Effect of supplementation with a synbiotic in pregnant and lactating rats

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Master's thesis

Interuniversity Master of Nutrition and Metabolism

(URV, UB and UIB)

2021-2022

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ABSTRACT

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The immune system is influenced by the diet. Pregnancy and lactation are periods of lifetime where nutritional requirements are increased, and the immune system undergoes different changes throughout both periods. It has been demonstrated that supplementation with prebiotics and probiotics has a positive impact on the immune system and is useful in different situations such as the prevention or amelioration of intestinal disorders. However, few studies address the impact of the supplementation during pregnancy and lactation. On this basis, we can hypothesize that the supplementation with a synbiotic during gestation and lactation has an impact on the immune system of the mothers and the offspring and is able to prevent the rotavirus (RV)-induced diarrhoea in early life. The aim of this study was to assess the effect of the supplementation with a synbiotic during gestation and lactation on the health of the dams, and to approach the impact of the supplementation with a synbiotic on the humoral immune response of dams in several compartments, including dams' milk.

For this, Lewis rats were administered with a synbiotic (SYM group) or the vehicle (REF group) during gestation and lactation. The effect of this supplementation was evaluated by different morphometric, hematologic and immune variables on the dams. Supplementation did not alter the body weight evolution during pregnancy and lactation, although it did increase the relative weights of the small intestine and the cecum. With regard to immune variables, an increase in IgG2c and in total IgG as well as a reduction in IgG1, IgG2b was observed in plasma, whey and salivary gland. Overall, an increase in Th1-associated IgG subclasses was observed without altering the Th1/Th2 ratio. Finally, supplementation did not change the proportion of mesenteric lymph nodes (MLN) lymphocyte populations.

In conclusion, this study shows that the maternal supplementation with a synbiotic during gestation and lactation is safe for dams and promotes changes at the immune level, thus being able to have a positive impact on the maturation of the immune system of their offspring.

Key words: immune system, supplementation, synbiotic, gestation, breast-feeding

1. Introduction

1.1 Immune system

The immune system consists of a wide variety of cells and molecules involved in protecting the body from invasion by pathogens and cancer. Its function involves two types of responses, innate immunity and acquired immunity, depending on the time and duration of the response, its specificity and its effector cell types (1,2).

Innate immune response is a nonspecific quick response (1-3) that includes different types of physical, chemical and microbial barriers and a lot of elements that provide an immediate defence against pathogens. Among elements involved in this response are neutrophils, monocytes, macrophages, complement, cytokines, and acute phase proteins (2).

Acquired immune response is a specific delayed response. This consists of a response to a specific antigen by T and B lymphocytes that can take from several days to weeks to develop. Nevertheless, thanks to the memory capacity of this response, future exposures will be vigorous and faster (1-3). Within the adaptive response there are two types of responses, humoral and cellular.

Humoral immunity is mediated by extracellular fluids' macromolecules such as secreted antibodies, complement proteins, and certain antimicrobial peptides. Its main mechanism is the production of antibodies also known as immunoglobulins (Ig), which are secreted by plasma cells. These are able to recognize specific antigens. The functions of each immunoglobulin are summarized in Table 1. IgA and IgM are present in high proportions in secretions such as saliva and breast milk, and IgG are able to cross the placenta. Therefore they will contribute to the defence system of the foetus.

Table 1. Functions of immunoglobulins

Immunoglobulins	Function
IgA	Antimicrobial defence in mucosal areas of gut, respiratory tract, urogenital tract, saliva, tears and breast milk
IgD	Antigen receptor on naïve B cells
IgE	Allergic reactions. Binds to allergens and triggers histamine release from mast cells and basophils Antiparasitic action
IgG	Principal antibody of secondary responses involved in opsonization and the neutralization of toxins Passive immunity to the foetus via placenta
IgM	Eliminates pathogens in the early stages of humoral immunity

Cellular immunity is involved in antigen-specific cytotoxic T cell activation as well as in the release of cytokines in response to an antigen attack (1).

Finally, the immune system associated with the mucous membranes, especially those associated with the gastrointestinal tract, constitutes a place of high immune activity, since from the point of view of the cells that compose it, it is the most complex and numerous.

In this mucosa, a modulation of the immune response is established with the main objective of inducing both local and systemic tolerance against innocuous antigens and vigilance against pathogenic microorganisms (4).

1.1.1 Interaction between the immune system and nutrition

There is a complex interaction between nutrition and the immune system. The function of the immune system can be influenced by a lot of factors such as the overall nutrition status, state of nourishment and pattern of food intake. In addition, the immune system can also impact the metabolism and its needs and it can also influence the physiological response to food.

Some of the factors that are most benefited by the dietary components is the microbiota, since elements such as fibres can serve as food for them. Furthermore, the fermentation of fibre by bacteria, can lead to the production of short-chain fatty acids that help maintain intestinal homeostasis by enhancing the epithelial barrier function, inhibiting pathogen-induced cytotoxicity and preventing colonization with pathogenic bacteria (5).

1.1.2 Immune system during pregnancy

Pregnancy is associated with alterations at the hormonal, immunological and microbial levels. Nevertheless, the processes by which these changes occur are not exactly known (6). Pregnant women are considered a special population group due to its increased susceptibility to pathogens and infective diseases caused by the unique “immunological” condition. This increased susceptibility is potentially harmful not only to the mother but also to the foetus, so it is important to determine when there is a risk of foetal transmission, as for example, viral infections are able to reach the foetus by crossing the placenta (7).

During pregnancy, several immune changes are seen that may extend beyond the placenta. Among these, there are a reduced immunological reactivity, microbial alterations which may cause both pathogenic and protective effects, hormonal, immunological and microbial alterations increasing the susceptibility to infection and inflammatory disease. Another factor that can contribute to this alteration of the susceptibility is the placenta, which is considered an additional immunological organ that can affect the mother’s global response to immunological infections, as well as its tropism for specific viruses and pathogens. Therefore, monitoring of infections and inflammatory disorders is necessary during pregnancy and postpartum. Indeed a careful balance between immunity against infectious agents and foetal antigens’ immunological tolerance needs to be maintained (6,7).

There is recent evidence suggesting that microbial alteration during pregnancy may help to maintain the homeostasis and the physiological changes required in this vital stage. On the contrary, it will produce an increase in vulnerability, especially to immunological and infectious diseases during pregnancy and postpartum. This immunological and microbial pattern changes fluctuate during pregnancy (6).

Findings referring to the immune system and microbiota include an increase in interleukin (IL)-6 levels as well as a reduction in intestinal microbial diversity during the third trimester of gestation. It is difficult to determine when the alterations produced during pregnancy are due to alterations in the microbiota or in the immune system. This is due to the fact that hormonal changes and the microbiota itself may produce fluctuations in the microbiota and the immune system, but also the interaction between the microbiota and the immune system can produce changes to each other (6).

Regarding the inflammatory response, an improvement has been observed in some cases, such as a reduction in the levels of proinflammatory cytokines (6).

During pregnancy, the human decidua contains a large number of immune system cells, such as natural killer (NK), regulatory T (Treg) cells and macrophages, as well as T lymphocytes. During the first trimester, these cells will infiltrate the decidua and confer protection against invading trophoblastic cells.

Finally, although at this stage there are changes at the immunological level that induce greater susceptibility to certain infections, it is appropriate to say that the immune status of pregnant women is modulated rather than suppressed (7). This is because, during pregnancy, the maternal immune system is characterized by a reinforced network of recognition, communication, trafficking and repair, that are able to rise alarm if it is necessary in order to protect the mother and the foetus' well-being by protection mechanisms including transplacental transfer of antibodies or anti-infective resistance factors in the amniotic fluid (7,8). At the same time the foetus provides a developing active immune system which modifies mother's response to the environment, providing this uniqueness immune system during pregnancy. These differential responses will vary during the different stages of pregnancy (7).

1.1.3 Immune system during breastfeeding

Neonatal period is also characterized by being a critical period as newborns are exposed to a high number of microorganisms. It is due to its low exposure to antigens before birth. The immunological immaturity inherent to this period is compensated by protection mechanisms provided by the mother throughout colostrum and milk. Through these two fluids, newborns receive large amounts of bioactive components and antibodies during this stage of immunological immaturity, especially for the mucous membranes' immune system (8).

Nutrition has a great impact on neonatal growth and early-life physiology, not only because it is a crucial period of development and adaptation but also for its long-lasting impact capacity. The relationship between nutrition and gut microbiota, mucosal homeostasis and immune programing has been reviewed (9).

1.1.3.1 Milk – Transference

There is plenty of evidence documenting the benefits of breast milk for infants including a reduction in morbidity and mortality, protection against specific infections and the stimulation of the innate and adaptive immune development (10).

Milk's composition and immunity change over time. Breastfeeding provides secretory IgA antibodies and other bioactive factors that confer protection against infections to infants, in addition to the nutrients necessary for optimal growth and development (8,10). These factors confer defence without causing inflammation, and some of these components are even anti-inflammatory.

In early stages of lactation, especially when the colostrum is secreted, IgA, anti-inflammatory factors and active cells provide support for the immature immune system of the newborn. That is the reason why it is said that colostrum is the most potent natural immune booster known. After this period, breast milk continuous to adapt to infant's needs referring immune protection and nutrition. Breastfeeding is recommended until 6 months of life for many reasons, such as the insignificant and delayed infant's secretory IgA (sIgA) production, incomplete physical and chemical barriers, reduced complement cascade function, poor innate effector cell function, and insufficient anti-inflammatory mechanisms of the respiratory and gastrointestinal tracts (8,10).

To sum up, there is a reduction in the quantity of immune factors of milk in parallel with the development of the infant's immune system (10).

Human milk composition is not only influenced by the infant development but also by maternal diet. Its composition changes depending on the time of the day, the feeding, over lactation as well as between mothers and populations as it is influenced by genetics and environmentally factors, by infant sex, infective status and maternal lifestyle including, as previously said, the maternal dietary habits (11).

1.1.3.1.1 Components of breast milk

In terms of composition, colostrum is also higher in protein, vitamin A, vitamin B_{12} and vitamin K levels than transition and mature milk (11). Transition milk has a decreasing number of proteins and Igs and an increasing amount of lactose, fat and soluble vitamins. This results in higher caloric density milk corresponding with the increased demands of the infant. Mature milk, remains constant after 6 weeks through the rest of the lactation period. Lysozyme amount in milk increases while Igs and lactoferrin decreases over the first 3-4 months.

If we focus on the different components of breast milk, there are plenty of multifunctional components such as enzymes, antimicrobial proteins/peptides (AMPs), growth factors, chemokines, antioxidants, anti-inflammatory elements, prebiotics, probiotics, and nutrients for the growing infant among others. These components interact with each other and also with elements of the infant gut.

Milk probiotics play an important role in the infant's gut microbiome. They can contribute to the proliferation of the intestinal microbiota, establishing a symbiosis between the microbiota and the infant. Gut microbiota is enhanced by essential nutrients present in milk such as human milk oligosaccharides (HMO). There is evidence that these probiotics could have an important effect on milk composition because of their positive impact on the secretion of sIgA and IL-6 in colostrum and on IL-10 and transforming growth factor β1 (TGF-B1) in mature milk (10).

HMO are a type of glycan and an important nutrient in human milk because of their function in pathogen binding and as a prebiotic facilitating the establishment of healthy infant microbiome and inhibiting the ability of the pathogen to bind to its host preventing them from infecting and causing symptoms such as diarrhoea (10,12,13). It also has an important role in promoting intestinal development and stimulating immune maturation (12).

Human milk glycoproteins such as lactoferrin, which is found in high amounts in colostrum and transitional milk, has also multiple functions in host defence through binding to bacterial membranes and iron resulting in a bacteriostatic effect, inhibition of tumour necrosis factor-alpha (TNF-α) and IL-1β or stimulating the activity and maturation of lymphocytes among others. Its peptides breakdown products also have antibacterial and antifungical functions. Another type of glycoprotein is lactadherin, which can inactivate viruses and limit inflammation by increasing phagocytosis of apoptotic cells. In addition, it is believed that its sialic acid component can interact with rotavirus (10,13).

Secretory IgA consists of 90% of Ig in milk. It can enter the alimentary canal of the infant and bind to enteric pathogens preventing diseases (13).

Fatty acids and monoglycerides present in milk have been found to have antiviral, antibacterial, and antiprotozoal properties, increasing the ability of the stomach to act as a barrier against ingested pathogens (13).

Human milk stem cells, which have a direct role in regeneration and repair, have also recently been discovered. Although the mechanisms by which they perform this effect are still being investigated (10).

1.2 Diet microbial modulators

As stated above, the mother's diet influences the establishment of the intestinal microbiota of both her and the infant, thus modulating the immune system of the pair. One way of enhancing this is through supplementation with microbial modulators such as prebiotics, probiotics or synbiotics.

1.2.1 Probiotics

According to the “International Scientific Association for Probiotics and Prebiotics”, probiotics are live microorganisms that provide health benefits to the host when ingested in adequate amounts (14). The delivery of beneficial bacteria to the intestine provides protection against pathogenic bacteria as well as some other benefits which are due to multiple mechanisms (14,15). Among these mechanisms, probiotics compete for mucosal adhesion, a fact that will prevent an overgrowth of pathogenic bacteria and viruses, in addition to allowing beneficial bacteria to adhere to the surface. Furthermore, the short-chain fatty acids produced by these will act by reducing the pH, thus creating a hostile environment for pathogenic organisms. Moreover, due to its immunomodulator character, it can act in different ways, including reducing intestinal inflammation (16).

There is scientific evidence in both animal and human models about the effects of these on numerous diseases such as diarrhoea (15,16). There are different interventional studies that support the effect of probiotics in the prevention and treatment of diseases such as

rotavirus or *Escherichia coli*-induced diarrhoea, since it has been observed that the administration of probiotics together with a standard rehydration therapy can help reduce the duration and the severity of diarrhoea (16,17). Furthermore, there is scientific evidence about the preventive effect of probiotics on community-acquired diarrhoea in infants.

Some of the most important bacteria associated with a higher incidence of intestinal infections are rotavirus, *Shigella, Salmonella, E. coli*, and *C. difficile*. Within these, rotavirus infection is known to be the leading cause of severe acute diarrhoea in children worldwide (17).

The recent and growing evidence provided by the study of the relationship between the gastrointestinal tract and commensal bacteria suggests that probiotics may be useful for the therapy of different diseases including diarrhoea. Although, the role of them in the prevention of diarrhoea needs to be defined (17).

1.2.2 Prebiotics

According to the "International Scientific Association for Probiotics and Prebiotics", a prebiotic is "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (18).

It has been demonstrated that the consumption of probiotics can improve some immune functions through different mechanisms of action. There is evidence that prebiotics can contribute to increase the protective microorganisms and therefore reduce the harmful bacteria population. Prebiotics can also induce the expression of immune molecules, especially cytokines. In addition, they serve as food for the microbiota, and their products, that is, short-chain fatty acids, produce the same positive effects mentioned in the previous point.

Fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) are two important groups of prebiotics with beneficial effects on human health. FOS have been associated with numerous implications at the immune level, for example by regulating the levels of some interleukins (IL-6), CD282+/TLR2+ myeloid dendritic cells, and a toll-like receptor 2-mediated immune response, as well as the prevention of some pathologies and intestinal manifestations such as diarrhoea (19). GOS have also been shown to have implications for the immune system, such as modulating some interleukins (IL-8 and IL-10), and protein C-reactive (PCR) which is involved in inflammation and NK cell function (18). Finally, there is evidence that the administration of FOS to a pregnant mouse model modified offspring microbiota (19).

1.2.3 Synbiotics

Synbiotics are composed by the combination of one or more prebiotics with one or more probiotics. This mixture causes an important synergy that multiplies the actions that both have separately causing beneficial effects to the host. Moreover, the combination of these will allow the survival of the probiotic through the gastrointestinal tract, and its implantation, allowing its action on the large intestine (20,21).

Synbiotics have multiple functions, among which, the most characterized is an increase in resistance against pathogens. This function is carried out through different mechanisms of

action shared with prebiotics and probiotics, such as the inhibition of pathogenic bacteria's growth by the production of short-chain fatty acids that create a hostile environment for them, through defence substances such as bacteriocins or by a competitive exclusion.

The study of synbiotics has been widely carried out and has shown an option to be used in combination with antibiotics or alone, in cases of pathologies or gastrointestinal manifestations, since there is evidence about a positive association between these and an improvement in gastrointestinal absorption and in the immune function.

In addition, these synbiotics are associated with many other health benefits such as an increase in the number of bifidobacteria, a reduction of blood cholesterol, a better glycemic control, balancing the intestinal microbiota which results in reducing constipation and/or diarrhoea, an improvement in intestinal permeability and a stimulation of the immune system (20).

2. Hypothesis and objectives

On the basis of the previous introduction, the researcher group hypothesized that the maternal supplementation with a synbiotic during gestation and lactation can enhance the immune system of the offspring and this can be evidenced in a better response to a virus infection in early life. To demonstrate such hypothesis, a project aimed to assess the influence of maternal supplementation with a synbiotic during gestation and lactation in the development of the offspring's immune system and in particular in the prevention of an infection with a rotavirus (RV) is developing.

Inside this project, the aims of the current study were:

- To assess the effect of the supplementation with a synbiotic during gestation and lactation on the health of the dams.
- To approach the impact of the supplementation with a synbiotic on the humoral immune response of dams in several compartments, including dams' milk.

3. Materials and methods

3.1 Animals and experimental design

Seven-week-old Lewis rats (16 female and 8 male) were obtained from Janvier Labs (La Plaine Saint Denis Cedex, France). After one week of acclimatization, each female rat was introduced into a male rat cage for one week. Animals were randomly distributed into two groups: Reference (REF, n=8) or Synbiotic (SYM, n=8). The females of the SYM group were supplemented with a synbiotic, daily, since mating with male in order to ensure that at the moment of fertilization the mothers were receiving the supplementation. The REF group received physiological saline solution.

After one week with male, female rats were individually caged and continued being supplemented during the entire gestation period (21 days) and the entire lactation period (21 days).

From the 16 female rats, 11 rats became pregnant and gave birth to 11 litters. After the birth (day D1), litters were unified to 9 pups per mother. Pups had free access to the nipples and rat diet during the entire study. On the 5th day of life all pups were orally

inoculated with rotavirus (RV) inducing an intestinal infection with diarrhoea during the following days. The RV strain used was simian SA-11 at a dose of $2x10^8$ TCID50/rat in 100 µL of PBS. It was obtained from the 'Enteric Virus Group of the University of Barcelona.

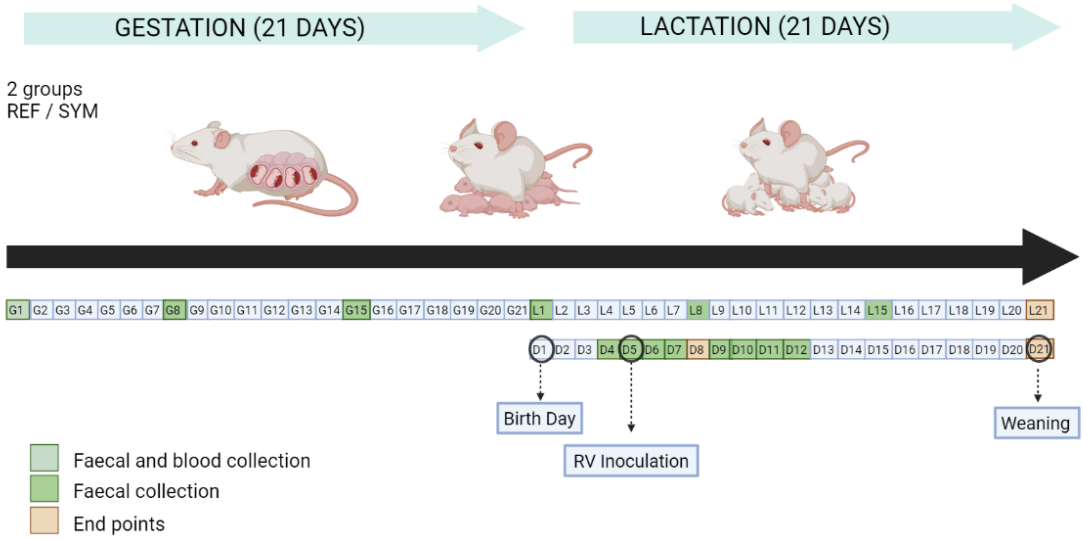

Figure 1. Experimental design. REF (reference); SYM (synbiotic); G (gestation period); L (lactation period); RV (rotavirus)

Throughout the study, different sampling days were established for dams and litters (not considered in the current study). After lactation, at the weaning day, milk, blood and tissues samples were collected from dams after intramuscular injection of ketamine (90 mg/kg) (Merial Laboratories S.A.) and xylazine (10 mg/kg) (Bayer A.G.).

During gestation and lactation, animals received water and feed ad libitum. Consumption of these was daily recorded. It is important to mention that at the beginning of the study, the consumption of these was shared with the male rat. The results were normalized by dividing the total consumption by two. Body weight was also daily recorded.

Animals were placed in a negative pressure chamber with controlled temperature and humidity at the Animal Facility of the Faculty of Pharmacy and Food Science, from the University of Barcelona. Light and dark cycles (12h/12h) were established. The project was previously approved by the Ethics Committee for Animal Experimentation (CEEA) of the University of Barcelona (Ref 240/19).

3.2 Synbiotic supplementation

Daily administration of the synbiotic or vehicle was performed. The synbiotic solution was extemporaneously prepared by the mixture of a prebiotic and a probiotic dissolved in physiological saline solution. The composition of the synbiotic cannot be deciphered due to confidential reasons of the delivering company.

One mL of synbiotic or saline solution was intragastrically administered through an oral catheter during pregnancy. In lactation the volume was increased to 1.5 mL. During the administration in the lactation period, mothers and their offspring were separated into different cages. The mothers were returned to the cages with their pups after each administration.

3.3 Data recorded and sample collection and processing

3.3.1 Animal morphometry

3.3.1.1 Body mass index and Lee index

Mothers were daily weighed during pregnancy and lactation. At the end of the study, their length was also determined in two different ways, from nose to tail and from nose to anus. These measurements were used to determine the Body Mass Index (BMI) and Lee index which allow analysing obesity (22).

$$\text{BMI} = \frac{weight\ (g)}{length^2\ (\text{cm}^2)}$$

$$\text{Lee Index} = \frac{\sqrt[3]{weight\ (g)}}{body - tail\ length\ (\text{cm})} * 1000$$

3.3.1.2 Organ weight

Different tissues were collected and weighed on the day of sacrifice. Among these, there were stomach, small intestine, large intestine, mammary gland, salivary glands, spleen, thymus, mesenteric lymph nodes (MLN), liver, right kidney and heart.

The relative organ weight was calculated following the formula:

$$Organs'\ \text{relative weight} = \frac{organ's\ weight\ (g)}{animal's\ weight\ (g)} * 100$$

3.3.2 Haematological variables

Counts of leukocytes, lymphocytes, monocytes, granulocytes, erythrocytes and platelets, as well as haemoglobin (HGB) concentration, haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) were obtained from blood samples obtained by cardiac puncture and collected in EDTA tubes. The blood tubes were kept in rotation (Roller Mix, Ovan, model RM20E) until the blood was analysed with an automated haematology analyser adapted to rat blood (Spincell, MonLab Laboratories).

3.3.3 Isolation of Mesenteric Lymph Node and spleen lymphocytes

MLN and spleen cells were isolated previous to flow cytometric analysis. First of all, organs were placed in a 15 mL sterile tube with 4 mL of RPMI (Roswell Park Memorial Institute) medium, previously weighed. A 40 µm mesh cell strainer was placed on a Petri dish on an ice surface. Subsequently, the tissues were introduced inside the strainer and were dissociated with the help of a plunger of a syringe. Resulting cell suspension was collected in a sterile 15 mL tube. The suspension was then centrifuged at 538 *g* for 10 min at 4 °C and resuspended in RPMI. In the case of spleen cells, another step was required in order to eliminate erythrocytes by using a hypotonic solution. Conditions were immediately restored to avoid lymphocytes death.

The viability and concentration of the resulting cells was analysed by Countess™ Automated Cell Counter (Invitrogen™, Thermo Fisher Scientific).

3.3.4 Flow cytometric analysis

Phenotypic characterization of spleen and MLN cells was performed by flow cytometric analysis. Monoclonal antibodies conjugated to different fluorochromes were used. These antibodies were added in three mix cocktails (*Table 2*). Each antibody was diluted 1/10 or 1/50 depending on the original concentration.

Table 2. Mix composition and conjugated fluorochromes

Mix 1	Mix 2	Mix 3	Fluorochromes
anti-TCRαβ (R73)	anti-TCRαβ (R73)	anti-TCRαβ (R73)	FITC
anti-NK (10/78)	anti-NK (10/78)	anti-CD62L (OX-85)	PE
anti-CD8α (OX-8)	anti-CD8α (OX-8)	anti-CD8α (OX-8)	PerCP
anti-CD4α (OX-35)	-	anti-CD4α (OX-35)	APC
anti-CD45RA (OX-33)	anti-TCRγδ (V65)	anti-CD45RA (OX-33)	BV421

FITC (Fluorescein isothiocyanate); PE (phycoerythrin); PerCP (Peridinin-Chlorophyll-Protein); APC (Allophycocyanin); BV421 (Brilliant™ Violet 421)

Before adding antibodies, 1 mL of PBS-FBS (2%) - NaN_3 (1%) was added to 500.000 cells. The solution was then centrifuged at 538 *g* for 5 min at 4 °C. The supernatant was removed and the corresponding mix cocktail was added. The tubes were vortexed and subsequently incubated for 20 min at 4 °C in the dark. After this, 1 mL of PBS was added and then centrifuged at 538 *g* for 5 min at 4 °C. Supernatants were discarded and the cells were resuspended with 300 µL of p-formaldehyde (0.5%) and kept at 4 °C in the dark until analysis.

Samples were analysed in the Scientific and Technological Centre of the University of Barcelona (CCiTUB) with a GalliosTM Cytometer (Beckman Coulter, Miami, FL, United States). Data was analysed by Flowjo v10 software (Tree Star, Inc., Ashland).

3.3.5 Small intestine and gut wash

Immediately after collecting, small intestine was weighed. After that, jejunum was opened lengthwise, cut into small pieces (about 1.5 cm) and sank in tubes containing 4 mL of PBS. Tubes were weighed before and after containing jejunum pieces and placed in a tub shaker (Thermo Fisher Scientific) with mild shaking at 37 °C for 10 min. Subsequently, tubes were centrifuged (Megafuge 1.0R, Heraeus) at 535 g and 4 °C for 10 min. Finally, supernatant was collected and frozen at -20 °C until further analysis.

3.3.6 Homogenization of the salivary gland, mesenteric lymph nodes and mammary gland

Homogenization of the salivary gland, MLN and mammary gland for antibody determination by ELISA technique was carried out. First of all, between 20 and 80 mg of tissue were weighed and subsequently deposited in a 10 mL tube. The exact weight of each tissue was recorded and PBS was added to the tissue in order to obtain the final concentration of 20 mg/mL.

Using a polytron previously cleaned with ethanol and PBS, the tissues were homogenized at power 7 for 10 s. This process was repeated twice per sample. Between samples, the tips of the polytron were washed twice using PBS.

Finally, samples were centrifuged (Megafuge 2.0) for 5 min at 538 *g* at 4 °C and the supernatant was collected and frozen at -20 °C until further analysis.

3.3.7 Extraction and homogenization of milk whey

For milk extraction, the pups were separated and the mothers were anesthetized using ketamine (100 µL/100g) intramuscularly in one limb. Thirty minutes after separation, 2 IU of oxotocin were intraperitoneally administered to allow milk to accumulate in the mammary glands. After 5-10 min, the nipples were pressed and the milk secretion was collected with a sterile tip of a pipette and collected in a sterile eppendorf and kept on ice.

The samples were then centrifuged (Thermo Heraeus Fresco 21) at 800 *g* for 10 min at 4 °C. Then, three different layers were observed: an upper layer formed by lipids, an intermediate layer called whey or aqueous phase, which is where Ig are found, and finally a lower layer or pellet formed by cells. With the help of a 2.5 mL syringe with a 23G needle, the whey was extracted and distributed in different aliquots that were kept at -20 °C or -80 °C until further analysis.

3.3.8 ELISA technique for IgA and IgM quantification

Secretory (s)IgA quantification of the gut wash, MLN, salivary gland, whey and mammary gland, and IgM quantification of gut wash were carried out through an Enzyme-Linked ImmunoSorbent Assay (ELISA) in 96 well plates NUNC Maxisorb (Labclinics).

Plate sensitization was performed with 100 µL of capture antibody (goat anti-rat IgA or IgM, Bethyl Laboratories) in carbonate buffer pH 8.6, with an overnight incubation in a humid chamber at 4 °C. After incubation, washing was performed with 200 µL of Tris-buffered solution-0.05% Tween (TBS-0.05% Tween). Blocking buffer (200 µL) consisting of TBS+BSA 1% (Bovine Serum Albumin) was added and subsequently incubated for 1 h at room temperature (RT) in a humid chamber. After this period, another wash was carried out.

Samples were diluted in TBS containing 0.05% Tween 20 and 1% BSA. One hundred µL of standard or sample were added in the assigned wells and incubated for 1 h at RT. After this, the plate was washed as previously described.

Consecutively, 100 µL of detection antibody (peroxidase-conjugated goat anti-rat IgA or biotin-conjugated goat anti-rat IgM, Bethyl Laboratories) were added. After 1 h of incubation at RT, another wash was performed.

ELISA technique for IgM quantification requires one more step before carrying out the enzymatic revelation. Extravidin–peroxidase (Sigma) in TBS-Tw-BSA was subsequently added (100 µL/well). After 30 min at RT in a humid chamber, the plate was washed again.

Finally, 200 µL of *o*-phenylenediamine dihydrochloride (OPD) (0.4 mg/mL) (Sigma) containing 0.04% hydrogen peroxide (H_2O_2) (30%) was added to each well. After 20-30

minutes, the reaction was stopped with 3 M sulphuric acid (H_2SO_4). Absorbance was read with Multiskan Ascent v2.6 software (Thermo Fisher Scientific SLU) at 495 nm.

3.3.9 ELISA technique for specific anti-rotavirus antibodies

Specific anti-rotavirus antibodies were determined in whey and plasma using an ELISA technique.

ELISA plates were sensitized with a rotavirus stock dilution (SA11 10^7). Thereafter blocking process was carried out following the same protocol as IgA and IgM determinations. After this process, a wash was performed with 200 µL of PBS-0.05% Tween.

The samples were then diluted in PBS containing 0.05% Tween 20 and 1% BSA. One hundred µL of the diluted samples or standard were added in the corresponding wells and incubated for 3 h at RT in a humid chamber. Next, another wash was performed.

After that, 100 µL of detection antibody (goat anti-rat IgG, IgA and IgM) were added and incubated for 2 h at RT in a humid chamber. Before carrying out the enzyme development, another wash was performed.

Substrate (200 µL of OPD containing 0.04% of H_2O_2) in phosphate-citrate buffer (pH 5) was finally added to each well. After 10-20 minutes under stirring, the reaction was stopped with 3 M H_2SO_4. Multiskan Ascent v2.6 software (Thermo Fisher Scientific SLU) at 492 nm was used in order to read the absorbance.

3.3.10 BEADS technique for Ig quantification

IgA, IgG1, IgG2a, IgG2b, IgG2c and IgM quantification in samples from plasma, MLN, salivary gland and whey was carried out through a ProcartaPlexTM Multiplex immunoassay (Thermo Fisher) in a 96-well flat bottom plate.

Dilution of the samples and beads was carried out following the manufacturer's instructions. After that, 25 µL of detection antibody mixture was added into each well and then incubated for 30 min in the dark. Then a wash was performed with 150 µL of wash buffer. Finally, 120 µL of reading buffer was added into each well and placed on a shaker in the dark at RT at 600 rpm.

MAGPIX® analyzer (Luminex Corporation, Austin, TX, USA) at the Cytometry Service of the Scientific and Technological Centers of the University of Barcelona (CCiT-UB) was used to determine the specific concentration of each type of Ig.

3.4 Statistical analysis

The IBM SPSS statistics program was used for the statistical analysis of the data previously processed through the Excel program. Shapiro-Wilk and Levene were used to determine the distribution and homogeneity of the obtained data. One way ANOVA statistical test was used for the results with a normal and homogeneous distribution. Instead, non-equal or non-normally distributed data were analysed with the Mann-Whitney U statistical test to analyse statistically significant differences between groups. Statistically significant differences were considered those with a $p<0.05$ and $p>0.05$ and $p<0.1$ was considered a statistical tendency.

4. Results

4.1 Feed and water consumption of dams

Feed (*Figure 2*) and water (*Figure 3*) consumption by dams was recorded during gestation and lactation. Feed consumption was quite regular during gestation but, on the day of delivery, an expected marked reduction was observed. During lactation, feed intake increased almost linearly. SYM group showed a significant reduction of food intake during gestation on days G12, G13, G16 and G17, in addition to a tendency towards reduction on days G19 and G20. In contrast, no significant differences were observed during lactation.

Water consumption during gestation and lactation followed a very similar distribution as feed intake. In this case, no significant differences in water consumption were observed except a slight increase ($p = 0.08$) on the 4^{th} day of gestation in the SYM group compared to the REF group.

4.2 Dams' body weight time course

The effect of the synbiotic on the dams' body weight was established during gestation and lactation (*Figure 4*). A lineal daily growth during gestation was observed in SYM and REF groups. On the day of labour, a significant reduction in body weight was observed in both groups. During lactation, body weight remained almost constant. No significant differences due to SYM intake neither in pregnancy nor in lactation were observed.

4.3 Dams' morphometric parameters

Different morphometric variables were measured to dams at the end of the lactation period (*Table 3*). SYM supplementation did not influence these morphometric parameters.

4.4 Organ relative weight

Figure 5 summarizes the relative weight of some organs at the end of the nutritional intervention. As it can be observed, the SYM group showed a higher small intestine and full cecum relative weights than the REF group (*Figure 5B*). However, no significant differences were observed between both groups for the empty stomach, empty cecum, spleen, liver, thymus, kidney, heart and salivary gland.

4.5 Haematological variables

With regard to haematological variables (*Table 4*), the supplement with SYM did not modify the counts of leucocytes, lymphocytes, granulocytes, erythrocytes, and platelet either HGB, HCT, MCV, or MCH. However, a tendency towards a lower monocytes count was observed in the SYM group compared to the REF group.

4.6 Immunoglobulin quantification

The concentration of IgG isotypes, IgA and IgM in plasma, mesenteric lymph nodes, salivary gland and whey, was analysed. Analysis of the concentration of sIgA from gut wash and mammary gland as well as sIgM of gut wash was carried out too.

Table 5 summarizes the plasma Ig concentrations of dams receiving the synbiotic or vehicle. As it can be observed, the group treated with SYM showed a tendency to increase plasma IgG concentration that was mainly due to an increase in IgG2c. The addition of

IgG2b and IgG2c concentrations was performed in order to determine antibodies associated with the Th1 response. IgG1 and IgG2a concentrations were also joined to assess antibodies associated with the Th2 response. Both Th1- and Th2-related antibodies showed an increase in SYM group compared to the REF group without an increase in Th1/Th2 ratio. When the results were expressed as relative values, an increase in IgG2c was observed, although IgG1 and IgG2b values were reduced in the SYM group in relation to the REF group. A small reduction in the SYM group compared to the REF group was also observed in IgA.

A similar pattern was observed in whey (*Table 6*). An increment in absolute values of IgG2c and consequently in total IgG was observed in the group that received the SYM. Th1 response was also higher in the SYM group compared to the REF group, however when Th2 and the ratio Th1/Th2 was analysed no statistical differences were found. When results were expressed as relative values, an increase in IgG2c and total IgG was observed in the SYM group, although IgG2b and IgA were reduced. A tendency to decrease IgG1 amount was also observed in the SYM group compared to the REF group.

With regard to the salivary gland (*Table 7*), no significant differences between the SYM and the REF group were observed when the results were analysed in absolute values. However, an increase in IgA and IgG2c contributing to an increased concentration in total IgG, as well as a reduction in IgG1 and IgG2b were observed in the SYM group compared to the REF group, when results were expressed as relative values.

Table 8 summarizes the mesenteric lymph nodes Ig concentrations of the SYM and REF groups. IgM was increased in mesenteric lymph nodes in the group treated with the SYM. A tendency to increase IgG2b and IgA concentrations was also observed in the SYM group compared to the REF group.
Finally, no significant differences between the two groups were found for sIgA from the mammary gland and sIgA and sIgM from gut wash (*Table 9*).

4.7 Specific anti-rotavirus antibodies quantification

Specific anti-rotavirus antibodies were analysed in whey and in plasma of dams at the end of lactation and are represented in *Figures 6* and *7*, respectively.

The supplementation with the SYM did not modify the concentration of specific anti-rotavirus antibodies in both plasma and whey of dams.

4.8 Lymphocyte composition of spleen and mesenteric lymph nodes

Table 10 summarizes the relative proportion of spleen lymphocyte subsets in dams that received synbiotic or vehicle. As it can be observed no differences were found between both groups. In both SYM and REF groups the different populations found in spleen showed the following distribution. The most abundant population was T cells, specifically CD4 or T helper cells (Th) followed by CD8 or T cytotoxic cells (Tc). Then, B cells were about 9% and in lower concentrations NK and NKT cells were found.

The effect of the synbiotic on the relative proportion of MLN cells in dams was also analysed (*Table 11*). The different lymphocyte populations followed a distribution similar to that found in the spleen. A reduction in the CD8+ cell proportion was observed in the SYM group compared to the REF one. The supplementation with the synbiotic did not show any effect on the rest of the different populations of T, B or NK cells.

The influence of the synbiotic supplementation on the presence of CD8+ co-receptor in TCRαβ+/ NK-, TCRγδ+, NK and NKT populations of the spleen and MLN was also analysed (*Figure 8*). The SYM supplementation did not modify these lymphocyte subpopulations in spleen.

Figure 9 summarizes MLN populations that express the CD8 co-receptor. An upward tendency to increase CD8+ proportion in NK population was observed in the SYM group compared to the REF group. The supplementation with the synbiotic did not modify the TCRαβ+, TCRγδ+ and NKT populations in the presence of this co-receptor in MLN.

The influence of synbiotic diet on cells expressing of αE integrin and CD62L was also analysed (*Figure 10*). These are important adhesion molecules involved in intestinal homing. A high proportion of CD62L+ cells (60-70%) was observed in the spleen, whereas the percentage of cells expressing αE integrin was very low. The supplementation with the SYM did not modify the pattern of these cells in the spleen.

A similar distribution of percentages of cells expression αE integrin and CD62L was observed in MLN (*Figure 11*). The proportions of cells expression αE integrin or CD62L were similar between the SYM and REF groups.

5. Discussion

Diet during pregnancy and lactation plays a very important role since it not only influences the nutritional and health status of the mother, but also influences the outcome of the pregnancy and the health of the newborn (23). These two vital moments are considered more easily influenced, so the study of them is really important. Although there is evidence about the positive impact of prebiotic and probiotic maternal supplementation in the immune system of their offspring (24), the up-to-date evidence on the safety of either probiotics, prebiotics or synbiotics on pregnant and lactating mothers is limited. Good safety data collection and reporting are important to avoid undesirable health outcomes, particularly for the most vulnerable populations (25). The aim of this study was to assess whether the combination of a prebiotic and a probiotic during gestation and lactation is safe and enhances the immune system of the mothers. This combination was chosen based on a previous study where the different prebiotics and probiotics included here were used (26). In this, a positive impact on the health of the offspring was observed, largely related to the promotion of intestinal maturation and enhancing neonatal immune responses (26). For this reason, the impact of this synbiotic in the mothers' health is evaluated in the current study.

During pregnancy and lactation, the effect of the synbiotic on feed and water consumption was evaluated. No standing differences were found in the water consumption. Considering feed intake, during pregnancy, a small reduction was observed on some specific days.

Prebiotic supplementation has been associated with an improvement in the subjective appetite ratings, leading to a reduction in energy intake (27,28). This would explain the punctual decreases observed during gestation, but these differences were not found here during lactation. Body weight time course of the mothers during gestation and lactation was also recorded. Dams' supplementation did not show an impact on their body weight in these periods. Moreover, no differences were found in other variables associated with weight, such as the Lee index or BMI measured at the end of the study. There is not much information regarding the effect of supplementation with a synbiotic on body weight. It has been described that lactating pups supplemented with a mixture of prebiotics and postbiotics, underwent an increase in body weight (29), but this different outcome may be due to the fact that the study was conducted on pups that were growing. Other studies using supplements with GOS/FOS prebiotics show changes in body weight values. Thus, this prebiotic supplementation increased body weight of pups (26), and also that of adult mice (30). Anyway, these studies were carried out with the only supplementation of the prebiotic, so the mix of prebiotic and probiotic could play a different role. In conclusion, the administration of the synbiotic used in the current study globally does not modify either hunger or thirst, nor the overall body weight of rats during gestation and lactation.

The weight of the different organs was recorded to analyse whether the daily synbiotic supplementation during 42 days (gestation plus lactation) had a toxic effect. There were no effect on the relative weight of heart, kidney, liver, salivary glands, immune tissues (spleen and thymus) and stomach. However, SYM group presented a higher relative weight of the small intestine and the full cecum compared to the REF group. Similar results were observed in a study performed in pups supplemented with a synbiotic, where an increase in the small intestine, as well as in the large intestine, were observed (29). Another study shows an intestinal trophic effect of various prebiotics, causing an increase in both small and large intestinal weight and promoting positive changes in the intestinal structure (26). The increase in the intestine weight found here could be due to the intestinal microbiota that would promote intestinal structure. To demonstrate such hypothesis, the composition of intestinal microbiota as well as the analysis of intestinal architecture of dams will be analysed in the future.

No significant differences were found in haematological variables. Only a tendency towards reduction in the case of monocytes was observed in the SYM group. These results are consistent with those obtained previously, where pups supplemented with a mixture of a prebiotic with a postbiotic did not modify similar haematological variables (29). Anyway, our results also allow reinforcing the synbiotic safety during gestation and lactation.

Humoral immunity was analysed by determining IgG subtypes, IgA and Ig concentrations in plasma and other compartments. The distribution of the Igs in dams' plasma showed the usual pattern found in rats: the most predominant was IgG (about 94% of total Igs), followed by IgM (about 4%) and finally IgA (around 2%). The concentration of these main classes of Igs was not statistically modified by the synbiotic supplementation. Such results do not agree with a study using a mix of probiotics in adult rats showing a higher plasma IgG and IgA concentrations (31). Nevertheless, when IgG subtypes were analysed, plasma

IgG2c concentration (that could be associated with Th1-immune response) in the SYM group was around twice-fold that in the control group. Little information is available about the impact of a daily supplementation with a synbiotic to mothers during gestation and lactation on plasma Igs. In a previous study in sucking rats that received prebiotics, postbiotics and their combination, a reduction in the total IgG of the supplemented pups was observed, together with a reduction in the isotypes related to the Th2 response and an increase in the isotypes related to the Th1 response such as IgG2b (29). These results obtained in sucking rats are somehow similar as those found in our study, suggesting that prebiotics and postbiotics increased antibodies related to Th1 immune response. On the contrary, a study carried out in dams supplemented with a probiotic, shows a reduction in IgG2c and an increase in IgG1 and IgG2a (30). In addition, a previous study in lactating dams supplemented with a probiotic shows no significant changes in the plasma IgG isotypes, although the Ig pattern associated with Th1 and Th2 responses, showed opposite results to those found here (24). These differences in the results could be due to the fact that we did not use only a probiotic, but also a mix of prebiotic and probiotic.

In addition to plasma, concentrations of IgG subtypes, IgA and IgM were quantified in milk and mucosal compartments. These Igs can arrive to the offspring throughout breast milk and, therefore, have an impact on their immune system (1,32). In whey, IgG was the predominant Ig (representing more than 80% of total Igs), followed by IgA (about 16%) and, in few proportion, IgM (around 1%). These results did not match with those described in human milk, where the most abundant Ig is IgA, but agree with those previously found in Wistar rats (32,33). Supplementation with the synbiotic induced a 2-fold increase of the amount of total IgG and a 2.5-fold of the number of IgG2c, which agree with those results found in plasma.

The Igs content in salivary glands and mesenteric lymph nodes, as well as in gut wash were also analysed as examples of mucosal tissues. In salivary glands, no changes were observed in the absolute values of either IgG subclasses, IgA (determined by two techniques) and IgM by SYM supplementation. Nevertheless, considering the proportion of Igs, they did change in a similar way to plasma and whey, increasing IgG proportion due to IgG2c increase. In the mesenteric lymph nodes, an increase in the IgA and IgG2b content was observed in the SYM group. Concerning IgA, similar results have been reported after supplementing dams with the probiotic *Lactobacillus fermentum* CECT5716 during gestation and lactation (24). These results emphasize the importance of prebiotics and prebiotics in the intestinal immunity. Nevertheless, although IgA content was higher in the SYM group in the mesenteric lymph nodes, we could not observe an increase in the gut wash sIgA of the same animals. This could be due to the fact that gut wash was obtained just from a part of the small intestine. Further studies focused on sIgA in the colon or even better in faecal samples will allow to confirm the role of the synbiotic in the intestinal immune compartment of dams. In fact, a study in dams supplemented with a *Lactobacillus fermentum* CECT5716 during gestation and lactation, showed higher faecal IgA content and the probiotic also induced an increase in the intestinal IgA of offspring (24).

In addition, specific anti-RV antibodies were also analysed in plasma and milk. The supplementation with the SYM did not modify the concentration of specific anti-RV

antibodies in these compartments. These results are in line with a previous study carried out in RV-infected suckling rats supplemented with prebiotics (26) and with another study carried out in lactating pups supplemented with a fermented milk/prebiotic mixture (34), where the specific anti-RV antibodies were not modified either.

Finally, a characterization of lymphocyte populations (B cells, T cells, NK cells and NKT cells) in the spleen and mesenteric lymph nodes was performed in order to see if the supplementation with the synbiotic was able to modify the lymphocyte composition in such immune compartments. In general, the supplementation with the synbiotic did not imply changes in the composition of these populations demonstrating its safe for this immune compartments. There is only a change in the mesenteric lymph nodes, where the synbiotic caused a significant reduction in T CD8+ cells, that could involve both TCRαβ+ and TCRγδ+ subpopulations. These results are in agreement with a lower proportion of CD8+ TCRαβ+ and TCRγδ+ cells in dams supplemented with *Lactobacillus fermentum* CECT5716 during gestation and lactation (24). CD8+ lymphocytes act as cytotoxic cells and play an important role in immunity against intracellular pathogens and tumours, as well as in the regulation against autoimmune and allergic disorders (35). Mesenteric lymph nodes are in constant interaction with the intestine and its surroundings and therefore with the microbiota. So this reduction could suggest a greater health of the microbiota and therefore a reduction in the inflammatory state or an absence of infection. Little information is available about the impact of the supplementation with a synbiotic in the mesenteric lymph nodes and spleen in lactating dams, but the results found in this study are in line with previous ones where no changes in terms of B cells, NK and NKT in the spleen and the mesenteric lymph nodes were found in a lactating pups supplemented with a probiotic (36).

Taking together all the results it is important to highlight that the supplementation is safe when administered to the mothers during the gestation and the lactation periods and is able to induce changes in the immune response. Further studies will be necessary to assess the effect on the offspring and to study whether the increase in Ig in breast milk is able to enhance the immune systems of the newborns to help fight against infectious diseases such as the RV-induced diarrhoea.

6. Conclusions

The supplementation with a synbiotic during gestation and lactation in rats:

- does not modify the weight or the body composition, with the exception of an increase in the relative the small intestine and the cecum and does not affect haematological variables either. In general rats supplemented with the synbiotic consume the same amount of feed and water as the ones supplemented with vehicle.
- was able to impact the humoral response by increasing the IgG2c in plasma and whey and the IgA content in mesenteric lymph nodes.
- does not change lymphocyte composition in the spleen and mesenteric lymph nodes. Only a decrease in CD8+ lymphocytes (both TCRαβ+ and TCRγδ+ cells) was found in mesenteric lymph nodes.

Overall, it can be said that the supplementation with the synbiotic during gestation and lactation is safe and does not affect the health of the dams and seems a good strategy to enhance the immune system of the mothers and the offspring. Further studies are needed to provide more knowledge about the role of the maternal diet during pregnancy and lactation and the impact on their offspring.

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