficient. Differences were considered significant at $P \leq 0{\cdot}05$.

Table 1. Sequences of primers used in the study*

Probes	Target	Primer (5′–3′) Forward	Primer (5′–3′) Reverse	Annealing temperature (°C)	Reference
Universal	16S	AGAGTTTGATCCTGGCTCAG	GGCTGCTGGCACGTAGTTAG	50	Kullen *et al.*[37]
Akkermansia muciniphila	16S	CAGCACGTGAAGGTGGGGAC	CCTTGCGGTTGGCTTCAGT	60	Collado *et al.*[38]
Bacteroides–Prevotella–Porphyromonas	16S	GGTGTCGGCTTAAGTGCCAT	CGGAYGTAAGGGCCGTGC	64	Rinttilä *et al.*[39]
Bifidobacterium genus	16S	GATTCTGGCTCAGGATGAACGC	CTGATAGGACGCGACCCCAT	60	Gueimonde *et al.*[40]
B. longum group	16S	TTCCAGTTGATCGCATGGTCTTCT	GGCTACCCGTCAAGCCACG	65	Gueimonde *et al.*[40]
B. catenulatum group	16S	GCCGGATGCTCCGACTCCT	ACCCGAAGGCTTGCTCCCGAT	64	Gueimonde *et al.*[40]
B. bifidum	16S	TGACCGACCTGCCCCATGCT	CCCATCCCACGCCGATAGAAT	61	Gueimonde *et al.*[40]
B. breve	16S	AATGCCGGATGCTCCATCACAC	GCCTTGCTCCCTAACAAAAGAGG	62	Gueimonde *et al.*[40]
B. animalis–lactis	16S	TCACGACAAGTGGGTTGCCA	GTTGATCGGCAGCTTGCCG	60	Sheu *et al.*[41]
Clostridium coccoides group	16S	AAATGACGGTACCTGACTAA	CTTTGAGTTTCATTCTTGCGAA	53	Matsuki *et al.*[42,43]
Clostridium leptum subgroup	16S	GCACAAGCAGTGGAGT	CTTCCTCCGTTTTGTCAA	60	Matsuki *et al.*[42,43]
Lactobacillus group	16S	AGCAGTAGGGAATCTTCCA	CACCGCTACACATGGAG	60	Walter *et al.*[44] and Heilig *et al.*[45]
Enterobacteriaceae family	16S	CATTGACGTTACCCGCAGAAGAAGC	CTCTACGAGACTCAAGCTTGC	63	Bartosch *et al.*[46]
Enterococcus group	16S	CCCTTATTGTTAGTTGCCATCATT	ACTCGTTGTACTTCCCATTGT	61	Rinttilä *et al.*[39]
Staphylococcus group	TUF	GGCCGTGTTGAACGTGGTCAAATCA	TIACCATTTCAGTACCTTCTGGTAA	60	Martineau *et al.*[47]
Streptococcus group	TUF	GTACAGTTGCTTCAGGACGTATC	ACGTTCGATTTCATCACGTT	61	Picard *et al.*[48]

* Y represents a (C/T) wobble nucleotide.

Results

Shifts in microbial colonisation patterns in neonatal gastrointestinal tract according to diet

The colonisation patterns of total bacteria and *Lactobacillus*, *Bifidobacterium*, *A. muciniphila* and *Streptococcus* groups in the breast-fed neonatal BALB/c mice were different from those in the formula-fed mice (Fig. 1). The colonisation patterns of total bacteria were significantly different throughout the gastrointestinal tract (from the oral cavity to the large intestine) of both the breast-fed ($P=0{\cdot}0001$) and formula-fed ($P=0{\cdot}0001$) mice, with the large intestine showing the highest number of bacteria. In the breast-fed mice, no differences were detected in the colonisation patterns of total bacteria in the stomach and small intestine ($P=0{\cdot}248$), while in the formula-fed mice, the colonisation patterns in the small intestine and oral cavity were similar ($P=0{\cdot}366$). The colonisation patterns of the *Lactobacillus* and *Streptococcus* groups in the gastrointestinal tract of the breast-fed mice were significantly different from those in the formula-fed mice. The colonisation patterns of *Bifidobacterium* from the stomach to the large intestine in the breast-fed group were significantly different from those in the formula-fed group, in which no differences were observed between the pattern in the stomach and that in the small intestine ($P=0{\cdot}564$). No significant differences in the colonisation pattern of *A. muciniphila* were observed in the breast-fed group ($P=0{\cdot}069$); however, in the formula-fed group, significantly higher amounts of *A. muciniphila* were observed in the large intestine than in the stomach and small intestine. There were no differences in the colonisation patterns in the stomach and small intestine ($P=0{\cdot}132$).

Interestingly, the numbers of bacteria of all the groups that were analysed were found to be increased throughout the intestinal tract, with the large intestine showing the highest numbers. However, this tendency was not observed for the *Streptococcus* group, the amounts of which were increased throughout the intestinal tract from the mouth to the small intestine, but were decreased in the large intestine (Fig. 1).

Microbial composition of the breast-fed and formula-fed neonatal BALB/c mice

Microbial composition in the oral cavity and stomach. Microbial composition in the oral cavity and stomach of the formula-fed BALB/c mice was different from that in the breast-fed mice. Higher DNA concentrations of total bacteria ($P<0{\cdot}001$) were observed in the oral cavity of the formula-fed mice than in that of the breast-fed mice. There were significantly higher amounts of total bacteria ($P=0{\cdot}0001$), bacteria of the *Lactobacillus* group ($P=0{\cdot}001$) and those of the *Streptococcus* group ($P=0{\cdot}008$) in the stomach of the breast-fed mice than in that of the formula-fed mice.

Microbial composition in the small intestine. Higher amounts of total bacteria ($P=0{\cdot}000$), *A. muciniphila* ($P=0{\cdot}005$),

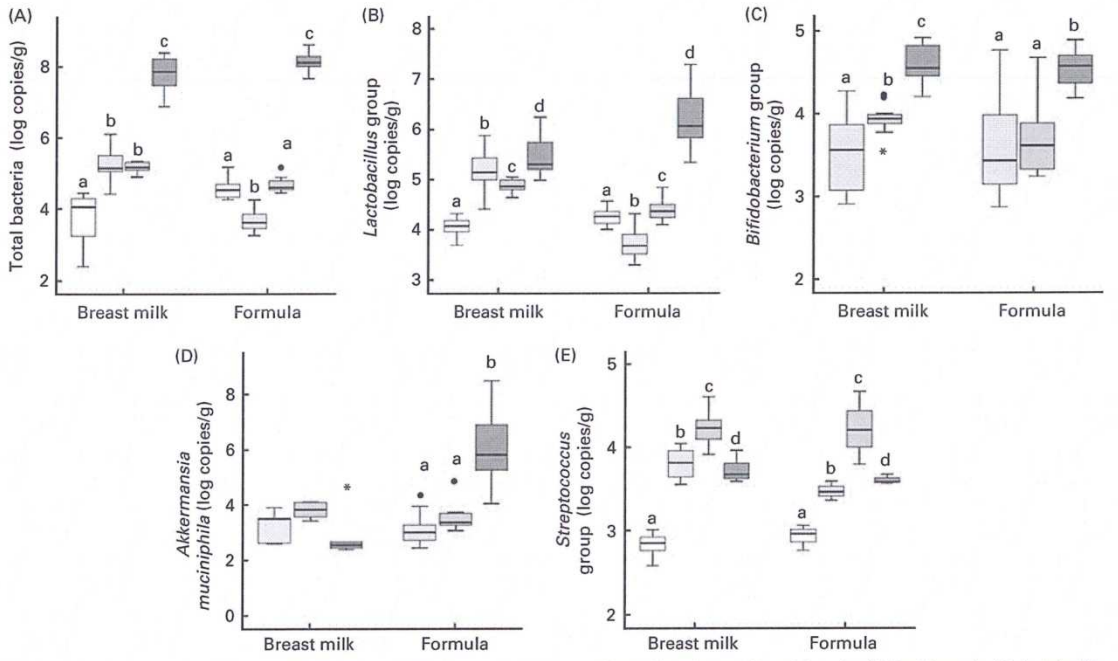

Fig. 1. Microbial colonisation in the gastrointestinal tract of BALB/c neonatal mice on different diets: breast milk and formula. (A) Total bacteria; (B) *Lactobacillus* group; (C) *Bifidobacterium* group; (D) *Akkermansia muciniphila*; (E) *Streptococcus* group. Differences among the sites were calculated using the Friedman test for related samples. ^{a,b,c,d}Values with unlike letters were significantly different among the areas for the same diet group ($P<0{\cdot}05$). □, Oral cavity; □, stomach; □, small intestine; ■, large intestine.

Bifidobacterium ($P=0{\cdot}012$), *Lactobacillus* ($P=0{\cdot}000$), bacteria of the *Bacteroides–Prevotella* group ($P=0{\cdot}007$) and *Streptococcus* ($P=0{\cdot}001$) were found in the breast-fed BALB/c neonatal mice than in the formula-fed mice. The prevalence of the bacteria of the *Enterococcus* group was higher in the formula-fed mice than in the breast-fed mice ($P=0{\cdot}005$), while *C. leptum, C. coccoides and Staphylococcus* were more common in the breast-fed mice ($P=0{\cdot}035$, $P=0{\cdot}041$ and $P=0{\cdot}004$, respectively).

Microbial composition in the large intestine. Higher amounts of *A. muciniphila* ($P=0{\cdot}000$), *Lactobacillus* ($P=0{\cdot}005$), bacteria of the *Bacteroides–Prevotella* group ($P=0{\cdot}000$), *Enterococcus* ($P=0{\cdot}007$), bacteria of the Enterobacteriaceae family ($P=0{\cdot}000$) and *C. leptum* ($P=0{\cdot}024$) were found in the formula-fed mice than in the breast-fed mice during early life. In general, a higher prevalence of bacterial groups was found in the breast-fed group.

Impact of polyamine supplementation on microbiota composition

The formulas enriched with different concentrations of polyamines had an impact on microbial composition based on the polyamine concentration used and gastrointestinal site. On comparing the impact of different concentrations of polyamines throughout the gastrointestinal tract – oral cavity and stomach (Table 1S, available online), small intestine (Table 2S, available online) and large intestine (Table 3S, available online) – it was found that the microbial composition of the formula-fed mice was modified by the polyamines and that it became similar to that of the breast-fed mice in the majority of cases.

Oral cavity and stomach. The amounts of total bacteria and *Lactobacillus* in the oral cavity of the low polyamine group were significantly different from those present in the oral cavity of the intermediate and high polyamine groups (Fig. 2). However, higher concentrations of polyamines in the formula were found to be correlated with lower amounts of total bacteria and bacteria of the *Lactobacillus* and *Streptococcus* groups in the oral cavity and with higher amounts of total bacteria and *Lactobacillus* and bacteria of the *Bacteroides–Prevotella* group in the stomach.

Small intestine. The amounts of bacteria of the Lactobacillus group in the intermediate polyamine group were significantly different from those in the other polyamine groups ($P=0{\cdot}001$) (Table 2S, available online). No differences were observed in the composition of other bacterial groups in the three polyamine groups. *B. animalis* was the most common *Bifidobacterium* species found in the intermediate and high polyamine groups, compared with *B. longum*, which was more prevalent in the formula-fed mice.

Increasing concentrations of polyamines in the formula were found to be correlated with higher amounts of total bacteria and *A. muciniphila* and bacteria of the *Bifidobacterium* group, *Lactobacillus* group, *Bacteroides–Prevotella* group and Enterobacteriaceae family in the small intestine (Fig. 3).

Large intestine. The supplementation of IF with polyamines had a significant impact on microbial composition throughout the gastrointestinal tract of the BALB/c mice after 4 d of intervention (Table 3S, available online). Formula supplemented with intermediate concentrations of polyamines showed lower amounts of total bacteria ($P=0{\cdot}018$) and higher amounts of *Bifidobacterium* ($P=0{\cdot}0001$) and *Lactobacillus*

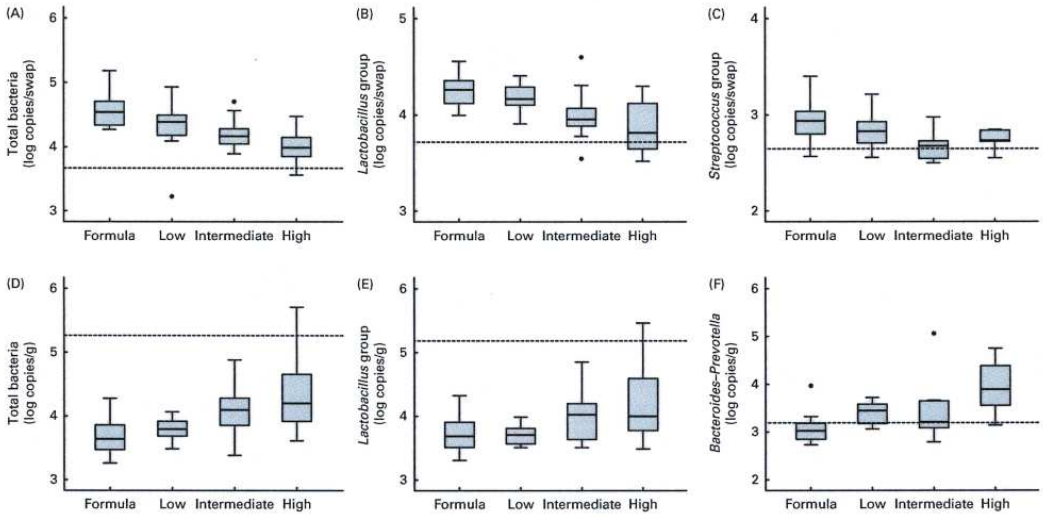

Fig. 2. Box-and-whisker diagrams and Spearman's rank test correlations among the microbial compositions in the samples of the oral cavity and stomach of BALB/c mice, from 0 to increasing quantities of polyamine mixture added to the infant formula. Each bar represents the smallest observation, lower quartile (Q1), median, upper quartile (Q3) and largest observation. The correlation coefficient and significance level are expressed as Θ. Oral cavity: (A) total bacteria ($\Theta=-0{\cdot}66$, $P=0{\cdot}0001$); (B) *Lactobacillus* group ($\Theta=-0{\cdot}55$, $P=0{\cdot}0001$); (C) *Streptococcus* group ($\Theta=-0{\cdot}45$, $P=0{\cdot}002$). Stomach: (D) total bacteria ($\Theta=0{\cdot}51$, $P=0{\cdot}001$); (E) *Lactobacillus* group ($\Theta=0{\cdot}42$, $P=0{\cdot}003$); (F) *Bacteroides–Prevotella* ($\Theta=0{\cdot}50$, $P=0{\cdot}003$). Dotted line represents the mean value for the breast-fed group.

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group (P=0·004) than the other groups of low and high concentrations of polyamines. We also analysed the *Bifidobacterium* species in the large intestine. *B. animalis* was the most common *Bifidobacterium* species found in all the groups included in the study, followed by *B. breve* and *B. catenulatum*. However, *B. bifidum* was detected in only one sample.

Higher concentrations of polyamines in the formula were found to be correlated with higher amounts of total bacteria, *Bifidobacterium* and *C. coccoides* and lower amounts of *A. muciniphila* (Fig. 4).

Discussion

The present study assessed the effect of infant feeding type on microbial colonisation patterns in the entire intestinal tract. The most predominant groups found in the intestinal contents of mice in the present study were similar to those found in human infants[14]. The bacterial populations in the breast-fed group could be considered to be present at normal levels during lactation in the BALB/cOlaHsd mice, with a non-altered mucus layer and without pathology. Significant differences in the predominant microbial groups between the breast-fed and IF-fed mice were observed, including in sites that have not been focused upon in previous research, such as the oral cavity and stomach. In general, we found significantly higher amounts of bacteria in the stomach and small intestine of the breast-fed mice than in those of the formula-fed mice. In particular, we found high amounts of total bacteria, bacteria of the *Lactobacillus and Bifidobacterium* groups, and *A. muciniphila*. An opposite scenario was found in the large intestine, with higher amounts of bacteria being found in the formula-fed group, mainly due to high amounts of *A. muciniphila* and bacteria of the Enterobacteriaceae family. These differences could be correlated with an altered intestinal microbial colonisation pattern. Due to general similarities in the mammalian diet during early life and the development of the gastrointestinal tract, something similar may occur in humans.

The present study provides novel data on the impact of polyamines on microbial composition. To our knowledge, this is the first study to show how an IF enriched with polyamines has a significant impact on early microbial composition in the entire gastrointestinal tract of neonatal BALB/c mice. In general, the addition of polyamines to the manufactured formula regulated microbial populations to amounts found in the breast-fed group.

The present study also expands our previous knowledge on the impact of polyamines on microbial composition in the intestine[35]. We had shown previously, using flow cytometry-fluorescence *in situ* hybridisation, that the amounts of bacteria of the *Bifidobacterium* group were significantly greater in the formula-fed mice following supplementation with polyamines (P<0·01). We confirmed these results by quantitative PCR in the present study, and found high amounts of bacteria of the *Bifidobacterium* group in the small and large intestines of pups fed the IF enriched with polyamines. The high amounts of bacteria of the *Bifidobacterium* group found in the small and large intestines of pups fed the IF enriched with polyamines could be a biological index of the health status of the intestinal tract[50]. Recent studies have reported that high numbers of bifidobacteria may correlate positively with the normalisation of inflammatory status,

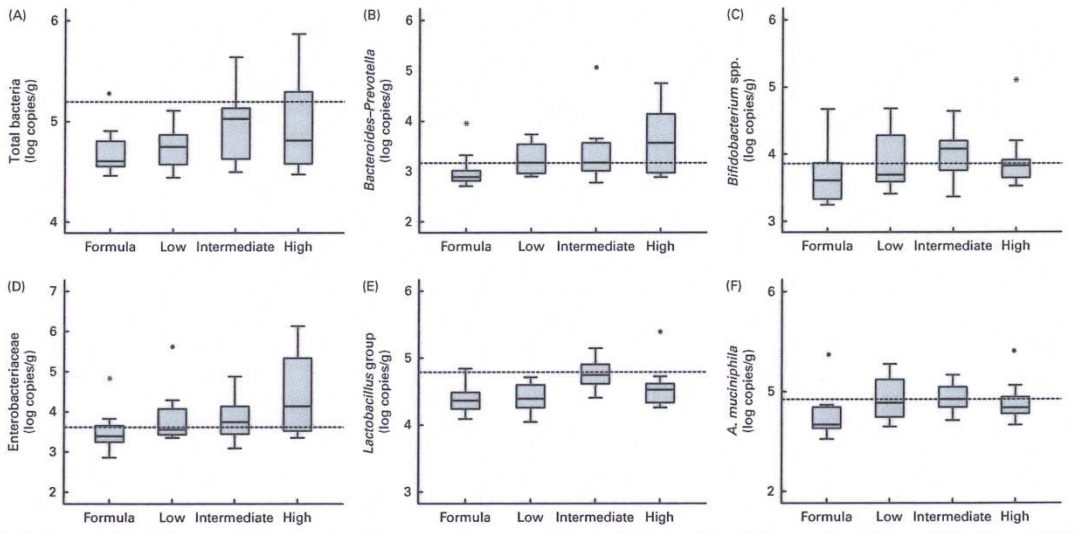

Fig. 3. Box-and-whisker diagram and Spearman's rank test correlations among the microbial compositions in the samples of the small intestine of BALB/c mice, from 0 to increasing quantities of polyamine mixture added to the infant formula. Each bar represents the smallest observation, lower quartile (Q1), median, upper quartile (Q3) and largest observation. The correlation coefficient and significance level are expressed as Θ. Small intestine: (A) total bacteria (P=0·046); (B) *Bacteroides–Prevotella* group (P=0·004); (C) *Bifidobacterium* spp. (Θ = 0·27, P=0·06); (D) Enterobacteriaceae (Θ = 0·40, P=0·004); (E) *Lactobacillus* group (P=0·007); (F) *Akkermansia muciniphila* (Θ = 0·31, P=0·035). The dotted line represents the mean value for the breast-fed group.

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Supplementary Data

Table 1S. Microbiota composition of oral cavity and stomach analysed by qPCR. Data are shown as prevalence, median, and interquartile range (IQR) of the logarithm of gene copies per swab for oral samples and per gram of gastric content. Statistical analysis was calculated using the Kruskal-Wallis test (P-value1 is the significance level for comparison between breast-fed formula; P-value2 is for comparison between formula feeding and polyamine supplementation; P-value3 is the significance of comparison between groups with polyamines supplementation).

	Log RNA gene copies /swab or g												
	Breastfed		Formula		Low		Intermediate		High				
Bacterial Group	**Prevalence**	**Median (IQR)**	**Prevalence**	**Median (IQR)**	**Prevalence**	**Median (IQR)**	**Prevalence**	**Median (IQR)**	**Prevalence**	**Median (IQR)**	**P-value 1**	**P-value 2**	**P-value 3**
Oral cavity													
Total Bacteria	12	4.06 (3.04-4.383)	12	4.54 (4.33-4.71)	12	4.40 (4.16-4.51)	12	4.17 (4.01-4.32)	12	3.99 (3.84-4.18)	0.000	0.000	0.011
Bifidobacterium	1	-	-	-	4	2.64 (2.53-2.88)	-	-	-	-	-	-	-
Akkermansia	4	2.32 (2.09-2.54)	2	2.05 (2.00-2.50)	3	2.15 (2.10-2.70)	2	-	2	-	-	-	-
Lactobacillus	9	4.08 (3.94-4.23)	12	4.26 (4.11-4.40)	12	4.17 (4.10-4.33)	12	3.95 (3.88-4.09)	10	3.82 (3.63-4.15)	0.082	0.002	0.010
Streptococcus	9	2.81 (2.70-2.92)	12	2.94 (2.78-3.05)	12	2.83 (2.70-2.94)	12	2.63 (2.52-2.75)	10	2.72 (2.55-2.81)	0.148	0.005	0.047
Enterococcus	10	2.71 (2.31-2.82)	11	2.38 (2.32-2.44)	12	2.90 (2.50-3.90)	9	2.58 (1.50-2.95)	10	2.29 (2.00-2.76)	0.152	0.033	0.145
Stomach													
Total Bacteria	12	5.15 (5.06-5.60)	12	3.63 (3.56-3.90)	12	3.80 (3.66-3.92)	12	4.08 (3.72-4.31)	12	4.18 (3.91-4.64)	0.000	0.005	0.024
Bifidobacterium	12	3.56 (3.02-3.87)	11	3.43 (3.11-4.05)	12	3.13 (2.85-3.96)	6*	2.63 (2.55-3.15)	12	3.23 (3.18-3.34)	0.755	0.03	0.042
Akkermansia	9	3.48 (2.83-3.60)	11	2.99 (2.70-3.30)	10	2.86 (2.72-3.54)	1*	-	11	2.82 (2.69-3.16)	0.370	0.382	0.253
Lactobacillus	12	5.15 (4.97-5.40)	12	3.67 (3.50-3.96)	12	3.70 (3.53-3.81)	12	4.01 (3.60-4.25)	12	3.98 (3.75-4.58)	0.000	0.029	0.040
Bacteroides_Prevotella	10	3.24 (3.18-3.40)	7	3.02 (2.84-3.33)	8	3.45 (3.15-3.60)	10	3.21 (3.07-3.65)	8	3.90 (3.34-4.45)	0.070	0.017	0.052
Streptococcus	12	3.84 (3.66-4.00)	12	3.42 (3.34-3.64)	12	3.40 (3.35-3.57)	12	3.32 (3.29-3.41)	12	3.53 (3.41-3.68)	0.008	0.004	0.002
Enterococcus	4	2.16 (2.05-2.22)	4	2.63 (2.13-3.00)	3	2.22 (2.15-2.50)	5	2.27 (2.17-3.00)	2	-	0.343	0.636	0.528
Enterobacteriaceae	-	-	1	-	-	-	1	-	3	2.22 (2.13-3.74)	-	0.766	0.655
Staphylococcus	-	-	2	-	2	-	1	-	1	-	-	-	-

Data was obtained from positive samples and are shown as median and interquartile range (IQR). Statistical analysis was calculated using the Kruskal-Wallis test. Statistical differences were corrected for a multiple comparison test using the Bonferroni adjustment, and significant differences among groups were considered as having a P-value 2 < 0.0125 (0.05/4) and P-value 3<0.017 (0.05/3).

* Statistical differences in the prevalence between low and intermediate polyamine diet, and intermediate and high polyamine diet. No significant differences were found between positive samples. Statistical analysis was calculated using the $\chi 2$ test. No significant differences were found between low and high.

Table 2S. Microbiota composition of the small intestine analysed by qPCR. Data are show as prevalence, median, and interquartile range (IQR) of the logarithm of gene copies per swab per gram of intestinal content. Statistical analysis was calculated by Kruskal-Wallis test (P-value1 is significance level for comparison between breast-fed formula; P-value2 is for comparison between formula feeding and polyamine supplementation; P-value3 is the significance of comparison between groups with polyamines supplementation).

	Log RNA gene copies /g												
	Breastfed		Formula		Low		Intermediate		High				
Bacterial Group	**Prevalence**	**Median (IQR)**	Prevalence	**Median (IQR)**	**Prevalence**	**Median (IQR)**	**Prevalence**	**Median (IQR)**	**Prevalence**	**Median (IQR)**	**P-value 1**	**P-value 2**	**P-value 3**
Total Bacteria	12	5.17 (5.10-5.33)	12	4.61 (4.54-4.82)	12	4.75 (4.55-4.88)	12	5.03 (4.63-5.14)	12	4.82 (4.57-5.37)	0.000	0.114	0.218
Bifidobacterium	12	3.93 (3.76-4.00)	12	3.61 (3.32-3.90)	12	3.70 (3.58-4.30)	12	4.09 (3.74-4.20)	12	3.83 (3.63-3.92)	0.012	0.109	0.531
B. longum	4	2.95 (2.55-3.22)	2	-	3	2.65 (2.60-2.70)	1	-	2	-	0.724	0.565	0.180
B. animalis	3	2.65 (2.50-3.58)	2	-	1	-	4	2.50 (2.45-2.90)	4	2.60 (2.50-2.86)	0.564	0.691	0.402
Akkermansia	12	3.95 (3.60-4.10)	12	3.34 (3.24-3.70)	12	3.78 (3.48-4.36)	12	3.86 (3.69-4.12)	12	3.68 (3.54-3.93)	0.005	0.032	0.645
Lactobacillus	12	4.83 (4.74-4.96)	12	4.36 (4.22-4.50)	12	4.39 (4.25-4.61)	12	4.75 (4.59-4.92)	12	4.53 (4.33-4.62)	0.000	0.001	0.004
Bacteroides-Prevotella	12	3.21 (3.07-3.40)	12	2.89 (2.80-3.02)	12	3.17 (2.95-3.57)	12	3.18 (3.00-3.61)	12	3.57 (2.95-4.25)	0.007	0.023	0.512
Streptococcus	12	4.32 (4.16-34.46)	12	3.77 (3.70-4.14)	12	3.86 (3.77-4.25)	12	4.07 (3.92-4.37)	12	3.82 (3.64-3.95)	0.001	0.028	0.024
Enterococcus	12	2.42 (2.35-2.54)	6‡	2.16 (2.04-2.40)	7	2.43 (2.20-2.66)	10	2.32 (2.12-2.55)	10	2.19(2.15-2.35)	0.151	0.406	0.491
Enterobacteriaceae	12	3.74 (3.53-3.80)	12	3.41 (3.22-3.66)	12	3.58 (3.41-4.13)	12	3.76 (3.44-4.20)	12	4.13 (3.51-5.44)	0.068	0.038	0.243
C.leptum subgroup	10	2.64 (2.53-2.80)	5‡	2.60 (2.55-2.72)	7	2.76 (2.62-2.89)	10	2.65 (2.52-2.81)	8	2.80 (2.60-3.02)	0.859	0.451	0.530
C.coccoides group	8	2.61 (2.50-2.84)	3‡	2.60 (2.56-2.61)	6	2.55 (2.50-2.78)	2	3.28 (2.76-3.80)	6	2.70 (2.60-2.82)	0.667	0.107	0.079
Staphylococcus	10	2.95 (2.63-3.08)	3‡	2.85 (2.52-3.70)	6	3.11 (2.66-3.30)	10	2.80 (2.68-3.10)	7	2.60 (2.50-3.15)	0.935	0.590	0.345

Data was obtained from positive samples and are shown as median and interquartile range (IQR). Statistical analysis was calculated using the Kruskal-Wallis test. Statistical differences were corrected for a multiple comparison test using the Bonferroni adjustment, and significant differences among the groups were considered as having a P-value2 < 0.0125 (0.05/4) and P-value3<0.017 (0.05/3).

‡Statistical differences in the prevalence between formula- and breast-fed mice. No significant differences were found between positive samples. Statistical analysis was calculated using the $\chi 2$ test. No significant differences were found between low and high, low and intermediate, and intermediate and high polyamine diet.

Table 3S. Microbiota composition present in BALB/c mice large intestines analysed by qPCR. Data are show as prevalence, median, and interquartile range (IQR) of the logarithm of gene copies per swab per gram of intestinal content. Statistical analysis was calculated using the Kruskal-Wallis test (P-value 1 is the significance level for comparison between breast-fed formula; P-value2 is for comparison between formula feeding and polyamine supplementation; P-value3 is the significance of comparison between groups with polyamines supplementation).

	Log RNA gene copies /g												
	Breastfed		Formula		Low		Intermediate		High				
Bacterial Group	**Prevalence**	**Median (IQR)**	Prevalence	**Median (IQR)**	**Prevalence**	**Median (IQR)**	**Prevalence**	**Median (IQR)**	**Prevalence**	**Median (IQR)**	**P-value 1**	**P-value 2**	**P-value 3**
Total Bacteria	12	7.86 (7.32-8.22)	12	8.11 (7.97-8.31)	12	8.68 (8.64-8.82)	12	8.25 (8.02-8.63)	12	8.66 (8.54-8.78)	0.143	0.000	0.018
Bifidobacterium	12	4.60(4.21-4.78)	12	4.56 (4.30-4.70)	12	4.51 (4.38-4.78)	12	5.01 (4.97-5.17)	12	4.64 (4.39-4.90)	0.630	0.000	0.000
B. longum	2	-	2	-	4	2.76 (2.34-3.00)	2	-	2	-	-	0.708	0.920
B.breve	11	2.71 (2.65-2.78)	11	2.76 (2.66-2.87)	12	2.80 (2.73-2.95)	12	2.98 (2.77-3.06)	10	3.15 (2.45-4.07)	0.374	0.124	0.285
B.bifidum	1	-	-	-	-	-	-	-	1	-	-	-	-
B.catenulatum	6	2.28 (2.25-2.40)	6	2.23 (2.20-2.35)	9	2.20 (2.16-2.24)	9	2.27 (2.25-2.54)	10	2.24 (2.20-2.28)	0.145	0.028	0.138
B.animalis	12	4.63 (4.51-4.75)	12	4.53 (4.50-4.57)	12	4.48 (4.42-4.52)	12	4.49 (4.40-4.60)	12	4.57 (4.48-4.77)	0.150	0.111	0.130
Akkermansia	7	2.48 (2.40-2.76)	12‡	5.74 (4.96-6.92)	12	5.58 (4.12-7.37)	12	3.00 (2.74-3.27)	12	3.31 (3.17-3.43)	0.000	0.000	0.000
Lactobacillus	12	5.36 (5.21-5.68)	12	6.06 (5.80-6.76)	12	5.95 (5.63-6.56)	12	6.75 (6.18-7.06)	12	5.75 (5.48-6.01)	0.005	0.011	0.004
Bacteroides-Prevotella	12	7.37(6.65-7.66)	12	8.07 (7.82-8.30)	12	8.20 (8.12-8.32)	12	8.11 (8.04-8.29)	12	8.20 (8.00-8.25)	0.000	0.507	0.506
Streptococcus	9	3.69 (3.62-3.80)	3‡	3.58 (3.56-3.67)	9	3.60 (3.52-3.73)	3*	3.60 (3.55-3.76)	7	3.50 (3.40-3.53)	0.100	0.340	0.224
Enterococcus	6	2.90 (2.71-3.20)	12‡	3.71 (3.22-4.14)	12	3.37 (3.20-3.73)	12	3.90 (3.32-4.12)	12	3.20 (2.80-3.60)	0.007	0.187	0.139
Enterobacteriaceae	12	3.18 (2.89-4.09)	12	7.41 (7.00-8.16)	12	8.11 (7.72-8.25)	12	7.97 (6.70-8.32)	12	7.73 (6.84-8.26)	0.000	0.497	0.613
C.leptum subgroup	12	6.51 (6.20-6.75)	12	6.97 (6.56-7.10)	12	7.26 (6.91-7.67)	12	6.71 (5.47-7.02)	12	7.01 (6.45-7.42)	0.024	0.042	0.050
C.coccoides group	12	5.96 (5.62-6.24)	12	5.54 (4.75-6.20)	12	6.43 (6.24-6.62)	12	6.22 (5.24-6.56)	12	6.52 (6.08-6.70)	0.294	0.004	0.196
Staphylococcus	5	2.23 (2.10-2.85)	4	2.25 (2.07-2.35)	6	2.70 (2.16-3.88)	4	2.43 (2.12-3.00)	7	2.28 (2.08-2.50)	0.479	0.590	0.905

Data was obtained from positive samples and are shown as median and interquartile range (IQR). Statistical analysis was calculated using the Kruskal-Wallis test. Statistical differences were corrected for a multiple comparison test using the Bonferroni adjustment and significant differences among the groups were considered as having a P-value2 < 0.0125 (0.05/4) and P-value3<0.017 (0.05/3).

‡Statistical differences in the prevalence between formula- and breast-fed mice. No significant differences were found between positive samples. Statistical analysis was calculated using the $\chi 2$ test.

* Statistical differences in the prevalence between low and intermediate polyamine diet. No significant differences were found between positive samples. Statistical analysis was calculated using the $\chi 2$ test. No significant differences were found between low and high and intermediate and high polyamine diet

4.3

Gómez-Gallego, C., Frias, R., Perez-Martínez, G., Bernal, M. J., Periago, M. J., Salminen, S., Ros, G., Collado, M. C.

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Polyamine supplementation in infant formula: Influence on lymphocyte populations and immune system-related gene expression in a Balb/cOlaHsd mouse model

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ABSTRACT

The aim of this work was to study whether the proportion of polyamine found in human milk, administered with a commercial infant formula, affected the maturation of the immune system in a BALB/cOlaHsd mouse model. Forty-eight pups (14-days old) were randomly assigned to four-day intervention groups: 1) breast-fed (normal lactation); 2) fed infant formula; and 3) two different groups fed with infant formula supplemented with two different amounts of polyamines. The influence of polyamine administration on lymphocyte populations in the blood, spleen, and mesenteric lymph nodes, as well as on the modulation of immune system-related gene expression in the small intestine, was analyzed. The results demonstrated that polyamine supplementation induced an increase in splenic B cells to levels observed during normal lactation when compared with formula without supplementation. The correlation coefficients for the splenic lymphocyte populations increased with polyamine supplementation, with a dose-dependent effect. Our results demonstrate that polyamines influence gene expression profile, mainly *Cd1d1*, *Cd40*, *Hdac5*, *Hdac7*, *Clcf1* and *Tlr4* compared with normal lactation. In general, the gene expression results verified that the expression of genes associated with immune system was similar in the group with high polyamine supplementation to that observed in the group with normal lactation.

1. Introduction

In the early period of life, both humans and rodents are exposed to a wide variety of microorganisms and dietary antigens, which drive the complete maturation of the intestinal and systemic immune system (Brandtzaeg, 2003).

Breast milk contains bioactive substances, such as polyamines, which are known to be important for the development of the neonate's immune system by providing protection against infections, promoting oral tolerance, and controlling inflammatory responses (Newburg & Walker, 2007). Specific polyamines, including spermine (SPM), spermidine (SPD), and putrescine (PUT) have been identified in the breast milk of mammalian species, but their levels in most infant formulas are significantly lower than those in human milk (Buts, De Keyser, De Raedemaeker, Collette, & Sokal, 1995; Pollack, Koldousky, & Nishioka, 1992; Romain, Dandrifosse, Jeusette, & Forget, 1992).

Dietary polyamines are rapidly absorbed in the small intestine (Seiler & Raul, 2007). They are essential for cell proliferation and differentiation (Löser, 2000) and are involved in DNA, RNA, and protein synthesis (Seiler & Raul, 2007). Previous studies have reported the essential role of polyamines in the development of the intestine (Sabater-Molina et al., 2009), the modulation of intestinal microbiota by polyamines (Gómez-Gallego et al., 2012), and positive effects on the developing spleen in mice (Jolois et al., 2002). The previously reported effects of milk polyamines on the maturation of the intestinal and systemic immune system (Pérez-Cano, González-Castro, Castellote, Franch, & Castell, 2010; Steege, Buurman, & Forget, 1997) suggest that supplementation of manufactured infant formulas with polyamines might improve immune functions of human infants in a manner similar to that observed by breastfeeding.

Only a few studies (Jolois et al., 2002; Pérez-Cano et al., 2010; Steege et al., 1997) on the effects of polyamines during lactation in rodents have been reported. These were performed by orally administering a single polyamine and the pups continuing to be fed by their mothers. Thus, they were not well controlled for real polyamine ingestion due

Abbreviations: FACS, fluorescence-activated cell sorting; FITC, Fluorescein isothiocyanate; HDAC, hystone deacetylase; PE, phycoerythrin; PUT, putrescine; SPD, spermidine; SPM, spermine.

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to the natural polyamine content in mother's milk that was not reported.

The aim of this work was to investigate whether the proportion of polyamine found in human milk, administered in combination with a commercial infant formula in early-weaned pups, affected the maturation of the immune system in a BALB/cOlaHsd mouse model. For this purpose, the current study evaluated the influence of orally administered polyamines in infant formula on lymphocyte populations in the blood, spleen, and mesenteric lymph nodes, as well as the on the modulation of immune system-related gene expression in the small intestine, in a BALB/cOlaHsd mouse model.

2. Material and methods

2.1. Animals and study design

A total of 48 BALB/cOlaHsd mouse pups were used in the study. The pups were born and raised in a semi-barrier facility of the Central Animal Laboratory, University of Turku as a result of breeding adult dams and sirs that were supplied by a reputed commercial vendor of laboratory rodents (Harlan Laboratories®, Horst, Netherlands).

The pups were housed in groups of two, and grouping was decided based on male–female pup availability from the breeding colony. All the mice were maintained in conventional stainless steel cages (370 cm^2; 26.7 × 20.7 × 14 cm), with solid bottoms and Aspen chips as bedding. Cage change was undertaken twice a week. The room temperature was 23 °C (±3 °C), the relative humidity was 45 to 65%, and artificial lightning was used with a 12 h light/dark cycle (lights on at 6:00 am). Prior to entering the breeding program, the adult mice were acclimatized for at least 30 days. Throughout the breeding period, all the adult mice were fed standard mouse chow (SDS, Special Diet Services, Whitham, Essex, UK) ad libitum, and tap water was provided without restrictions in polycarbonate bottles. The entire mouse colony was judged to be healthy on the basis of the absence of clinical signs and the results of microbiological screening routinely performed on the colony in accordance with European recommendations (Nicklas et al., 2002).

The experimental protocol was approved by the National Ethics Committee for Animal Experiments in Finland (ESLH-2009-04845/Ym-23) and conformed to the regulations and requirements of the European Union concerning the protection of animals used for scientific purposes.

2.2. Polyamine supplementation

Treatment and handling were performed as described previously (Gómez-Gallego et al., 2012). Fourteen-day-old pups were randomly assigned to one of the following four dietary groups according to dietary treatment: normal lactation group (NL, n = 12), unweaned pups, infant formula group (IF, n = 12), weaned pups fed with commercial infant formula, and weaned pups fed on infant formula enriched with low (T1, n = 12) and high (T2, n = 12) concentrations of polyamines.

PUT (D13208, Aldrich, Steinheim, Alemania), SPD (2626, Sigma, Steinheim, Alemania), and SPM (85590, Fluka, Steinheim, Alemania) were orally administered in the following proportions: 3.38%, 35.48%, and 61.14%, respectively. The concentration levels tested in the two polyamine supplementation groups were: treatment I) 2.10 μg/day PUT, 22.05 μg/day SPD, and 38.00 μg/day SPM (T1); treatment II) 8.40 μg/day PUT, 88.20 μg/day SPD, and 152.00 μg/day SPM (T2). The manufactured formula employed was a commercial infant formula targeted for babies during the first six months, fortified with nucleotides, α-lactalbumin, and ω−3 and ω−6 fatty acids supplied by HERO España S.A., (Alcantarilla, Spain) (infant formula composition was described in Gómez-Gallego et al., 2012). The nonenriched formula and the formula with polyamines (100 μl) were made with warm water following the manufacturer's instructions and given to the pups twice daily by oral gavage as previously described. Early-weaned animals were fed a porridge made with the same IF without the enrichment in polyamines being the polyamines only supplied by oral gavage.

2.3. Sample collection

After the four-day diet intervention, the animals were anesthetized with isoflurane, and blood, mesenteric lymph node, spleen, and small intestine samples were obtained. The blood, lymph node, and spleen were conserved in saline buffer (Dulbecco's phosphate-buffered saline, 1% fetal calf serum, and 0.1% sodium azide) until flow cytometer analysis. The whole small intestine was emptied of the contents for microbiota analysis (not reported here), and the tissue was kept at −80 °C using Trizol® reagent (15596-026, Invitrogen, Paisley, UK) according to the manufacturer's instructions for RNA purification.

2.4. Immunofluorescence and flow cytometry

These analyses were performed as described by Alam, Valkonen, Ohls, Törnqvist, and Hänninen (2010), on the same collection day. Single-cell suspensions of spleen cells and mesenteric lymph node cells were acquired by gently pressing the tissues through a metal mesh. Erythrocytes of spleen and blood samples were removed by hypotonic lysis using 0.2% (wt/vol.) NaCl solution.

Anti-mouse B220 (CD45R) allophycocyanin conjugated (FAB1217A, R&D Systems, Minneapolis, USA), CD4 PE-conjugated, and CD8a FITC-conjugated (Immunotools, Friesoythe, Germany) were used to examine the surface expression of markers. Flow cytometry was performed using fluorescence activated cell sorter (FACS) Calibur® flow cytometer (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) and Cell Quest (Becton, Dickinson and Company) software. The cells were characterized according to their light and immunofluorescence characteristics. A single-cell suspension of isolated cells was analyzed by FACS for each sample, and the tissue was assayed. To determine the appropriate distribution of the cell populations, the cell suspension was stained with appropriate combinations of monoclonal antibodies. A maximum of 20,000 gated events were recorded, and the proportions of CD4+, CD8+, and B cells were quantified.

2.5. RT-PCR analysis

The whole small intestine was homogenized in Polytron® PT 10-35 GT (Kinematica, Littau/Lucerne, Switzerland).

For purification of total RNA and cleanup, the RNeasy® Mini Kit (Qiagen, Duesseldorf, Germany) was used. The total RNA concentrations and A260/A280, and A260/A230 ratios were determined using a NanoDrop ND1000 (NanoDrop Technologies, Wilmington, USA) spectrophotometer to assure the integrity and the purity of the mRNA.

The expression of genes encoding T-cell and B-cell activation, as well as Toll-like receptors (TLRs), in the small intestinal tissues was assessed using a RT2 First Strand Kit (C-03, SABiosciences Corporation, Frederick, USA) according to the manufacturer's instructions. The real-time PCRs were carried out using 96-well PCR arrays designed for the evaluation of mouse T-cell and B-cell proliferation and the differentiation genes (PAMM-053, SABiosciences) and using a Roche LightCycler 480 (Roche Diagnostics Corporation, Indianapolis, USA) as a cycler platform with a fluorescence detection system. The Mouse T-cell and B-cell Activation RT^2 Profiler™ PCR Array profiles the expression of 84 genes representing T-cell and B-cell activation, a key part of adaptive immunity. This array includes genes involved in B-cell activation, as well as genes involved in B-cell proliferation and differentiation. Genes involved in the activation of T cells and their proliferation and differentiation are also represented, along with genes regulating Th1 and Th2 development and T-cell polarization. Genes involved in the activation of macrophages, neutrophils, and natural killer cells are also included. For cDNA synthesis, 5 μg of total RNA was used with the RT2 First Strand Kit (SA Biosciences)

Table 1
Lymphocyte populations in percentage analyzed in blood, spleen and mesenteric lymph node. Data are shown as median, and interquartile range (IQR). Statistical analysis was calculated using the Kruskal–Wallis test for blood samples and one-way ANOVA for spleen and lymph node samples.

Group	Blood			Spleen			Node		
	CD4+ cells	CD8+ cells	B cells	CD4+ cells	CD8+ cells	B cells	CD4+ cells	CD8+ cells	B cells
NL	11.34 (9.44–14.28)	4.52 (3.32–5.61)	13.34 (10.87–15.03)	11.25 (10.55–11.94)	4.20 (3.97–4.89)	39.84 (36.05–45.21)	46.95 (45.64–48.48)	19.06 (17.44–20.67)	29.47 (25.55–30.38)
IF	2.71 (1.60–4.00)	0.68 (0.46–1.09)	0.75 (0.40–0.84)	14.77 (14.06–15.88)	4.92 (4.47–5.34)	30.99 (25.99–33.56)	52.47 (49.82–54.76)	17.10 (14.63–19.58)	16.92 (13.08–20.46)
T1	3.05 (2.49–4.38)	0.85 (0.72–1.29)	0.68 (0.55–0.91)	15.95 (14.72–19.53)	4.77 (4.40–5.38)	34.15 (32.88–38.55)	55.54 (51.15–58.64)	19.08 (16.32–20.12)	14.57 (12.83–17.60)
T2	2.16 (1.5–2.92)	0.44 (0.32–0.71)	1.13 (0.88–1.52)	20.22 (18.86–21.61)	7.17 (6.07–9.12)	52.07 (43.86–54.46)	52.18 (47.04–55.35)	18.10 (16.81–22.47)	18.95 (14.93–21.3)
p-Value	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.003**	0.323	**0.000**

Statistical differences were corrected for a multiple comparison test using the Bonferroni adjustment, and significant differences among groups (bold numbers) were considered as having a p-value <0.0125 (0.05/4).

as described by the manufacturer. The relative gene expression was determined according to the comparative C_t method.

2.6. Statistical analysis

2.6.1. Cell population

For samples of spleen and lymph node one-way ANOVA and relevant post-hoc test were used with SPSS 15.0 software. For blood samples, due to non-normal distribution, comparisons among data were done by applying the Kruskal–Wallis test. Correlations between variables were determined by applying the Pearson's or Spearman test correlation depending on normal or non-normal distribution. Significant differences were considered at $p \leq 0.05$.

2.6.2. Gene expression profiles

Genes and treatment groups were analyzed using the RT^2 Profiler™ PCR Array Data Analysis (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php March 27th, 2013). The integrated web-based software package for the PCR Array System automatically performs all ΔΔCt based fold-change calculations from your uploaded raw threshold cycle data. Gene transcription results were considered significant at ≥1.5-fold or ≤−1.5-fold change and p-value ≤0.05 in Student's *t* test comparing with normal lactation group.

3. Results

3.1. Differences in lymphocyte population in infant formula and formula supplemented in polyamines

We found statistically significant differences in the lymphocyte populations in the three areas sampled (Table 1). The blood analysis demonstrated that the percentages of lymphocytes in breast feeding group were significantly higher than those observed in the formula and polyamine-supplemented groups. However, in the spleen samples, higher levels of Cd4+, Cd8+, and B cells were observed in the T2 group. The node samples showed no clear tendencies in terms of the levels of lymphocytes. In the blood, the main differences were between the breastfed and the three formula feeding groups. Considering the effect of the enrichment of infant formulas with polyamines, the data showed a significant correlation between an increase in the B lymphocyte population and an increase in polyamine supplementation (Fig. 1).

A comparison between the differences in the distribution of the cells is shown in Fig. 2. In the spleen, animals that received high polyamine supplementation showed high proportions of CD4+ and CD8+ lymphocytes compared to the normal lactation and formula-fed pups. The polyamine supplementation induced an increase in B cells compared with formula without supplementation, with levels similar to

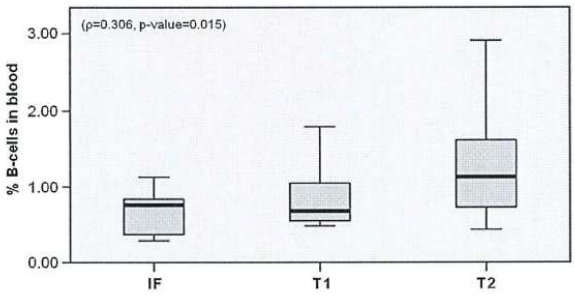

Fig. 1. Box-and-whisker diagram and Pearson's correlation (ρ) among the B cells in the blood samples. Each bar represents the smallest observation, lower quartile (Q1), median, upper quartile (Q3), and largest observation. Significant differences among the percentages of the cells are shown with letters: The groups with different letters have statistically significant differences at the level $p < 0.05$. *R* denotes the regression coefficient and the significance level of the correlation.

Table 2
Immune system proliferation and development gene changes (fold change) in formula and formula enriched in polyamines feeding mouse small intestine comparing with normal lactation.

Gene	Gene description	IF	T1	T2	General gene related function[a]
Cd1d1	CD1d1 antigen	***−2.17***	−1.79	−1.09	Regulator of T-cell activation and differentiation. Macrophage activation.
Cd40	CD40 antigen	**3.20**	−1.26	−1.14	Involved in Th1/Th2 differentiation, T-cell differentiation and B-cell activation.
Hdac5	Histone deacetylase 5	**−3.51**	−2.51	−1.58	B-cell differentiation
Hdac7	Histone deacetylase 7	1.03	***−2.10***	−1.39	
Il27	Interleukin 27	−1.74	**−2.17**	−1.7	T-cell differentiation

Bold numbers indicate gene transcriptional significant differences ($p < 0.05$).
Bold italic numbers indicate gene transcriptional significant differences ($p < 0.01$).
[a] According to array manufacturer.

The expression of *Cd1d1* was lower in the formula-fed group than in the normal lactation group. However, the expression of *Cd1d1* was not significantly different in the groups fed the infant formula supplemented with polyamines compared with that in the normal lactation group, being the most similar expression in the group with high polyamine supplementation.

The expression of *cd40* was higher in the nonenriched formula group than that in the normal lactation group, and it did not differ significantly between the polyamine supplementation and the normal lactation groups. The expression of *hdac5* was down-regulated in the early-weaned groups compared to that in the normal lactation group. The expression was lower despite the polyamine supplementation, but the difference in expression was not significant compared to that of the polyamine supplementation groups. Moreover, the differences were smaller than those observed in the formula-fed group without supplementation. With regard to *hdac7*, the down-regulated expression was significantly lower in the group with low polyamine supplementation (T1). The expression of *il27* was down-regulated in the early-weaned groups compared to that in the normal lactation group, and the difference in expression was statistically significantly different only in the T1 group.

Table 3
Fold regulation on immune system genes in formula and formula enriched in polyamine groups comparing with normal lactation.

Gene symbol	Fold regulation		
	IF	T1	T2
Ap3b1	−2.33		
Cd1d1	−2.17		
Cd2	−2.02		
Cd40	3.20		
Cd40lg	−2.24	−4.18	
Cd74		−3.11	−4.50
Cd93		−2.94	−2.68
Clcf1		−2.65	−2.60
Cxcr4	−3.23	−2.66	−2.83
Egr1		−3.90	
Flt3	2.02		
Hdac5	−3.51	−2.51	
Hdac7		−2.10	
Hsp90aa1	5.93	4.93	5.08
Icosl		−2.02	
Il11			−2.19
Il27		−2.17	
Impdh2		−2.28	
Irf4			3.48
Jag2	2.92		
Ms4a1	4.35	4.30	
Prkcq		2.35	
Ptprc	4.07	2.91	
Tlr1		2.60	
Socs5	−2.11		
Tlr4	3.00		
Tnfrsf13c	3.92		
Tnfsf13b	2.50	2.64	
Tnfsf14		2.90	
Traf6	2.21		
Was	−2.07	−2.37	−2.42

The table represents fold differences minor than −2 and higher than 2.

To estimate the similarity in gene expression between the samples, two clusters of the differentially regulated genes were performed (Fig. 4). Cluster map profiling revealed that compared with normal lactation, the most different gene expression was associated with infant formula diet, being placed in the left side of the cluster. These data were confirmed by clustering the whole array and comparing the gene expression of all the genes checked (Fig. 4). The results showed that gene expression profile of the high polyamine supplementation group was most similar to that of the normal lactation group.

The gene expression profile comparison between the nonsupplemented and supplemented formula feeding shows significant fold change down-regulation in five genes (Table 4). The gene expression profile of the low and high polyamine supplementation groups was similar, and no significant differences were found, with one exception, *Il2ra*, which was down-regulated in the T1 group, with a fold change of 1.9 (p-value = 0.03).

3.3. Associations between lymphocyte population in mesenteric lymph node and gene-expression in the small intestine

In the normal lactation group, higher CD4+ levels in the mesenteric lymph node were related to the down-regulation of the expression of cd1d1 ($\rho = -0.997$, p-value = 0.049) and cd40 ($\rho = -0.999$, p-value = 0.013). The presence of CD8+ was related to up-regulation of the hdac5 gene ($\rho = 0.988$, p-value = 0.037) and down-regulation of the il27 gene ($\rho = -0.990$, p-value = 0.007).

B-cells were related to higher expression of the hdac7 gene in the polyamine-supplemented groups ($\rho = 0.999$, p-value = 0.031). Moreover, up-regulated genes, such as cd1d1, hdac5 and il27, were related to higher levels of B-cells in mesenteric lymph nodes. No other significant associations were found.

4. Discussion

This study demonstrates that the supplementation of manufactured infant formula in polyamines induces changes during lactation in lymphocyte populations and gene expression. These changes mainly relate to genes associated with immune system development in the BALB/cOlaHsd mouse model. Such modifications are likely to reflect the maturation of the immune system in agreement with previous reports in mouse (Jolois et al., 2002) and rat studies (Pérez-Cano et al., 2010). The main difference of this study in relation to previous studies was the fact that the pups were weaned early and the only polyamine source was the enrichment of the infant formula. Due to complications in early-weaned pup handling, the study was performed only for four

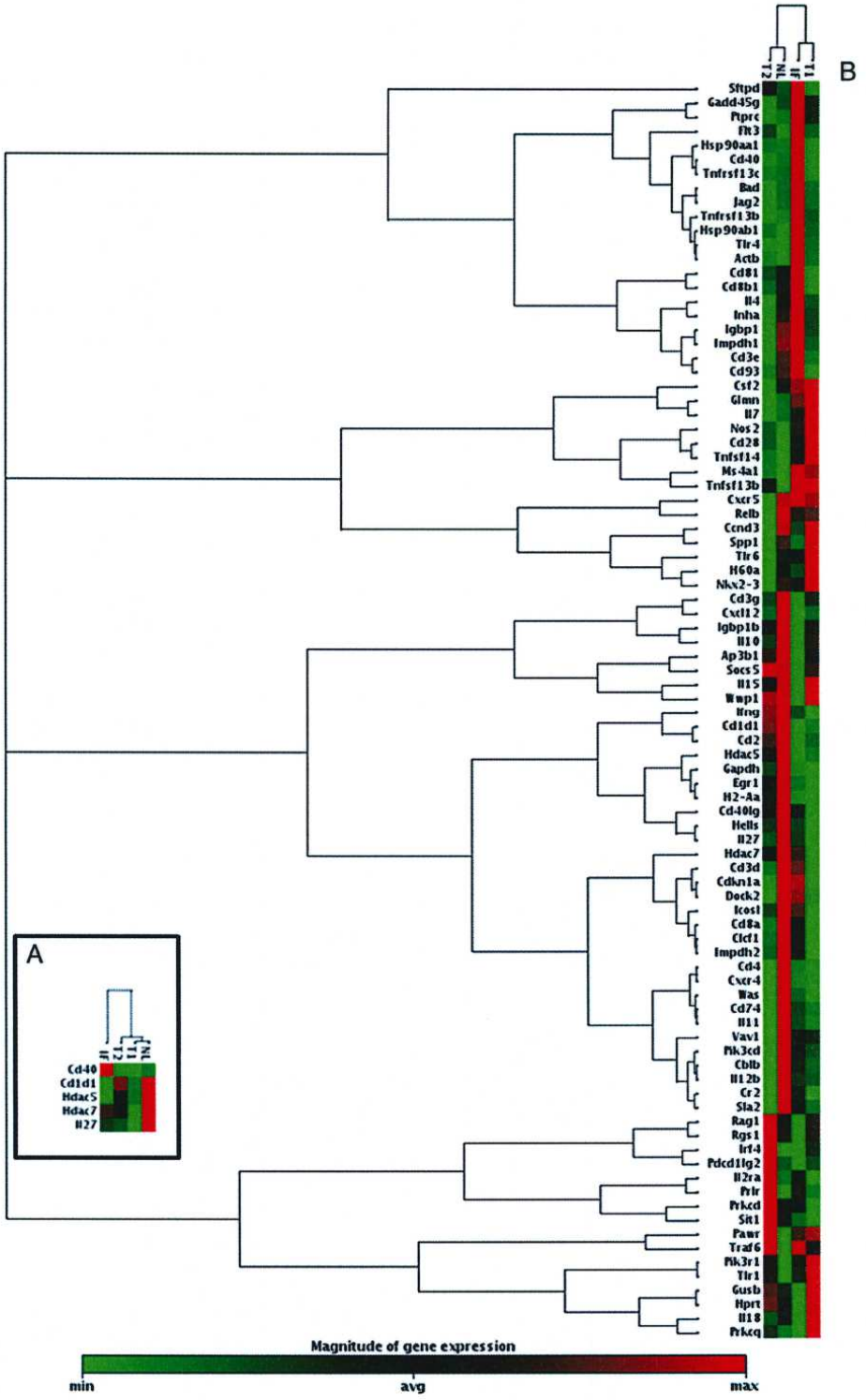

Fig. 4. Gene expression and cluster diagram showing genes that were up- or down-regulated (A) in the small intestine samples and in the full array (B). Similar expression profiles are grouped together to form a clustering tree. The color map represents the normalized intensity values (normalized to the median of all samples) of the genes (rows), where red and green represent the up- and down-regulated genes, respectively.

Table 4
Immune system proliferation and development gene significant (p-value < 0.05) fold changes comparing infant formula in polyamines and supplemented infant formulas (T1 and T2).

Gene	Gene description	T1	T2	General gene related function[a]
Cd40	CD40 antigen	−4.03	−3.64	Involved in Th1/Th2 differentiation, T-cell differentiation and B-cell activation.
Clcf1	Cardiotrophin-like cytokine factor 1	−2.11	−2.08	B-cell differentiation
Jag2	Jagged 2	−2.44	−2.60	T-cell differentiation
Tnfrsf13c	Tumor necrosis factor receptor superfamily, member 13c	−6.30	−4.72	T and B-cell proliferation
Tlr4	Toll-like receptor 4	−2.54	−2.81	Macrophage and mast cell activation, regulator of Th1 and Th2 development

[a] According to array manufacturer.

days at the end of the lactating period. Despite mice and humans having some differences, the available data suggest similar patterns of immune development (Holladay & Smialowicz, 2000). Thus, similar phenomena might occur in humans during lactation, and these should be further characterized.

The influence of the supplementation of polyamines on immune system was examined, taking into consideration representative compartments of the immune system. The important differences identified in the present study in the lymphocyte populations in the blood, with around 10-fold more cells in the breastfed pups, could be due to two factors: 1) stress induced by early weaning and/or 2) the absence of other immunomodulatory dietary factors in infant formula different than polyamines. As reported previously in lamina propria and epithelium in rats, the blood results suggest that the effects of polyamines on T cells depend on continuous and early intake (Pérez-Cano et al., 2010). Moreover, the increase in circulating B lymphocytes in response to polyamine supplementation indicates that this population is more sensitive than T cells to polyamines.

With regard to the spleen, a representative tissue from the systemic immune system, the mice spleen B lymphocyte population increased with polyamine supplementation to a level close to that of normal lactation. Levels of CD4+ and CD8+ T cells also increased in response to high polyamine supplementation with levels higher than those of normal lactation group. This positive effect on splenic lymphocytes could be explained by findings of earlier studies, which showed that SPD and SPM induce premature development of the spleen morphology and the cell contents in mice (Jolois et al., 2002), and induce an increase in the percentage of splenic B lymphocytes in rats (Pérez-Cano et al., 2010).

Regarding gene expression, there was a general tendency of similar expression between the normal lactation group and the high polyamine supplementation group, except for *Il27*. *Il27* gene expression was downregulated for all the groups fed the infant formulas, suggesting that polyamines do not modulate the expression of this gene.

It is of interest to note that there were significant changes observed in the expression of the *Cd1d1* gene. Cd1d1 is a glycolipid antigen-presenting major histocompatibility complex-like molecule. When expressed, it could regulate mucosal commensalism and the colonization of the intestines (Nieuwenhuis et al., 2009) through the activation of NKT cells (Geng, Laslo, Barton, & Wang, 2005). The differences in *cd1d1* expression explain our previous observations (Gómez-Gallego et al., 2012), which showed that polyamine-enriched formula alters microbial colonization patterns during lactation in a similar way to that seen in normal lactation and different to that observed with formula feeding without polyamine supplementation. As was reported by other authors (Choque-Delgado, Cunha-Tamashiro, Maróstica Junior, Moreno, & Pastore, 2011) the immune system and the intestinal microbiota are interrelated, and the combination of the results of polyamine supplementation of infant formulas suggests that polyamines can modulate both, immune system development and intestinal microbial colonization pattern in a similar way that milk polyamines in normal lactation do.

Dysregulation of *Cd40* expression is associated with gastrointestinal disorders, such as necrotizing enterocolitis (Xu et al., 2011) or celiac disease (Di Sabatino et al., 2011). Breastfeeding has proved to have a protective effect against these disorders (Mezoff & Aly, 2013; Sherman, 2013). The increase observed in the expression of *Cd40* in the formula-fed group compared with the breastfed pups and the normalized expression when the infant formula was supplemented with polyamines confirmed the immunoprotective effect of milk polyamines during lactation. Compared to infant formula feeding, the polyamine supplementation down-regulated the expression of the *Clcf1* and *Tlr4* genes. *Clcf1* is a cytokine belonging to the interleukin-6 (IL-6) family. Therefore, the down-regulation in the polyamine-supplemented group could be correlated with low inflammatory status and correct development of intestinal mucosa. In humans, IL-6 plasmatic levels and *Tlr4* mRNA expression are related to innate immune system activation. They begin to decrease after birth and stabilize at six months (Liao, Yeh, Lai, Lee, & Huang, 2013). As the expression of *Clcf1* and *Tlr4* was reduced in the polyamine-supplemented groups, polyamine supplementation in formula during lactation seems to improve immune system maturation and healthy intestinal mucosa development. However, further study is needed to clarify this point.

The class IIa histone deacetylases (HDACs), including HDAC5 and 7, have crucial roles in the development of the immune system (Parra & Verdin, 2010). B cell development is absolutely dependent on HDACs, and HDAC7 has proved to have a key role in thymocyte development and T cell proliferation (Sweet, Shakespear, Kamal, & Fairlie, 2012). Although HDAC5 and HDAC7 have been shown to have a critical role in epigenetic control of chromatin structure and gene expression in B cells mediated by protein kinase D activity (Matthews et al., 2006), their functional consequences remain unknown (Shakespear, Halili, Irvine, Fairlie, & Sweet, 2011). Our data suggest that the high B cell expression of *Hdac5* and *Hdac7* in the breastfed and high polyamine-supplementation groups might be related to higher percentages of B cells. This important finding suggests that dietary polyamines may act as epigenetic regulators of the immune system during lactation. Moreover, alterations in the expression of HDACs have been shown to have a key role in immune-mediated inflammatory disease development (Grabiec, Tak, & Reedquist, 2011). Therefore, balanced expression of *Cd40*, *Hdac5*, and *Hdac7* might be required for healthy development of the intestinal mucosa and the intestinal immune system. The addition of functional ingredients to infant formula might contribute to improved immune development during lactation.

In conclusion, the enrichment of infant formula with polyamines enhanced the maturation of the systemic and intestinal immune systems. The present study contributes to confirming the functional role of polyamines in breast milk in neonatal immune development, favoring the acquisition of more mature acquired immune system, potentially by epigenetic regulation. As the differences in immune system in mice are significant, it will be important to assess the impact of breastfeeding and formula feeding on immune system development in human infants during lactation and to assess the role of polyamine supplementation in infant formulas.

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5. OVERALL SUMMARY

The role of breastfeeding in promoting general health, growth and development and the significant reduction in the risk of acute and chronic diseases has long been demonstrated (Horta *et al*., 2007). Currently, considerable documented differences with respect to functionally short- and long-term effects between human milk and infant formulas require more research with the aim of improving infant formula composition.

Technological processes used in infant formula manufacturing affect the functional properties of the final product. Our data shows an increase in protein digestibility, reflected in the increase in non-protein nitrogen after digestion *in vitro* with a reduced amount of enzymes and time of digestion to simulate similar conditions to those of breastfeeding children, and the disappearance of β-lactoglobulin and α-lactalbumin bands in gel electrophoresis (see annex 9.1). But the estimated digestibility of the infant formula only reached 44.9%, maybe due to the soft gastrointestinal digestion conditions. From the beginning, these results seemed positive, but taking into account that infant formula has a higher protein content than human milk (Raikos and Dassios, 2014), even this relatively low protein digestibility could imply an increase in amino acid uptake, which, according to Early Programming Theory, could be responsible for the differences between breastfed children and infant formula-fed children in their short- and long-term susceptibility to some diseases. Moreover, protein profiles are different, so bioactive peptides released after gastrointestinal digestion may have different profiles and properties. Our results for the profile of majority peptides released after gastrointestinal digestion *in vitro* of infant formula shows some fragments originating from β-lactoglobulin, a protein absent in human milk. In addition, two of the peptides from β-lactoglobulin that were found—RVY and IPAVFK—had reported ACE-inhibitory and antibacterial activity respectively (see annex 9.1). Therefore, protein compositional dissimilarity between human milk and infant formula, and the functionality of these proteins and their peptides, could be responsible for some of the differences in health reported between breastfed and formula-fed children. Further studies are important to address the effects of the manufacturing process on protein digestibility and the peptide profile during formulation of infant formulas, for potential implications for human health.

According to previous data (Pollack *et al*., 1992; Romain *et al.*, 1992; Buts *et al*., 1995), manufacturing process technologies also reduce the polyamine content in infant formulas due to a relative high polyamine oxidase activity during the process (see annex 9.1). The consequence is a low polyamine uptake in children fed with infant formula compared with children who are breastfed.

In a mouse model, our results show that early weaned mice fed with infant formula without polyamine supplementation had significantly greater differences in intestinal microbiota, immune system populations and immune-related gene expression compared to mice with normal lactation (chapters 4.2–4.4).

Regarding microbiota composition, independently of the analysis methods, our results show for the first time, to our knowledge, that supplementation of infant formula with polyamines modulates *Bacteroides-Prevotella*, *Bifidobacterium* spp., *Lactobacillus* spp. and *Akkermansia*-like bacteria groups, including *A. muciniphila*, to levels closely related to normal lactation groups, in a dose-dependent manner (see chapter 4.2 and 4.3). Moreover, significant differences in the predominant microbial groups between those who were breastfed and formula-fed and those fed formula supplemented with polyamines were observed along the whole gastrointestinal tract, including in sites that have not been focused upon in previous research, such us the oral cavity and stomach. The mechanism by which milk polyamines modulate the intestinal microbiota remains unclear, and the similar effect should be further studied in human infants.

Lower levels of *Bacteroides-Prevotella*, *Bifidobacterium* spp., *Clostridium perfringens/histolyticum*, *Lactobacillus-Enterococcus* spp. and *Akkermansia*-like bacteria groups were found by fluorescence *in situ* hybridization (FISH) coupled with flow cytometry in infant formula-fed mice than those observed in the group with normal lactation (see chapter 4.2). Increasing amounts of polyamine supplementation appeared to increase the relative population of all microorganisms studied, in similar amounts to what we reported for normal lactation. However, we observed different tendencies in *A. muciniphila* populations in the large intestine, using the quantitative PCR molecular technique (see chapter 4.3), with lower levels occurring in the large intestine in a dose-dependent manner. The fact that PCR results exhibit contradictory results could be related to the fact that FISH analysis covers great bacterial groups, while PCR is very selective. The main result is that significant differences in microbiota composition between normal lactation and infant formula feeding were observed using both analysis methods at two levels, for bacterial groups and bacterial species inside these groups.

PCR analysis also confirms the results of FISH in *Bifidobacterium* spp. in the large intestine, with an increase of the *Bifidobacterium* spp. population with higher levels of polyamine supplementation, with *B. animalis* being the most common *Bifidobacterium* species found, followed by *B. breve* and *B. catenulatum*. Our results suggest that polyamines increase the number of *Bifidobacterium* spp. in the intestine and promote a healthy mucosal status. A previous study reported that high numbers of bifidobacteria may correlate positively with the normalization of inflammatory status (Cani *et al*., 2007), and in animal models, high levels of *A. muciniphila* have been associated with allergic diarrhoea while lower levels were associated with lower allergic symptoms (Sonoyama *et al*., 2010). This supports our hypothesis that polyamines could contribute to a healthy mucosal status, improving microbial colonization and immune system development.

PCR analysis also showed that polyamine supplementation reduced the populations of the *Lactobacillus* group and the *Streptococcus* group in the oral cavity and produced higher amounts of the *Lactobacillus* and *Bacteroides-Prevotella* groups in the stomach. Increasing concentrations of polyamines in the formula also correlated with higher amounts of Enterobacteriaceae, the *Bacteroides-*

Prevotella group, *Lactobacillus* and the *Bifidobacterium* group in the small intestine and correlated with higher amounts of *Clostridium coccoides* in the large intestine.

In parallel with the microbiota results, early weaned mice fed with infant formula showed a low percentage of CD4+, CD8+ and B-cells in the blood and a low percentage of B-cells in the spleen and mesenteric lymph node, compared to mice fed through normal lactation (chapter 4.4); this could be related to a low maturation of the immune system (Perez-Cano *et al*., 2010). Our study also shows gene expression differences in the small intestine with a fold change less than -2 and higher than 2 in 18 genes related to T- and B-cell differentiation and proliferation, including surface antigens, transcriptional regulator proteins, histone deacetylases, intercellular signalling proteins, toll-like receptors and tumour necrosis factor receptors, with a strong significance in the differences in *Cd1d1*, *Cd40* and *Hdac5* gene expression.

Just as for the microbiota, the supplementation of manufactured infant formula with polyamines induces changes in lymphocyte populations and gene expression, increasing B-cells in blood and CD4+, CD8+ y B-cells in the spleen in a dose-dependent manner, and reducing differences in gene expression.

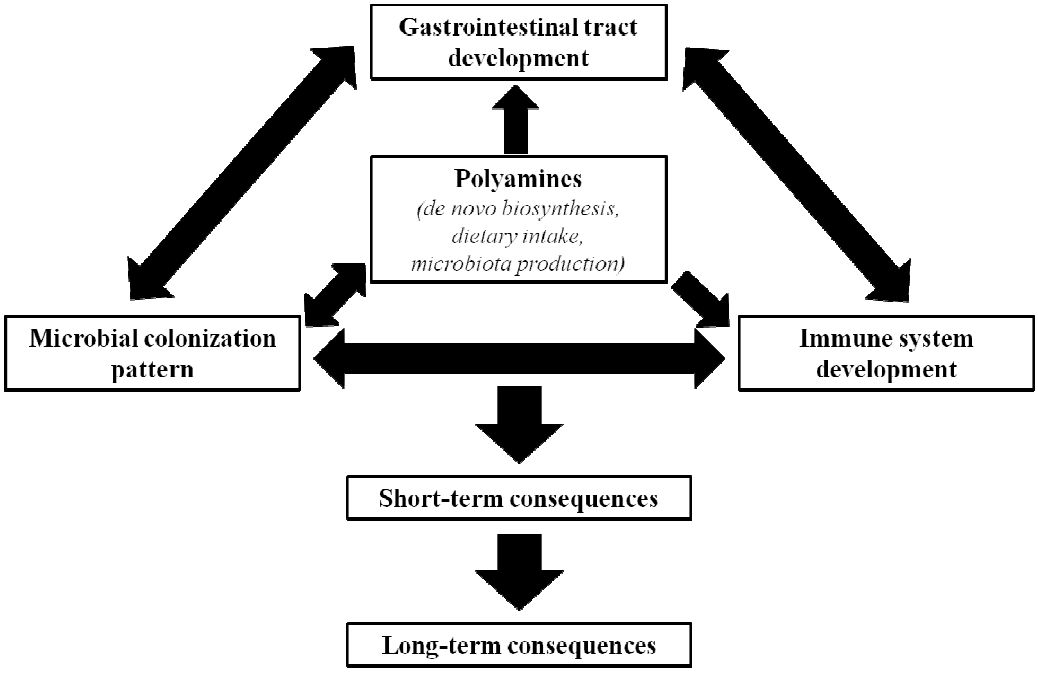

Figure 5. 1. Influence of polyamines diagram over gastrointestinal tract development, immune system development and microbial pattern colonization. Several studies show that these processes must be closely related and that the modulation of one of them could have an influence on the other, resulting in short- and long-term consequences on health.

The similar behaviour of polyamine supplementation on immune system development and intestinal microbial colonization patterns, taken together with data reported by other authors, demonstrates the key role of polyamines in the maturation and development of the gastrointestinal tract (Sabater-Molina

et al., 2009) and associated organs such as the liver and pancreas (Dandrifosse *et al*. 1999), demonstrating that these processes are closely related (Figure 5.1).

It is of interest to note that there were significant changes observed in the expression of the *Cd1d1* gene. CD1d1 is a glycolipid antigen-presenting major histocompatibility complex-like molecule. When expressed, it can regulate mucosal commensalism and the colonization of the intestines (Nieuwenhuis *et al*., 2009) through the activation of NKT cells (Geng *et al*., 2005). The differences in *Cd1d1* expression might explain why polyamine-enriched formula alters microbial colonization patterns during lactation in a similar way to that seen in normal lactation and different from that observed with formula feeding without polyamine supplementation. As was reported by other authors (Choque-Delgado *et al*., 2011), the immune system and the intestinal microbiota are interrelated, and the combination of the results of polyamine supplementation of infant formulas suggests that polyamines can modulate both immune system development and intestinal microbial colonization patterns in a similar way to milk polyamines in normal lactation.

Consequently, shown by our results, mainly related to changes in gene expression of histone deacetylases genes—*Hdac5* and *Hdac7*—low polyamine content of infant formula can lead to changes in immune system development. Further studies are necessary to discern whether the effect on histone deacetylases genes is a direct effect of polyamines, because microbial fermentation products have been shown to affect the enzymes involved in modifications of histone proteins and gene expression (Kim *et al*., 2010), providing a possible epigenetic mechanism which relates microbiota and immunological status (Amarasekera *et al*., 2013). What is certain is that changes in immune system and intestinal microbiota can potentially lead to more profound short- and long-term health effects.

The influence on these processes of dietary polyamines during lactation might play an important role in the modulation of the development of allergies in children or adults (Dandrifosse *et al*., 2000). It is now generally accepted that the intestines of healthy term infants are more permeable to food proteins during their first months than later in life. Oral administration of polyamines has been demonstrated to induce precocious intestinal postnatal maturation, diminishing intestinal permeability (Motyl *et al*., 1995), increasing the specific activity of proteases contained in the pancreas or associated with the enterocyte brush borders (Romain *et al*., 1998; Dandrifosse *et al*., 1999), thereby potentially leading to better digestion and a diminishing exposure of allergenic proteins. The ingestion of polyamines could also influence the development of the immune system, as mentioned previously, which could contribute to a lower susceptibility to allergy. One of the most important environmental changes implicated in early immune dysregulation and the rise in allergic disease is the change in the microbiome, which could also be modulated by polyamines.

Thus, as proposed by other authors, the relevant food safety organizations should consider including polyamines in manufactured formulas after suitable scientific evidence has been obtained supporting its safety for human babies. Whilst further data are needed, polyamines seem to have toxicity at

concentrations higher than normal physiological levels in foods (Til *et al*., 1997). Their relatively low toxicity could be related to the control of polyamine metabolism in mammalian cells and the fast oxidation by PAO (Seiler, 1995), so their addition to infant formulas at the same concentration as in human milk might be safe.

The inclusion of additional bioactive factors in currently available milk formulations would make these more similar to human milk, thereby promoting better growth and development of infants.

The results described in this thesis highlight the complex interplay between nutrition and early programming of the immune system and provide new perspectives on how diet-induced alterations to microbial colonization patterns can have an effect on the whole organism.

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6. CONCLUSIONS*/CONCLUSIONES*

1. The results of the study carried out in animal models confirm differences between breastfed and formula-fed mice in different areas along the whole intestinal tract in microbial colonization patterns.

1. Los resultados del estudio con animales prueban la existencia de diferencias en el patrón de colonización microbiana entre los ratones alimentados con leche materna y los alimentados con fórmulas infantiles en diferentes áreas a lo largo de todo el tracto gastrointestinal.

2. Supplementation of infant formula with polyamines modulates microbiota along the intestinal tract in the *Bacteroides-Prevotella*, *Bifidobacterium*, *Streptococcus*, *Lactobacillus* and Clostridia subgroups, *perfringens*/*Histolyticum* and *C. coccoides* groups, *Akkermansia*-like bacteria, including *A. muciniphila*, *Enterococcus* groups and the Enterobacteriaceae family, to levels closely related to those for breastfeeding, in a dose-dependent manner.

2. La suplementación de la formula infantil con poliaminas modula las poblaciones microbianas a lo largo del tracto gastrointestinal afectando a los grupos Bacteroides-Prevotella, Bifidobacterium, Streptococcus, Lactobacillus *y Clostridia subgrupos,* perfringens/Histolyticum *y* C. coccoides, Akkermansia-*like bacteria, incluida* A. muciniphila, Enterococcus *grupo y la familia Enterobacteriaceae, hasta niveles similares a los encontrados en el grupo con lactancia materna, en función de la concentración de poliaminas ingerida.*

3. The results show differences in the immune system between breast- and formula-feeding *in vivo*. A low percentage of CD4+, CD8+ and B-cells in blood and a low percentage of B-cells in the spleen and mesenteric lymph node were found in formula-feeding groups compared to mice fed with normal lactation. Gene expression differences in the small intestine with a fold change less than -2 and higher than 2 appeared in 18 genes related to T- and B-cell differentiation and proliferation, including surface antigens, transcriptional regulator proteins, histone deacetylases, intercellular signalling proteins, Toll-like receptors and tumour necrosis factor receptors; the differences in *Cd1d1*, *Cd40* and *Hdac5* gene expression are strongly significant.

3. Los resultados del estudio in vivo *muestran diferencias en el sistema inmune entre la alimentación con leche materna y fórmulas infantiles. En los grupos alimentados con fórmula se encontraron bajos porcentajes de linfocitos CD4+, CD8+ y B en sangre, y bajos porcentajes de linfocitos B en bazo y nódulo linfático mesentérico, comparado con aquellos ratones con lactancia normal. Se encontraron diferencias de expresión (*fold change*) inferiores a la mitad (-2) y mayores del doble (2) en 18 genes relacionados con la diferenciación y proliferación de linfocitos T y B, entre los que se encontraban antígenos de membrana, proteínas reguladoras de la transcripción, histona deacetilasas, proteínas de comunicación intercelular, receptores tipo Toll y receptores del factor de*

necrosis tumoral; siendo especialmente significativas las diferencias en la expresión de los genes Cd1d1, Cd40 *and* Hdac5.

4. Supplementation of manufactured infant formula in polyamines increases lymphocyte populations in the spleen and B-cells in blood and modulates the expression of immune system-related genes during lactation. B lymphocytes in the spleen and the gene expression of the group fed with infant formula supplemented with a high concentration of polyamines were similar to those for the breastfeeding group.

4. La suplementación de formulas infantiles con poliaminas incrementa las poblaciones de linfocitos en bazo y de linfocitos B en sangre, y modula la expresión de genes relacionados con el sistema inmune durante la lactancia. Los porcentajes de linfocitos B en bazo y las niveles de expresión génica obtenidos en el grupo alimentado con la formula infantil suplementada con la concentración mayor de poliaminas fueron similares a los observados en el grupo con lactancia materna.

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8. ABSTRACT

As has been recommended by a large number of health or breastfeeding organisations, whenever feasible, infants should be fully breastfed for at least six months. If full breastfeeding is not possible, safe and suitable infant formula should be used, and that formula should be similar to mature human milk in terms of its fat, carbohydrate, mineral and vitamin content. However, infant formulas do not have the functional compounds (immunoglobulins, enzymes, hormones, etc.) that are found in human milk, nor do they have the same protein composition and amino acid profile as human milk. Although human milk is considered to be the best alimentary option for children, more than 70 per cent of children in Western Europe are being fed with manufactured formula from the twelfth week of life, thus the impact that this different type of feeding can have on health must be taken into consideration.

According to the early programming theory, environmental exposure, including nutritional exposure during the intrauterine stage and during the perinatal months of life, might make children more susceptible to some diseases later in life. Indeed, it has been demonstrated that infants who have been breastfed have a lower susceptibility to some diseases than infants who were fed with artificial manufactured formulas, including a lower risk of gastrointestinal and respiratory diseases and a lower risk of obesity and diabetes in adult life. The fact that these changes are extended into adult life suggests than early exposure to nutritional factors are associated with epigenetic changes.

Correct development of the human immune system can be seen as an integral part of the pathogenesis of these diseases and it may be sensitive to nutritional changes. Furthermore, changes in diet have been associated with changes in the gut microbiome, which can modulate immune system development, because certain bacterial strains can modulate immune response and inflammation. In humans, there is also evidence that infants who go on to develop allergic diseases have an altered pattern of gut microbiota in early life, indicating the importance of a balanced microbial colonization pattern in early programming.

The differences in health status between breastfed and formula-fed children could be dependent upon compounds that are part of the peptide and non-protein nitrogen fraction of human milk, which includes bioactive peptides and polyamines.

Enzymatic digestion of proteins results in protein fragments with biological properties that are different from the properties exhibited by the intact precursor molecule. These fragments, called bioactive peptides, consist of 3-20 amino acids, and their biological activities are dependent upon their sequence, and they may have opiate, antithrombotic, antihypertensive, antimicrobial, antioxidant, immunomodulating or mineral absorption activities.

Polyamines are organic polycations that are present in all mammalian cells. They have significant interest due to their reported biological roles in eukaryotic cells, because they are essential to cell

proliferation and differentiation. Their presence has been demonstrated in human milk as being the most abundant spermidine and spermine polyamines.

Therefore, the main objective of the present thesis was to evaluate if the addition of polyamines after processing could improve immune system development and the microbial colonization pattern in a similar way that breast feeding do.

In a previous study, the influence that processing had on polyamines and peptide release after the digestion of commercial infant formula designed for children during the first months of life was examined. Samples of milk were taken at different representative stages throughout the manufacturing process. The results show an increase in protein digestibility, which was reflected in the increase in non-protein nitrogen after digestion and the disappearance of the β-lactoglobulin and α-lactalbumin bands in the gel electrophoresis. Moreover, the protein composition of infant formula is different from the protein composition of human milk, so the bioactive peptides that are released after gastrointestinal digestion of formula and human milk have different profiles and properties. Depending upon the sample, between 22 and 87 peptides were identified by high-performance liquid chromatography (HPLC)-tandem mass spectrometry after *in vitro* gastrointestinal digestion of infant formula. A peptide from β-casein, fragment 98-105, with the sequence VKEAMAPK and antioxidant activity, appeared in all of the samples. The results of the majority of the peptides released after gastrointestinal digestion of infant formula shows some fragments originating from β-lactoglobulin, a protein that is absent in human milk. In addition, two of the peptide released from β-lactoglobulin, RVY and IPAVFK, had been reported to show ACE-inhibitory and antibacterial activity respectively. Therefore, protein compositional dissimilarity between human milk and infant formula, and the functionality of these proteins and their peptides, could have potential implications for human health.

Additionally, manufacturing process technologies also reduce the polyamine content in infant formulas due to relatively high persistent polyamine oxidase activity in all of the samples during processing. After HPLC analysis, the final concentrations of putrescine, spermidine and spermine were: 0.317 ppm, 0.075 ppm and 0.061 ppm, respectively, but the average concentrations of these polyamines in human milk are around 0.058 ppm, 0.580 ppm and 0.825 ppm for putrescine, spermidine and spermine, respectively. The consequence is a low polyamine uptake for children fed with infant formula compared with breastfed children.

Consequently, the next step was to evaluate if the addition of polyamines to a commercial infant formula may have an impact on intestinal microbiota and immune system development. A total of 60 mice pups (14-days old) were randomly assigned to four-day intervention groups as follows: 1) breastfed unweaned pups; 2) early weaned pups fed with infant formula; and 3) three different groups of early weaned pups fed with infant formula supplemented with increasing levels of polyamines (a mixture of putrescine, spermidine and spermine), following the proportions found in human milk and lower than the non-observed adverse effect levels (NOAEL). After a four-day diet intervention, the

animals were anesthetised with isoflurane, and blood, mesenteric lymph node and spleen samples were obtained and conserved in saline buffer until flow cytometer analysis. The entire intestinal tract was removed from the pups. Samples of oral mucosa were taken with a sterile swab. The contents of the stomach, small intestines and large intestine, including the caecum, were emptied for microbiota analysis. Whole small intestine tissue was kept at -80°C using Trizol® reagent, according to the manufacturer's instructions for RNA purification and gene expression analysis.

Microbiota composition was analysed by fluorescent *in situ* hybridization (FISH) coupled with flow cytometry detection, and by quantitative PCR targeted at 14 bacteria genus and species. The lymphocyte populations in the blood, spleen and mesenteric lymph nodes were analysed by fluorescence activated cell sorting (FACS); moreover, the expression of genes encoding T-cell and B-cell activation, proliferation and differentiation, as well as Toll-like receptors (TLRs), in the small intestinal tissues was assessed using a 96-well RT-PCR arrays.

In a mouse model, our results showed that early weaned mice fed with infant formula without polyamine supplementation had significantly greater differences in intestinal microbiota, immune system populations and immune-related gene expression compared to mice with normal lactation.

Regarding the microbiota composition, independently of the analysis methods, our results showed, for the first time to our knowledge, that supplementation of infant formula with polyamines modulates Bacteroides-Prevotella, *Bifidobacterium* spp., *Lactobacillus* spp. and *Akkermansia*-like bacteria groups, including *A. muciniphila*, to levels closely related to normal lactation group, in a dose-dependent manner. Moreover, significant differences in the predominant microbial groups between the breastfed mice, the formula-fed mice and the formula supplemented with polyamines-fed mice were observed along the entire gastrointestinal tract, including sites that have not been focused upon in previous research, such us the oral cavity and the stomach. The mechanism by which the milk polyamines modulate intestinal microbiota remains unclear and the similar effect should be further studied in human infants.

Lower levels of Bacteroides-Prevotella, *Bifidobacterium* spp., *Clostridium perfringens/histolyticum*, *Lactobacillus-Enterococcus* spp. and *Akkermansia*-like bacteria groups were found by FISH analysis in the infant formula-fed mice than those observed in the group with normal lactation. Increasing amounts of polyamine supplementation appeared to increase the relative population of all the microorganisms studied in similar amounts to what we reported for normal lactation. Our results suggest that polyamines increase the number of *Bifidobacterium* spp. in the intestine, together with *A. muciniphila* results, which supports our hypothesis that polyamines could contribute to a healthy mucosal status, thereby improving microbial colonization and immune system development.

However, we observed different tendencies in *A. muciniphila* populations in the large intestine with other molecular techniques, such as quantitative PCR, and found lower levels of that type of bacteria in the large intestine in a dose-dependent manner. The fact that the PCR analysis exhibited

contradictory results could be related to the fact that FISH analysis covers a large range of bacterial groups while PCR is more selective. The main result is that significant differences were observed in the microbiota composition between normal lactation and infant formula feeding groups using both analysis methods at two levels: bacterial groups and the bacterial species inside those groups.

Moreover, PCR analysis confirms the results of FISH in *Bifidobacterium* spp. in the large intestine, with an increase in the *Bifidobacterium* spp. population with higher levels of polyamine supplementation; *B. animalis* was the most common *Bifidobacterium* species found, followed by *B. breve* and *B. catenulatum*. Furthermore, PCR analysis showed that polyamine supplementation reduced the *Lactobacillus* group population and the *Streptococcus* group population in the oral cavity, whereas larger *Lactobacillus* and *Bacteroides-Prevotella* populations were found in the stomach. Increasing concentrations of polyamines in the formula were also correlated with larger populations of the Enterobacteriaceae, *Bacteroides-Prevotella* group, the *Lactobacillus* and *Bifidobacterium* group, in the small intestine; and that correlated with higher numbers of *Clostridium coccoides* in the large intestine. A statistically significant different ($P< 0.05$) in microbial colonization pattern was observed between breastfed and formula-fed mice along the entire gastrointestinal tract by PCR analysis. Hence, our findings demonstrate that supplementation of polyamines regulates the total bacteria, *Akkermansia muciniphila*, *Lactobacillus*, *Bifidobacterium*, *Bacteroides-Prevotella* and *Clostridium* groups to levels that are, generally, more similar to those found in the breastfed group than in the formula-fed group.

In parallel with the microbiota results, the early weaned mice fed with infant formula showed a low percentage of CD4+, CD8+ and B-cells in blood and a low percentage of B-cells in the spleen and the mesenteric lymph node compared to the mice fed by normal lactation. Moreover, our study shows gene expression differences in the small intestine with a fold change less than -2 and greater than 2 in 18 genes related to T-cell and B-cell differentiation and proliferation, including surface antigens, transcriptional regulator proteins, histone deacetylases, intercellular signaling proteins, toll-like receptors and tumour necrosis factor receptors; and these differences were found to be strongly significant in *Cd1d1*, *Cd40* and *Hdac5* gene expression.

Similar to microbiota, the supplementation of manufactured infant formula in polyamines induces changes in lymphocyte populations and gene expression, increasing the percentage of B-cells in blood and CD4+, CD8+ and B-cells in the spleen in a dose-dependent manner, and it reduces differences in the expression of *Cd1d1*, *Cd40*, *Hdac5*, *Hdac7*, *Clcf1* and *Tlr4* genes compared with normal lactation.

Combined with data reported by other authors, this study's findings that polyamine supplementation has a similar impact on immune system development and intestinal microbial colonization pattern, demonstrates the key role that polyamines play in the maturation and development of the gastrointestinal tract. Moreover, it is interesting to note that significant changes were observed in the expression of the *Cd1d1* gene, which is able to regulate mucosal commensalism and colonization of the intestines through the activation of NKT cells. The differences in *Cd1d1* expression might help to

explain why polyamine-enriched formula alters microbial colonization patterns during lactation in a similar way to what is seen in normal lactation and in ways that are different from what is observed with formula feeding without polyamine supplementation.

Consequently, primarily due to changes in the gene expression of histone deacetylases genes, our results demonstrate that the low polyamine content of infant formula can lead to changes in immune system development. Further studies are necessary to discern if the impact on the histone deacetylases genes is a direct effect of the polyamines, because microbial fermentation products have been shown to affect the enzymes involved in modifications of histone proteins and gene expression, providing a possible epigenetic mechanism that relates microbiota and immunological status. What is certain is that changes in immune system and intestinal microbiota may potentially lead to more profound short- and long-term health effects, including susceptibility to allergy.

The immune system and the intestinal microbiota are interrelated, and the results of polyamine supplementation of infant formulas suggests that polyamines can modulate both immune system development and intestinal microbial colonization patterns in a way that is similar to milk polyamines in normal lactation. The results obtained from this study highlight the complex interplay between nutrition, the immune system and microbial colonization patterns during lactation. Such an effect requires further investigation in human infants because supplementation of an infant formula in polyamines might contribute to healthy gastrointestinal tract development in children fed with commercial formula.

RESUMEN

La lactancia materna es la forma de alimentación recomendada, siempre que sea posible, al menos durante los primeros seis meses de vida. Si esto no es posible, existen fórmulas artificiales con las que poder cubrir los requerimientos nutricionales de los recién nacidos, siendo similares a la leche materna en cuanto al contenido en grasas, carbohidratos, minerales y vitaminas. No obstante, estas fórmulas infantiles no son capaces de imitar a la leche materna en cuanto al contenido en compuestos funcionales (inmunoglobulinas, enzimas digestivas, hormonas, etc.), ni son similares a la leche humana en relación al tipo de proteínas y al perfil de aminoácidos que contienen. A pesar de ser la mejor opción para la alimentación de los recién nacidos, más del 70% de los niños en Europa occidental son alimentados con fórmulas infantiles desde la duodécima semana de vida por lo que el impacto que sobre la salud puede tener este tipo de alimentación debe ser tenido en cuenta.

Según la "Teoría de la Programación Temprana", la exposición a determinados factores ambientales, incluidos factores nutricionales, durante el periodo intrauterino y los meses posteriores al nacimiento podría determinar la susceptibilidad a determinadas enfermedades en etapas posteriores de la vida. De hecho, se ha demostrado una menor susceptibilidad a determinadas enfermedades en niños alimentados con leche materna frente a aquellos que lo han sido con fórmulas infantiles, lo que incluye menor riesgo de enfermedades gastrointestinales y respiratorias, y menor riesgo de obesidad y diabetes en la edad adulta. El hecho de que estos cambios se prolonguen hasta la edad adulta sugiere que la exposición temprana a factores nutricionales podría estar asociada a cambios epigenéticos.

El correcto desarrollo del sistema inmune va a tener una gran influencia sobre la patogénesis de estas enfermedades, siendo susceptible a los cambios nutricionales. Además, cambios en la alimentación van a estar relacionados con cambios en la microbiota intestinal lo que también va a afectar al desarrollo del sistema inmune, ya que se ha visto que determinados grupos bacterianos son capaces de modular la respuesta inmune y la inflamación. Se ha demostrado en humanos que los niños que desarrollan alergias tienen una microbiota intestinal alterada en etapas tempranas de la vida, lo que indica la importancia del patrón de colonización microbiana en la programación temprana.

Las diferencias encontradas en la susceptibilidad a enfermedades entre niños alimentados con lactancia materna y fórmulas artificiales, podría estar condicionada entre otros factores por compuestos que son parte de la fracción peptídica y de la fracción nitrogenada no proteica de la leche humana, lo que incluiría a los péptidos bioactivos y a las poliaminas.

La digestión enzimática de proteínas origina una serie de fragmentos, algunos de los cuales pueden exhibir propiedades diferentes a la de la proteína de origen. Estos fragmentos, denominados péptidos bioactivos, tienen secuencias comprendidas entre los 3 y los 20 aminoácidos, y su actividad biológica va a depender de su secuencia, pudiendo presentar actividad opiácea, antitrombotica,

antihipertensiva, antimicrobiana, antioxidante, inmunomoduladora o incrementar la absorción de minerales.

Las poliaminas son policationes orgánicos presentes en todas las células de mamíferos. Su interés es debido a su función en las células eucariotas, siendo esenciales para la proliferación y la diferenciación celular. Su presencia ha sido demostrada en la leche humana siendo las poliaminas más abundantes la espermidina y la espermina.

Por lo tanto, el objetivo de la presente Tesis ha sido evaluar si la adición de poliaminas tras el procesado podría mejorar el desarrollo del sistema inmune y producir un patrón de colonización microbiana similar al de la leche materna.

En un estudio previo, se estudió la influencia del procesado sobre el contenido en poliaminas y en los péptidos liberados tras la digestión de fórmulas infantiles comerciales desarrolladas para la alimentación de niños durante los primeros meses de vida. Se tomaron muestras de leche en diferentes etapas representativas a lo largo del proceso de elaboración. Los resultados muestran un incremento en la digestibilidad de proteínas, lo que se refleja in un incremento en el nitrógeno no proteico después de la simulación de la digestión gastrointestinal y la desaparición de las bandas correspondientes a la β-lactoglobulina y a la α-lactoalbúmina. Además, la composición de proteínas de las fórmulas infantiles es distinta a la de la leche humana, por lo que los péptidos bioactivos liberados tras la digestión gastrointestinal de las fórmulas y de la leche humana tendrán un perfil y propiedades diferentes. Dependiendo del tipo de muestra, entre 22 y 87 péptidos fueron identificados por HPLC-MS tras la digestión gastrointestinal in vitro *de las fórmulas infantiles. Un péptido procedente de la β-caseína, el fragmento 98-105, con la secuencia VKEAMAPK y actividad antioxidante, aparece en todas las muestras. Los resultados del perfil de péptidos mayoritarios liberados tras la digestión gastrointestinal muestran varios fragmentos originados a partir de la β-lactoglobulina, una proteína que está ausente en la leche humana. Además, dos de los péptidos liberados de la β-lactoglobulina, el RVY y el IPAVFK, muestran actividad inhibidora de la ACE y antibacteriana respectivamente. Por lo tanto, las diferencias en cuanto al tipo de proteínas entre la leche humana y las fórmulas infantiles, y la funcionalidad de esas proteínas y sus péptidos, podrían tener implicaciones en la salud humana.*

Además, el procesado reduce el contenido en poliaminas de las fórmulas infantiles debido a una relativamente alta actividad poliamino oxidasa en todas las muestras a lo largo del procesado. El análisis por HPLC muestra concentraciones finales de putrescina, espermidina y espermina de 0.317 ppm, 0.075 ppm y 0.061 ppm, respectivamente. Pero las concentraciones medias de estas poliaminas en la leche humana tienen valores aproximados de 0.058 ppm, 0.580 ppm y 0.825 ppm para putrescina, espermidina y espermina respectivamente, lo que lleva una menor ingesta de poliaminas en los niños alimentados con formula infantil frente a los de lactancia materna.

En base a estos resultados, la siguiente etapa recogida en esta teisis, fue evaluar si la adición de poliaminas a una fórmula de inicio podría tener un efecto en la microbiota intestinal y en el desarrollo del sistema inmune. Para ello, 60 crías de ratón de 14 días de edad fueron distribuidas aleatoriamente en cuatro grupos: 1) ratones sin destetar con lactancia normal; 2) ratones destetados precozmente alimentados con fórmulas infantiles; y 3) tres grupos distintos de ratones destetados precozmente alimentado con una fórmula infantil suplementada con concentraciones crecientes de una mezcla de putrescina, espermidina y espermina, según las proporciones encontradas en la leche humana e inferiores a los niveles sin efecto tóxico (NOAEL). Tras el tercer día de dieta, los animales fueron anestesiados con isoflurano y se obtuvieron muestras de sangre, nódulo mesentérico y bazo que fueron conservadas en solución salina hasta el análisis por citometría d flujo. Se extrajo el tracto gastrointestinal completo y se tomaron muestras de la mucosa oral con una torunda estéril. Se vació el contenido del estómago, el intestino delgado y el grueso, incluido el ciego, para el análisis de microbiota intestinal. El intestino delgado completo, sin su contenido, se conservó a -80ºC en Trizol®, según las instrucciones del fabricante para la extracción de ARN para el análisis de expresión génica.

La microbiota intestinal fue analizada mediante hibridación in situ *de fluorescencia (FISH) acoplada con un detector por citometría de flujo, y por PCR cuantitativa, analizándose 14 géneros bacterianos y especies. El análisis de las poblaciones linfocitarias en sangre, bazo y nódulo mesentérico fue analizado mediante* fluorescence activated cell sorting *(FACS); además, la expresión de genes responsables de la activación, proliferación y diferenciación de linfocitos T y B, así como de receptores tipo Toll (TLRs) en tejido del intestino delgado fueron analizados mediante RT-PCR arrays.*

En ratón, nuestros resultados muestran que el destete precoz y la alimentación con fórmulas de inicio sin suplementación en poliaminas producen grandes diferencias en la microbiota intestinal, en poblaciones de células del sistema inmune y en la expresión de genes relacionados con el sistema inmune cuando se comparan con ratones no destetados con lactancia normal.

En relación a la microbiota, independientemente del método de análisis, nuestros resultado muestran, por primera vez que nosotros sepamos, que la suplementación de las fórmulas infantiles con poliaminas modula las poblaciones bacterianas de Bacteroides-Prevotella, Bifidobacterium spp., Lactobacillus spp. *y bacterias similares a* Akkermansia, *incluida* A. muciniphila, *hasta niveles parecidos a los del grupo alimentado con lactancia normal en un efecto que parece ser dependiente de la dosis de poliaminas administrada. Además, se encontraron diferencias estadísticamente significativas en los grupos microbianos predominantes a lo largo de todo el tracto gastrointestinal entre los ratones con lactancia normal, alimentados con fórmulas infantiles y aquellos alimentados con fórmulas con poliaminas añadidas, incluidos sitios en los que investigaciones anteriores no habían incidido, como la cavidad oral y el estómago. El mecanismo por el cual las poliaminas de la*

lecha afectan a la microbiota intestinal no parece claro y habría que estudiar si el mismo efecto ocurre en humanos.

En ratones alimentados con fórmulas infantiles, mediante FISH se encontraron niveles más bajos de Bacteroides-Prevotella, Bifidobacterium spp., Clostridium perfringens/histolyticum, Lactobacillus-Enterococcus spp. *y bacterias similares a* Akkermansia *que en aquellos ratones con lactancia normal. La suplementación de la fórmula infantil en cantidades crecientes de poliaminas incrementa las poblaciones relativas de esos grupos bacterianos hasta niveles similares a los encontrados con lactancia normal. Nuestros resultados sugieren que las poliaminas incrementan el número de* Bifidobacterium spp. *en el intestino grueso, lo que junto a los resultados encontrados para* A. muciniphila, *refuerza la hipótesis de que las poliaminas pueden contribuir a un desarrollo saludable de la mucosa intestinal, mejorando el patrón de colonización microbiana y el desarrollo del sistema inmune.*

Sin embargo, se encontraron tendencias contradictorias en las poblaciones de A. muciniphila *en el intestino grueso mediante PCR cuantitativa, encontrándose niveles más bajos en intestino grueso conforme se incrementaba la concentración de las poliaminas suplementada. El hecho de que los análisis por PCR muestren resultados contradictorios puede estar relacionado con el hecho de que los análisis mediante FISH engloban grandes rangos de grupos bacterianos mientras que el análisis mediante PCR es más selectivo. Pero el resultado principal es que se observan diferencias en la composición microbiana entre el grupo con lactancia normal y los grupos alimentados con poliaminas en los dos métodos de análisis a dos niveles: grupos bacterianos y especies bacterianas dentro de esos grupos.*

Además, los análisis mediante PCR confirman los resultados encontrados mediante FISH en Bifidobacterium spp. *en intestino grueso, con un incremento de las poblaciones de este género conforme se incrementa la concentración de poliaminas suplementada;* B. animalis *fue la especie más común de bifidobacteria encontrada, seguido de* B. breve *y* B. catenulatum. *El análisis mediante PCR también mostro que la suplementación de la fórmula con poliaminas disminuye las poblaciones de lactobacilos y estreptococos en la cavidad oral, mientras que incrementa las poblaciones de lactobacilos y* Bacteroides-Prevotella *en el estómago. La suplementación de poliaminas en concentraciones crecientes está correlacionadas con cantidades mayores de enterobacterias,* Bacteroides-Prevotella, *lactobacilos y bifidobacterias en intestino delgado; y con cantidades mayores de* Clostridium coccoides *en intestino grueso. Además, se encontraron diferencias estadísticamente significativas ($P< 0.05$) a lo largo de todo el tracto gastrointestinal entre el grupo de lactancia normal y el alimentado con formula infantil. Nuestros resultados demuestran que la suplementación de poliaminas afecta al número de bacterias totales y a las poblaciones de* Akkermansia muciniphila, Lactobacillus, Bifidobacterium, Bacteroides-Prevotella *y* Clostridium *hasta niveles que son, generalmente, más parecidos a los encontrados en ratones alimentados con lactancia normal que a los encontrados en los ratones alimentados con fórmulas infantiles.*

En paralelo con los resultados encontrados para la microbiota, los ratones alimentados con fórmulas infantiles muestran bajos porcentajes de linfocitos CD4+, CD8+ y B en sangre, y de linfocitos B en bazo y nódulo mesentérico comparado con aquellos ratones alimentados con lactancia normal. Además, nuestro estudio muestra diferencias en la expresión génica en intestino delgado en 18 genes relacionados con la diferenciación y la proliferación de linfocitos T y B, incluyendo antígenos de membrana, proteínas reguladoras de la transcripción, histonas deacetilasas, proteínas de señalización intracelular, receptores tipo Toll y receptores del factor de necrosis tumoral; siendo esas diferencias estadísticamente significativas en la expresión de los genes Cd1d1, Cd40 *y* Hdac5.

De forma similar a lo que ocurría con la microbiota intestinal, la suplementación de la fórmula infantil con poliaminas induce cambios en las poblaciones linfocitarias y la expresión génica, incrementándose el porcentaje de linfocitos B en sangre, y linfocitos CD4+, CD8+ y B en bazo, en una proporción dependiente de la dosis de poliaminas; y reduce las diferencias en la expresión de los genes Cd1d1, Cd40, Hdac5, Hdac7, Clcf1 *y* Tlr4 *cuando se compara con lactancia normal.*

Junto con datos publicados por otros autores, los resultados de este estudio con un efecto similar de las poliaminas sobre el estatus del sistema inmune y el patrón de colonización microbiana, demuestra el papel clave que las poliaminas juegan en la maduración y el desarrollo del tracto gastrointestinal. Además, es interesante señalar que los cambios significativos en la expresión del gen Cd1d1, *que es capaz de regular la colonización microbiana a través de la activación de células NKT. Estás diferencias de expresión podría ayudar a explicar porque las fórmulas enriquecidas en poliaminas afectan a las poblaciones microbianas durante la lactancia de forma similar a lo que ocurre con lactancia normal y diferente a lo observado en la alimentación con fórmulas sin suplementación en poliaminas.*

De modo que, principal y probablemente debido a los cambios en la expresión de los genes de las histonas deacetilasas, nuestros resultados demuestran que el bajo contenido en poliaminas de las fórmulas infantiles puede alterar el desarrollo del sistema inmune. Es necesario profundizar sobre si el efecto en los genes de las histonas deacetilasas es un efecto directo de las poliaminas, porque algunos productos de la fermentación microbiana parecen afectar a enzimas relacionadas con la modificación de las histonas y la expresión génica, aportando un posible mecanismo epigenético que relaciona microbiota y estatus inmunológico. Lo que es cierto es que cambios en el sistema inmune y en la microbiota intestinal podrían, potencialmente producir profundos efectos sobre la salud a corto y largo plazo, incluyendo la susceptibilidad a alergias.

El sistema inmune y la microbiota intestinal están interrelacionadas, y el efecto que ejerce la suplementación de fórmulas infantiles con poliaminas, sugiere que las poliaminas pueden afectar tanto al sistema inmune como al patrón de colonización microbiana de forma similar a como lo hacen las poliaminas de la leche durante la lactancia materna. Los resultados de este estudio arrojan luz sobre la compleja interrelación entre la nutrición, el sistema inmune y el patrón de colonización

microbiana. Estos resultados requieren de más investigaciones que conduzcan a dilucidar el efecto que la suplementación de fórmulas infantiles con poliaminas puede tener en niños humanos, ya que podrían contribuir a un mejor desarrollo del tracto gastrointestinal en aquellos niños alimentados con fórmulas.

9. ANNEXES

9.1. ANALYZED DATA ABOUT THE EFFECT OF PROCESSING ON POLYAMINE CONTENT AND BIOACTIVE PEPTIDES RELEASED AFTER GASTROINTESTINAL DIGESTION IN COMMERCIAL INFANT FORMULAS.

Breast milk has a complex composition of nutrients and bioactive components designed to fulfil the needs of the growing infant. In recent years, the infant food industry has made an effort to develop infant formulas that are more similar to human milk to improve the nutrition of infants who are not breastfeeding.

Protective compounds, such as cytokines, oligosaccharides, and even microbes, in breast milk provide the newborn with the means to adapt to the environment.[1,2] Among the bioactive compounds found in breast milk are polyamines, such as, spermidine, spermine, and putrescine, as well as bioactive peptides released during milk protein digestion. Polyamines have a positive effect on the development of gastrointestinal tract[3] and immune system.[4,5] The levels and effects of these compounds in infant formulas compared with human milk are of special interest, as their concentrations are lower than in human milk.[6] Moreover, dietary proteins are a source of biologically active peptides that are inactive within the sequence of parent protein and can be released during gastrointestinal digestion or food processing. Once bioactive peptides are liberated, they may act as regulatory compounds. Bioactive peptides are widely distributed among milk protein sequences,[7] which can be released during digestion *in vivo*. However, changes that take place in protein structure during the manufacturing of infant formulas can influence protein digestion and peptide liberation.[8]

Technological processes used in food manufacturing affect the functional, nutritional, and biological properties of food components. Heating is the most common and most widely used method capable of modifying proteins during infant formula manufacturing. Depending on the intensity of the heat treatment, the nutritive value of proteins can be affected in a positive or negative way[8] and, by extension, can affect other related compounds.

The aims of this study were to evaluate how formula processing influences polyamine content and peptide release after digestion. Furthermore, the behavior of the polyamine oxidase activity of the milk and the digestibility of proteins was studied. The results of this work could be a preliminary step to improving infant formula composition, which could promote better health status of children fed with infant formulas during the first months of life.

MATERIALS AND METHODS

Samples

The infant formula samples used in this study were supplied by Hero España S.A. (Alcantarilla, Spain) at different representative stages along the manufacturing process. Figure 1 shows the flow diagram of the infant formula manufacturing process and the steps at which the samples were taken. The samples

were: F1) cow milk used as raw material in infant formula processing; F2) cow milk after skimming and the first thermal treatment; F3) concentrated milk after the second thermal treatment; F4) concentrated infant formula after the last thermal treatment; F5) the infant formula final product; and S) milk whey used as an ingredient. Whey was added as ingredient to increase milk serum proteins in the final product to 60% of total proteins.

One kg of powder of final formula (F5) and 500 ml of liquid samples (F1, F2, F3, F4, and S) were taken from five separate batches. Liquid samples were lyophilized, and all of the samples were preserved at -20 °C until analysis.

Determination of humidity and nitrogen

Humidity (method 964.22) in the samples along the infant formula manufacturing process and nitrogen (N) content in the samples and digestions using the micro-Kjeldahl procedure (method 955.04) were determined using official AOAC methods[9]. Protein calculations were made using 6.25 as the conversion factor. Non-protein nitrogen (NPN) in the samples and digestions was estimated using the micro-Kjeldahl method after dissolving 20 g of the sample in 100 ml of 15% trichloroacetic acid (Merck, Darmstadt, Germany) for milk protein precipitation and filtration.

Analysis of polyamines

A high-performance liquid chromatography (HPLC) method using a diode array detector was used. The HPLC system consisted of a Waters 2690 system connected to a Waters 910 detector. The analytical column was a Spherisorb® 5.0-µm ODS2, 4.6 mm × 150 mm (Waters, Milford, CT). Detection was performed at 254 nm.

The samples were diluted ten times with a solution of trichloroacetic acid (TCA) (Merck, Darmstadt, Germany) as described by Nishibori *et al*.,[10] but the concentration was adjusted to 15% for milk protein precipitation. The samples were homogenized using gentle agitation for 30 min.[11] After centrifugation at 13000 × g for 15 min at 4 °C, the supernatants were filtered using 0.45-µm membrane filters (Whatman, Brentford, England) and dansylated by adapting the method described by Buts *et al*.[6] Clear supernatant (1 ml) was basified by adding 250 µl of saturated Na_2CO_3 and 1 ml of dansyl chloride solution (10-mg/ml acetone; Fluka, Steinheim, Germany).

After incubation, 200 µl of L-proline solution (Scharlau, Barcelona, Spain) was added to clean the excess dansyl chloride.[12] Extraction of dansyl derivatives was performed twice with cyclohexane, and organic phases were collected and evaporated in nitrogen air flow. Residues of dansyl derivatives were dissolved in 1-ml acetonitrile (Merck, Darmstadt, Germany) and filtered with 0.45-µm HPLC filters (Upchurch Scientific, Oak Harbor, WA). Aliquots of 20 µl were injected in the HPLC. Samples were run for 30 min according to a linear gradient method including two mobile phases: water and acetonitrile (Table 1).

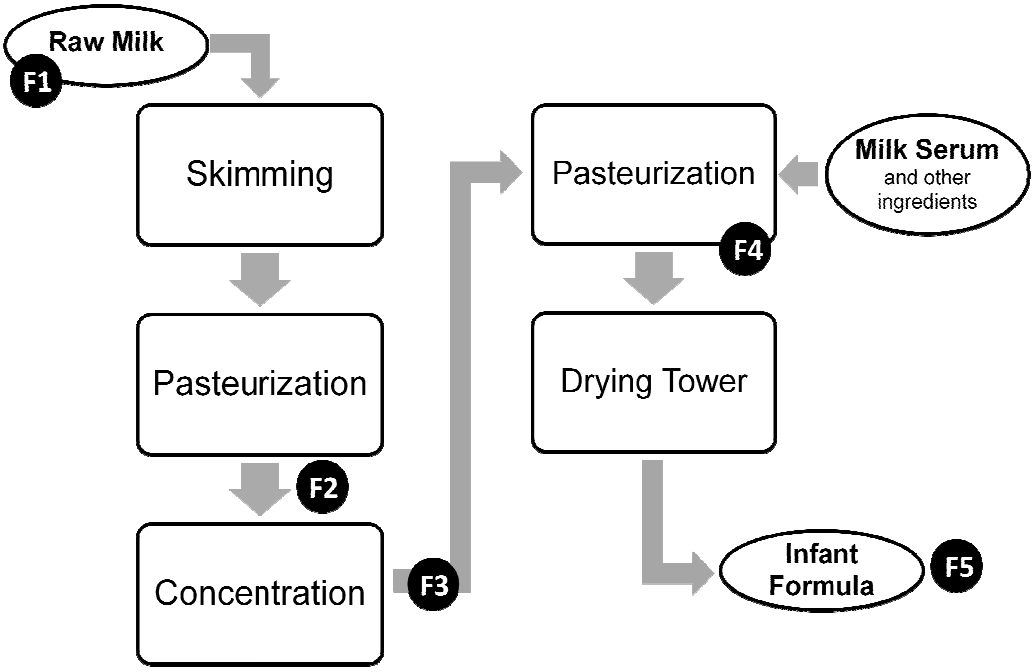

Figure 1. Diagram of infant formula manufacturing process. Samples were taken in the steps labeled with a black circle, from originally raw milk (F1) to final infant formula ready for sale (F5). In the first concentration stage, the product was concentrated at 30-40%. In the drying tower the water was removed to a final humidity lower than 3%.

Each sample was analyzed in duplicate. Quantification of the polyamine concentration was made by comparing the integrated surface areas of peaks with areas of dansylated polyamines of known concentration on a standard curve. To avoid mistakes due to loss of dansylated polyamines during manipulation, 1,3-diaminopropane (Aldrich, Steinheim, Germany) was used as the internal standard.[13] Polyamine concentrations were expressed in parts per million.

Table 1. Gradient of acetonitrile:water employed by dansylated polyamines separation

Time (min)	% Acetonitrile	% Water
0	60	40
5	60	40
15	70	30
20	95	5
25	98	2
26	98	2
26.1	61	39
30	60	40

Analysis of polyamine oxidase activity

The polyamine oxidase (PAO) activity assay was performed using the method described by Suzuki *et al*.,[14] in which hydrogen peroxide formed by polyamine oxidase is measured fluorometrically by converting homovanillic acid to a highly fluorescent compound by peroxidase, adding pargyline and semicarbazide for monoamine oxidase and diamine oxidase inhibition, respectively. A multi-mode microplate reader (Synergy 2; Bio-Tek, Winooski, VT); fluorescence filters with excitation and emission wavelengths of 360 and 460 nm respectively; and 96 well black, flat bottom microplates were used to carry out this assay. Each sample was analyzed in triplicate. Results were expressed as µmoles H_2O_2/mg dry extract/30 min.

In vitro gastrointestinal digestion of samples

The hydrolysis procedure was based on the method described by Miller *et al*.[15] and comprised the simulation of gastric and intestinal digestion of the samples by *in vitro* enzymatic treatment. The modifications introduced by different authors [16, 17, 18, 19, 20] reduced the concentrations of digestive enzymes and the time of digestion, to simulate gastrointestinal conditions during lactation. In the first step, 3.9 g of the dry extract of the sample were dissolved in 10 mL of Milli-Q water obtained using a Milli-Q water purification system (Millipore, Molsheim, France). Then, the pH was adjusted to 4.0 with 1M HCl, and the samples were hydrolyzed with pepsin (from porcine gastric mucosa; Sigma, St. Louis, MO) at a ratio of 10.7 mg of pepsin/g of sample, for 30 min at 37 °C. Directly afterward, the pH of the digested sample was raised to 5.0 using $NaHCO_3$ 1M, and the samples were hydrolyzed with pancreatin (from porcine pancreas; Sigma) and bile salts (Fluka), at a ratio of 2-mg pancreatin/g of the sample and 6.25-g bile salts/g of pancreatin. Intestinal digestion with pancreatin was performed at 37 °C for 90 min. Digestion was carried out in a thermally controlled incubator under constant stirring. The reaction was stopped, the sample was put on ice, and the pH was adjusted to 7.2 with $NaHCO_3$ 1M. The enzymes were inactivated by heating at 95 °C for 15 min in a water bath, followed by cooling to room temperature. A fraction of the digestions were run in gel electrophoresis to check for the presence of proteins and changes in protein composition. Digestibility and RP-HPLC-MS/MS analyses were performed to evaluate influence of the processing in peptide release after digestion. In vitro gastric and intestinal digestions and subsequent analyses were carried out at least in duplicate.

Gel electrophoresis

To investigate the protein composition of milk fractions during digestion, the molecular weights of the proteins were analyzed by gel electrophoresis in sodium dodecyl sulfate (SDS). The analysis was conducted using a PhastSystem™ electrophoresis system (Pharmacia, Uppsala, Sweden) as described by Jiménez-Saiz *et al*.,[21] with precast homogenous gels 20% (GE Healthcare, New York, NY) and PhastGel™ SDS buffer strips (Amersham Biosciences Corp., Uppsala, Sweden), following the manufacturer's instructions for the electrophoretic and Coomassie staining conditions. The samples

were dissolved in 10-mM Tris-HCl buffer, pH 8.0, containing 2.5% SDS and 10 mM EDTA, and heated at 95 °C. They were then analyzed in the presence of 5% 2-β-mercaptoethanol.

Analysis by on-line RP-HPLC-MS/MS

A water-soluble hydrolysate extract was obtained by centrifugation at 20000 × g for 30 min at 5 °C and by filtration through a Whatman no. 41 filter (GE Healthcare Bio-Sciences, Pittsburgh, PA). The water-soluble extract was subjected to ultrafiltration through a hydrophilic 1000-Da cutoff membrane (Amicon Inc., Beverly, MA).

The hydrolysates were injected into an HPLC system (Agilent, Santa Clara, CA), which was connected on-line to an Esquire-LC quadrupole ion trap instrument (Bruker Daltonik, Bremen, Germany), according to the method of Hernández-Ledesma *et al*.[22] The column used in these experiments was a 250-mm × 4.6-mm Discovery BIO Wide Pore C_{18} column (Bio-Rad, Richmond, CA). A Nova-Pak® C18 guard column 20 mm × 3.9 × 4 µm (Waters Corp., Milford, MA) was used to protect the analytical column. The injection volume was 50 µl. Solvent A was a mixture of water and trifluoroacetic acid (1000:0.37, v/v), and solvent B contained acetonitrile and trifluoroacetic acid (1000:0.27, v/v). Peptides were eluted with a linear gradient of 0–45% solvent B over 60 min at a flow rate of 0.8 ml/min. The signal threshold to perform auto MS(n) analyses was 1,000, and the precursor ions were isolated within a range of 4.0 m/z and fragmented with a voltage ramp going from 0.35–1.4 V. The m/z spectral data were processed using Data Analysis 3.0 (Bruker Daltonik) and transformed to spectra representing mass values. MS(n) spectra were processed in BioTools 2.1 (Bruker Daltonik) to perform peptide sequencing.

Statistical analysis

Statistical analyses were conducted using ANOVA and relevant post-hoc tests with SPSS 15.0 software. Differences between means were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Impact of processing on polyamine content and polyamine oxidase activity of infant formulas

The concentrations of the polyamines detected in the analyzed samples varied over a wide range, probably reflecting differences in the stages of the process from where the samples were taken, the original raw milk of the batch, and the PAO activity of the sample. The results are summarized in Table 2. Overall, polyamine content was lower in the first steps of the processing than in the last steps or final product. This may have been due to the PAO activity in these samples (Table 3) influenced for whey addition before the last concentration stage. Compared with the PAO activity of the raw milk, the final infant formula retained more than 60% of the enzymatic activity, which was not inactivated

due to the high resistance of the enzyme. This persistent PAO activity reduces polyamine concentrations over time and during infant formula reconstitution.

Table 2. Polyamine concentration (ppm) in dry samples along manufacturing process

Sample	Putrescine		Spermidine		Spermine	
F1	**0.650**	0.576-0.749	**0.057**[a]	0-0.144	**0.065**	0-0.130
F2	**1.047**	0.860-1.280	**0.126**[a]	0.116-0.141	**0.125**	0.124-0.129
F3	**1.858**	0.189-3.082	**0.966**[a,b]	0.078-1.629	**0.469**	0-1.054
F4	**2.961**	1.619-6.747	**1.081**[b]	0.731-1.529	**0.706**	0.376-1.087
F5	**2.441**	1.489-3.462	**0.576**[b]	0.281-0.903	**0.466**	0.277-1.127
S	**2.837**	1.729-4.196	**0.347**[a,b]	0-0.751	**0.763**	0-1.685

Each value represents the mean (bold) and the range
Differents letters in the same column indicates statistical significant differences at the level of $p<0.05$

Table 3. PAO activity (µmoles H_2O_2/mg dry extract/30min) in samples along manufacturing process and evolution of the percentaje of relative PAO activity comparing with raw milk

Sample	PAO actvity (mean ± SD)		Relative PAO activity (%)
F1	**21.59**[a]	± 2.31	**100.00**
F2	**25.27**[a]	± 0.72	**117.04**
F3	**11.94**[b]	± 1.43	**55.30**
F4	**11.98**[b]	± 3.58	**55.49**
F5	**14.48**[b]	± 0.45	**69.38**
S	**15.44**[b]	± 3.23	**71.51**

Differents letters in the same column indicates statistical significant differences at the level of $p<0.001$

As reported in previous studies,[6, 11, 23] the polyamine content in infant formula is around ten times less than that of human milk. In our samples, if the formula was reconstituted according to the manufacturer's instructions, the final concentrations of putrescine, spermidine, and spermine would be 0.317, 0.075, and 0.061 ppm, respectively. These amounts were similar to those reported by Romain *et al.*,[23] with higher levels for putrescine. Previous studies[6, 11, 23, 24] established a great variation in milk composition from one mother to another, and even between the left and right breasts,[6] but the average concentrations of polyamines were around 0.058, 0.580, and 0.825 ppm for putrescine, spermidine, and spermine, respectively. Thus, the concentrations of spermidine and spermine, the most active

polyamines[3] in manufactured formulas, were much lower than in human milk, although the concentration of putrescine was higher than in breast milk.

This finding reveals the lack of an important functional compound in infant formulas compared with breast milk, and due to the role of polyamines on immune system and intestinal microbiota development we recently reported,[5, 25] the development of infant formulas with a high but safe polyamine content must be taken into consideration.

Impact of processing on digestibility of milk proteins in infant formulas

The protein composition of fractions was further characterized by gel electrophoresis. We found that the processing of infant formulas had an influence on the protein structure. The comparison between raw milk (F1) and infant formula (F5) shows that processing wide and diffused bands on SDS-gels (Figure 2; lanes 1 and 2, versus 5 and 6). As reported by other authors,[26] heat treatment induced susceptibility to hydrolysis, thereby facilitating protein digestion, which was reflected in band comparison after gastric and gastrointestinal digestion (Figure 2; lanes 3 and 4, versus 7 and 8).

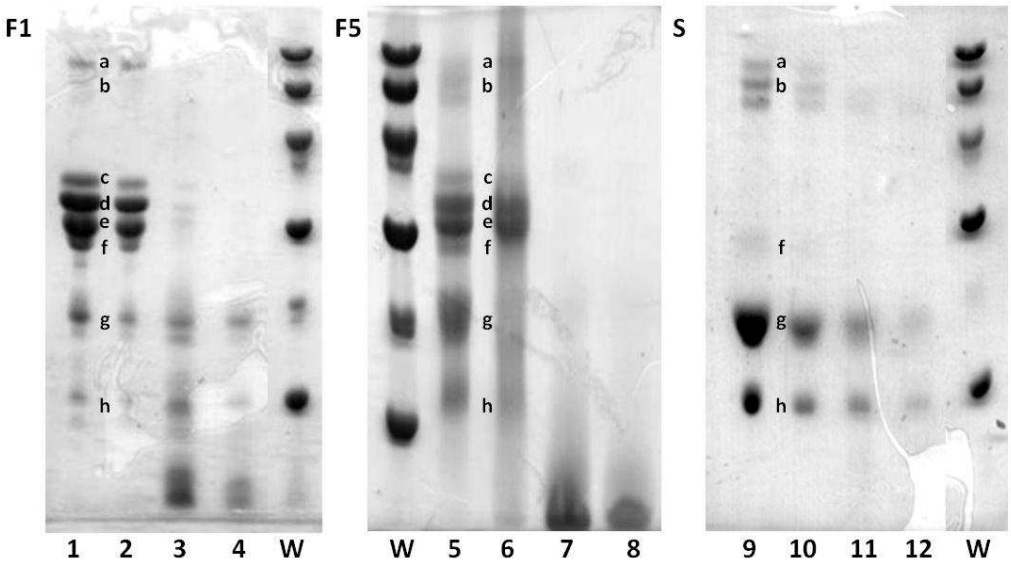

Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of raw milk (F1); infant formula (F5); and whey (S) proteins and their fractions along gastrointestinal digestion: lane 1, raw milk; lane 2, raw milk at infant gastric pH (pH = 4); lane 3, raw milk after infant gastric digestion; lane 4, water-soluble extract of raw milk after infant gastrointestinal digestion (at pH = 7); lane W, molecular weight marker (97 kDa, *phosphorylase b from rabbit muscle*; 60 kDa, *bovine serum albumin*; 45 kDa, *ovalbumin*; 30 kDa, carbonic anhydrase from bovine erythrocytes; 20.1 kDa, *soybean trypsin inhibitor*; 14.4 kDa, *bovine alpha lactalbumin*); lane 5, infant formula reconstituted per manufacturer's indications; lane 6, infant formula at infant gastric pH (pH = 4); lane 7, infant formula after infant gastric digestion; lane 8, water-soluble extract of infant formula after infant gastrointestinal digestion (at pH = 7); lane 9, milk serum; lane 10, serum at infant gastric pH (pH = 4); lane 11, serum after infant gastric digestion; lane 12, water-soluble extract of milk serum after infant gastrointestinal digestion (at pH = 7). The positions of lactoferrin (a), serum albumin (b), αs2-casein (c), αs1-casein (d), β-casein (e), κ-casein (f), β-lactoglobulin (g), and α-lactalbumin (h) are indicated.

It is mainly evident in the β-lactoglobulin and α-lactalbumin bands, which persist in raw milk after gastrointestinal digestion and practically disappear during digestion in infant formula samples. In raw milk, β-lactoglobulin and α-lactalbumin remain undegraded due to the partial resistance to pepsin and pancreatin in the soft acidic conditions of infant *in vitro* digestion.[8] In the whey samples, proteins seemed to have relatively high resistance to the conditions of digestion, which was reflected in the persistence of the bands for β-lactoglobulin and α-lactalbumin, even after gastrointestinal digestion (Fig 2; lane 12).

Digestibility was related with the increase in NPN that resulted from enzymatic digestion is shown in Table 4. Protein digestibility, related with the increase in NPN was lower in whey (S), that supported the results confirmed by gel electrophoresis. It appeared that manufacturing procces exerted whey protein denaturation, which facilitated its digestion.

Although protein bands in gel electrophoresis were notably reduced at the end of the final infant formula digestion, estimated *in vitro* digestibility of the infant formula (F5) increase from 17'9% of the cows milk (F1) to 44.9%, calculating it as ΔNPN x 6.25 x 100/protein content of undigested infant formula. As reported by Rudloff and Lönerdal[27], this low digestibility could be due to the soft gastrointestinal digestion conditions during the first months of life as well as lipid-protein or peptide interactions. But our results were even lower than those reported by these authors.

It is important to consider that *in vitro* digestibility is only an approximation of the physiological process and does not take into account other characteristics of real digestion, such as gastric emptying, intestinal fluid, intestinal motility, and mucosal enzymes. However, it could be considered a warning regarding real protein digestion during lactation.[28]

Table 4. Increase in NPN after gastrointestinal digestion in samples along infant formula manufacturig process.

	Humidity (%)	Protein in dry extract (mg/g)	NPN before digestion in dry extract (mg/g)	NPN after digestion in dry extract (mg/g)	ΔNPN (mg/g)
F1	88.17	26.84	0.50 ± 0.21	1.27 ± 0.21	0.77
F2	91.18	37.45	0.35 ± 0.11	1.23 ± 0.16	0.88
F3	60.38	10.89	0.18 ± 0.12	0.97 ± 0.20	0.79
F4	61.11	11.23	0.24 ± 0.03	1.03 ± 0.21	0.79
F5	1.69	10.57	0.19 ± 0.02	0.95 ± 0.20	0.76
S	79.14	69.72	0.90 ± 0.09	1.45 ± 0.08	0.55

Data of NPN represents mean ± SD

Identified peptides after gastrointestinal digestion

The ultrafiltration permeates of the digested samples were obtained and subjected to tandem MS for peptide identification. A total of 49 fragments could be identified for raw milk (F1) (Table 5), 29 for skimmed milk (F2) (Table 6), 22 for concentrated milk (F3) (Table 7), 23 for milk serum (S) (Table 8), 55 for concentrated manufactured formula (F4) (Table 9), and 87 for infant formula (F5) (Table 10).

There is growing evidence that the peptide sequences released during digestion can vary depending on the manufacturing process of infant formulas. As suggested by other authors who studied other foods,[77] digestion experiments with infant formulas may provide information regarding the susceptibility of proteins to gastrointestinal conditions, hydrolysis, and bioactive peptide release in infant formulas.

The comparison of the majority peptide profiles in the 1000 Da permeates showed that changes in the profile of peptides released and identified was greatly dependent on the processing stage of the infant formula. Our results showed that the percentage increases of peptides originating from whey proteins during processing—from 33.3% in raw milk (F1) to 49.9% in final infant formula (F5)—may have been due to the addition of whey (S) as an ingredient.

Table 5. Milk protein-derived peptides identified in the permeate obtained from raw milk samples (F1) subjected to a hydrolysis process that simulates gastrointestinal digestion.

Detection time (min)	Observed mass (Da)	Protein fragment	Sequence	Activity	References
12.8	460.3	Serum albumin (421-424)	TLVE		
13.5	617.2	αs1-casein (7-10)	VSSS		
13,5-13,8	501,2-501,4	Serum albumin (530-533)	ELLK		
16.7	543.4	Lactoferrin (96-100 y 437-441)	AVVKK		
17	437.1	β-lactoglobulin (100-102) and αs1-casein (102-104)	KKY		
17.3	516.3	κ-casein (111-114)	KKNQ		
17.4	346.2	β-casein (164-169)	LQS	Fragment from LQSW (ACE-inhibitory activity)	29
17.6	543.3	αs1-casein (100-103)	RLKK		
17,6-17,8	445.2	κ-casein (91-94)	QPTT		
18.5	648.3	β-casein (164-169)	SLSQSK	Fragment from PPQSVLLSLSQSKVLPVPE (ACE-inhibitory activity)	30
18.5	484.3	κ-casein (74-79)	KPAAV		
18,8-19,0	648.3	Lactoferrin (644-649)	NTECLA		
19	616.4	β-casein (94-99)	GVSKVK		
19.3	616.4	Serum albumin (440-444)	PESER		
19.4	660.3	Lactoferrin (258-263)	RSVDGK		
20,1-20,3	516,2-516,3	β-casein (101-105)	AMAPK	Fragment from VKEAMAPK (antioxidant activity)	31
20,6-20,9	449.2	Lactoferrin (372-376)	ATAST		
21,7-22,2	645.3	β-casein (100-105)	EAMAPK	Fragment from VKEAMAPK (antioxidant activity)	31
22.8	488.3	β-casein (94-98)	GVSKV		
23,4-24,1	674.3	β-casein (1-5)	RELEE	Fragment from RELEELNVPGEIVESLSSSEESITR (immunomodulatory activity)	32
23,8-24,0	872.4	β-casein (98-105)	VKEAMAPK	Antioxidant activity	31
24,3-24,4	416.2	β-casein (1-3) and α-lactalbumin(10-12)	REL		
24.9	400,2-400,3	αs1-casein (1-3)	RPK		
24.9	449.3	αs1-casein (151-153)	RQF		
25.1	598.3	β-casein (50-54)	HPFAQ	ACE-inhibitory activity and fragment from IHPFAQTQ (anti-amnestic activity)	33, 34
25.4	656.4	αs1-casein (99-103)	LRLKK		
25.8	656.4	Lactoferrin (350-355)	VGPEEQ		
27.2	528.4	αs1-casein (136-140)	IGVNQ		
27,4-27,5	528.4	β-casein (25-28)	RINK	Fragment from RELEELNVPGEIVESLSSSEESITRINK (immunomodulatory activity)	35
28	807.3	β-lactoglobulin (62-68)	ENDECAQ		36
28.3	807.4	κ-casein (18-24)	FSDKIAK	Antibacterial and ACE-inhibitory activity	37
28.7	685.2	Serum albumin (370-375)	STVFDK		
29.5	635.3	α-lactalbumin (69-74)	SSNICN		
29,5-29,7	803,3-803,4	κ-casein (162-169)	VQVTSTAV		38
29,9-30	780.4	β-casein (169-175)	KVLPVPQ	ACE-inhibitory activity	29
31,1-31,5	612.2	αs1-casein (151-154)	RQFY		
32,3-32,4	501.3	αs2-casein (164-167)	LKKI	fragment from LKKISQ (ACE-inhibitory activity)	39
32.7	673.3	β-casein (184-189)	DMPIQA		
33.4	596.3	αs1-casein (150-153)	FRQF	ACE-inhibitory activity	38
33.7	583.3	Lactoferrin (493-498)	PGADPK		
34,8-34,9	400,2-400,3	Lactoferrin (570-572)	RLL		
40.5	786.4	κ-casein (74-79)	LQWQVL		
42.3	786.3	κ-casein (117-123)	TEIPTIN		
43,1-43,2	627,2-627,3	αs1-casein(91-95)	YLGYL	Fragment from RYLGYLE (α-casomorphin with opioid activity)	7
48.7	462.2	Serum albumin (426-429)	SRSL		
51,3-51,6	675.3	αs1-casein (29-34)	PEVFGK	Fragment from FPEVFGK (ACE-inhibitory activity)	40
52,8-53	741,3-741,4	Serum albumin (569-575)	VEGPKLV		
57.4	474.1	β-casein (90-93)	PEVM		

Table 6. Milk protein-derived peptides identified in the permeate obtained from skimmed milk samples (F2) subjected to a hydrolysis process that simulates gastrointestinal digestion.

Detection time (min)	Observed mass (Da)	Protein fragment	Sequence	Activity	References
13.2	501.2	Serum albumin (530-533)	ELLK		
15.1	387.2	Lactoferrin (108-110)	QLQ		
15.8	574.2	Lactoferrin (500-504)	RLCAL		
16.4	502.2	αs2-casein (167-170)	ISQR	Fragment from LKKISQRYQKFALPQY (antibacterial activity)	41
16.7	543.3	Lactoferrina (96-100 y 437-441)	AVVKK		
17.3	516.3	Serum albumin (520-523)	KQIK	Light antifungal activity	42
17.6	445.2	Lactoferrin (620-623)	KNGK		
17.9	801.3	β-lactoglobulin (77-83)	KIPAVFK		
18.5	648.3	Lactoferrin (644-649)	NTECLA		
19.0	616.4	Serum albumin (440-444)	PESER		
20.1	516.3	Serum albumin (76-80)	KVASL		
20.5	449.2	Lactoferrin (372-376)	ATAST		
21.7	645.3	β-casein (100-105)	EAMAPK	Fragment from VKEAMAPK (antioxidant activity)	31
22.5	488.1	β-casein (94-98)	GVSKV		
23.7	872.5	β-casein (98-105)	VKEAMAPK	Antioxidant activity	31
25.0	488.1	Serum albumin (248-251)	DLLE		
25.4	656.3	αs1-casein (99-103)	LRLKK		
27.4	528.4	β-casein (25-28)	RINK	Fragment from RELEELNVPGEIVESLSSSEESITRINK (immunomodulatory activity)	35
27.9	963.4	κ-casein (96-103)	ARHPHPHL	Fragment from ARHPHPHLSFM (antioxidant activity)	31
29.6	819.3	αs1-casein (94-104)	LRLKKY		
30.1	779.4	β-lactoglobulin (133-138)	LEKFDK		43
32.3	501.2	αs2-casein (164-167)	LKKI	Fragment from LKKISQ (ACE-inhibitory activity)	39
33.2	596.3	αs1-casein (150-153)	FRQF	ACE-inhibitory activity	38
33.6	583.3	Serum albumin (383-387)	PQNLI		
38.3	746.3	Lactoferrin (559-564)	DWAKNL		
42.6	786.3	κ-casein (74-79)	LQWQVL		
43.0	627.2	αs1-casein (91-95)	YLGYL	Fragment from RYLGYLE (α-casomorphin with opioid activity)	7
44.4	834.4	αs2-casein (55-60)	GSSSEE		
51.6	675.3	αs1-casein (29-34)	PEVFGK	Fragment from FPEVFGK (ACE-inhibitory activity)	41

Table 7. Milk protein-derived peptides identified in the permeate obtained from concentrated milk samples (F3) subjected to a hydrolysis process that simulates gastrointestinal digestion.

Detection time (min)	Observed mass (Da)	Protein fragment	Sequence	Activity	References
8.0	468.2	Serum albumin (82-85)	ETYG		
13.3	501.3	Serum albumin (530-533)	ELLK		
17.7	445.2	κ-casein (91-94)	QPTT		
18.7	648.3	β-casein (164-169)	SLSQSK	Fragment from PPQSVLLSLSQSKVLPVPE (ACE-inhibitory activity)	30
19.9	376.3	κ-casein (166-169)	STAV	Fragment from VTSTAV (ACE-inhibitory activity)	44
20.5	449.2	α-lactalbumin (110-113)	LCSE	Fragment from ALCSEK (antibacterial activity)	45
21.7	645.2	β-lactoglobulin (137-142)	DKALKA		
22.2	614.2	αs1-casein (120-124)	LHSMK		
22.6	445.2	Serum albumin (515-518)	LPDT		
23.7	872.4	β-casein (98-105)	VKEAMAPK	Antioxidant activity	31
24.1	674.2	β-casein (1-5)	RELEE	Fragment from RELEELNVPGEIVESLSSSEESITRINK (immunomodulatory activity)	32
25.0	449.2	αs1-casein (151-153)	RQF		
26.2	819.4	β-lactoglobulin (2-8)	IVTQTMK		46
26.5	507.3	αs1-casein (90-93)	RYLG	Fragment from RYLGYL (opioid antagonist)	47
32.4	501.3	αs2-casein (164-167)	LKKI	Fragment from LKKISQ (ACE-inhibitory activity)	39
32.8	673.3	β-casein (184-189)	DMPIQA	Fragment from RDMPIQAF (ACE-inhibitory activity)	30
33.4	596.3	Serum albumin (89-93)	DCCEK		
34.9	400.4	Lactoferrina (570-572)	RLL		
37.3	673.3	β-caseína (157-162)	FPPQSV		22
43.3	627.2	αs1-caseína (91-95)	YLGYL	Fragment from RYLGYLE (α-casomorphin with opioid activity)	7
43.9	544.2	κ-caseína (76-79)	WQVL		48
73.0	354.3	αs1-caseína (128-130)	HAQ		

Table 8. Milk protein-derived peptides identified in the permeate obtained from milk serum employed as ingredient for infant formula manufacturing (S) subjected to a hydrolysis process that simulates gastrointestinal digestion.

Detection time (min)	Observed mass (Da)	Protein fragment	Sequence	Activity	References
7.2	468.1	Serum albumin (82-85)	ETYG		
7.6	468.1	κ-casein (109-112)	PPKK	Fragment from MAIPPKK (antithrombotic activity)	49
9.1	555.1	κ-casein (62-67)	AKPAAV		
18.2	493.2	β-lactoglobulin (24-28)	MAASD	Fragment from WYSLAMAASDI (antioxidant activity)	50
19.0	616.3	β-casein (94-99)	GVSKVK		
19.3	445.2	β-lactoglobulin (125-128)	TPEV		
19.8	376.3	κ-casein (166-169)	STAV	Fragment from VTSTAV (ACE-inhibitory activity)	44
20.6	439.3	αs1-casein (150-152)	FRQ	Fragment LFRQ (ACE-inhibitory activity)	51
23.0	633.3	κ-casein (162-167)	VQVTST		52
23.3	574.3	κ-casein (159-163)	INTVQ		
25.1	776.3	κ-casein (138-145)	AVESTVAT		
25.7	646.3	β-casein (53-58)	AQTQSL	Fragment from AQTQSLVYP (ACE-inhibitory activity)	53
28.6	549.3	Serum albumin (407-410)	IVRY		
29.0	514.3	Serum albumin (456-459)	LNRL		
29.5	803.4	κ-casein (162-169)	VQVTSTAV		38
30.1	801.3	β-lactoglobulin (83-89)	KIDALNE		54
30.8	452.2	β-lactoglobulin (20-23)	YSLA	Fragment from WYSLA (antioxidant activity)	18
32.6	643.4	α-lactalbumin (96-101)	LDKVGI		
32.8	537.4	β-lactoglobulin (146-149)	HIRL	β-lactoquinine (ACE-inhibitory activity)	55
34.0	400.3	Lactoferrin (570-572)	RLL		
35.8	675.2	β-lactoglobulin (25-31)	AASDISL		
39.1	805.3	β-casein (155-161)	VMFPPQS		
87.9	356.4	Lactoferrin (29-32)	LGAP		

Table 9. Milk protein-derived peptides identified in the permeate obtained from concentrated manufactured formual (F4) subjected to a hydrolysis process that simulates gastrointestinal digestion.

Detection time (min)	Observed mass (Da)	Protein fragment	Sequence	Activity	References
8.2	468.2	Serum albumin (82-85)	ETYG		
8.3	447.3	Lactoferrin (177-180)	GENQ		
13.5	501.3	Serum albumin (94-97)	QEPE		
13.5	501.3	αs1-casein (121-124)	HSMK		
14.3	273.1	Lactoferrin (344-345) and β-lactoglobulin (40-41)	RV		
16.6	502.2	Lactoferrin (428-431)	RPTE		
17.5	437.1	αs1-casein (103-105)	KYK		
17.7	516.2	Serum albumin (541-544)	EQLK		
18.4	661.3	αs1-casein (135-140)	MIGVNK		
18,8-18,9	648.3	β-casein (164-169)	SLSQSK	Fragment from PPQSVLLSLSQSKVLPVPE (ACE-inhibitory activity)	30
19.2	470,2-470,3	as1-casein (105-108) and serum albumin (413-417)	KVPQ	Fragment from KKYKVPQ (ACE-inhibitory activity)	18
19,9-20,0	376,2-376,3	κ-casein (166-169)	STAV	Fragment from VTSTAV (ACE-inhibitory activity)	44
20,2-20,3	516.2	Lactoferrin (4-7)	KNVR	Fragment from APRKNVRW (antibacterial activity)	56
20.7	449.3	α-lactoalbumin (110-113)	LCSE	Fragment from ALCSEK (antibacterial activity)	45
20.7	449.3	Serum albumin (10-12)	RFK		
21.8	645.2	Lactoferrin (88-92)	PQTHY	Fragment from AGIYGTKESPQTHYY (immunomodulatory activity	57
21.9	645.3	β-casein (100-105)	EAMAPK	Frafment from VKEAMAPK (antioxidant activity)	31
22,4-22,5	614.3	αs1-casein (120-24)	LHSMK		
22.7	445.2	Serum albumin (515-518)	LPDT		
23,2-23,3	572,1-572,3	β-lactoglobulin (71-75)	IIAEK	Lactostatin (hipocholesterolemic, inhibitor of cholesterol absorption and ACE-inhibitory activity)	58
23.3	572.1	Lactoferrin (146-151)	QGAVAK		
23.9	872.4	β-casein (98-105)	VKEAMAPK	Antioxidant activity	31
24.1	510.1	Serum albumin (565-569)	ACFAV		
24.3	674.3	β-lactoglobulin (78-83)	IPAVFK	Antibacterial activity	59
25.2	449.2	αs1-casein (151-153)	RQF		
25.2	604.2	Serum albumin (457-461)	NRLCV		
25.4	488.1	β-casein (205-208)	FPII		
25.5	1336.6	αs1-casein (80-90)	HIQKEDVPSER	Antioxidant activity	60
26,7-26,8	507.3	αs1-casein (90-93)	RYLG	Fragment from RYLGYL (opioid antagonist)	47
27.6	528.4	αs1-casein (99-102)	LRLK		
28.2	469.3	κ-casein (26-29)	IPIQ	Fragment from IPIQYVL (antioxidant activity)	61
29.6	803,3-803,4	κ-casein (162-169)	VQVTSTAV		38
30.6	833.3	β-lactoglobulin (148-154)	RLSFNPT		
31-31,1	452.2	β-lactoglobulin (20-23)	YSLA	Fragment from WYSLAM (antioxidant activity)	18
31.7	398.3	Lactoferrin (591-594)	VAPN		
31.7	398.2	Lactoferrin (409-412)	PVLA		
32.5	501.3	αs2-casein (164-167)	LKKI	Fragment from LKKISQ (ACE-inhibitory activity)	39
32.6	501.3	β-casein (27-30)	NKKI		62
32.8	627.3	β-casein (61-66)	PFPGPI	Cathepsin B inhibitor	63
32.9	673.3	κ-casein (125-131)	IASGEPT		
32.9	673.3	β-casein (177-182)	AVPYPQ	Fragment from AVPYPQR (ACE-inhibitory and antioxidant activity)	
33.7	583.3	Serum albumin (383-387)	PQNLI		
33.8	583.2	Lactoferrin (493-498)	PGADPK		
34,1-35,0	400,3-400,4	Lactoferrin (570-572)	RLL		
37.1	520.2	κ-casein (53-56)	NQFL		
38.4	583.3	β-lactoglobulin (20-24)	YSLAM	Fragment from WYSLAM (antioxidant activity)	18
38.8	6103	Serum albumin (450-454)	DYLSL		
40,2-40,3	680.3	κ-casein (103-108)	LSFMAI		23
43.4	627,2-627,3	αs1-casein (91-95)	YLGYL	Fragment from RYLGYLE (α-casomorphin with opioid activity)	7
43.9	544.2	κ-casein (76-79)	WQVL		48
46.4	391.3	κ-casein (25-27)	YIP	Fragment from YIPIQYVLSR (immunomodulatory, opiod antagonist and ACE-inhibitory activity)	
53	741,3-741,4	Serum albumin (569-575)	VEGPKLV		
57.4	474.1	β-casein (90-93)	PEVM		
72.2	372.2	Serum albumin (569-575)	EPQ		
72.8	354.3	αs1-casein (128-130)	HAQ		

Table 10. Milk protein-derived peptides identified in the permeate obtained from final infant formula (F5) subjected to a hydrolysis process that simulates gastrointestinal digestion.

Detection time (min)	Observed mass (Da)	Protein fragment	Sequence	Activity	References
8	468.2	Serum albumin (82-85)	ETYG		
8,0-8,5	446,2-446,3	κ-casein (137-140)	EAVE		
10.8	399.2	αs1-caseína (1-3)	RPK	ACE-inhibitory activity	64
13,5-13,7	501,2-501,4	Serum albumin (530-533)	ELLK		
14.3	273.2	Lactoferrin (344-345) and b-lactoglobulin (40-41)	RV	Fragment from RVY (ACE-inhibitory activity)	65
15.5	387.3	Serum albumin (114-116, 273-275, 375-377)	KLK	Fragment from KLKLLLLLKLK with antimicrobial and immunomodulatory activity	66
16.2	287.1	Serum albumin, Lactoferrin, αs1-casein, αs2-casein and β-lactoglobulin	RL		55
16.5	5933	Lactoferrin (277-281)	KFGKN	Fragment from lactoferrampin (WKLLSKAQEKFGKNKSR) with antibacterial activity	67
16.6	502,2-502,3	Lactoferrin (428-431)	RPTE		
16.8	502.2	αs2-casein (42-45)	EVVR		
17.3	524.3	αs1-casein (78-81)	QKHI		
17.4	524.3	β-casein (59-62)	VYPF	Fragment from VYPFPG (ACE-inhibitory activity)	68
17.4	623.2	κ-casein (98-102)	HPHPH		
17.6	617.2	αs1-casein (35-39)	EKVNE		
17,6-17,8	445.2	κ-casein (91-94)	QPTT		
18	515.2	Serum albumin (273-276)	KLKE		
18.2	801.3	αs2-casein (183-188)	VYQHQK	Fragment of casocidin-I (antibacterial activity)	69
18.3	661.2	Lactoferrin (200-206)	QDGAGDV		
18.4	661.3	αs2-casein (140-144)	DMEST		
18.7	648.3	Serum albumin (181-185)	IETMR		
18,8-19,0	648.3	Lactoferrin (644-649)	NTECLA		
19.2	470.2	αs1-casein (105-108) and serum albumin (413-417)	KVPQ	Fragment from KKYKVPQ (ACE-inhibitory activity)	18
19,4-19,7	398.1	β-lactoglobulin (78-81)	IPAV	Fragment from IPAVFK (antibacterial activity)	59
19,4-20,0	376,2-376,3	κ-casein (166-169)	STAV	Fragment from VTSTAV (ACE-inhibitory activity)	44
20,1-20,3	516,2-516,3	β-casein (101-105)	AMAPK	Fragment from VKEAMAPK (antioxidant activity)	31
20,6-20,8	449.2	Lactoferrin (372-376)	ATAST		
20.9	449.4	β-lactoglobulin (78-81)	ECAQ		
21.7	643.3	β-lactoglobulin (78-81)	ENSAEP		
21,7-21,9	645,2-645,3	β-casein (100-105)	EAMAPK	Fragment from VKEAMAPK (antioxidant activity)	31
22.5	445.2	Serum albumin (515-518)	LPDT		
22,4-22,7	614.3	αs1-casein (120-24)	LHSMK		
22.3	572.3	κ-casein (71-75)	AQILQ	Fragment from PAAVRSPAQILQ (antibacterial activity)	37
23.2	572.3	Lactoferrin (579-583)	KPVTE		
23,2-23,3	572,1-572,4	β-lactoglobulin (71-75)	IIAEK	Lactostatin (hipocholesterolemic, inhibitor of cholesterol absorption and ACE-inhibitory activity)	58
23.3	572.4	Lactoferrin (146-151)	QGAVAK		
23.6	436.3	β-lactoglobulin (40-42)	RVY	ACE-inhibitory activity	70
23,9-24,0	872.4	β-casein (98-105)	VKEAMAPK	Antioxidant activity	31
24.1	518.2	αs1-casein (57-60)	IKQM		
24.3	488.2	αs1-casein (151-153)	NENL	Fragmen from VLNENLLR (antibacterial activity)	35
24,3-24,4	674,2-674,3	β-lactoglobulin (78-83)	IPAVFK	Antibacterial activity	59
24.7	673.3	κ-casein (117-122)	TEIPTI		
25,2-25,5	449.2	αs1-casein (151-153)	RQF		
25.7	1336.6	αs1-casein (80-90)	HIQKEDVPSER	Antioxidant activity	60
25.9	646.3	Lactoferrin (402-408)	AGKCGLV		
26.3	819.4	Serum albumin (229-235)	FGERALK		
26.3	819.3	Serum albumin (363-369)	DDPHACY		
26,6-26,8	507.3	αs1-casein (90-93)	RYLG	Fragment from RYLGYL (opioid antagonist)	47
27.1	633.3	αs1-casein (104-108)	YQVPQ		
27.3	615.3	α-lactoalbumin (54-58)	QINNK	Modulates functions of non classical cadherin	71
27.4	615.3	αs1-casein (184-189)	NPIGSE	Fragment from SDIPNPIGSENSEK (antibacterial activity)	35
27.5	528.4	αs1-casein (97-100)	QLLR		
27,4-27,5	528.4	β-casein (25-28)	RINK	Fragment from RELEELNVPGEIVESLSSSEESITRINK (immunomodulating activity)	35
28.4	567.2	β-lactoglobulin (142-146)	ALPMH	Fragment from ALPMHIR (ACE-inhibitory activity)	72
28,7-28,9	695.2	Serum albumin (370-375)	STVFDK		
29.3	672.3	Serum albumin (77-88)	VASLRE		
29.5	672.3	β-lactoglobulin (8-13)	KGLDIQ		
29,6-29,7	803,3-803,4	κ-casein (162-169)	VQVTSTAV		38

30,0-30,1	779.4	β-lactoglobulin (133-138)	LEKFDK		43
30.9	452.2	Serum albumin (485-488)	PCFS		
31-31,1	452.2	β-lactoglobulin (20-23)	YSLA	Fragment from WYSLAM (antioxidant activity)	18
31.6	398.1	β-lactoglobulin (78-81)	IPAV	Fragment from TKIPAV	23
31,7-31,8	584.2	Lactoferrin (144-149)	PLQGAV		
32.1	526.3	β-lactoglobulin (77-81)	KIPAV	Fragment from TKIPAV	22
32.5	501.2	β-casein (27-30)	NKKI		62
32.9	673.3	Lactoferrin (232-237)	LNNSRA		
32,7-32,9	627,2-627,4	β-lactoglobulin (76-81)	TKIPAV		23
32,9-33,1	673,2-673,3	β-casein (177-182)	AVPYPQ	Fragment from AVPYPQR (antioxidant and ACE-inhibitory activity)	73
32,8-32,9	627.3	β-casein (61-66)	PFPGPI	Cathepsin B inhibitor	63
33.3	532.3	Lactoferrin (491-496)	CAPGAD		
33,2-33,5	596.3	αs1-casein (150-153)	FRQF	ACE-inhibitory activity	38
33.7	583.3	Serum albumin (383-387)	PQNLI		
33.8	596.2	Lactoferrin (211-215)	ETTVF		
34,1-35,0	400,2-400,4	Lactoferrin (570-572)	RLL		
34.6	514.2	αs1-casein (91-94)	YLGY	Fragment from RYLGYLE (α-casomorphin with opioid activity)	7
35.9	583.3	Lactoferrin (478-483)	TGSCAF		
37	596.3	αs1-casein (113-117)	PNSAE		
37.3	759.3	αs1-casein (150-154)	FRQFY		
39.2	805.3	β-lactoglobulin (150-156)	SFNPTQL		74
40.2	680.3	β-lactoglobulin (142-147)	ALPMHI	Fragment from ALPMHIR (ACE-inhibitory activity)	72
40,2-40,3	680.3	κ-casein (103-108)	LSFMAI		22
42,5-42,8	414,2-414,3	αs1-casein (197-200)	PLW	ACE-inhibitory activity	75
43,3-43,5	627.3	αs1-casein (91-95)	YLGYL	Fragment from RYLGYLE (α-casomorphin with opioid activity)	7
45.7	688.3	Serum albumin (485-488)	LVNELT		
46.3	391.4	β-casein (190-192)	FLL		
52,8-53	741,3-741,4	Serum albumin (569-575)	VEGPKLV		
73	372.3	αs1-casein (185-188)	PIGS	Fragment from SDIPNPIGSENSEK (antibacterial activity)	76

With the exception of the whey samples (S), a peptide from β-casein f(98-105) with the sequence VKEAMAPK and antioxidant activity[31] appeared in all of the samples. Other abundant peptides in the samples were ETYG, ELLK, STAV, LPDT, AVVKK, ATAST, IIAEK, EAMAPK, RELEE, RYLG, LRLK, LRLKK, LKKI, VQVTSTAV, YLGY, YSLA, YLGYL, VEGPKLV, and HAQ. Among these peptides, the α_{s1}-casein fragment f(91-94), with the sequence YLGY, had a structure potentially able to act as an opioid receptor ligand. This peptide is a fragment of α-casomorphin[7], which maintains an amino-terminal tyrosine for essential opioid activity[77] and an aromatic amino acid in the third or fourth position that favors peptide union to opioid receptor.[53] Further studies are necessary to confirm the potential opioid activity of this fragment, because it may have an important role in sleeping pattern and the development and function of the gastrointestinal tract in infants depending on its opioid agonistic or antagonistic activity.[78]

Additionally, some angiotensin I-converting enzyme (ACE)-inhibitory peptides, were found in F1, F2 and F5 samples. Their sequences were also described in human milk proteins and can be expected that breast-feed infants as well as infants fed with milk-based formulas will obtain ACE-inhibitory peptides, which may play a role in cardiovascular health. Has been proposed than the immnaturity related higher serum ACE activity in early life could program cardiovascular disease later in life but that ACE-inhibitory peptides present in milk might improve this negative effect[78]. So their presence in formulas and their activity compared with breast milk must be object of further studies.

Moreover, in the profile of majority peptides released after gastrointestinal digestion of the final manufactured formula (Table 10), peptides with antioxidant (HIQKEDVPSER, VKEAMAPK),[31, 60] antimicrobial activity (IPAVFK),[59] ACE-inhibitory peptides (FRQF, PLW, RPK, RVY, IIAEK),[38, 58,

[64, 70, 75] a peptide modulator of non classical cadherins functions (QUINNK)[71] and a cathepsin B inhibitor (PFPGPI)[63] were found. It is important to highlight that our results shows some fragments originating from β-lactoglobulin, a protein absent in human milk. Two of this peptides—RVY and IPAVFK—had reported ACE-inhibitory and antibacterial activity respectively. Additional studies are necessary to determine whether these peptides have similar functional properties to human milk, defined for a different majority peptide profile.[78] In addition, the fragments of many previously characterized bioactive peptides were found after infant formula (F5) gastrointestinal digestion. Some of these fragments may have preserved similar bioactivity of the origin sequence and must be taken in consideration for further studies.

Antimicrobial and immunomodulatory peptides, together with polyamines, may be responsible for the different susceptibility to some diseases between formula-fed and breast-fed infants. ACE-inhibitory peptides may play a role in cardiovascular health,[78] and antioxidant peptides may protect infants from oxidative stress-associated diseases, such as necrotizing enterocolitis.[79]

The present study confirms that the infant formula manufacturing process determines the polyamine content and peptidic profile after digestion of the infant formula. Therefore, compositional dissimilarity between human milk and infant formula in polyamines and proteins, and the functionality of these proteins and their peptides, could be responsible for some of the differences in health reported between breast-fed and formula-fed children. These changes must be taken into consideration because they may have a great impact on infant nutrition and development.

Further studies are important to address the effects of manufacturing process in protein digestibility, peptides profile and polyamine content during formulation of infant formulas for the potential implication in human health.

ABBREVIATIONS

ACE, angiotensin I-converting enzyme; **HPLC**, high-performance liquid chromatography; **MS**, mass spectrometry; **N**, nitrogen; **NPN**, non-protein nitrogen; **PAO**, polyamine oxidase; **SDS**, sodium dodecyl sulphate.

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9.2. A METHOD TO COLLECT HIGH VOLUMES OF MILK FROM MICE (*Mus musculus*).

Collection of milk from mice (*Mus musculus*) may be a critical part for a variety of preclinical studies such as mammary gland biology, lactation, infant nutrition, and toxicological evaluation of novel drugs (Jolois et al., 2001; Case and Domant, 2012; Demon et al., 2012). Protocols describing how to collect milk from a mouse are scarce in scientific literature. Common features of such protocols are the manually generated vacuum and the small sample volume that is normally collected (De Peters and Hovey, 2009). The aim of our study was to develop a milking protocol for mice that is practical and produces a higher volume of milk.

MATERIALS AND METHODS

Mice, housing and husbandry

A total of 20 female adult mice—7 outbred HsdWinin:NMRI (bred and supplied by a breeding colony of the Central Animal Laboratory at University of Turku, Finland); 3 outbred Sca:NMRI mice (bred and obtained from Scanbur Ltd., Sollentuna, Sweden), and 10 inbred BALB/cOlaHsd mice (supplied by Harlan Laboratories®, Horst, Netherlands and bred in a facility of the Central Animal Laboratory, University of Turku, Finland)—were used in this study. The mice were selected based on availability of dams with a nursing litter from the breeding colony. At the commencement of the milking process, the dams ranged in body weight between 28 and 47 g. All the mice were maintained and milked in a facility of the Central Animal Laboratory, University of Turku, Finland. The mice were housed in top-filtered stainless steel cages (365 x 207 x140 cm) with solid bottoms and Aspen chips as bedding (Tapvei Ltd, Kaavi, Finland), with substantial nesting material. Cages were changed twice a week. The environment in the mouse room consisted of a temperature range of 22 to 27 °C, a relative humidity of 50 to 60%, and artificial illumination with a 12-h light/dark cycle (lights on at 06:00 am). Throughout the study period, all the mice were fed a standard mouse chow (SDS, Special Diet Services, Witham, Essex, UK) *ad libitum*, and tap water was provided without restrictions in polycarbonate bottles. Prior to the milking, all the dams were determined to be healthy based on clinical observations, and were considered pathogen-free based on the results of routine microbiological screening performed in the colony in accordance with current European recommendations (Nicklas et al., 2002). This study was part of the pilot experiments performed to optimise handling and treatment of another study approved by the National Ethics Committee for Animal Experiments in Finland (ESLH-2009-04845/Ym-23).

Construction of the milking machine

An electric off-the-shelf human breast pump was acquired from a local infant supply store. The original tubing of the milking machine was partially replaced with smaller gauge tubes. The flap valve unit from the machine was modified to function without the original collection vessel. The original breast cup was fitted with diameter reducers and connected to a new collection vessel. All connections and contacting surfaces were sealed using Pechiney Plastics Parafilm M* laboratory wrapping film to avoid pressure loss due to leakage (Figures 1 and 2).

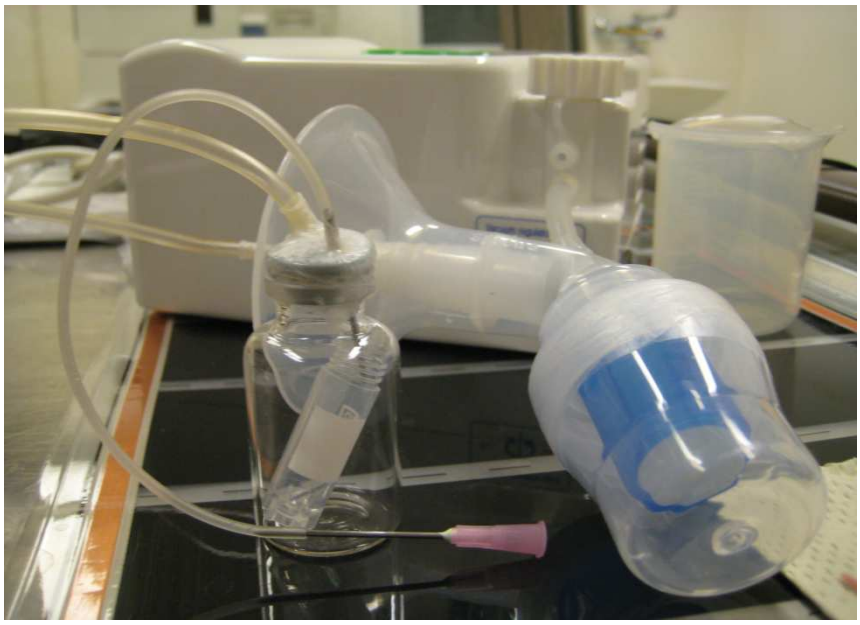

Figure 1. Modified electric breast pump and a glass vial for milk collection.

A new collection vessel fit for small liquid volumes was constructed from a small empty glass bottle, a rubber cap for the vial, a 2.0 mL collection tube and two 18G hypodermic needles. All parts were sterilized using 70% ethanol. Pre-sterilized parts in autoclave were used when possible.

A small hole punctured the rubber cap. The tube connected to the milking machine was inserted through the hole. A hypodermic needle, without a plastic syringe attachment, was inserted through the vial cap. The tube was attached to this needle. Another needle was inserted into the free end of the tube to provide a suction cup for a nipple.

The milk collection tube was placed inside the bottle, needle piercing the bottle cap was aimed at the collection tube and the cap was closed. The cap was sealed using Pechiney Plastics Parafilm M* laboratory wrapping film (Figure 3).

The milking machine was turned on and all seals were checked for possible pressure leaks by listening for an audible hiss. Suction pressure was checked by placing the suction cup against a hand covered in a laboratory glove, to which the cup should stick if proper suction was present.

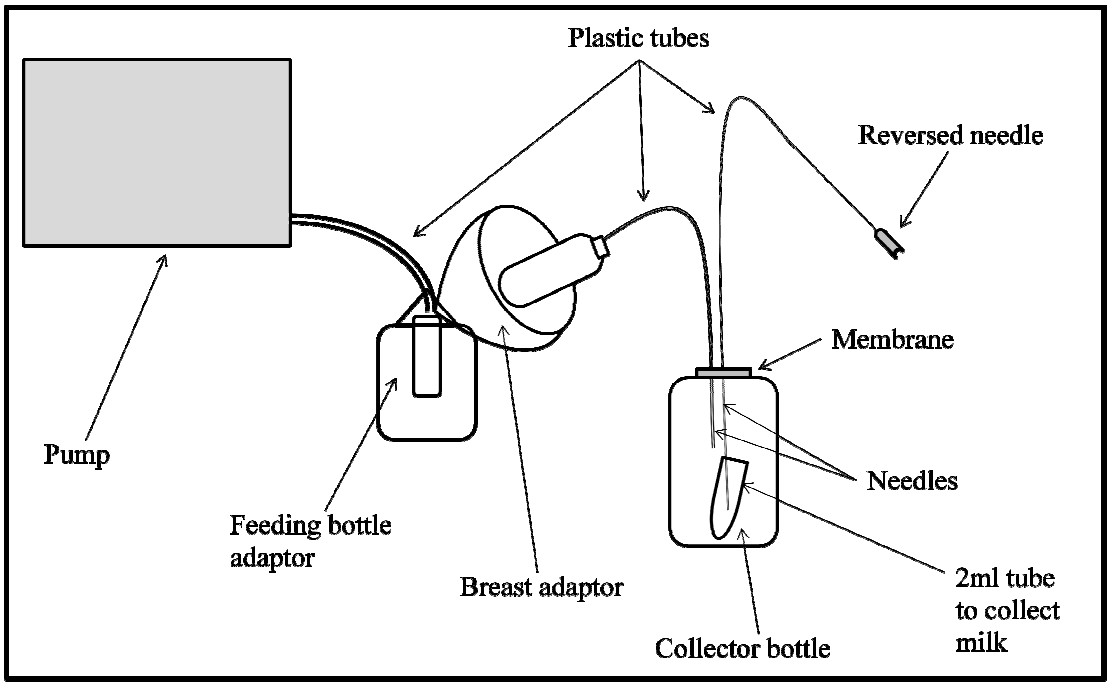

Figure 2. Milking machine diagram. The different parts are out of scale.

Figure 3. A glass vial containing a collection tube. The tube with larger diameter (left) is connected to the milking machine, and the tube with smaller diameter (right) is connected to an inverted needle serving as a suction cup.

Milking protocol

Milking was carried out during the days of higher milk production, which is between days 7-12 after the parturition. The pups were separated from their mothers for 6-12 h before milking to allow the milk to accumulate in the mammary glands.

Oxytocin was prepared by pipetting 0.8-0.9 ml oxytocin (Partoxin® Vet. 17 µg/ml, 10 U.I./ml) into 1.5 ml Eppendorf tubes. To avoid acidosis of the animal, the pH of the oxytocin was neutralized by adding bicarbonate buffer to the oxytocin until pH is approximately 7. The amount of bicarbonate buffer needed depends on molarity of the buffer and the acid used in the commercial oxytocin solution. The pH was measured with specific strips and adjusted before milking.

A mixture of midazolam (Dormicum® 5 mg/ml) and ketamine (Ketalar® 10 mg/ml) to produce a solution containing 5 mg midazolam/kg bodyweight and 75 mg ketamine/kg bodyweight was prepared to be used as injectable anaesthesia of the milking dam. The animal to be milked was weighed and the amount of solution needed for anaesthesia then calculated.

The anaesthetic solution was injected subcutaneously. After the injection, the animal was placed back in its cage until the effects of anaesthesia were observed. When the animal was unconscious, it was placed on the heating mat to maintain the animal's warmth during the milking process. The depth of anaesthesia was confirmed by checking for lack of pedal reflex.

If the animal started to regain consciousness during the milking an injectable anaesthetic, approximately 1/3 of the initial amount of the anaesthetic, was injected intraperitoneally to the right side of the animal. When the animal is held on its back with its abdomen facing up, its right side will be on the left. This was done to avoid damage to the cecum of the animal.

When using injectable anaesthetics, animals were placed in their respective cages after the milking procedure, and the cage was left on a heating pad until the animal showed signs of recovery (i.e. was aware of its surroundings and started moving around).

Alternatively, inhalational agent isoflurane (Isoflurane®) was used for anaesthesia. For induction, a concentration of 4-5% was used, and for maintenance, the concentration of isoflurane was lowered to approximately 2-3%.

After an animal was anesthetized, 2-8 IU of oxytocin was administered intraperitoneally to induce milk flow. Half of the volume was injected between the right inguinal nipples and the other half was injected between the left inguinal nipples (Figure 4).

After milking, the animal was rehydrated using 0.5 ml of a solution consisting of 0.40 ml glucose solution (50 mg/ml) and 0.1 ml physiological saline (9 mg/ml) (Figure 5).

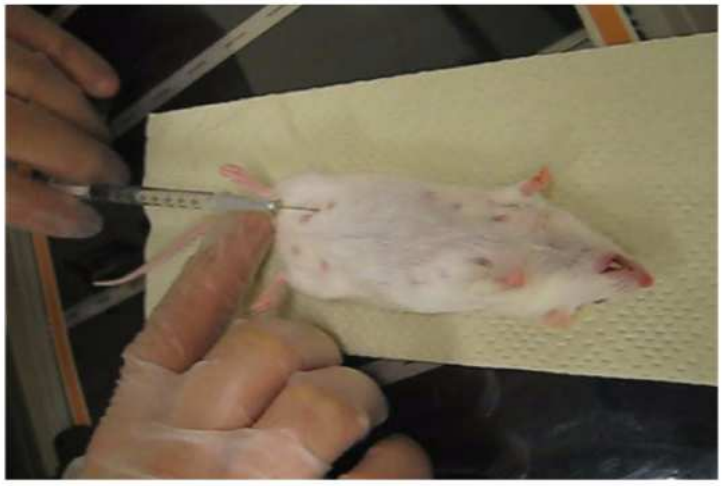

Figure 4. IP administration of oxytocin between inguinal nipples.

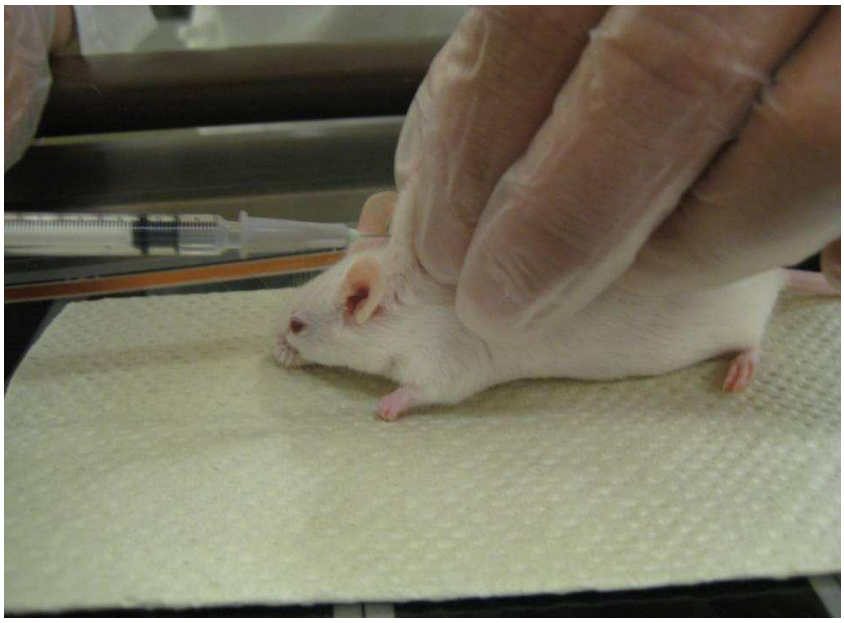

Figure 5. Rehydration of the dam after milking.

Collection of milk

Milk started flowing within 4-10 min after the oxytocin injection. Then the milking machine was turned on and the amount of suction was adjusted. The suction head of the milking machine was placed on one of the inguinal nipples (Figure 6). Four nipples were used for milking. When the amount of milk received decreases, another nipple was milked. Nipples that have been already milked can be returned to later on.

When no additional milk was produced from the nipples, the milking machine was stopped and the milk stored.

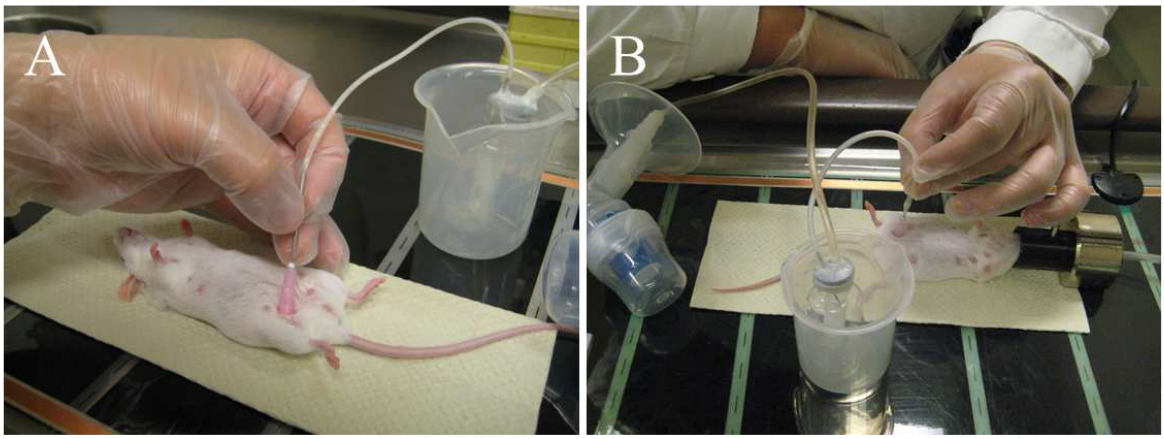

Figure 6. Milking a mouse using A) injectable (midazolam-ketamine); or B) inhalational anesthesia.

As isoflurane anaesthesia exerted worse results in volume of milk collected and mouse recovery compared with injectable anaesthesia, this method was obviated. The negative effects could be due to the reported inhibitory effect of the inhalational hydrocarbonated ethers in contractions induced with oxytocin (Yildiz et al., 2005; Gultekin et al., 2006) and the reduction of prolactin levels in blood (Chassagne et al., 2000).

The milk (Figure 7) can be stored up to 3 h in 4ºC, and 5 months in -20ºC and 8 months in -80ºC, with the aim to preserve initial levels of vitamins and long chain polyunsaturated fatty acids (Romeu-Nadal et al. 2008).

Figure 7. Milk collected from a HsdWinin:NMRI dam.

For outbred NMRI mice the volume range of milk collected was between 0.5-1.5 ml, and for inbred BALB/cOlaHsd mice the volume collected was between 0.2-0.4 ml. This difference may be in relation

with the size of the animal, with a weight between 25-40 g and 15-20 g respectively, or with the genetic background of these two different mouse strains.

About possible anatomical, physiological or histological abnormalities in the mammary glands such as mastitis, we recommend further studies to check the sensibility of the specific strain to the milk collection. If abnormalities in the mammary gland of the mice are suspected, it is recommended to treat the animals in accordance with good veterinary practice.

In the present methodology, an effective milking method to extract high volumes of milk from mice is described. With this protocol, the total amount of milk collected from dams yielding milk in each milking ranged between 0.2 and 1.5 ml. In particular, maximal milk production was achieved from outbred and multiparous dams with higher bodyweight. In these dams, the better milk extraction was under inhalational anaesthesia while for inbred dams, with less weight, the best response was obtained under injectable anaesthesia.

In summary, the milking method described above, provides a valuable means for acquiring substantial amounts of mouse milk. Nevertheless, careful selection of a suitable dam is required, because considerably higher amounts of milk are generally collected from outbred and multiparous in comparison with inbred and primiparous dams.

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