CHARLES UNIVERSITY

Faculty of Pharmacy in Hradec Králové

Department of Biological and Medical Sciences

The effect of dietary prebiotic supplementation on neonatal development in rats

Diploma thesis

Supervisor: PharmDr. Eva Doleželová, Ph.D.

Consultant: Dr. Francisco José Pérez Cano, Ph.D.

Hradec Králové, 2017

Martina Medveďová

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Acknowledgement

I would like to express my sincere gratitude to Dr. Francisco José Pérez Cano, Ph.D. for giving me the unforgettable opportunity to work with his amazing research group, for supervising and all useful advices. Then, a big thank you belongs to all members of the Autoimmunity and Tolerance Research Group at the University of Barcelona, who perfectly cooperated as a team and were a great inspiration and support for me not only when performing the experiments. Namely, a special note is dedicated to Malén Massot-Cladera and Ignasi Azagra-Boronat for their kind, patient and invaluable assistance and willingness to answer any of my questions. Furthermore, I would like to thank to my Czech supervisor, PharmDr. Eva Doleželová, Ph.D., for her professional approach and help with the correction and formal aspects of my diploma thesis. Last but not least, I would like to thank to my family for all their support during my studies including Erasmus in Barcelona.

Abstract

Charles University, Faculty of Pharmacy in Hradec Králové, Department of Biological and Medical Sciences

University of Barcelona, Faculty of Pharmacy and Food Science, Department of Biochemistry and Physiology

Student: Martina Medveďová

Supervisor: PharmDr. Eva Doleželová, Ph.D.

Consultant: Dr. Francisco José Pérez Cano, Ph.D.

Study program: Pharmacy

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Human breast milk is considered a gold standard in infant nutrition due to its multiple evident benefits on human health. However, breastfeeding is not always possible for various reasons; thus, it is a huge challenge to provide infant formulae which best mimic the composition and effects of human milk. Prebiotics added to infant formulae may greatly contribute to these prospective notable improvements in infant nutrition. The aim of this study was to evaluate the effect of a novel dietary prebiotic supplementation (under patent) on growth, fecal consistency, and immune maturation in suckling rats. G14 Lewis suckling rats were distributed into 2 experimental groups (n = 21/group): reference and prebiotic group. Vehicle or the prebiotic was administered by oral gavage daily from day 2 to 16 of life. Animals were weighed daily and their size and related variables were measured on day 8 and 16. For the detection and quantification of serum immunoglobulin isotypes and several intestinal cytokines in the gut wash on day 8 and 16, immunoassays based on the Luminex® xMAP® technology were used. The prebiotic intervention showed growth promoting effect without influencing the fecal consistency. Based on measurements of serum immunoglobulin and intestinal cytokine levels, the supplementation significantly stimulated immunoglobulin (IgG2a, IgG2b, IgG2c, and IgA) production, increased the Th1/Th2 Ig as well as the IL-10/TNFα ratios and reduced the levels of most detected intestinal cytokines. According to the results, the tested prebiotic demonstrated the immunomodulatory impact on neonatal immune development in this preclinical rat model and showed a potential to become a beneficial component of a new infant formula.

Abstrakt

Univerzita Karlova, Farmaceutická fakulta v Hradci Králové, Katedra biologických a lékařských věd

University of Barcelona, Faculty of Pharmacy and Food Science, Department of Biochemistry and Physiology

Studentka: Martina Medveďová

Školitel: PharmDr. Eva Doleželová, Ph.D.

Konzultant: Dr. Francisco José Pérez Cano, Ph.D.

Studijní obor: Farmacie

Název diplomové práce: Vliv suplementace prebiotikem na neonatální vývoj u potkanů

Mateřské mléko je považováno za zlatý standard v kojenecké výživě díky jeho mnohým zjevným přínosům pro lidské zdraví. Kojení však z různých důvodů není vždy možné, proto je obrovskou výzvou poskytnout kojeneckou formuli, která nejlépe napodobuje složení a účinky lidského mléka. Prebiotika přidaná do kojenecké formule mohou výrazně přispět k těmto potenciálním důležitým zlepšením výživy kojenců. Cílem této studie bylo zhodnotit vliv suplementace novým prebiotikem (pod patentovou ochranou) na celkový růst, fekální konzistenci a neonatální imunitní vývoj u potkanů. G14 Lewis kojená mláďata potkanů byla rozdělena do 2 experimentálních skupin (n = 21/skupinu): referenční a prebiotická skupina. Vehikulum nebo prebiotikum bylo denně podáváno perorální žaludeční sondou od 2. do 16. dne života. Zvířata byla denně vážena a jejich velikost a související proměnné byly měřeny v den 8 a 16. Pro detekci a kvantifikaci sérových izotypů imunoglobulinů a vybraných intestinálních cytokinů v střevním obsahu v den 8 a 16 byly provedeny testy založené na technologii Luminex® xMAP®. Intervence prebiotikem prokázala účinek na růst potkanů bez ovlivnění konsistence stolice. Na základě měření hladin sérových imunoglobulinů a střevních cytokinů suplementace významně stimulovala produkci imunoglobulinů (IgG2a, IgG2b, IgG2c a IgA), zvýšila Th1/Th2 Ig jako i IL-10/TNFα poměry a snížila hladiny většiny detekovaných střevních cytokinů. Vzhledem k výsledkům testované prebiotikum prokázalo imunomodulační vliv na vývoj neonatálního imunitního systému u tohoto preklinického modelu potkanů a ukázalo potenciál stát se prospěšnou složkou nové kojenecké formule.

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List of abbreviations

AIN	American Institute of Nutrition
APC	antigen-presenting cell
BCR	B-cell receptor
BMI	body mass index
CD	cluster of differentiation
DP	degree of polymerization
ELISA	enzyme-linked immunosorbent assay
FOS	fructo-oligosaccharide
Foxp3	forkhead box P3
GOS	galacto-oligosaccharide
HIV	human immunodeficiency virus
HMO	human milk oligosaccharide
IFN	interferon
Ig	immunoglobulin
IL	interleukin
MHC	major histocompatibility complex
NK cell	natural killer cell
PBS	phosphate buffered saline
PRE	prebiotic
REF	reference
RT	room temperature
SCFAs	short-chain fatty acids
scFOSs	short-chain fructo-oligosaccharides

SIgA	secretory immunoglobulin A
Tc cell	cytotoxic T cell
TCR	T-cell receptor
Th cell	T helper cell
TNF	tumor necrosis factor
TOS	transgalacto-oligosaccharide
Treg cell	regulatory T cell
WHO	World Health Organization

1 Introduction

Since a prebiotic concept was introduced to the scientific community, prebiotics have gradually gained increasing attention and up to now stimulate research in many areas owing to their various promising health effects including inhibition of gut pathogens, immune stimulation and growth improvement. At present, prebiotics are conceived as substrates selectively utilized by host microorganisms, and therefore conferring benefits upon health (Gibson *et al.*, 2017). There are currently known several types of prebiotics, mostly nondigestible carbohydrates, while numerous candidate prebiotics are still being investigated.

The significance of prebiotics in human nutrition, especially in early life, is supported by the fact that they naturally occur in human breast milk and are called human milk oligosaccharides. Breastfeeding plays unquestionably a superior and unique role in infant nutrition. However, it is not always possible to breastfeed due to multiple reasons. Thus, we face a huge challenge to provide the best suitable alternative to human milk in a form of infant formula. Prebiotics added to infant formula may greatly contribute to these prospective significant improvements in infant nutrition and health.

In this work, which was carried out at the University of Barcelona by the Autoimmunity and Tolerance Research Group, we investigated the effects of a novel prebiotic (subject to patent protection) intended to be used as a dietary supplement in a new infant formula. We focused particularly on the principal potentially beneficial effects of the prebiotic on neonatal development that included assessment of growth and immune maturation. In regard to certain limitations of interventions in human neonates, we chose suckling rats as the appropriate animal model for this preclinical immunonutrition study in early life.

2 Theoretical part

2.1 Prebiotics

2.1.1 Definitions of prebiotics

The first studies of prebiotics date back to the end of the 1970s when Japanese researchers demonstrated *in vitro* that several carbohydrates, especially fructo-oligosaccharides, were selectively fermented by bifidobacteria and had the capacity to stimulate their growth (Yazawa *et al.*, 1978).

The results of these investigations were confirmed by Gibson and Roberfroid who proposed the first prebiotic definition in 1995: *"A prebiotic is a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health."*

However, in 2004 Gibson *et al.* revised the concept and redefined a prebiotic as *"a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host wellbeing and health"*. In comparison to the original definition, this one considers not only microbial changes in the colonic ecosystem but also extrapolates this into other areas that may benefit from a selective targeting of particular microorganisms. In addition to this, three clear criteria for a food ingredient to be classified as a prebiotic were established: the ability to resist host digestion; being fermented by intestinal microorganisms; and selective stimulation of the growth and/or activity of intestinal bacteria associated with health and well-being.

Different international organizations such as the Food and Agricultural Organization of the United Nations also proposed a broader definition of a prebiotic in 2008: *"a nonviable food component that confers a health benefit on the host associated with modulation of the microbiota"* (Pineiro *et al.*, 2008). Since the important criterion regarding selective fermentation was removed, the definition was criticized for not excluding antibiotics (Gibson *et al.*, 2017).

Prebiotics at that time were perceived mostly from the gastrointestinal point of view but further exploration regarding possible modulations of other microbial ecosystems (the oral cavity, skin and the urogenital tract) by a prebiotic approach were expected. Therefore, the need for a narrower category of a "dietary prebiotic" occurred. It was defined in 2010 as *"a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health"* (Gibson *et al.*, 2010).

Eventually, in December 2016, a panel organized by the International Scientific Association for Probiotics and Prebiotics reviewed and updated the definition of a prebiotic to the most recent one: "*a substrate that is selectively utilized by host microorganisms conferring a health benefit"*. This enabled non-carbohydrate substances and various categories other than food to be included in the prebiotic concept while the selective microbiota-mediated requirement for prebiotics was sustained (Gibson *et al.*, 2017).

2.1.2 Types of prebiotics

Based on the established criteria, the substances that are nowadays considered as prebiotics include inulin-type prebiotics (inulin, oligofructose), galacto-oligosaccharides (GOSs), and lactulose (Bellei and Haslberger, 2013; Gibson *et al.*, 2017; Kelly, 2008). However, there exist several other potential prebiotics that have not shown enough scientific support and evidence in human studies yet. These are various different oligosaccharides (isomalto-, xylo-, gluco-oligosaccharides, soybean oligosaccharides, and human milk oligosaccharides), lactosucrose, polydextrose and resistant starch (Gibson *et al.*, 2010; Rastall, 2010; Watson and Preedy, 2015).

2.1.2.1 Inulin-type prebiotics

Inulin-type prebiotics belong to a larger group called "fructans" in which the majority of glycosidic bonds is constituted by one or more fructosyl–fructose linkages (Roberfroid, 2005). Thus, fructans represent a huge heterogeneous group of linear or branched fructose polymers. In addition to that, they can contain one or more fructosyl-glucose linkages (Kelly, 2008).

An oligomer or polymer chain of inulin-type fructans compose mainly of d-fructose units that are joined by a β(2→1) glycosidic linkage. Other chemical components may include disaccharides and d-glucose which is also a terminal unit (Figure 1). There occurs a certain inconsistency in the division of inulin-type prebiotics among many researchers. Most frequently, inulin-type fructans are subdivided according to their degree of polymerization (DP) into a higher-molecular-weight group represented by inulin (DP between 10 and 65), and lower-molecular-weight group known as oligofructose (DP between 3 and 10) (Cho and Finocchiaro, 2010; Gibson *et al.*, 2010; Roberfroid, 2005).

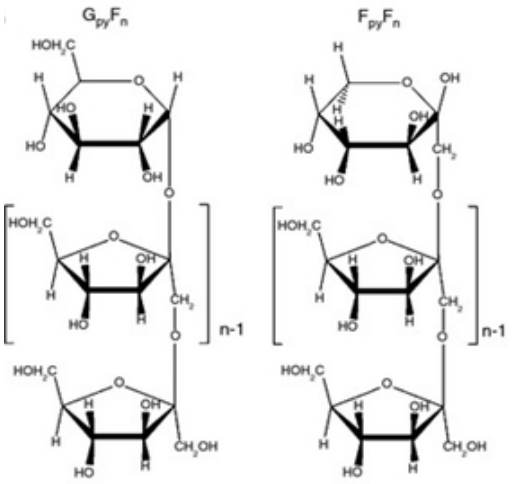

Figure 1: Chemical structures of inulin-type prebiotics (G, glucose; F, fructose). Modified from (De Vuyst *et al.*, 2014).

Inulin is a soluble dietary fiber which is a naturally occurring polysaccharide, and its indigestible nature enables it to pass intact through the upper gastrointestinal tract (Yoo and Kim, 2016). However, similarly to other prebiotics, inulin undergoes bacterial fermentation process in the colon. Since even in minimal doses it stimulates the growth of *Bifidobacterium* species demonstrably more than of other gut organisms, it is considered bifidogenic (Kelly, 2008; Roberfroid, 2007). It can be found in foods like onions, bananas, and garlic while higher concentrations exist in herbs like dandelion root and chicory root (Bellei and Haslberger, 2013). Inulin is stable at any pH above 4 and temperature below 10°C, therefore, the failure to meet these conditions leads to hydrolysis and the eventual formation of fructose (Zhang *et al.*, 2015).

Oligofructose is also referred to as fructo-oligosaccharide (FOS). These linear indigestible oligosaccharides are usually generated by incomplete enzymatic hydrolysis

of inulin. Interestingly, FOS has a sweetening capacity of 30 – 35% compared to table sugar while the calorie content of 1.5 kcal/g is lower than that of sucrose. Besides, it is worth noting that FOS is understandably more soluble than inulin and this leads to their different functional properties (Castro *et al.*, 2009).

The FOS group includes an important subgroup called short-chain fructo-oligosaccharides (scFOSs). It is commercially synthesized from sucrose by enzymatic transfructosylation resulting in the formation of linear chains with only 3 to 5 units, terminated by glucose (Cho and Finocchiaro, 2010; Hidaka *et al.*, 1986). Nevertheless, the short chains appear also at low levels naturally in onion, artichoke, garlic, wheat, and banana (Cho and Finocchiaro, 2010).

2.1.2.2 Galacto-oligosaccharides

Galacto-oligosaccharides (GOSs) can be produced enzymatically through conversion of lactose which is usually purified from bovine milk. These GOSs are also sometimes called transgalacto-oligosaccharides, transgalactosylated oligosaccharides, TOS, or oligogalactosyl-lactose (Cho and Finocchiaro, 2010). Typically, GOSs comprise β-d-galactose units arising from consecutive transglycosylation synthesis (β(1→4) glycosidic bond) with a terminal unit of β-d-glucose (Figure 2). The degree of GOS polymerization can range from 2 to 8 depending on the type of enzyme and the degree of lactose conversion. GOS can be found naturally in green bananas and raw potatoes (Patel and Goyal, 2012).

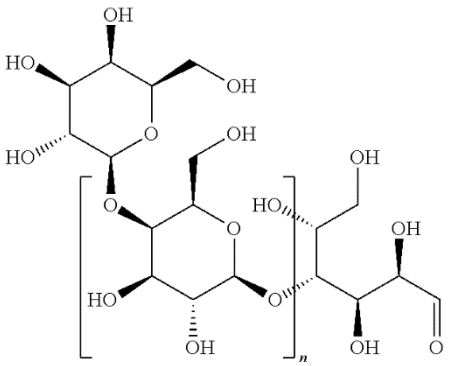

Figure 2: The general structure of a β(1→4) linked galacto-oligosaccharide molecule. Adapted from (Jeong *et al.*, 2011).

2.1.2.3 Lactulose

Lactulose is currently categorized as a candidate prebiotic in some literature sources (Cho and Finocchiaro, 2010; Gibson *et al.*, 2017). As shown in Figure 3, it is a disaccharide obtained through chemical synthesis by combining fructose and galactose (Slavin, 2013). Its mild laxative effect is mainly caused by the osmotic effect of fermentation products (short-chain fatty acids (SCFAs)) which draw fluid into the bowel and consequently stimulate intestinal peristalsis (Alexiou and Frank, 2008). Additionally, due to several related mechanisms including the acidification of the colon, blood ammonia levels decrease (Slavin, 2013).

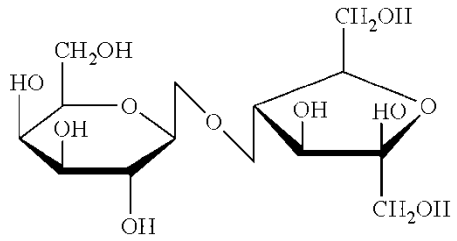

Figure 3: The chemical structure of lactulose. Adapted from (Fallon and Feltenstein, 2014).

2.1.3 Benefits of prebiotics

Since the introduction of the prebiotic concept, many various health benefits have been discovered regarding the dietary prebiotic supplementation and the research still continues. Most of these health end points proven in human trials are presented in Table 1.

Table 1: Proven health end points targeted in multiple human trials of orally administered prebiotics. Modified from (Gibson *et al.*, 2017).

Health end point	Prebiotic used
Metabolic health: overweight and obesity; type 2 diabetes mellitus; metabolic syndrome and dyslipidemia; inflammation	Inulin, GOS, FOS
Satiety	FOS
Stimulation of neurochemical-producing bacteria in the gut	GOS
Improved absorption of calcium and other minerals, bone health	Inulin, FOS
Skin health, improved water retention and reduced erythema	GOS
Allergy	FOS, GOS
Inflammatory bowel disease	Inulin, lactulose
Urogenital health	GOS
Bowel habit and general gut health in infants	GOS, FOS,
Infections and vaccine response	FOS, GOS, polydextrose
Necrotizing enterocolitis in preterm infants	GOS, FOS
Irritable bowel syndrome	GOS
Traveler's diarrhea	GOS
Constipation	Inulin
Immune function in elderly individuals	GOS

FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides.

Nevertheless, in this literature review, we will intentionally focus on the specific effects of prebiotics on neonatal growth and immune development. A neonate normally encounters prebiotics and receives them from the time it is born owing to its breastfeeding mother. This is because the human breast milk is rich in certain bioactive components called human milk oligosaccharides (HMOs) which act similarly to other well-known prebiotics and are crucial for a child's health and well-being (Patel and Goyal, 2012). However, the changing lifestyles of the 21st century have created a need for prebiotic supplements to promote growth and development of the infant.

Prebiotics contribute to a stronger immune system by encouraging the development of a desirable microbiota which can prevent the formation of pathogenic microorganisms through competitive inhibition and production of mucins as well as secretory immunoglobulin A (SIgA) which prevents pathogen colonization (Slavin, 2013). Besides, prebiotics block the binding of pathogens by acting as a lure since they resemble the binding sites for pathogens. The resemblance makes pathogens bound to prebiotics and consequently pathogens are excreted from the body. This effect is what

happens for example to *Salmonella* or *Vibrio cholerae* hence reducing the risk of diarrhea. The activities stimulated by prebiotics lead to maturation of the child's immune system since immune development is highly dependent on the interaction of commensal bacteria and prebiotics (Bellei and Haslberger, 2013).

During the early years of life, infants undergo a lot of growth changes which ensure that they have the physical, emotional and cognitive capability to help them navigate their way through life. It is proven that prebiotics play a significant role in the growth of a child. For instance, prebiotics promote maturation of infant's gut since their fermentation products (short-chain fatty acids (SCFAs)) proliferate and differentiate epithelial cells hence boosting gut barrier function (Mugambi *et al.*, 2012). Additionally, a study by Grenoy *et al.* (2017) to unearth the impact of prebiotic supplements on malnourished children in Uganda concluded that prebiotic supplements increase height and weight gain.

2.2 Human breast milk

2.2.1 Breastfeeding

Neonates are not fully grown and developed including their immune system what makes them vulnerable in many ways. Therefore, they naturally rely on mother's provision of all needed nutrients essential for survival through breastfeeding. Based on available scientific evidence, majority of health organizations, such as the World Health Organization (WHO), highly recommend and establish as public health policy early initiation of breastfeeding within 1 hour of birth and exclusive breastfeeding for the first 6 months of life in both developing and developed countries. Moreover, WHO endorses serving complementary foods in combination with continued breastfeeding up to 2 years of age or beyond ("Infant and young child feeding," 2017; Kramer and Kakuma, 2012).

However, there occur some rare cases when breastfeeding is contraindicated. These include infants suffering from galactosemia or few other metabolic diseases (for instance phenylketonuria), mother's untreated active tuberculosis, or her HIV infection Nevertheless, other risk factors such as environmental contaminants, maternal smoking or abuse of illegal drugs, moderate alcohol consumption, or the use of most prescription and over-the-counter drugs are not considered as an absolute contraindication to

breastfeeding and therefore should not prevent mothers from breastfeeding ("Breastfeeding and the Use of Human Milk," 2012; Wilson and Temple, 2016).

Besides above-mentioned contraindications, there exist also various personal, social, financial and other reasons why breastfeeding is not possible. As a result, the infant formula appears to be the most acceptable alternative to human milk. (Wilson and Temple, 2016). Therefore, it is crucial to provide the appropriate new infant formulae, which not only best mimic the composition of human milk but also minimize current differences in performance between formula-fed and breastfed infants (Hernell, 2011).

2.2.2 Composition of breast milk - bioactive components

Human milk, a complex and dynamic fluid, changes in composition during specific periods of infant life as it is seen in distinct differences between colostrum, transitional milk, and mature milk. Moreover, nutrient content can even vary within the same feed, between mothers and populations (Ballard and Morrow, 2013; Wilson and Temple, 2016). In general, it seems that poor maternal diet does not significantly influence the protein, lipid, lactose, mineral (except for selenium), most water-soluble vitamin, or caloric content of breast milk. On the contrary, almost all fat-soluble vitamins and fatty acids are notably affected by the differences in maternal nutrient intakes (Lonnerdal, 1986; Lonnerdal, 2000).

However, breast milk consists not only from well-known macronutrients (proteins, lipids, and lactose) and micronutrients (vitamins, macroelements, and microelements) but also contains a lot of bioactive components. They play numerous roles in infant‘s body leading primarily to its protection and proper development (Ballard and Morrow, 2013).

So far, a huge variety and number of bioactive components have been discovered in human milk including cells, cytokines and other immunological factors, growth factors, hormones, antimicrobial agents, oligosaccharides, nucleotides, antioxidants, and enzymes (Jensen, 1995). Origin of bioactive components found in human milk is various, since some of them are expressed and secreted by mammary epithelial cells, but some are produced by milk cells (Garofalo, 2010), and others are transported to mammary glands by circulatory system either from the maternal gut (entero-mammary pathway) (Fernández *et al.*, 2013) or directly from maternal blood.

Bioactive components in breast milk play numerous, diverse roles and often act synergistically. They participate in milk synthesis and influence its composition, support bioavailability of different substances, help to protect and develop the infant properly (Ballard and Morrow, 2013). However, only some bioactive components which are primarily responsible for neonate's immune protection, development, and healthy microbial colonization are described below.

2.2.2.1 Leukocytes

The main leukocytes detected in human milk are all present in an activated phenotype and include macrophages, neutrophils, T-cells, and B-cells. Their concentrations decrease notably during the lactation periods (Jensen, 1995; Hosea Blewett *et al.*, 2008). According to Lewis *et al.* (2017), leukocytes together with immunoglobulins, cytokines, and oligosaccharides belong to the group of immune components in breast milk, which show both, protective and developmental benefits on the immune system of the infant. This might be also possible because maternal leukocytes from human milk are probably able to reach circulation as it has already been proven on animal models (Zhou *et al.*, 2000).

2.2.2.2 Cytokines

Cytokines are chemically characterized as peptides, eventually proteins or glycoproteins, and have multiple autocrine/paracrine functions (Garofalo, 2010). These important signaling molecules are bioactive even in minute quantities and interfere with each other as well as with other agents contained in human milk (Jensen, 1995; Hosea Blewett *et al.*, 2008).

Since neonates have limited ability to produce their own cytokines (Field *et al.*, 2001), cytokines received from breast milk play a significant role in either enhancement or reduction of inflammation (Garofalo, 2010). Cytokines detected in human milk include interleukin-1β (IL-1β), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, tumor necrosis factor alfa (TNFα), interferon gamma (IFNγ), granulocyte colony-stimulating factor, and macrophage colony-stimulating factor (Jensen, 1995; Hosea Blewett *et al.*, 2008). In general, levels of pro-inflammatory cytokines such as TNFα or IFNγ in human milk mutually correlate, are lower and decrease over lactation (Ustundag *et al.*, 2005).

2.2.2.3 Immunoglobulins

The immunoglobulins (Ig) are glycoproteins composed of 2 large heavy chains and 2 small identical light chains. Mammalian heavy chains exist in five types (α, δ, ε, γ, and μ) and determine five immunoglobulin isotypes (IgA, IgD, IgE, IgG, and IgM) (Janeway *et al.*, 2001).

The main immunoglobulin transported from the mammary gland to infant thanks to breast milk is secretory IgA (SIgA), which features more than 90 % of all milk-borne immunoglobulin isotypes. Other antibodies detected in breast milk include IgG and IgM (Jakaitis *et al.*, 2014). SIgA acts as a major immune defense factor in infant‘s intestine in multiple ways such as prevention of pathogens from attaching to mucosal surfaces, microbial toxins’ neutralization, and enhancement of virus excretion (van de Perre, 2003).

Similarly to cytokine production, neonates produce generally low quantities of immunoglobulins, and therefore the importance of breast milk in early nutrition increases. (Hanson *et al.*, 2002). Nevertheless, these immunoglobulin concentrations decrease abundantly in human milk during the first month of lactation as a result of the infant‘s gradual immune maturation and thus lower antibody requirement. In addition to that, during the first few days of a neonate’s life, its gut is able to absorb whole proteins including Ig, but later gut permeability to macromolecules decreases (Andreas *et al.*, 2015).

2.3 Immune system in early life

The function of the immune system is to protect the individual against pathogens or other foreign agents. The immune system is also responsible for the tolerance of the organism to its own elements, food and other environmental components. Generally, the immune system is classified into innate and adaptive (acquired) immunity. The first includes skin and mucous barriers, such as the intestinal barrier, the phagocytosis performed by neutrophils, monocytes and macrophages, and the cytotoxicity mediated by natural killer cells (NK), among others. Adaptive immunity corresponds mainly to the activity performed by T helper (Th) and T cytotoxic (Tc) lymphocytes and B lymphocytes resulting in a production of antibodies and cytokines (Beck and Habich, 1996).

The development of the human immune system begins during pregnancy and is not fully functional until the first years of life (Chirico, 2014; Melville and Moss, 2013). Although the immune system progressively develops its defensive capacity against pathogens after birth, this is still a period characterized by a high incidence of infections, especially in preterm infants (Adams-Chapman *et al.*, 2012; Melville and Moss, 2013). This immaturity of the newborn's immune system has a strong impact on adaptive immunity which, thus, has to develop its specificity and memory characteristics (Hannet *et al.*, 1992). Therefore, the innate immune system, even though still immature, will be the main responsible for the neonate's immune response (Marodi, 2006).

2.3.1 Neonatal innate immunity

Unlike the adaptive immune response, neonatal innate immunity does not require a genetic reordering or the prior contact with the antigen.

Neutrophils are the most abundant leukocytes in the blood (70-75% of leukocytes), they represent the main component of the innate immune system and are responsible for capturing and destroying pathogens during infection. It has been reported that newborns have deficits in neutrophils, both quantitative and qualitative, in relation to adults (Melvan *et al.*, 2010).

NK cells are large granular lymphocytes and play a key role in the control of viral infections (Jost *et al.*, 2013). Functionally, they are characterized by their cytolytic activity and by the production of interferon γ (IFNγ) (Guilmot *et al.*, 2011).

Antigen-presenting cells (APC) process and display antigens bond to major histocompatibility complexes (MHCs) on their surfaces what enables T cells to recognize them owing to their T cell receptors (TCRs). Thus, antigen-presenting cells such as macrophages, B cells and dendritic cells are considered as key elements for the induction of the adaptive immune response and for the acquisition of tolerance (Dowling *et al.*, 2008; Steinman, 2012).

2.3.2 Neonatal adaptive immunity

B and T lymphocytes play crucial and complex role in neonatal adaptive immunity, which is antigen-specific and requires the acquisition of immune memory

(Melville and Moss, 2013). Historically, the adaptive immune response of the newborn has been considered immature. This immaturity can be attributed to the fact that during gestation, the exposure of the immune system to antigens is very limited. T and B lymphocyte populations are smaller than in adults and require clonal expansion, which occurs during the first weeks of life (Walker *et al.*, 2011). At the time of birth, most T and B lymphocytes are virgin and, therefore, the population of memory lymphocytes is very limited in relation to the adult (Adkins, 2007). In this sense, newborns have been described by the activation of T lymphocytes and the production of cytokines, by the production of antibodies by B lymphocytes, and also by the interactions between these two lymphocyte populations (Basha *et al.*, 2014; Melville and Moss, 2013).

In relation to T cell lymphocytes, involved in cellular immunity, it has been described that neonates have poor functionality, which is attributed to a very high proportion of circulating virgin T lymphocytes and a few memory T lymphocytes (Walker *et al.*, 2011). T cell cytotoxic lymphocytes (Tc or CD8+) are important in the defense against intracellular infections and are effector cells of antiviral and antitumor immunity (Barry *et al.*, 2002).

Regarding T helper lymphocytes (Th or CD4+), once activated, they can be differentiated fundamentally in the subpopulations Th1 and Th2, which secret different cytokine profiles. The Th1 phenotype, classified as inflammatory, produces IFNγ, interleukin-2 (IL-2) and tumor necrosis factor (TNF). The Th2 phenotype, considered anti-inflammatory, produces cytokines such as IL-4, IL-5, IL-13 and IL-10. It has been reported that neonatal lymphocytes show a clear polarization towards a Th2 response and a reduced production of cytokines of type Th1, which could explain the predisposition of newborns to infections (Marodi, 2006; Melville and Moss, 2013).

Recent studies have reported that Th lymphocytes can be differentiated in the subpopulation of Th17 lymphocytes. These lymphocytes secret proinflammatory cytokines IL-17A, IL-17F, IL-21 and IL-22; and are involved in the protection against infections by extracellular bacteria and fungi at the level of epithelial barriers (Cosmi *et al.*, 2008). Because newborns have a deficit in Th1 lymphocyte-mediated immunity, the development of the Th17 population could compensate for this defect. In any case, it has been demonstrated that IL-4 secretion by Th2 lymphocytes inhibits the development of the Th17 response in newborn mice (Debock and Flamand, 2014).

Part of the subpopulations Th1, Th2 and Th17 lymphocytes involved in the development of the immune response against intracellular pathogens and extracellular parasites can be differentiated into regulatory T (Treg) cells. These lymphocytes, which express CD4, CD25 and Foxp3, are essential for immune tolerance and limit excessive immune responses by Th1, Th2 and Th17 lymphocytes (Basha *et al.*, 2014). In relation to adults, newborns exhibit a high percentage of Treg cells (Burt, 2013).

In summary, the infant's T cell immunity is characterized by a defect in the differentiation of Th1, Th17 lymphocytes and by an increase in the Th2 and Treg lymphocyte populations (Fig. 3).

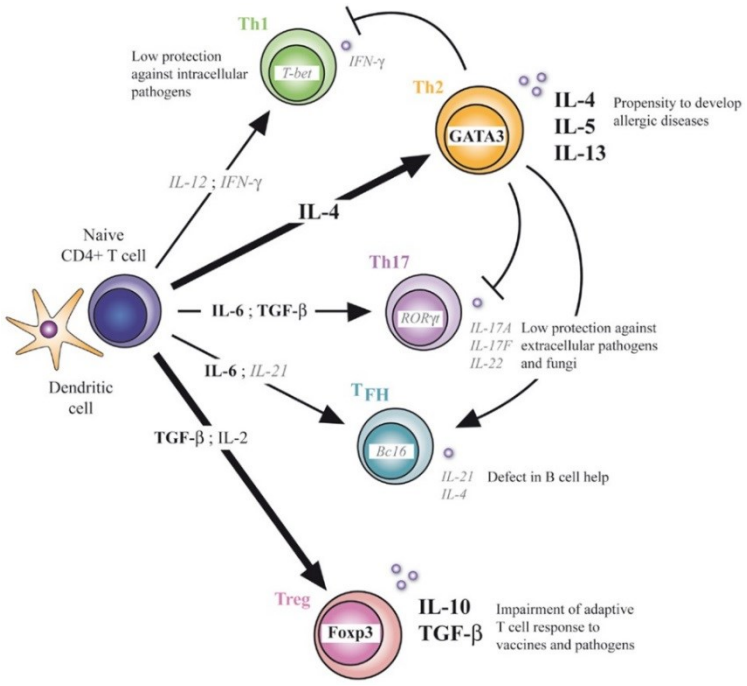

Figure 3: CD4+ T cell differentiation in early life - defect in Th1, Th17 differentiation and enhanced Th2 and Treg differentiation. Transcriptional factors and cytokines are positively (bold) or negatively (italic) regulated. Adapted from (Debock and Flamand., 2014).

Regarding newborn B lymphocytes, and as discussed above for T lymphocytes, they are fundamentally virgin due to a lack of exposure to the antigen. In this first stage of life, antibody production is very limited, which is attributed to both intrinsic and extrinsic factors. Among the intrinsic factors, it is necessary to consider the immaturity of the B cells, their limited differentiation to plasma cells and the reduction in signaling through their antigen-specific receptors (BCR, B-cell receptor) (Basha *et al.*, 2014; Dowling *et al.*, 2014).

As was already mentioned, the plasma cells secrete immunoglobulins which are in human divided into 5 isotypes and each Ig isotype performs its specific function in concrete compartment of the body. IgM is the first antibody produced in a humoral immune response since it does not require isotype switching of B cells for expression. Structurally, IgM molecules are pentamers with 10 antigen-binding sites which enable IgM to bind simultaneously to multivalent antigens such as bacterial capsular polysaccharides despite to relatively low affinity of the IgM monomers. IgM is present in the blood and the lymph where it plays important role in activating the complement system (Janeway *et al.*, 2001).

Other Ig isotypes are usually found as monomers (IgG, IgE, IgD) or dimers (IgA) and therefore diffuse easily into other tissues. Unlike IgM, they have higher affinity of the individual antigen-binding sites for the corresponding antigen (Janeway *et al.*, 2001). IgA is the principal isotype in mucosal areas, such as the intestinal, respiratory or urogenital tract and functions primarily as a neutralizing antibody preventing colonization by pathogens (Underdown and Schiff, 1986). On the other hand, IgG in its four forms is contained in the blood and extracellular fluid where it is responsible for opsonization of pathogens for engulfment by phagocytes and complement activation (Pier *et al.*, 2004). IgD is often present as an antigen on B cells that have not been exposed to antigens and influences basophils and mast cells to produce antimicrobial factors (Geisberger *et al.*, 2006). Lastly, IgE is mostly detected on mast cells that are found beneath the skin and mucosa and along blood vessels in connective tissue and binds to antigens what induces the release of chemical mediators such as histamine from mast cells and basophils. Thus, it is involved in allergy and protection from several parasites (Janeway *et al.*, 2001; Pier *et al.*, 2004).

3 Aim of the study

This work is a part of a large research project conducted by the Autoimmunity and Tolerance Research Group at the University of Barcelona. The group investigates the effects of a novel prebiotic intended to be used as a dietary supplement in a new infant formula on neonatal development in rats. Since the substance is still under research and is subject to patent protection, its nature and properties can not be further specified and described. For this reason, in this diploma thesis, it is generally referred to as a prebiotic.

The aim of this study was to evaluate the effect of this dietary prebiotic supplementation on growth, fecal consistency, and immune development in suckling rats. From the immunological point of view, we focused especially on the analysis of the serum antibody production and the intestinal cytokine production on day 8 and day 16 of life. For this purpose, Luminex® xMAP® technology was used.

4 Experimental part

4.1 Materials

4.1.1 Animals

G14 pregnant Lewis rats were obtained from Janvier (France). The rats were monitored daily and allowed to deliver at term. The day of birth was registered as a day 1 of life. Litters were unified to 7 pups per lactating dam. After the first day of environmental adaptation, animals were individually identified by labeling with a permanent marker.

Animals were housed in 1 cage per dam under controlled temperature and humidity conditions in a 12 h light–12 h dark cycle. Cages were located in a special safe isolated room of the Animal Service of the Faculty of Pharmacy, the University of Barcelona. This safe room had been specifically designed to conduct work under biosafety level 2 conditions; therefore, it was maintained under negative pressure relative to corridors or adjacent non-laboratory areas.

The dams were fed a commercial diet specially formulated for the growth, pregnancy, and lactation phases of rodents corresponding to the American Institute of Nutrition (AIN)-93G (Reeves *et al.*, 1993). Food and water were available *ad libitum.* Pups had a free access to nipples and a rat diet.

Studies were performed in accordance with institutional guidelines for care and use of laboratory animals established by the Ethics Committee for Animal Experimentation of the University of Barcelona and the Catalonian Government (CEEA-UB ref. 165/11, DAAM: 5871).

4.1.2 Devices

Centrifuge - Heraeus Megafuge 2.0 (Thermo Fisher Scientific Inc., USA)
Centrifuge for Eppendorfs - Heraeus Fresco 21 (Thermo Fisher Scientific Inc., USA)
Crushed Ice Maker - AF 20 (Scotsman, UK)
Freezer - Balay 3KF4930N (BSH Home Appliances Group, Spain)

Luminex MAGPIX (Luminex Corporation, USA)
Precision Balance - Kern EW 150-3M (Kern & Sohn GmbH, Germany)
Refrigerator - LKexv 5400 MediLine (Liebherr, Switzerland)
Vibrating Platform Shaker - Heidolph Titramax 100 (Heidolph Instruments, Germany)
Vortex Mixer - Heidolph REAX 2000 (Heidolph Instruments, Germany)
Water Bath - P14 (Thermo Fisher Scientific, USA)

4.1.3 Tools and glassware

The tools and glassware used in this research work included glass graduated beaker 50, 100, 250 ml; plastic graduated beaker 500, 1000 ml; bottle for washing 500 ml; glass graduated bottle 500, 1000 ml; volumetric flask 1 l; stainless steel forceps; dissection scalpel; stainless steel scissors; magnetic stir bar; glass graduated pipette 10, 20 ml; manual 8-channel pipette 20–200 µl; single channel pipette 0.1–2 µl, 2–20 µl, 20–200 µl, 200–2000 µl; glass plate; polystyrene box; non-extensible measuring tape.

4.1.4 Disposables

We used following disposable tools and materials: aluminum foil; Parafilm M® film; nitrile gloves; sterile needle; 23-gauge gavage needle; sterile short-volume syringe 5–100 µl; sterile syringe 1, 2.5, 5 ml; pipette tip 0.5–10 µl, 10–200 µl, 100–1000 µl; Petri dish; graduated transfer pipette 3 ml; Eppendorf® microtubes 1.5ml; conical centrifuge tube 15, 50 ml; PCR8-tube strip; 96-well flat bottom plate; black microplate lid; plate seals.

4.1.5 Reagents

The reagents involved in the research work are ethanol 96%, ethanol 70%, distilled water, phosphate buffered saline (PBS) pH 7.2 (stored at 2–8°C), and ketamine hydrochloride/xylazine hydrochloride solution (stored at 2–8°C).

The reagents supplied by Thermo Fisher Scientific Inc. included in 2 different ProcartaPlex™ multiplex immunoassay kits (stored at 2–8°C):

- Antigen Standards, premixed;
- Detection Antibody, premixed (50x);
- Detection Antibody Diluent;

- Magnetic Beads, premixed (1x);
- Streptavidin-PE (1x);
- Wash Buffer Concentrate (10x);
- Universal Assay Buffer (10x);
- Reading Buffer.

4.1.6 Software

The software used in the study included Luminex MAGPIX system - xPONENT software (Luminex Corporation), Microsoft Office 2013 (Microsoft Corporation), PASW Statistics 22 software package (SPSS Inc.), SigmaPlot 12.0 software package (Systat Software Inc.).

4.2 Methods

4.2.1 Experimental design

The animals were distributed into 2 experimental groups (Figure 5), both consisting of three litters with seven pups (n = 21/group). The first group (reference – REF group) received vehicle and the second one (prebiotic group – PRE group) was treated with a specific prebiotic (under patent). Vehicle (mineral water) or the prebiotic supplementation was administered to the suckling rats by oral gavage daily throughout the suckling period (from day 2 to 16), as previously described (Pérez-Cano *et al.*, 2007).

After terminal intramuscular anesthesia with ketamine/xylazine, the animals were euthanized by transcardial perfusion at two different time points: three animals of each litter were sacrificed on day 8 (n = 9/group), whereas the rest continued receiving the diet until day 16 (n = 12/group).

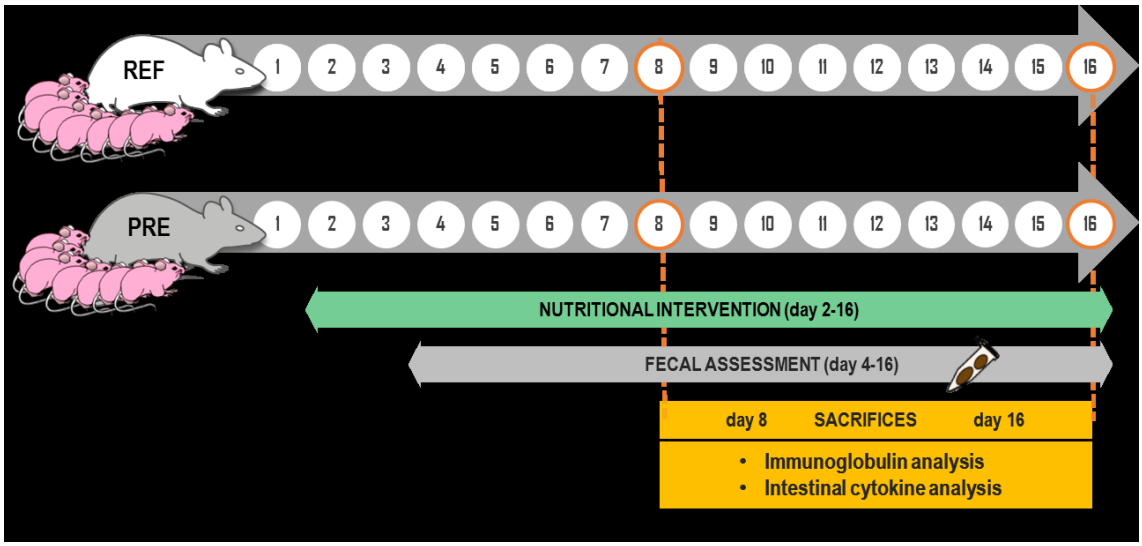

Figure 5: Experimental design followed to assess the effect of prebiotic supplementation on neonatal development in rats.

4.2.2 Body growth and related variables

Rats were weighed and monitored daily to obtain data regarding the influence of nutritional intervention on body weight and growth. In addition to that, size of the animals (body and tail length), weight of some organs (spleen, thymus, liver, and intestines) and length of the large and small intestine were measured on the days when the animals were sacrificed (day 8 and 16).

Body weight, body length (measured as nose-anal length) and tail length were used to calculate the following variables:

- Body weight gain at specific time periods was expressed as a percentage (%) and determined as:

$$\frac{Animal\ weight\ difference\ (final\ -\ initial\ day\ of\ the\ period)\ (g)}{Animal\ weight\ (initial\ day\ of\ the\ period)\ (g)} \times 100$$

- Body/tail ratio was estimated as follows:

$$\frac{Nose - anal\ length\ (cm)}{Tail\ length\ (cm)}$$

- Body mass index (BMI) was calculated according to the formula (Novelli *et al.*, 2007):

$$\frac{Animal\ weight\ (g)}{Nose-anal\ length^2\ (cm^2\ )}$$

- Lee index, an obesity index used in rodents, which corrects body weight for individual differences in body length (Lee, 1929; Simson and Gold, 1982), was determined as:

$$\frac{\sqrt[3]{Animal\ weight\ (g)}}{Nose-anal\ lenght\ (cm)} \times 1000$$

4.2.3 Fecal specimen collection and evaluation

Fecal sampling was performed in order to investigate the prebiotic effect on fecal consistency and weight. It was evaluated from the day 4 until the end of the study once a day. Feces were collected by gently pressing and massaging the abdomen. Specimens were immediately weighed and examined for the purpose of establishment of a fecal score.

The fecal score was assessed by scoring stools from 1 to 4 on the basis of color, texture, and amount as follows:

- normal feces (fecal score = 1),
- loose yellow-green feces (fecal score = 2),
- totally loose yellow-green feces (fecal score = 3),
- the high amount of watery feces (fecal score = 4).

Fecal scores ≥ 2 indicate diarrheic feces.

4.2.4 Blood and intestinal content sample collection

Blood and intestinal content samples were collected on sacrifice days. Blood samples were obtained by cardiac puncture. Serum was separated after a clot formation followed by centrifugation and stored at -20°C until immunoglobulin analysis.

Small and large intestines were excised, measured and weighed. Gut wash was performed by incubating the distal portion of the small intestine in phosphate-buffered

solution (PBS) for 10 minutes at 37°C with shaking. After centrifugation, supernatants were stored at -80°C until cytokines quantification.

4.2.5 Luminex® xMAP®

For the detection and quantification of all rat immunoglobulin isotypes (IgG1, IgG2a, IgG2b, IgG2c, IgM and IgA) in serum samples as well as of several intestinal cytokines (IL-1β, IL-4, IL-6, IL-10, IL-12, IFNγ and TNFα) in the gut wash on day 8 and day 16 of life, immunoassays based on the Luminex® xMAP® (multi-analyte profiling) technology were chosen. In this study, ProcartaPlex™ multiplex immunoassays (Invitrogen brand assays of Thermo Fisher Scientific) were used, specifically the Antibody Isotyping 6-Plex Rat Panel for the immunoglobulin analysis and the Mix&Match Rat 6 Plex for the cytokine analysis.

Principle: The Luminex® xMAP® technology is a relatively new bead-based multiplex technology that enables the simultaneous detection and quantification of a huge variety and number of protein targets in a single sample of serum or other bodily fluids. In comparison to widely performed traditional enzyme-linked immunosorbent assay (ELISA), multiplex immunoassays require significantly fewer sample amounts, are faster, more efficient and offer reproducible results.

The Luminex® assay is based on polystyrene beads with diameter 5.6 µm impregnated with 2 fluorescent dyes in different ratios for easy detection of up to 100 different bead identities. In addition, each bead can be chemically coated with specific capture reagents. An analyte binds to them and creates a complex that can be specifically visualized (Martins *et al.*, 2004). Identification of these bead-antigen/antibody complexes is performed with a Luminex® analyzer which resembles a dual-laser flow cytometer. One laser allows the bead identification according to the different intrinsic fluorophore emission profile. The other laser excites the reporter fluorophore bounded to each bead and generates quantitative information relative to the analytes of the sample (Martins *et al.*, 2004; Waterboer *et al.*, 2006).

Procedure: The analysis was carried out following the instructions provided by the manufacturer. Prior to the assay, we prepared needed reagents which included a dilution of wash buffer (1X), universal assay buffer (1X) and detection antibody (1X). Samples, as well as antigen standards, were reconstituted and diluted. A 4-fold serial

dilution of standards was performed for the cytokine analysis and a 3-fold serial dilution was used in case of the Ig analysis.

The first step represented the addition of magnetic beads solution to each well of the 96-well flat bottom plate (50 µl/well). Then we proceeded to a washing phase. Firstly, the plate was securely inserted into the hand-held magnetic plate washer. After 2 minutes, the plate was emptied without removing it from the magnetic plate washer and blotted onto several layers of paper towels. 150 µl/well of wash buffer was added and it was discarded after 30 seconds, similarly to the previous step. Eventually, the plate was removed from the magnetic plate washer. Consequently, the addition of 50 µl of prepared samples, standards, and blanks to specific wells took place. The plate was sealed and covered with a black microplate lid and shook at 500 rpm for 2 h at room temperature (RT). After the incubation, the plate was washed 3 times using the previously described steps. Later, 25 µl of detection antibody mixture was added to each well and incubated 30 min on a plate shaker at RT at 500 rpm. Then the washing phase took place (3 washes). In case of immunoglobulin detection, an additional step was needed. It consisted of an addition of Streptavidin-PE solution (50 µl) to each well followed by 30-minute incubation with shaking (RT, 500 rpm). The final washing of the plate was performed. Finally, 120 µl/well of reading buffer was added and the sealed and covered plate was incubated for 5 minutes on a plate shaker. The reading was carried out with Luminex® MAGPIX® system at the Scientific and Technological Centers of the University of Barcelona.

4.2.6 Statistical analysis

Statistical analyses were carried out by using PASW Statistics 22 software package. The Kolmogorov–Smirnov test was applied to assess normal distribution, followed by Levene's test to determine the equality of variances. Results were consequently evaluated by Student´s *t*-test (mean cytokine levels) or by the Mann–Whitney *U* test in case non-normal distribution or different variance was found (mean immunoglobulin levels, body weight, etc.). Differences were considered significant at p-values <0.05. Data are presented as means values with their standard errors. Calculations were performed using Microsoft Excel 2013 and graphs were created with the SigmaPlot 12.0 software package.

5 Results

5.1 Body weight

The effect of the prebiotic (PRE) supplementation on growth was evaluated on the basis of monitoring weight of the animals daily throughout the study (Figure 6).

A

Day	REF	PRE
1	6.23 ± 0.10	5.74 ± 0.17*
2	6.95 ± 0.12	6.48 ± 0.16
3	7.92 ± 0.12	7.31 ± 0.20
4	9.09 ± 0.12	8.25 ± 0.19*
5	10.3 ± 0.1	9.64 ± 0.26
6	11.5 ± 0.1	10.8 ± 0.3*
7	12.2 ± 0.2	12.2 ± 0.3
8	13.3 ± 0.5	13.9 ± 0.3
9	16.3 ± 0.4	15.8 ± 0.5
10	18.5 ± 0.4	18.3 ± 0.4
11	21.0 ± 0.5	21.1 ± 0.6
12	23.2 ± 0.6	23.8 ± 0.7
13	25.5 ± 0.6	26.5 ± 0.7
14	27.6 ± 0.8	29.2 ± 0.7
15	29.6 ± 1.0	31.4 ± 0.6
16	31.4 ± 0.9	33.9 ± 0.5*

B

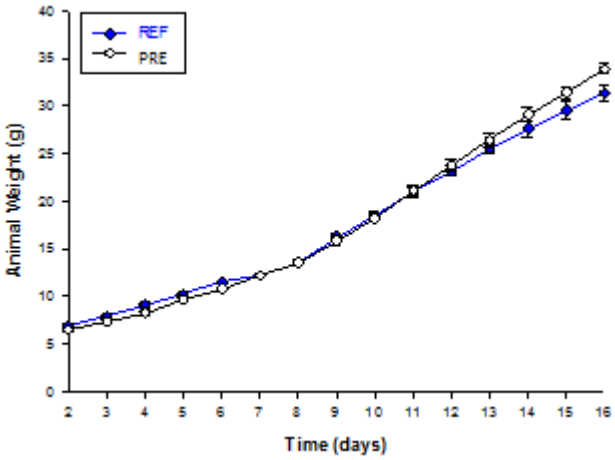

Figure 6: Data of the body weight (g) of the rats during the study for REF and PRE experimental groups (A) and graphical representation (B). Results are expressed as mean values (*n* = 12–21 animals per group), with their standard errors. Statistical significance is shown only in part A: *p<0.05 *vs* REF (by Mann–Whitney *U* test).

We observed a statistically significant lower body weight of the PRE group on certain days with respect to the reference (REF) group (day 1, day 4, and day 6) but this occurred because the PRE litters were born with lower body weight. Surprisingly, on the last day of the study (day 16), a significantly higher body weight of the PRE group compared to the REF one was noticed.

Body weight was also analyzed as a weight gain in three periods along suckling (Table 2). We observed a growth promoting effect of the PRE in the two last periods studied.

Table 2: Body weight gain (%) in specific periods along the study for REF and PRE experimental groups. Results are expressed as mean ± SEM (*n* = 12–21 animals per group). Statistical significance: * p<0.05 *vs* REF (by Mann–Whitney *U* test).

Day interval	REF	PRE
2–5	47.98 ± 1.38	48.99 ± 2.26
5–9	59.08 ± 2.90	71.75 ± 2.49*
9–16	94.61 ± 2.84	115.4 ± 5.0*

5.2 Animal size and related variables

Evaluation of body weight, body length (nose-anal length) and tail length allowed calculating the variables such as the body/tail ratio, BMI and Lee index presented in Table 3.

Table 3: Animal size and related variables on day 8 and day 16 of life for REF and PRE experimental groups. Results are expressed as mean ± SEM (*n* = 9–12 animals/group). Statistical significance: *p<0.05 *vs* REF (by Mann–Whitney *U* test).

	REF (d8)	PRE (d8)	REF (d16)	PRE (d16)
Body/tail ratio	2.14 ± 0.07	2.28 ± 0.04*	1.77 ± 0.02	1.86 ± 0.02*
BMI	0.301 ± 0.010	0.288 ± 0.012	0.351 ± 0.010	0.372 ± 0.001
Lee Index	355 ± 3	347 ± 4	334 ± 3	336 ± 2
Tail (cm)	3.14 ± 0.09	3.04 ± 0.03*	5.34 ± 0.06	5.18 ± 0.05
Body (cm)	6.67 ± 0.11	6.93 ± 0.12	9.43 ± 0.09	9.64 ± 0.08
Tail+Body (cm)	9.81 ± 0.15	9.97 ± 0.11	14.8 ± 0.1	14.8 ± 0.1
Weight (g)	13.3 ± 0.5	13.9 ± 0.3	31.4 ± 0.9	33.9 ± 0.5*

No significant differences except for the already mentioned body weight on day 16 and the body/tail ratio on both days were observed. The PRE group showed in both cases higher body/tail ratio in comparison to the REF group.

5.3 Organ weight and size

Weight of some organs (spleen, thymus, liver, and intestines) and length of the large and small intestine were measured on both days of sample obtaining (Table 4).

Table 4: The relative weight of organs expressed as a percentage (%) with respect to the body weight on day 8 and day 16 of life for REF and PRE experimental groups. Length of the large and small intestine was also measured and expressed as cm/g with respect to body weight. Results are expressed as mean ± SEM (*n* = 9–12 animals/group). Statistical significance: * p<0.05 *vs* REF (by Mann–Whitney *U* test).

	REF (d8)	PRE (d8)	REF (d16)	PRE (d16)
Spleen/body weight ratio (%)	0.57 ± 0.03	0.63 ± 0.02	0.47 ± 0.02	0.52 ± 0.03
Thymus/body weight ratio (%)	0.34 ± 0.01	0.29 ± 0.02	0.41 ± 0.02	0.42 ± 0.03
Liver/body weight ratio (%)	3.0 ± 0.1	3.0 ± 0.1	3.7 ± 0.1	3.6 ± 0.1
Large intestine/body weight ratio (%)	0.60 ± 0.01	0.63 ± 0.05	1.1 ± 0.0	0.99 ± 0.01
Small intestine/body weight ratio (%)	3.1 ± 0.1	3.2 ± 0.1	2.9 ± 0.1	3.1 ± 0.1
Large intestine length/body weight ratio (cm/g)	0.36 ± 0.01	0.33 ± 0.01	0.21 ± 0.01	0.19 ± 0.01
Small intestine length/body weight ratio (cm/g)	1.9 ± 0.1	1.9 ± 0.1	1.1 ± 0.1	0.96 ± 0.05

No clear differences between REF and PRE group were observed in any of the organ weight and/or length obtained at any of the days studied.

5.4 Fecal consistency

The fecal consistency represented by the fecal score was evaluated for the REF and PRE groups according to the scoring system described in Methods. Results are shown in Figure 7.

A

Day	REF	PRE
4	1.08 ± 0.04	1.18 ± 0.10
5	1.02 ± 0.02	1.18 ± 0.08*
6	1.00 ± 0.00	1.08 ± 0.05*
7	1.02 ± 0.02	1.00 ± 0.00
8	1.00 ± 0.00	1.00 ± 0.00
9	1.00 ± 0.00	1.11 ± 0.07*
10	1.02 ± 0.02	1.06 ± 0.06
11	1.00 ± 0.00	1.04 ± 0.04
12	1.00 ± 0.00	1.00 ± 0.00
13	1.00 ± 0.00	1.00 ± 0.00
14	1.00 ± 0.00	1.00 ± 0.00
15	1.00 ± 0.00	1.00 ± 0.00
16	1.00 ± 0.00	1.00 ± 0.00

B

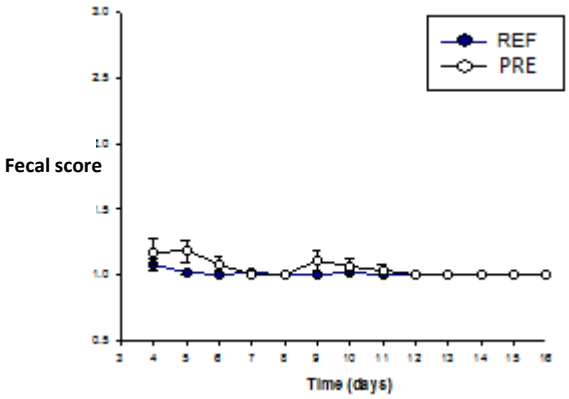

Figure 7: Data of the fecal consistency score throughout the study for REF and PRE experimental groups (A) and its graphical representation (B). Results are expressed as mean values of obtained samples from 12–21 animals/group (*n* = 4-26 samples/group), with their standard errors. Statistical significance is shown only in part A: *p<0.05 *vs* REF (by Mann–Whitney *U* test).

It can be observed that the fecal score in the PRE group is highly similar to the REF group since the fecal score is practically 1 (normal feces) throughout the study. PRE supplementation did not show any notable impact on the stool consistency.

5.5 Fecal weight

Fecal weight was analyzed at 3 intervals along the studied period (Table 5).

Table 5: Mean fecal weight (mg) in three periods of suckling for REF and PRE experimental groups. Results are expressed as mean values of obtained samples from 12–21 animals/group (*n* = 11–62 samples/group/period), with their standard errors. Statistical significance: *p<0.05 *vs* REF (by Mann–Whitney *U* test).

Day interval	REF	PRE
4–5	3.23 ± 0.30	4.65 ± 0.44
6–9	5.62 ± 0.30	6.17 ± 0.41
10–16	6.07 ± 0.43	7.20 ± 0.76

A pattern of increasing fecal weight was observed for both groups throughout the study without significant differences between them suggesting that the PRE intervention did not affect fecal weight.

5.6 Serum immunoglobulin production

The levels and distribution of the different classes (IgA, IgM, and IgG) and subclasses (IgG1, IgG2a, IgG2b and IgG2c) of rat antibodies were quantified using a ProcartaPlex™ multiplex immunoassay (Table 6 and Table 7).

Table 6: Mean serum levels (µg/ml) of IgG class and subclasses, their distribution into Th1- and Th2-associated Ig and Th1/Th2 Ig ratio on day 8 and day 16 for REF and PRE experimental groups. Results are expressed as mean ± SEM (*n* = 8 animals/group/day). Statistical significance: *p<0.05 *vs* REF at the same age (by Mann–Whitney *U* test).

	REF (d8)	PRE (d8)	REF (d16)	PRE (d16)
IgG1	112.5 ± 3.0	105.4 ± 7.1	273.6 ± 6.7	296.8 ± 33.2
IgG2a	476.7 ± 24.1	484.0 ± 12.7	722.8 ± 17.4	**884.2 ± 25.9***
IgG2b	558.2 ± 51.1	**834.3 ± 37.1***	1056 ± 27	**2452 ± 166***
IgG2c	975.9 ± 78.5	1031 ± 28	1641 ± 170	**2099 ± 79***
Total IgG	2123 ± 78	**2454 ± 76***	3693 ± 180	**5732 ± 284***
Th1 Ig (IgG2b+IgG2c)	1534 ± 76	**1865 ± 62***	2697 ± 176	**4551 ± 243***
Th2 Ig (IgG1+IgG2a)	589.2 ± 25.4	589.3 ± 15.9	996.4 ± 20.2	1181 ± 56
Th1/Th2 Ig	2.603 ± 0.183	**3.164 ± 0.047***	2.706 ± 0.176	**3.853 ± 0.165***

First, we focused on the total IgG (determined indirectly as a sum of all IgG isotype levels) and its subclass levels which also allowed us to calculate their distribution into typical Th1-associated Ig (IgG2b+IgG2c in rat) and typical Th2-associated Ig (IgG1+IgG2a in rat) as well as their ratio.

The levels of any of the IgG types or the total IgG analyzed on day 16 were significantly increased in comparison to their values on day 8 in both experimental

groups. This age-associated increase was similar in both groups: values on day 16 were 1 to 2 times greater than those on day 8.

The animals supplemented with PRE during 7 days had significantly higher values of IgG2b with respect to the REF animals, while the other subclasses were not affected. Due to this increase, the total IgG, Th1-associated Ig levels as well as the Th1/Th2 Ig ratio were significantly increased, too.

However, on day 16 of life PRE supplementation showed a positive influence on the quantities of more subclasses of IgG than on day 8. Specifically, the PRE group pups had significantly higher values of IgG2a, IgG2b, and IgG2c compared to the REF group ones. These changes caused again a significant increase in the overall value of IgG, the Th1-associated Ig levels and consequently the Th1/Th2 Ig ratio.

Regarding IgM (Table 7), no changes owing to the diet were found either on day 8 or day 16 even though the age-associated effect was evident. Serum IgA levels were increased after the PRE intervention on both days, although results were only significant on day 16 ($p<0.05$) (Table 7).

Table 7: Mean serum IgM and IgA class levels (µg/ml) on day 8 and day 16 for REF and PRE experimental groups. Results are expressed as mean ± SEM (*n* = 8 animals/group/day). Statistical significance: *p<0.05 *vs* REF at the same age (by Mann–Whitney *U* test). Superscript "a" means a p value of 0.09.

	REF (d8)	PRE (d8)	REF (d16)	PRE (d16)
IgM	5.65 ± 0.19	5.74 ± 0.31	19.5 ± 0.8	18.8 ± 1.1
IgA	38.9 ± 3.1	**45.4 ± 1.9[a]**	63.3 ± 1.5	**90.2 ± 3.4***

When analyzing the data more carefully, we observed a marked "mother effect" in both experimental groups, since pups from the same mother had very similar quantities of Ig classes and subclasses, whereas pups from different mothers showed notably different values (data not shown).

5.7 Intestinal cytokine production

The presence of cytokines in the gut wash on day 8 and day 16 was evaluated by a ProcartaPlex™ multiplex immunoassay (Table 8).

Table 8: Mean cytokine levels (pg/ml of PBS/g of intestinal tissue) and IL-10/TNFα ratio on day 8 and day 16 for REF and PRE experimental groups. Results are expressed as mean ± SEM (*n* = 6 animals/group/day). Statistical significance: $^{\delta}p<0.05$ *vs* day 8 in the same group, *p<0.05 *vs* REF at the same age (by Student's *t*-test).

	REF (d8)	PRE (d8)	REF (d16)	PRE (d16)
IL-1β	232.5 ± 37.3	280.8 ± 57.8	624.0 ± 116.9$^{\delta}$	314.1 ± 23.2*
IL-4	28.31 ± 5.67	31.71 ± 7.95	88.68 ± 17.69$^{\delta}$	43.00 ± 2.96*
IL-6	375.6 ± 81.0	439.4 ± 114.8	1439 ± 293$^{\delta}$	692.0 ± 67.2$^{\delta*}$
IL-10	916.0 ± 125.2	950.3 ± 165.2	1901 ± 288 $^{\delta}$	1125 ± 56
IL-12	1453 ± 184	1466 ± 250	2774 ± 521$^{\delta}$	1529 ± 92 *
IFNγ	579.0 ± 118.3	657.1 ± 156.2	1972 ± 386 $^{\delta}$	978.5 ± 77.9$^{\delta*}$
TNFα	115.0 ± 27.5	153.9 ± 36.2	418.6 ± 81.1$^{\delta}$	211.3 ± 15.8*
IL-10/TNFα	6.639 ± 0.391	6.532 ± 0.404	4.748 ± 0.283$^{\delta}$	5.397 ± 0.223*

When we compared the levels of the cytokines on day 8 with those on day 16, we could observe that there was an increase of all of them in both experimental groups. This age-associated increase seemed to be higher in the REF group (values on day 16 were 2 to 4 times greater than those on day 8) rather than in the PRE group (not reaching 1 time greater values). This implies that these age-associated changes in levels of all analyzed cytokines in the REF group were statistically significant whereas only 2 types of cytokines (IL-6 and IFNγ) were significantly increased in the PRE group.

The rats supplemented with PRE during 7 days did not show any differences in any of the studied cytokines with respect to those from the REF group. On the contrary, on day 16 of life, the PRE supplementation significantly reduced the levels of cytokines compared to those in the REF animals in almost all cases (with exception of the IL-10).

However, the IL-10/TNFα ratio was significantly higher in the PRE group, suggesting a better anti-inflammatory/pro-inflammatory profile owing to the diet.

As commented before for the Ig analysis, a marked “mother effect” was detected again. This means that pups from the same mother had very similar cytokine levels while pups from different mothers showed notably different values (data not shown).

6 Discussion

Nowadays, human breast milk is considered a gold standard in infant nutrition due to its multiple proven benefits on human health. However, breastfeeding is not always possible for various reasons; thus, it is a huge challenge to formulate infant formulae, which best mimic the composition and effects of human milk. As a result, dietary oligosaccharides, some of them also naturally occurring in human milk, are usually added to infant formulae as prebiotic components. In regard to certain limitations of controlled interventional studies in human neonates, in this preclinical study, we chose the suckling rats as the suitable animal model (Pérez-Cano *et al.*, 2012) to evaluate the effects of the novel prebiotic on growth, fecal consistency, and immune maturation.

The effect of the prebiotic (PRE) supplementation on neonatal growth in rats was assessed on the basis of daily weight monitoring of the animals and analysis of related variables on day 8 and 16. Due to lower mean body weight of PRE litters at birth, it was only on the last day of the study (day 16), that we noticed a significantly higher body weight of the PRE group compared to the reference (REF) group. Therefore, we decided to analyze the body weight gain in three periods along suckling which led to clear observation of a weight gain promoting effect of the PRE in the two last periods studied. In addition to that, the PRE group showed higher body/tail ratio on both analyzed days (day 8 and 16) in comparison to the REF group without any influence on BMI or Lee index. These favorable results regarding growth promotion induced by this PRE intervention only partially correlate with those of studies conducted in infants, since most of them conclude that the supplementation with formulae enriched with prebiotics had no influence on growth (length and weight gain) compared to breastfed group (Holscher *et al.*, 2012; Veereman-Wauters *et al.*, 2011). Moreover, other prebiotics tested on rotavirus infection suckling rat model also did not show any notable impact on growth of the animals after intervention (Rigo-Adrover *et al.*, 2016; Rigo-Adrover *et al.*, 2017).

Based on the evaluation of organ weights (spleen, thymus, liver, and intestines) and length of the large and small intestine, we have not recognized any clear differences between REF and PRE group at any of the days studied. Together with similar organ appearance between groups, these outcomes imply no side effects or signs of abnormalities in tested organs induced by the PRE supplementation.

Regarding the fecal consistency, the established fecal score in the PRE group was very similar to the REF group throughout the study. It might be useful to mention, that although the fecal score is widely used for this type of studies, it may cause certain bias due to subjective evaluation. Therefore, the fecal weight could be a more objective indicator of the stool characteristics concerning water content in feces. Anyway, we did not observe significant differences between groups in fecal weight either. Thus, this denotes that the PRE supplementation did not affect the stool consistency including its weight. However, in previous studies, the research group observed a direct effect of some different prebiotics (e.g., short-chain galacto-oligosaccharides/long-chain fructo-oligosaccharides) on the fecal consistency (Rigo-Adrover *et al.*, 2016; Rigo-Adrover *et al.*, 2017). This is also supported by several clinical trials in which infants fed formula containing a prebiotic tended to have softer stools closer to the breast-fed infant pattern (Vandenplas, 2010; Veereman-Wauters *et al.*, 2011; Westerbeek *et al.*, 2011). On the other hand, Holscher *et al.* (2012) found out in their randomized controlled trial that the prebiotic supplementation in infant formula did not alter stool patterns.

In order to evaluate the potential immunomodulatory effect of the PRE particularly on the systemic compartment, levels and distribution of the different classes (IgA, IgM, and IgG) and subclasses (IgG1, IgG2a, IgG2b, and IgG2c) of rat antibodies were analyzed. IgGs were divided into typical rat Th1-associated Ig (IgG2b+IgG2c) and Th2-associated Ig (IgG1+IgG2a).

When comparing these analyses from day 8 and 16 of life, we noticed the anticipated age-associated increase of each IgG subclass as well as IgA and IgM concentrations in both groups as it had been also previously reported in rats of the same strain (Pérez-Cano *et al.*, 2007). We can suppose that certain amounts of these detected immunoglobulins were transferred to fetal rats during prenatal period and some were obtained from maternal milk, but the observed increase of serum IgG levels is most probably caused by proper antibody production in neonate‘s lymph nodes or bone marrow, which still needs to be investigated (Pérez-Cano *et al.*, 2007).

IgG represented doubtlessly the predominant immunoglobulin in the serum of both groups with levels in unsupplemented group (REF) similar to those measured by Pérez-Cano *et al.* (2007), whereas IgM and IgA concentrations and their trends differ widely from that previous study. The initial IgM levels were found equivalent in both

studies but the antecedent showed a more distinct age-associated increase. Although IgA is understood as the last Ig isotype to appear in rat serum, we quantified higher amounts of IgA in comparison to IgM in both groups already on day 8. In addition to that, in the referred study, serum IgA levels remained almost unchanged during the first 2 weeks of life, while we noticed an obvious progressive increase of these levels. What is even more unforeseen, serum IgA concentrations in unsupplemented animals on day 8 were approximately 500 times higher than those formerly recorded in rats of the corresponding age (Pérez-Cano *et al.*, 2007).

In our experiment, only after 7 days of PRE supplementation, IgG2b levels rose significantly with respect to the REF group resulting in remarkable increase of the total IgG and Th1-associated Ig levels as well as of the Th1/Th2 Ig ratio. Other subclasses were not affected. However, on day 16 of life, supplementation showed a positive influence on the quantities of more IgG isotypes than on day 8. Specifically, the PRE group pups had significantly higher values of IgG2a, IgG2b, and IgG2c compared to the REF group ones. These changes caused again a notable increase in the overall value of IgG, the Th1-associated Ig levels and consequently the Th1/Th2 Ig ratio. Regarding IgM concentrations, no diet-associated changes were found either on day 8 or day 16. Nevertheless, the administration of the PRE only during 16 days of life was enough to exhibit a markedly enhancing effect on serum IgA production.

Despite the fact that blood immunoglobulin concentrations can be useful indicators of immune development, there is currently a lack of studies evaluating the effect of prebiotic supplementation on these Ig levels in non-infected or otherwise unaffected neonatal rat model. In this sense, any comparison of results is difficult. Anyway, for instance, a study in newborn female calves indicated no significant differences in plasma IgG1 concentration after prebiotic intervention agreeing with our findings in rats (Roodposhti and Dabiri, 2012).

When analyzing the data more carefully, we observed a “mother effect” in both experimental groups, since pups from the same mother had very similar quantities of Ig classes and subclasses, whereas pups from different mothers showed notably different values. It can not be denied that this “mother effect” could somehow influence the final results and therefore it would be desirable to further analyze this phenomenon in order to minimize the impact of this potential variation on overall validity.

As was already mentioned, we studied also rat Th1- and Th2-associated Ig distribution. This classification is based on the ascertainments that synthesis of rat IgG1 and IgG2a is controlled by Th2 stimulated immune response whereas rat IgG2b and IgG2c are known to be dependent on Th1 cells and related cytokines (Cetre *et al.*, 1999; Gracie and Bradley, 1996; Saoudi *et al.*, 1999). Rats, similarly to human neonates, usually develop Th cell responses skewed toward Th2 and thus produce more antibodies corresponding to Th2 and unlike an adult rats fail to develop Th1-associated antibodies (Pérez-Cano *et al.*, 2012). However, this relative deficiency in Th1 population and immune response can be partially corrected by the use of various substances including prebiotics which have demonstrated to enhance systemic Th1-dependent immune responses for instance in a murine infection or vaccination model (Schijf *et al.*, 2012; Vos *et al.*, 2007). In this study, we observed the predominance of Th1-associated Ig even in REF group but this Th1 response was much more intensified after PRE supplementation on both analyzed days by the significantly increased Th1/Th2 Ig ratio in the absence of specific stimulation.

To sum up, these results show the immunoenhancing effect of this PRE supplementation on the production of systemic antibodies, namely IgG2a, IgG2b, IgG2c and IgA, and lead to the increased Th1/Th2 Ig ratio, what could ultimately reflect the activation of a Th1 immune response.

Besides the evaluation of the effect of the PRE on the systemic compartment, we also monitored its influence on an intestinal level by cytokine detection and quantification in the gut wash on day 8 and day 16. When we compared the levels of the cytokines on day 8 with those on day 16, we could observe an increase of all of them in both experimental groups. This age-associated increase seemed to be much more accentuated in the REF group. This implies that these age-associated changes in levels of all analyzed cytokines in the REF group were statistically significant whereas only 2 types of cytokines (IL-6 and IFNγ) were significantly increased in the PRE group.

The animals supplemented with PRE during 7 days did not show any differences in any of the studied cytokines with respect to those from the REF group. On the contrary, on day 16 of life, the PRE intervention significantly reduced the levels of almost every cytokine (with exception of the IL-10). However, it is necessary to point out, that the

IL-10/TNFα ratio was significantly higher in the PRE group, suggesting a better anti-inflammatory/pro-inflammatory profile owing to the diet.

As it was explained in the theoretical part, human breast milk, as well as rat's milk, naturally contains multiple cytokines which reach the neonate's gut thanks to breastfeeding. Thus, we can assume that the range and amount of cytokines detected in gut wash correspond principally to the cytokines secreted by mother and received in breast milk. This presumption is also supported by the observation of a marked "mother effect" on found intestinal cytokine levels.

Nevertheless, these measurements of cytokine levels in gut wash represent only one part of the experiments conducted by the research group and therefore analyses of intestinal lymphocyte phenotype, intestinal gene expression or antibody secretion in the gut are needed in order to objectively assess the impact of PRE supplementation on intestinal immune development in suckling rats.

7 Conclusion

In this study, we evaluated the effect of dietary prebiotic supplementation on growth, fecal consistency, and immune development in suckling rats. The prebiotic intervention caused growth promoting effect and did not influence the fecal consistency. Based on analyses of serum immunoglobulin and intestinal cytokine levels, the prebiotic supplementation significantly stimulated immunoglobulin (IgG2a, IgG2b, IgG2c, and IgA) production, increased the Th1/Th2 Ig as well as the IL-10/TNFα ratios and reduced the levels of most detected intestinal cytokines.

Thus, this novel prebiotic demonstrated the immunomodulatory impact on neonatal immune development in this preclinical rat model and showed a potential to become a beneficial component of a new infant formula. Further studies are needed and are currently carried out in order to better understand the overall effect of this prebiotic supplementation on neonatal development including the improvement of intestinal microbiota composition or epithelial barrier function.

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