UNIVERSITAT DE BARCELONA

# Maternal synbiotic supplementation during gestation and lactation: effects on the mother–infant immune system and on the prevention of rotavirus infection in a preclinical model

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| | |
|---|---|
| **2'-FL** | 2'- Fucosyllactose |
| **3'-FL** | 3' - Fucosyllactose |
| **3'-SL** | 3'-Sialyllactose |
| **6'-SL** | 6'-Sialyllactose |
| **Abs** | Antibodies |
| **Ags** | Antigens |
| **APCs** | Antigen Presenting Cells |
| **BAT** | Brown Adipose Tissue |
| **BM** | Breast Milk |
| **BMI** | Body Mass Index |
| **BSH** | Bile Salt Hydrolases |
| **CD62L** | L-Selectina |
| **CFU** | Colony-Forming Units |
| **CG** | Chorionic Gonadotropin |
| **CKs** | Cytokines |
| **Cldn** | Claudin |
| **DCs** | Dendritic Cells |
| **DNA** | Deoxyribonucleic Acid |
| **EFSA** | European Food Safety Authority |
| **EMA** | European Medicines Agency |
| **EV** | Enterovirus |
| **FcRn** | Neonatal Fc Receptor |
| **FDA** | Food And Drug Administration |
| **FFAR** | Free Fatty Acid Receptors |
| **FUT2** | Fucosyltransferase 2 |
| **GALT** | Gut Associated Lymphoid Tissue |
| **(sc)GOS** | (Short-chain) Galacto-oligosaccharides |
| **HBGAs** | Histo-Blood Group Antigens |
| **hCG** | Human Chorionic Gonadotropin |
| **HDAC** | Histone Deacetylase Inhibition |
| **HMOs** | Human Milk Oligosaccharides |
| **HSC** | Hematopoietic Stem Cells |
| **IECs** | Intestinal Epithelial Cells |
| **IFNy** | Interferon-γ |
| **Igs** | Immunoglobulins |
| **Ig-CB** | Immunoglobulin - Coated Bacteria |
| **ILCs** | Innate Lymphoid Cells |
| **ILs** | Interleukins |
| **(lc)FOS** | (Long-Chain) Fructo-Oligosaccharides |

| | |
|---|---|
| **LN** | Lymph Nodes |
| **LNT** | Lacto-N-tetraose |
| **LPS** | Lipopolysaccharides |
| **MDA-5** | Melanoma Differentiation-Associated Gene 5 |
| **MHC** | Major Histocompatibility Complex |
| **MLN** | Mesenteric Lymph Nodes |
| **mRNA** | Messenger RNA |
| **Muc** | Mucin |
| **NF-Kβ** | Nuclear Factor NF-kβ |
| **NKs** | Natural Killer |
| **NMDS** | Non-Metric Multidimensional Scaling |
| **NSVP** | Non-Structural Viral Proteins |
| **Ocln** | Occludin |
| **PGlyRP3** | Peptidoglycan Recognition Protein 3 |
| **PRRs** | Pattern Recognition Receptors |
| **RIG-I** | Retionoid Acid-Inducible Gene |
| **RNA** | Ribonucleic Acid |
| **RV** | Rotavirus |
| **SCFAs** | Short Chain Fatty Acids |
| **SEM** | Standard Error Of The Mean |
| **sIgA** | Secretory Iga |
| **Treg** | T Regulatory |
| **Tc** | T Cytotoxic |
| **TCR** | T Cell Receptor |
| **TGF** | Transforming Growth Factor |
| **Th** | T Helper |
| **TJ** | Tight Junction |
| **TLRs** | Toll-Like Receptors |
| **TNFα** | Tumor Necrosis Factor- α |
| **VP** | Viral Proteins |
| **WAT** | White Adipose Tissue |
| **WHO** | World Health Organization |
| **Zo** | Zonula Occluden |
| **β-Gal** | Beta-Galactosidases |

# Abstract

The first 1000 days of a baby's life are crucial for their development and long-term health. This period encompasses pregnancy through the second year of life, emphasizing the importance of the maternal nutrition during both gestation and lactation for the child's growth. Maternal supplementation with synbiotics during pregnancy and lactation could potentially improve the immune functions and intestinal health of both the mother and the baby.

This thesis examines the effects of maternal supplementation with *Bifidobacterium breve* M-16V and short-chain galacto-oligosaccharides (scGOS) / long-chain fructo-oligosaccharides (lcFOS) during pregnancy and lactation on the maternal and offspring immune systema and microbiota composition in a preclinical model. Additionally, this thesis analyzed the potential effect of the maternal synbiotic supplementation to counteract early life infections.

Firstly, the analysis of the effects of the maternal synbiotic supplementation on the mother indicated that synbiotic supplementation during pregnancy and lactation positively affects the colonization of the maternal microbiota and modulates the maternal immune system, enhancing the immunoglobulin profile at the end of lactation. Furthermore, the maternal synbiotic supplementation also modulates the bioactive components and microbiota composition of the breast milk.

Secondly, the analysis of the maternal supplementation on the offspring during the rotavirus (RV) infection in early life suggested that maternal synbiotic supplementation ameliorates the infectious process. *Bifidobacterium breve* M-16V and scGS/lcFOS reduced the incidence and severity of the viral process. Furthermore, the maternal supplementation modulates the newborn immune system and the microbiota colonization process.

Thirdly, the effects of the maternal supplementation on the offspring were tested at the end of the suckling period. Herein, it was observed that maternal supplementation influences positively the intestinal immune system and the microbiota colonization of the infant. In particular, the supplementation leads into increased proportions of *Bifidobacterium* and improves the intestinal structures and functionality of the intestine at the end of the suckling period.

Finally, it was evaluated the effects of the maternal supplementation until weaning and after one week of direct supplementation post-weaning. This approach confirmed that synbiotics in early life contribute to the maturation of the infant, with highlighted benefits on the gut. Specifically, synbiotics improve the integrity, defense and functionality of the gastrointestinal tract and enhances the gut microbiota colonization, increasing the presence of beneficial bacteria like *Bifidobacterium*.

Taking together the principal findings of this thesis, it can be concluded that there is a vertical transmission of bioactive component like immunoglobulins and microbiota from the mother to the infant.

Overall, this thesis shed light on the maternal supplementation with *Bifidobacterium breve* M-16V and scGOS/lcFOS during pregnancy and lactation shows great promise in improving the immune and gastrointestinal health of offspring. These studies collectively suggest that synbiotics can enhance immune system development, reduce infection severity, and promote a healthier gut microbiota in developing infants. These findings underscore the importance of maternal nutrition in shaping infant health outcomes and highlight the potential for synbiotics as a preventive strategy against early-life infections and for promoting long-term health.

# Resumen

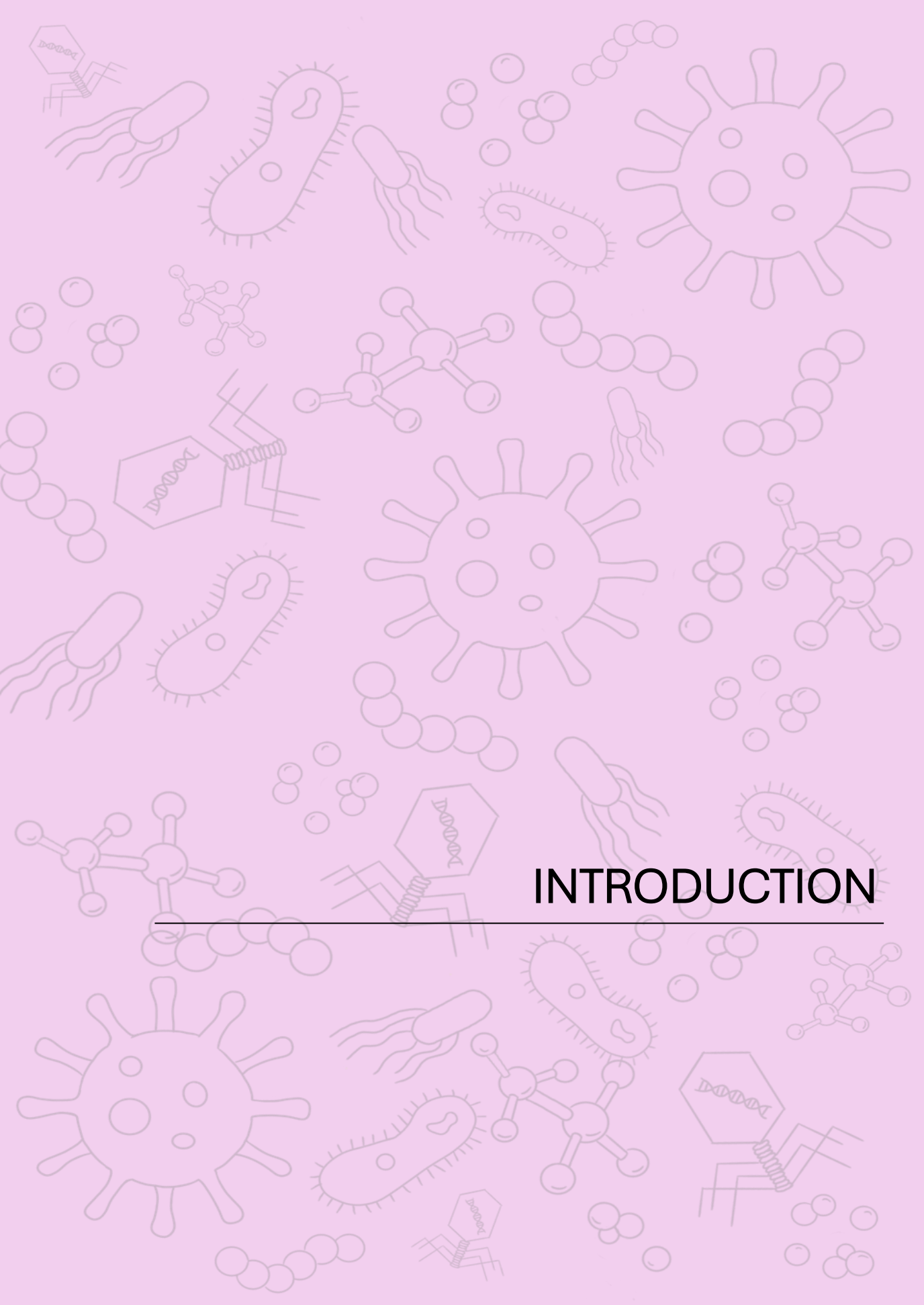
Los primeros 1000 días de vida de un bebé son fundamentales para su desarrollo y salud a largo plazo. Este periodo engloba desde el embarazo hasta el segundo año de vida, por lo que la nutrición materna durante la gestación y la lactancia es crucial para el desarrollo del niño. La suplementación materna con sinbióticos durante la gestación y la lactancia podría mejorar la capacidad defensiva tanto de la madre como del bebé.

Esta tesis examina los efectos de la suplementación materna con *Bifidobacterium breve* M-16V y galacto-oligosacáridos de cadena corta (scGOS) / fructo-oligosacáridos de cadena larga (lcFOS) (9:1) durante el embarazo y la lactancia sobre el sistema inmunitario, la función de barrera intestinal y la composición de la microbiota de la madre y el bebé en un modelo preclínico. Además, evalúa el efecto de la suplementación sinbiótica materna para contrarrestar la infección por rotavirus en las primeras etapas de vida del neonato.

Los primeros resultados preclínicos mostraron que la suplementación sinbiótica materna durante la gestación y la lactancia modula positivamente el sistema inmunitario y la composición de la microbiota materna. La suplementación materna también afecta a los componentes bioactivos y a la microbiota de la leche materna.

Los análisis realizados sobre el efecto de la suplementación materna en la descendencia confirmaron que la ingesta de este sinbiótico durante el embarazo y la lactancia mejora la colonización de la microbiota de la cría. Además, durante las primeras etapas de la vida, la suplementación materna fortalece el sistema inmunitario y la función de barrera intestinal, contribuyendo a reducir la incidencia y la gravedad de la infección por rotavirus.

En general, esta tesis doctoral aporta evidencias sobre el impacto de la suplementación materna en su propio sistema inmunitario y en el de su descendencia, ayudándo a protegerlo de infecciones en las primeras etapas de la vida.



# INTRODUCTION

# 1. MATERNAL-FETAL PHYSIOLOGICAL AND IMMUNOLOGICAL DYNAMICS

The immune system is constituted by the innate (non-specific) and adaptive (specific) responses, playing both together essential roles in host defense[1]. It is designed to exert protective responses against pathogens or dangerous antigens (Ags) that may alter the healthy status of the host.

The innate response includes non-specific mechanism and different types of physical, chemical, and microbial barriers. Among others, the innate components include the phagocytic system, the acute-phase response, and the complement, as well as some cells like natural killer (NK) cells and dendritic cells (DCs). Both together cooperate to detect the pathogenic Ags and trigger the activation of the adaptive response[2].

The hallmark of the adaptive immunity is the immunological memory which aids in combating future infections by recognizing and responding to the same Ag[3]. The principal elements of the adaptive immune system are B cells and T cells, which are responsible for humoral and cellular immunity, respectively. B cells protect the host by secreting antibodies (Abs), while T cells respond to the presentation of Ags. T cells can destroy infected cells and activate other B or T lymphocytes through the release of cytokines, coordinating the immune response[3].

Regarding the plasticity of the immune cells, macrophages, NK cells and T cells, concretely the T helper (Th) subsets, modulate their phenotypes depending on pro-inflammatory or anti-inflammatory responses (Figure 1). In pro-inflammatory responses, macrophages polarize towards a M1 phenotype, Th cells towards a Th1 phenotype and NK cells towards a NK1 phenotype. Generally, pro-inflammatory phenotypes are followed by pro-inflammatory cytokines (CKs) or interleukins (ILs) such as IL-1β, Tumor Necrosis Factor-α (TNF-α) or interferon-ɣ (IFN-ɣ). On the contrary, in anti-inflammatory responses, macrophages polarize towards a M2 phenotype, Th cells towards Th2 and NK towards a NK2 phenotypes. The CKs associated to anti-inflammatory responses are IL-4, IL-6, IL-10, IL-13[4,5].

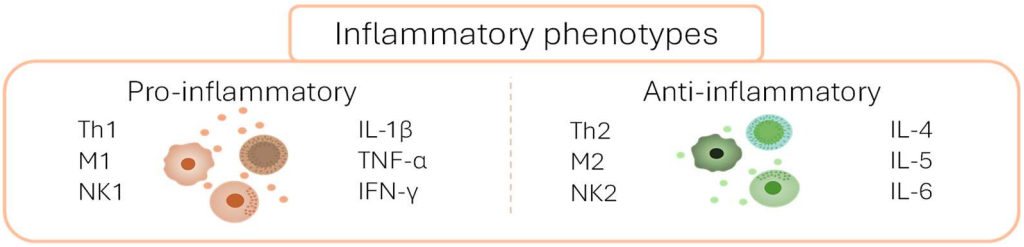



**Figure 1.** Inflammatory phenotypes of the immune system cells. Th, T helper; M, macrophage; NK, natural killer; IL, interleukin; TNF- α, Tumor Necrosis Factor-α; IFN- ɣ interferon-ɣ.

## 1.1. INSIGHTS INTO MATERNAL CHANGES

During pregnancy, maternal immune system undergoes to several changes at endocrine, metabolic, and immune levels. All of them participate in preventing rejection and ensuring the fetus survival.

### 1.1.1. MATERNAL DECIDUALIZATION

Following fertilization, the zygote undergoes different processes including implantation, decidualization, trophoblast differentiation and finally placentation and embryo development[6]. The embryo carries a combination of paternal and maternal genes, being a semi-allogenic fetus which will grow in the maternal-fetal interface. The maternal-fetal interface is found in the uterus where the blastocyst invades the maternal decidua, and fetal and maternal cells coexist[4,5]. The maternal decidua refers to the uterine mucosal layer (endometrium) which will undergo the decidualization (remodelation of the uterus to allow embryo development)[4]. In decidualization, the blastocyst first attaches to the endometrial luminal epithelium, then the external cells of the blastocyst, trophoblasts, invade the maternal decidua. Consequently, the uterine blood flow increases to ensure the sufficient delivery of maternal nutrients and oxygen to the placenta[7].

### 1.1.2. MATERNAL IMMUNE CHANGES IN PREGNANCY AND TOLERANCE

In 1953 *Billingham et al*. stablished the concept of the maternal tolerance during pregnancy. This phenomenon allows the survival of the semi-allogenic fetus, carrying paternal Ags, within the maternal environment. To achieve this, the maternal immune system regulates its activity to prevent rejection and ensure successful fetal development[4]. One of the key aspects of the regulation of the maternal immune system is the pro-inflammatory and anti-inflammatory balance. Notably, during pregnancy, the immune system is biased to an anti-inflammatory state, thus with a polarization of immune cells towards M2, Th2 and NK2 phenotypes. Additionally, T regulatory (Treg) and Th17 cells actively participate in the environmental tolerance suppressing the activity of other immune cells that could implicate a threat to the fetus[8].

Although the most important part of the maternal tolerance is stablished in the maternal-fetal interface, the overall maternal immune system also suffers changes required for the fetus development, including metabolic and immune changes at systemic and mucosal level[7]. Additionally, not only the maternal immune system is regulated, the fetal trophoblast expresses specific receptors on its surface to avoid the recognition and destruction by maternal cells[4] (*reviewed in section 3.1.)*.

The maternal immune tolerance requires a degree of immunosuppression of the innate and adaptive immune systems while maintaining them sufficiency active to fight infections[4]. As mentioned above, in pregnancy, the maternal immune system changes towards a Th2 type responses, being a clear polarization towards a T cells (Th2), NK cells towards a NK2 phenotype and macrophages towards a M2 phenotype[5]. However, the

polarization of the immune cells depends on the gestational stage. For implantation, a moderate inflammation requiring M1 polarization is essential. Later, a Th2, M2 and NK2 polarization is established for the fetal survival, and at the end of pregnancy, to induce labor a soft switch toward the pro-inflammatory phenotypes Th1 and M1 is required, which is linked with an increase in IL-1β, TNF-α or IFN-γ[4] (Figure 2).

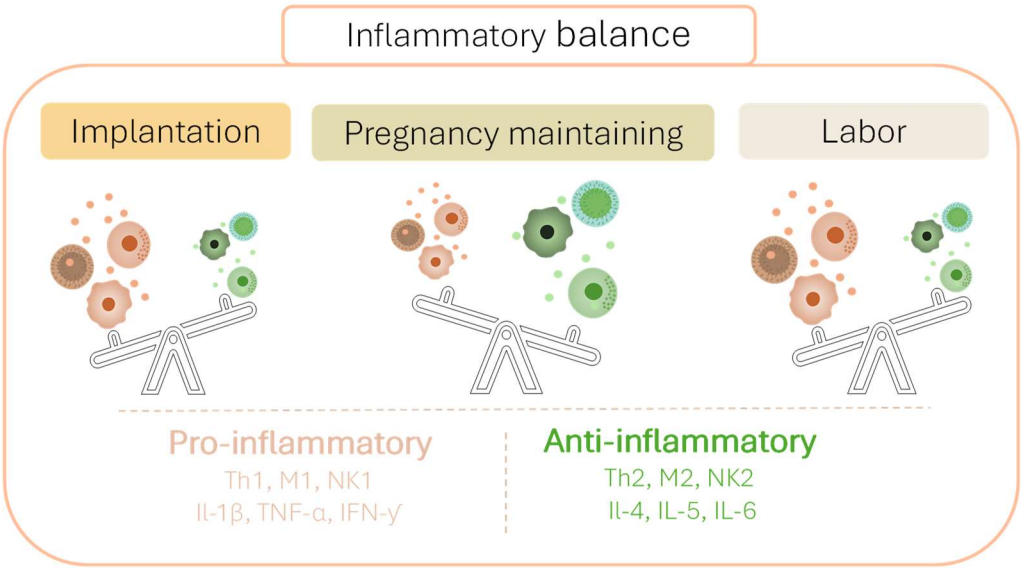



**Figure 2.** Evolution of the inflammatory balance throughout pregnancy. Th, T helper; M, macrophage; NK, natural killer; IL, interleukin; TNF- α, Tumor Necrosis Factor-α; IFN- γ interferon-γ.

In the Th2 response, anti-inflammatory CKs stimulate the naïve T cells to differentiate them into Th2 cells in the maternal decidua. At the same time, migration of peripheral Th2 cells towards the maternal decidua happens[4]. During the gestational period the increase in the anti-inflammatory CKs contributes to a successful pregnancy.

First, analyzing some innate immune cells, pregnancy leads to an increase in the maternal blood cells. The crosstalk between the maternal blood cells and the fetal cells through the placenta activates some maternal immune cells. As a result, the number of total leukocytes increase, mainly associated to the higher circulating monocytes and granulocytes (neutrophils)[7]. Monocytes and granulocytes are innate immune cells that, upon Ag detection migrate to the lymph nodes to initiate an immune response[9,10]. This process provides a link between the innate and adaptive immune system. The role of neutrophils during pregnancy is not properly defined. However, it has been postulated that they assume an immune regulatory role, modulating the anti- or pro-inflammatory state[11]. During pregnancy the number of not all cell types are increased, the proportion of

circulatory DCs and NKs is reduced, probably due to its accumulation in the maternal-fetal interface[7].

Secondly, analyzing the adaptive immune cells, Th17 and Treg cells are also implicated in the regulation of the immune system during pregnancy. The literature is quite controversial about Th17 and Tregs. At the beginning it was though that Treg proportion increases during gestation[7], however, later studies demonstrated that there are several types of Treg phenotypes, which vary their proportions depending on the gestational stage[8]. Some authors indicated that their levels remain constant as in non-pregnant women while some others reported that Th17 cells are increased during pregnancy[7]. Despite this data, Th17 and Tregs cells collaborate in maintaining a healthy pregnancy and defend against pathogens. Th17 cells produce IL-17, a CK with pro-inflammatory properties that plays specific roles in host defense against certain pathogens and in organ specific autoimmunity[5]. Additionally, other Th17-related CKs promote the expression of depressive and anxiety symptoms during pregnancy. For these reasons, the role of Th17 and Tregs has to be checked to ensure a correct development of the fetus[6].

Several studies have demonstrated that pregnancy is linked to a reduction in peripheral B, T helper (Th), and T cytotoxic (Tc), and NK cells[12]. Some of these changes in the cell proportions may persist until about 1 year after delivery. Thus, the serum levels of immunoglobulins (Ig), IgG, IgM and IgA decrease during pregnancy[12]. Additionally, around 2 months after delivery, IL-4, IL-10 and IL-2 increase in the peripheral blood to counteract the pro-inflammatory CKs released for delivery[12].

All above suggests that pregnancy is associated with a reduction in the overall activity of the maternal immune system, alterations in the proportions of different cell types[12,13]. As a consequence of all these adaptations, pregnant women are more sensitive to certain infections that depends on pro-inflammatory responses. Also, some autoimmune diseases ameliorate during pregnancy as a result of the immune response suppression followed by a rebound worsening after labor[7,12].

The gastrointestinal tract is also affected during pregnancy, as a result of the increased nutrient requirements, the intestinal weight during pregnancy rises[13]. These adaptations including the uterine and placental mucosa, many immune cells such as NK, macrophages, T regs and γδ T cells are already recruited before implantation and increase their presence after implantation [7].

of the intestine are maintained after labor. Regarding the immune cells in the mucosa,

### 1.1.3. MATERNAL CYTOKINES

CKs are the most important molecules implicated in the modulation the immune system during pregnancy at both, systemic and mucosal level. Depending on the pregnancy stage, the different CKs profiles drive the Th polarization towards a Th1 or Th2 response. For implantation some Th1 CKs such as IL-1 are essential. Then, to ensure the fetal

survival the Th2 CKs (IL-4, IL- 5, IL-6) increase their presence to shift towards a Th2 response. Finally, at the end of the third trimester, an increase in pro-inflammatory CKs is necessary to induce the delivery. Overall, at systemic level, the Th1 CKs, such as IFN-ɣ and IL-2 are systematically impaired during pregnancy. On the contrary, the secretion of Th2 CKs, such as IL-4, IL-6, and IL-10 are increased[14]. Even though the immune response is biased towards a Th2 response some of the Th1 CKs such as IFN-γ or TNF-α play an important role in placental development[7].Indeed, the maternal fetal interface is a CK productor site, mainly produced by the NK cells, suggesting a potential role to regulate the Th1/Th2 balance[15].

### 1.1.4. MATERNAL HORMONES

During pregnancy dramatic changes in hormone levels happen to ensure the survival and tolerance of the fetus (Figure 3). In early pregnancy, when fetal organogenesis takes place, hormones are involved in the neurodevelopment of the fetus. And, in the late pregnancy, they are involved in preparing the maternal body for childbirth and breast for lactation[16].

Among the most relevant hormones in pregnancy, steroid hormones like progesterone and oestrogens (estradiol, estrogen and estriol) are found. In humans, both progesterone and oestrogens follow similar evolutions during conception, increasing their levels gradually, achieving the higher levels in the last trimester and dropping soon after birth[17,18]. Progesterone during pregnancy can inhibit the development of Th1 responses and the production of proinflammatory cytokines, such as IFN-γ. Furthermore, progesterone promotes Th2 immune responses, inducing the synthesis of anti-inflammatory CKs such as IL-4, IL-5, and IL-10[19]. Oestrogens are linked to the adipose metabolism and collaborates in maintaining pregnancy for a correct fetal development[20].

Prolactin is other important pregnancy hormone which promotes the multiple maternal adaptions that occur during gestation. In humans, the levels of prolactin increase during pregnancy achieving the peak at labor[21]. Chorionic gonadotropin (CG) is a hormone exclusively secreted during pregnancy. That of humans (hCG) promote an immunologic state to ensure fetal survival and growth[22].

Some other hormones are adipokines, including mainly adiponectin and leptin, which are synthetized in the adipose tissue. Alterations in their levels have been correlated with pregnancy complications[23].

Despite the importance of hormones during pregnancy, disruptions in hormone levels can raise the mother's risk of cancer such as ovarian and breast cancer[24]. Additionally, external factors like smoking could affect the hormones concentrations. For example, in smoking-pregnant women, the levels of estradiol and testosterone are higher in the third trimester[24].

In rats, hormonal changes may differ with humans. For example, the changes in the progesterone levels are not as linear as in humans. Their higher levels are achieved at the end of the second week of pregnancy and reduce its levels in the third week until delivery[18]. The prolactin levels in rats go up and down abruptly during early pregnancy and then achieve the peak in the late pregnancy[21]. However, the estradiol fluctuation is quite similar in humans and rats (Figure 3).

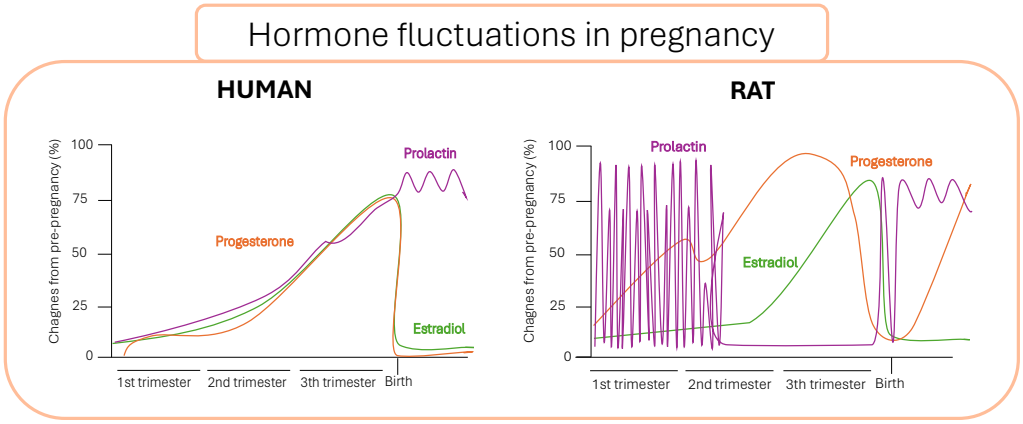



**Figure 3**. Fluctuation of the principal hormones during pregnancy in humans and rats. Adapted from[18].

### 1.1.5. MATERNAL MICROBIOTA

The development of the baby's gut microbiota starts prior to being born. It has been concluded that maternal microbiota during pregnancy and lactation could affect the immune system development and the intestine colonization of the fetus, neonate and infant.

During pregnancy the diversity of the intestinal maternal microbiota is altered, specifically, the alpha diversity (intraindividual bacterial diversity) is reduced, and the beta diversity (interindividual bacterial diversity) is increased[25]. Commonly, a reduction in the alpha diversity is associated with health disorders such as obesity. However, during pregnancy this is a regular change[26]. Additionally, the proportion of the opportunistic pathogens increases in the late pregnancy [27].

In healthy physiological conditions, the human microbiota is dominated by Firmicutes, followed by Bacteroidetes and in minority proportions Proteobacteria, Actinobacteria and Verrucomicrobia[28]. In early pregnancy the intestinal microbiota remains similar to a non-pregnant women[26]. Later, in the first trimester there is an increase in the abundance of Firmicutes (*Ruminococcaceae* and *Lachnospiraceae)* and Bacteroidetes (*Prevotellaceae* and *Bacteroidaceae)*[19]. In the second trimester, Proteobacteria (*Enterobacteriaceae)* and Actinobacteria (*Bifidobacteriaceae)* start to predominate, being

clearly increased at the end of the gestation. Finally, in the third trimester, the abundances of Proteobacteria and Actinobacteria, especially the *Enterobacteriaceae* family and *Streptococcus* genus, increase substantially[19,29,30]. The increase in the Proteobacteria at the end of the gestation seems to stimulate the immune system and enhances local inflammatory responses, collaborating to induce labor[26] (Figure 4).

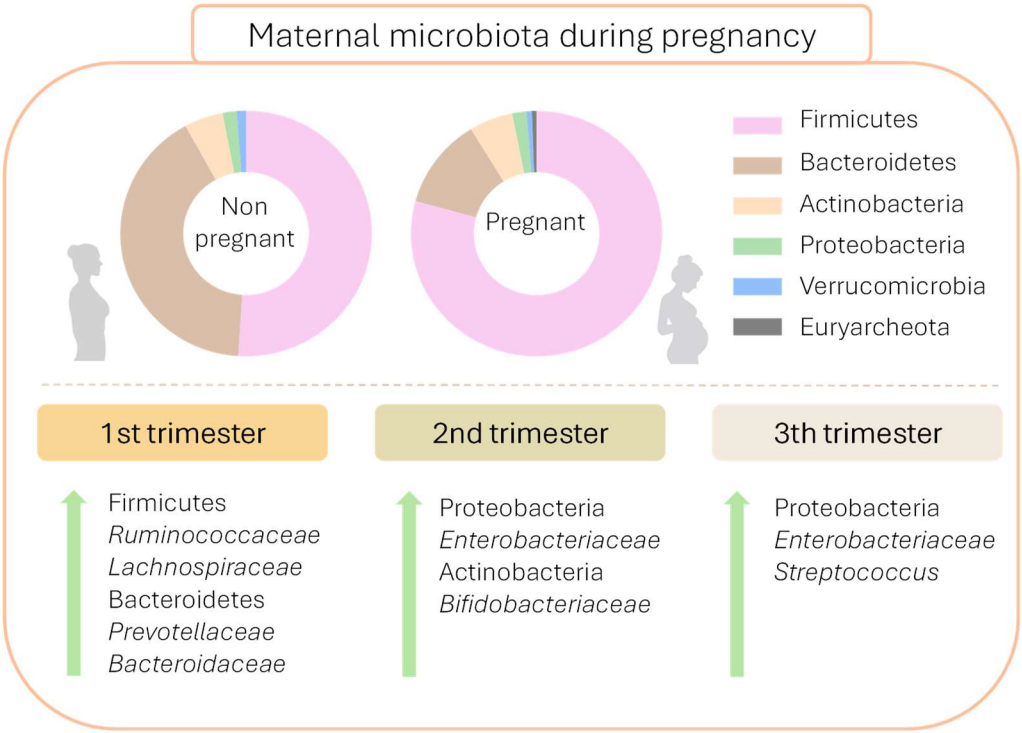



**Figure 4.** Main microbiota changes induced during pregnancy in humans.

One of the main communication channels between the intestinal microbiota and the immune system is through the production of short chain fatty acids (SCFAs). These molecules via immunostimulatory signals activate innate and adaptive immune responses. SCFAs penetrate the placental barrier, being critical to ensure the fetal development and growth[19] . In pregnancy, the abundance of *Lactobacillus,* the main lactic acid producer, is increased while that of *Faecalibacterium*, the main butyrate producer is reduced. Also, intestinal microbiota is involved in changes in the peripheral immune cells during pregnancy. However, the specific mechanisms involving the intestinal immune cells modulation are still unknown[26].

The placenta has been considered as sterile for many years. However, genetic material from non-pathogenic bacteria have been detected in the placenta, umbilical blood and meconium of healthy newborns. The placenta has a low microbiota abundance, but it is highly metabolically active[27,31]. Additionally, it is a source of *Escherichia*, explaining the

higher abundance of *Escherichia* in the meconium at birth[19]. Placental microbiota is closely correlated with the oral microbiota of the mother which is composed of Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes, and Fusobacteria phyla[32]. Some pathogens can also be present in the placenta before labor such as *Streptococcus agalactiae*[19].

Microbiota is highly correlated with hormones, for instance, hormone alterations can be associated with inflammation and immune changes[19,33]. The progesterone plasma concentration increases during pregnancy and is positively correlated with several bacterial species in the gut, such as those from the *Bifidobacterium* genus. This change collaborates to establish a healthier microbiota of pregnant mothers and contributes to the bacterial transmission to the neonate[19]. Vaginal and oral microbiota during pregnancy are also modulated in this period. Both are highly susceptible to hormone variations modulating the microbiota proportions[34–36]. The changes of the intestinal and vaginal microbiota can be used as markers for the pregnancy evolution. To date, some vaginal communities in early pregnancy are associated with preterm birth[19].

## 1.2. DECIPHERING HUMAN EMBRYO AND FETAL DEVELOPMENT

The embryo development starts from a single-cell zygote until forming a blastocyst that will evolve into a fetus by the process of gastrulation and organogenesis. In the first 3 weeks of gestation, the blastocyst rearranges itself in different layers (endoderm, ectoderm, and mesoderm) that will evolve in different parts required for a properly fetus development. In human, organogenesis takes around 5 weeks, from week 3 to week 8 of the gestation[4].

The fetal organogenesis starts in the yolk sac where hematopoietic stem cells (HSC), NK cells, erythroid cells, mast cell, innate lymphoid cells (ILCs) and macrophages are found at week 4 of gestation. All these cells contribute to the colonization of other tissues such as the liver, brain, lungs and epidermis, and participates in the protection and the remodelation of the fetus and the decidua[5]. Later on, the HSC are produced in the liver, spleen and bone marrow. The liver and spleen production stops prior to birth whereas while the bone marrow cell production continues until birth[5].

The fetal immune cell phenotypes may differ with respect to adult phenotypes. During fetal development and at birth, the DCs are CD4-, CD8-, CD11-, this phenotype is reduced after birth until almost absent in adults[5]. B and T cells appear around week 7-9 of gestation in the liver, and migrate to the spleen and thymus, respectively, to mature and then being widespread to other tissues and organs.

The functionally of each cellular type vary. The DCs stimulate T cell differentiation, propagation, and activation, promoting the secretion of IL-4, which will contribute to the fetal tolerance. B cell functionality is mainly focus on the Ig production, and its

proportions are higher in fetus than in adults. During gestation large quantities of IgG and IgM are secreted, suggesting that B cells highly contribute to the first line of defense in the newborn[5].

In all the stages, the trophoblast contributes to maintain the tolerogenic environment, the principal strategies include, among others, restricted major histocompatibility complex (MHC) protein expression, the presence of immunosuppressive proteins, immunomodulatory adhesion molecules, the expression of apoptosis-inducing proteins, and complement regulatory proteins. This selective expression contributes to the tolerance status in the maternal-fetal interface[37,38].

## 1.3. IMMUNE-COMMUNICATION BETWEEN MOTHER AND FETUS DURING PREGNANCY

During gestation is established an active communication between the mother and the fetus. In this period the transference of molecular and cellular components contributes to program the neonatal immune system. The principal function of the placenta is the maternal-fetal transport of nutrients and waste. The principal mechanism involved is diffusion. The efficiency of the diffusion depends on the molecular properties and concentration of the solutes, for example, the oxygen passes via passive diffusion. Active transport using transporters also happens, participating in the transference of hydrophobic molecules, proteins and amino acids[39].

Considering the active transporters, humoral immunity is transferred through receptor-mediated mechanisms to confer passive immunity to the fetus. In humans, the unique Ig with the ability to cross the placenta is the IgG. The maternal IgG is transferred with the aid of the neonatal Fc receptor (FcRn). The FcRn has a high affinity for the IgG which is monomeric and binds to it under acidic conditions. The FcRn is located inside endosomes of the maternal interface which will internalize the IgG, when the FcRn – IgG bind happens, the complex is liberated into the neutral pH of the fetal stroma[40]. The IgG transference starts in the first semester and achieve its highest concentration at birth. Apart from the placental transfer, the amniotic fluid also collaborates in cellular pinocytosis, diffusion, and absorption of IgG across the fetal gut[40]. Thus, boosting the maternal immunity during gestation could improve the infant immune responses in early life[41]. In humans, there are different subtypes of IgG and, the FcRn has more affinity to transport the IgG1, followed by IgG4, then IgG3 and finally IgG2[40].

Inflammatory mediators like CKs and SCFAs can be also transferred from the mother to the fetus, however, the mechanisms involved remain ambiguous. Different studies indicate that CKs are produced by the placenta and fetal tissues but do not cross the placenta[40,42]. The unique CK identified in *ex vivo* studies which is transported across the maternal-fetal compartment is IL-6[43]. The placenta also contains SCFAs and their

receptors[44], indicating a potential role of microbial metabolites during gestational development[31].

The umbilical cord blood is vital for fetal survival and development. During pregnancy, the transport of nutrients and other factors play a role in fetal development. Several correlations have been done with the maternal immunological profile and the cord blood profile[45]. As mentioned before, for delivery a pro-inflammatory response is needed. This pro-inflammatory status is associated with higher levels of inflammatory CKs such as IL-1β, IL-6, IL-8, and TNF-α. To date, the inflammatory status is mainly induced locally in the fetal environment. For this reason, higher levels of these CKs are found in the cord blood with respect to maternal plasma[45]. When maternal infections happen, a pro-inflammatory status is stablished, which modulates the CK levels in cord blood[40].

As reported in the *1.1.5 section*, DNA fragments has been detected in the placenta. Additionally, the presence of microbes on the amniotic fluid, the umbilical cord blood, the fetal membranes, and the meconium suggests a transference of the maternal microbiota to the fetus during gestation[46]. One of the mechanisms of transference of maternal microbiota to the fetus is mediated by DCs which internalize the bacteria from the intestinal epithelium, then enter in the maternal blood flow and arrive to the fetus contributing to its colonization[25]. Additionally, to the direct microbiota translocation, DCs can also transfer microbiota derived molecules or compounds such as SCFAs, lipopolysaccharide (LPS) or flagellin[25].

## 1.4. ANIMAL MODELS FOR UNDERSTANDING HUMAN PHYSIOLOGY IN THIS PERIOD

Animal models have provided huge advantages in a wide range of knowledge in nutrition, immune diseases, rheumatoid arthritis, epilepsy, Alzheimer's disease, cardiovascular diseases, atherosclerosis and, diabetes, among others[47–49]. Additionally, they have demonstrated being appropriate models for studying the embryo and placental development[50]. For obvious ethical reasons, the study of pregnancy adaptations in humans are highly limited. Thus, rodents have been extremely helpful to study in depth into the physiological changes during pregnancy[45]. The short gestation period and lifespan of rodents allow a quick understanding of the physiological changes and the exposure effects on the offspring[51].

The main differences between human and rat fetal development are described in Table 1. The maternal and offspring body weight and the gestation length are the most notorious differences[51].

The degree of the fetus maturation at birth (regarding bone, muscle and nervous system) can be divided in precocial and altricial. Humans at birth have well-developed sensory and

locomotor skills, typically from precocial. However, rats at birth are undeveloped, relatively immobile, have their eyes closed and lack of hair, being classified as altricial[51].

Regarding the ontogeny of the immune system in rats, it is delayed compared to humans due to their shorter gestation period, this delayed maturation can be observed in the intestinal compartment by analyzing intestinal, length, weight, enzymatic activity, crypts, villi and microbiota[49]. Additionally, in rats the functionality of the T and B cells is reduced being less competent at birth in rats compared to humans[49].

**Table 1**. Human and rat pregnant features

| | **Human** | **Rat** |
|---|---|---|
| **Pre-gestation weight (g)** | 6000 | 280 |
| **Neonate birth weight (g)** | 2800 | 6 |
| **Gestation length (weeks)** | 40 | 3 |
| **Litter size** | 1 | 10-16 |
| **Neonatal maturity** | Precocial | Articial |
| **Breastfeeding length (days)** | 730 | 21 |

In spite of all these differences between humans and rodents, researchers agree that the use of rodents to understand the maternal changes induced during pregnancy and the embryo and infant development is a good strategy. This affirmation is mainly due to the easy housing, the low cost of the overall model and the short gestation[49,50]. Additionally, the immaturity of rodents, especially rats, at birth provides the perfect opportunity to evaluate the potential of various immunomodulators, including diet, to accelerate the physiological maturation process[49,52].

## 2. IMMUNE CROSSTALK BETWEEN THE NEWBORN AND THE MOTHER AFTER BIRTH: BREAST MILK

After birth, an active communication is established between the mother and the newborn through the breast milk (BM). BM is an extremely complex and variable fluid full of bioactive compounds which contribute to the infant development after birth, BM is the most relevant element for the metabolic and immunological programming of the infant. The beneficial immunological effects of breastfeeding during early life are linked to short-term effects such as reducing gastrointestinal and respiratory infections[53], and long-term effects such as reducing obesity and type 2 diabetes[54]. The production of BM, called lactogenesis, starts at the end of the pregnancy, and the first milk fluid is called colostrum[55].

Besides water, BM is composed of macronutrients (carbohydrates, lipids and proteins), micronutrients (oligosaccharides, vitamins and minerals), bioactive compounds (Igs, antimicrobial peptides, growth factors, microRNA), commensal bacteria and other microbial modulatory components (i.e. with prebiotic and postbiotic activity) (Figure 5). Even though all the components are important for the infant nutrition, their proportions vary depending on the lactation stage. In the colostrum the immunological components are prioritized including secretory IgA (sIgA), lactoferrin, leukocytes, CKs and other developmental factors[56]. Later, the transitional milk starts to be secreted (from day 4-5 to 5-6 weeks postpartum). It is enriched in lactose, fat and water-soluble vitamins. Then, after 5 or 6 weeks, the milk is considered fully mature, and its composition remains relatively similar over the lactation period[56,57]. All the BM compounds contribute to the development of the neonate, but the human milk oligosaccharides (HMOs), the microbiome and the immunoglobulinome have exceptional functions (Figure 5).

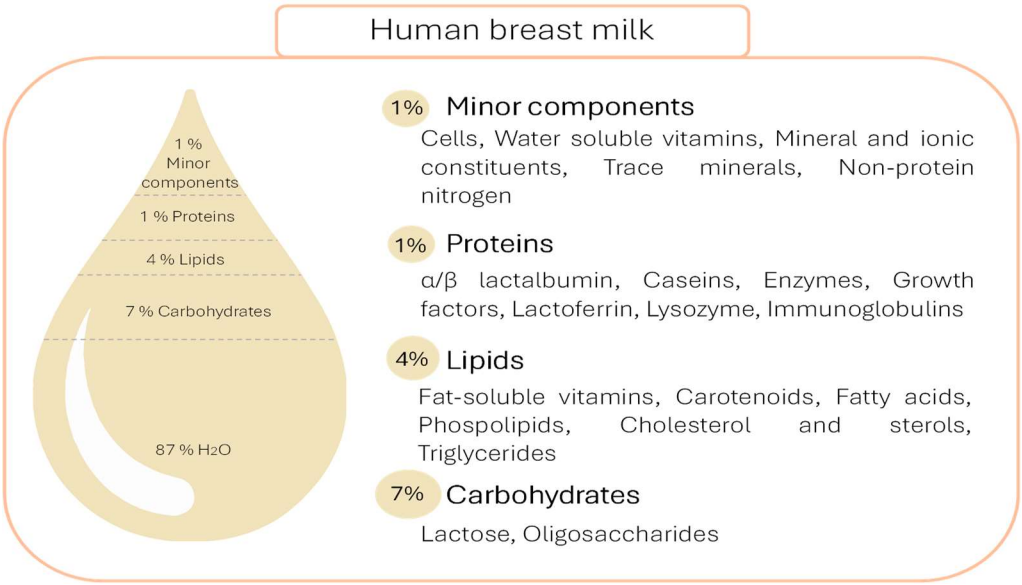



**Figure 5**. Principal components and their proportions in human breast milk.

## 2.1. HUMAN MILK OLIGOSACCHARIDES

HMOs are the most abundant solid component of BM after lactose and fat[58]. HMOs are complex carbohydrates composed of multiple combinations of glucose, galactose, N-acetylglucosamine, fucose and/or sialic acid[59]. It has been described more than possible 200 combinations, where the lactose structure can be elongated the other molecules that from the HMOs. The maternal genotype and the lactation stage contribute to provide about different 150 HMOs in BM[60]. The most abundant HMOs are those fucosylated (45-83%), followed by the acetylated (6-35%) and the sialyated (6-21%) ones. Among the fucosylated HMOs, the 2' - Fucosyllactose (2'-FL) and 3' - Fucosyllactose (3'-FL) are highlighted. In the acetylated group, the Lacto-N-tetraose (LNT) is the most abundant and in the sialyated group, the 3'-sialyllactose (3'-SL) and 6'-sialyllactose (6'-SL) are the most common[61]. In general, the majority of the HMOs decrease in concentration over the course of lactation, with exception of the 2'-FL, which remains constant with time[62] (Figure 6). Despite the widely accepted statement of the 2'-FL as the most abundant HMOs, this only happens in women who actively express the enzyme express *fucosyltransferase 2* (*FUT2*), also referred to as secretor mothers[63].

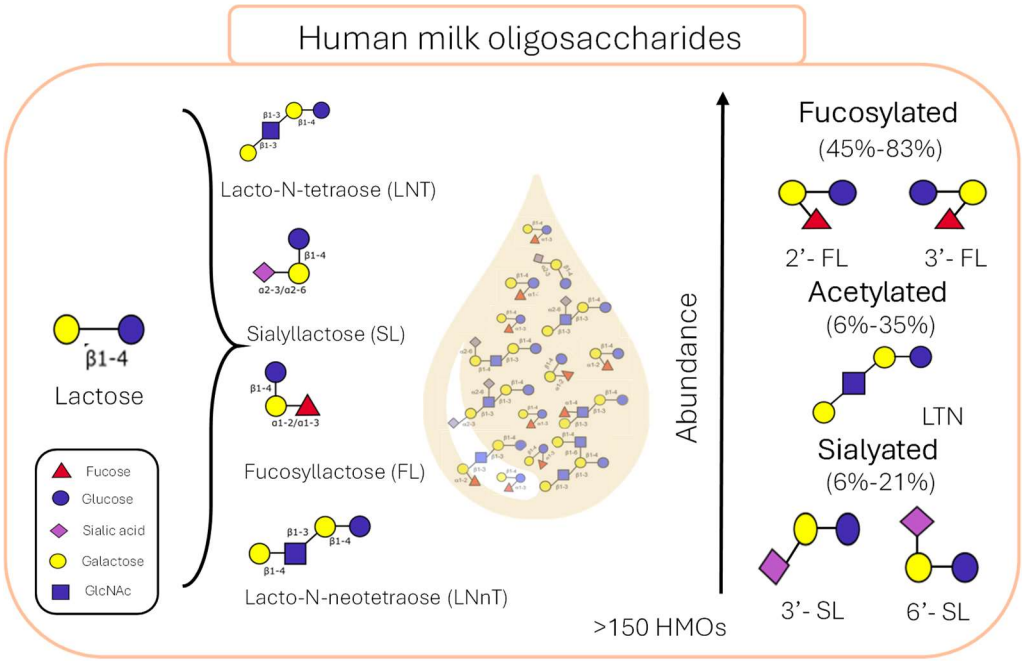



**Figure 6.** Structure of the human milk oligosaccharides (HMOs) and their abundance in human breast milk.

HMOs are considered prebiotic compounds, because they are not digested by the infant and, serve as substrate for the bacteria communities promoting their growth and colonization[64]. Specifically, HMOs promote Bifidobacteria-dominated gut microbiota. Therefore, for the degradation of the HMOs, microbial enzymes are required. Some species such as *Bifidobacterium infantis, Bacteroides fragilis, and Bacteroides vulgatus* have all the necessary enzymes for all HMOs metabolization while some others like *Bifidobacterium breve* and *Bifidobacterium longum* are only able to metabolize some subsets of HMOs[65]. HMOs have also been linked to the blockage of pathogen adhesion to the intestinal epithelium, intestinal cell maturation and enhancement of barrier function[66], and the prevention of gastrointestinal and respiratory infections[67–69]. Due to their reported benefits, HMO have been added to infants' formulas to resemble BM composition[70].

## 2.2. MICROBIOTA

The human BM microbiota comprises bacteria, archaea, microeukaryotes and viruses. However, the bacterial composition is the most studied, and contributes to the infant gut colonization[65]. It is still unclear where the microbes in human BM originate from, but they might derive from the mother's skin, the infant's oral cavity, or from the mothers' gut through an entero-mammary connective pathway[71]. Like the composition of the BM, the milk microbiome also changes depending on the lactational stage, gestational age,

maternal weight, and antibiotic use[72,73]. There are large differences in the reported microbes present in human BM; however, *Streptococcus, Staphylococcus Corynebacteria*, *Propionibacteria*, *Lactobacillus spp* and *Bifidobacterium spp* are generally predominant[71,74]. Breastfeeding contributes to lower microbial diversity in breastfed infants, considered as optimum in early life, with higher proportions of *Bifidobacterium*[65] (Figure 7).

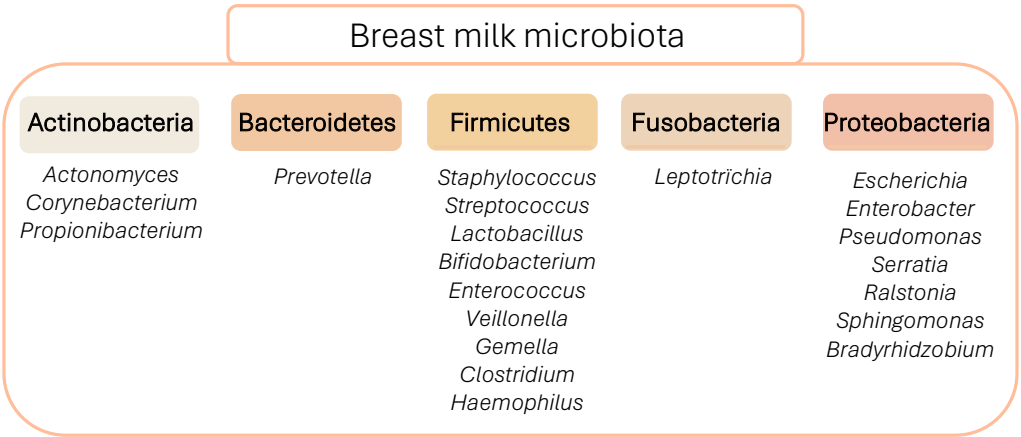



**Figure 7**. Most relevant bacteria found in the human milk.

Animal models have been used to understand human BM dynamics. Like human BM, the rat milk is also highly variable depending on the stage of lactation is dominated by *Proteobacteria* and *Firmicutes*[75]. Among the most abundant genera founded in rat milk *Pasteurella*, *Rodentibacter*, *Rhotia* and *Lactobacillus*[76]. Additionally, previous studies have demonstrated that almost the 65% of the cecal and BM genera are shared, suggesting that there is a clear transmission of the intestinal microbiota to the BM[76].

## 2.3. IMMUNOGLOBULINOME

Among the bioactive compounds of the BM, the Igs confers passive immunization to the neonate while its immune system matures[75]. The composition of the Ig profile of the BM changes according to the species and vary across the lactation stages. In humans, sIgA is the most abundant followed by IgM and IgG, concretely, in the early breastfeeding, there is a higher proportion of IgA and IgM compared to mature milk. Conversely, the proportion of IgG remains relatively stable throughout lactation[75]. In contrast, in rats the IgG predominates followed by IgA and IgM. Through lactation, the IgM did not suffer significant changes while the IgG and IgA increase at the end of lactation[75,77].

The BM Igs come from their local Ig production by the B cells in the mammary gland or their transference from the maternal plasma. Then, they are transferred to the mucosa of the neonate conferring protection until the neonate is able to produce its own Igs[78]. The relevance of the BM Igs has been studied. Particularly, the presence of the sIgA has been

analyzed in depth whereas the importance of IgM, IgG and IgE has been less studied[79]. The sIgA, plays an important role in the maintenance and clearance of microbes, it has been shown that target rotavirus, poliovirus, and other enteric pathogens previously encountered by the mother through infection or vaccination[80–82]. Apart from the passive immunization capacity of the sIgA, it interacts with the gut microbiota, being involved in damping immune responses in early life preventing inflammation, overactive responses, and allergy susceptibility[83]. As mentioned before, in spite of the relevance of the sIgA, in rats, the IgG is the most abundant. It participates in short-term systemic immune response, specifically by its absorption in the neonatal intestine by a receptor-mediated endocytosis[75] (Figure 8).

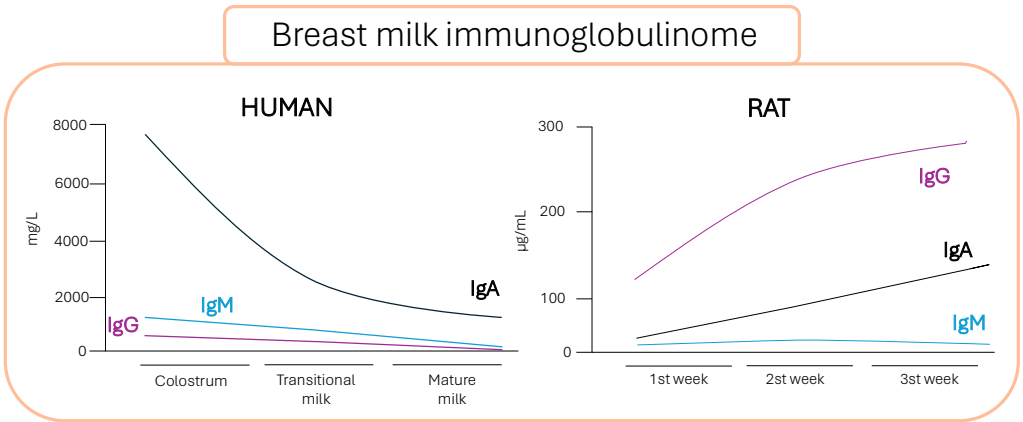



**Figure 8.** Comparison of the immunoglobulin profiles in human and rat breast milk. *Ig, immunoglobulin*.

## 2.4. FACTORS INFLUENCING MILK COMPOSITION

As reported above, the microbiota, Ig profiles and HMOs proportion, among others, are highly influenced by external factors (Figure 9). The majority of BM modulators depend intrinsically on the mother, like the gestational age, the maternal genetics, the health status, the diet and the lifestyle[84,85]. At birth, the delivery mode also influences the composition of the BM, mainly modulating the microbiome[86]. After birth the dietary pattern in breastfeeding is one of the most studied factors. It's been concluded that it does not only modulate the microbiota composition, but also other components of the BM such as HMOs, lactoferrin, fatty acids and vitamins[84,87] (Figure 9).

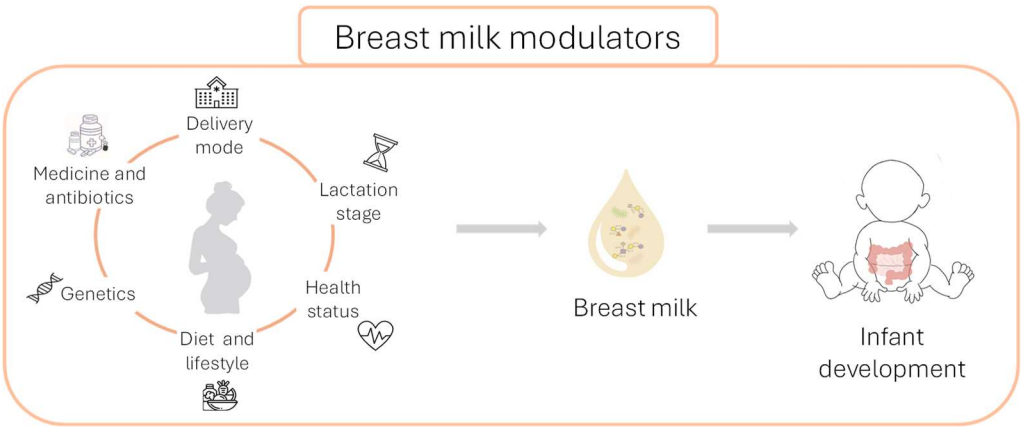



**Figure 9.** Principal breast modulators of the human breast milk composition.

The composition of the microbiome is also highly influenced by all the mentioned factors. In particular, the enrichment of the BM microbiota has been classified as a good approach to improve the infant gut colonization. For example, the ingestion of specific probiotic strains during pregnancy and breastfeeding are effective in promoting *Bidifobacterium* colonization of the neonate[88]. Also, maternal supplementation with a probiotic (*Limosilactobacillus reuteri*) promoted the presence of the administered probiotic in BM and infant feces[88]. It has been also confirmed at preclinical level, specially, for *Lactobacillus fermentum* CETC5716 in rats[89].

During breastfeeding, bacterial infections can occur in the nursing mother, requiring the use of antibiotics. The use of this drug is checked to make sure the resolution of the maternal infection and the infant health. Some of them are considered suitable while others are recommended to be avoided or to cease breastfeeding while taking them due to some adverse effects[90] (*reviewed in section 4.2.*).

## 2.5. INFANT FORMULAS

Infant formulas are effective substitutes of the maternal BM when breastfeeding is not possible, suitable or adequate. To ensure the appropriate infant development, the infant formula composition tries to mimic the BM composition as much as possible[91]. Although infant formulas have become more similar to breast milk over the years, there is still a developmental gap between formula-fed infants and breastfed infants. Formula-fed infants remain more susceptible to metabolic and cardiovascular risks[44]. To prepare the infant formulas, cow milk or soymilk are the most common used bases which are then supplemented with different components to approximate their composition to the BM[92]. Infant formulas are mainly enriched in bioactive compounds present in BM, such as vitamins, nucleotides, certain fatty acids, oligosaccharides, probiotics and prebiotics. Data reported that the supplementation of the infant formula with these compounds generates a similar growth rate and same immunological parameters as BM fed-infants[70].

One of the main reasons for adding oligosaccharides to the infant formulas is due to their prebiotic effect, contributing to the infant gut colonization. The production of the HMOs at large-scale is not an easy process, and in order to solve this difficulty, the addition of some prebiotics, such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), (used single or combined), acidic oligosaccharides, oligofructose, inulin has shown positive results in preclinical and clinical studies. For example, GOS and FOS are well known to increase the abundance of *Bifidobacterium,* also called as bifidogenic effect[44].

# 3. IMMUNE AND MICROBIAL DEVELOPMENT OF THE NEWBORN

## 3.1. IMMUNE SYSTEM MATURATION

In the first days of life, the newborn is exposed to a huge number of microorganisms which activate its immune system. At birth, the mucosal and systemic immune system remains immature but most infants are able to respond to immunization[93,94].

The innate immune system is the first line of defense against infections, the main involved cells are neutrophils, Ag presenting cells (APC) and NK cells. Neutrophils play an important role in bacterial clearance during infection. However, at birth, they have lower functionality compared to adults, linked to a reduction of the expression of some surface markers and receptors such as L-selectin (CD62L) and Toll-like receptors (TLR) 2 and TRL 4[95]. APCs are also present in all the required tissues, and similar to neutrophils, they have an "immature" phenotype reducing their functionality[95]. The complement system is important for eliminating microorganisms and, the newborn completement system is immature because all its components are present but in lower concentrations compared to adults[96]. The NK population changes from the fetal stage (it increases its cytolytic function though fetal stage) and at birth, the proportion of the NK increases although the cytotoxic activity suffers a reduction[97].

The newborn adaptive immune system remains a bit naïve at birth as it has been exposed to a little number of Ag *in utero*, for this reason to counteract infections the innate immune system plays a more important role[98].

The T cells, including Tc (CD8+) and Th (CD4+) polarized to Th1 or Th2, are in lower proportions compared to adults. During the first weeks of life, T cells suffer clonal expansion increasing their levels, however the ratio of the naïve T cells remains proportionally higher due to the immaturity of the APCs to present Ag[99,100]. Thus, during early life the characteristic polarization towards a Th2 response in fetal stages is reverted towards an equilibrated Th1/Th2 response. As a consequence, neonates on some occasions are more susceptible to viral infections and experiment some difficulties to fight against them because the Th1 response is suppressed[96].

Regarding to the B cell functionality at birth, they exert reduced activity in secreting Ig because the interaction with the APCs is diminished. As the exposition to Ag is low due to the low efficacy of the APC- B cells interaction, the isotype switching capacity from IgM to IgG is lower[98]. Although, the own production of Ig is limited, the neonate has higher levels of Igs in all the compartments due to the maternal acquisition through the placenta or through the BM after birth. Additionally, to the acquired Igs from the mother, during the first year of life, the infant starts to produce his/her own Igs[101] (Figure 10).

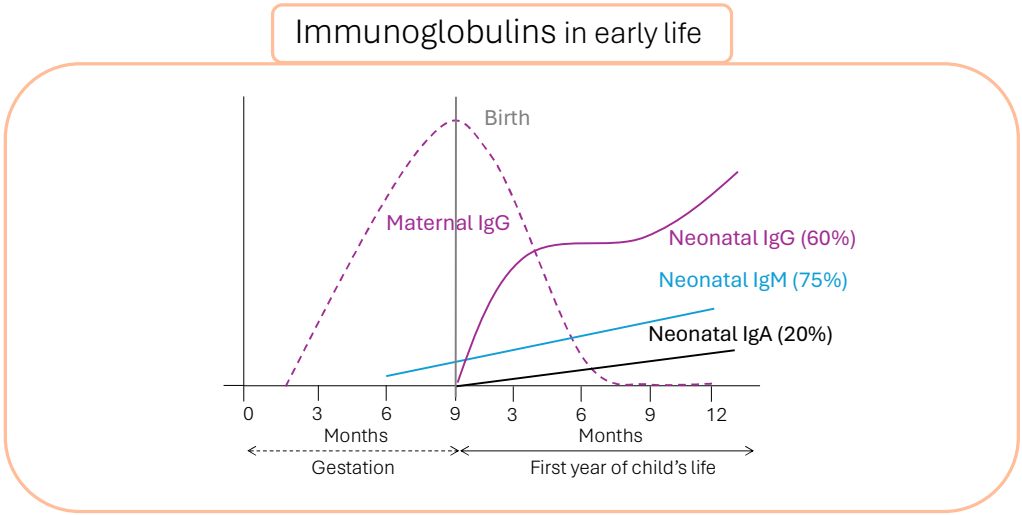



**Figure 10**. Evolution of the fetal and infant immunoglobulin levels in early life. Adapted from[101]. *Ig, immunoglobulin*.

In early life as the newborn faces environmental and external Ags, both T cellular and B humoral responses continue their maturation. One of the principal routes of Ag exposure is through the intestinal barrier[102].

## 3.2. NEONATAL MICROBIOTA

The timeline of the initial microbial seeding is controversial. Over a century ago, the sterile womb theory was accepted. Then, the detection of bacterial DNA opened the window about the presence of live bacteria in the placenta[103]. However, in the recent years, microbial products and fragments of bacterial DNA have been detected in the placenta, amniotic fluid, umbilical cord blood, fetal membrane and meconium. These findings have set a new theory of the microbial colonization and supports the *in utero* colonization hypothesis. Even though, the literature of this data remains ambiguous due to the methodological limitations to obtain samples[104].

Following this last hypothesis, the neonatal microbiota is influenced prior to birth by among other maternal factors, the maternal stress and the maternal diet. The exposure to maternal stress before delivery may impact on the vaginal microbiome which may lead into a stressed microbiota phenotype in the offspring[105]. Similar results are found when the maternal diet is analyzed. Higher intakes of fiber or sugar are associated with beneficial or harmful changes, respectively[106,107]. During birth, the gestational age, the mode of delivery and the term or pre-term birth also affect to the neonatal microbiota.

For instance, vaginal delivery exposes the neonate to the vaginal microbiota affecting the intestinal microbiota[108]. Even though, the material vaginal microbes usually do not settle the infant gut, their direct contact is linked to a higher colonization of bifidobacteria and

*Bacteroides*. In contrast, the gut microbiota of infants born by C-section presents low abundance of bifidobacteria and almost total lack of *Bacteroides*[108,109]. And, after birth, the infant nutrition (BM or infant formula), the antimicrobial exposure or infections continue to shape the microbiota development[110,111].

The neonatal microbiota at birth has a low diversity and is rich in aerobes and facultative anaerobes, characterized by Bacteroides, Actinobacteria (*Bifidobacterium*) and Proteobacteria (mainly *Escherichia*, *Staphylococcus* and *Enterococcus*)[112,113]. The proportion of Proteobacteria in the neonate seems to be linked to the increased proportion of Proteobacteria in the maternal gut. During infancy, the microbiota continues its colonization process by increasing the diversity, raising the proportions of *Bifidobacterium* and Bacteroides and reducing the Proteobacteria abundance. In the childhood, the diversity continues growing, increasing strict anaerobes and facultative anaerobes, characterized by *Bacteroidaceae* and *Ruminococcaceae* [112] (Figure 11).

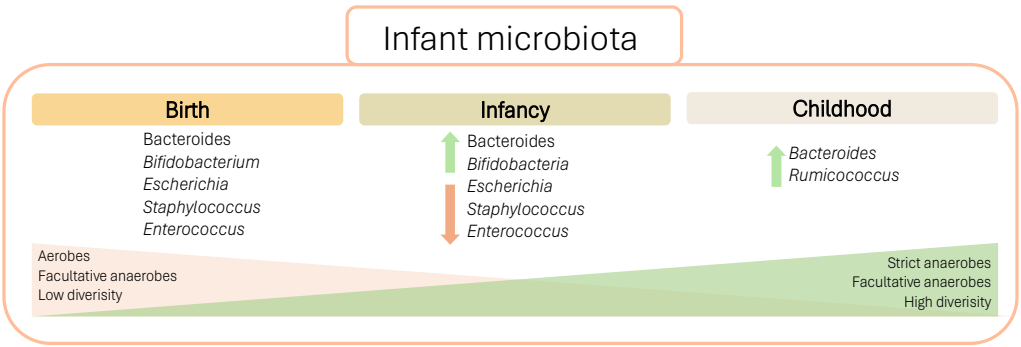



**Figure 11**. Evolution of gut microbiota in early life.

One of the mechanisms of microbiota transference from the mother to the infant after birth is through the maternal BM and the maternal skin. Nourishing during early life with BM, provides selective advantages to different microbes mainly to the *Bifidobacteriaceae* family. Breast-fed infants present higher abundance of Bifidobacteria and lower numbers of enterobacteria and clostridia after 1 month of age. Compared to them, the formula-fed infants, undergo a slower colonization of Bifidobacteria and the proportions of *Enterococci*, *Lactobacilli* and *Clostridia* are higher[108].

The introduction of solid food results in a large increase in bacterial numbers and evolution towards that of an adult individual. This microbiota shift is named as *weaning reaction* and contributes to the correct development of the immune system where *Bifidobacterium* proportions decrease until adult proportion and other species such as *Lachnospiraceae, Bacteroidaceae* and *Ruminococaceae* increase its presence[31,114,115]. The reduction of the abundance of *Bifidobacterium* in adulthood explain why this genus is more associated with early life as contribute to the metabolization of the HMOs[31].

Overall, the intestinal microbiota plays an important role in the maintenance of a healthy status[25]. Neonatal microbiota exhibits high adaptability and plasticity. However, the disruption of the early life microbiota is linked to necrotizing enterocolitis, obesity, diabetes, inflammatory bowel disease, allergies, asthma, atopy, autoimmune diseases and neurological diseases later in life[31,116].

# 4. EARLY LIFE EXPOSOME

During fetal and early life stages, the fetus and the newborn are exposed to several factors that could modulate the offspring outcomes. In early life the offspring is exposed to multitude of external factors englobed as the “exposome”. In particular, in early life, the exposure to certain conditions or factors may affect to infant outcomes contributing to the denominated “early life programming”[117] The influencing factors of the early life programming can be grouped into environmental, maternal and specific of the infant[118,119].

Environmental factors comprise the geographical location and exposure to harmful substances. Maternal factors include everything that may influence pregnancy and lactation such as health status, infections, medication intake, genetics, dietary habits, delivery method, and breastfeeding decisions. Among the infant factors, the incidence of infections, the antibiotic treatments and the diet are found[119–121] (Figure 12). In this chapter, these highlighted factors will be discussed.

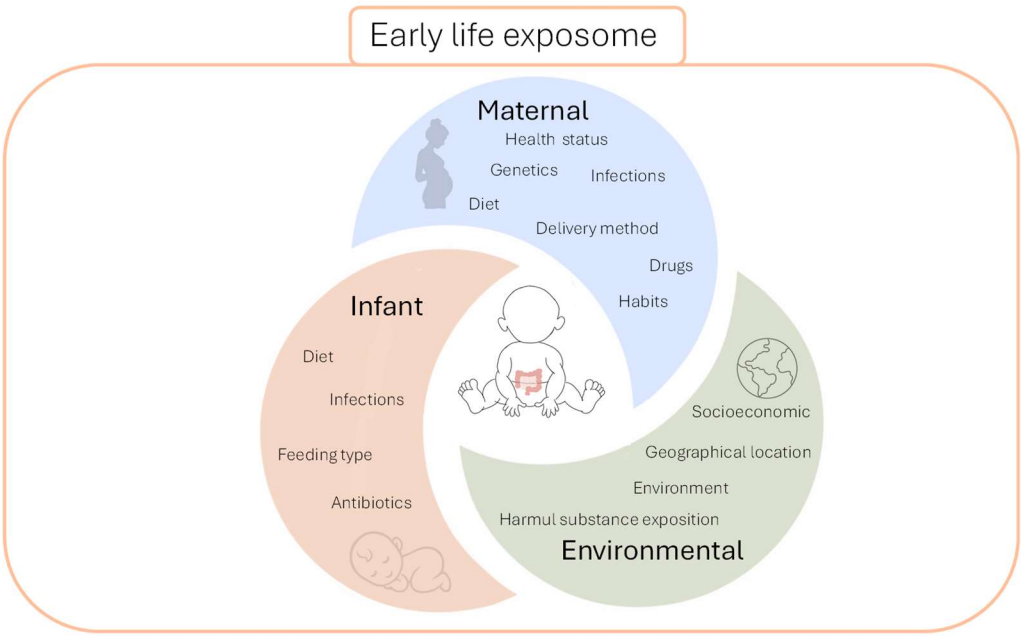



**Figure 12**. Principal factors influencing the early life exposome classified as maternal, environmental and infant factors.

## 4.1. DIET

Nutrition is considered as one of the most critical environmental factors influencing the embryo and fetal development. In fact, the maternal diet in pregnancy has been established as one of the most important factors in the “early life programming” of the offspring due to its long-term implications. The effect of the maternal diet influences the

infant development during the preconception period, conception period and postnatal period.

In pre-pregnancy, a high-quality diet helps to decrease the risk of preterm birth, gestational diabetes mellitus development, infant obesity and behavioral problems, among others[122,123].

Later, during the perinatal period, diet alterations including undernutrition or overnutrition, influences the central nervous system development and the metabolism, outcoming with the alteration of the cognitive processes, obesity, endocrine alterations, etc[124]. Also, deficiencies in macronutrients and micronutrients have been linked to an increased risk of infertility, fetal structures abnormalities and long-term illnesses. Macronutrients, proteins, fats and carbohydrates, contribute to the correct nourishment of the fetus during pregnancy, and an impairment in their acquisition may result in alterations in the body weight, growth and brain development[125–127]. To date, the relevance of the carbohydrates relies on its digestibility, for this reason, they are classified in digestible and non-digestible (dietary fiber). The dietary fiber is fermented by the gut microbiota resulting mainly in the production of SCFAs[128,129]. Regarding to micronutrients, deficiencies in iron, iodine, calcium, vitamin D and folic acid, are the most examined during pregnancy. Iron participates in the $O_2$ transport to the tissues, deficiencies in iron trigger anemia, affects the lipid peroxidation, the glucose metabolism and the oxidative stress. Iodine contributes to hormone secretion and tissue and organ formation, deficiencies in iodine affects the circulating thyroid hormones leading to spontaneous abortion, neurological disorders and higher rate of perinatal mortality. Calcium is critical in the bone mass formation. Thus, deficiencies in calcium lead to preterm delivery and blood pressure alterations. At the same time, calcium supplementation reduces gestational hypertension and preeclampsia. Vitamin D regulates CKs that collaborate into the embryo implantation and hormone secretion. And, vitamin D deficiency is associated with preeclampsia, gestational diabetes mellitus, impaired skeletal development and allergic disease. Acid folic participates in the biosynthesis of the DNA and RNA, and its deficiency led to neural tube defects[127].

After birth, maternal diet also influences the baby by modulating her BM composition. The mother's intake of protein, fatty acids, carbohydrates and vitamins has been reported to affect the BM composition[84]. Some studies have concluded that higher BM protein content is due to a high-protein diet, however, some others did not correlate the BM protein with the maternal protein intake[130,131]. Contrary to the controversial data about the protein composition of the BM, the milk fats are strongly correlated with the maternal dietary intake, mainly trans and polyunsaturated fatty acids[132–134].

Once of all these effects of nutrition on the newborn, at pre-conception, pregnancy or nursing, researchers and clinics agree that a healthy diet contributes to a correct fetal and neonate development. In particular, mediterranean diet, rich in fruits, vegetables, whole

grain cereals, legumes, extra virgin olive oil, fish and nuts, is one of the most suitable nutritional patterns during pregnancy as it contributes to control the most common health problems in pregnancy, like gestational diabetes mellitus, insulin sensitivity, obesity and abnormalities in the nervous system development[124,135] . To ensure the accomplished of the nutritional requirements during pre-conception, conception, and breastfeeding, the supplementation of the maternal diet with folic acid, iodine and calcium are universally recognized as beneficial for pregnant women[136]. Additionally, researchers agree that complementing the maternal diet with other supplements such as probiotics, prebiotics and synbiotics could improve the maternal and fetal status, specifically the gut colonization, during these compromising periods[137] (*reviewed in section 5*).

## 4.2. ANTIBIOTICS

During pregnancy, the immune system is biased towards a Th2-response to allow the correct fetal development. This fact also induces a certain immune system suppression, leading to a higher susceptibility to infections[46]. To solve these pregnancy infections, the use of antibiotics is a risk-versus-benefit decision because antibiotics destroy large parts of the microbiome, mitigating the bacterial infection but also inducing a loss of health-promoting bacteria. Subsequently reducing the expression of antibacterial products, increasing the susceptibility to infections[138]. In spite of this, the non-treatment with antibiotics could evolved in significant fetal risk including abortion, prematurity and low birth weight[139]. For these reasons, the World Health Organization (WHO) consider antibiotic intake during pregnancy and after labor only following medical indications and using the narrowest antibacterial spectrum and the simplest effective dose[140].

The prenatal exposure to antibiotics may result in microbiota alterations that may impact in short and long-term, such as childhood obesity, neurologic disease, atopic disease, asthma[31]. During labor, the antibiotic exposure impacts the microbiota composition of the neonate, with lower abundance of Actinobacteria, especially *Bifidobacteriaceae* and overrepresentation of Proteobacteria[46]. Afterwards, in early life, the continuous antibiotic intake may affect to the proportion of the commensal intestinal microbiota which could lead into long-term consequences for host immunity such as increasing the risk of metabolic disorders and immune-mediated diseases[141].

## 4.3. EARLY LIFE INFECTIONS

Early life infections include the maternal infections during pregnancy and their transmission to the fetus as well as postnatal infections. All of them will influence the offspring development.

The most common studied infections during gestation are the ones that may cause complications to the pregnancy and may be vertically transmitted to the fetus, and infections that can lead into birth defects. Among them, viral and bacterial infections are highlighted[116]. Viral perinatal infections increase the risk of severe illness and mortality for

both the mother and the fetus[142]. The effects of the viral infections during pregnancy on the neonate are highly diverse. For instance, herpes simplex virus and cytomegalovirus infections could implicate the transmission of the infection to the infant. Varicella zoster virus is associated with maternal and fetal morbidity and mortality[142]. Bacterial infections are also common in pregnancy including urinary tract infections, pelvic inflammatory disease, bacterial vaginosis, acute diarrheal infections and upper respiratory tract infections[143–145]. Similar to viral infections, the most common adverse outcomes of these infections are preterm birth, birth defects and miscarriage[116]. To fight the bacterial infections, antibiotic treatments must be analyzed, as some of them are not recommended during pregnancy as it may affect the fetal development (*reviewed in section 4.2*.).

After birth, maternal infections may also affect the infant development if the infant is nourished with BM. It is true that the transmission of pathogenic organisms through maternal breast to infant oral cavity after birth is relatively rare compared to the transmission during prenatal period or during delivery. However, some microorganisms are able to infect the neonate through maternal-infant breast contact, such as *Staphylococcus aureus* or *Streptococcus agalactiae*. Both pathogens are common in the mother during lactation and may cause an impaired development of the neonate[146,147]. However, to reaffirm that maternal infections do not typically reach the infant through BM, recent studies have evaluated the presence of SARS-COV-2 RNA in the BM during maternal infection[148]. As predicted, SARS-CoV-2 RNA was not detected in BM and, consequently, lactation is safe for the infant.

Finally, during the neonatal period, regarding the first 28 days of life, the lack of structural barriers and an immature immune system also increase the rate of infections and mortality to the neonates. Indeed, 48% of the deaths during the neonatal periods are attributable to infections[149], including bacterial, fungal and viral. The most frequent bacteria agents have not change significantly over recent years and remain dominated by Group B *Streptococcus* and *Escherichia coli*[150]. The most common fungi that cause infection are *Candida spp*[151]. And, the most viral relevant pathogens are parenchovirus, enterovirus (EV) and rotavirus (RV), being this last one of particular interest in this thesis[152].

### 4.3.1. ROTAVIRUS

RV is the leading cause of acute gastroenteritis which have more severe manifestations in in infants compared to adults, being a major cause of mortality in low-income countries and a significant cause of morbidity in developed countries[153]. RV infections still have a high incidence among children <5 years old[154]. Additionally, in 2019, RV infections still accounted for almost 20% of deaths from diarrhea[155]. There are 9 species of RV (from A to I) and RV A provokes more than the 90% of RV infections in humans[156]. RV is highly contagious among children. Repeated infections with different viral strains are possible with several episodes of gastroenteritis in the first year of life. Infections may be

asymptomatic, may cause self-limited watery diarrhea, or may result in severe dehydrating diarrhea with vomiting, fever and abdominal pain. RV is transmitted by fecal-oral contact and by contaminated surfaces and hands, and also respiratory spread[156].

RV belongs to *Reoviridae* family and are non-enveloped viruses that have three layers of proteins that contains 11 segments of genomic dsRNA encoding for six structural and six non-structural viral proteins, VP1-7 and NSP1-6, respectively[156,157]. *In vitro* experiments showed that trypsin cleavage of VP4 into VP5 and VP7 is necessary for virus entry into cells. Analysis of the structural domains of VP5 and VP7 reveals some information about the early interaction between the virus and the host cell[158] (Figure 13).

RVs infects and replicates in the mature, non-dividing enterocytes in the middle and tip of the villi and in enteroendocrine cells in the small intestine[159]. The intestinal cell invasion leads to disruption of the intestinal epithelial cell (IEC) homeostasis due to villus atrophy, increases epithelial cell turnover, enhances apoptosis and formation of large vacuoles in enterocytes. All this will induce the activation of the innate and adaptive humoral and cellular response[160].

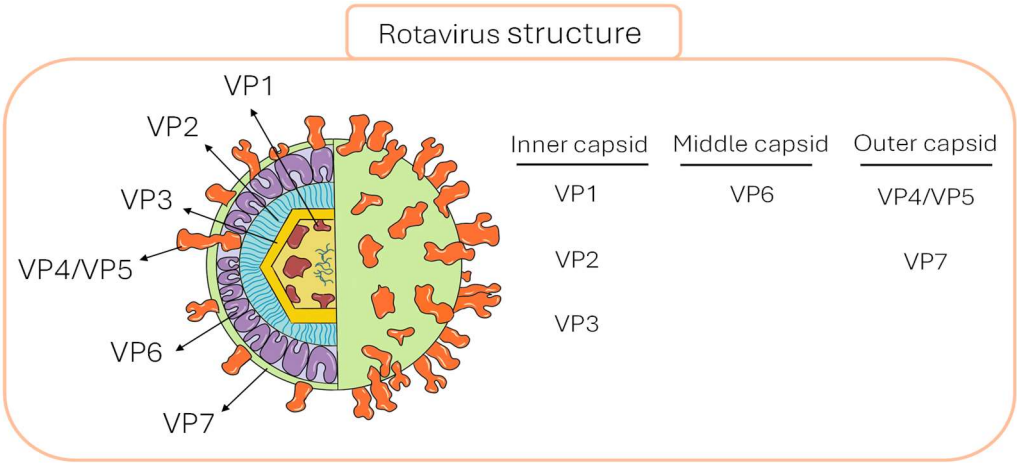



**Figure 13**. Rotavirus structure and representation of the proteins of each capsid. *VP, viral proteins.*

### 4.3.2. ROTAVIRUS INFECTION AND REPLICATION CYCLE

The RV entry in the host cells is mediated by the VPs, which attach the RV to the cell surface. In the attachment, the RV recognize different surface molecules such as sialic acid, α2β1, and histo-blood group Ag (HBGAs) and the VP4 and VP7 will directly participate in this step. Later, the viral particle is internalized into the host cell by endocytosis, following the clathrin-dependent pathway. Once inside the endocytic vesicle, the outer layer capsid disintegrates, and the double-layer particles are released into the cytoplasm. These particles contain the viral RNA, which will use the host cell mechanism for

translation and replication of the viral proteins and RNA. Then, the viral proteins and RNA start to assembly the viral particles to continue its organism dissemination through membrane protrusions[158,161].

### 4.3.3. ROTAVIRUS IMMUNE RESPONSE

RV immune response starts first in the mucosal compartment. In the intestine, the innate immune cells recognize the viral particles through the pattern recognition receptors (PRRs). The most common PRRs involved are the retionoid acid-inducible gene (RIG-1), the melanoma differentiation-associated gene 5 (MDA-5) and TLRs, mainly TLR3. After the RV detection, the innate immune cells activate its antiviral status by inducing the production of IFN type I (IFN a/b) and type III (IFNy) to control the viral infection. Thus, the secretion of CKs such as IL-18 and IL-22 collaborate in the viral clearance and activate the adaptive immune response[162–164]. The activation of the adaptive response, mediated by the innate cells and the secreted CKs, produces the expansion of RV-specific B cells and T cytotoxic (T CD8+) cells. B cells start to secrete large amounts of Igs, mainly IgA and IgG against VPs. The stimulation of the adaptive immune response, mainly mediated by the intestinal IgA, will contribute to reduce the severity of further RV infections[165,166] (Figure 14). Additionally, to the local immune response, the RV can spread from the gut to the entire organism through the viral attachment to specific surface receptors, entering into the bloodstream to arrive to the central nervous system[167].

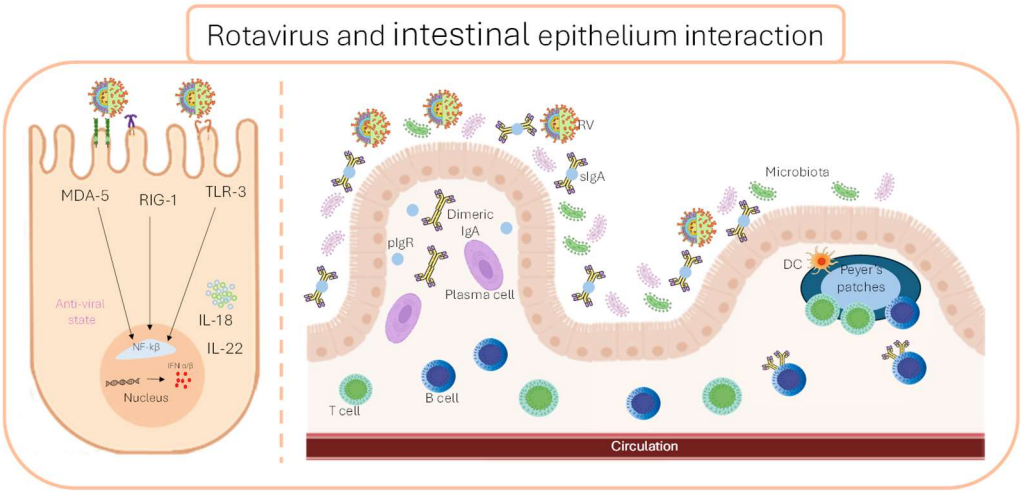



**Figure 14**. Mechanism of host defense against rotavirus infection in the intestinal epithelium. *MDA-5; melanoma differentation-associated gene 5; RIG-I, retinoid acid-inducible gene 1, NF-κB, nuclear factor-κB; IFN, interferon; pIgR, polymeric Ig receptor; sIgA, secretory IgA; DC, dendritic cells*.

Although the host immune response has many pathways to counteract the RV infection. The viral particles have evolved in several strategies to escape from the host immunity and

ensure their survival and spread. One of the most studied mechanisms is the interruption of the IFN-mediated response at different steps[157].

### 4.3.4. THERAPEUTIC STRATEGIES

The oral and intravenous rehydration remains the most used RV treatment today, however, this solution only reduces the symptomatic manifestations without affecting the course and severity of the infection[158]. In 2009, the WHO made a recommendation for the global use of the approved RV vacccines, Rotarix® (RV1) and RotaTeq ™ (RV5), particularly in the developing countries with high mortality rate due to this infectious diarrhea[168]. The introduction of RV immunization programs has reduced mortality by 60%, however vaccine efficacy in developing countries is still low[158].

The Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have not approved any drugs for the RV gastroenteritis. One of the most recommended guidelines for the treatment and prevention includes probiotic supplementations[169–172] (*reviewed in section 5.2*). In addition to this, as the majority of infections happens in early life, breastfeeding is recommended due to its richness in oligosaccharides and proteins that inhibit RV infection of the host and modulate host immune function[173].

# 5. BIOTICS: PROBIOTICS, PREBIOTICS AND SYNBIOTICS

The principal and most used biotic supplements which stimulate the immune system are probiotics, prebiotics and synbiotics. However, postbiotics beneficial substances resulting from certain fragmentation or metabolism, have been also studied due to their beneficial effects on the host and on the microbiota[174,175]. Here, we will discuss the benefits and mechanisms of action of probiotics, prebiotics and synbiotics.

## 5.1. PREBIOTICS

Prebiotics were defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" [176]. To classify a substrate as a prebiotic, it should be resistant to the acidic pH of the stomach and to the hydrolytic enzymes, and not be absorbed in the gastrointestinal tract, it should be fermented by the intestinal microbiota and should stimulate its growth and/or activity, enhancing the host's health[177]. The most common beneficial effects of the prebiotics are changes in the gut microbiota, health maintenance, protection against gastrointestinal disorders, and improvement of the immune system functions are found[178].

There are different types or prebiotics, and among them carbohydrates are highlighted. These are mainly composed of fructans, galactans, starch and glucose-derived oligossacharides and other oligossacharides (including pectin) and non-carbohydrate oligossacharides (cocoa-derived flavanols)[178].

### 5.1.1. MECHANISMS OF ACTION

The mechanisms of action of the prebiotics can be dependent or independent of the microbiota (Figure 15).

**Microbiota-dependent mechanisms**

The mechanisms dependent on the prebiotic microbiota are linked to the stimulation of the microbiota's composition or activity[179]. Prebiotic substrates serve as "food" for commensal bacteria. Prebiotics encourage the activities of the host microbiota that have beneficial functions, including those commonly used as probiotics (*Lactobacillus* and *Bifidobacterium*). Prebiotics are selectively used by intestinal bacteria leading to changes in the microbiota composition. *Bifidobacterium* are the most common stimulated bacteria after prebiotic intake, although in some cases the increase of lactobacilli is also stimulated[180].

The final products of prebiotic fermentation are SCFAs, lactate, pyruvate, and succinate. The principal SCFAs are acetic, butyric, and propionic acids. According to this, the most relevant microbiota-dependent mechanism of action of the prebiotics are due to the immunomodulatory effects of the SCFAs and their interaction with the gut epithelial cells and immunological cells[181]. Additionally, as a consequence of the release of the SCFAs,

the intestinal pH becomes more acidic and facilitate the absorption of some minerals and ions[182,183].

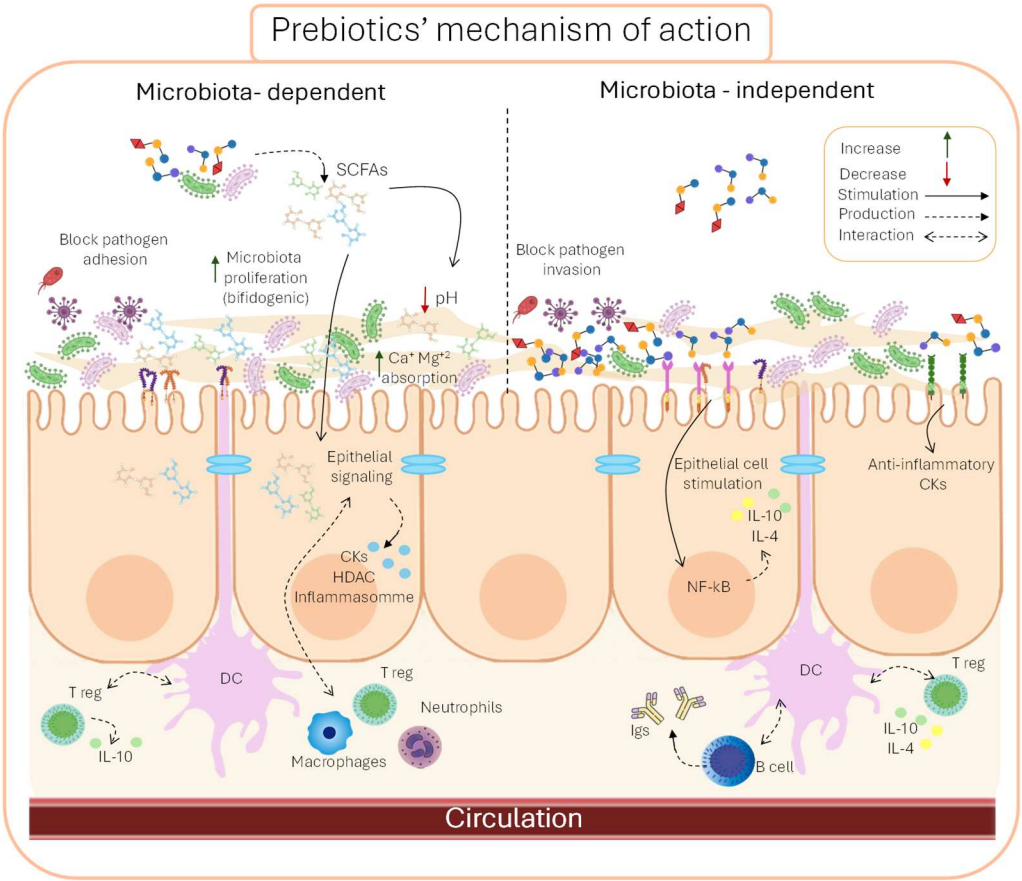



**Figure 15**. Microbiota dependent or independent mechanisms of action of prebiotics. *SCFAs, short-chain fatty acids; CKs, cytokines; HDAC, histone deacetylase; DC, dendritic cell; T reg, regulatory T cell; IL, interleukin; NF-κB, nuclear factor- κB; Ig, immunoglobulin*.

Gut epithelial cells uptake the SCFAs through three primary pathways: surface signaling receptors, passive diffusion, and soluble transporters. Surface signaling involves receptors such as free fatty acid receptors (FFAR) 2 and 3[184]. These uptake mechanisms collectively result in changes of the epithelial signaling, which ultimately regulates the immune system. The changes induced in the immune system by SCFAs include the activation of intestinal cells to produce chemokines and CKs, epigenetic modifications via histone deacetylase (HDAC) inhibition, and the activation of the inflammasome to maintain the integrity of the epithelial barrier[184–186] .

The interaction of the SCFAs with the immune system is mediated in the GALT by the DCs, macrophages and T reg cells[181]. DCs recognize SCFAs via TLR2 and TLR4 leading to the up-

regulation and down-regulation of membrane molecules, and the secretion of CKs. This process promotes the polarization of T cells towards a Treg phenotype and the production of IL-10[187]. Additionally, SCFAs directly interact with naïve T cells, inducing their polarization into Tregs. Furthermore, SCFAs modulate macrophage phenotypes, promoting M2 polarization, enhance NK cell activity and block neutrophil functionality inhibiting their pro-inflammatory activities[187–189].

**Microbiota-independent mechanisms**

As mentioned above, some of the immunomodulatory effects of the prebiotics are linked to the SCFAs produced by the microbiota. However, the use of germ-free mice and *in vitro* models have shown that prebiotics also exert beneficial effects in a microbiota-independent way. These mechanisms primarily involve the ability of prebiotics to interact with gut epithelial and immune cells through various surface receptors, such as TLRs[181].

Gut epithelial cells recognize prebiotics through TLR4 and TLR2[190,191]. Ligation of TLR4 and TLR2 induces the nuclear factor-κB (NF-κB) activation and, the production of anti-inflammatory CK such as IL-4 and IL-10[181].

In the interaction between the immune cells, DCs, macrophages and monocytes, and the prebiotics, surface receptor are also involved. TLR4 induces the activation of the NF-κB to lastly secrete IL-4 and IL-10 and promotes Treg cells polarization[192,193]. Also, other surface molecules such as the peptidoglycan recognition protein 3 (PGIyRP3) participates in prebiotics recognition leading to the production of anti-inflammatory CKs[194].

As a results of the direct interaction of the prebiotics with the host cells, an anti-inflammatory environment is induced. The anti-inflammatory milieu joined to the stimulation of the surface receptors helps reduce the interactions with pathogens. As consequence, pathogen virulence and adherence possibility are diminished.

## 5.2. PROBIOTICS

Probiotics were defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”. To define bacteria as a probiotic it has to be a safe strain and has to maintain viability and bioactivity during processing and storage. Additionally, it has to survive and resist to gastric acid and bile acids among others[195].

Probiotics offer a wide range of health benefits, with a primary focus on gastrointestinal health. Despite of the general benefits of probiotics, they are strain specific. Specifically, some of the conferred benefits are shared among different species or among bacteria from the same species while some others are specifics from each strain[196–199].

One of the most relevant benefits of probiotics are due to their immunomodulatory effects. Probiotics interact with the intestinal epithelial cells and with the immune cells of

the GALT. As consequence, they potentiate the intestinal epithelial barrier by enhancing its functionality and defense functions, and then reducing the possibility of pathogen invasion. Additionally, probiotics mainly collaborate in the induction of anti-inflammatory milieu and contribute to the modulation of the microbiota composition of the gastrointestinal tract, which is involved in the maintenance of a healthy well-being. Moreover, probiotics improve the clinical outcomes of some pathologies. For example, probiotics contribute to reduce the incidence of antibiotic-associated diarrhea, reduce colic symptoms in breastfed infants, reduce necrotizing enterocolitis in preterm infants and reduce acute infectious diarrhea in early life[33,200].

### 5.2.1. MECHANISMS OF ACTION

The defensive mechanisms of action of the probiotics can be mediated by their direct effect on pathogens or through their interaction with host cells (Figure 16).

**Direct mechanisms**

Probiotics are able to interact directly with the host immune system and microbiota. When probiotics arrive to the intestinal epithelium attach to it, competing with the pathogens for the adhesion places and reducing the possibility of pathogen adhesion[201]. Therefore, probiotics also inhibit bacterial invasion. Once in the intestinal epithelium, probiotic bacteria start to ferment the un-digestible carbohydrates producing as final products SCFAs. As mentioned before, the release of SCFAs in the gut reduce the luminal pH, limiting the growth of pathogens[200]. Additionally, the produced SCFAs exert immunomodulatory effects in the host as reviewed in *5.1. section*.

Apart from the SCFAs, probiotic bacteria release bacteriocins. These molecules attack membrane of the pathogenic bacteria inducing cell permeabilization and pore formation. Therefore, inactivating the harmful bacteria [202]. There are four types of bacteriocins based on their structures, molecular weights and post-translational modifications. Their effects are directly on pathogenic agents such as *Helicobacter*, *C. difficile*, *E. coli* and RV, among others, inhibiting their growth[203]. Additionally, probiotics codify for different enzymes which contribute to host nutrient digestion. The most common enzymes are bile salt hydrolases (BSH), beta-galactosidases (β-Gal) and lactocepin[204]. BSH are microbial enzymes that participate in the bile acid metabolism being involved in changes in the microbiota and in the lipid's metabolism[205]. β-Gal enzymes participate in lactose digestion through its broken down to galactose and glucose[203]. And, lactocepin which interacts with CKs, specifically, selectively degrading pro-inflammatory CKs[206].

**Indirect mechanisms**

Probiotics interact in the gut with the intestinal epithelial cells and with the immune cells of the GALT like DC, macrophages and lymphocytes.

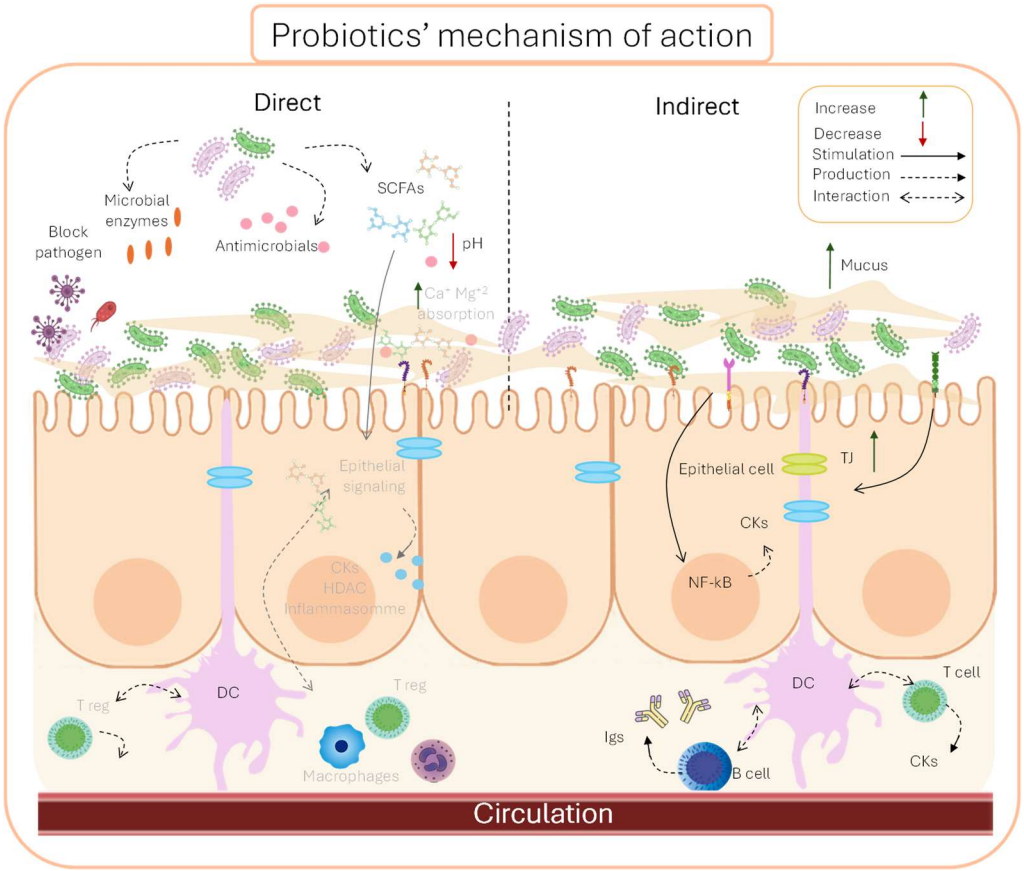



**Figure 16**. Mechanisms of action of probiotics. *SCFAs, short-chain fatty acids; CKs, cytokines; HDAC, histone deacetylase; DC, dendritic cell; T reg, regulatory T cell; NF-κB, nuclear factor- κB; TJ, tight junction; Ig, immunoglobulin*

One of the most described benefits of prebiotics is the reinforcement of the intestinal barrier. To achieve this, probiotics contribute to the preservation of the intestinal structure by modulating the expression of TJ proteins such as *zonula occludens* 1 (Zo-1) and occludin (Ocln). Additionally, stimulate epithelial cells to produce mucus, mainly upregulating the expression of *Muc2* and *Muc3*, to protect the intestinal epithelium from the direct contact with bacteria in the intestinal lumen[207].

Intestinal epithelial cells recognize the luminal bacteria using cell surface receptors such as TLR2 and TLR4. The host cells are able to detect the probiotic bacteria and the pathogenic bacteria. In case of detecting pathogenic bacteria, the immune system activates the NF-B pathway to lastly produce inflammatory CKs[201]. However, the detection probiotic bacteria block the NF-κB pathway and stimulate the production of protective CKs that enhance the epithelial cell regeneration and inhibit epithelial cell apoptosis[201,208]. The probiotic interaction with host cells also led to the production of antimicrobial

peptides like defensins. These molecules act as immune cell stimulators to produce CK and chemokines and recruit effector cells [209].

The immune cells of the intestinal surroundings form the GALT. Probiotic bacteria can be recognized by immune cells of the GALT. For example, DCs detect the microbial-associated molecular patterns (MAMPs) using the TLR2 and TLR4. This interaction between the DCs and the probiotic bacteria will lead to the activation and polarization of the T cells towards a Th1, Th2 or Treg phenotype[201,210]. Apart from the polarization of the T cells, probiotics exerts changes in their distribution, stimulating their migration towards the MLN. B cells can also detect the probiotic bacteria and induce the production of Ig to potentially neutralize pathogens[201].

Furthermore, probiotics exert modulatory effects on the endocrine and nervous system through the nervous receptors and intestinal molecules such as serotonin, melatonin, and histamine among others, that participate in the gut-brain axis[211].

## 5.3. SYNBIOTICS

Synbiotics were defined as "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host"[212].

The benefits of the synbiotics come from the synergistic effects of probiotics and prebiotics, reviewed in *sections 5.1* and *5.2*. In general, synbiotics are used to address a range of health conditions, including obesity, insulin resistance syndrome, type 2 diabetes, gastrointestinal disorders and diarrheas, dermatitis, issues related to oxidative metabolism, lactose intolerance, and infections[213–222]. Concretely, they can exert positive effects on humans increasing the proportion of *Lactobacillus spp* and *Bifidobacterium spp*, maintaining the intestinal balance, and preventing of bacterial translocation to reduce infections [223].

### 5.3.1. MECHANISMS OF ACTION

Synbiotics exert their beneficial effects through two distinct pathways. First, they enhance host health through the combined consumption of prebiotic and probiotics. The prebiotic serves as "food" for the probiotic bacteria, ensuring probiotic survival. Second, they promote the growth of indigenous beneficial bacteria, particularly exert a bifidogenic activity, increasing the *Bifidobacterium* proportions[224,225]. These mechanisms collectively improve the survival and implantation rate of probiotics in the colon, selectively stimulate the growth or activate the metabolism of health-promoting bacteria and enhance the overall microbial composition of the gastrointestinal tract[226,227].

Synbiotics can modulate the functionality of the intestine, maintain intestinal homeostasis, and promote the growth of beneficial bacteria while inhibiting the growth of potential pathogens. This stimulation of beneficial bacteria leads to an increased

production of SCFAs, which directly modulate the immune system (*reviewed in 5.1. section*).

## 5.4. MOST RELEVANT BIOTIC COMPOUNDS IN EARLY LIFE

Once analyzed the general characteristics of the prebiotics, probiotics and synbiotics and their mechanisms of action. Here, we will discuss the most used prebiotics, probiotics and synbiotics to boost the infant immune system and their principal benefits focusing on infancy and maternal effects during pregnancy and lactation.

### 5.4.1. PREBIOTICS

Prebiotics has been prescribed for a wide variety of alterations, including chronic obesity, diabetes, degenerative disease, cancer and coronary disease. Therefore, they are also recommended to reinforce the immune system of healthy people and reduce the risk of infections. Herein, we will discuss the effects of prebiotics in infancy and in pregnant mothers.

In infancy, *in vitro* and *in vivo* studies have demonstrated that 2'-FL (*previously reviewed*) increases the relative proportion of *Bifidobacterium* and other bacteria that produce SCFAs, and reduces the adhesion of *Clostridium difficile*, *Campylobacter jejuni*, enteropathogenic *E. coli*, and *Pseudomonas aeruginosa*[228,229].

In addition to the natural prebiotics, the most used prebiotics in infant formulas are GOS and FOS. GOS are the more frequently added in infant formulas because they exert a bifidogenic effect, limit the adhesion of pathogens to epithelial cells, stimulate the Treg and Th1 cells, induce anti-inflammatory and regulatory effect, stimulate the intestinal barrier function and the production of SCFAs[229,230]. FOS share some properties with GOS, like the bifidogenic effect, the limitation of pathogen adhesion, the stimulation of the intestinal barrier and the Th1 immune response[229].

Joining both, the mix GOS/FOS at ratio 9:1 has been widely analyzed for its properties of reducing the diarrheal and infection episodes. Clinical and preclinical researchers concluded that this mix tends to boost the Igs in the intestinal compartment, reduces the incidence of infectious gastroenteritis, reduces the use of antibiotics, approaches the microbiota colonization to a breast-fed infant (bifidogenic activity), reduces the rate of atopic dermatitis and, increases the SCFA production[231–234]. Focusing on the reduction of the gastrointestinal infections, the combination of GOS/FOS has demonstrated to be effective in the preclinical prevention of the RV-induced diarrhea[235–237].

Other commonly used prebiotic substance is certain types of fiber. This prebiotic substance derived from plants is fermented by the intestinal bacteria as the host did not have the necessary enzymes to metabolize it[238]. Dietary fiber can be divided into two categories, soluble and insoluble. Inulin is one of the most important insoluble dietary fiber and it has demonstrated greats benefits on human health as reducing hyperlipidemia

and hyperglycemia, regulating gut microbiota, improving constipation, enhancing mineral absorption, and inhibiting inflammation[239–241]. Moreover, *in vivo* studies have demonstrated that inulin intake during early life contribute to enhance the intestinal microbiota and animal growth[242].

The use of prebiotics in pregnancy and lactation has been raising in the last decade. Their benefits can be reflected in the mother and in the infant. As prebiotics modulate the microbiota composition, prebiotics intake could contribute to counteract to the reduction of the bacterial diversity associated with pregnancy. The supplementation with prebiotics restores this process and collaborates to boost the *Bifidobacterium* colonization, improves the glucose and lipid metabolism and attenuates inflammation. Additionally, the use of prebiotics in pregnancy reduces the predisposition to gestational diabetes, infections, pre-eclampsia, and other pregnancy complications which impact negatively the fetus development[243,244]. Apart from the modulation of the mother immune system, the intake of prebiotics may also improve the BM composition, increasing the beneficial bacteria and enriching it with bioactive compounds. These changes induced with the prebiotics intake during pregnancy and lactation will contribute to the infant maturation[245].

The GOS/FOS intake during pregnancy has been associated with beneficial changes in microbiota composition and SCFAs profile of both the mother and the infant[246]. Also, inulin intake during pregnancy modulates infant development, improving fetal development, reducing asthma outbreaks, and modulating the offspring microbiota colonization[247–249].

### 5.4.2. Probiotics

Probiotics as mentioned above, are linked with benefits in health and disease condition in all stages of life, infancy, adulthood, pregnancy and elderly[226]. The most used in early life are *Bifidobacterium spp* and *Lactobacillus spp,* isolated mainly from the maternal milk and gut of healthy infants and adults. Their beneficial effects are strain-dose dependent[226]. Herein, we will discuss the intake of probiotics in early life and their effects on the maternal immune system during pregnancy and lactation.

In early life, several studies have evaluated the impact of probiotic supplementation to counteract infectious process[169,170,172,173]. *Bifidobacterium spp* and *Lactobacillus spp* have demonstrated a positive impact stimulating the immune system to fight against the viral particles, reduce the incidence and severity of the infection[250–254].

In pregnancy the intake of probiotics has demonstrated beneficial effects on the maternal immune system and on the offspring. First, regarding the maternal outcomes, probiotic supplementation with *Lactobacillus rhamnosus* HN001 demonstrated to reverse the dysbiosis typical from pregnancy which leads to some pregnancy alterations such as gestational diabetes mellitus, eczema and allergy [255,256]. *Lactobacillus rhamnosus GG* and

*Bifidobacterium lactis* reduce the gestational weight gain[257], the risk of gestational diabetes mellitus[258] and control the lipid metabolism[259]. Other mix of *Lactobacillus spp* and *Bifidobacterium spp* reduce the risk of pre-eclampsia[260] and the pre-term birth[261]. Secondly, considering the infant outcomes, mix of both *Lactobacillus spp* and *Bidifidobacterium spp* contribute to regulate the body weight gain[262], reduce the allergies[263,264] and prevent necrotizing enterocolitis[264]. These positive effects on the offspring can be done to the modulation and colonization of the infant microbiota through changes in the BM. To date, on some cases, probiotics supplemented during pregnancy have been detected in the BM and infant's intestine, corroborating the hypothesis that BM is active maternal-infant way of communication[89].

### 5.4.3. SYNBIOTICS

Once deciphered the individual benefits of the prebiotics and probiotics, the combination of them in synbiotics mix have been linked with synergistic effects. The most common synbiotics include *Bifidobacterium* or *Lactobacillus* strains and HMOs, GOS, FOS and inulin[226,227]. Herein, we will discuss the principal applications of synbiotics, and their use in pregnant mothers and children.

In infancy, the neonatal immaturity led the infant susceptible to infections. Synbiotic supplementation in infancy has demonstrated benefits to prevent adverse outcomes. For example, *Lactobacillus plantarum* and FOS has been used to prevent neonatal sepsis[265], *B. breve* M-16V and scGOS/lcFOS to improve microbiota colonization[266], to reduce asthma-like symptoms[267] and to reduce early life infections[268–271]. To date, *B. breve* M-16V and scGOS/lcFOS (9:1) has been used for gastrointestinal infections such as RV infections, reducing the incidence and ameliorating the RV-induced diarrhea at preclinical level[235].After the synbiotic supplementation, the cecal microbiota resemble more to a breastfed infant, increasing the proportion of *Bifidobacterium*[271].

The impact of synbiotic supplementation during pregnancy and its effects on the offspring have been less studied. Some researchers have been focused on the synbiotic supplementation on the changes induced in the maternal microbiota. For example, supplementation with xylo-ligosaccharide, *Lactobacillus plantarum* and *Saccharomyces cerevisiae* modulates the maternal fecal microbiota and the composition of the BM[272]. Similar to the impact of the maternal intake of prebiotics or probiotics alone, maternal synbiotic supplementation also impacts on the offspring. For instance, the maternal intake of xylo-oligosaccharides, *Lactobacillus plantarum* and *Saccharomyces cerevisiae* modifies beneficially blood parameters, colonic microbiota composition and metabolic activity in suckling piglets[273].

# HYPOTHESIS AND OBJECTIVES

The first 1000 days of life which include gestation, breastfeeding and the period of solid food introduction, represent a crucial window for the correct growth and development of the fetus and the infant. Maternal diet influences both the fetal and the infant immune system maturation. Therefore, the modulation of the maternal diet during gestation and lactation can be a powerful strategy to reduce postnatal infections. Breast milk contains, among others, microorganisms and active compounds that participate in gut colonization, intestinal and immune system maturation and, prevents early life infections. The use of the synbiotics *Bifidobacterium bre*ve M-16V and scGOS/lcFOS (9:1) during early life induced immune maturation and prevented rotavirus (RV) infection.

Based on this background, the **hypothesis** of this thesis is that "the supplementation of the mother with this synbiotic during gestation and lactation will prevent the RV infection in early life by modulating both the maternal and infant immunity, as well as the breast milk composition".

Therefore, considering this hypothesis, the **general objective** of this thesis is "to stablish the efficacy of the maternal supplementation with the synbiotic *Bifidobacterium bre*ve M-16V and scGOS/lcFOS (9:1) to modulate the maternal and infant immune system and its capacity to counteract infant infections in early life". To achieve this general goal, the following specific objectives are stablished:

**Objective 1:** To study the immunomodulatory effects of a maternal supplementation with a synbiotic during gestation and lactation on the maternal immune system.

The results of this objective are found in the following work:

> **Article 1:** Impact of maternal *Bifidobacterium bre*ve M-16V and scGOS/lcFOS supplementation during pregnancy and lactation on the maternal immune system and milk composition (Accepted).

**Objective 2:** To establish the effect of biotic supplementation following two different approaches, direct or through the mother, to counteract the RV infection in early life.

1) To analyze the genetic changes induced by rotavirus infection and to evaluate the modulation of these pathways by a direct infant prebiotic supplementation.

The results of this sub-objective can be found in the following work:

> **Article 2:** Sáez-Fuertes, L., Azagra-Boronat, I., Massot-Cladera, M., Knipping, K., Garssen, J., Franch, À., Castell, M., Pérez-Cano, F. J., & Rodríguez-Lagunas, M. J. (2023). Effect of Rotavirus Infection and 2'-Fucosyllactose Administration on Rat Intestinal Gene Expression. *Nutrients*, *15*(8), 1996. https://doi.org/10.3390/nu15081996

2) To explore the efficacy of a maternal synbiotic supplementation during gestation and lactation to counteract a rotavirus infection of the offspring in the first days of life.

The results of this objective are found in the following work:

**Article 3:** *Bifidobacterium breve* M-16V and scGOS/lcFOS supplementation to dams ameliorates infant rotavirus infection in early life.

**Objective 3:** To evaluate the influence of a synbiotic supplementation to the mothers during gestation and lactation and after weaning on infant immune development. Two specific sub-objectives were established:

1) To analyze the effects of a maternal synbiotic supplementation during pregnancy and lactation on the offspring at the end of suckling.

The results of this sub-objective are found in the following work:

**Article 4:** Maternal synbiotic supplementation with *Bifidobacterium Breve* M-16V and scGOS/lcFOS shape offspring immune development and gut microbiota of suckling rats.

2) To assess the immunomodulatory effects of synbiotic exposure throughout the first 1000 days of life, including maternal supplementation during pregnancy and lactation, followed by direct supplementation to the offspring one week after.

The results of this sub-objective are found in the following work:

**Article 5:** Enhancing gastrointestinal immunity and microbial diversity in young rats by *Bifidobacterium breve* M-16V and scGOS/lcFOS supplementation in early life.

# RESULTS

# ARTICLE 1

## "Impact of maternal *Bifidobacterium bre*ve M-16V and scGOS/lcFOS supplementation during pregnancy and lactation on the maternal immune system and milk composition"

Sáez-Fuertes, Laura; Kapravelou, Garyfallia; Grases-Pintó, Blanca; Massot - Cladera, Malen; Bernabeu, Manuel; Knipping Karen; Garssen, Johan; Bourdet-Sicard, Raphaëlle; Castell, Margarida; Rodríguez-Lagunas, Maria José; Collado, María Carmen; Pérez-Cano, Francisco José

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- **VI workshop Anual INSA-UB.** Barcelona (February 2022). **Sáez-Fuertes, L.**; Rio-Aige, K.; Grases-Pintó, B.; Melero, C.; Galindo S.; Cabré, P.; Gironés, A.; Zhan S.; Castell, M.; Collado, M.C.; Rodríguez-Lagunas, M.J.; Pérez-Cano, F.J. "Impact of a synbiotic administration during gestation and lactation to dams in the offspring's development".
- **VXI Congreso de la Sociedad Catalana de Inmunología.** Barcelona (November 2022). **Sáez-Fuertes, L.**; Grases-Pintó, B.; Castell, M.; Collado, M.C.; Rodríguez-Lagunas, M.J.; Pérez-Cano, F.J. "Changes in the immunoglobulin profiles after synbiotic administration in pregnant and lactating rats".
- **XIII Workshop de la Sociedad Española de Microbiota, Probióticos y Prebióticos (SEMiPyP).** Valencia (June 2022). **Sáez-Fuertes, L.**; Rio-Aige, K.; Grases-Pintó, B.; Melero, C.; Galindo S., Cabré, P.; Gironés, A.; Zhan S.; Castell, M.; Collado, M.C; Rodríguez-Lagunas, M.J.; Pérez-Cano, F.J. "La

administración simbiótica durante la gestación y la lactancia mejora las infecciones por rotavirus en la descendencia en las primeras etapas de vida"

- **XIV Workshop de la Sociedad Española de Microbiota, Probióticos y Prebióticos (SEMiPyP).** Pamplona (March 2023). **Sáez-Fuertes, L.;** Rio-Aige K.; Grases-Pintó, B.; Castell, M.; Collado, M.C.; Rodríguez-Lagunas, M.J.; Pérez-Cano, F.J. "Impacto inmunológico de la suplementación simbiótica en ratas embarazadas y lactantes"
- **XV Workshop de la Sociedad Española de Microbiota, Probióticos y Prebióticos (SEMiPyP).** Sevilla (February 2024). **Sáez-Fuertes, L.**; Rio-Aige, K.; Grases-Pintó, B.; Castell, M.; Collado, M.C.; Rodríguez-Lagunas, M.J.; Pérez-Cano, F.J. "Efectos de la suplementación materna de un simbiótico en la microbiota de la madre y de su descendencia y bajo la infección por rotavirus".

## Abstract

**Aim**: This study explored the immunomodulatory effects of *B. breve* M-16V and short chain galacto-oligosaccharides (scGOS)/long chain fructooligosacharides (lcFOS) on the maternal immune system at the end of suckling.

**Methods**: Pregnant rats were administered with *B. breve* M-16V and scGOS/lcFOS during gestation and lactation. At weaning, different mucosal and systemic samples were collected to analyze the maternal immune system and the microbiota composition. Additionally, breast milk was collected to characterize the immunoglobulin profile and the microbiota composition.

**Results**: The intervention modified the immunoglobulinome profile showing higher levels of IgG2c in plasma and milk, as well as rise in sIgA in feces at the end of suckling. The supplementation improved lipid metabolism as it enhanced brown adipose tissue activity and it reinforced the intestinal barrier by increasing the expression of *Muc3*, *Cldn4*, and *Ocln*. The higher production of short chain fatty acids in the cecum and increased *Bifidobacterium* counts in the intestine suggest a potential positive impact on the gastrointestinal tract.

**Conclusion**: The obtained results indicate that the intervention during pregnancy and lactation with *B. breve* M-16V and scGOS/lcFOS modulates the maternal immune system. Synbiotic supplementation not only affects the immunological profile of the mothers but also the immune components of the milk, which aims to support infant development.

# Impact of maternal *Bifidobacterium breve* M-16V and scGOS/lcFOS supplementation during pregnancy and lactation on the maternal immune system and milk composition

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**Keywords: *Bifidobacterium breve M-16V*, short chain galacto-oligosaccharides (scGOS), long chain fructo-oligosachairdes (lcFOS), pregnancy, breastfeeding.**

**Abstract**

Maternal synbiotic supplementation during pregnancy and lactation can significantly influence the immune system. Prebiotics and probiotics have a positive impact on the immune system by preventing or ameliorating among others intestinal disorders. This study focused on the immunomodulatory effects of *B. breve* M-16V and short chain galacto-oligosaccharides (scGOS)/long chain fructo-oligosachairdes (lcFOS), including systemic and mucosal compartments and milk composition. Lewis rats were orally administered with the synbiotic or vehicle during pregnancy (21 days) and lactation (21 days). At the weaning day, small intestine (SI), mammary gland (MG), adipose tissue, milk, mesenteric lymph nodes (MLN), salivary gland (SG), feces and cecal content were collected from the mothers. The immunoglobulinome profile showed increased IgG2c in plasma and milk, as well as elevated sIgA in feces at weaning. The supplementation improved lipid metabolism through enhanced brown adipose tissue activity and reinforced the intestinal barrier by increasing the expression of *Muc3*, *Cldn4*, and *Ocln*. The higher production of short chain fatty acids in the cecum and increased *Bifidobacterium* counts suggest a potential positive impact on the gastrointestinal tract. These findings

indicate that maternal synbiotic supplementation during gestation and lactation improves their immunological status and improved milk composition.

## 1 Introduction

Pregnancy and lactation are crucial for a correct development of the infant. These two periods are highly influenced by external stimuli including environmental, nutritional, and lifestyle factors. Nutrition, including the nourishment status and pattern of food intake, impacts on physiological and metabolic responses (1,2). During gestation the placenta serves as a bridge for nutrients, hormones, cytokines (CK), immunoglobulins (Igs) and other bioactive molecules. In humans, IgG crosses the placenta through an active mechanism due to the presence of the neonatal fragment crystallizable receptor (FcRn) expressed by the cells of the syncytiotrophoblast (3). During lactation breast milk (BM) works as a transference vehicle for nutrients and bioactive compounds including Ig and CK. Its composition is dynamic and changes to supply the nutritional requirements of the infant (4). Thus, during these periods the maternal nutrition influences the composition of the BM (5).

The use of food complements such as probiotics, prebiotics, synbiotics and postbiotics has been growing during the last few years (6) for different purposes such as their anti-infective action. Probiotics reduce the colonization of pathogens, prebiotics promote the growth of the beneficial microbes of the intestine and, synbiotics act as a combination of both taking the advantages of probiotics and prebiotics (6). In 2020, synbiotics were defined as "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host" (7). Clinical and preclinical studies have verified that the supplementation with these microbial modulators improves the gastrointestinal health and infection resolution (8,9). In some studies, these effects have been associated with the microbiota and its impact on the immune system (6,9).

Until now, only a few studies have reported beneficial effects of probiotics and prebiotics supplementation during gestation and lactation on the newborn (10). However, less information is known about the impact of synbiotic supplementation on the maternal immunity. Hence, this study aims to evaluate the impact of a synbiotic mixture, particularly composed of *Bifidobacterium breve* M-16V ($10^9$ CFU) and short-chain galacto-oligosaccharide (scGOS) and long-chain fructo-oligosaccharide (lcFOS) at ratio 9:1 during gestation and lactation on the maternal immune system. These probiotic and prebiotic have been linked to immunomodulatory effects in previous studies (11–16).

## 2 Materials and Methods

### 2.1 Animals

Seven-week-old Lewis rats (16 females and 8 males) were obtained from Janvier Labs (La Plaine Saint Denis Cedex, France). After one week of acclimatization, females were randomly distributed into two groups: Reference (REF, n=8) or Synbiotic (SYN, n=8). At the same day, females were introduced into the male cages for one week, and then separated into individual cages. Since the mating day, the female rats of the SYN group were supplemented daily during gestation (21 days) and lactation (21 days) with the synbiotic mixture, and the REF group received a matched volume of saline solution. Animals were fed with a commercial diet corresponding to the American Institute of Nutrition 93G formulation (17) and water *ad libitum*. Rats were allowed to deliver naturally, and the day of birth was considered as day 1 of pups. Pups had free access to the nipples and rat diet during the entire study.

The experiment was finally executed with the 5 dams of the SYN group and 6 dams of the REF group that became pregnant.

Animal room conditions (temperature and humidity) were controlled in a 12 h light – 12 h dark cycle in a negative pressure chamber at Animal Facility of the Diagonal Campus, Faculty of Pharmacy and Food Science, from the University of Barcelona. All the experimental procedures were previously approved by the Ethics Committee for Animal Experimentation (CEEA) of the University of Barcelona (UB) (Ref 240/19) and from the Catalan Government (Ref.10933).

### 2.2 Synbiotic supplementation

Daily administration of the synbiotic or vehicle was performed during the gestation and lactation periods always at the same time range of the day. The synbiotic solution was obtained by mixing *Bifidobacterium breve* M-16V ($10^9$ CFU) with scGOS/lcFOS 9:1 concentration. Bifidobacterium breve M-16 V has been purchased from Morinaga Milk Industry, Tokyo, Japan. GOS/FOS is a mixture of GOS (Vivinal GOS, Borculo Domo, Zwolle, The Netherlands) with a degree of polymerization (dp) of 3–8, as well as long-chain FOS (Raftiline HP, Orafti, Wijchen, The Netherlands; average dp > 23) in a 9:1 ratio. The products had a purity of 47.6% for GOS and 94.5% for FOS. The dose of scGOS/lcFOS was approximately 2 % of a established daily food intake of 40 g. The mix was extemporaneously prepared by the mixture of the prebiotic and the probiotic dissolved in physiological saline solution. One mL of the synbiotic ($10^9$ CFU/rat/day) or saline solution was intragastrically administered through an oral gavage during pregnancy. After birth, dams were separated from the pups for animal handling and the volume administered was increased to 1.5 mL. All supplements were kindly provided by Danone Nutricia Research (Utrecht, The Netherlands). The control group received the same volume of saline with same amount of corn starch as the treated group.

### 2.3 Sample collection and processing

Animal body weights (BW), and food and water consumption were monitored daily, and feces were collected weekly during the study. The relative humidity and the pH of the feces was monitored in fresh after collection. At the weaning day, dams were isolated from the pups 1 h before milk extraction to allow the milk to accumulate in the mammary gland (MG). Then, dams were anesthetized with 10 mg/100 g of ketamine (Merial Laboratories S.A., Lyon, France) and administered intraperitoneally with 2 Ul of oxytocin (Syntocinon 10 U.I./mL, Alfasigma S.L., Bologna, Italia) thirty min after administering oxytocin, the milking process began by gently and manually stimulating the teat from its base to the top. The milk was collected with an automatic pipette in sterilized tubes, centrifuged (12000 *g*, 5 min, 4 °C) and then the lactic serum (LS) was obtained and stored at -80 °C.

Finally, dams were re-anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg; Bayer A.G., Leverkusen, Germany). First, blood was obtained by cardiac puncture and collected in heparin tubes for the haematologic analyses. Then, samples were centrifuged (1000 *g*,10 min 4ºC). Also,, intestinal and adipose tissues, intestinal and cecal contents, salivary glands (SG), mesenteric lymph nodes (MLN), MGand spleen were collected and immediately processed or stored at -20 °C or -80 °C for future analysis.

BW was monitored daily and at the end point body and tail lengths were measured to calculate the body mass index (BMI) ($weight/length^2$ (g/cm$^2$)) and the Lee Index ($weight^{0.33}/length) \times 1{,}000$ (g$^{0.33}$/cm). The weight of different organs was recorded including thymus, spleen, liver, heart, kidney, large intestine, and small intestine (SI) (length and wide were also measured).

### 2.4 Isolation of Mesenteric Lymph Nodes and spleen lymphocytes

MLN and spleen cells were isolated as previously described (18). For splenic cells, an additional step was required to eliminate erythrocytes by an osmotic lysis. Conditions were immediately restored by adding PBS (Phosphate-buffered saline) to avoid lymphocytes death (19). Cell viability and concentration was analyzed by Countess™ Automated Cell Counter (Invitrogen™, ThermoFisher Scientific, Barcelona, Spain) based on Trypan Blue staining.

### 2.5 Small intestine sampling

SI was processed for diverse analysis. Two portions of 1 cm from the middle part of the intestine were collected for histomorphometry and gene expression analysis. For gene expression analysis the intestine portion was immersed in RNAlater (Ambion, Life technologies, Madrid, Spain), kept at 4 °C for 24 h and then stored at -20 °C. The remaining proximal part of the SI was opened lengthwise and cut in 0.5 cm pieces and incubated with PBS in a shaker (37 °C for 10 min) to recover the gut wash (GW). The content of the distal part of the SI (intestinal content, IC) was collected for microbiota analysis.

### 2.6 Histology

The SI and adipose tissues (white adipose tissue (WAT) and brown adipose tissue (BAT)) were fixed in 4 % buffered formaldehyde for 24 h at room temperature. Then, samples were rinsed in PBS solution for 3 h until dehydrated in graded ethanols (70 %, 90 % and 100 %) and permeated in xylene (Panreac Química SLU, Barcelona, Spain). Afterwards, samples were embedded in melted paraffin (Merck, Madrid, Spain). Paraffin sections (5 µm) were stained using hematoxylin-eosin (HE). Observation of the samples was performed under the microscope (Olympus BX41 and Camera Olympus XC50, Olympus Barcelona, Spain). Representative photos were made for each sample of WAT (20x), BAT (40x) and intestine (10x) and were analyzed using Image J (Image Processing and Analysis in Java, National Institute of Mental Health, Bethesda, MD, USA). In SI, the length and width of microvilli were measured. In WAT, adipocyte area as well as the number of adipocytes per section were quantified. In BAT, the number of nuclei, the area of LDs as well as the number of LDs with a size greater than 50 $\mu m^2$ were measured.

### 2.7 Immunoglobulin quantification

Different tissues and samples were processed for Ig quantification by Enzyme-Linked ImmunoSorbent Assay (ELISA) (Bethyl, Laboratories Inc., Montgomery, TX, USA) and/or ProcartaPlex™ Multiplex immunoassay (eBioscience, San Diego, CA, USA).

Secretory (s)IgA quantification was performed by a sandwich ELISA technique in milk, salivary and mammary glands, MLN, cecal and fecal homogenates. Additionally, sIgA and IgM were evaluated in GW. Both Igs were quantified following the previous described protocol (20), and absorbance results were measured with a microplate photometer (Labsystems Multiskan, Helsinki, Finland) at 495 nm, and data were analyzed by Multiskan Ascent v2.6 software (Thermo Fisher Scientific SLU, Barcelona, Spain). The lower limits of detection were 1.95 ng/mL for sIgA and IgM.

IgA, IgM, IgG and IgG isotypes (IgG1, IgG2a, IgG2b, IgG2c) were quantified in plasma, milk, and SG and MLN homogenates by ProcartaPlex™ Multiplex immunoassay. Briefly, 96 well flat bottom plates were used to prepare samples following manufacturer's instructions, as in previous studies (21). Data were acquired by MAGPIX® analyzer (Luminex Corporation, Austin, TX, USA) at the

Cytometry Service of the Scientific and Technological Centers of the University of Barcelona (CCiT-UB). The lower limits of detection were: 0.58 ng/mL for IgA, 1.70 ng/mL for IgG1, 1.73 ng/mL for IgG2a, 2.67 ng/mL for IgG2b, 3.67 ng/mL for IgG2c and 0.2 ng /mL for IgM. The relative abundance of IgG subtypes was analyzed considering total IgG. Thus, Th1 and Th2 responses were evaluated adding the levels of IgG subtypes, IgG2b + IgG2c and IgG1 + IgG2a, respectively.

### 2.8 Cell subset staining and flow cytometry analysis

Phenotypic population analysis was performed in MLN and splenic cells by flow cytometry analysis using fluorescent mouse anti-rat monoclonal antibodies (mAbs) conjugated to different fluorochromes. All the chosen mAbs were purchased from BD Biosciences (San Diego, CA, USA), Serotec (Kidlington, Oxford, UK) and Caltag (Burlingame, CA, USA): anti-TCR αβ (R73), anti-CD103 (OX-62), anti-NK (10/78), anti-CD62L (OX-85), anti-CD8α (OX-8), anti-CD4 (OX-35), anti-CD45RA (OX-33), anti-TCRγδ (V65). The staining combination and gates strategy is showed in Supplementary Figure 1. The staining technique was performed following the protocol previously described by Marín-Gallen *et al* (22). Analyses were performed with a Gallios™ Cytometer (Beckman Coulter, Miami, FL, United States) in the CCiT-UB and data were analyzed by Flowjo v10 software (Tree Star, Inc., Ashland, OR, USA).

### 2.9 Cecal bacteria and Ig-coated bacterial analysis

The proportion of cecal bacteria and Ig-coated bacteria (Ig-CB) was determined as previously described (23) with slight modifications, only 10 µL of the homogenized cecal sample was used. A Cytek Aurora (Cytek Biosciences, Inc., CA, USA) flow cytometry equipment was used in the CCTi-UB. The acquisition parameters were adjusted to obtain a maximum of 25.000 counts. Data analysis was performed using the FlowJo v.10 software. The total bacterial and the Ig-CB proportions were evaluated as Massot *et al.* established before (24).

### 2.10 Gene expression analysis

SI and WAT samples kept in RNAlater were thawed and homogenized for RNA extraction and gene expression analysis. Samples were placed in lysing matrix tubes (MP biomedicals, Illkirch, France) and homogenized using a FastPrep-24 instrument (MP biomedicals, Illkirch, France). RNeasy Mini Kit (Qiagen, Madrid, Spain) was used for RNA extraction following the manufacturer's instructions. RNA purity and concentration was determined with a NanoPhotometer (BioNova Scientific S.L., Fremont, CA, USA) and cDNA obtained using TaqMan Reverse Transcripiton Reagents (Applied Biosystems, AB, Weiterstadt, Germany). Then, Real Time (RT) – PCR for target genes (Supplementary Table 1) was performed with ABI Prism 7900 HT quantitative RT-PCR system (AB). Results were normalized using the housekeeping gene *Gusb* (β-glucuronidase, Rn00566655_m1, I) and analyzed using the 2-ΔΔCt method, as previously described (25). Data is shown as the percentage of expression in each experimental group normalized to the mean value obtained for the REF group, which was set at 100 %.

### 2.11 Detection of *B. breve* M-16V

*B. breve* M-16V detection was carried out in fecal and mammary gland samples by qPCR technique following the protocol previously described by Gil-Campos *et al* (26). Genomic DNA was extracted from ~ 100 mg of fecal or tissue samples using the FastDNA kit (MP biomedicals Inc., Santa Ana, CA, USA) following the manufacturer instructions. The probiotic genome was detected by Taq-Man based PCR assay. The forward, reverse and probe used were previously designed by Phavichitr *et al.* (27).

The PCR was performed with ABI Prism 7900 HT quantitative RT-PCR system (AB) at the CCiT-UB services.

### 2.12 Microbial profiling of rat samples analysis

Total DNA was isolated from fecal (100 mg) and milk samples (500 µL-1 mL) using an automated assisted method based on magnetic beads (Maxwell® RSC Instrument coupled with Maxwell RSC Pure Food GMO and authentication kit, Promega, Spain) following the manufacturer's instructions with previous treatments to improve the DNA extraction. In brief, samples were treated with lysozyme (20 mg/mL) and mutanolysin (5 U/mL) for 60 min at 37°C and a preliminary step of cell disruption with 3-µm diameter glass beads during 1 min at 6 m/s by a bead beater FastPrep 24-5 G Homogenizer (MP Biomedicals). After the DNA extraction, DNA was purified using the DNA Purificaton Kit (Macherey-Nagel, Duren, Germany) following the recommended protocol and the final DNA concentration measured using Qubit® 2.0 Fluorometer (Life Technology, Carlsbad, CA, USA). Microbial profiling was assessed by amplicon V3-V4 variable region of the 16S rRNA gene. Libraries were prepared following the 16S rDNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15044223 Rev. A). The libraries were then sequenced using 2x300 bp paired-end run on a MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain). Negative and positive mock community (Zymobiomics) communities were also included.

Raw reads were then processed with the integrated dada2 method for denoising, amplicon sequence variance (ASV) clustering and chimeral removal. Reads were trimmed at 270 and 210 nucleotides in forward and reverse reads, respectively. Resulted ASV were then taxonomically assigned using Silva v.138. No rarefaction was done and also, samples with less than 4500 reads were removed and data was normalized using Centered-log-ratio (CLR). Beta diversity was based on Bray-Curtis distances and Permutational Analysis of Variance (PERMANOVA) was performed. Alpha-diversity indexes Chao1 and Shannon were also calculated and differences by group were assessed by Mann-Whitney and/or Kruskal-Wallis non-parametric test. Besides this, the Kruskall-Wallis test on the CLR normalized data were also assessed with Benjamini-Hochberg false discovery rate (FDR) correction. Taxa tables at phylum, family and genus level were provided to integrate to the other data obtained in the study. Negative binomial regression as implemented by DESeq2 tool was used for differential abundance analysis in order to estimate the fold-change of genus taxa (28). Plots were generated using MicrobeAnalyst platform v.2 (29).

### 2.13 Microbial metabolites profiling by SCFA analysis

SCFA analysis was performed using gas chromatography–mass spectrometry (GC-MS), following the method described by Eberhart et al. (30). An internal standard solution (3-Methylvaleric acid) was added to the samples that were processed and finally centrifuged at 4000 rpm for 2 min at 4 °C according to the protocol. The final supernatant was collected, filtered-sterilized (0.22 µm PES size filter, Sarstedt SA) and then, injected in the Agilent GC 7890B–5977B GC-MS with a multipurpose sampler (Gerstel MPS, Mülheim, Germany). The GC column used was Agilent DB-FATWAX, 30 m × 0.25 mm × 0.25 µm, operated in split mode (20:1). The oven temperature program was set as follows: 100 °C for 3 min, ramped to 100 °C at a rate of 5 °C min−1, then to 150 °C for 1 min, further ramped to 200 °C at a rate of 20 °C min−1, and finally held at 200 °C for 5 min. Helium was used as the carrier gas at a flow rate of 1 mL min−1, with an inlet temperature of 250 °C. The injection volume was 2 µL. Standards curves for acetate, butyrate, and propionate were used for quantifying the SCFAs.

### 2.14 Statistical analysis

SPSS Statistics 22.0 software package (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. To assess normal distribution and homogeneity of variance of the data, Shapiro-Wilk and Levene test were used. Normal and homogeneous results were analyzed by one-way ANOVA. Kruskal-Wallis test was performed when results did not follow a normal and equal distribution to assess significant differences among groups ($p<0.05$). Spearman correlation coefficient was used to search correlation between variables. Non-metric multi- dimensional scaling (NMDS) in Rstudio using the R package vegan (31) was used to search clusters of similarities between samples in terms of immune factor composition. Besides, the function "envfit" assess the association of factors with the ordination of the samples in the NMDS. Differences were considered statistically significant when $p$ value $< 0.05$.

## 3 Results

### 3.1 Animal body weight

Synbiotic supplementation during gestation and lactation did not affect either the body weight gain or the overall daily food and water intake during pregnancy or lactation. Only punctual changes were observed in the food intake at the middle of the gestation (Figure 1). As expected, in all groups the body weight exponentially increased during gestation and showed a sharped decrease on the delivery day (Figure 1a). The overall intake showed a distinct pattern associated to the growth gain during gestation and the higher requirements of food and water during lactation (Figure 1b, 1c).

### 3.2 Organ size and growth parameters

At the weaning day, dams were measured and weighted, and different organs were obtained and weighted (Supplementary Table 2). No changes were observed due to the synbiotic supplementation in the body size evaluation. However, an increase in the relative weight of the SI was observed in the SYN dams.

### 3.3 Adipose tissue

The SYN administration during gestation and lactation provoked some changes in the adiposity of the rats (Figure 2). The nutritional intervention did not modify the relative weight of the adipose tissue from different body locations (Figure 2a). Representative images of histologic sections of WAT and BAT are shown in Figure 2b and Supplementary Figure 2. The number of adipocytes and the adipocyte area of the parametric-WAT were not modified with the supplementation (Figure 2c). In contrast, the BAT of the SYN group showed an increase in the number of the nuclei associated with a reduction of the area of the lipid droplets (LD) and the number of LD bigger than 50 $\mu m^2$ (Figure 2d). Additionally, synbiotic supplementation showed a tendency to increase the relative gene expression of the *Ucp-1* gene ($p$=0.09) in WAT without affecting *Cidea*, *Prdm16*, *Pparγ*, free fatty acid receptor (*Ffar2)* and IL-1β gene expression (Figure 2e), suggesting a higher thermogenesis of the BAT.

### 3.4 Hematological variables

The last day of suckling, different hematological variables were analyzed in maternal blood samples (Supplementary Table 3). Synbiotic supplementation during gestation and lactation showed no effect on hematological parameters.

### 3.5 Fecal sample analysis

Feces from the SYN group showed an increase in the total IgA at the end of the lactation (Figure 3a). Regarding to the pH and water content, feces SYN dams had overall lower pH and water content (during pregnancy) compared to REF (Figure 3b, 3c).

### 3.6 Immunoglobulin quantification

To evaluate the impact of *B. breve* M-16V and scGOS/lcFOS during gestation and lactation on Ig levels, IgM, IgA and IgG concentration was determined in plasma, milk, salivary gland and MLN (Figure 4). Both in plasma (Figure 4a) and milk (Figure 4d) the total levels of IgG were increased due to supplementation without changes in IgA or IgM. Conversely, in MLN IgM was higher and IgA tend to be increased without changes in IgG (Figure 4j). The synbiotic supplementation did not influence the Ig levels in the salivary gland (Figure 4g). The relative proportion of IgG subtypes (IgG1, Ig2a, Ig2b, IgG2c) was also evaluated. Synbiotic supplementation modified the IgG subtype profiles in plasma, milk and SG (Figure 4b, e, h, respectively), mainly by increasing the relative proportion of IgG2c without changing the relative proportion of Th1/Th2associated response (Figure 4m). Moreover, non-metric multi-dimensional scaling (NMDS) graphs of each compartment were plotted with the Igs data and, in plasma (Figure 4c) and milk (Figure 4f) samples, different clusters appeared ($p<0.01$ with the ANOSIM test). Furthermore, plasma levels of IgG2c and IgG2b were highly correlated with the corresponding Ig in milk and in SG, respectively (Figure 4n). Additionally, the sIgA and IgM levels were quantified in the intestinal compartment in GW. Synbiotic supplementation did not affect either the sIgA (REF: 36.56 ± 6.72 µg/g; SYN: 28.98 ± 2.69 µg/g) or the IgM (REF: 0.14 ± 0.02 µg/g; SYN 0.09 ± 0.02 µg/g).

### 3.7 Phenotypic characterization of MLN and spleen cells

The relative proportion of MLN and spleen cell subsets was analyzed at the end of the study (**Table 1**). Spleen and MLN lymphocyte populations are rich in T cells with a low proportion of B lymphocytes. In terms of T helper (Th) and T cytotoxic (Tc) cells, Th predominates in both compartments. Regarding the minor populations of NK and NKT cells, the NK subset dominates in the spleen while the NKT subset prevails in the MLN. Synbiotic supplementation did not alter the proportion of B or T lymphocytes either in the spleen or in MLN. Additionally, the expression of CD8 was analyzed in the different subsets of lymphocytes, and only a punctual reduction in the proportion of Tc CD8+ cells occurred in MLN due to the supplementation. Additionally, adhesion molecules important in the intestinal homing such as αE integrin and CD62L were also assessed (Supplementary Figure 3). As expected, in both tissues CD62L was highly expressed and αE integrin was very low expressed. The synbiotic intervention did not modify the pattern expression of any of these molecules in the spleen or in MLN.

### 3.8 Gene expression analysis

The effect of *B. breve* M-16V and scGOS/lcFOS on gene expression in the intestine and mammary gland was assessed at the end of the lactation period (Figure 5). In the intestinal samples, genes implicated in the activity of the immune system and gut barrier were evaluated. Synbiotic supplementation increased the mRNA levels of *Muc3, Cldn4* and *Ocln*. No changes in TLR gene expression were observed in the intestine. Regarding the mammary gland, the SYN supplementation did not modify the expression of *IgA* or *Ffar2*.

### 3.9 IgA and Ig-CB cecal analysis

In the cecum, IgA is the most abundant Ig and can be bound to the resident bacteria (Figure 6). Synbiotic supplementation during gestation and lactation did not affect the IgA levels in the cecum (Figure 6a). The proportion of Ig-CB was evaluated in the cecum (Figure 6b), and after the supplementation, neither the total bacteria nor the relative proportion of Ig-coated bacteria were affected. However, the total Ig-CB tended to be increased ($p$=0.05). Furthermore, the amount of Ig coating of each bacteria was measured, by means of the Mean Fluorescence Intensity (MFI) of this population which was highly increased after the SYN supplementation (Figure 6c).

### 3.10 Intestinal histomorphometry

The impact of the synbiotic on the intestinal architecture showed that the synbiotic supplementation during gestation and lactation induced histological changes in the intestinal epithelium (Figure 7 and Supplementary Figure 4). Specifically, the villi height and the crypts depth were increased in the SYN group. On the contrary, the villi width was lower in the supplemented animals (Figure 7b). The synbiotic did not change the number of goblet cells responsible for mucus secretion.

### 3.11 B. breve M-16V detection in feces and mammary gland

The presence of *B. breve* M-16V in feces and MG was studied also (Figure 8). In feces, both at the end of the gestation and lactation periods the number of *B. breve* M-16V UFC was increased 1000xin the supplemented dams with respect to the basal levels at the beginning of the intervention represented with the horizontal dotted line. The detection of *B. breve* M-16V in REF animals during gestation and lactation was always at basal levels (~$10^3$). In order to explore the gut-mammary pathway its presence was also evaluated in MG. However, *B. breve* M-16V was not detected either in the SYN or the REF MG.

### 3.12 Impact of SYN supplementation on the rat intestinal and milk microbiota

Significant differences between groups and sample types were found (CC, CI and milk samples) as reported in the beta-diversity Bray-Curtis PERMANOVA and also, alpha-diversity indexes as well as taxonomical composition (Supplementary Figure 4).

SYN supplementation had an impact on the CC microbiota profile. Statistically significant differences were detected in the microbiota profiles of REF and SYN (permutational multivariate analysis of variance (PERMANOVA) *Bray-Curtis F-value*=3.1912; *R-squared*=0.26176; $p$=0.004) (Figure 9a), as well in the alpha-diversity indexes as measured by the Chao1 ($p$=0.01) and Shannon indices ($p$=0.662), respectively, (Figure 9b, 9c).

Regarding the taxonomy of the CC, differences were found in the phylum, family and genera. In the SYN group, the proportion of *Firmicutes* tend (p=0.06) to be reduced while the abundance of *Desulfobacteria* was significantly reduced. The family analysis revealed that *Desulfovibrionaceae* and the *Suterellaceae* families were reduced in the SYN group (Figure 10). Thus, specific microbial genera were significantly present in SYN group including *Bifidobacterium*, *Faecalibaculum* (*Erysipelotrichidae* family) and *Marvinbruantia* (*Lachnospiraceae* family) and other butyrate producers such as *Blautia*, *Ruminoclostridium* and also, other *Lachnospiraceae* (Table 2).

The impact of the SYN supplementation was also observed in the intestinal content (IC) as two distant groups were identified. The beta-diversity analysis with Bray-Curtis distances reported significant differences (PERMANOVA] F-value: 2.6694; R-squared: 0.22875; p-value: 0.007) while no significant differences were observed in alpha-diversity indexes (Figure 9). The taxonomic analysis of

the IC showed that the *Firmicutes* phylum was reduced and the *Actinobacteria* phylum tend to be higher in the IC of the SYN supplemented animals ($p$=0.07). With regard to the family diversity, the *Bifidobacteriaceae* tend to be increased in the SYN group ($p$=0.07) (Figure 10). The specific genera were overrepresented in SYN compared to REF group, including again the presence of *Bifidobacterium*, *Faecalibaculum* and *Blautia* (Table 3).

Regarding the milk microbiota profile, the SYN supplementation had no effect on the microbiota profile. No differences were found in beta-diversity and alpha-diversity between SYN and REF groups (Figure 9 g-i). In general, milk microbiota in rats is characterized by a higher presence of *Firmicutes*. Our results indicated that the SYN supplementation tend to reduce the proportion of *Firmicutes* and *Actinobacteria* phylums ($p$=0.084 and $p$=0.088, respectively). In the family analysis, the *Bifidobacteriaceae* tend to be higher ($p$=0.07) in the SYN group (Figure 10). Finally, the genus analysis confirmed that SYN group had higher levels of *Bifidobacterium* compared to those observed in the REF group (6 % *vs*. 1 %, respectively but p-value FDR>0.05).

### 3.13 Cecal SCFA content

SCFA are the communication channel of the intestinal microbiota and the immune system. For this reason, the main SCFA were evaluated in CC at the end of the study (Figure 11). After the SYN supplementation, the total amount of SCFA was increased in the cecum (Figure 11a). This increase was mainly due to an increase in the total amount of acetic, propanoic, butanoic and isovaleric acids (Figure 11b).

### 3.14 Correlations between microbiota, SCFA and Igs

Considering the importance of SCFA derived from the microbiota in the overall intestinal health, the correlation between SCFA-microbiota-Ig was evaluated (Figure 12). Acetic, propanoic, and butanoic acids were positively correlated with the cecal *Bifidobacterium* detected by sequencing the 16s gene (0.76, $p$=0.01; 0.79, $p$=0.006; 0.84, $p$=0.002, respectively). Likewise, acetic and propanoic acids were positively correlated with the cecal *Blautia* (0.73, $p$=0.01; 0.84, $p$=0.002, respectively). After the microbiota analysis, the correlation between the *Bifidobacterium* and the IgG2c of the plasma, milk, SG and MLN was performed. The cecal *Bifidobacterium* was positively correlated with the plasma and milk IgG2c. Furthermore, the correlation between the cecal SCFA levels and the concentration of IgG2c in plasma, milk, SG and MLN was assessed. Results suggest that plasmatic and milk IgG2c were positively correlated with the increased acetic and propanoic acids. Additionally, a positive correlation was observed between the IgG2c of the MLN with the valeric acid.

## 4 Discussion

During gestation and lactation, the maternal immune system undergoes significant changes to adapt to this critical period (32). To protect the fetus, a delicate balance between tolerance and defense is established, mediated by among others a unique Th1/Th2 response equilibrium. In early pregnancy, a shift towards a Th2 dominant immune response occurs, preventing fetal rejection. As gestation progresses, the immune response gradually becomes more balanced between Th1 and Th2, with Th1 primarily defending the fetus against pathogens. Maintaining this equilibrium is vital to ensure immune tolerance towards the fetus while allowing the maternal immune system to combat infections (33). After labor, breastfeeding further supports the infant's immune balance by adjusting its composition based on the infant's needs (34). This bridge between maternal and infant immune systems during gestation and lactation plays a vital role in safeguarding both mother and child's health.

Additionally, maternal immunological status impacts the offspring's development. In the last decades, probiotics, prebiotics and synbiotics have demonstrated beneficial effects in adult individuals (35). The research of synbiotics for immunological health improvement has been focused on prophylactic or complementary treatments for different diseases such as infections or antibiotics-induced diarrhea (9). However, few studies have evaluated the impact of synbiotic supplementation during gestation and lactation on the maternal and newborn immune system (36).

Here, we have demonstrated that the supplementation with a synbiotic composed of *B. breve* M-16V and scGOS/lcFOS during pregnancy is safe for the dams, since it does not affect the weight gain, food or water intake and the hematological parameters at the weaning day.

Physiologically, the expansion of adipose tissue during pregnancy aims to provide necessary nutrients for the correct development of the fetus (37). Dietary habits during pregnancy play an important role in the offspring health. Maternal high fat diet causes reprogramming of adipose tissue including increasing adipogenic and lipogenic markers in both WAT and BAT (38). The overexpansion of the adipose tissue during gestation may increase the adverse outcomes in the later life of both the mother and the offspring, mainly associated with glucose and insulin metabolism (39–41). In this study, supplementation with scGOS/lcFOS and *B. breve* M-16V during pregnancy, increased the number of nuclei and decreased the area of LDs in BAT. Moreover, the slight increase of *Ucp1* expression may be able to justify the obtained results pointing out the activation of this tissue (39,42). BAT is specialized in dissipating excess energy into heat (non-shivering thermogenesis) through mitochondrial uncoupling protein 1 (*Ucp1*) (43). It is well documented that bioactive compounds like antioxidants and dietary fiber enhance the expression of thermogenic genes in BAT (44–46). In fact, BAT is involved in heat generation for maintaining the body temperature, and it is diminished in obese individuals. Thus, a higher number of nuclei and a reduction of the area of the LDs indicate a higher activation of the BAT (42). Joining these approaches, our results suggest that synbiotic supplementation influences positively the adipose tissue during gestation and lactation by increasing the BAT activity.

Different samples from the gastrointestinal tract were analyzed such as the SI, the cecum, and feces at weaning. *B. breve* M-16V and scGOS/lcFOS induced macroscopic and microscopic trophic effects on the SI. The relative weight of the SI and the villi and crypts length increased at the end of lactation, suggesting a higher nutrient-absorptive surface that could contribute to a healthier gastrointesinal tract and overall health (47). In addition to the observed morphologic changes resulting from supplementation, we also examined the immunological maternal status at systemic and gastrointestinal levels. The systemic immunological status was assessed by measuring the concentration of various Igs in different compartments, including plasma and milk. The supplementation of *B. breve* M-16V and scGOS/lcFOS enhanced the presence of IgG2c in both plasma and milk. In addition, a positive correlation between IgG2c levels in plasma and milk was found, suggesting the improvement of the immunological composition of milk. Human milk is known to contain high levels of IgA, which provides protection to the gastrointestinal tract. In contrast, rat milk primarily consists of IgG, which enhances short-term systemic immune response through receptor-mediated endocytosis in the neonatal intestine, facilitating its absorption (48). Similar to our results, previous studies have demonstrated that supplementation with the human milk oligosaccharide 2'-Fucosyllactose increases neonatal plasmatic IgG2c during early life (18) which also correlates in the maternal Ig profile of plasma and milk (49).

After infections IgGs are the main contributors to long-term immunity (50). Little is known about the role of dams IgG2c. However, in mice IgG3 (analog of rat IgG2c) (48) has been linked to regulatory responses in the neonate intestine to translocate microbes and might be involved in long-term

immunity (51). Considering the increase of IgG2c in plasma and milk, we suggest that maternal synbiotic supplementation induces the IgG2c isotype switching to promote the long-term passive immunization to the infant through the breastfeeding.

The study of the gastrointestinal tract revealed that the synbiotic supplementation not only modified the microstructures of the SI but also the gene expression levels. The synbiotic supplementation increased *Muc3, Cldn4* and *Ocln* intestinal expression at the weaning day. *Muc3* is a transmembrane mucin that exerts protective roles in inflammatory bowel conditions (52). *Cldn4* and *Ocln* are epithelial tight junction (TJ) proteins involved in the passage of ions and macromolecules across the intestinal epithelium (53). The effects of the probiotic *B. breve* M-16V or the prebiotic scGOS/lcFOS have been widely studied by separate, suggesting anti-inflammatory and protective roles, respectively (54,55). However, little information is known about the combination of both. Overall, these results indicate a direct relationship between the synbiotic supplementation and the improvement of the gut barrier function.

IgA is the most abundant Ig in the mucosal compartment and contributes to the development of the immune response (56). The analysis of Ig-CB has been controversial due to its imbalance in healthy and disease conditions (57). In heathy people, IgA-coated microbiome plays a homeostatic role in the gut favoring host-microbiome symbiosis while in inflamed gut it may exacerbate the inflammation (58). In our model in healthy conditions, the synbiotic supplementation, did not impact the sIgA, however it increased the Ig-CB in cecum, corroborating previous results that suggest the participation in the maintenance of the gut homeostasis (56,59–62). The production of cecal IgA can be induced by endogenous or pathogenic bacteria, pathogen-induced IgA is considered to have high-affinity and specificity (63–65). This fact suggests that even though the IgA is not increased, the increased Ig-CB can be linked to the higher affinity induced by pathogenic species, facilitating its elimination.

Although there is limited information about the impact of synbiotics on cecal and fecal features during gestation and lactation, the effect of probiotic supplementation has been studied during these periods (10,56). In our case, synbiotic supplementation reduced the fecal pH during gestation. Probiotics have been linked to a reduction of the intestinal pH due to the production of organic acids (62). As the cecum is characterized by the presence of high amounts of SCFA, and the synbiotic supplementation led to an overall increase in SCFA levels, primarily attributed to the rise in acetic, propanoic, butanoic, and isovaleric acids, both facts could be connected. In addition, numerous studies have shown that SCFAs have beneficial effects on energy metabolism, intestinal structure and integrity, as well as immunological regulation of anti-inflammatory activities (66). Overall, our study suggests that synbiotic supplementation enhance the production of SCFA which exert a reduction of the fecal pH.

Previously, it has been demonstrated that a maternal supplementation with a probiotic is able to influence the milk composition (67). The exact mechanisms involved are not yet fully understood, but both a direct and indirect effect could be participating. Regarding the direct effect of the probiotic, it could be due to its arrival to the breast milk by the entero-mammary pathway (68). This pathway enables the movement of commensal bacteria from the intestine to the mammary gland and milk, and consequently microorganisms are able to reach the infant intestine and therefore influencing the colonization of the newborn's microbiota (67). This is not the case in our study, where we did not observe the presence of *B. breve* M-16V in the mammary gland.

Pregnancy leads to dramatic changes in the gut microbiome. In the last decades it has been demonstrated that during pregnancy a healthy microbiota undergoes a shift to a more dysbiotic one (69). In general, pregnancy reduces the alpha diversity and increases the beta diversity (70).

Considering our results of the cecal microbiota, the synbiotic supplementation modified the beta diversity and also, the Chao1 diversity index and the cluster aggrupation of the supplemented group was different compared to the non-supplemented one. The SYN intervention also influenced the beta-diversity but not the alpha-diversity indexes in the IC. It is also relevant that SYN intervention did not influence the milk microbiota profile. However, the supplementation with the synbiotic influences the phylum, family and genus composition of the CC, IC and milk. In general, during gestation *Faecalibacterium* is reduced in the CC and IC (71). It has to be noted that *Faecalibacterium* is one of the main producers of butyrate (72). Our results indicated that the maternal synbiotic supplementation is able to counteract this reduction during this period, which can be confirmed with the levels of SCFA, as butyrate was increased in the SYN group. In addition, one of the most remarkable microbiota changes is the increase of the *Bifidobacteriaceae* and *Bifidobacterium* in the milk, suggesting that the maternal supplementation may be able to modify the milk composition through the entero-mammary route (68), as this change is also found in the CC. The importance of *Bifidobacterium* is due to the positive impact on the infant development, improving the nutrients absorption and regulating the immune system (73,74). These findings confirm that this particular maternal synbiotic supplementation reduces microbiota alterations during gestation while enriches milk composition. These facts will contribute to the infant colonization and immune system maturation through the breastfeeding.

The obtained results point out that maternal nutritional intervention with *B. breve* M-16V and scGOS/lcFOS modulates maternal immune system during pregnancy and lactation. Synbiotic supplementation not only modulates the immunological profile of dams, but also the immune components of the milk composition which targets to encourage the infant development. Although the beneficial results of the study are clear, there are still some limitations and gaps that require further investigation. In this sense, although animal models have been highly useful in understanding the physiological changes induced after conception, there are significant differences between humans and animals, in this case rats, regarding pregnancy features. As consequence, integrating pre-clinical results into clinical knowledge should be performed to ensure translation. Besides this, to complete the study, further research is needed to determine whether supplementation during pregnancy or breastfeeding has a greater impact on modulating the maternal immune system and the composition of breast milk. Additionally, it is important to analyze the impact of B. breve M-16V and scGOS/lcFOS separately to evaluate if any of them interacts more with the maternal immune system. Finally, the last step required would be to analyze the impact of these maternal interventions on their offspring and study the transmission of the bioactive components from the mother to the infants.

## 5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Johan Garssen is part time employee/scientific advisor of Danone Nutricia Research. Raphaëlle Bourdet is employee of Danone Nutricia Research, and Karen Knipping was also at time of the experiment.

## 6 Author Contributions

Conceptualization, M.J.R.-L., M.C.C. and F.J.P.-C.; formal analysis and investigation, L.S.-F., B.G.-P., M.M.-C., G.K., M.B.; writing-original draft preparation; L.S.-F.; writing-review and editing, R.B.-S., M.J.R.-L., M.C.C., M.C. and F.J.P.-C.; funding acquisition, M.C.C. and F.J.P.-C. J.G. and K.K. played a key role in this public private research program and organized and explained the

ingredients for intervention including background knowledge on the synbiotics used. All authors have read and agreed to the published version of the manuscript.

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## 9 Legends

**Figure 1.** Effect of SYN supplementation on dams' growth and daily intake during gestation and lactation. (a) Dams' body weight (BW) evolution. (b) Dams' chow intake. (c) Dams' water intake. Data are expressed as mean ± standard error of the mean (S.E.M.). Statistical differences: $^{*}p< 0.05$ *vs* REF. (n=5-6).

**Figure 2.** Impact of SYN supplementation on the adipose tissue. (a) Relative weight of the adipose tissue from different locations. (b) Hematoxylin and eosin-stained sections of parametric WAT (P-WAT) and BAT. Images were captured at 200x and 400x magnification, respectively. (c) Analysis of P-WAT: adipocyte area and number of adipocytes. (d) Analysis of BAT: number of nuclei, area of lipid droplets (LD), and number of LD (>50µm$^2$). (e) Relative gene expression analysis of BAT calculated with respect to REF, which corresponded to 100% of gene expression (represented with a horizontal dotted line). Data (a, c and d) are expressed as mean ± S.E.M. Statistical differences: $^{*}p< 0.05$ *vs* REF. (n=5-6). WAT, white adipose tissue; BAT, brown adipose tissue; P, parametric; R, retroperitoneal; D, dorsal; I, Inguinal.

**Figure 3.** Fecal sample analysis throughout gestation and lactation. (a) IgA levels. (b) pH. (c) Relative water content. Data are expressed as mean ± S.E.M. Statistical differences: $^{*}p< 0.05$ *vs* REF. (n=5-6). G, gestation; L, lactation.

**Figure 4.** Effect of SYN supplementation on the Ig profiles in different compartments. Total Igs levels (IgA, IgM, and IgG) in (a) plasma, (d) milk, (g) SG and (j) MLN. Relative proportion of IgG subtypes in (b) plasma, (e) milk, (h) SG and (k) MLN. Analysis of non-parametric multidimensional scaling (NMDS) for the Ig profiles based on the Bray-Curtis distance in (c) plasma, (f) milk, (i) SG and (l) MLN. (m) Analysis of the Th1/Th2 ratio at the end of suckling in the different compartments. (n) Correlation between the Ig profile of plasma with respect to the Ig profiles of milk, SG and MLN. Data (a, d, g, j) are expressed as mean ± S.E.M. $^{*}p< 0.05$ *vs* REF (by Kruskal Wallis test). (n=5-6). Each point represents an animal in figures (c, f, i, l) (n=11) (by ANOSIM test). The Spearman correlation coefficient is represented in the heat map following the color in the legend. Correlations with statistical significance ($p< 0.05$) are shown in a bold frame. SG; salivary gland; MLN, mesenteric lymph nodes.

**Figure 5.** Gene expression in small intestine and mammary gland. Relative gene expression analysis in the small intestine of (a) mucins, (b) TJ proteins and (c) Toll-like receptors. (d) Relative gene expression of *IgA* and *GPR43* (*Ffar2*) genes in mammary gland. Relative gene expression was calculated with respect to REF, which corresponded to 100 % of transcription (represented with a horizontal dotted line). Statistical differences: $^{*}p<0.05$ *vs* REF. (n=5–6). *Muc2*, mucin2; *Muc3*, mucin3; *Zo-1*, Zonula occludens-1; *Cldn2*, claudin 2; *Cldn4*, claudin 4; *Ocln*, occludin; *TLR*, Toll-like receptor; *IgA*, immunoglobulin A; *Ffar2*, free fatty acid receptor 2.

**Figure 6.** Evaluation of the cecal samples. (a) IgA concentration in cecum. (b) Representative dot-plots of Ig-CB. (c) Total bacterial counts in the cecum, proportion of Ig-CB, total Ig-CB and the mean fluorescence intensity (MFI) of the Ig-CB. Data are expressed as mean ± S.E.M. Statistical differences: $^{**}p< 0.05$ *vs* REF. (n=5-6).

**Figure 7.** Effect of SYN on intestinal architecture. (a) Representative images of the small intestine stained with hematoxylin and eosin, 100X. (b) Height, width, and area of the intestinal villi. Results are expressed as mean ± S.E.M. Statistical differences: $^{*}p< 0.05$ *vs* REF. (n=5-6).

**Figure 8.** Detection of *B. breve* M-16V in fecal samples during gestation (G21) and lactation (L21) and in mammary gland (MG). The horizontal dotted line represents the levels of the REF group, which were considered as basal levels. Results are expressed as mean ± S.E.M. $^{*}p< 0.05$ *vs* basal levels (n=5-6).

**Figure 9.** Beta- and alpha-diversity in rat microbiota depending on SYN intervention. Beta-diversity analysis using Bray-Curtis distance in CC (a), IC (d) and milk samples (g). Alpha-diversity indexes (Shannon index) and richness (Chao1 index) for CC (b, c), IC (e, f) and milk (h, i) samples. Statistical testing was performed by PERMANOVA using Bray Curtis distances and the Mann-Whitney test was used for alpha-diversity indexes. (n=5-6).

**Figure 10.** Microbiota composition of dams at weaning in cecal content (CC), intestinal content (IC) and milk. Relative proportions (a) phylum and (b) families present in the CC. Relative proportions (c) phylum and (d) families present in the IC. Relative proportions (e) phylum and (f) families present in the milk. Results are expressed as relative proportions of population. Statistical differences: $^{*}p< 0.05$ *vs* REF; $^{\#}p< 0.1$ *vs* REF. (n=5-6).

**Figure 11.** Cecal SCFA composition at the end of the weaning. (a) Total SCFA. (b) Acetic, formic, propionic, isobutanoic, butanoic, isovaleric, valeric and hexanoic acids were quantified by HS-GC-MS. Results are expressed as mean ± S.E.M. Statistical differences: $^{*}p< 0.05$ *vs* REF. (n=5-6).

**Figure 12.** Correlation between the levels of Ig2c in plasma, milk, SG, and MLN with respect to the *Bifidobacterium* (16s sequence) and cecal SCFA. The Spearman correlation coefficient is represented in the heat map following the color in the legend. Correlations with statistical significance ($p< 0.05$) are shown in a bold frame. (n=5-6). SG; salivary gland; MLN, mesenteric lymph nodes; CC, cecal content.

## 10 Tables

**Table 1**. Effect of *B. breve* M-16V and scGOS/lcFOS on the spleen and MLN immune cells proportion at the end of suckling period.

| | SPLEEN | | MLN | |
|---|---|---|---|---|
| **%** | **REF** | **SYN** | **REF** | **SYN** |
| **B cells (CD45RA+)** | 9.13 ± 1.89 | 9.91 ± 4.09 | 13.64 ± 5.27 | 4.44 ± 5.71 |
| **T cells (TCRαβ+NK- and TCRgδ+)** | 72.23 ± 7.38 | 64.08 ± 8.67 | 79.90 ± 2.80 | 70.44 ± 17.04 |
| TCRαβ+ NK- | 64.87 ± 8.33 | 60.40 ± 10.76 | 77.83 ± 3.27 | 68.80 ± 17.35 |
| % CD8+ | 27.83 ± 1.83 | 27.06 ± 1.52 | 22.63 ± 1.87 | 16.51 ± 4.31 |
| TCRgδ+ | 2.09 ± 0.55 | 3.68 ± 2.11 | 2.61 ± 0.69 | 1.64 ± 0.32 |
| % CD8+ | 2.56 ± 0.84 | 1.54 ± 0.35 | 3.42 ±2.28 | 0.72 ± 1.43 |
| CD4+ CD8- | 49.15 ± 7.39 | 41.83 ± 9.31 | 61.88 ± 2.64 | 56.11 ± 7.32 |
| CD8+ CD4- | 24.72 ± 2.80 | 24.28 ± 3.68 | 20.28 ± 0.34 | **18.4 ± 0.72*** |
| CD4+ CD8+ | 2.76 ± 0.59 | 1.45 ± 0.45 | 1.33 ± 0.26 | 1.00 ± 0.25 |
| **NK (TCRαβ- NK+)** | 4.45 ± 2.01 | 5.33 ± 2.73 | 0.85 ± 0.16 | 1.14 ± 0.54 |
| % CD8+ | 55.86 ± 9.45 | 40.26 ± 18.52 | 21.47 ± 7.11 | 30.42 ± 11.90 |
| **NKT (TCRαβ+ NK+)** | 2.81 ± 0.85 | 3.77 ± 0.92 | 1.81 ± 0.44 | 1.80 ± 0.28 |
| % CD8+ | 79.16 ± 5.46 | 86.30 ± 2.00 | 60.35 ± 7.37 | 64.80 ± 7.51 |
| **αE+** | 2.72 ± 0.40 | 4.47 ± 2.20 | 3.88 ± 0.76 | 8.04 ±2.60 |
| **CD62L+** | 77.72 ± 0.33 | 72.81 ± 8.14 | 69.07 ± 10.40 | 52.90 ± 5.86 |

Data are expressed as mean percentage ± S.E.M. (n=5-6). Statistical differences: $^{*}p< 0.05$ vs REF. mAbs: fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), allophycocyanin (APC), brilliant violet 421 (BV421), and phycoerythrin-Cyanine 7 (PE-Cy7).

627

628 **Table 2**. Differential microbial genera between REF and SYN in CC.

| Genera | log2FC | lfcSE | p-values | FDR |
|---|---|---|---|---|
| ***g__Anaeroplasma*** | -23.783 | 2.4324 | 0.000 | 0.000 |
| ***g__Bifidobacterium*** | 8.6122 | 1.2076 | 0.000 | 0.000 |
| ***g__Faecalibaculum*** | 9.2997 | 1.3103 | 0.000 | 0.000 |
| ***g__Marvinbryantia*** | 8.8595 | 1.3216 | 0.000 | 0.000 |
| ***g__Eubacterium_ruminantium_group*** | -8.72 | 1.3289 | 0.000 | 0.000 |
| ***g__Colidextribacter*** | -1.2643 | 0.28954 | 0.000 | 0.000 |
| ***g__otherMuribaculaceae*** | 0.98846 | 0.33762 | 0.003 | 0.037 |
| ***g__Harryflintia*** | 1.2993 | 0.45857 | 0.005 | 0.044 |
| ***g__Ruminiclostridium*** | 1.627 | 0.61146 | 0.008 | 0.066 |
| ***g__Incertae_Sedis*** | 1.5033 | 0.57876 | 0.009 | 0.066 |
| ***g__Blautia*** | 4.9847 | 1.9217 | 0.009 | 0.066 |
| ***g__Lachnospiraceae_NK4A136_group*** | 1.2904 | 0.54075 | 0.017 | 0.108 |

629 DESeq2 results showing the log2 fold-change values of bacteria at genus level between REF and SYN groups (positive
630 means more represented in SYN vs REF, and negative values means more represented in REF than in SYN).

631 **Table 3.** Differential microbial genera between REF and SYM in IC.

| Genera | log2FC | lfcSE | p-values | FDR |
|---|---|---|---|---|
| ***g__Faecalibaculum*** | 9.9933 | 1.7576 | 4.945E-7 | 1.3013E-8 |
| ***g__Bifidobacterium*** | 9.6098 | 1.8423 | 3.4689E-6 | 1.8257E-7 |
| ***g__Eubacterium_ruminantium_group*** | -10.295 | 2.1721 | 2.7154E-5 | 2.1437E-6 |
| ***g__Blautia*** | 7.8482 | 2.284 | 0.0050207 | 5.8997E-4 |
| ***g__Colidextribacter*** | -6.5453 | 1.922 | 0.0050207 | 6.6061E-4 |
| ***g__Lactococcus*** | -4.3031 | 1.5419 | 0.033301 | 0.0052581 |
| ***g__Ruminococcus_gauvreauii_group*** | 3.8752 | 1.8731 | 0.20928 | 0.038552 |
| ***g__Bacteroides*** | -4.3033 | 2.2519 | 0.23335 | 0.056004 |

632 DESeq2 results showing the log2 fold-change values of bacteria at genus level between REF and SYN groups (positive
633 means more represented in SYN vs REF, and negative values means more represented in REF than in SYN).

634

635 ## 11 Reference styles

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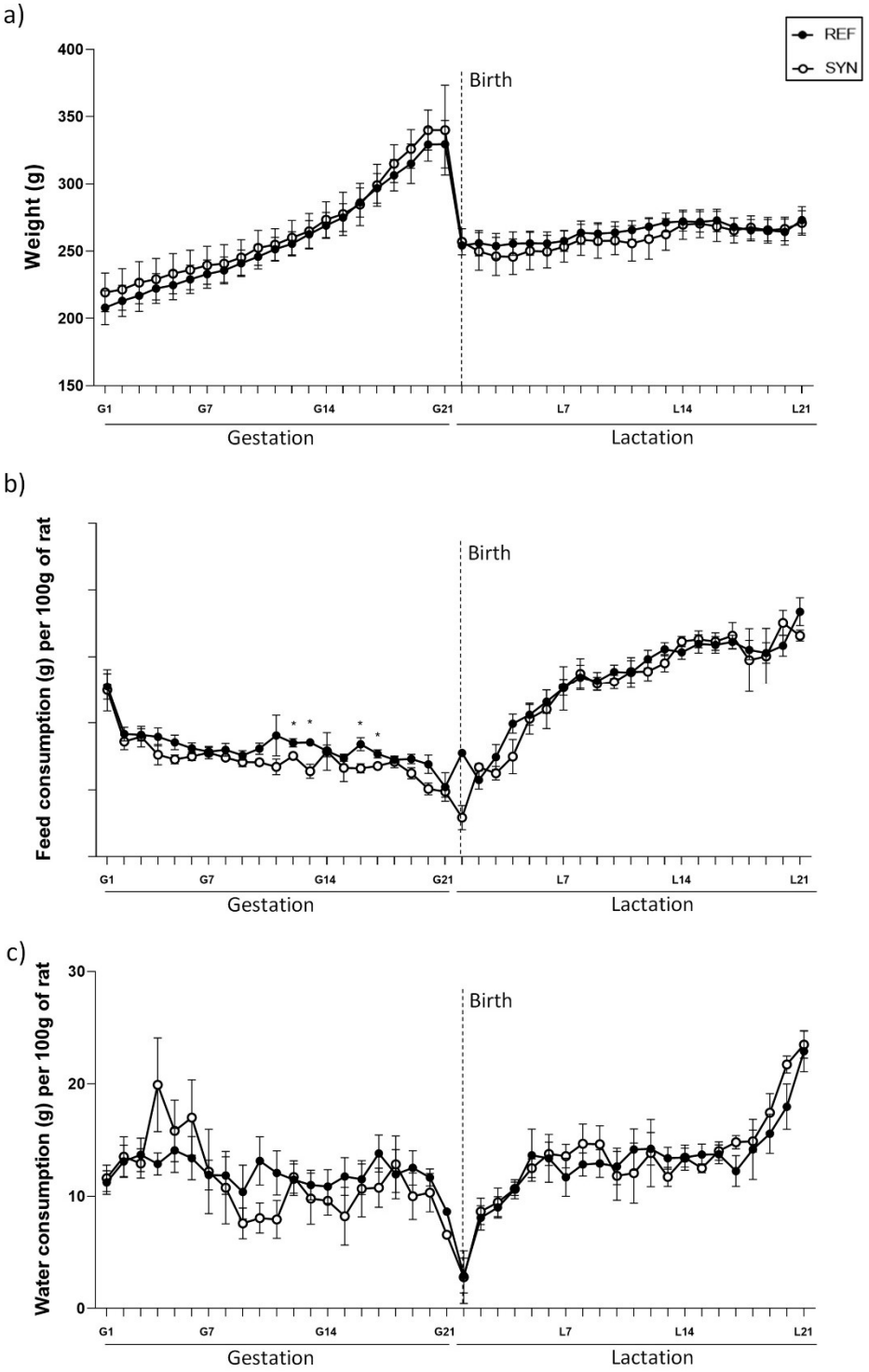
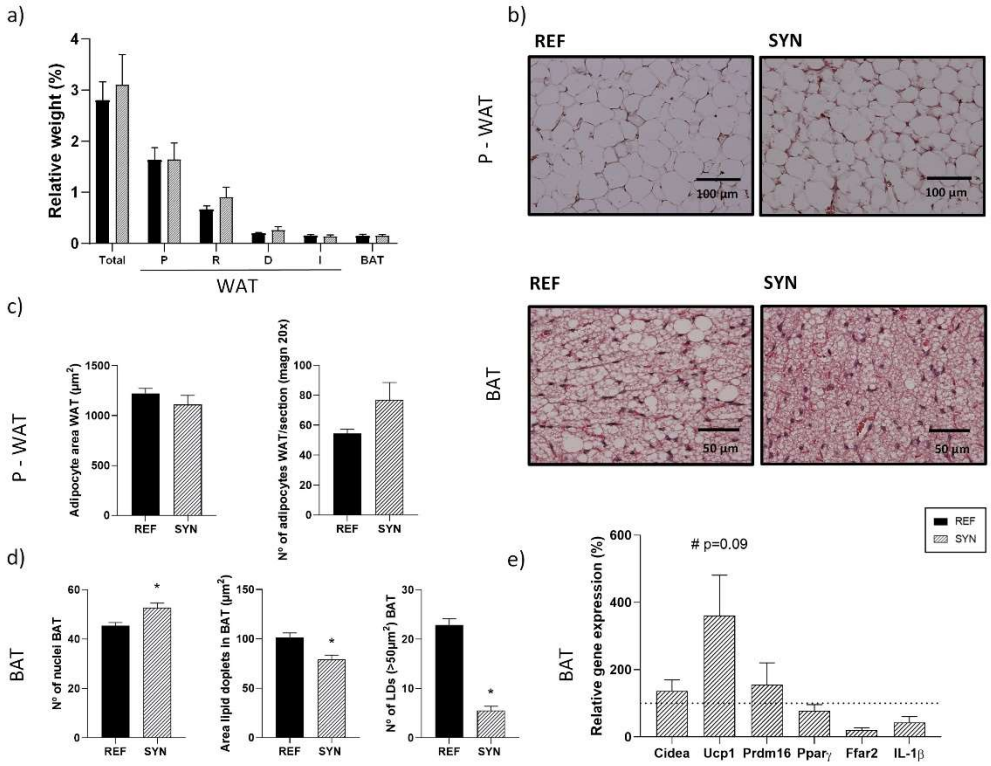
## 12 Supplementary Material

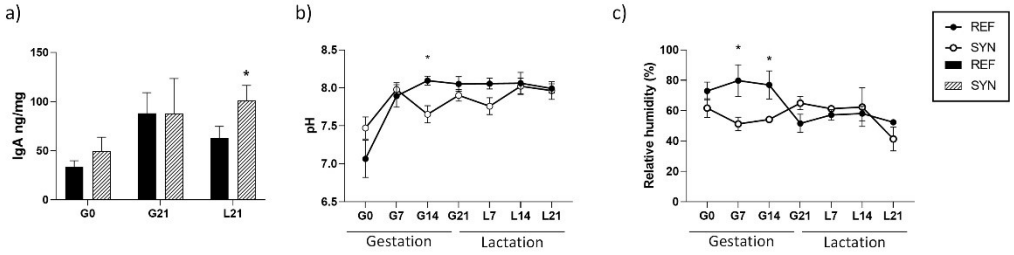
The Supplementary Material for this article can be found online at: XXX

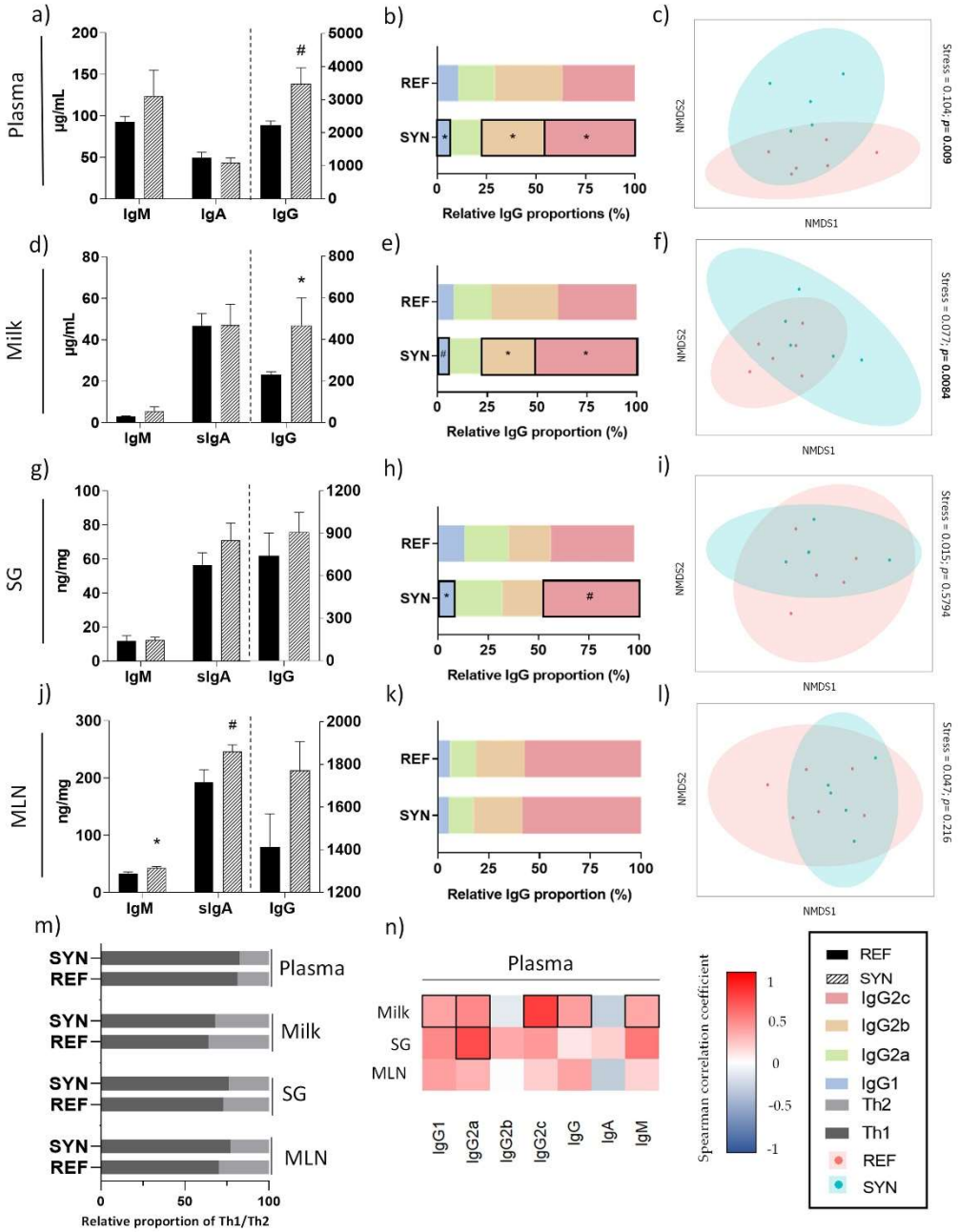
## 12 Data Availability Statement

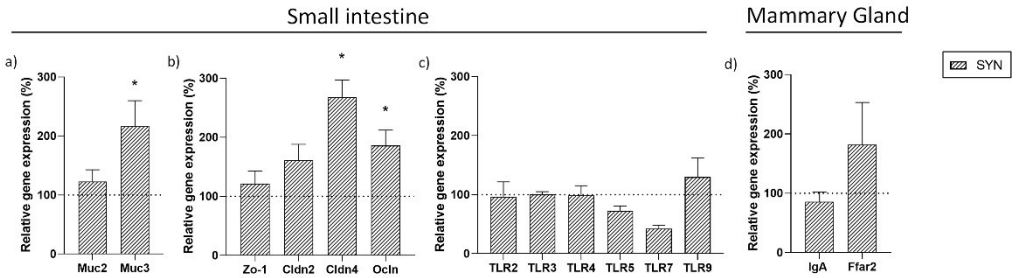
868 The datasets [GENERATED/ANALYZED] for this study can be found in the [NAME OF
869 REPOSITORY] [LINK]. Please see the "Availability of data" section of Materials and data policies
870 in the Author guidelines for more details.

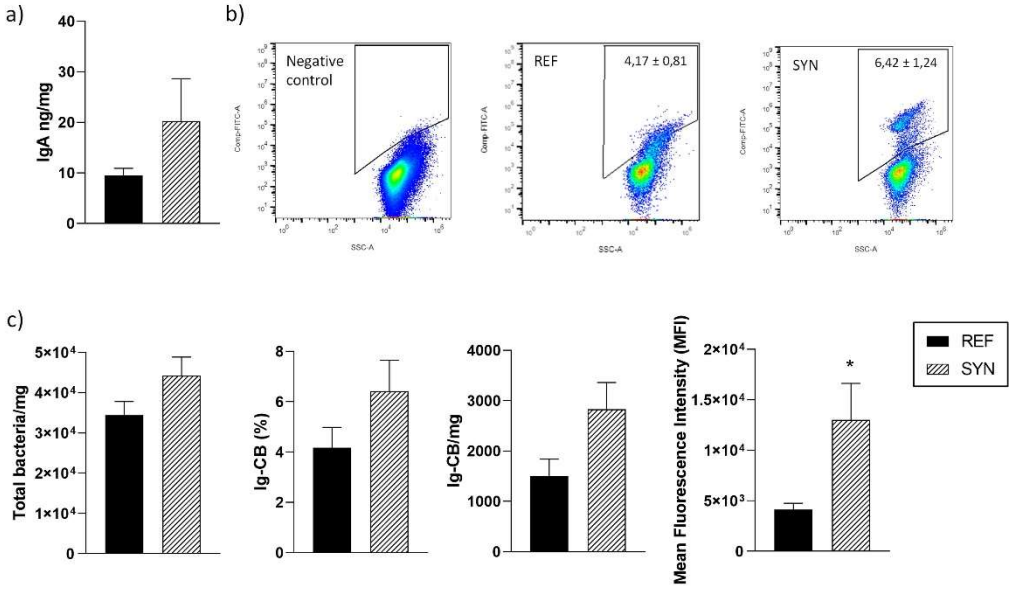

a)
REF
SYN
Birth
Weight (g)
400
350
300
250
200
150
G1
G7
G14
G21
L7
L14
L21
Gestation
Lactation
b)
Birth
Feed consumption (g) per 100g of rat
Gestation
Lactation
c)
Birth
Water consumption (g) per 100g of rat
30
20
10
0
Gestation
Lactation

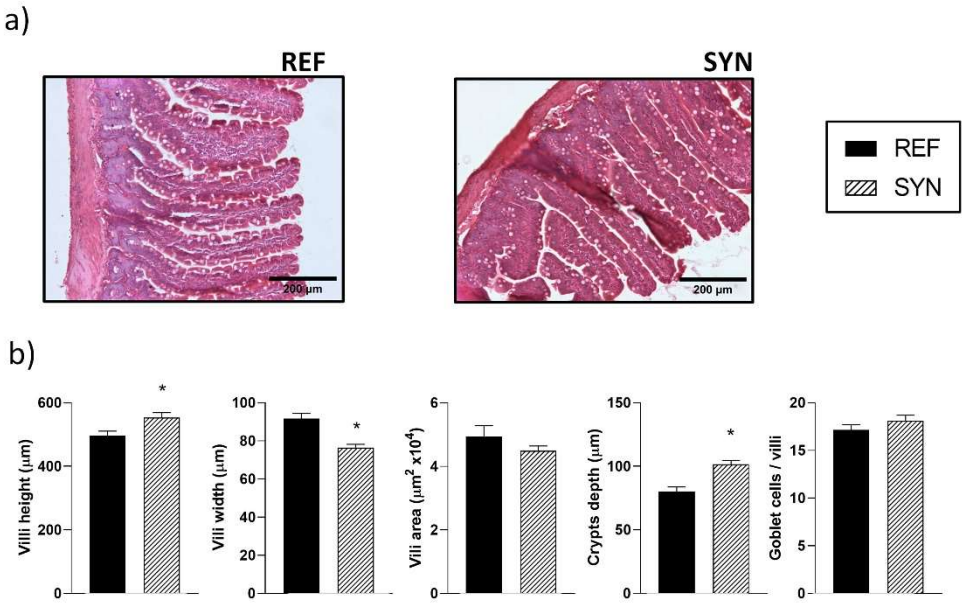

a)
Relative weight (%)
Total
P
R
D
I
BAT
WAT
b)
REF
SYN
P - WAT
100 µm
BAT
50 µm
c)
Adipocyte area WAT (µm²)
N° of adipocytes WAT/section (magn 20x)
d)
N° of nuclei BAT
Area lipid doplets in BAT (µm²)
N° of LDs (>50µm²) BAT
e)
# p=0.09
Relative gene expression (%)
Cidea
Ucp1
Prdm16
Pparγ
Ffar2
IL-1β

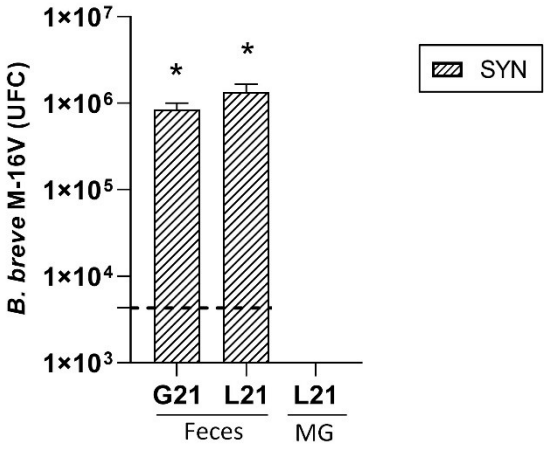

a)
IgA ng/mg
150
100
50
0
G0
G21
L21
*
b)
pH
8.5
8.0
7.5
7.0
6.5
G0 G7 G14 G21 L7 L14 L21
Gestation
Lactation
*
c)
Relative humidity (%)
100
80
60
40
20
0
G0 G7 G14 G21 L7 L14 L21
Gestation
Lactation
*
*
REF
SYN
REF
SYN

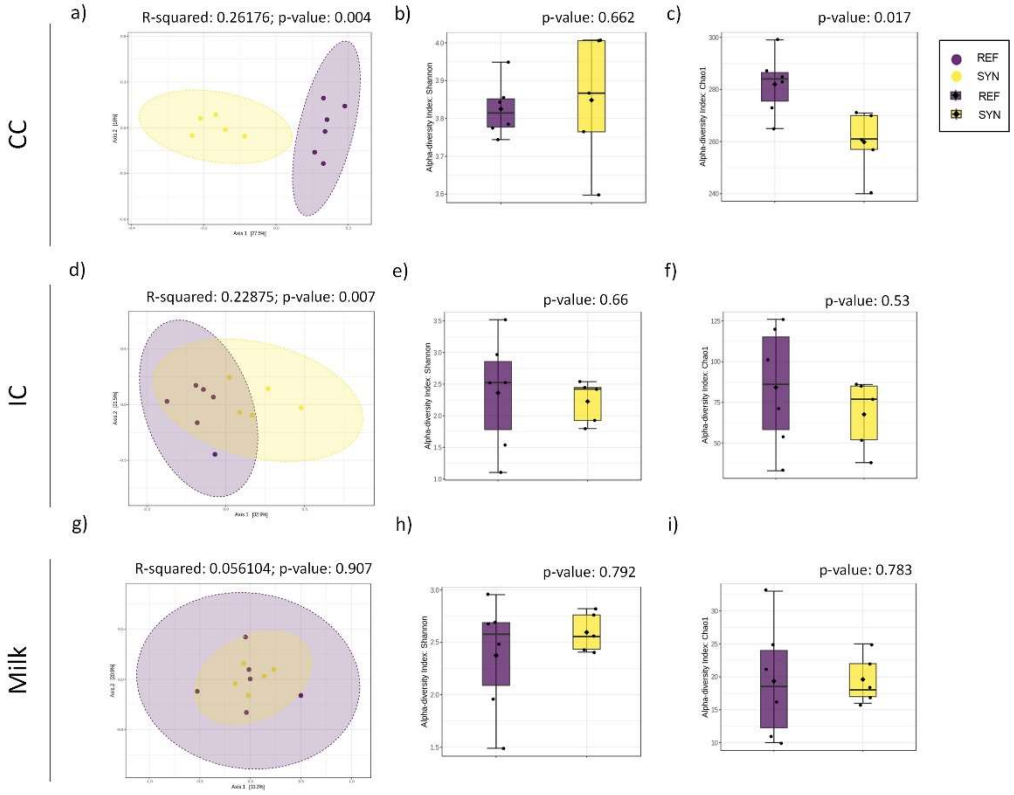

a)
Plasma
µg/mL
IgM
IgA
IgG
b)
REF
SYN
Relative IgG proportions (%)
c)
NMDS2
NMDS1
Stress = 0.104; p= 0.009
d)
Milk
µg/mL
IgM
sIgA
IgG
e)
REF
SYN
Relative IgG proportion (%)
f)
NMDS2
NMDS1
Stress = 0.077; p= 0.0084
g)
SG
ng/mg
IgM
sIgA
IgG
h)
REF
SYN
Relative IgG proportion (%)
i)
NMDS2
NMDS1
Stress = 0.015; p= 0.5794
j)
MLN
ng/mg
IgM
sIgA
IgG
k)
REF
SYN
Relative IgG proportion (%)
l)
NMDS2
NMDS1
Stress = 0.047; p= 0.216
m)
SYN
REF
Plasma
Milk
SG
MLN
Relative proportion of Th1/Th2
n)
Plasma
Milk
SG
MLN
IgG1
IgG2a
IgG2b
IgG2c
IgG
IgA
IgM
Spearman correlation coefficient
REF
SYN
IgG2c
IgG2b
IgG2a
IgG1
Th2
Th1
REF
SYN

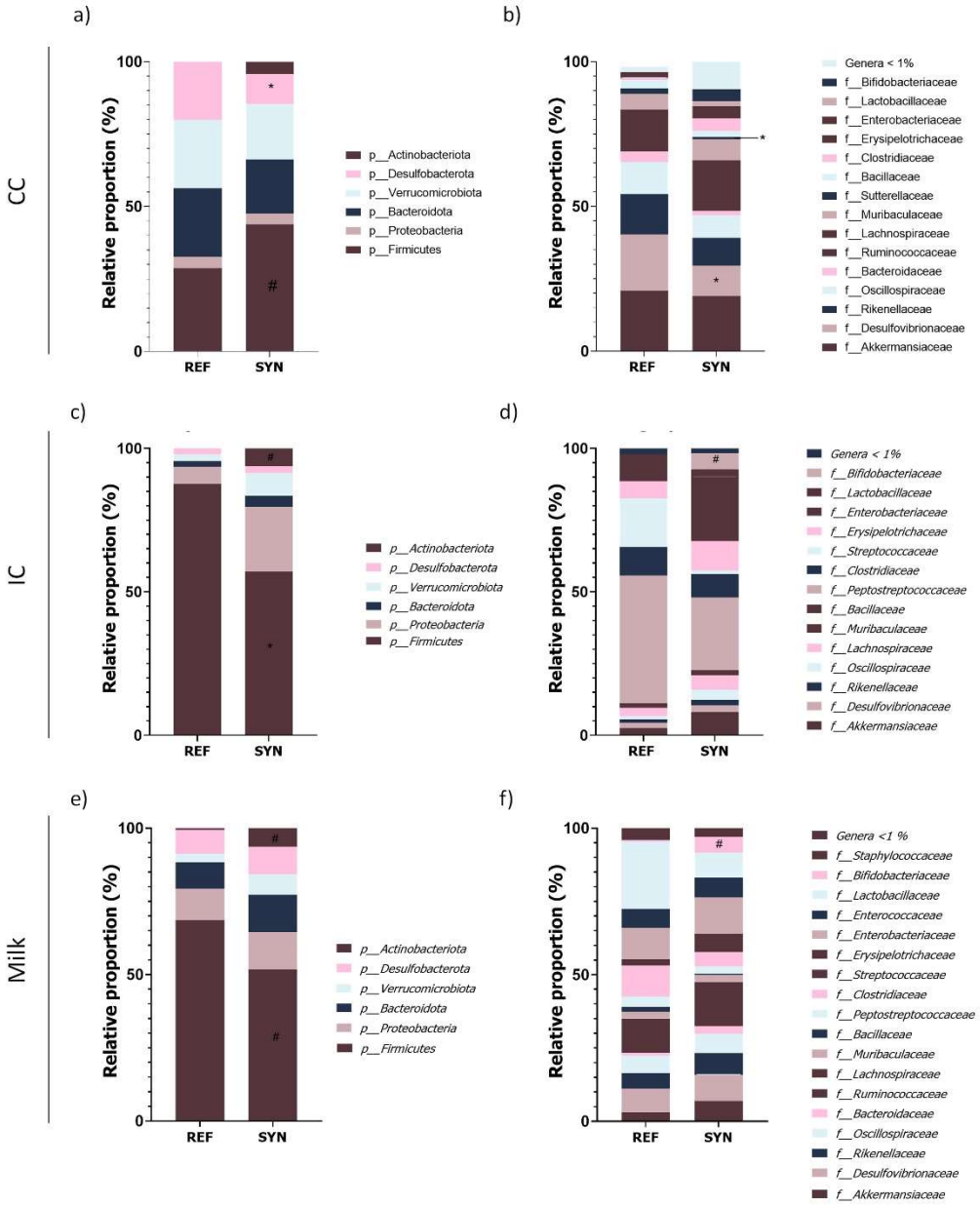

Small intestine
Mammary Gland
SYN
a)
b)
c)
d)
Relative gene expression (%)
300
200
100
0
*
Muc2
Muc3
Zo-1
Cldn2
Cldn4
Ocln
TLR2
TLR3
TLR4
TLR5
TLR7
TLR9
IgA
Ffar2

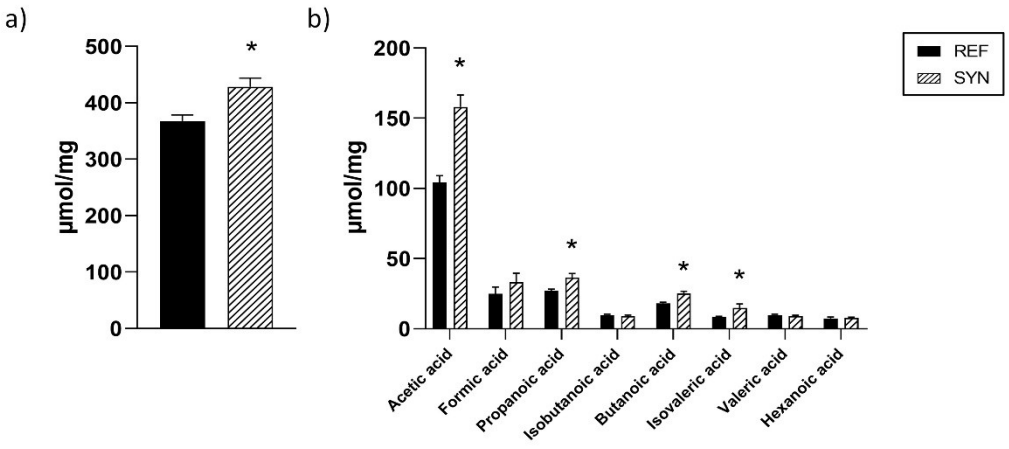

a)
IgA ng/mg
40
30
20
10
0
b)
Negative control
REF
4,17 ± 0,81
SYN
6,42 ± 1,24
SSC-A
c)
Total bacteria/mg
5×10⁴
4×10⁴
3×10⁴
2×10⁴
1×10⁴
0
Ig-CB (%)
8
6
4
2
0
Ig-CB/mg
4000
3000
2000
1000
0
Mean Fluorescence Intensity (MFI)
2×10⁴
1.5×10⁴
1×10⁴
5×10³
0
*
REF
SYN

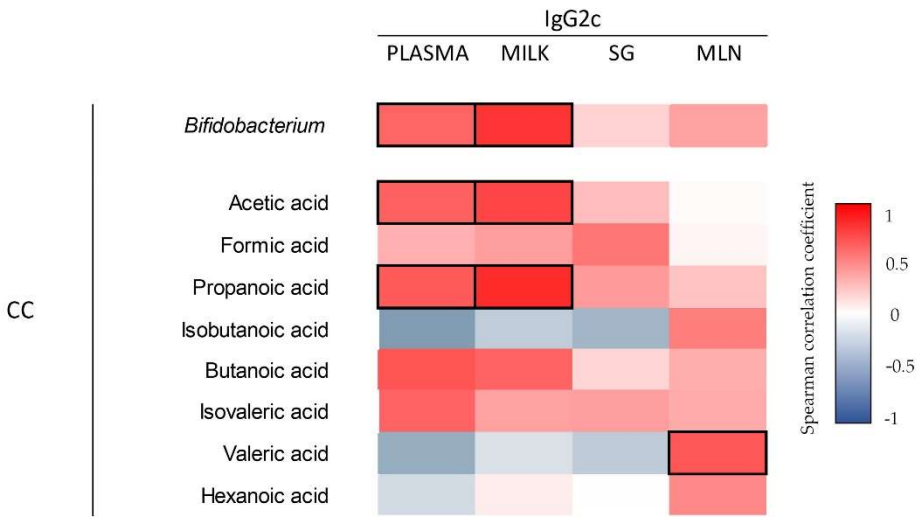

a)
REF
SYN
200 µm
200 µm
REF
SYN
b)
Villi height (µm)
Villi width (µm)
Vili area (µm² x10⁴)
Crypts depth (µm)
Goblet cells / villi
*
*
*


1×10⁷
1×10⁶
1×10⁵
1×10⁴
1×10³
B. breve M-16V (UFC)
*
*
SYN
G21
L21
L21
Feces
MG


a)
R-squared: 0.26176; p-value: 0.004
b)
p-value: 0.662
c)
p-value: 0.017
REF
SYN
REF
SYN
CC
d)
R-squared: 0.22875; p-value: 0.007
e)
p-value: 0.66
f)
p-value: 0.53
IC
g)
R-squared: 0.056104; p-value: 0.907
h)
p-value: 0.792
i)
p-value: 0.783
Milk


a)
b)
c)
d)
e)
f)
CC
IC
Milk
Relative proportion (%)
100
50
0
REF
SYN
p__Actinobacteriota
p__Desulfobacterota
p__Verrucomicrobiota
p__Bacteroidota
p__Proteobacteria
p__Firmicutes
Genera < 1%
f__Bifidobacteriaceae
f__Lactobacillaceae
f__Enterobacteriaceae
f__Erysipelotrichaceae
f__Clostridiaceae
f__Bacillaceae
f__Sutterellaceae
f__Muribaculaceae
f__Lachnospiraceae
f__Ruminococcaceae
f__Bacteroidaceae
f__Oscillospiraceae
f__Rikenellaceae
f__Desulfovibrionaceae
f__Akkermansiaceae
Genera <1 %
f__Staphylococcaceae
f__Enterococcaceae
f__Peptostreptococcaceae
*
#


a)
b)
µmol/mg
500
400
300
200
100
0
*
µmol/mg
200
150
100
50
0
*
*
*
*
REF
SYN
Acetic acid
Formic acid
Propanoic acid
Isobutanoic acid
Butanoic acid
Isovaleric acid
Valeric acid
Hexanoic acid

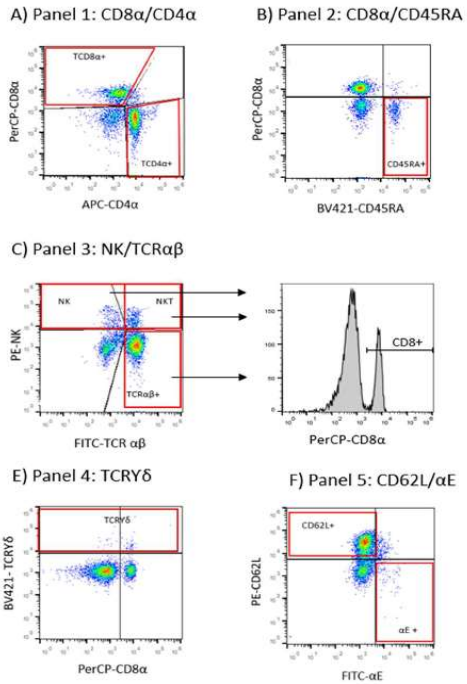

IgG2c
PLASMA
MILK
SG
MLN
Bifidobacterium
Acetic acid
Formic acid
Propanoic acid
Isobutanoic acid
Butanoic acid
Isovaleric acid
Valeric acid
Hexanoic acid
CC
Spearman correlation coefficient
1
0.5
0
-0.5
-1

frontiers

# *Supplementary Material*

## 1 Supplementary Figures

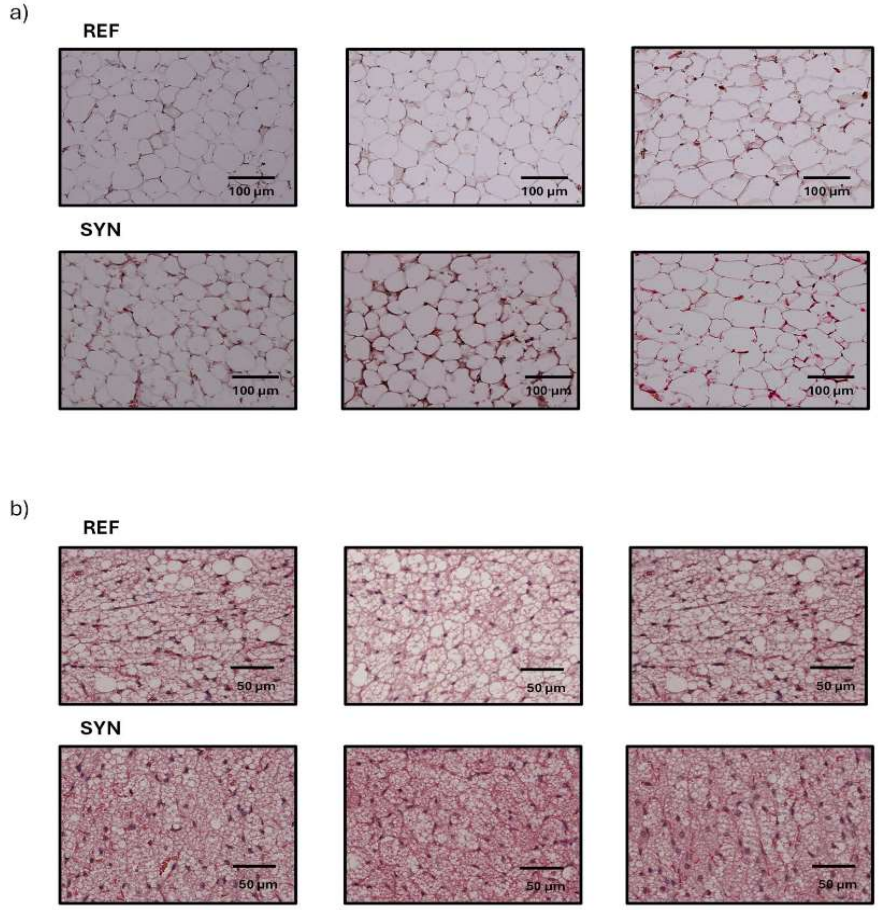
### 1.1 Supplementary Figure 1




**Supplementary Figure 1.** Example of the gating strategy used for MLN and spleen lymphocytes.

Different panels were used to identify the different lymphocyte subsets in MLN and spleen samples. A) In panel 1, the different populations of T helper lymphocytes (TCD8α+ and TCD4α+ cells) were identified. B) In panel 2, the proportion of B cells (CD45RA+) were analyzed. C) In panel 3, NK and TCRαβ cells split, also the proportion of NKT cells was evaluated and in those, the proportion of CD8+ expressing cells. D) In panel 4, the relative proportion of TCRγδ was identified, including the TCD8a+TCRγδ+. E) In panel 5, the αE integrin/CD62L expression pattern was evaluated in populations from panel A and B.

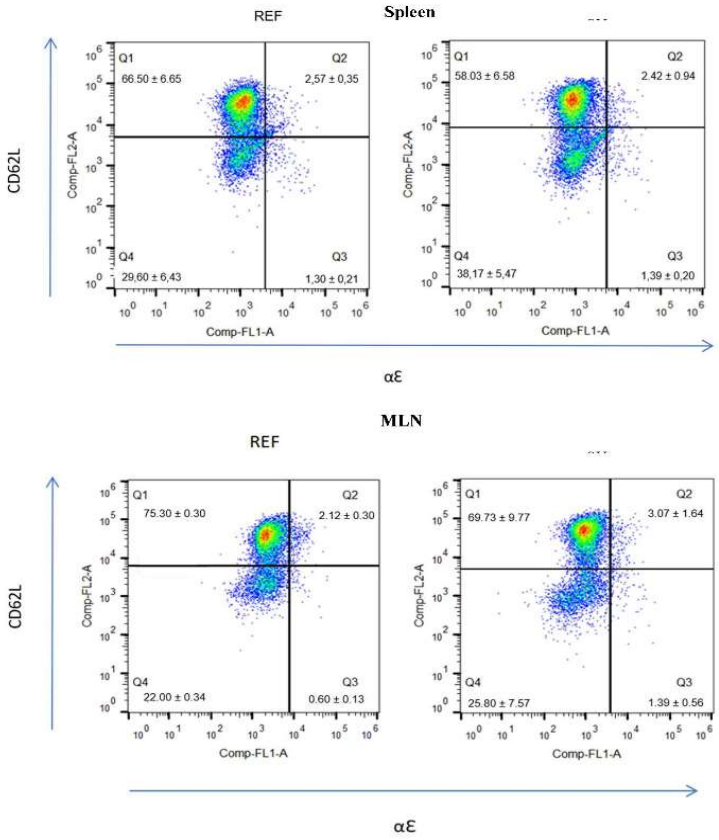
### 1.2 Supplementary Figure 2




**Supplementary Figure 2.** Representative images of the (a) parametric adipose white adipose tissue (P-WAT) and (b) brown adipose tissue (BAT). Three representative images per group of hematoxilin and eosin-stained sections of WAT and BAT. Images were captured at 200x and 400x, respectively.

Supplementary Material

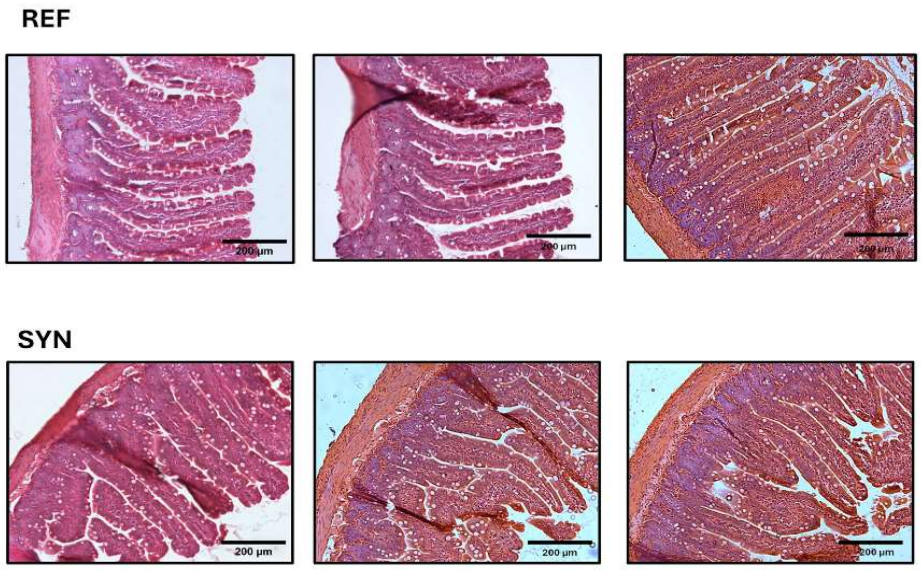
### 1.3 Supplementary Figure 3




**Supplementary Figure 2.** Assessment of αE integrin/CD62L expression patterns in the spleen and MLN. Molecular pattern of αE integrin/CD62L in the total lymphocytes of the spleen and mesenteric lymph nodes (MLN) at the weaning day. Data are expressed as mean ± S.E.M. (n=5-6).

3

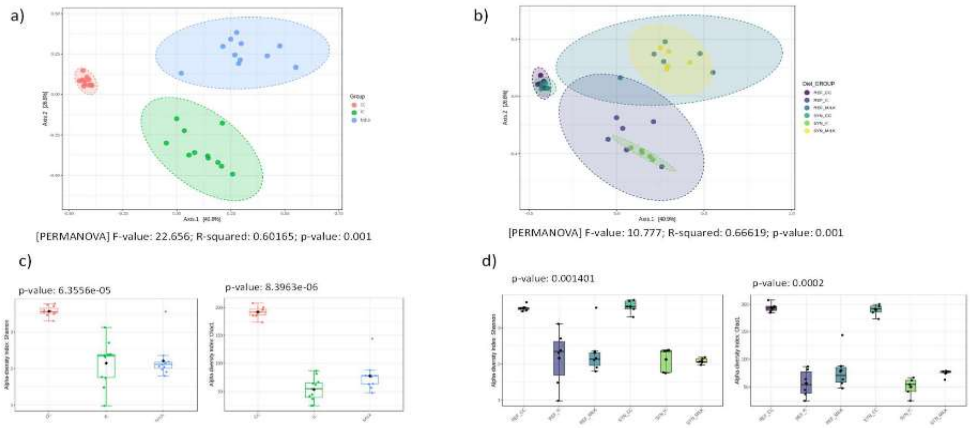
### 1.4 Supplementary Figure 4




**Supplementary Figure 4**. Representative images of the intestinal structures. Three representative images per group of hematoxylin eosin-stained sections of the small intestine captured at 100x.

Supplementary Material

### 1.5 Supplementary Figure 5




**Supplementary Figure 3.** Microbiota composition and diversity in cecal content (CC), intestinal content (IC) and milk samples from Beta-diversity analysis using Bray-Curtis distances according to (a) sample-type and also, (b) depending on the intervention group (REF *vs* SYN). Alpha-diversity indexes (Shannon index) and richness (Chao1 index) for according to (a) sample-type CC, IC and milk and (b) depending on the intervention group (REF *vs* SYN). Statistical testing was performed by PERMANOVA using Bray Curtis distances and the Mann-Whitney test was used for alpha-diversity indexes (n=5-6).

## 2 Supplementary Tables

### 2.1 Table 1. Description of the specific TaqMan primers AB.

| Gene | Reference |
|---|---|
| *Tlr2* | Rn02133647_s1, I |
| *Tlr3* | Rn01488472_g1, I |
| *Tlr4* | Rn00569848_m1, I |
| *Tlr5* | Rn04219239_s1, I |
| *Tlr7* | Rn01771083_s1, I |
| *Tlr9* | Rn01640054_m1, I |
| *Muc2* | Rn01498206_m1, I |
| *Muc3* | Rn01481134_m1, I |
| *Ocln* | Rn00580064_m1, I |
| *Cldn2* | Rn02063575_s1, I |
| *Cldn4* | Rn01196224_s1, I |
| *ZO1* | Rn02116071_s1, I |
| *IgA* | 331943, made to order |
| *Cidea* | Rn04181355_m1, I |
| *Ucp1* | Rn00562126_m1, I |
| *Prdm16* | Rn01516224_m1, I |
| *IL-1β* | Rn00580432_m1, I |
| *Pparγ* | Rn00440945_m1, I |
| *Ffar2* | Rn02345824_s1, I |
| *Fcgrt* | Rn00566655_m1, I, encoding for FcRn |
| *Gusb* | β-glucuronidase, Rn00566655_m1, I |

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Supplementary Material

## 2.2 Supplementary Table 2. Organ size and growth associated parameters at weaning

| | | REF | SYN |
|---|---|---|---|
| Body size | **Body length (cm)** | 21.43 ± 0.39 | 21.22 ± 0.34 |
| | **Body/tail length ratio** | 1.19 ± 0.02 | 1.16 ± 0.03 |
| | **BMI (g/cm²)** | 0.58 ± 0.03 | 0.60 ± 0.01 |
| | **Lee index ($g^{0.33}$/cm × $10^3$)** | 300.64 ± 6.91 | 305.38 ± 3.39 |
| Relative organ size (%) | **Spleen** | 0.14 ± 0.03 | 0.12 ± 0.04 |
| | **Thymus** | 0.14 ± 0.01 | 0.14 ± 0.00 |
| | **Kidney** | 0.36 ± 0.01 | 0.37 ± 0.02 |
| | **Heart** | 0.33 ± 0.05 | 0.38 ± 0.02 |
| | **Liver** | 5.52 ± 0.12 | 5.67 ± 0.17 |
| | **Salivary gland** | 0.14 ± 0.05 | 0.08 ± 0.00 |
| | **Stomach** | 0.57 ± 0.03 | 0.56 ± 0.02 |
| | **Cecum** | 0.32 ± 0.04 | 0.30 ± 0.05 |
| | **Small intestine** | 3.54 ± 0.05 | 4.50 ± 0.07* |

Data are expressed as mean ± S.E.M. (n=5-6). Statistical differences: $^{*}p < 0.05$ *vs* REF.

**2.3 Supplementary Table 3. Hematological variables at weaning day.**

| Hematological variables | REF | SYN |
|---|---|---|
| Leucocytes ($x10^9$ /L) | 7.83 ± 1.07 | 6.95 ± 1.29 |
| Lymphocytes ($x10^9$ /L) | 2.30 ± 0.41 | 2.78 ± 0.19 |
| Monocytes ($x10^9$ /L) | 0.42 ± 0.06 | 0.30 ± 0.04 |
| Granulocytes ($x10^9$ /L) | 4.58 ± 0.89 | 3.88 ± 1.30 |
| Erythrocytes ($x10^{12}$ /L) | 9.28 ± 0.51 | 9.53 ± 0.37 |
| HGB (g/L) | 162.17 ± 8.29 | 168.75 ± 7.48 |
| HCT (%) | 47.82 ± 2.72 | 49.40 ± 1.83 |
| MCV (fL) | 51.57 ± 0.30 | 51.90 ± 0.33 |
| MCH (pg) | 17.47 ± 0.27 | 17.68 ± 0.31 |
| Platelets ($x10^9$ /L) | 177.83 ± 47.20 | 232.25 ± 25.32 |

Data are expressed as mean ± S.E.M. (n=5-6). HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin.

# ARTICLE 2

## “Effect of Rotavirus Infection and 2’- Fucosyllactose Administration on Rat Intestinal Gene Expression”

Sáez-Fuertes, Laura; Azagra-Boronat, Ignasi; Massot-Cladera, Malén; Knipping, Karen; Garssen, Johan; Franch, Àngels; Castell, Margarida; Pérez-Cano, Francisco J; Rodríguez-Lagunas, María J.

**Nutrients 15 (8):1998**

(DOI: 10.3390/nu15081996)

Open access journal
Impact factor (2022): 5.9
Category: *Immunology (Q1) 17/88*

## Abstract

**Aim**: This study aimed to assess the changes in intestinal gene expression after rotavirus (RV) infection in a preclinical model, and the effect of 2'-fucosyllactose (2'-FL) on this process.

**Methods**: To accomplish this objective Lewis pups were supplemented from days 2 to 8 of life, with the 2'-FL or vehicle. At day 5, pups were infected with the RV. Fecal samples were collected during the RV infectious process to calculate the incidence and severity of the RV-induced diarrhea. At the peak of the infection, day 8, a section of the small intestine was processed for gene expression evaluation by a microarray technique and qPCR.

**Results**: The supplementation with the 2'-FL during the RV infection exerts a preventive effect reducing the incidence and severity of the RV-induced diarrhea. The clinical impact of the RV is linked with changes in the intestinal gene expression levels. The non supplemented animals exhibited an upregulation of host antiviral genes (e.g., *Oas1a, Irf7, Ifi44, Isg15*) and a downregulation of genes involved in absorptive processes and intestinal maturation (e.g., *Onecut2*, and *Ccl19*). The infection and the 2'-FL supplementation induced changes in some immunity/maturation markers that were differentially expressed (e.g., *Ccl12* and *Afp*).

**Conclusion**: The effect of the supplementation with 2'-FL during early life attenuating RV-induced diarrhea seems to be linked to its ability to alter the expression of certain genes. Additionally, the change in the expression of key genes may be used as biomarkers in the evaluation of the efficacy of nutritional interventions or treatments for RV infection.

*nutrients*

MDPI

*Article*

# Effect of Rotavirus Infection and 2′-Fucosyllactose Administration on Rat Intestinal Gene Expression

**Laura Sáez-Fuertes [1,2], Ignasi Azagra-Boronat [1,2], Malén Massot-Cladera [1,2], Karen Knipping [3,4], Johan Garssen [3,4], Àngels Franch [1,2], Margarida Castell [1,2], Francisco J. Pérez-Cano [1,2,*] and María J. Rodríguez-Lagunas [1,2]**

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3 Danone Nutricia Research, 3584 CT Utrecht, The Netherlands
4 Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, 3584 CA Utrecht, The Netherlands
* Correspondence: franciscoperez@ub.edu

**Abstract:** Viral infections are described as modifying host gene expression; however, there is limited insight regarding rotavirus (RV) infections. This study aimed to assess the changes in intestinal gene expression after RV infection in a preclinical model, and the effect of 2-fucosyllactose (2′-FL) on this process. From days 2 to 8 of life, rats were supplemented with the dietary oligosaccharide 2′-FL or vehicle. In addition, an RV was inoculated on day 5 to nonsupplemented animals (RV group) and to 2′-FL-fed animals (RV+2′-FL group). Incidence and severity of diarrhea were established. A portion from the middle part of the small intestine was excised for gene expression analysis by microarray kit and qPCR. In nonsupplemented animals, RV-induced diarrhea upregulated host antiviral genes (e.g., *Oas1a*, *Irf7*, *Ifi44*, *Isg15*) and downregulated several genes involved in absorptive processes and intestinal maturation (e.g., *Onecut2*, and *Ccl19*). The 2′-FL-supplemented and infected animals had less diarrhea; however, their gene expression was affected in a similar way as the control-infected animals, with the exception of some immunity/maturation markers that were differentially expressed (e.g., *Ccl12* and *Afp*). Overall, assessing the expression of these key genes may be useful in the evaluation of the efficacy of nutritional interventions or treatments for RV infection.

**Keywords:** rotavirus; infection; 2-fucosyllactose; oligosaccharide



**Citation:** Sáez-Fuertes, L.; Azagra-Boronat, I.; Massot-Cladera, M.; Knipping, K.; Garssen, J.; Franch, À.; Castell, M.; Pérez-Cano, F.J.; Rodríguez-Lagunas, M.J. Effect of Rotavirus Infection and 2′-Fucosyllactose Administration on Rat Intestinal Gene Expression. *Nutrients* **2023**, *15*, 1996. https://doi.org/10.3390/nu15081996

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## 1. Introduction

The World Health Organization (WHO) has established that human milk (HM) is the most suitable option to meet nutritional requirements during the first period of life [1]. HM starts to be produced during the late gestational phase, and its composition changes throughout the lactation period [2]. Colostrum is the first milk produced after delivery and is rich in immunological components [2]. Five days postpartum, milk composition starts to change to adapt to the nutritional and developmental needs of the infant, which is called transitional milk. Then, after two to three weeks postpartum, the human milk composition is considered mature [3].

HM has several components, which, in addition to providing nutrition, contribute to protecting infants from diseases. These components are classified into macronutrients (~99%) and micronutrients (~1%). Macronutrients include water, carbohydrates, proteins, and lipids [4]. During the milk maturation process, the proportions of macronutrients such as proteins, fats, and carbohydrates vary [2]. Within carbohydrates, human milk oligosaccharides (HMOs) are a complex group of indigestible carbohydrates, with more than 200 structures identified so far; they present prebiotic activities [5] due to their beneficial effects on the host microbiota [6]. *Bifidobacteria* and *Bacteroidetes* are the main microorganisms

able to metabolize HMOs [7]. The most abundant HMO in human milk is 2′-fucosyllactose (2′-FL). This HMO has been widely studied to evaluate its impact on offspring development and its potential to reduce infections during early life [8–10]. In this regard, this HMO has been included in infant formula to come closer to HM composition and improve and modulate the immune system [11] and gut microbiota [12].

Rotavirus (RVs) is a nonenveloped double-stranded RNA (dsRNA) virus whose genome is protected by a 3-capsid structure and has the information needed for six structural proteins and five nonstructural proteins. Viral particles are able to reach the small intestine and replicate within the enterocytes [13], which disrupts the absorptive functions and fluid secretion, inducing severe dehydration and diarrhea in children, mainly before 5 years of age [14]. Once the enterocytes are infected, different cellular mechanisms recognize viral components in endosomal compartments (Toll-like receptor (*TLR*) 3) and in the cytoplasm (retinoic acid-inducible gene I (*RIG-I*) and melanoma differentiation-associated gene-5 (*MAD-5*)) [15,16]. To sum up, after RV particles reach the small intestine and infect enterocytes, the immune system cells will trigger an immunological response by changing gene expression profiles to produce cytokines and chemokines for the recruitment of immune cells needed to attack and remove the viruses [15].

Due to the high incidence of RV infections in infants, multiple studies have been conducted searching for alternatives to reduce RV infections and/or improve immune resistance to the virus. Among the options, dietary supplementations with probiotics and prebiotics have proven to impact and reduce RV-induced diarrhea [17,18]. To explain and understand the reduction in RV-induced diarrhea, molecular mechanisms have been studied, including changes in intestinal gene expression of particular receptors involved in viral resistance and immunity, such as the TLR, mucins, or tight junction proteins, among others [10]. However, concrete data on gene expression in an RV infection model and its modulation by dietary components have not been gathered.

In a previous study, it was demonstrated that daily supplementation of 2′-FL prevented several pathological effects in an RV infection rat model [10], and some mechanisms were suggested. The aim of this study was to evaluate the impact on small intestine gene expression after supplementation with 2′-FL during early life in an RV infection model. This study aims to explain the molecular pathways activated during RV infection and the mechanism underlying the reduction in RV-induced diarrhea after 2′-FL supplementation in rats.

## 2. Materials and Methods

### *2.1. Animals*

Twelve G15 pregnant Lewis rats were obtained from Janvier Labs (Le Genest-Saint-Isle, France). The day of birth was considered as day 1 of life, and pups were randomly distributed into the four experimental groups. Litters were unified to eight pups with free access to maternal milk, and dams were fed ad libitum. From day 2 to day 16 of life, pups were separated daily from dams for oral administration and then reunited again.

From the day of birth, all litters were housed in cages in an isolated room under biosecurity level 2 conditions at the Animal Facility of the Faculty of Pharmacy and Food Science at the University of Barcelona (UB). Animals were under temperature and humidity-controlled conditions in a 12 h/12 h light/dark cycle [19–21]. All procedures of care and use of animals were approved and conducted in accordance with the Ethical Committee of the UB and Catalonia Government (CEEA-UB, Ref. 74/05 and DAAM 3046, respectively).

### *2.2. Experimental Design and Sample Collection*

Newborn rats were distributed into four study groups (each group constituted by three litters of 8 pups each): reference (REF) group, 2′-fucosyllactose (2′-FL) group, rotavirus-infected (RV) group and rotavirus-infected group with 2′-FL supplementation (RV + 2′-FL). The number of animals per group was calculated taking into account that there was at

*Nutrients* **2023**, *15*, 1996 

least one animal from the three different litters in each group, due to the strong maternal influence in the process [22].

Suckling rats were orally administered in the first hours of the light phase, as previously described by Rigo-Adrover et al. [23]. The 2′-FL group and the RV + 2′-FL were supplemented from the day of birth with 0.2 g of 2′-FL/100 g of body weight (4.5 µL/g/day of a 4.5 g/mL solution), while REF and RV groups were administered with a matched volume of water [10].

The RV (simian SA-11) strain inoculated to animals was provided by the Virus Entèrics group from the UB. The infection was performed on day 5 of life at a dose of $4 \times 10^8$ tissue culture infectious dose 50 [$TCID_{50}$]/rat, similar to previous studies [24]. To assess the impact of RV infection, fecal samples and clinical evaluation were performed [17]. Fecal samples were obtained daily by massaging the abdomen and scored to analyze the severity of diarrhea. The scoring scale (diarrhea index, DI) ranged from 1 to 4, with 1 being normal feces and 4 being watery feces.

At day 8 of life, during the maximum peak of diarrhea, selected pups from each experimental group and at least one from each litter ($n = 4$) were anesthetized by using a mix of ketamine (90 mg/kg) and xylazine (10 mg/kg). After the euthanasia, exsanguination by cardiac puncture was performed, and a 1 cm section of the central part of the small intestine was collected in RNA later to analyze gene expression.

### 2.3. RNA Extraction and Microarray Procedure

Representative samples were homogenized as Massot-Cladera et al. described [20]. Afterward, RNA isolation was performed following the manufacturer's recommendations by using RNAeasy Mini Kit (Qiagen, Madrid, Spain) and quantified with a NanoDrop spectrophotometer and NanoDrop IVD-1000 v.3.1.2 software (NanoDrop Technologies, Wilmington, DE, USA). To analyze the results, Agilent 2100 Bioanalyzer with the RNA 6000 LabChip kit was used. Only samples with RNA integrity number $\geq 9$ were selected.

The study of the differential expression profiling was carried out with a SurePrint-G3 Rat GE $8 \times 60$ K microarray kit (ID 028279, Agilent Technologies, Madrid, Spain), following a loop experimental design in which a pairwise comparison was performed in collaboration with BA Microarray (Alicante, Spain). Quadruplicate samples at day 8 of the design for each experimental condition (REF, RV, and RV+2′-FL) were used, and dye swaps (Cy3 and Cy5) were performed on the RNA amplified from each sample. RNA quality was assessed using a TapeStation (Agilent Technologies). RNA concentration and dye incorporation were measured using a UV-VIS spectrophotometer (Nanodrop 1000, Agilent Technologies, Wilmington, DE, USA). Labeling and hybridization to microarray were conducted following the manufacturer's two-color protocol (Two-Color Microarray-Based Gene Expression Analysis v. 6.5, Agilent Technologies), using LowInput QuickAmp Labeling Kit and Agilent Microarray Hybridization Chamber Kit for labeling and hybridization, respectively. Microarray chips were then washed and immediately scanned using a DNA microarray scanner (Model G2505C, Agilent Technologies) by the Genetic Diagnostic Bioarray facilities (Bioarray, Alicante, Spain).

### 2.4. Microarray Data Analysis

Data extraction was performed with Agilent Feature Extraction Software v.10.7 (Agilent Technologies). Bioinformatic analysis was performed with Bioconductor software under R environment, using the following packages: limma (v.3.16.1) for background correction and normalization; Marray and pcaMethods for quality control plots; RankProd for differential expression; and finally GOstats (v.2.26.0.) and GSEABase for gene ontology functional analysis. The latest gene annotations available were used. Raw feature intensities were background-corrected using the normexp background correction algorithm. Within-array normalization was performed using spatial- and intensity-dependent loess. Quantile normalization was used to normalize between arrays. The expression of each gene was reported as the base 2 logarithm of ratio of the value obtained of each condition relative

to the control condition (REF group or RV group). A gene was considered differentially expressed if it displayed a PFP (percentage of false prediction, equivalent to the false discovery rate, FDR) of less than 0.05 by rank product nonparametric method (RankProd). Finally, up- and downregulated genes were analyzed in terms of gene ontology by using a hypergeometric analysis (GOStats).

*2.5. Validation of Gene Expression by Real-Time PCR*

Two µg of total RNA were converted to cDNA. Specific PCR TaqMan® primers and probes (Applied Biosystems, AB, Weiterstadt, Germany) were used to measure selected targets, depending on the results raised. In addition to the three studied groups (RV, RV, and RV+2′-FL), the PCR was also performed in the 2′-FL group in order to observe whether some effects induced by 2′-FL in the context of infection (RV+2′-FL) were also observed in a health context (2′-FL). Quantitative real-time PCR assays were performed for eight samples/group using an ABI PRISM 7900HT Sequence Detection System (AB). The specific PCR TaqMan® primers (AB) were *Isg15* (Rn01519614_m1_I), *Oas1a* (Rn04219673_m1_I), *Irf7* (Rn01450778_g1_I), *Ifi44* (Rn01523064_m1_I), *Ccl19* (Rn01439563_m1_I), *Ccl12* (Rn01464638_m1_I), *OneCut2* (Rn01265320_m1_I), and *Afp* (Rn00560661_m1_I). Quantification of the genes studied was normalized to the housekeeping gene *Gusb* (Rn00566655_m1, I). The SDS v2.4 software (AB) was used to analyze the expression data. Results were expressed as the fold change in the amount of target mRNA relative to the endogenous control expression calculated using the standard 2-ΔΔCt method for the different experimental groups in relation to values from the REF or RV group, which represent a onefold change in gene expression.

*2.6. Statistical Analysis*

PCR results were statistically analyzed using the software package SPSS 22.0 (SPSS, Inc., Chicago, IL, USA). To evaluate variance equality and normal distribution, Levene's and Kolmogorov–Smirnov tests were conducted. Kruskal–Wallis and Mann–Whitney U (MWU) tests were performed when results did not follow a normal and equal distribution to assess significant differences among groups ($p < 0.05$). To compare the frequencies of diarrhea incidence (%DA), the chi-square test was performed. Results are expressed as mean ± standard error of the mean (S.E.M).

## 3. Results

*3.1. Clinical Results*

Clinical evaluation carried out at the peak of the infection (day 8) by scoring feces from 1 to 4 allowed the calculation of the incidence and the severity of RV-induced diarrhea in the subgroup of animals selected for the gene expression analysis in the present study (Figure 1). The results showed a lower percentage of animals having diarrhea (%DA) and lower severity ($p < 0.05$) in the RV+2′-FL group compared to the RV group, demonstrating that supplementation with 2′-FL ameliorates rotavirus-induced diarrhea in similar and representative proportions of those obtained from the full cohort in previous studies [10].

*3.2. RV Effect on Overall Intestinal Rat Gene Expression*

The infection with RV in both groups (RV and RV+2′-FL) impacted the intestinal gene expression during the peak of diarrhea by means of the overall gene ontology (GO) approximation (Figure 2). Both groups displayed a number of genes being statistically modified due to the infection. Most of these genes were related to stress and immunity GO biological processes.

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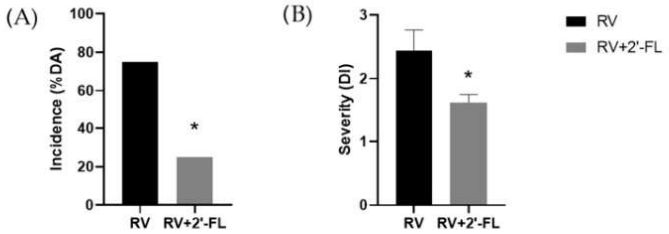


**Figure 1.** Preventive effect of 2′-FL on the RV infection. (**A**) Incidence (%DA, Diarrheic Animals) and (**B**) Severity (DI, Diarrhoea Index) on day 8 of the selected animals of the array study ($n$ = 4, from three different litters). Results are expressed as unique values of % for incidence (derived from all animals each day) and as mean ± S.E.M. for severity. Statistical differences: * $p < 0.05$ compared to RV group by chi-square test and by MWU test, respectively.

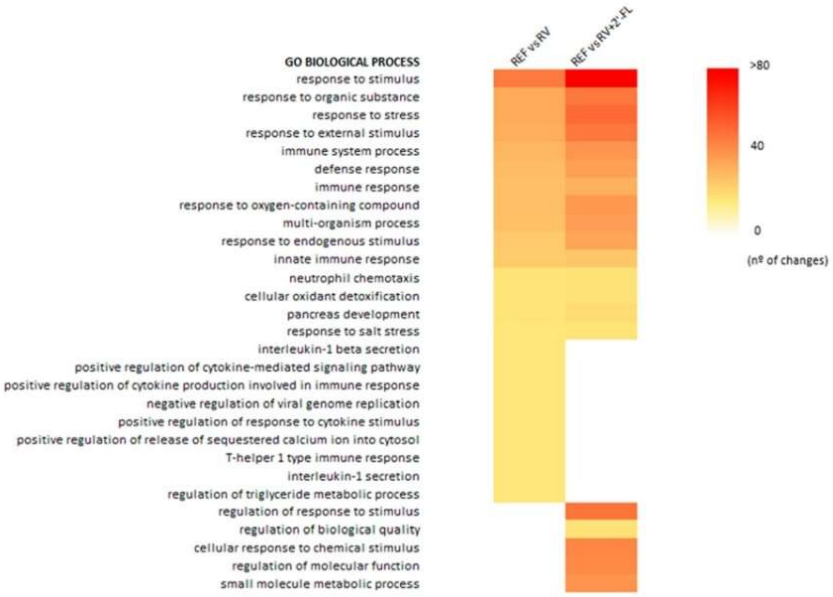


**Figure 2.** List of the gene ontology (GO) pathways in which higher numbers of statistically affected genes are counted in the array due to the RV infection with respect to the REF group ($n$ = 4/group).

Among the upregulated GO processes, the defense response to the virus, the cellular response to the type-I interferon, and the positive regulation of innate immune response were demonstrated. In contrast, the main downregulated GO processes were associated with the positive regulation of neutrophil chemotaxis and intestinal absorption. These results are in line with the main effects of RV infection in the small intestine.

The RV+2′-FL affected not only a higher number of genes in pathways already modified by the RV but also induced changes in other pathways, such as the regulation of response to stimulus, biological quality, molecular function, and small molecule metabolic processes (Figure 2).

### 3.3. Gene Expression Changes Due to RV Infection and 2′-FL Supplementation

In order to evaluate the impact of RV infection on gene expression in the small intestine of 8-day-old pups, the fold change for the genes that were modified significantly was studied (Table 1). The genes are ordered based on the fold change comparing RV vs. REF and RV+2′FL vs. REF, depending on whether they were upregulated (Table 1A) or downregulated (Table 1B). RV infection significantly upregulated the expression of several genes ($n$ = 30), which were mainly involved in the defense response against the virus and the innate immune response, such as *Oas1a* (*2′-5′ oligoadenylate synthetase 1A*), *Oas1k* (*2′-5′ oligoadenylate synthetase 1K*), and *Usp18* (*ubiquitin specific peptidase 18*) genes. Another upregulated gene was *Rpl391* (*60S ribosomal protein L39*), involved in innate immune responses in the mucosa and in viral transcription processes. In contrast, many genes were downregulated ($n$ = 40) by the RV infection, such as *Tmprss15* (*transmembrane serine protease 15*), *Onecut2* (*one cut homeobox 2*), and *Afp* (*alpha-fetoprotein*). These genes are involved in nutrient digestion processes and produced in the duodenum (*Tmprss15*) or associated with anatomical morphogenesis.

**Table 1.** List of partial genes (A) upregulated and (B) downregulated.

| (A) Upregulated | | | (B) Downregulated | | |
|---|---|---|---|---|---|
| **Gene** | **RV** | **RV+2′-FL** | **Gene** | **RV** | **RV+2′-FL** |
| *Oas1a* | 2.21 | 2.04 | *Ccl19* | −0.57 * | - |
| *Oas1k* | 2.1 | 1.87 | *Slpi* | −0.77 | −1.06 |
| *Usp18* | 2.06 | 1.75 | *Lpin1* | −0.82 | - |
| *Zbp1* | 1.71 | 1.79 | *Alpk3* | −0.83 | −1.02 |
| *Irf7* | 1.59 | 1.51 | *LOC103691469* | −0.84 | −1.08 |
| *Tmigd1* | 1.53 | 1.89 | *Kng2l1* | −0.85 | −1.01 |
| *Ifi44* | 1.52 | 1.38 | *Gkap1* | −0.87 | −0.96 |
| *Cfb* | 1.51 | 1.58 | *S100g* | −0.88 | −0.97 |
| *Rpl39l* | 1.45 * | - | *Xcl1* | −0.9 | - |
| *Dhx58* | 1.4 | 1.2 | *Abca8a* | −0.94 | −1.14 |
| *Ifi27* | 1.32 | 1.44 | *Gpcpd1* | −0.95 | −0.93 |
| *LOC679368* | 1.24 | 1.89 | *Apoa4* | −0.95 | −0.95 |
| *Isg15* | 1.21 | - | *Ribc2* | −0.96 | −0.9 |
| *LOC690082* | 1.19 | 1.03 | *Selenop* | −0.97 | −0.96 |
| *Aqp3* | 1.17 | 1.36 | *Aoah* | −1.01 | - |
| *Capn3* | 1.12 | 1.16 | *Cyp3a62* | −1.03 | −1.2 |
| *Rasa4* | 1.11 | 1.07 | *Sla2* | −1.03 | - |
| *Tmem52* | 1.1 | - | *Fcgrt* | −1.08 | −1.21 |
| *Fyb2* | 1.07 | 1.05 | *LOC102555026* | −1.09 | −1.67 |
| *Chdh* | 1.07 | 1.24 | *Igfals* | −1.11 | −1.35 |
| *Samd9* | 1.06 | 1 | *Gpx3* | −1.13 | −1.32 |
| *Upk1b* | 1.02 | 1.21 | *Ephb6* | −1.13 | −1.2 |
| *Samd9* | 0.99 | - | *Cd36* | −1.17 | −1.1 |
| *Ces1e* | 0.93 | 0.92 | *LOC691352* | −1.2 | −1.11 |
| *MGC108823* | 0.91 | 0.92 | *Ankrd29* | −1.23 | −1.44 |
| *Oas1i* | 0.9 | - | *Ptprr* | −1.23 | −1.28 |
| *Mcpt4* | 0.9 | - | *Hoxc11* | −1.26 | −1.41 |
| *LOC100912658* | 0.86 | - | *Tacr3* | −1.31 | −1.63 |
| *Lgals3bp* | 0.83 | - | *Srd5a2* | −1.32 | - |
| *Slc37a4* | 0.73 | 0.83 | *C8g* | −1.52 | −1.53 |
| | | | *Dapl1* | −1.54 | −1.82 |
| | | | *Ggh* | −1.58 | −1.49 |
| | | | *Slc3a1* | −1.63 | −1.09 |
| | | | *Pdx1* | −1.66 | −1.8 |
| | | | *Kcne1* | −2.33 | −2.63 |
| | | | *Aqp8* | −2.35 | −2.85 |

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**Table 1.** *Cont.*

| (A) Upregulated | | | (B) Downregulated | | |
|---|---|---|---|---|---|
| **Gene** | **RV** | **RV+2′-FL** | **Gene** | **RV** | **RV+2′-FL** |
| | | | *RGD1562699* | −2.66 | - |
| | | | *Afp* | −3.29 * | −5.39 |
| | | | *Onecut2* | −3.75 | −3.71 |
| | | | *Tmprss15* | −5.13 | −5.86 |

Results are expressed as fold change. All genes in the table are significantly modified in the RV group vs. the REF group. (-) non-modified compared to REF. * $p < 0.05$ compared to REF ($n = 4$/group).

In addition, the effect of the supplementation with 2′-FL was evaluated in RV infection. The main up- and downregulated genes are also shown in Table 1A,B, respectively. In these two lists, it can be observed that many changes were shared in both groups, and similarly to what was found without the supplementation, *Oas1a* and *Oas1k* genes were the most abundant upregulated genes. Comparing the most downregulated genes, it was observed that *Tmprss15* and *Onecut2* followed the same expression pattern as in the RV group, whereas *Afp* was highly downregulated after the supplementation with 2′-FL in RV infection. Only three genes were differentially expressed between groups: *Rpl391* (upregulated in the RV group but not affected in the RV+2′-FL group), *Ccl19* (*C-C motif chemokine ligand 19*, downregulated in the RV group but not affected in the RV+2′-FL group), and *Afp*, the downregulation of which is higher in the case of the RV+2′-FL group.

Additionally, Figure 3 shows different approaches to better understand the impact of RV infection and 2′-FL on intestinal gene expression. First, the Venn diagram (Figure 3A) shows that RV infection modifies a total of 70 genes (30 up- and 40 downregulated), with 55 genes (22 upregulated and 33 downregulated) being shared in the RV and RV+2′-FL groups.

In order to evaluate which genes were exclusively modified by RV infection, a plot displaying these genes was created (Figure 3B). It can be observed that *Ccl19* is found among the most downregulated genes, whereas *Isg15* (*Isg15 ubiquitin-like modifier*) and *Rpl39* are the most upregulated genes, as already observed in Table 1.

The Venn diagram also shows that 105 genes were exclusively modified in the RV+2′-FL group (20 genes up- and 85 downregulated). To zoom in on the exclusive changes due to the 2′-FL intervention, the 15 most affected genes in each case were also identified (Figure 3C,D). The three most upregulated genes exclusively present in the RV+2′-FL group were the uncharacterized proteins LOC501038 and RGD1562699 and F12, also known as coagulation factor XII. On the other hand, the three most downregulated genes exclusively present in the RV+2-'FL group were the ribosomal RNA gene *Rn5-8s*, the *solute carrier family 5 member 4* (SLC5A4), also known as SGLT3, and the enzyme that cleaves an insulin-like growth-factor-binding protein, also known as Pappa2 (pregnancy-associated plasma protein-A2).

Finally, as the previous results were found by comparing both infected groups with respect to the REF group, another approach was taken to evaluate the impact of the 2′-FL under RV conditions (Figure 4). For this, the 15 most upregulated and downregulated genes of the RV+2′-FL group were evaluated compared to the RV group. The results confirmed previous data showing that, whereas *Ccl12* (chemokine (C-C motif) ligand 12) and Rn5-8s were the most upregulated genes, *Afp* and *Rpl39l* were the most downregulated genes with the 2′-FL supplementation.

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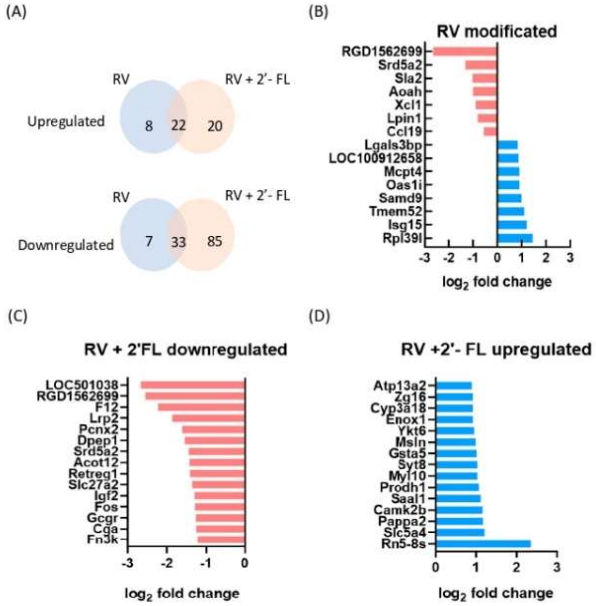


**Figure 3.** Impact of RV infection and 2′-FL on intestinal gene expression. (**A**) Venn diagram displaying statistically differential up- and downregulated genes with respect to the REF group; (**B**) genes exclusively modified in the RV group; (**C**) the 15 (from the 20) most downregulated genes exclusively modified in the RV+2′-FL group; and (**D**) the 15 (from the 85) most upregulated genes exclusively modified in the RV+2′-FL group ($n$ = 4/group).

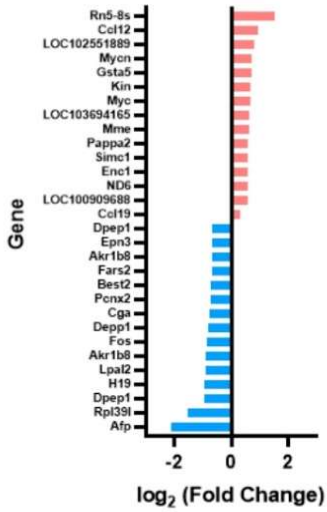


**Figure 4.** Changes in gene expression between RV and RV+2′-FL groups. The 15 most upregulated genes (red) and the 15 most downregulated genes (blue) comparing the RV+2′-FL group vs. RV group ($n$ = 4/group).

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### 3.4. PCR Confirmation of Key Genes

To confirm the array results, qPCR of key genes was performed (Figure 5). The main upregulated genes in both groups were *Isg15*, *Oas1a*, *Irf7*, and *Ifi44*. All of them are involved in the type-I interferon pathway and were confirmed by qPCR. The gene expression of two chemokines was also analyzed (*Ccl19* and *Ccl12*). Only *Ccl12* was confirmed in the RV+2′-FL group by qPCR. *OneCut2* and *Afp* genes were highly modified in microarray results in both groups. However, qPCR results only confirmed *Afp* gene expression in the RV+2′-FL group.

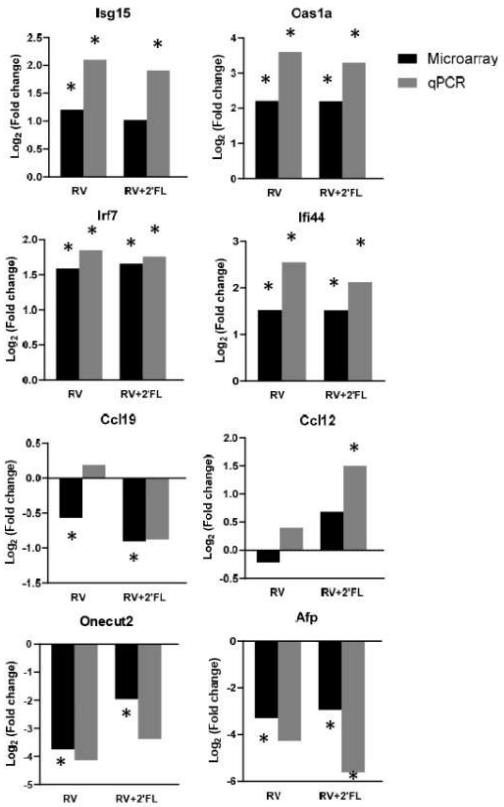


**Figure 5.** Fold change in selected target genes with respect to REF group by the array results and by Taqman PCR ($n$ = 4/group in the array and $n$ = 8/group in the qPCR). Statistical differences: * $p < 0.05$ vs. REF.

### 3.5. Gene Expression Changes with 2′-FL Supplementation

After the microarray and qPCR evaluation of the RV and RV+2′-FL group, the most interesting genes were analyzed in the 2′-FL group by qPCR as well (Figure 6).

The results from Figure 6 showed that the expression of *Ccl12* was increased with 2′-FL supplementation, similar to what was found in the RV+2-FL. Apart from Ccl12, the upregulation of the gene expression of *Isg15*, *Oas1a*, *Irf7*, and *Ifi44* and the downregulation of *Ccl19* and *OneCut2* after the 2′-FL supplementation were similar to the RV+2′-FL group.

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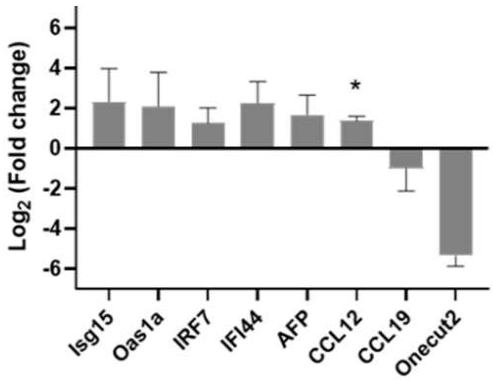


**Figure 6.** Fold change in selected target genes in the 2′-FL group with respect to REF by Taqman PCR ($n$ = 4–8/group). Statistical differences: * $p < 0.05$ vs. REF.

## 4. Discussion

Since 2020, COVID-19 has placed viral infections and the necessity to understand its physiopathology in the spotlight. However, it should not be forgotten that many other viruses also affect human health. Among them, RVs are one of the most infectious agents in children, especially in low-income countries. In this context, more studies to identify underlying mechanisms for RV-induced pathophysiology are essential and aim to produce better tools to prevent and treat RV infections in humans.

Previous in vitro and in vivo studies have reported that RV particles can change the expression of several genes, especially in the small intestine [10,15]. The majority of these studies are focused on potential and specific target genes associated with inflammation [25]. In this regard, it might be that specific dietary interventions using, e.g., pro- or prebiotics or even specific HMOs such as 2′-FL can interfere with these processes, leading to changes in severity and incidence of RV-related health features (i.e., diarrhea, inflammation, and tissue damage) [26–28].

To elaborate on our previous study showing the positive effect of 2′-FL on RV-induced diarrhea, in the present study, we moved into more molecular details using specific microarray technologies. In the end, we aimed to evaluate the role of RV infection in gene modifications and the potential of 2′-FL supplementation to reduce the impact of RV infection. 2′-FL is the most abundant oligosaccharide in human milk and has been associated with beneficial functions, including the reduction in inflammatory responses by modulating gene expression [9,10] and intestinal microbiota [29]. Our findings indicate that supplementation with 2′-FL to rats during early life attenuates the incidence and severity of the RV infection. These results are in line with previous nutritional interventions in our laboratory study [10] and by others where a mix of HMOs was used [30,31].

Having demonstrated that specific dietary supplementation reduces viral infections, different approaches were utilized to evaluate its impact on the genetic expression located in the small intestine and in order to create a mechanistic understanding. As can be observed in the Venn diagrams, RV infection induced several changes in intestinal gene expression. However, the supplementation with 2′-FL under RV infection induced even more changes in the small intestine tract at this gene level. These exclusively modified genes after nutritional supplementation under RV conditions may be associated with the protective role of 2′-FL in these preclinical disease models.

In general, RV infection modifies different genes grouped in GO biological processes. The upregulated GO processes are involved in the activation of viral immunity mediated by type-I interferon. In contrast, the most downregulated GO processes are linked to intestinal

absorption but also neutrophil chemotaxis. These results are in accordance with previous reports of intestinal infections [32,33].

The genes that are the most modified by this RV infection are linked to GO biological processes of the response to external stimulus, and are especially associated with viruses aimed to activate viral immunity. In this type of infection, the RV viral particles are recognized by pattern recognition receptors (PRRs) and trigger the activation of the innate immune viral response, inducing the expression of pro-inflammatory genes. One of the main pro-inflammatory-activated genes is interferon, including type-I and type-III [34]. During viral infections, both interferon families, interferon-stimulated genes (ISGs) and interferon-regulatory factors (IRFs), collaborate to produce an efficient virus clearance [35]. Type-I (α/β) IFN and type-III (λ) *IFN* coordinate and overlap some of their functions during viral infections, but there are specific functions associated with each one. To date, *IFN*-λ is expressed in intestinal epithelial cells and displays a protective role in mucosal surfaces, while *IFN*-α/β is expressed in systemic tissues and organs (liver, spleen, and kidney), avoiding the systemic spread of the infection [34]. However, changes in *IFN* gene expression at the intestinal level were not found in this model. However, the genes involved in IFN pathways, such as the OAS family, *Irf7*, *Ifi44*, and *Isg15*, were found to be affected by RV infection. The OAS (*Oas1*, *Oas2*, *Oas3*, and *OasL*) family are IFN-stimulated proteins that are highly induced by type-I *IFN*. This family is involved in viral RNA degradation to facilitate viral recognition by *RIG-I* and *MDA5* pathways. In particular, *Oas1a* has been associated with the induction of IFN signals [36]. Our microarray results showed that the most upregulated gene was *Oas1a*, indicating that its induction is involved in the inflammatory state and the IFN activation pathway initiated. The RV+2′-FL showed similar results to the RV group, suggesting that the reduction in RV-induced diarrhea in the RV+2′-FL group is not due to changes in this particular gene.

Similarly to the OAS family, Ifi44 is one of the first ISGs after viral infections to stimulate PPRs. Its expression results in the activation of different transcription factors, such as *Irf3* and *Irf7*, which promotes IFN signaling [37]. In this context, it is suggested that *Ifi44* acts in the first defense line of the viral immune response, while *Irf7* is involved in the later stage of IFN induction [38]. *Irf7* stimulation will contribute to the activation of innate immune responses. In our case, microarray and qPCR results showed that RV and RV+2′FL induced the gene expression of *Ifi44* and *Irf7* in the small intestine, thus activating antiviral responses. Comparing the RV and RV+2′-FL groups, no statistical differences were observed between them in terms of *Ifi44* or *Irf7* gene expression under RV conditions. Although our results are in line with previous studies [37], it is important to mention that many viruses, including RV, have developed multiple evasion mechanisms. The main evasion pathway relies on the degradation of different IRFs, such as *Irf3*, *Irf5*, and *Irf7* [39], which have not been found to be statistically modified in this approach either.

Another upregulated gene by RV infection was *Isg15*, which works together with *Usp18*. The mechanism of action of the *Isg15* gene is based on the conjugation of Isg15 with different molecules (ISGylation), which stimulates the IFN type-I pathway [40]. In the beginning, Usp18 was associated with a reverse function of ISGylation, reducing the inflammatory IFN pathways [41]. However, a later study reported that Usp18 plays a double role in inducing or blocking IFN signaling. *Usp18* can directly activate the JAK/STAT signaling pathway to promote IFN responses [42] or may bind to interferon receptor 2 (IFNAR2) and inhibit the JAK/STAT [43]. Considering the above, the overexpression of *Isg15* promotes IFN signals. In contrast, the role of *Usp18* is not yet clear. Our results indicate that RV infection upregulates the expression of *Isg15*, whereas the supplementation of 2′-FL is not able to modify its expression with or without RV conditions. With regard to *Usp18* mRNA expression, both RV and RV+2′-FL increased it. These results are in accordance with Ye et al. [42] under dengue infection. Nevertheless, further studies are needed to elucidate the positive or negative impact on RV clearance.

In addition to the changes in genes involved in the IFN pathway, many other genes were modified, including chemokines, transmembrane proteins, and ribosomal proteins.

After viral or bacterial infections, different chemokines collaborate to induce an effective response. To fight against pathogens, the expression of pro-inflammatory, anti-inflammatory, or homeostatic chemokines can be stimulated. Among the changes in the homeostatic chemokines, *Ccl12* and *Ccl19* are included [44]. They play a critical role in the first line of defense, and their functions are linked with immune cell recruitment [45]. Our microarray and qPCR results showed that *Ccl19* was reduced under RV conditions, suggesting that the downregulation of this gene limits the mechanisms of action of the immune system to recruit immune cells. In contrast, the microarray results revealed that *Ccl12* was upregulated in the RV+2′-FL, and the qPCR results of the 2′-FL group showed that the expression of this gene was also upregulated in noninfected conditions. Combining these results, it can be confirmed that the supplementation with 2′-FL during early life is able to modify the expression of *Ccl12* in the small intestine to modulate immunity and enhance the defense against pathogens such as RV.

*Tmprss15* was the most downregulated gene in both the RV and RV+2′FL groups. This gene is a transmembrane protease involved in trypsin metabolism. *Tmprss15* collaborates in the proliferation process during viral infections, such as the influenza A virus [46]. Additionally, a deficiency of this enzyme is associated with enterokinase deficiency (EDK), characterized by severe chronic diarrhea after birth [47]. Similarly to EDK, the main manifestation of RV infection is diarrhea, suggesting that diarrhea is highly linked to the downregulation of *Tmprss15*. In our case, the supplementation of 2′-FL reduced RV-induced diarrhea, but the expression of *Tmprss15* did not change, indicating the modulation of these genes cannot explain the diarrhea reduction by the 2′-FL supplementation.

Comparable to *Tmprss15*, the expression of *OneCut2* was highly downregulated in the RV and RV+2′-FL groups. The *OneCut* family of transcription factors is involved in organ development, such as that of the gut endoderm, liver, and pancreas [48]. Since the identification of this family, most of the related studies have focused on neuronal and liver development diseases [49]. No information is known about the relationship between *OneCut2* and viral infections in early life. For this reason, further studies are needed to clarify its relation and whether infection can affect intestinal development.

Another upregulated gene was *Pappa2*. The down-expression of this gene has been associated with changes in growth parameters [50,51]. In our case, the supplementation with 2′-FL under RV conditions upregulated the expression of this gene. However, the previously published results showed that the growth evolution of these animals was not affected during the nutritional intervention [10,52], and thus did not affect postnatal growth.

Finally, among the gene expression modifications, the genes that were differentially modified comparing RV and RV+2′-FL groups will be discussed. The expression of *Rpl39l* and Afp genes was differentially influenced by 2′-FL under RV conditions. To date, there have been no correlations between *Rpl39l* and *Afp* expression patterns and viral infections. Changes in the expression of *Rp39l* have been associated with hepatocellular carcinoma tumors [53], while *Afp* expression is important during fetal growth, contributing to appropriate gastrointestinal development [54]. Our microarray results demonstrated that 2′-FL attenuates the overexpression of *Rpl39* and boosts the down-expression of *Afp*. Although little information is known about the relation between *Rpl39l*, *Afp*, and RV infections, we suggest that the mechanism of action of 2′-FL under RV conditions to reduce diarrhea is linked to intestinal development. However, more studies are required to clarify the role of 2′-FL in early life, including its mechanism of action to mitigate the impact of RV infections.

Thus, further research is needed to elucidate the role of 2′-FL on intestinal maturation and its influence on *Afp*, *OneCut*, and *Pappa2* gene expression. Additionally, the impact of 2′-FL supplementation on the expression of genes involved in the immune response (chemokines and transmembrane proteins) will provide a better understanding of its protective effects. All these analyses will contribute to completing the gaps in the knowledge of 2′-FL.

A current limitation of this research is the lack of a wide gene expression study of the 2′-FL in early life under physiological development. Only the most interesting genes raised by the microarray were studied.

## 5. Conclusions

In this work, we identified the main gene expression modifications during RV infections in a preclinical disease model. The effect of the supplementation with 2′-FL during early life seems to be linked to its ability to attenuate RV-induced diarrhea through changes in different identified genes involved in the IFN pathway and intestine maturation involved in the RV pathology. However, as this study is a preclinical approach, more data and investigations, including human studies, are needed to validate these effects and those from other HMOs in viral infections.

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# ARTICLE 3

## "*B. breve* M-16V and scGOS/lcFOS supplementation to dams ameliorates infant rotavirus infection in early life"

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## Abstract

**Aim:** This study aimed to evaluate the effect of a synbiotic (*Bifidobacterium breve* M-16V and scGOS/lcFOS, 9:1)supplementation to dams during gestation and lactation to prevent the rotavirus (RV) infections in their offspring in early life.

**Methods:** Pregnant rats were administered with the synbiotic during gestation and lactation. At day 5 of life pups were infected with RV. To analyse the evolution of the infection, at day 8 of life, blood, small intestine, stomach content, caecal content and faeces were collected. Diarrea, immune response, intestinal defence and microbiota composition was studied.

**Results:** The RV induced diarrea was partially prevented in the neonatal rats whose mothers received the symbiotic during gestation and lactation. In addition, infected rats displayed higher levels of immunoglobulin (Ig) IgG2c in plasma and IgA in the digested milk of the stomach content. The synbiotic supplementation also enhanced the maturation of the intestine and upregulated the intestinal gene expression of certain toll like receptors (TLRs), such as *Tlr2* and *Tlr7 and other barrier and defensive molecules such as Ocln*, *Muc2* and *Muc3***.**

**Conclusion:** These findings indicate that the administration of the synbiotic to gestating and lactating mothers ameliorated the incidence and severity of the diarrhoea caused by the RV infection by improving the gastrointestinal immunity of the pups.

# *B. breve* M-16V and scGOS/lcFOS supplementation to dams ameliorates infant rotavirus infection in early life

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**Keywords**: *Bifidobacterium breve* M-16V, short chain galacto-oligosaccharides (scGOS), long chain fructo-oligosaccharides (lcFOS), rotavirus, infancy

## ABSTRACT

The immune system of newborns is underdeveloped, leaving them susceptible to infections like rotavirus (RV). Despite vaccines, RV remains a leading cause of child mortality, especially in developing countries. Maternal immunity is transferred during pregnancy and breastfeeding to the offspring protecting against RV infection. This study aims to explore how maternal diet can enhance the newborn's ability to fight early infections. Pregnant rats received orally *B. breve* M-16V and short chain galacto-oligosaccharides (scGOS)/long chain fructo-oligosaccharides (lcFOS). At day 5 of life pups were infected with RV. At day 8, samples were collected for analysis. Pups whose mothers received the synbiotic had lower RV infection severity. Immunoglobulins (Ig) IgG2c and IgA were raised in pups' plasma and digested milk of the stomach content, respectively. Synbiotic supplementation improved intestinal maturation and increased gene expression of immune-related genes. In conclusion, the administration of the synbiotic to gestating and lactating mothers ameliorated the incidence and severity of the pups diarrhoea caused by the RV infection by improving their immunity.

## 1. INTRODUCTION

Maternal immunological status significantly impacts infants' early development, particularly during gestation and lactation, which shape neonatal maturation [1,2]. Both stages are extensively shaped by maternal environmental factors including diet and lifestyle[3] having a balanced diet during pregnancy and lactation a counteracting effect in early life infections in the offspring.[1]

Dietary ingestion of prebiotics [4], probiotics [5] and synbiotics [6] are of interest for a healthy lifestyle. Synbiotics combine the beneficial effects of probiotics and prebiotics in stimulating the growth of probiotic microorganisms by providing them with the appropriate substrates. One of the most common cited benefits of synbiotics is the modulation of the immune system while inhibiting pathogenic invasions.[7] Bifidobacteria combined with fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) is one of the most used synbiotic combinations.[7]

Rotaviruses (RV) are the main etiological agent responsible for acute gastroenteritis in children under 5 years of age worldwide, associated with 20-30% of cases of diarrhoea that require hospitalization.[8] The watery diarrhoea is caused by the destruction of enterocytes, deregulation of liquid transport proteins, and disruption of tight junctions (TJs) between these epithelial cells.[9] To prevent RV infections, oral vaccines have been developed [10] , however, RV continues to cause more than 200,000 deaths annually, mainly in developing countries, due to the low rates of vaccination. [11]

For these reasons, it is necessary to develop alternatives to treat and prevent RV infections. It is known that the microbiota plays a role in modulating host immunity, especially against enteric pathogens, therefore RV infection could be controlled by boosting infant's immunity or promoting the presence of beneficial microbiota.[12] However, the use of supplements in mothers to prevent neonate's infection has not been properly studied. Thus, we aimed to evaluate the effect of synbiotic supplementation to dams during gestation and lactation to counteract RV infections in the of offspring in early life. Clinical and molecular parameters associated to the RV-induced diarrhoea were evaluated.

## 2. EXPERIMENTAL SECTION

### 2.1. Animals

Seven-week-old Lewis rats (16 females and 8 males) were obtained from Janvier Labs (La Plaine Saint Denis Cedex, France). After one week of acclimatization, the females were placed into the males' cages for one week, and then were separated into individual cages. From the mating day, the female rats were divided into two experimental groups (reference (REF) and synbiotic (SYN)) and received the synbiotic supplementation (or vehicle) during gestation (21 days) and lactation (21 days), and at weaning, pups were supplemented for 1 week. The animals were given access to a commercial diet that corresponded to the American Institute of Nutrition 93G formulation [13] and water ad libitum. The rats were allowed to deliver naturally, and the day of birth was considered as day 1 for the pups. All litters were culled up to 10 pups per lactating dam. The pups had free access to the nipples and rat diet throughout the study.

The animal room conditions, including temperature and humidity, were carefully controlled in a 12 h light – 12 h dark cycle within a negative pressure chamber at the Animal Facility of the Diagonal Campus of the University of Barcelona (UB). Approval for all experimental procedures was obtained from the Ethics Committee for Animal Experimentation (CEEA) of the UB (Ref. 240/19).

### 2.2. Experimental design

Female rats were randomly assigned into two groups: REF (n=8) or SYN (n=8). Nutritional intervention lasted from the first day of the gestation (G1) until the end of the lactation (L21). The SYN dams were orally administered once a day with 1 mL of the synbiotic containing $10^9$ UFC/mL of *Bifidobacterium breve* M-16V and short chain galacto-oligosaccharides (scGOS) / long chain fructo-oligosaccharides (lcFOS) during gestation and 1.5 mL during lactation. The REF dams received the matching volume of saline solution in the same conditions.

On day 5 of life, pups were inoculated with a RV strain (simian SA-11) at $4x10^8$ tissue culture infectious dose, TCID50/rat. The SA-11 provided by Enteric Virus Group (UB) was inoculated as

previously described.[14] Animal body weight (BW) was monitored daily, and associated growth parameters such as the body mass index (BMI) and the Lee Index. A representative number of pups (4 pups/litter) were euthanized on day 8 to evaluate the impact of maternal nutritional intervention during the RV infection. Additionally, the anti-RV immunoglobulin (Ig) response was also studied in pups' plasma at day 21 and day 28 (3 pups/litter at each time), and also in dams' plasma and milk at day 21. The milk extraction was performed as previously described.[15] The stomach content of day 8 was stored at −80 °C until use. On day 21, the rest of the pups of each litter (3 pups) were directly supplemented with 0.2 mL of the synbiotic for 1 week (until day 28 of life).

### 2.3. Synbiotic preparation

Daily administration of the synbiotic or vehicle was performed during the gestation and lactation periods in the same time frame each day. The synbiotic solution was prepared daily by mixing *Bifidobacterium breve* M-16V ($10^9$ CFU) with scGOS/lcFOS in physiological saline solution. The dose of scGOS/lcFOS (ratio 9:1) was 2% of an established daily food intake of 40 g. One mL of the synbiotic ($10^9$ CFU/rat/day) or saline solution was intragastrically administered through an oral gavage during pregnancy, and after birth the volume was increased to 1.5 mL. For the pups from day 21 of life until day 28 of life the dose was fixed at 2 * $10^7$ CFU/rat/day. All supplements were kindly provided by Danone Research & Innovation (Utrecht, The Netherlands). The control group received the same volume of saline with same amount of corn starch as the treated group.

### 2.4. Sample collection

On day 8 of life, pups were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg; Bayer A.G., Leverkusen, Germany) and blood, intestinal samples and caecal content (CC) were collected and immediately processed or stored at −20 °C or −80 °C for future analysis. The weight of different organs was measured.

Blood samples were immediately analysed using an automated hematologic analyser (Spincell, MonLab Laboratories, Barcelona, Spain). Blood was also centrifuged (10.000 *g*, 10 min, 4 °C) to obtain plasma for total Ig and anti-RV Ig quantification.

SI samples were collected for gene expression analysis and Ig quantification. One cm of a central portion of the SI was conserved in RNAlater® and frozen at −20 °C until PCR analysis. To obtain gut wash (GW), the remaining parts of the intestine were opened lengthwise, cut into 5 mm pieces, incubated with 2 mL of Phosphate Buffered Saline (PBS), and centrifuged.

Digested milk from the pups' stomach was obtained and homogenized with PBS for Ig quantification. Caecal content was used for microbiota profiling.

## 2.5. Clinical assessment

RV infection induced diarrhoea from day 4 to day 11. Faecal samples were obtained by gently massaging the abdomen, then were weighted and frozen at −20 °C for further analysis. The severity of diarrhoea was assessed by analysing faecal weight and a scoring system ranging from 1 to 4 (diarrhoea index [DI]). The scoring system was based on the colour, texture, and the amount of faecal samples, with scores of 1 indicating normal faeces and scores of 4 indicating a high amount of watery faeces. Scores of 2 or higher indicated the presence of diarrhoea, while scores below 2 indicated the absence of diarrhoea.[14] Furthermore, other parameters associated to the diarrhoea were calculated. The area under the curve of severity (S-AUC), the incidence of diarrhoea was expressed as the percentage of diarrheic faeces (% DF) and the percentage of diarrheic animals (% DA) in each group. The AUC of the incidence (I-AUC) of diarrhoea during the whole period was measured as global values of incidence. The maximum incidence (MI) and severity (MS) were also calculated, which represent the highest values during the diarrhoea period. The days when MI (MId) and MS (MSd) were achieved were also analysed. Additionally, the interval between the beginning day of diarrhoea (BDD) and the final day of diarrhoea (FDD) was measured to calculate the diarrhoea period (DP) for

each animal, and finally the number of days with diarrhoea within the diarrhoea period was calculated (days with diarrhoea, DwD).

## 2.6. Faecal SA-11 shedding

On day 6 of life, 1-day post-infection (DPI), the collected faecal samples were homogenized and centrifuged and the SA-11 particles quantification was performed by ELISA.[16] For the standard curve, titrated dilutions of inactivated SA-11 particles (from $10^6$ to $10^4$) were used.

## 2.7. Specific humoral response and immunoglobulin quantification

Plasma, milk and digested milk from stomach content of day 8 of life were used to quantify the total anti-RV Ig (IgM, IgA and IgG) by ELISA technique. The Ig assessment was performed as previously described.[17]. Plasma samples were analysed for IgA, IgM, IgG, and IgG isotypes (IgG1, IgG2a, IgG2b, IgG2c) using ProcartaPlex™ Multiplex immunoassay [18] at the Cytometry Service of the Scientific and Technological Centres of the University of Barcelona (CCiT-UB). Th1 and Th2 responses were evaluated by adding the relative proportions of IgG subtypes, IgG2b + IgG2c and IgG1 + IgG2a, respectively.

Secretory (s)IgA and IgM were quantified in digested milk and GW of day 8 pups. The quantification of both Igs was performed using the protocol previously described.[19]

## 2.8. Small intestine gene expression

RNA extraction from SI samples frozen in RNAlater was performed in a FastPrep-24 instrument (MP biomedicals, Illkirch, France) and the RNeasy Mini Kit (Qiagen, Madrid, Spain). cDNA was obtained using TaqMan Reverse Transcription Reagents (Applied Biosystems, AB, Weiterstadt, Germany). Real Time (RT) - PCR was performed using the ABI Prism 7900 HT quantitative RT-PCR system (AB).

The specific TaqMan primers AB used and the housekeeping gene Gusb (β-glucuronidase) are specified in the **Table S1**. Data was analysed using the -2ΔΔCt method.[20] The data are presented as the percentage of expression in each experimental group normalized to the mean value obtained for the REF group, which was set at 100%.

The identification of *B. breve* M 16-V was conducted in the faeces on day 10 and 11 and in CC on day 21 of life.[21] The Taq-Man-based forward, reverse, and probe sequences were designed by Phavichitr *et al.*[22]

## 2.9. Small intestine histology

The central section of the SI was fixed in 4% formalin for 24 h, was rinsed with PBS, dehydrated in a graded series of ethanol and xylol, and then embedded in melted paraffin (Merck, Madrid, Spain). Hematoxylin-eosin (HE) staining was performed on 5 µm-thick paraffin sections. Representative photos were taken at 100x in an Olympus BX41 microscope. Villi height, width and area were measured with the Image J software (Image Processing and Analysis in Java, Bethesda, MD, USA)[23].

## 2.10. Caecal microbiota profiling

Total DNA was isolated from CC samples using an automated assisted method based on magnetic beads (Maxwell® RSC Instrument coupled with Maxwell RSC Pure Food GMO and authentication kit, Promega, Spain), lysozyme (20 mg/mL) and mutanolysin (5 U/mL) for 60 min at 37 °C and a preliminary step of cell disruption with 3 µm diameter glass beads during 1 min at 6 m/s by a bead beater FastPrep 24-5 G Homogenizer (MP Biomedicals). DNA was purified using the DNA Purification Kit (Macherey-Nagel, Duren, Germany) and the final DNA concentration was measured using Qubit® 2.0 Fluorometer (Life Technology, Carlsbad, CA, USA). Microbial profiling was assessed by amplicon V3-V4 variable region of the 16S rRNA gene. Libraries were prepared following the 16S rDNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15044223 Rev. A). The libraries were then sequenced using 2x300 bp paired-end run on a MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain). Negative and positive mock community (Zymobiomics) communities were also included. Bioinformatic processing and analysis was performed as detailed in **Figure S1**.

### 2.11. Statistical analysis

SPSS Statistics 22.0 software (SPSS Inc., Chicago, IL, USA) was employed for statistical analysis. Normality and homogeneity of variance were assessed with Shapiro-Wilk and Levene tests, respectively. Data meeting these underwent one-way ANOVA, while non-normally distributed and unequal data underwent Kruskal-Wallis ($p < 0.05$). Spearman correlation coefficients were calculated to explore variable associations. Non-metric multidimensional scaling (NMDS) in Rstudio with the 'vegan' package[24] package uncovered sample similarities based on immune factor composition, and 'envfit' function was applied to evaluate factors' associations ($p < 0.05$).

## 3. RESULTS

### 3.1. Growth evolutione

Pups BW was monitored from day 2 to the end of the diarrhoea period (**Figure 1**). SYN had lower birth weights (REF = 7.02 ± 0.15 and SYN = 6.14 ± 0.14), due to larger litter sizes in the SYN group (13.0 ± 0.71 *vs*. 10.0 ± 1.10) ($p$=0.07). Therefore, the total weight gain adjusted for litter size was lower in SYN dams (9.72 ± 0.69) compared to REF dams (13.70 ± 1.08) ($p$=0.01). However, the BW gain was not modified due to the maternal supplementation during the study (**Figure 1**). The growth-associated parameters and the relative organ sizes were not affected with the maternal intervention (**Table S2**).

### 3.2. Haematological variables

RV infection in the SYN group increased the total count of leucocytes, driven by elevated lymphocytes and granulocytes. In contrast, the red blood cell variables were not modified (**Table S3**).

### 3.3. Clinical evaluation

After RV inoculation, diarrhoea was evaluated in terms of incidence and severity (**Figure 2**). RV infection resulted in mild diarrhoea with an average score in both groups below 2.5 (**Figure 2a**). The severity curve of the SYN group was always below the REF one, however only on day 10 of life this result was significantly for lower diarrhoea. The mean S-AUC was calculated as a good indicator of

the global process and pups whose mothers received the synbiotic showed a lower diarrhoea S-AUC than that of the REF group (**Figure 2b**).

During the period from day 4 to day 13, the percentage of animals with diarrhoea (%DA) progressively increases, reaching a peak on day 9 in both groups, and then decreasing to almost no animals presenting diarrhoea around day 12 (**Figure 2c**). Specifically, on day 6, a tendency to lower %DA in the SYN group ($p$=0.06) was observed compared to the REF group, and on day 10, this effect was significant. The I-AUC calculated as the overall incidence value shows that the SYN group has a lower I-AUC than the REF group, indicating a global reduction in the incidence of diarrhoea because of the maternal synbiotic supplementation (**Figure 2d**).

Other variables associated to the severity, incidence and duration of the diarrhoea were calculated (**Table 1**). The dMI was similar in both groups, around day 8, while the dMS was achieved later in the SYN group. Regarding the variables related to the duration of diarrhoea studied, the BDD and FDD were similar between both groups. However, the duration of the DP and the DwD were significantly lower in the group supplemented with the SYN compared to the REF group, indicating the shortening symptoms effect of the intervention.

### 3.4. Faecal sample analysis

The faecal elimination of the SA-11 viral particles was quantified at day 6 (**Figure 3**). The SYN eliminated around 1 x $10^8$ RV particles/mg, similarly to the REF group, which clearance was 5 x $10^7$ (p=0.21) (**Figure 3a**). Additionally, the collected samples were separated into pre-diarrhoea period (pre-D, day 4-5), diarrhoea period (D, day 6-9) and post-diarrhoea period (post-D, day 10-13) (**Figure 3b**). As expected, during the RV infection the faecal consistency increased with respect to the pre-D period in both groups. At the end of the infectious process, in the post-D period the faecal weight was reduced in the SYN pups.

### 3.5. Anti-RV antibody response

Anti-RV antibody (Ab) response was assessed in plasma samples from pups at different time points, and in plasma and milk from dams (**Figure 4**). Maternal synbiotic supplementation had no effect on dams' specific anti-RV levels in plasma or milk. Synbiotic supplementation did not alter either specific Ab production in pups during the RV infection (day 8), at the end of lactation (day 21) or 1 week post-weaning (day 28). Notably, a significant increase in total anti-RV antibodies was observed from 8- to 21-days-old animals, indicating a distinct Ab response during this period.

### 3.6. Pups immunoglobulinome

The Ig profile of 8-day-old pups was examined in digested milk, mucosal (small intestine), and systemic (plasma) compartments (**Figure 5**). In pups' stomach milk obtained on day 8 (during RV infection), synbiotic supplementation did not induce a change in total IgM, whereas total IgA was increased with respect to REF (**Figure 5a**). In the GW, IgM levels tended to increase ($p$=0.06) in the SYN group, contrasting with unchanged sIgA levels (**Figure 5b**). Systemically, total IgM, IgA, and IgG remained unaffected (**Figure 5c**). However, IgG subtype analysis revealed decreased IgG1 and IgG2b proportions and increased IgG2c in the SYN group (Figure 5d). The overall profile was analysed by non-metric multi-dimensional scaling (NMDS) (**Figure 5e**) and the Th1/Th2 ratio (**Figure 5f**). In the NMDS potential cluster differences ($p$=0.09) between REF and SYN groups were found, while the Th1/Th2 ratio remained unchanged with the synbiotic supplementation.

### 3.7. Small intestine gene expression

Biomarkers of the immune system and gut barrier function of the SI were studied by q-PCR (**Figure 6**). During RV infection, maternal supplementation increased mRNA levels of Toll-like receptors (*Tlr2* and *Tlr7*) in the SYN group (**Figure 6a**). Synbiotic supplementation also enhanced the expression of genes associated with epithelial barrier function (*Ocln, Muc2*, and *Muc3*) (**Figure 6b**), while intestinal maturation markers (*IgA*, *Blimp1, FcRn* and *Afp*) remained unaltered (**Figure 6c**).

### 3.8. Small intestine histology

The intestinal morphology was evaluated during the RV infection (**Figure 7**). Synbiotic supplementation exerted a trophic effect on the villi by increasing their height and area. The villi width also tended to be increased ($p$=0.07) in the SYN group.

### 3.9. Ceacal microbiota composition

The impact of the RV infection on the caecal microbiota was analysed at day 8 of pups' life (**Figure 8**). No impact of maternal synbiotic intervention on the pup caecal microbial richness (Chao1 and observed species) and diversity (Shannon) (**Figure 8a-c**) was observed. Beta-diversity showed two distinct microbial clusters depending on the maternal intervention (PERMANOVA test F-value: 3.0566; R-squared: 0.12706; $p$=0.011, **Figure 8d**). The individual profiles showed that SYN intervention normalized the caecal microbial profile toward *E.coli/Shigella* and *Ligilactobacillus* compared to REF (**Figure S1**) and the LEfSe test also demonstrated the role of those two microbial genus depending on SYN intervention (**Figure 8e**). Additionally, the results of the bacteria proportions in terms of phylum, family and genera were analysed. On day 8 of life the beta diversity of the cecum was really low, the phylum bacteria proportions were mainly divided in Firmicutes and Proteobacteria, without being modified by the maternal synbiotic supplementation (**Figure 8f**). Regarding the family proportions, only a reduction of the *Enterococcaceae* was observed in the SYN group (**Figure 8g**). At genera levels, the maternal synbiotic supplementation increased the proportion of *Escherichia*/Shigella, and reduced the proportion of *Enterococus* and *Enterobacter* members (**Figure 8h**). DESEq tests showed the differential presence of specific microbial genus. Enterobacter was depleted (FDR $p$=0.023) and enriched in *E. coli/Shigella* (FDR $p$=0.06) in the SYN intervention. In addition, SYN intervention increased the presence of Streptococcus (FDR $p$<0.001), *Ruminococcus_gauvreauii*_group (FDR $p$<0.001); and Erysipelatoclostridium (FDR $p$<0.001) in the pup caecum content (although relative abundances were lower than 1-0.50%). The tracking of the B. *breve* M-16V in the faeces indicated that it was present in a 50% of the analysed samples, being identified at 2 $*10^8$ UFC/mg.

## 4.DISCUSSION

This study shows that supplementation with *B. Breve* M-16V and scGOS/lcFOS to dams during gestation and lactation improves the newborn immune response and ameliorates the RV infection. Our results suggest that breast milk (BM) would act as the main source of immunological compounds to the protection the newborn.

Breastfed infants have lower early-life infection rates than formula-fed infants [25], attributed to bioactive compounds in BM which enhance the adaptive immunity.[26] Recent studies examined how maternal nutrition during gestation and lactation affects offspring development [27] and milk composition.[28] For example, maternal diet and probiotic intake influence milk immunoglobulin profile, increasing sIgA and altering oligosaccharide levels. [15,29,30]

Maternal transmission of immunological components positively impacts infant immune maturation and reduces early-life infections. [31] However, little research explores how enhancing maternal immunological status through diet affects infant development. Our study shows that supplementation with *B. Breve* M-16V and scGOS/lcFOS during gestation and lactation improves newborn immune responses, reducing RV infection. These findings emphasize the potential of maternal nutritional intervention to decrease infection severity and incidence in neonates, with synbiotic supplementation aiding recovery, consistent with previous studies. [12]

Our study assessed RV infection parameters and immunological variables. According to previous findings, the RV-induced diarrhoea was not accompanied by BW loss. Unlike previous findings, RV-induced diarrhoea didn't lead to weight loss. [32] Furthermore, no variations were detected in relative organ weights, suggesting a safe influence of the synbiotic when administered to the mothers.

RV infection targets small intestine enterocytes, disrupting absorptive and fluid secretion functions, leading to diarrhoea.[8] In this preclinical study, maternal synbiotic supplementation

demonstrated efficacy in mitigating RV infection by reducing severity, incidence, and diarrhoea duration in the offspring. Previous RV infection model studies have observed an increase in watery faeces, intestinal dysbiosis, and altered immune responses during RV infection.[12,17,23] Probiotics and prebiotics, like *Lactobacillus, Bifidobacterium, Saccharomyces* strains [33], and scGOS/lcFOS, are utilized to alleviate diarrhea. [34] Their combination displays promising results in modulating and preventing RV gastroenteritis. [34,35] Our study confirms that synbiotic supplementation to dams enhances pups' immune systems, ameliorating RV infection. This is an additional step forward to enhance the newborn health before birth to counteract early life infections.

Maternal synbiotic supplementation affected haematological variables, revealing higher total leucocyte counts in SYN pups. This increase was associated with elevated total lymphocytes and granulocytes during RV infection. Viral infections typically induce lymphocytosis [36] and granulocytosis.[37] The observed increase is probably due to a higher mobilization that may be linked to a better resolution of the process in the SYN group.

Throughout RV infection, innate and adaptive cells work together against the virus. [38] While plasma Ig composition remained relatively stable, NMDS analysis suggested potential differences in global Ig profiles between groups. However, the increase in IgG2c, mice analogue IgG3c, suggests enhanced long-term immunity and regulatory responses in neonatal intestines. [39,40] At the mucosa, sIgA offers protection by recognizing viral particles [41], while mucosal IgM aids the initial humoral response and tissue homeostasis.[42] Our data revealed a potential effect to modulate the IgM levels without changes in the sIgA.

Although viral infections trigger the memory immune system, inducing the production of specific Ab production[43] , the synbiotic did not modify the pup's specific anti-RV Ab levels after the experimental RV infection. Hence, maternal synbiotic supplementation induce the production of specific Ab responses but other mechanisms are involved in the observed protective effects, such as the changes in the composition of BM changes according to the

infant's requirements.[44] Our results indicated that synbiotic maternal supplementation increases the total milk IgA levels during infant's RV infection suggesting an enhanced protection for infants against the RV.

RV targets intestinal enterocytes [9], inhibiting host immune responses [45] and modulating gene expression for viral replication. [46] Maternal synbiotic supplementation upregulated *Tlr2, Tlr7, Ocln, Muc2,* and *Muc3* expression. RV infections have been linked to TLR and mucin changes [47,48] and TJ disruption [49], potentially impacting the protective mucus and tissue integrity. Probiotics improve the intestinal barrier by modulating TLR and TJ protein expression and mucin secretion. Thus, the overexpression of TLR, mucins and TJ proteins in the SYN group indicates that maternal supplementation enhances the infant intestinal epithelial barrier function, contributing to viral defense.[50]

RV infection alters the small intestine architecture, reducing villi height.[51] Here, maternal synbiotic supplementation induced a trophic effect on small intestine villi, increasing height and area, along with a tendency to a higher width, aiding in nutrient and water absorption essential for both infection or healthy maturation.[52]

At the peak of the infection independently of the maternal intervention, *Enterobacteriaceae* family dominated in both groups, although a depletion on *Enterococcaceae* family proportion in the SYN group was observed. Additionally, the proportions of *Escherichia/Shigella* were higher in the SYN while *Enteroccocus* and *Enterobacter* genera were lower. RV infection has been linked to an increase in the *Escherichia-Shigella* abundance,[53] but here, the maternal synbiotic supplementation was not able to reduce the RV-induced effect. However, the reduction of the *Enterococcus* and *Enterobacter* was previously linked with synbiotics.[54] Our results did not agree with the available data indicating a reduction in the proportion of *Escherichia Shigella.*[55] However, the maternal synbiotic supplementation promotes the growth of *Ligilactobacillus,* which is an anti-infective probiotic due to its antimicrobial activity.[56] Altogether, maternal synbiotic supplementation modulates the microbiota

composition in early life as previously described [57], specifically in our case contributing to reduce the RV incidence and severity.

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## 6. Conflict of Interest

The authors declare no conflicts of interest.

## 7. Author Contributions

L.S.-F., K.R.-A., M.M.-C., M.C., K.K., J.G., R.B.-S., M.J.R.-L., M.C.C and F.J.P.-C participated in the conceptualization and critical analysis of the results. All authors have contributed to the manuscript revision and agreed to the published version of the manuscript.

## 8.Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## FIGURE LEGENDS

**Figure 1.** Body weight gain of neonatal rats from day 2 to day 13 of life. Results are expressed as mean increase with respect to day 2 (%) ± standard error of the mean (SEM). (n=36-39 until day 8 and 15-17 until day 13). Statistical differences: **p*< 0.05 vs REF

**Figure 2.** Clinical indices of diarrhoea. a) The incidence of diarrhoea is indicated by the percentage of animals with diarrhoea (%DA), which is calculated based on the percentage of animals in each group that have a DI score of 2 or higher. b) I-AUC: area under the incidence curve. c) The diarrhoea severity is measured by using the Diarrhoea Index (DI), which involves grading faecal samples on a scale of 1 to 4 based on their colour, texture, and amount. A DI score of 2 or greater indicates the presence of diarrhoea, while scores below 2 indicate absence. d) S-AUC: area under the severity curve. (n=15-17). **p*< 0.05 *vs* REF; #*p*< 0.1 *vs* REF.

**Figure 3**. Faecal samples analysis. a) The viral shedding was assessed at the peak of viral elimination (1 Day post-infection (DPI)). b) The fecal weight, as an objective indicator of the severity of diarrhoea, during the pre-diarrhoea (pre-D), diarrhoea (D), and post-diarrhoea (post-D) periods. Data are expressed as mean ± SEM. (n=15-17). **p*< 0.05 *vs* REF.

**Figure 4.** Concentration of total anti-RV antibodies in milk and plasma of pups and dams. Results are expressed in Arbitrary Units/mL. Data are expressed as mean ± SEM. (n=15-17).

**Figure 5.** Immunoglobulin profile in pups at day 8 of life. a) Total levels of IgM and sIgA in digested milk. b) Total levels of IgM and sIgA in the gut wash (GW). c) Total Ig levels (IgM, IgA and IgG) in

plasma. d) Relative proportion of IgG subtypes (IgG1, IgG2a, IgG2b, IgG2c). e) Analysis of the Th1/Th2 ratio. f) Analysis of non-parametric multidimensional scaling (NMDS) for the Ig profiles based on the Bray-Curtis distance. Data (a - e) are expressed ad mean ± SEM. Each point represents an animal (f) by ANOSIM test. (n=15-17). $^{*}p< 0.05$ *vs* REF; $^{\#}p< 0.1$ *vs* REF. GW, gut wash.

**Figure 6.** Effect of synbiotic supplementation on the intestinal gene expression. a) Toll-like receptors. b) Intestinal barrier molecules. c) Intestinal maturation. Relative gene expression was calculated with respect to REF, which corresponded to 100% of transcription (represented with a horizontal dotted line). Statistical differences: *p< 0.05 vs REF. (n=15-17). Tlr, Toll-like receptor; Muc2, mucin2; Muc3, mucin3; Ocln, occludin; IgA, immunoglobulin A; Blimp1, B-lymphocyte-induced maturation protein 1; FcRn, neonatal Fc receptor; Afp, alpha-fetoprotein.

**Figure 7.** Effect of synbiotic supplementation to dams on pups' small intestine architecture. a) Representative images of histological sections of the small intestine stained with haematoxylin and eosin, 100x. b) Villi height. c) Villi area. d) Villi width. Data are expressed as mean ± SEM. (n=15-17). $^{*}p< 0.05$ *vs* REF; $^{\#}p< 0.1$ *vs* REF.

**Figure 8**. Caecal microbial alpha- and beta-diversity of dams at weaning day. Microbial richness measured by (a) number of observed species and (b) Chao1 index. c) Microbial diversity measured by Shannon index. d) Beta diversity as a principal coordinate analysis (PCoA) plot based on Bray-Curtis dissimilarity. e) LEfSe test of *Enterobacter* and *Escherichia Shigella*. f) Relative proportions of caecal phylum. g) Main families present in the CC. h) Main genera present in the CC. Statistical differences: $^{*}p< 0.05$ SYN vs REF. (n=11-12). CC, caecal content.

**TABLE LEGENS**

**Table 1.** Clinical variables associated with the RV-induced diarrhoea.

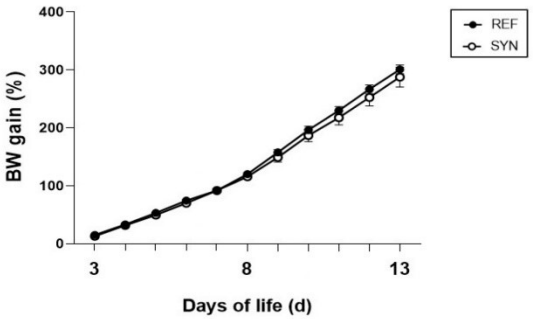


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Figure 1

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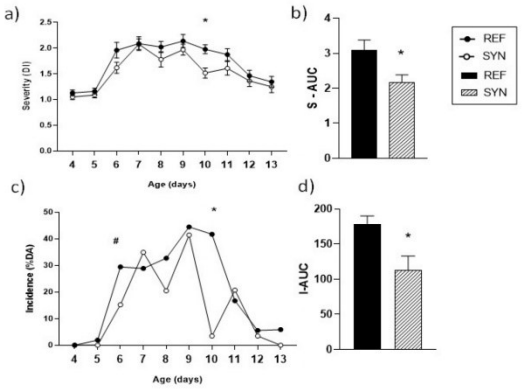


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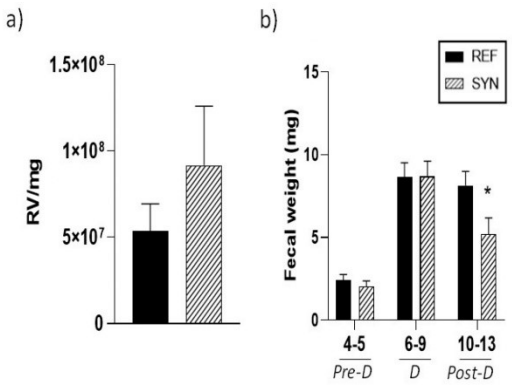


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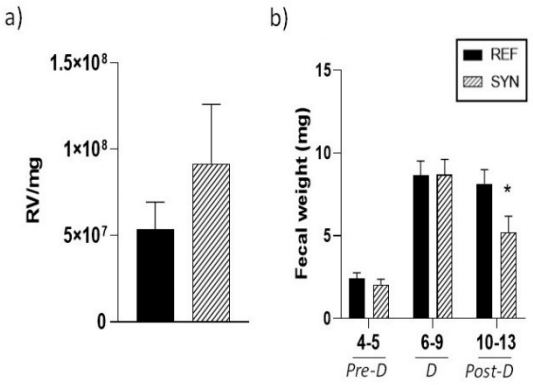


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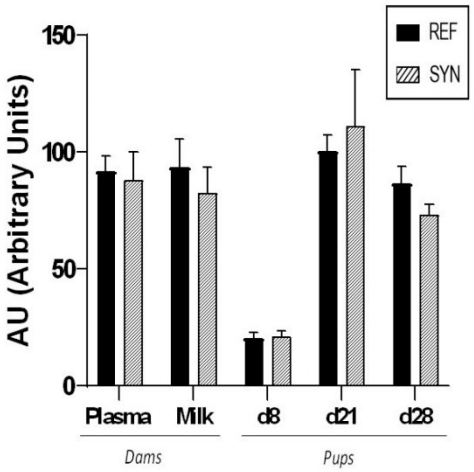


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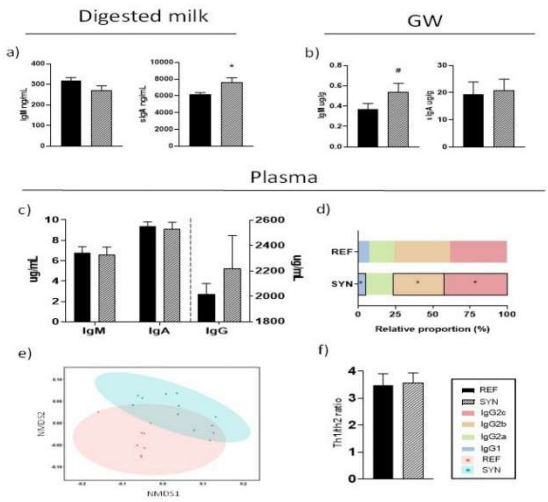


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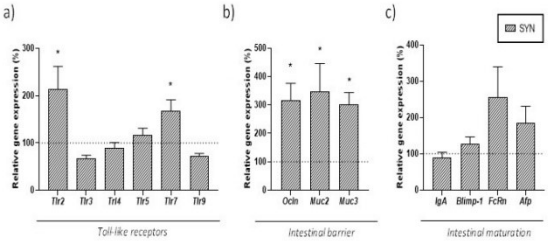


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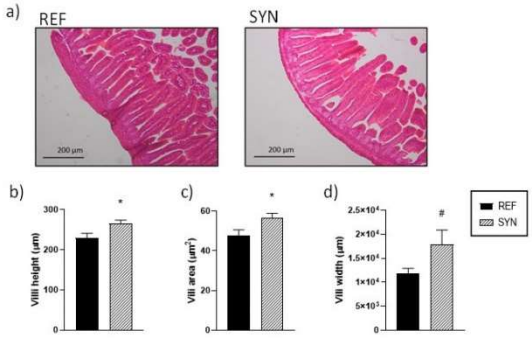


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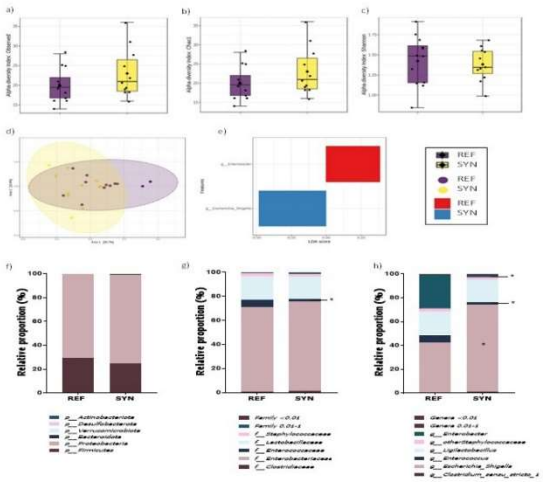


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Figure 8

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**Table 1.** Clinical variables associated with the RV-induced diarrhoea.

| | REF | SYN |
|---|---|---|
| | Incidence | |
| MI (%) | 44.4 | 41.38 |
| dMI | 7.67 ± 0.76 | 8.00 ± 0.45 |
| | Severity | |
| MS (1-4) | 2.27 ± 0.12 | 1.90 ± 0.13 |
| dMS | 7.12 ± 0.27 | 7.97 ± 0.26* |
| | Duration | |
| BDD | 7.28 ± 0.20 | 7.65 ± 0.25 |
| FDD | 9.05 ± 0.27 | 8.59 ± 0.27 |
| DP | 2.16 ± 0.24 | 1.64 ± 0.17* |
| Dwd | 1.49 ± 0.19 | 1.02 ± 0.13* |

Data are expressed as mean ± SEM. (n=15-17). *$p < 0.05$ *vs* REF. MI: maximum index; dMI: day of maximum incidence; I- AUC: area under the Incidence curve; MS: maximum severity; dMS: day of maximum severity; S-AUC: area under the severity curve; BDD: beginning day of diarrhoea; FDD: final day of diarrhoea; DP: diarrhoea period; DwD: days with diarrhoea.

# ARTICLE 4

## "Maternal synbiotic supplementation with *B. Breve* M-16V and scGOS/lcFOS shape offspring immune development and gut microbiota at the end of suckling"

Sáez-Fuertes, Laura; Kapravelou, Garyfallia; Grases-Pintó, Blanca; Bernabeu, Manuel; Knipping, Karen; Garssen, Johan; Castell, Margarida; Rodríguez-Lagunas, María José; Collado, María Carmen; Pérez-Cano, Francisco José

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The results showed in this paper have been presented in the following congresses:

- **VI workshop Anual INSA-UB** Barcelona (February 2022). **Sáez-Fuertes, L.**; Rio-Aige, K.; Grases-Pintó, B.; Melero, C.; Galindo S.; Cabré, P.; Gironés, A.; Zhan S.; Castell, M.; Collado, M.C.; Rodríguez-Lagunas, M.J.; Pérez-Cano, F.J. "Impact of a synbiotic administration during gestation and lactation to dams in the offspring's development".
- **15ª Reunión de la red española de bacterias lácticas: Bacterias Lácticas en Alimentación y Salud. Valencia** (May 2022). **Sáez-Fuertes, L.**; Rio-Aige, K.; Grases-Pintó, B.; Melero, C.; Galindo S.; Cabré, P.; Gironés, A.; Zhan S.; Castell, M.; Collado, M.C.; Rodríguez-Lagunas, M.J.; Pérez-Cano, F.J. "Efecto preclínico de la suplementación materna con un simbiótico durante la gestación y lactancia en el sistema inmunitario del lactante".
- **XV Workshop de la Sociedad Española de Microbiota, Probióticos y Prebióticos (SEMiPyP).** Sevilla (February 2024). **Sáez-Fuertes, L.**; Rio-Aige, K.; Grases-Pintó, B.; Castell, M.; Collado, M.C.; Rodríguez-Lagunas, M.J.; Pérez-Cano, F.J. "Efectos de la suplementación materna de un simbiótico en la microbiota de la madre y de su descendencia y bajo la infección por rotavirus".

# Abstract

**Aim**: The objective of the present study was to evaluate the impact of the maternal supplementation during gestation and lactation with *Bifidobacterium breve* M-16V ($10^9$ CFU) and short-chain galacto-oligosaccharide (scGOS) and long-chain fructo-oligosaccharide (lcFOS) in a 9:1 proportion on the offspring immune system at the end of suckling.

**Methods**: Pregnant rats were administered with the synbiotic during gestation and lactation. At the end of weaning, day 21 of pups' life, blood, adipose tissue, small intestine (SI), mesenteric lymph nodes (MLN), salivary gland (SG), cecum, and spleen were collected to analyse the immune system development and the microbiota colonization.

**Results**: In terms of intestinal barrier, the expression of the genes of *Tlr9, Muc2, IgA* and *Blimp1* were upregulated in the SI of those rats whose mothers received the symbiotic. This increase in IgA gene expression was further confirmed at protein level in the gut wash. Maternal synbiotic supplementation also positively impacted the microbiota composition in both the small and large intestines of their offspring resulting in higher proportions of *Bifidobacterium* genus, among others. In addition, in these animals there was an increase in butanoic, isobutanoic, and acetic acids concentrations in the cecum but a reduction in the small intestine. At the systemic level, the maternal synbiotic supplementation resulted in higher levels of immunoglobulin IgG2c in plasma, SG and MLN of their infants but did not modifiied the main lymphocyte subsets in the spleen and MLN.

**Conclusion**: These findings indicate that maternal synbiotic supplementation positively influence the immune system development, particularly at the intestinal level, and the microbiota colonization of the suckling offspring.

*nutrients*

MDPI

*Article*

# Maternal synbiotic supplementation with *B. Breve* M-16V and scGOS/lcFOS shape offspring immune development and gut microbiota at the end of suckling

**Sáez-Fuertes, Laura[1,2]; Kapravelou, Garyfallia[1]; Grases-Pintó, Blanca[1,2]; Bernabeu, Manuel[3]; Knipping, Karen[4,5]; Garssen, Johan[4,5]; Bourdet-Sicard, Raphaëlle[6]; Castell, Margarida[1,2,7]; Collado, María Carmen[3†]; Pérez-Cano, Francisco José[1,2†*]; Rodríguez-Lagunas and María José[1,2]**

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[2] Nutrition and Food Safety Research Institute (INSA-UB), 08921 Santa Coloma de Gramenet, Spain

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†These authors shared senior authorship

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**Abstract:** Immune system development during gestation and suckling is significantly modulated by maternal environmental and dietary factors. Breastfeeding is widely recognized as the optimal source of nutrition for infant growth and immune maturation, and its composition can be modulated by the maternal diet. In the present work, we investigated whether the oral supplementation with *Bifidobacterium breve M-16V* and short-chain galacto-oligosaccharide (scGOS) and long-chain fructo-oligosaccharide (lcFOS) to rat dams during gestation and lactation has an impact on the immune system and microbiota composition of the offspring at day 21 of life. On that day, blood, adipose tissue, small intestine (SI), mesenteric lymph nodes (MLN), salivary gland (SG), cecum, and spleen were collected. Synbiotic supplementation did not affect the overall body or organ growth of the pups. The gene expression of *Tlr9, Muc2, IgA* and *Blimp1* were upregulated in the SI, and the increase in IgA gene expression was further confirmed at protein level in the gut wash. Synbiotic supplementation also positively impacted the microbiota composition in both the small and large intestines resulting in higher proportions of *Bifidobacterium* genus, among others. In addition, there was an increase in butanoic, isobutanoic, and acetic acids concentrations in the cecum but a reduction in the small intestine. At the systemic level, synbiotic supplementation resulted in higher levels of immunoglobulin IgG2c in plasma, SG and MLN, but not modify the main lymphocyte subsets in the spleen and MLN. Overall, the synbiotic maternal supplementation is able to positively influence the immune system development and microbiota of the suckling offspring, particularly at the gastrointestinal level.

**Keywords:** Breastfeeding, *Bifidobacterirum Breve* M-16V, scGOS/ lcFOS, immune system

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## 1. Introduction

In humans, the newborn starts its development in the placenta, although it remains immature at birth. During pregnancy, maternal health status and environment directly influence fetus development, [1] and the placenta serves as a communication bridge between the mother and the fetus by supplying the necessary nutrients [2]. Breastfeeding has been widely recognized as the most suitable nutrition for newborns The World Health Organization (WHO) suggests that newborns should be exclusively fed breast milk (BM) until 6 months of age [3]. During breastfeeding, an active communication is established between mother and infant through BM [4]. In addition, BM not only provides nourishment for the infant, but also plays a role in the maturation of the infant's immune system, contributing to the "early life programming" [5]. BM is rich in bioactive components, including metabolites, vitamins, oligosaccharides, immunoglobulins (Igs), microbes and microbial products which are part of the passive immunity transfer to the newborn [6].

BM is a dynamic, complex fluid, and its composition changes depending on the lactation stage, infant's needs, maternal diet, and environmental factors [7]. Over the last decade, research has proved that maternal nutrition modulates BM composition [8–10]. These approaches are aligned with the hypothesis that an intervention in the maternal diet is a potent alternative to support neonatal development through BM modulation[11]. In this sense, maternal diet supplemented with either probiotics, prebiotics, or synbiotics has been performed to study their impact on infant development [12].

Probiotics, prebiotics, and synbiotics have gained popularity in recent years. Probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [13]. Prebiotics are "a substrate that is selectively utilized by host microorganisms conferring a health benefit" [14]. Synbiotics are "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host" [15]. All of them contribute to maintain and improve the gastrointestinal and systemic health, and all are naturally present in BM [16].

The mechanism of action of pro-, pre-, and synbiotics first targets the gastrointestinal tract. To highlight some aspects, probiotics interact with the immune system by producing short chain fatty acids (SFCA) and inhibiting pathogen colonization [13]. Prebiotics work as immune modulators by promoting the proliferation of beneficial and probiotic bacteria and improving the gastrointestinal transit [14]. And synbiotics combine both strategies to promote the gastrointestinal health and gut barrier function [15].

Studies have shown that during pregnancy and lactation, maternal gut microbiota may reach the mammary gland through the enteromammary pathway and modulate BM composition [17]. This mechanism of communication between the mother and the infant contributes to infant gut colonization and the immune system maturation. Different studies have demonstrated the ability of probiotics to reach the mammary gland, influencing the infant's fecal microbiota composition [18–20]. However, these studies have focused their research on the microbiota transference from the mother to the infant without evaluating the impact on the global infant immune system.

Suckling rats are still developing their immature immune system and become a good model to evaluate the impact of the administration of a bioactive component [21], as performed in previous studies, either directly to the pups [22,23] or through maternal intervention [20]. It is hypothesized that the maternal intervention with the synbiotic will led to an earlier acquisition of an immune pattern in line with more adult animals.

For this reason, the objective of the present study was to evaluate the impact of the maternal supplementation during gestation and lactation with *Bifidobacterium breve* M-16V ($10^9$ CFU) and short-chain galacto-oligosaccharide (scGOS) and long-chain fructo-oligosaccharide (lcFOS) at a ratio of 9:1 on the infant immune system at the end of suckling.

## 2. Materials and Methods

### 2.1. Animals and experimental design

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Seven-week-old Lewis rats (16 females and 8 males) were obtained from Janvier Labs in La Plaine Saint Denis Cedex (France). Upon arrival, the rats had a one-week acclimatization period. After this period, the female rats were placed into the males' cages for one week for mating. Then, the females were separated and placed into individual cages.

The female rats were then divided into two experimental groups: reference (REF) and synbiotic (SYN). Animals from the different groups were supplemented with a synbiotic (SYN, n=8) or vehicle (REF, n=8) during gestation (21 days) and lactation (21 days). The pups were allowed to born naturally, and the day of birth was designated as day 1 of life for the pups. Litters were unified up to 10 pups per litter. Pups were divided to evaluate the impact at different ages, 4 pups/litter were euthanized on day 8 of life, 3 pups/litter and dams were euthanized on day 21 of life and the resting 3 pups/litter were euthanized on day 28 of life. Here, the results are focused on the pups euthanized on day 21 of life. Throughout the study, the rats had unrestricted access to a commercial diet formulated according to the American Institute of Nutrition 93G formulation [24]. They were also provided with water *ad libitum* and the pups had free access to their mother's nipples for nursing.

The animal room conditions (temperature and humidity) were controlled. The room followed a 12-h light - 12-h dark cycle within a biosafety chamber at the Campus Diagonal Animal Facility of the Faculty of Pharmacy and Food Science at the University of Barcelona (UB). The experimental procedures conducted in this study were carried out with the necessary ethical approvals. The research received its approval from the Ethics Committee for Animal Experimentation (CEEA) of the University of Barcelona (Ref. 240/19) and from the Catalan Government (Ref. 10933).

The nutritional intervention started on the first day of gestation (G1) and continued until the end of the study, which was day 21 of the pups' life (d21). The SYN group dams were orally administered 1 mL of a synbiotic mix daily during the gestation period and 1.5 mL during the lactation period while the REF dams received an equivalent volume of a saline solution under similar conditions. Briefly, the synbiotic suspension was constituted by a mix of *Bifidobacterium breve* M-16V ($10^9$ CFU/mL) with scGOS/lcFOS (9:1 proportion, 7.6 mg/mL and 0.08 mg/mL, respectively). The dose was calculated in basis of a daily food intake of 40 g diet of a 2 % prebiotic mixture. All supplements were kindly provided by Danone Research & Innovation (Utrecht, The Netherlands).

## 2.2. Sample collection and processing

The body weight of the animals was monitored daily, and at the end of the study the length of the animals was also measured. To assess the animals' development and overall body composition the Body Mass Index (BMI) ($(weight/length^2$ (g/cm$^2$)) and the Lee Index ($(weight^{0.33}/length) \times 1{,}000$ (g$^{0.33}$/cm) were calculated. In the middle of lactation (day 12 to day 14), feces were collected and frozen for probiotic tracking. Pups were anesthetized on day 21 to evaluate the impact of the maternal nutritional intervention during the breastfeeding period.

At the weaning day (day 21), pups were anesthetized with ketamine (90 mg/kg; Merial Laboratories S.A., Lyon, France) and xylazine (10 mg/kg; Bayer A.G., Leverkusen, Germany). Blood samples were obtained by cardiac exsanguination and analyzed in an automated haematologic analyzer (Spincell, MonLab Laboratories, Barcelona, Spain) or centrifuged to obtain plasma. Also, intestinal samples, adipose tissues (AT) (epididymal, parametric, dorsal, retroperitoneal, inguinal for white adipose tissue (WAT) and brown adipose tissue (BAT)), cecal content (CC), salivary gland (SG), mesenteric lymph nodes (MLN) and spleen were collected and immediately processed or stored at −20 °C or −80 °C for future analysis.

AT, both BAT and WAT (epidydimal or parametric, for males or females respectively were chosen as representative of WAT), and small intestine (SI) were collected for histomorphometry. The central section of the SI was collected for gene expression analysis, embedding it in RNAlater (Ambion, Life Technology, Madrid, Spain), kept at 4 °C for 24

h, and stored at –20 °C later. The rest of the proximal part of the SI was opened lengthwise and cut into 0.5 cm pieces and incubated with PBS in a shaker (37 °C for 10 min) to obtain the gut wash (GW) for Ig quantification, and the content of the distal part of the intestine (IC) was collected for microbiota analysis.

### 2.3. Immunoglobulin quantification

Plasma and homogenized MLN and SG were processed for ProcartaPlex™ Multiplex immunoassay (eBioscience, San Diego, CA, USA). Total IgA, IgM, IgG and IgG isotypes (IgG1, IgG2a, IgG2b, IgG2c) were quantified following the manufacturer's instructions as previously described [25] using a MAGPIX® analyzer (Luminex Corporation, Austin, TX, USA) at the Cytometry Service of the Scientific and Technological Centers of the UB (CCiT-UB). The relative abundance of IgG subtypes was calculated with respect to the total IgG levels. IgG2b and IgG2c proportions allow to assess the Th1 response whereasIgG1 and IgG2a proportions allow to obtain the Th2 response in rats [26].

Secretory (s)IgA in CC homogenates and GW samples was assessed by sandwich ELISA technique (Bethyl, Laboratories Inc., Montgomery, TX, USA). Also, IgM was quantified in GW by following the previously described protocol [27].

### 2.4. Tissue histology

The central section of the SI and the AT, selected for histomorphometry, were treated by immersing it in 4 % buffered formaldehyde solution for 24 h at room temperature. Afterwards, the samples were rinsed in a phosphate-buffered solution (PBS) until dehydration in consequent graded ethanol solutions (70 %, 90 %, and 100 %). After the dehydration and permeation steps in xylene, the samples were embedded in melted paraffin. Paraffin sections (5 μm) were stained using hematoxylin-eosin (HE). Olympus BX41 and Camera Olympus XC50, Olympus (Barcelona, Spain) was used to examine the samples. For each sample of white adipose tissue (WAT) (20x), brown adipose tissue (BAT) (40x), and intestine (10x), representative photos were taken. All histology samples were analyzed using Image J (Image Processing and Analysis in Java, National Institute of Mental Health, Bethesda, MD, USA).

### 2.5. Gene expression analysis

RNAlater stored SI samples were thawed for tissue homogenization by using lysing matrix tubes and FastPrep-24 instrument (MP biomedicals, Illkirch, France) as previously described [28]. RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Madrid, Spain) following the manufacturer's instructions. RNA purity and concentration were determined with a NanoPhotometer (BioNova Scientific S.L., Fremont, CA, USA) and cDNA obtained using TaqMan Reverse Transcripiton Reagents (Applied Biosystems, AB, Weiterstadt, Germany). Then, Real Time (RT) – PCR was performed with ABI Prism 7900 HT quantitative RT-PCR system (AB). Toll like receptors (TLRs), barrier and immune related genes listed in Supplementary Table 1 were used.

After normalizing the results with the -2ΔΔCt method [29], data was showed as a percentage of expression in each experimental group with respect to the mean value obtained for the REF group, which was set at 100 %.

Additionally, the identification of *B. breve* M-16V was conducted in the feces from day 12 to 14 and CC at the end of suckling by extracting the DNA [26]. The Taq-Man-based forward, reverse, and probe sequences were designed by Phavichitr *et al.* [30].

### 2.6. MLN and spleen lymphocytes isolation

To isolate MLN and spleen lymphocytes, the tissues were passed through a sterile 40 μm mesh cell strainer (Thermo Fisher Scientific, Barcelona, Spain) by using Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, Madrid, Spain) enriched with 10 % fetal bovine serum (FBS, Sigma-Aldrich), 100 IU/mL streptomycin-penicillin

(Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), and 0.05 mM 2-β-mercaptoethanol (Merck Millipore, Darmstadt, Germany). The cell suspension was centrifuged at 538 *g* for 10 min at 4 °C. The resulting pellet was then resuspended in the RPMI enriched medium. For splenic cells, an additional step was required to eliminate erythrocytes by osmotic lysis. Conditions were immediately restored by adding PBS to avoid lymphocyte death [31]. The number and viability of the cells were assessed using the Countess™ Automated Cell Counter (Invitrogen™, Thermo Fisher Scientific).

2.7. Cell subset staining and flow cytometry analysis

MLN and spleen phenotypic populations were characterized by flow cytometry analysis using fluorescent mouse anti-rat monoclonal antibodies (mAbs) conjugated to different fluorochromes: fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), allophycocyanin (APC), brilliant violet 421 (BV421), and phycoerythrin-Cyanine 7 (PE-Cγ7). All the mAbs were acquired from BD Biosciences, Serotec, and Caltag, respectively: anti-TCR (R73), anti-CD103 (OX-62), anti-NK (10/78), anti-CD62L (OX-85), anti-CD8 (OX-8), anti-CD4 (OX-35), anti-CD45RA (OX-33), and anti-TCR (V65). The staining technique was performed following the protocol previously described by Torres-Castro *et al* [32]. Data were obtained with Gallios™ Cytometer (Beckman Coulter, Miami, FL, United States) in the CCiT-UB and analyzed by Flowjo v10 software (Tree Star, Inc., Ashland, OR, USA).

2.8. Cecal bacteria and Ig-coated bacterial analysis

Homogenized cecal samples were processed by flow cytometry to characterize the proportion of cecal bacteria and Ig-coated bacteria (Ig-CB) as in previous studies [33]. Data was collected with Cytek Aurora (Cytek Biosciences, Inc., CA, USA) in the CCTi-UB and analyzed using FlowJo v.10 software.

2.9. Short chain fatty acids (SCFAs) microbial metabolite profile

SCFAs determination was performed using gas chromatography–mass spectrometry (GC-MS) as described previously by Eberhart *et al.* [34]. Samples with the addition of the internal standard solution (3-Methylvaleric acid) were centrifuged (1800 *g*, 2 min, 4 °C). The supernatant was filtered-sterilized (0.22 µm, Sarstedt SA) and then, injected in the Agilent GC 7890B–5977B GC-MS with a multipurpose sampler (Gerstel MPS, Mülheim, Germany). The Agilent DB-FATWAX, 30 m × 0.25 mm × 0.25 µm GC column was used in split mode (20:1). The oven temperature program was 100 °C for 3 min, ramped to 100 °C at a rate of 5 °C/min, then to 150 °C for 1 min, then ramped to 200 °C at a rate of 20 °C/min, and finally held at 200 °C for 5 min. Helium was the carrier gas and it was used at a flow rate of 1 mL/min, with an inlet temperature of 250 °C. The volume of injection was 2 µL. For the quantification of the SCFAs, standards curves for acetate, butyrate, and propionate were used.

2.10. Cecal Microbiota profiling by 16S rRNA amplicon sequencing

Total DNA was isolated from small intestine and CC (100-200 mg) using an automated assisted method based on magnetic beads (Maxwell® RSC Instrument coupled with Maxwell RSC Pure Food GMO and authentication kit, Promega, Spain) following the manufacturer's instructions with previous treatments to improve the DNA extraction. In brief, samples were treated with lysozyme and mutanolysin (20 mg/mL and 5 U/mL, respectively) for 60 min at 37°C. After a preliminary step of cell disruption with 3-µm diameter glass beads during 1 min at 6 m/s by a bead beater FastPrep 24-5 G Homogenizer (MP Biomedicals), the DNA obtained was purified using the DNA Purificaton Kit (Macherey-Nagel, Duren, Germany) and DNA concentration was measured using Qubit® 2.0 Fluorometer (Life Technology, Carlsbad, CA, USA). Microbial profiling was assessed by targeted amplicon V3-V4 variable region of the 16S rRNA gene. Libraries were prepared

following the 16S rDNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15044223 Rev. A). The libraries were then sequenced using 2x300 bp paired-end run on a MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain). Negative and positive mock community (Zymobiomics) communities were also included. Raw reads were then processed with the integrated dada2 method for denoising, amplicon sequence variance (ASV) clustering and chimeral removal. Resulted ASV were then taxonomically assigned using Silva v.138.

### 2.11. Statistical analysis

The statistical analysis utilized SPSS Statistics 22.0 software (SPSS Inc., Chicago, IL, USA). Normality and variance homogeneity of the data were evaluated using the Shapiro-Wilk and Levene tests, respectively. When the data followed normal and homogeneous distributions, a one-way ANOVA was conducted for analysis. In cases where the data did not follow normal and equal distributions, the Kruskal-Wallis test was employed to identify significant differences among groups ($p < 0.05$). Variable correlations were explored using the Spearman correlation coefficient. Non-metric multidimensional scaling (NMDS) was executed in R studio, employing the 'vegan' package [35], to identify clusters of sample similarities based on immune factor composition. The 'envfit' function was used to assess the association of factors with the ordination of samples in the NMDS plot. Statistical significance was established when the *p-value* < 0.05.

For microbiota composition analysis, no rarefaction was done and samples with less than 4500 reads were removed and data was normalized using Centered-log-ratio (CLR). Beta diversity analysis was based on Bray-Curtis distances matrix and Permutational Analysis of Variance (PERMANOVA) was performed. Alpha-diversity indexes Chao1 and Shannon were also calculated and differences by group were assessed by Mann-Whitney and/or Kruskal-Wallis non-parametric test. Besides this, the Kruskall-Wallis test on the CLR normalized data were also assessed with Benjamini-Hochberg false discovery rate (FDR) correction. Negative binomial regression as implemented by DESeq2 tool was used for differential abundance analysis to estimate the fold-change of genus taxa [36]. Plots were generated using MicrobeAnalyst platform v.2 [37].

## 3. Results

### 3.1. Growth and morphometry

The body weight evolution was assessed throughout the study (Figure 1) and both groups revealed a similar growth pattern, without differences between sexes.

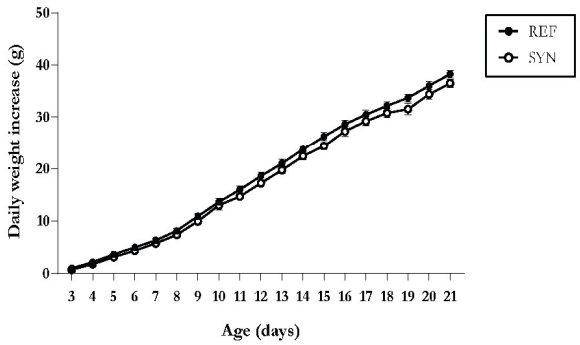

**Figure 1.** Daily weight increase with respect to day 2 of neonatal rats during the suckling period. Results are expressed as mean ± standard error of the mean (S.E.M.) Statistical differences: $^{*}p < 0.05$ vs REF (n=11-16).

At the end of suckling, pups were also measured to calculate different growth associated parameters such as the body/tail length ratio, the BMI and the Lee Index (Supplementary Table 2). The SYN group did not exhibit any significant changes in the measured parameters compared to the REF group. After exsanguination, organ weights were recorded, and the maternal synbiotic supplementation did not affect the overall values. However, in the SYN group, there was a decrease in the relative weight of the cecum and an increase in both relative weight and length of the SI (Supplementary Table 2).

### 3.2. Intestinal morphology

To follow the trophic effect in the SI of 21-day-old rats after the maternal supplementation during gestation and lactation, a histomorphometric analysis of the SI was conducted (Figure 2). In the SYN group, no significant changes were observed in the villi height and area, crypts depth or goblet cells abundance. However, a reduction in villi width was observed at the end of the suckling period.

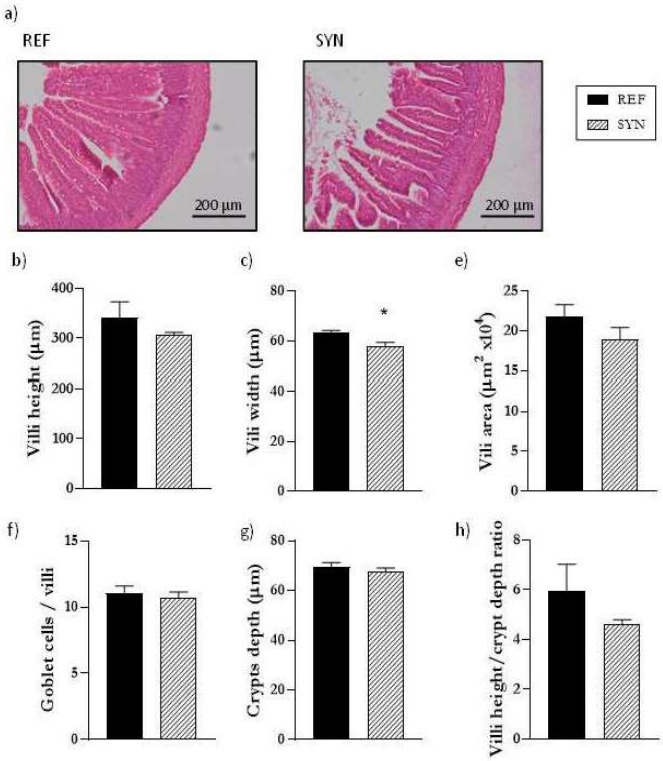


**Figure 2.** Effect of the maternal synbiotic supplementation on the pups' intestinal morphology. (a) Representative images of the small intestine stained with hematoxylin and eosin, 10x. (b) Height, (c) width, (e) area of the intestinal villi, (f) ratio of goblet cells/villi,

(g) crypts depth and (h) ratio of the villi height/crypt depth. Results are expressed as mean ± S.E.M. Statistical differences: *$p < 0.05$ vs REF (n=11-16).

### 3.2. Intestinal gene expression

The expression levels of genes involved in defense, mucosal barrier, and immunity were also assessed at day 21 (Figure 3). The supplemented group displayed an increase in the gene expression of the *Tlr9* whereas the *Tlr2*, *3*, *4*, *5*, and *7* remained unaffected (Figure 3a). The analysis of mucins revealed that *Muc2* gene expression was upregulated in the SYN group (Figure 3b). The proteins associated with epithelial barrier functions, such as tight junction (TJ) proteins, were not modulated by maternal supplementation (Figure 3c). Among the genes involved in the immunological intestinal status (*IgA* and *Blimp1*), its gene expression was increased at the end of the study (Figure 3d).

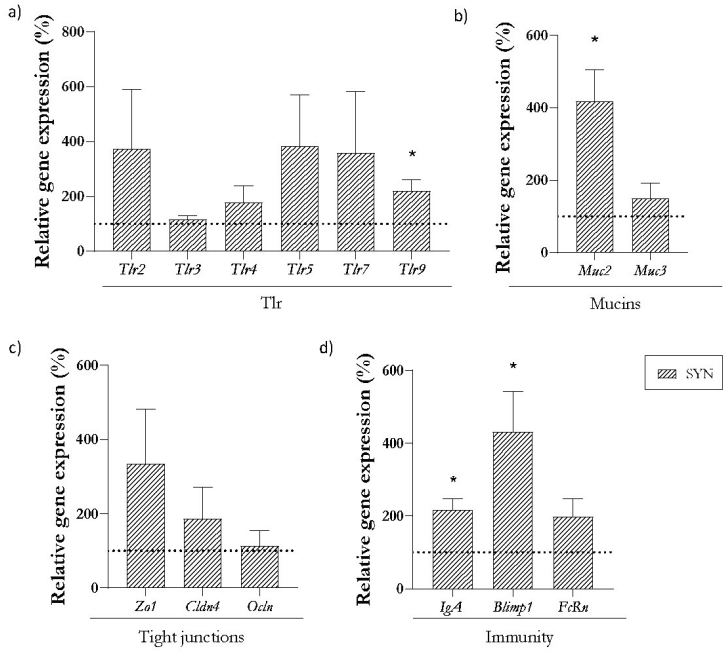


**Figure 3**. Impact of the maternal synbiotic supplementation in the small intestine. Relative gene expression analysis in the small intestine of (a) Toll-like receptors (TLR), (b) mucins and (c) Tight Junctions (TJ) proteins and (d) immunological proteins. Relative gene expression was calculated with respect to REF, which corresponded to 100 % of transcription (represented with a horizontal dotted line). Statistical differences: *$p < 0.05$ vs REF (n=11-16). *TLR*, Toll-like receptor; *Muc*, mucin; *Zo-1*, zonula occludens-1; *Cldn4*, claudin 4; *Ocln*, occludin; *IgA*, immunoglobulin A; *Blimp1*, B lymphocyte-induced maturation protein-1; *FcRn*, neonatal Fc receptor.

### 3.3. Small and large intestinal immunity Ig profile

First, the analysis of the SI was completed by studying the Ig composition of the GW (Figure 4). The SYN group exhibited higher sIgA levels in the intestine at the end of lactation than those in the REF group, whereas IgM remained unaffected (Figure 4a-b).

Second, the analysis of the cecal composition was performed (Figure 4). Differentially to that found in the SI, the total levels of cecal sIgA were not modulated with the maternal nutritional intervention (Figure 4c). As cecal Igs bind to cecal bacteria to promote its neutralization and elimination [38], the number of total bacteria and the proportion of Ig-CB in the CC were also evaluated. No changes were observed neither in the total bacteria nor in the relative Ig-CB. However, a clear tendency toward an increase ($p = 0.06$) of the total Ig-CB was found in the SYN group (Figure 4d).

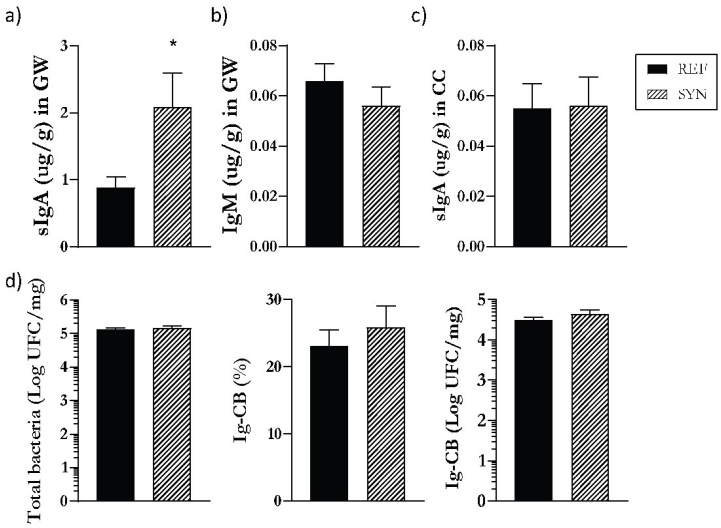


**Figure 4**. Impact of maternal supplementation at the end of the lactation on the gastrointestinal tract of pups. Quantification of (a) IgA and (b) IgM in the gut wash and (c) IgA in the cecum. (d) Analysis of cecal bacteria composition counts of total bacteria, proportion of Ig-CB, total Ig-CB. Data are expressed as mean ± S.E.M. Statistical differences: $^{*}p < 0.05$ vs REF (n=11-16).

### 3.4. Microbial SCFAs production

The main mechanism of communication between the cecal microbiota and the immune system is through the production of SCFAs. Thus, the quantification of the SCFAs levels serves as an indicator of microbiota functionality [39]. Even though SCFAs predominate in the cecum, the intestinal levels were also analyzed (Figure 5). The composition of the IC was evaluated and although no changes were observed in the total amount of SCFAs, a reduction in the acetic, isobutanoic, butanoic and hexanoic acids was observed at the end of the study in the SYN group (Figure 5a). In contrast, in the cecum, the total production of the SCFAs increased at weaning in the SYN group, and this increase was mainly due to an increase in the acetic, propanoic, and butanoic acids (Figure 5b).

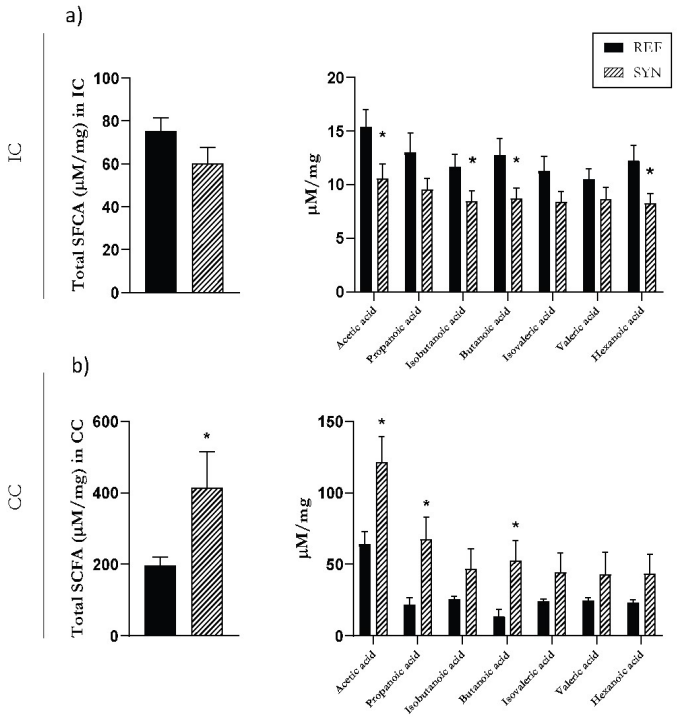


**Figure 5**. Short-chain fatty acids (SCFAs) production in the pups' rat gastrointestinal tract. (a) SCFAs in intestine content (IC) and (b) in cecal content (CC). Data are expressed as mean ± S.E.M. Statistical differences: $^{*}p < 0.05$ vs REF (n=11-16).

### 3.5. Small intestinal and cecal microbiota

The analysis of the microbiota composition was performed in terms of phylum, family, and genus (Figure 6 and Figure 7).

Alpha diversity and beta-diversity analysis showed significant differences depending on the sample type (intestine *vs.* cecum) and depending on the intervention (REF *vs.* SYN). In detail, regarding the alpha-diversity metrics, cecum showed significantly higher microbial diversity (Shannon index) and richness (Chao1 index) compared to the intestine. SYN increased significantly the microbial richness (Chao1) and diversity (Shannon) in CC ($p$=0.0053 and $p$= 0.015, respectively) however no effects were found in the small intestine (CI, p= 0.829 and $p$=0.413, respectively) (Figure 6a-b).

Beta-diversity showed two general distinct microbial clusters depending on sample-type CC vs IC, accounting for 54.4 % of the variation (PERMANOVA test F-value: 14.0; R-squared: 0.50; $p$=0.001) (Figure 6c). In addition, when groups were compared considering the intervention, we observed a significant impact of the SYN supplementation on the cecum (PERMANOVA test F-value: 4.1159; R-squared: 0.164; $p$=0.001) and on the intestine (PERMANOVA] F-value: 2.69; R-squared: 0.113; $p$=0.013) (Supplementary Figure 1). DESEq tests showed the differential presence of specific microbial genera. An enrichment in *Bifidobacterium* genus (FDR $p$<0.001), *Faecalibaculum* (FDR $p$<0.001), *Lactobacillus* (FDR $p$=0.027) and *Turicibacter* genus (FDR $p$=0.036) was observed after the SYN intervention in

CI. In CC, the SYN intervention also increased the *Prevotellaceae*_UCG_001 (FDR $p<0.001$). LEfSe test also demonstrated the role of those these microbial genera depending on SYN intervention (Figure 6d).

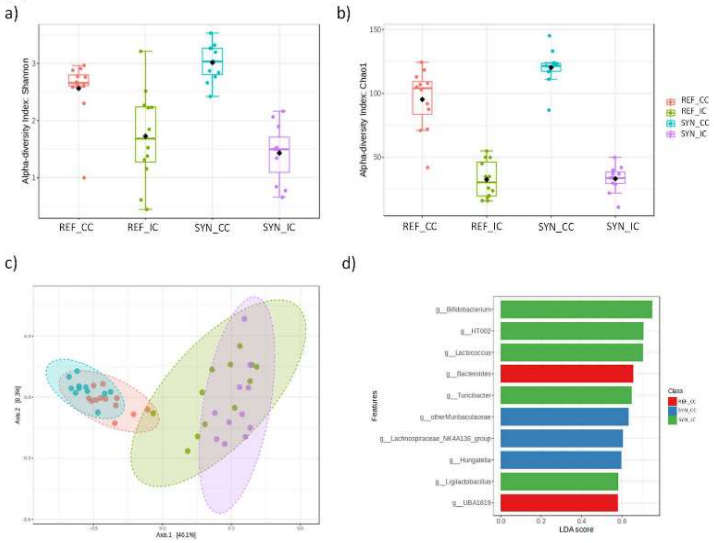


**Figure 6.** Alpha-diversity indexes (a) (Shannon index) and (b) richness (Chao1 index) for CC and IC. (c) Beta-diversity in cecal content (CC) and intestinal content (IC) microbiota depending on SYN intervention. (d) Linear Discriminant Analysis (LDA) Effect Size (LEfSe) plot of taxonomic genera identified in CC and CI. Statistical testing was performed by PERMANOVA using Bray Curtis distances and the Mann-Whitney test was used for alpha-diversity indexes. Statistical differences: $^{*}p< 0.05$ vs REF; $^{a}p< 0.1$ vs REF (n=5-6).

Regarding bacterial proportions, in the intestine, the most abundant phylum was Firmicutes (mainly characterized by *Escherichia Shigella* and *Ligilactobacillus*) in the REF group. However, in the SYN group, the supplementation induced an increase in the Actinobacteria phylum (mainly by *Bifidobacterium* genus) (Figure 7a). Evaluating the family abundance, *Bifidobacteriaceae* proportion increased up to a 35 % in the SYN ($p$=0.261) group compared to the REF (Figure 7c). The analysis of the genus proportion confirmed the family results, showing higher proportions of *Bifidobacterium* genus at weaning in the SYN group (Figure 7e).

In the cecum, Firmicutes and Desulfobacterota were the most abundant phyla and, the Firmicutes increased significantly after the SYN group (Figure 7b). The family analysis revealed that maternal supplementation modified the composition of the offspring CC and, *Bifidobacteria* were only present in the SYN group and *Lanchospiracea* and *Muribacteriaceae* proportions were increased (Figure 7d). The genus analysis revealed that *Bifidobacterium* and *Faecalibaculum* were only detected in the SYN group, and *Muribaculaceae* was increased after the maternal nutritional intervention (Figure 7f). Additionally, the *B. breve* M-16V was detected by qPCR in the CC of the SYN group at ~ $3.5 * 10^{9}$ UFC/mg at the end of suckling. However, at the middle of lactation, the probiotic was detected only in a 30 % of the analyzed fecal samples and at very low levels (>$10^{7}$ UFC/mg).

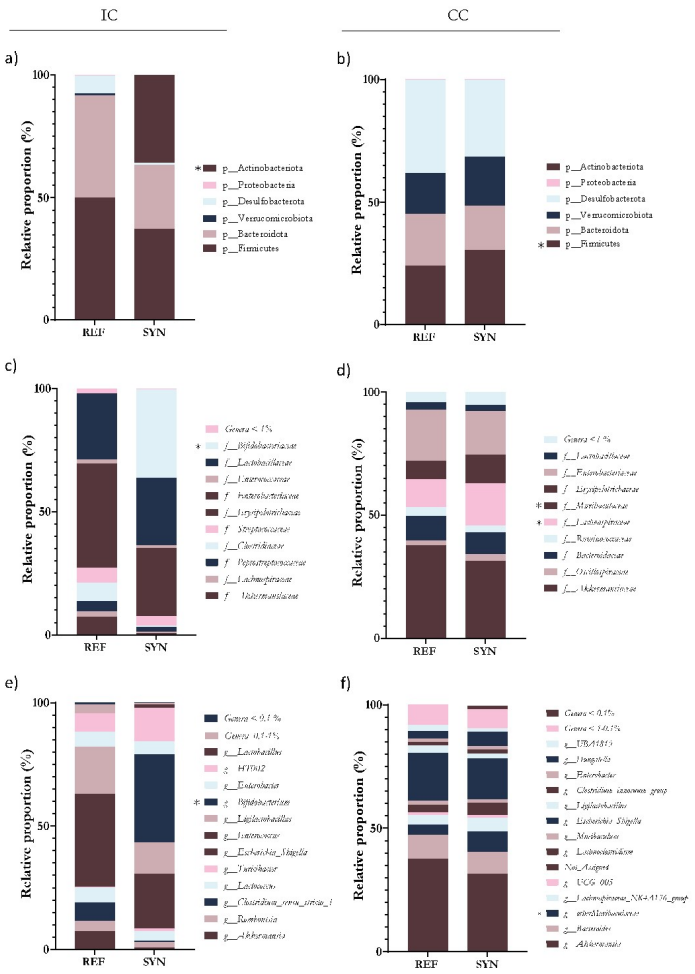


**Figure 7**. Microbiota analysis of the small intestinal content (IC) and cecum content (CC). Relative bacterial proportions at phylum level in (a) IC and (b) CC. Relative proportions of IC microbiota at family level in (c) IC and (d) CC. Relative proportions of IC microbiota at genus level in (e) IC and (f) CC. Results are expressed as relative proportions for each population. Statistical differences: *$p$< 0.05 vs REF; #$p$< 0.1 vs REF (n=5-6).

### 3.6. Lymphocyte populations

The synbiotic supplementation to dams affects the infant's gastrointestinal tract during lactation, potentially influencing lymphocytic populations at intestinal level, but also spreading to the systemic compartment.

At intestinal level, the MLN cells/populations were studied, detailed data are shown in Table S3. The proportion of B cells accounted for ~20 % whereas the T cells were ~70 %.

None of them were affected at the weaning day due to the synbiotic intervention. The T cells subsets, T CD4+ and T CD8+, were around ~ 47 % and ~ 17 %, respectively. The NK cell proportion was higher in the SYN group at the end of suckling with respect to the REF group whereas NKT were not affected. Additionally, two migration markers (CD62L+ and αE+) were assessed in the total lymphocyte subsets, in B cells, in T CD4+ cells and in T CD8+ cells (Figure 8). The results indicated that they were not altered with the maternal synbiotic supplementation.

To enrich the analysis of the lymphocyte populations, the evaluation of the lymphocytes was done also in the spleen, a secondary lymphoid tissue representative of systemic immune system (Table S3). B cells and T cells in this tissue constituted equally approximately 30 % of the lymphocytes, and none of these populations were affected by maternal supplementation. When all lymphocytes are analyzed, including both B and T populations, the proportion of cells expressing f CD62L+ was higher in SYN than in REF group in the spleen (Figure 8a)Regarding B cells, neither activation levels (CD25+) nor the expression of migration markers (CD62L+ and αE+) were altered in the SYN group (Figure 8b). In terms of T cells, the relative proportion of T CD4+ or T CD8+ was not affected. However, the expression of the CD62L selectin was lower in the T CD4+ population and higher in the T CD8+ from the SYN animals than in the REF ones (Figure 8c-d). Additionally, the proportion of αE+ in T CD8+ from SYN animals was lower than that in REF group.The NK and NKT population percentages were not modified either.

In addition, the analysis of the blood cell composition was done (Supplementary Table S4) and no significant changes were observed in leukocyte counts, erythrocytes or platelets.

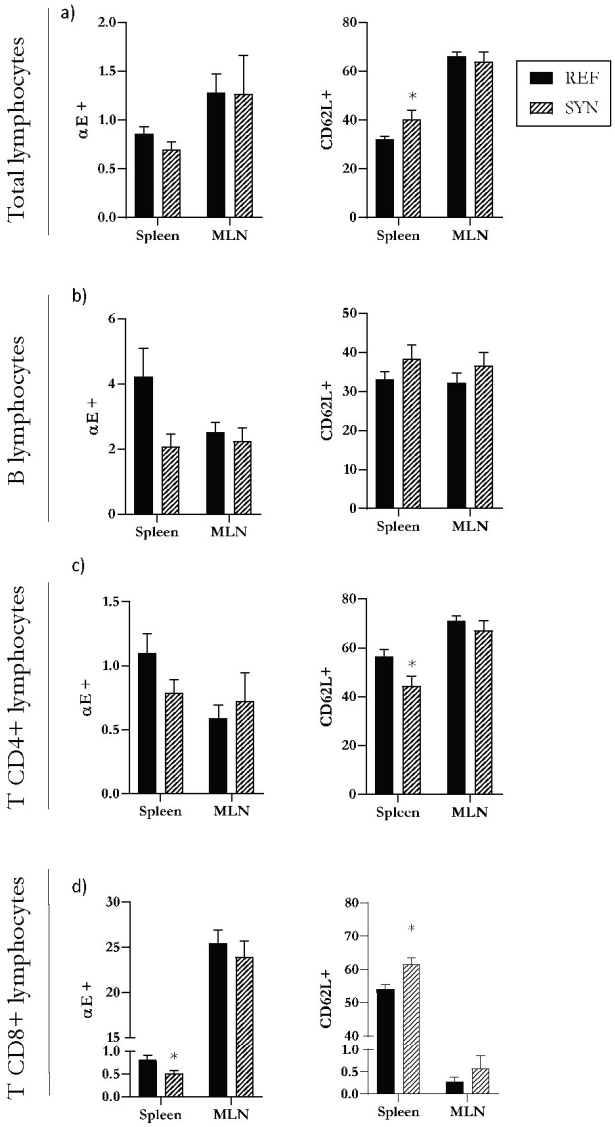


**Figure 8.** Analysis of the expression of migration markers CD62L and αE in different lymphocytes subsets in the spleen and mesenteric lymph nodes (MLN). Evaluation of the expression of CD62L and αE in the total lymphocytes (a), in B cell lymphocytes (b), in T CD4+ lymphocytes (c) and T CD8+ lymphocytes (d). Data are expressed as mean ± S.E.M. Statistical differences: *$p < 0.05$ vs REF (n=11-16).

### 3.7. Immunoglobulin profile in different tissues

Igs levels, as valuable markers of the immunological status [40], were assessed at the end of suckling in systemic and mucosal compartments (Figure 9). Total levels of IgM, IgA, and IgG in plasma, SG, and MLN were unaffected by maternal supplementation (Figure 9a-c). However, there were variations in the relative proportions of IgG subtypes. Notably, IgG2c showed a higher proportion in SYN compared to REF animals in all three compartments (Figure 9d-f). The Th1/Th2 ratio was evaluated using the levels of the IgG2b and IgG2c (representing Th1) and IgG1 and IgG2a (representing Th2). Overall, the synbiotic nutritional intervention did not affect the Th1/Th2 balance in the offspring (Figure 9g-i). After assessing Ig levels in each compartment, NMDS graphs were generated to identify distinct clusters based on global Ig profiles (Figure 9j-l). In this study, maternal synbiotic supplementation did not lead to the differentiation of clusters in any of the evaluated compartments.

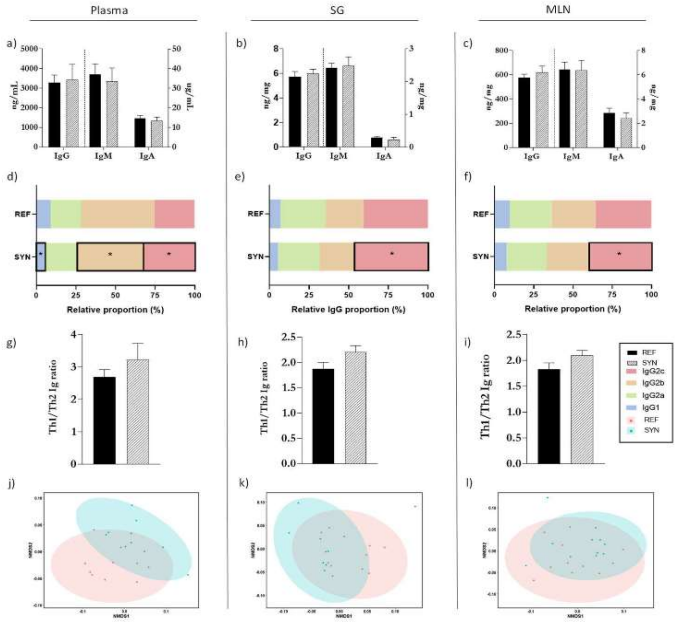


**Figure 9.** Immunoglobulin quantification in pups at day 21 of life in different compartments. Total Igs levels (IgA, IgM, and IgG) in (a) plasma, (b) SG, and (c) MLN. Relative proportion of IgG subtypes in (d) plasma, (e) SG and (f) MLN. Analysis of the Th1/Th2 ratio in plasma (g), SG (h) and MLN (i). Analysis of non-parametric multidimensional scaling (NMDS) for the Ig profiles based on the Bray-Curtis distance in (j) plasma, (k) SG and (l) MLN. Data (a-i) are expressed as mean ± S.E.M. Each point in NMDS (j-l) represents an animal (c) by ANOSIM test. Statistical differences: '$p < 0.05$ vs REF (n=11-16).

### 3.8. Adipose tissue (AT) analysis

The impact of the maternal supplementation was also evaluated on the AT of their descendants at the end of suckling (Figure 10). The nutritional intervention during

gestation and lactation did not affect the relative AT weight of the pups (Figure 10a). Representative images of histologic sections of WAT and BAT are shown in Figure 10b. The quantification of the number of adipocytes and their area were not modified in the WAT tissue (Figure 10c). Likewise, the number of nuclei and the area of the lipid droplets in the BAT were not altered. However, the big lipid droplets, considered as those larger than 50 μm² were more abundant in the BAT of the SYN animals than in the REF ones (Figure 10d).

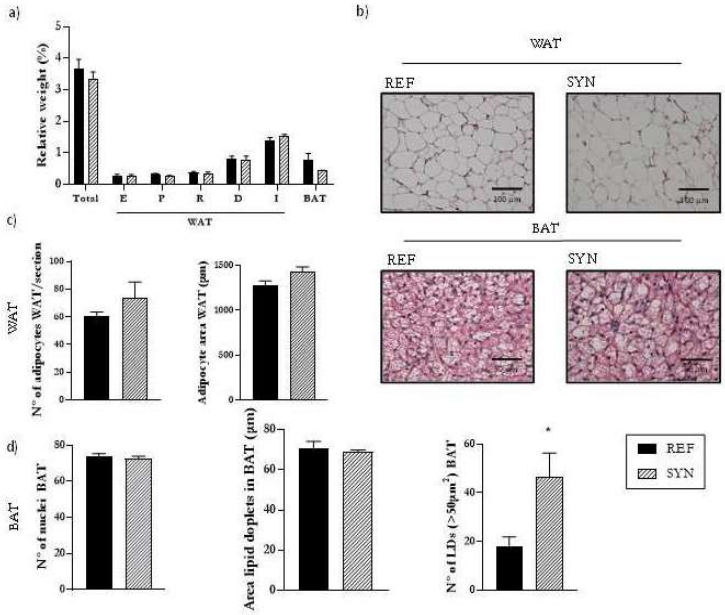


**Figure 10.** Influence of maternal supplementation on AT. (a) Relative weight of the adipose tissue from different locations. (b) Histological analysis of parametric WAT (P-WAT) and BAT in hematoxylin and eosin-stained sections. Images were captured at 20x and 40x magnification, respectively. (c) Analysis of WAT, epididymal or parametric, for males or females respectively were chosen as representative of the WAT: adipocyte area and number of adipocytes. (d) Analysis of BAT: number of nuclei, area of lipid droplets (LD), and number of LD (> 50 μm²). Data are expressed as mean ± S.E.M. Statistical differences: *$p < 0.05$ vs REF (n=11-16). WAT, white adipose tissue; BAT, brown adipose tissue; E, epididymal; P, parametric; R, retroperitoneal; D, dorsal; I, Inguinal.

## 4. Discussion

Good nutritional habits during pregnancy and breastfeeding present an opportunity to improve the health of both mother and child [41–44]. Among the maternal environmental factors, diet before conception and during pregnancy and breastfeeding has been linked to the modulation of breast milk composition, which in turn influences the immunological status of both the mother and offspring [45–47]. Concretely, prebiotic supplementation during pregnancy and lactation has been targeted as a potential strategy to boost both maternal and offspring immune system and microbiota composition [20].

However, there is very little information available about the potential impact of maternal supplementation with probiotics, prebiotics, or their combination to mothers and their infants during gestation and lactation on the overall infant development. Our results

clearly indicate that a supplementation of maternal diet with *Bifidobacterium breve* M-16V and scGOS/lcFOS during gestation and lactation improves the mucosal immune system and microbiota composition of the neonate at the end of suckling. The observed effects may be due to the direct impact of these components on several maternal variables by strengthening the intestinal barrier, improving the microbial composition and promoting an improved immune response. All these effects could be derived by indirect effects on the pups during gestation through placenta or breast milk (i.e. by higher metabolites or Ig transfer). This indirect beneficial effect on the offspring was previously confirmed when a probiotic was administered to the mothers [20].

To assess the influence of the maternal synbiotic supplementation on offspring growth, we initially examined its effects on macroscopic and morphologic variables. As the daily growth pattern was similar between the groups, it can be suggested that the maternal synbiotic supplementation did not affect the overall growth until weaning. In line with this, *Rigo-Adrover et al*. did not find any differences on the body weight evolution when the same synbiotic was administered directly to the pups during early life [23]. In the present study, the analysis of growth-related parameters and the relative organ weights confirmed that maternal synbiotic supplementation did not have an effect. However, a trophic effect in the SI by its increasing weight and length was observed, suggesting an increase of the intestinal surface, and favoring the absorption capacity of nutrients of the pups [48]. Similar results were found when the scGOS/lsFOS was administered to rotavirus infected pups in early life [49].

After evaluating the macroscopic effects of the SYN supplementation on the SI, we proceeded to analyze its impact at microscopic level to validate the observed trophic effect. Our findings indicated that, while both the length and weight of the SI increased, most of the microscopic structures remained unaffected (including the villi height and area, the crypts depth, the villi height and the crypt depth ratio and the amount of goblet cells). Only a slight reduction in villi width was observed at weaning. The SI acts as a barrier between the external environment and the host. Its primary function is nutrient absorption, whereas the villi and microvilli of the intestine optimize this absorption [50]. For this reason, an increase of the area of villi and microvilli structures is expected to enhance nutrient absorption. The supplementation with probiotics and prebiotics has been associated with positive effects on the homeostasis and integrity of the gastrointestinal tract. Many studies have analyzed the impact of the biotics on the intestinal structures. Most of them were focused on the direct administration of the pro-, pre- or synbiotics [22,51,52]. However, few studies have focused on the effect of maternal supplementation on the offspring intestine. *Wang K et al*. supplemented pregnant pigs during the gestation with a synbiotic mix (*Lactiplantibacillus Plantarum*, *Saccharomyces cerevisiae* and xylo-oligosaccharides), and they observed that the maternal supplementation influenced the intestinal structures of the offspring by increasing the villi height [53]. Our results are not in accordance with these data; however, it needs to be taken under consideration the host difference and that the effect of the synbiotics are dose and strain dependent.

In addition to the analysis of morphological structures, we evaluated the effect of maternal synbiotic supplementation on the gene expression levels of relevant intestinal markers, including TLRs, mucins, TJ proteins and maturation genes. The synbiotic supplementation induced higher *Tlr9, Muc2, IgA* and *Blimp1* gene expression. *Tlr9* plays a pivotal role in innate immunity, safeguarding internal homeostasis against danger signals. [54]. *Muc2* is the main constituent of the small intestine's mucus layer, offering protection to the intestinal tract against self-digestion and facilitating nutrient retention for efficient nutritional uptake [55,56]. Previous studies have demonstrated that synbiotic pups' supplementation with a *Bifidobacterium* strain and 2'-Fucosyllactose also increased *Muc2* gene expression in the intestine [57]. In healthy conditions an increase of sIgA is considered as a positive outcome as sIgA interacts with pathogenic organisms, preventing their penetration into the intestinal barrier [58]. The enhancement of this sIgA agrees with the one described by *Wang K et al.* who demonstrated that a maternal synbiotic

supplementation in pigs during gestation and lactation also increased the intestinal sIgA of the offspring [53]. Finally, *Blimp1* maturation marker is expressed during fetal and neonatal periods, and its expression decreases at weaning [59]. However, our results did not reveal such decrease at the end of suckling. In general, our results suggest that the maternal synbiotic supplementation induced a positive effect on the SI by increasing the expression of *Tlr9*, *Muc2* and *IgA*. These genes will contribute to maintain the intestinal homeostasis and to avoid pathogen colonization. Nonetheless, further studies are needed to evaluate the impact of maternal nutrition intervention on the intestinal maturation and reveal the mechanism that leads to this increase in the supplemented mothers.

To corroborate the intestinal gene expression, the sIgA and IgM protein levels was assessed. According to the RT-PCR results, sIgA was increased in the SYN pups, suggesting higher pathogen neutralization capacity [58]. Intestinal IgM interacts with different antigens by opsonizing (coating) them, favoring their destruction. Interestingly, we did not detect any significant changes in intestinal IgM levels following synbiotic supplementation. These results remained consistent even after the SYN mix was administered directly to pups, as described in previous studies [23].

The importance of cecal immune composition has been rising in the last decade due to the established link between the microbiota and the immune system. Within the cecum, sIgA plays a crucial role by coating cecal bacteria and participating in their neutralization to facilitate their subsequent elimination [38]. Maternal synbiotic supplementation did not exert any effect on the total sIgA nor in the Ig-CB at the end of suckling in this compartment. Ongoing studies on the impact of such mixture on the dams allow to observe that this change observed in the pups was also found at maternal level, and then, somehow also transferred to the pups.

Besides the immune observations at intestinal level, the microbiota composition and activity were also modulated by the maternal supplementation. Despite observing no changes in the total amount of bacteria in the cecum, we examined the proportion of the different microbiota proportions in the intestine and cecum. Notably, the SYN group exhibited higher proportions of *Bifidobacterium* in the intestinal compartment, with the detection of *Bifidobacterium* and *Faecalibaculum* exclusively in the cecum. *Bifidobacterium spp.*, particularly *B. breve*, *B. bifidum*, and *B. longum subsp. infantis*, are predominant, known for their positive impact on infant development, including immune system maturation [60–62]. *Faecalibaculum spp.* are novel species of potential beneficial microorganism productors of SCFAs. Additionally, *Facecalibaculum* contributes to reduce the colon inflammation by stimulating Treg cells [63]. These results suggest that synbiotic maternal supplementation during gestation and lactation boosts the colonization of the offspring gut during suckling.

Thus, although the supplemented probiotic strain *B. breve* M-16Vused was not detected in the supplemented BM from supplemented dams nor in all the feces at the middle lactation, it was detected in the CC of the SYN pups at the end of suckling. It is noteworthy that some days before day 21 of life, the pups had already initiated chewing. Consequently, the SYN pups might chew the maternal feces acquiring the administered probiotic, explaining why the strain is detected in pups at day 21, but not in pups at the middle of lactation. It's important to highlight that *B. breve* M-16V was not detected in the REF group in the feces nor in the CC. Similar data were found when a probiotic (*L. fermentum* CECT5716) was supplemented to the dams, only some samples of the offspring were strain positive at the middle lactation [20]. These data can not confirm that maternal synbiotic supplementation may be transferred to the offspring through their mother's milk, as it was not detected after analysis, but it cannot be discarded either and require further studies focused on the transmission pathway.

SCFAs are recognized as mediators of communication between the intestinal microbiota and the immune system. The major SCFAs include formic, acetic, propionic and butyric acids [64]. Our findings indicated that maternal synbiotic supplementation induced an increase of the total SCFAs in the offspring cecum. This rise was linked to higher levels

of acetic, propanoic and butanoic acids. In the SI, some of the SCFAs were lower although the total levels were not affected. However, it must be considered that the production of SCFAs is mainly linked to the cecum. The production of each specific acid is linked to different bacterial groups [39]. Butyric acid is known for its antioxidant and anti-inflammatory properties, contributing to the maintenance of digestive and immune homeostasis [65]. Additionally, an increase in butyric acid has been linked with an increase of the intestinal *Muc2* gene expression via the selective acetylation/methylation of histones. This observation agrees with our results where maternal synbiotic supplementation induced an increase of the butyric acid and the intestinal *Muc2* gene expression in the offspring [66]. Acetic acid production is linked to the protection of epithelial cells and the promotion of probiotic bacteria growth [67], and it is positively correlated with fecal sIgA [68]. Similar results were obtained in our study, where the maternal synbiotic supplementation increased the acetic acid in the cecum and the intestinal IgA in the offspring during pregnancy. Propionic acid has been associated with anti-proliferative effects and improvements in lipid metabolism and serves as a precursor of gluconeogenesis in the liver [69–71]. Formic acid has been less studied, but it is known to be linked to methanogenesis and inflammation [72,73]. Numerous studies have consistently shown that probiotic supplementation provokes an increase in the production of SCFA [39,74–76]. Joining the microbiota and the SCFAs results in the IC, it is important to focus on the reduction of some of the SCFAs, and it can be associated to the reduced bacterial diversity on the IC. However, in the CC, the increase of the cecal butyrate, acetate and propionate can be linked to the increase of the Firmicutes phylum on the CC, as they are the main producers of these acids [77]. All this data supports our hypothesis that the maternal synbiotic supplementation during gestation and lactation positively impacts the infant cecum microbiota and its SCFAs production due to the observed increase the end of suckling. In the future, more research is needed to investigate whether higher levels of SCFAs derived from the synbiotic supplementation are transferred to the fetus through the placenta.

Regarding immunity, the influence of maternal synbiotic supplementation extended beyond the biomolecular level, affecting various lymphocyte subset populations in both mucosal (MLN) and systemic (spleen) compartments. In general, maternal synbiotic supplementation did not exert great changes on those lymphocytes' populations. It can be highlighted that in the spleen, an increase in the migration marker CD62L was observed at weaning due to the synbiotic supplementation. CD62L is a migration marker linked to cells that are ready to migrate to the intestinal compartment [78]. Little information is known about the impact of diet supplementation on lymphocyte subsets populations. Only a few studies are available; in those cases, the supplementation was typically administered directly to the animals rather than being provided as maternal supplementation [79]. Thus, it is a new unexplored field that should be addressed in the future, as it can elegantly show the potential of the maternal diet on the lymphocyte cell numbers and even activity.

To complete the analysis, the Ig profile of different samples was performed. The impact of pro-, pre- and synbiotics on the Ig production has been documented [80]. Our results indicated that maternal synbiotic intervention, administered both during gestation and lactation, did not lead to significant modifications in the IgA, IgM and IgG isotypes in offspring plasma, SG or MLN. However, the IgG subtypes proportions in pups were modulated after the maternal nutritional intervention. Mainly, the IgG2c levels were increased in the plasma, SG and MLN samples. Whereas limited information is available regarding the role of IgG2c in rats, in mice, its analog IgG3 has been associated with regulatory responses in the intestine and is involved in long-term immunity [81,82]. The observed rise in IgG2c levels is consistent with previous findings in which increased IgG2c levels were documented in both the milk and plasma of dams following the administration of the same SYN mix (unpublished results).

Finally, the impact of the maternal synbiotic supplementation on the morphology of the adipose tissue was performed. In recent years, there has been more and more research

focused on the influence of the maternal nutrition during pregnancy on the offsprings' adipose tissue [83,84]. However, there is a lack of information on the influence of synbiotic supplementation during pregnancy and lactation. Our results suggest that maternal nutritional intervention did not modulate the adipose tissue metabolism; only a punctual rise in high size lipid droplets of the BAT was observed. *Lukaszewski MA et al.* found that the maternal dietary pattern influences the adipose tissue programming in the offspring. However, they did not review the impact of synbiotic supplementations [85]. Therefore, further studies are needed.

Maternal supplementation with *Bifidobacterium breve* M-16V and scGOS/lcFOS on the offspring exerts positive effects on the offspring, however, there are some limitations to this study. Specifically, there is a need to further determine the relative contribution of gestation and lactation periods to the immune maturation of the pups. Additionally, while the health benefits of the synbiotics are highly documented, future research should aim to elucidate whether *B. breve* M-16V or scGOS/lcFOS plays a more pivotal role in immune system modulation. Furthermore, the study did not explore all possible mechanisms underlying immune system modulation. In addition, although the *in vivo* studies in rats contribute to understanding the mechanisms in humans, in some cases, the results' integration may result difficult. And finally, the study has been focused on physiological conditions of a healthy pregnancy and normal development of the offspring. Now, with the effects found in the pups after the maternal supplementation established under healthy conditions of the mother and the offspring, a future approach could involve investigating the synbiotic impact in pathological or infectious conditions in both the mother and the offspring.

## 5. Conclusions

Maternal synbiotic supplementation during both gestation and lactation exerts a positive influence on the infant's immune system development. The main remarkable effects are observed within the gastrointestinal tract, in which maternal supplementation enhances the integrity and function of the intestinal barrier and microbiota composition. Thus, this strategy appears to be a useful tool to improve the mucosal immunity and the intestinal barrier of the offspring. Nevertheless, further research is necessary to assess the long-term implications of maternal synbiotic supplementation during pregnancy and lactation on both, mothers and offspring.

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**Data Availability Statement:** The Illumina sequencing raw data was uploaded to the NCBI database. 711 712

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**Supplementary Materials**

**Supplementary Table 1.** Description of the specific TaqMan primers AB

| Gene | Reference |
|---|---|
| *Tlr2* | Rn02133647_s1, I |
| *Tlr3* | Rn01488472_g1, I |
| *Tlr4* | Rn00569848_m1, I |
| *Tlr5* | Rn04219239_s1, I |
| *Tlr7* | Rn01771083_s1, I |
| *Tlr9* | Rn01640054_m1, I |
| *Muc2* | Rn01498206_m1, I |
| *Muc3* | Rn01481134_m1, I |
| *Ocln* | Rn00580064_m1, I |
| *Cldn2* | Rn02063575_s1, I |
| *Cldn4* | Rn01196224_s1, I |
| *Tjp1* | Rn02116071_s1, I, encoding for Zo1 |
| *IgA* | 331943, made to order |
| *Prdm1* | Rn03416161_m1, I, encoding for Blimp1 |
| *Fcgrt* | Rn00566655_m1, I, encoding for FcRn |
| *Gusb* | Rn00566655_m1, I |

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**Supplementary Table 2.** Growth-associated parameters and organ's size at the weaning day.

| **Body size** | **REF** | **SYN** |
|---|---|---|
| Body lenta (cm) | 18.67 ± 0.14 | 18.27 ± 0.22 |
| Body/tail length ratio | 1.57 ± 0.02 | 1.62 ± 0.02 |
| BMI ($g/cm^2$) | 0.35 ± 0.01 | 0.34 ± 0.01 |
| Lee index ($g^{0.33}/cm \times 10^3$) | 317.47 ± 5.76 | 314.97 ± 6.72 |

| **Relative organ size** | **REF** | **SYN** |
|---|---|---|
| Spleen (%) | 0.37 ± 0.02 | 0.35 ± 0.01 |
| Thymus (%) | 0.50 ± 0.01 | 0.52 ± 0.01 |
| Kidney (%) | 3.65 ± 0.11 | 3.63 ± 0.15 |
| Heart (%) | 0.62 ± 0.01 | 0.61 ± 0.02 |
| Liver (%) | 0.60 ± 0.02 | 0.60 ± 0.03 |
| Salivary gland (%) | 0.15 ± 0.01 | 0.15 ± 0.02 |
| Stomach (%) | 0.69 ± 0.05 | 0.69 ± 0.03 |
| Caecum (%) | 0.41 ± 0.02 | 0.30 ± 0.02* |
| Small intestine (%) | 3.40 ± 0.08 | 4.12 ± 0.24* |
| Small intestine length (cm) | 79.66 ± 1.87 | 88.79 ± 3.10* |

Data are expressed as mean ± S.E.M. (n=11-16). Statistical differences: *$p < 0.05$ vs REF.

**Supplementary Table 3.** Effect of maternal synbiotic supplementation on the spleen and MLN lymphocyte population at weaning.

| | Spleen | | MLN | |
|---|---|---|---|---|
| | REF | SYN | REF | SYN |
| B cells (CD45RA+) | 35.63 ± 1.47 | 31.89 ± 1.06 | 21.43 ± 1.75 | 23.02 ± 3.18 |
| % CD25+ | 12.08 ± 1.10 | 13.00 ± 1.45 | 5.25 ± 0.37 | 4.72 ± 0.71 |
| T cells (TCRαβ/TCRγδ) | 26.38 ± 1.50 | 30.33 ± 4.31 | 73.67 ± 2.03 | 71.47 ± 2.89 |
| TCRαβ+ NK- | 28.06 ± 3.61 | 28.19 ± 2.75 | 69.96 ± 1.99 | 68.33 ± 3.02 |
| % CD8+ | 69.94 ± 0.91 | 65.95 ± 1.14* | 75.48 ± 0.48 | 76.35 ± 0.65 |
| TCRγδ+ | 2.72 ± 0.38 | 3.53 ± 0.30 | 3.71 ± 0.33 | 3.14 ± 0.46 |
| % CD8+ | 2.27 ± 0.12 | 2.65 ± 0.27 | 2.13 ± 0.12 | 1.92 ± 0.20 |
| CD4+ CD8- | 25.27 ± 2.97 | 29.20 ± 2.58 | 52.24 ±2.11 | 46.51 ± 4.49 |
| % CD25+ | 6.19 ± 0.59 | 5.39 ± 0.84 | 11.47 ± 0.57 | 13.30 ± 2.28 |
| CD8+ CD4- | 12.4 ± 0.61 | 13.01 ± 1.06 | 17.77 ± 0.69 | 16.59 ± 1.51 |
| % CD25+ | 6.2 ± 1.24 | 6.93 ± 2.70 | 7.99 ± 0.60 | 8.41 ± 1.57 |
| CD4+ CD8+ | 2.86 ± 0.22 | 4.01 ± 0.50* | 2.26 ± 0.24 | 2.75 ± 0.49 |
| % CD25+ | 6.20 ± 1.34 | 4.40 ± 1.08 | 41.39 ± 3.32 | 44.43 ± 7.99 |
| NK (TCRαβ- NK+) | 2.97 ± 0.34 | 3.74 ± 0.46 | 1.22 ± 0.13 | 1.75 ± 0.23* |
| % CD8+ | 21.13 ± 2.16 | 22.62 ± 2.15 | 11.99 ± 1.66 | 10.68 ± 1.85 |
| NKT (TCRαβ+ NK+) | 3.06 ± 0.13 | 3.30 ± 0.35 | 1.95 ± 0.12 | 2.20 ± 0.12 |
| % CD8+ | 72.64 ± 3.01 | 78.65 ± 10.6 | 54.64 ± 1.36 | 46.00 ± 2.95* |

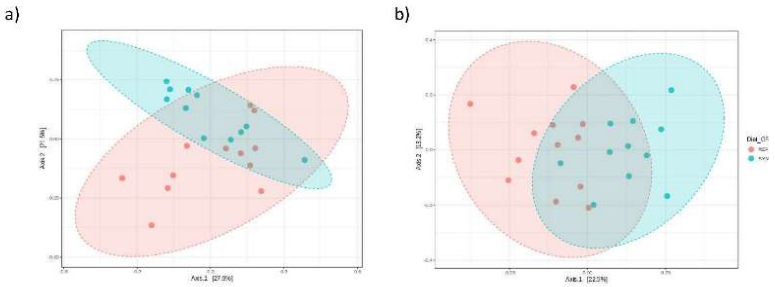
Data are expressed as mean ± S.E.M. Statistical differences: *$p < 0.05$ vs REF (n=11-16).

**Supplementary Table 4.** Haematological variables at day 21 of pups' life.

| Haematological variable | REF | SYN |
|---|---|---|
| Leucocytes (x$10^9$ /L) | 2.95 ± 0.59 | 4.36 ± 1.01* |
| Lymphocytes (%) | 69.81 ± 1.79 | 69.20 ± 2.50 |
| Monocytes (%) | 5.39 ± 0.58 | 5.76 ± 0.74 |
| Granulocytes (%) | 24.80 ± 1.50 | 25.04 ± 1.92 |
| Lymphocytes (x$10^9$ /L) | 1.58 ± 0.10 | 2.94 ± 0.71# |
| Monocytes (x$10^9$ /L) | 0.13 ± 0.03 | 0.23 ± 0.09 |
| Granulocytes (x$10^9$ /L) | 0.88 ± 0.23 | 1.20 ± 0.28 |
| Erythrocytes (x$10^{12}$ /L) | 4.25 ± 0.08 | 4.10 ± 0.32 |
| HGB (g/L) | 77.93 ± 1.49 | 151.82 ± 86.52 |
| HCT (%) | 23.11 ± 0.58 | 22.28 ± 1.75 |
| VCM (fL) | 54.49 ± 0.62 | 54.64 ± 0.87 |
| MCH (pg) | 18.31 ± 0.22 | 18.42 ± 0.28 |

Data are expressed as mean ± S.E.M (n=11-16). Statistical differences: *$p < 0.05$ vs REF. HGB, Hemoglobin; HCT, Hematocrit; MCV, Mean Cell Volume; MCH, Mean Cell Hemoglobin.

**Supplementary Figure 1.**




**Supplementary Figure 1.** Analysis of non-parametric multidimensional scaling (NMDS) for the microbiota profiles based on the Bray-Curtis distance in (a) IC and (b) CC. Each point in NMDS represents an animal by ANOSIM test. Statistical differences: *$p < 0.05$ vs REF (n=11-16).

# ARTICLE 5

## "Enhancing gastrointestinal immunity and microbial diversity in young rats by *Bifidobacterium breve* M-16V and scGOS/lcFOS supplementation in early life"

Sáez-Fuertes, Laura; Kapravelou, Garyfallia; Grases-Pintó, Blanca; Bernabeu, Manuel; Knipping, Karen; Garssen, Johan; Castell, Margarida; Rodríguez-Lagunas, María José; Collado, María Carmen; Pérez-Cano, Francisco José

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- **VI workshop Anual INSA-UB** Barcelona (February 2022). **Sáez-Fuertes, L.**; Rio-Aige, K.; Grases-Pintó, B.; Melero, C.; Galindo S.; Cabré, P.; Gironés, A.; Zhan S.; Castell, M.; Collado, M.C.; Rodríguez-Lagunas, M.J.; Pérez-Cano, F.J. "Impact of a synbiotic administration during gestation and lactation to dams in the offspring's development".
- **XV Workshop de la Sociedad Española de Microbiota, Probióticos y Prebióticos (SEMiPyP).** Sevilla (February 2024). **Sáez-Fuertes, L.**; Rio-Aige, K.; Grases-Pintó, B.; Castell, M.; Collado, M.C.; Rodríguez-Lagunas, M.J.; Pérez-Cano, F.J. "Efectos de la suplementación materna de un simbiótico en la microbiota de la madre y de su descendencia y bajo la infección por rotavirus".

## Abstract

**Aim**: This study explored the impact *Bifidobacterium breve* M-16V and a combination of short-chain galacto-oligosaccharide (scGOS) and long-chain fructo-oligosaccharide (lcFOS) 9:1 proportion on rats. The impact was evaluated after its indirect effect through maternal supplementation from gestation to lactation, and after its direct effect when extending the supplementation to early childhood.

**Methods**: Pregnant rats were administered with the synbiotic during gestation and lactation. After weaning, the pups were directly supplemented from day 21 to day 28 of life with the same mix. At day 28 of pups' life, blood, adipose tissue, small intestine (SI), mesenteric lymph nodes (MLN), salivary gland (SG), cecum, and spleen were collected to analyze the immune system development and the microbiota colonization.

**Results**: The supplementation did not influence the overall growth while a trophic effect was observed in the small intestine. Specifically, a higher intestinal weight, length, width, and changes in villi was also observed due to the synbiotic. Gene expression analysis in these animals indicated a reduction in *FcRn* and *Blimp1* mRNA levels and an increase in *Zo1* and *Tlr9*, suggesting enhanced maturation and barrier function. Intestinal immunoglobulin (Ig) A levels remained unaffected, while cecal IgA amounts were decreased. The synbiotic supplementation led to both higher total bacteria abundance and Ig-coated bacteria in the cecum. *Bifidobacterium* abundance increased in both the intestine and cecum of the animals receiving the synbiotic. Short-chain fatty acid levels in the intestine and cecum are lower and higher, respectively, than the reference animals. Systemically, the Ig profiles remained unaffected due to the synbiotic supplementation.

**Conclusion**: These results indicate that animals from mothers receiving synbiotic supplementation during gestation and lactation, and directly after in the following week positively influenced intestinal immunity, demonstrating potential benefits to enhance the maturation and functionality of the intestinal barrier and microbiota of developing infants.

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MDPI

*Article*

# Enhancing gastrointestinal immunity and microbial diversity in young rats by *Bifidobacterium breve* M-16V and scGOS/lcFOS supplementation in early life

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**Abstract:** Immunonutrition, focusing on specific nutrients in breast milk and post-weaning diets, plays a crucial role in supporting the infant's immune system development. This study explored the impact of maternal supplementation with *Bifidobacterium breve* M-16V and a combination of short-chain galacto-oligosaccharide (scGOS) and long-chain fructo-oligosaccharide (lcFOS) from pregnancy through lactation, extending into early childhood of the offspring. The synbiotic supplementation's effects were examined at both mucosal and systemic levels. While the supplementation did not influence overall growth or intake consumption, a trophic effect was observed in the small intestine, enhancing weight, length, width, and microscopic structures. Gene expression analysis indicated a reduction in *FcRn* and *Blimp1* and an increase in *Zo1* and *Tlr9*, suggesting enhanced maturation and barrier function. Intestinal immunoglobulin (Ig) A levels remained unaffected, while cecal IgA decreased. The synbiotic supplementation led to increased total bacteria abundance and Ig-coated bacteria in the cecum. *Bifidobacterium* abundance increased in both the intestine and cecum. Short-chain fatty acid production decreased in the intestine but increased in the cecum due to the synbiotic supplementation. Systemically, the Ig profiles remained unaffected. In conclusion, maternal synbiotic supplementation during gestation, lactation, and early life positively influences gastrointestinal immunity, demonstrating potential benefits to enhance the maturation and functionality of the intestinal barrier and microbiota of developing infants.

**Keywords:** *Bifidobacterium breve* M-16V, galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), immunonutrition, immune system.

## 1. Introduction

Immunonutrition is defined as the capacity of specific nutrients to regulate or influence the activity of the immune system [1]. Immunonutrients include vitamins, minerals, amino acids, and fatty acids that play a direct role in supporting or regulating the immune system [2]. Although some of these components in the maternal diet during gestation reach the fetus through the placenta, breast milk (BM) serves as the primary source of immunonutrients for neonates and infants. The bioactive compounds of the BM stimulate the maturation of the immune system, contribute to immune tolerance, and aid in the colonization of the intestinal microbiome [3,4]. Human BM is considered the optimal nutrition for infants during the first 6 months of life and facilitates immune system development. After weaning, healthy nutritional habits continue to enhance the infant's immune system, reducing susceptibility to infections and diseases [5].

Probiotics, prebiotics, and synbiotics are not classified as immunonutrients, but they exert a modulatory effect on the immune system [6,7]. The immunomodulatory effects of pro-, pre- and synbiotics are strain-specific and dose-dependent [6]. *Bifidobacteria* and *lactobacilli* are commonly used probiotic strains that help reduce gut inflammation and maintain intestinal balance [8,9]. Prebiotics, among other actions, serve as specific substrates for the intestinal microbiota, encouraging its proliferation and avoiding pathogen colonization through their antiadhesive properties. Short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) are common prebiotics known for promoting the growth of beneficial gut bacteria when added to infant formulas [9,10]. Synbiotics, a blend of pro- and prebiotics, yield a synergistic effect by ensuring the viability of the probiotic bacteria and their growth stimulation by the delivered substrates. Supplementation with synbiotics has been linked to a reduction in the incidence of eczema, asthma development, infectious diseases, respiratory issues, and dysentery [11–15]. These findings emphasize the essential role of these compounds in strengthening and optimizing the immune system, consequently promoting overall health and well-being.

Supplementing maternal diets with synbiotics during both gestation and lactation positively impacts maternal immune health and contributes to the development of the neonate immune system [16,17]. In our research, we assessed the impact of maternal synbiotic supplementation from gestation through lactation and continuing into the offspring's early childhood for one week. The synbiotic mix consisted of *Bifidobacterium breve* M-16V and scGOS/lcFOS. This overall period complies in rat the equivalent of the first 1000 days of life in humans, considered as essential in infant development [18,19]. We examined its immunomodulatory effects on both systemic and mucosal immunity, its influence on various lymphocyte populations, and on the microbiota composition, diversity, and activity.

## 2. Materials and Methods

### *2.1. Animals and experimental design*

Sixteen female and eight male Lewis rats were obtained from Janvier Labs in La Plaine Saint Denis Cedex, France. After one week of acclimatization, the females were placed in the males' cages for one week for mating. Then the females were separated and placed in individual cages.

Afterwards, the female rats were divided into two experimental groups (n=8/group): reference (REF) and synbiotic (SYN). Animals from the two groups were supplemented daily with a synbiotic during gestation (21 days) and lactation (21 days). Pups were allowed to be born naturally. After weaning, the pups were directly supplemented from day 21 to day 28 of life with the same mix. The day of birth was designated as day 1 for the pups and litters were unified up to 10 pups. From these, 3 pups/litter were kept until day 28 of life, which is described in the present study. The pups had free access to their mother's nipples for nursing (until day 21) and were also provided with the rat diet used

in the study. Throughout the study, the rats had unrestricted access to a commercial diet formulated according to the American Institute of Nutrition 93G formulation [20]. They were also provided with water *ad libitum*.

The synbiotic was administered daily by oral gavage to the dams during gestation and lactation, and to the pups after weaning. The synbiotic was freshly prepared by mixing *Bifidobacterium breve* M-16V ($10^9$ CFU) with scGOS/lcFOS in a physiological saline solution. For the dams, the dose of scGOS/lcFOS was approximately 2% of an established daily food intake of 40 g, the administered volume was fixed at 1 mL during gestation and at 1.5 mL during lactation. For the pups, from day 21 to day 28 of life the dose was fixed at $2 * 10^7$ UFC/rat/day, administered in 0.2 mL of volume. The REF dams and the REF pups received an equivalent volume of a saline solution under the same conditions. All supplements were kindly provided by Danone Research & Innovation (Utrecht, the Netherlands).

The animal room conditions (temperature and humidity) were controlled. The room followed a 12-h light – 12-h dark cycle within a negative pressure chamber at the Diagonal Campus Animal Facility of the Faculty of Pharmacy and Food Science at the University of Barcelona (UB). The experimental procedures conducted in this study were carried out according the necessary ethical approvals. The research received approval from the Ethics Committee for Animal Experimentation (CEEA) of the University of Barcelona (Ref. 240/19) and from the Catalan Government (Ref. 10933).

### 2.2. *Sampling and processing*

From birth until day 28 of the pups' life, animal weight was measured, and diet intake was followed from weaning (day 21) to day 28. Then the pups were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg; Bayer A.G., Leverkusen, Germany). They were then measured to evaluate their overall development according to the Body Mass Index (BMI) ($(weight/length^2$ (g/cm$^2$)) and the Lee Index ($weight^{0.33}/length) \times 1{,}000$ (g$^{0.33}$/cm).

Cardiac exsanguination was performed to obtain blood samples. Intestinal samples, adipose tissues (epididymal, parametric, dorsal, retroperitoneal, inguinal for white adipose tissue (WAT) and brown adipose tissue (BAT)), cecal content (CC), salivary gland (SG), mesenteric lymph nodes (MLNs), and spleen were collected and immediately processed or stored at −20 °C or −80 °C for future analysis.

Blood samples were analyzed using an automated hematologic analyzer (Spincell, MonLab Laboratories, Barcelona, Spain) and centrifuged to obtain plasma.

Small intestine (SI) and adipose tissue were collected for histological evaluation. Additionally, the central section of the SI was collected for gene expression analysis. One centimeter of the central section of the SI was embedded in RNAlater (Ambion, Life Technology, Madrid, Spain), kept at 4 °C for 24 h, and then stored at −20 °C. The remaining portion of the proximal SI was opened lengthwise and incubated in shaking (37 °C, 10 min) with phosphate-buffered saline (PBS), and the supernatant (gut wash (GW)) was recovered for immunoglobulin (Ig) quantification. The content of the distal part of the SI (intestinal content, IC) was collected for microbiota analysis.

Part of the MLNs and the spleen were processed as previously described to obtain splenic and MLN lymphocytes [21]. Briefly, tissues were ground using a 40 µm mesh cell strainer (Thermo Fisher Scientific, Barcelona, Spain) by using enriched Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, Madrid, Spain). The obtained suspension was centrifuged, and the resulting pellet was resuspended in enriched RPMI.

The remaining MLNs, SG, and CC were frozen. Then they were thawed and homogenized for Ig quantification using Enzyme-Linked ImmunoSorbent Assay (ELISA), ProcartaPlex™ Multiplex immunoassay (eBioscience, San Diego, CA, USA) or flow cytometry. The weight of different organs was recorded, including thymus, spleen, liver, heart, kidney, large intestine, and SI (length and width were also measured).

### 2.3. *Tissue histology*

The chosen sections of the SI and both BAT and WAT (epididymal or parametric for males or females, respectively, were chosen as representative of WAT) were initially fixed in a 4% buffered formaldehyde solution for 24 h at room temperature. Subsequently, the samples were rinsed in PBS solution and dehydrated using a series of graded ethanol solutions (70%, 90%, and 100%) and permeated in xylene (Panreac Química SLU, Barcelona, Spain). Following this, the samples were embedded in melted paraffin (Merck, Madrid, Spain). Paraffin sections of 5 μm thickness were obtained with a microtome (Leica biosystems, Nußloch, Germany) and stained using hematoxylin-eosin (HE). Samples were examined using an Olympus BX41 microscope and Olympus XC50 camera. Representative photos were taken at 20x magnification for WAT, 40x for BAT, and 10x for intestine sections. All histological samples were analyzed using Image software (National Institute of Mental Health in Bethesda, MD, USA).

*2.4. Gene expression analysis*

RNAlater stored SI samples were thawed for RNA extraction and gene expression analysis. Samples were homogenized using lysing matrix tubes and a FastPrep-24 instrument (MP Biomedicals, Illkirch, France), as previously described [22]. RNA extraction was performed following the manufacturer's protocol using the RNeasy Mini Kit (Qiagen, Madrid, Spain), and quantified with a NanoPhotometer (BioNova Scientific S.L., Fremont, CA, USA). cDNA was obtained using TaqMan Reverse Transcription Reagents (Applied Biosystems, AB, Weiterstadt, Germany). Then, real-time (RT) – PCR was performed with an ABI Prism 7900HT quantitative RT-PCR system (AB) in the Scientific and Technological Centers of the University of Barcelona (CCiT-UB).

The specific TaqMan primers AB used are found in Supplementary Table 1. The housekeeping gene *Gusb* (β-glucuronidase) was used to normalize the obtained results. Data were analyzed using the -2ΔΔCt method, as previously described [23]. The data are presented as a percentage of expression in each experimental group, normalized to the mean value obtained for the REF group, which was set at 100%.

The detection of the *B. breve* M 16-V was performed in the CC after the DNA extraction, as previously described [24]. The Taq-Man-based forward, reverse, and probe were previously designed by Phavichitr et al. [25].

*2.5. Immunoglobulin quantification*

Plasma, GW, and homogenized MLNs, SG, and CC were used for Ig quantification. Secretory (s)IgA was quantified by ELISA. Additionally, IgM was also evaluated in GW. sIgA and IgM were quantified following the previous procedures [26]. Absorbance data were acquired using a microplate photometer (Labsystems Multiskan, Helsinki, Finland) at 495 nm. Results were analyzed by Multiskan Ascent v2.6 software (Thermo Fisher Scientific SLU, Barcelona, Spain). The lower limits of detection were 1.95 ng/mL for sIgA and IgM.

In plasma and homogenized MLNs, and SG, a ProcartaPlex™ Multiplex immunoassay was performed to quantify IgA, IgM, IgG, and IgG isotypes (IgG1, IgG2a, IgG2b, IgG2c). Briefly, 96-well flat-bottom plates were used to prepare samples following the manufacturer's instructions, as described in previous studies [27]. Data were acquired by MAGPIX® analyzer (Luminex Corporation, Austin, TX, USA) at the Cytometry Service of the CCiT-UB. The lower limits of detection of each Ig are found in Supplementary Table 2. Th1 and Th2 responses were evaluated adding the levels of IgG subtypes, IgG2b + IgG2c and IgG1 + IgG2a, respectively.

*2.6. Cecal bacteria and Ig-coated bacteria analysis*

The proportion of cecal bacteria and Ig-coated bacteria (Ig-CB) was determined as previously described [28]. Data acquisition was done using Cytek Aurora (Cytek Biosciences, Inc., CA, USA) flow cytometry equipment at the CCiT-UB. FlowJo v.10 software

(Tree Star, Inc., Ashland, OR, USA) was used to analyze data by following the protocol described by Massot et al. [29].

### *2.7. Cell subset staining and flow cytometry analysis*

Mesenteric and splenic lymphocyte subsets were characterized by flow cytometry analysis using fluorescent mouse anti-rat monoclonal antibodies (mAbs) conjugated to different fluorochromes, as previously described [30]. Data acquisition was performed in the CCiT-UB using a Gallios™ Cytometer (Beckman Coulter, Miami, FL, United States) and analyzed by FlowJo v.10 software.

### *2.8. Short-chain fatty acids quantification*

Short-chain fatty acids (SCFAs) analysis was performed using gas chromatography–mass spectrometry (GC-MS), following the method described by Eberhart et al. [31]. An internal standard solution (3-methylvaleric acid) was added to the samples that were processed and finally centrifuged at 1500 *g* for 2 min at 4 °C, according to the protocol. The final supernatant was collected, filtered-sterilized (0.22 µm PES size filter, Sarstedt SA, Nümbrecht, Germany) and injected in the Agilent GC 7890B–5977B GC-MS with a multipurpose sampler (Gerstel MPS, Mülheim, Germany). The GC column used was an Agilent DB-FATWAX, 30 m × 0.25 mm × 0.25 µm, operated in split mode (20:1). The oven temperature program was set as follows: 100 °C for 3 min, ramped up to 100 °C at a rate of 5 °C min−1, then to 150 °C for 1 min, further ramped up to 200 °C at a rate of 20 °C min−1, and finally held at 200 °C for 5 min. Helium was used as the carrier gas at a flow rate of 1 mL min−1, with an inlet temperature of 250 °C. The injection volume was 2 µL. Standard curves for acetate, butyrate, and propionate were used for quantifying the SCFAs.

### *2.9. Microbiota analysis by 16S rRNA amplicon-sequencing*

Total DNA was isolated from the IC and CC (100–200 mg) using an automated assisted method based on magnetic beads (Maxwell® RSC Instrument coupled with Maxwell RSC Pure Food GMO and authentication kit, Promega, Spain) following the manufacturer's instructions, with previous treatments to improve the DNA extraction. In brief, samples were treated with lysozyme (20 mg/mL) and mutanolysin (5 U/mL) for 60 min at 37 °C and a preliminary cell disruption with a bead beater FastPrep 24-5 G Homogenizer (MP Biomedicals). After DNA purification (Purification Kit, Macherey-Nagel, Duren, Germany), DNA concentration was measured using a Qubit® 2.0 Fluorometer (Life Technology, Carlsbad, CA, USA).

Libraries targeting the amplicon V3–V4 variable region of the 16S rRNA gene were prepared following the 16S rDNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15044223 Rev. A) and then sequenced using 2x300 bp paired-end run on a MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain). Negative and positive mock communities were also included. Raw reads were then processed with the integrated dada2 [32] method for denoising, amplicon sequence variance (ASV) clustering and chimeral removal. The resultant ASV were then taxonomically assigned using Silva v.138.

### *2.10. Statistical analysis*

The statistical analysis was performed using SPSS Statistics 22.0 software (SPSS Inc., Chicago, IL, USA). Normality and variance homogeneity were assessed using the Shapiro–Wilk and Levene tests, respectively. When the data exhibited normal and homogeneous distributions, a one-way ANOVA was performed for analysis. In cases where the data did not adhere to normal and equal distribution assumptions, the Kruskal–Wallis test was used to identify significant differences ($p < 0.05$). To explore variable correlations, the Spearman correlation coefficient was calculated.

To identify clusters of sample similarities based on immune factor composition, non-metric multidimensional scaling (NMDS) was employed in Rstudio, utilizing the 'vegan' package [33]. To assess the association of factors with the ordination of samples in the NMDS plot, the 'envfit' function was used. Statistical significance was considered when the $p$-value < 0.05.

Regarding the microbiota analysis, no rarefaction was done, samples with less than 4500 reads were removed and data were normalized using centered log-ratio (CLR). Beta diversity analysis was based on the Bray–Curtis distances matrix and permutational analysis of variance (PERMANOVA) was performed. Alpha-diversity indexes Chao1 and Shannon were also calculated and differences by group were assessed by Mann–Whitney and/or Kruskal–Wallis non-parametric test. In addition, the Kruskall–Wallis test on the CLR normalized data were also assessed using the Benjamini–Hochberg false discovery rate (FDR) correction. Negative binomial regression as implemented by the DESeq2 tool was used for differential abundance analysis to estimate the fold-change of genus taxa [34].

## 3. Results

### *3.1. Body weight and intake consumption*

Animal growth evolution was monitored daily from day 2 until the last day of study (day 28 of life) (Figure 1). From birth, SYN pups showed a similar growth pattern to the REF group. At weaning (day 21) both groups showed a decrease in body weight as a consequence of the separation from their dams (Figure 1a). After pup separation from the dams, the intake consumption was monitored daily. Food consumption increased in line with animal growth from ≈ 5 g at day 21 to ≈ 12 g at day 28 (Figure 1b) and water consumption was maintained constant throughout the study, with overall values of ≈ 13 mL (Figure 1c). Generally, the synbiotic supplementation did not modify the global intake consumption.

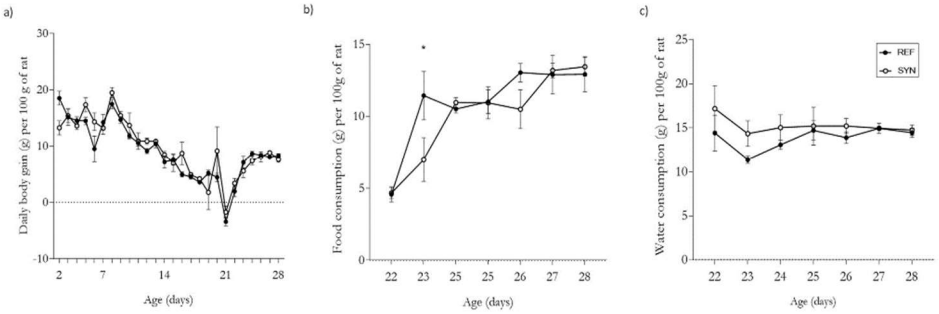



**Figure 1.** Growth evolution until day 28. (a) Daily body gain; (b) Food consumption from day 2 to day 28 of life; (c) Water consumption from day 2 to day 28 of life. Results are expressed as mean ± standard error of the mean (SEM). Statistical differences: *$p$ < 0.05 vs. REF (n=9-11).

### *3.2. Growth parameters and organs' weight*

On day 28 of life, the pups were measured and weighed to calculate the growth-associated parameters including the BMI and the Lee Index (Table 1). Additionally, the weights of different organs were assessed, and the majority showed no significant changes. However, the synbiotic supplementation triggered a trophic effect in the SI leading to an increase in its weight, length, width, and area.

**Table 1.** Animal growth parameters

| **Body** | **REF** | **SYN** |
|---|---|---|
| Body length (cm) | 13.58 ± 0.11 | 13.26 ± 0.15 |
| Body/tail length ratio | 1.42 ± 0.02 | 1.55 ± 0.14 |
| BMI (g/cm$^2$) | 0.36 ± 0.01 | 0.35 ± 0.05 |
| Lee Index (g$^{0.33}$/cm × 10$^3$) | 298.82 ± 1.80 | 295.93 ± 3.15 |

| **Organs** | **REF** | **SYN** |
|---|---|---|
| Spleen (%) | 0.36 ± 0.01 | 0.36 ± 0.01 |
| Thymus (%) | 0.44 ± 0.02 | 0.44 ± 0.02 |
| Kidney (%) | 0.62 ± 0.01 | 0.59 ± 0.01 |
| Heart (%) | 0.58 ± 0.01 | 0.60 ± 0.03 |
| Liver (%) | 4.49 ± 0.04 | 4.09 ± 0.46 |
| Salivary gland (%) | 0.24 ± 0.09 | 0.16 ± 0.01 |
| Stomach (%) | 0.71 ± 0.02 | 0.73 ± 0.02 |
| Cecum (%) | 0.33 ± 0.02 | 0.30 ± 0.01 |
| Small intestine (%) | 4.18 ± 0.07 | 4.61 ± 0.19* |
| Intestine length (cm) | 67.04 ± 2.14 | 72.39 ± 2.95# |
| Intestine width (cm) | 0.85 ± 0.07 | 1.15 ± 0.05* |
| Area (cm$^2$) | 56.26 ± 5.03 | 83.90 ± 6.59* |

Data are expressed as mean ± SEM. Statistical differences: *$p$ < 0.05 vs. REF, #$p$ < 0.1 vs. REF (n=9–11).

### 3.3. Hematologic variables

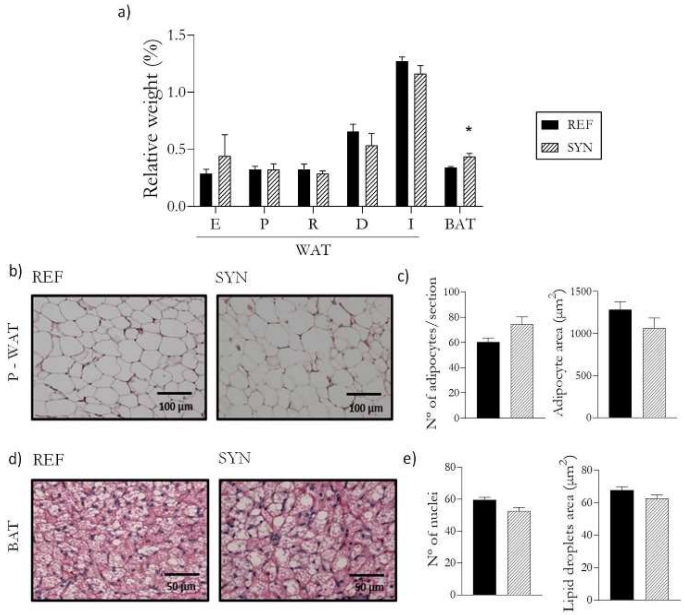
After synbiotic supplementation (day 28), the hematological variables were assessed (Table 2). Synbiotic supplementation lowered the counts in the total leukocyte population, primarily attributable to reductions in lymphocytes and granulocytes, without affecting the erythrocytes and platelets measurements.

**Table 2.** Hematological variables at day 28 of life

| | REF | SYN |
|---|---|---|
| Leukocytes (x10$^9$ /L) | 4.84 ± 0.93 | 2.78 ± 0.2 * |
| Lymphocytes (x10$^9$ /L) | 3.09 ± 0.42 | 1.89 ± 0.18 * |
| Monocytes (x10$^9$ /L) | 0.27 ± 0.12 | 0.11 ± 0.01 |
| Granulocytes (x10$^9$ /L) | 1.47 ± 0.40 | 0.78 ± 0.08 * |
| Erythrocytes (x10$^{12}$ /L) | 5.41 ± 0.25 | 5.38 ± 0.12 |
| HGB (g/L) | 101.79 ± 4.02 | 101.22 ± 2.47 |
| HCT (%) | 29.76 ± 1.47 | 29.79 ± 0.93 |
| MCV (fL) | 55.01 ± 0.62 | 55.34 ± 0.69 |
| MCH (pg) | 18.99 ± 0.40 | 18.74 ± 0.11 |
| Platelets (x10$^9$ /L) | 234.54 ± 28.88 | 182.89 ± 41.62 |

Data are expressed as mean ± SEM. Statistical differences: *$p$ < 0.05 vs. REF (n=9–11). HGB, Hemoglobin; HCT, hematocrit; MCV, Mean Cell Volume; MCH, Mean Cell Hemoglobin.

### 3.4. *Adipose tissue*




The influence of the synbiotic administration on adipose tissue examined on day 28 of life (Figure 2) revealed an increase in the relative weight of the BAT (Figure 2a). Histological analysis using HE staining on both WAT (Figure 2b, c) and BAT (Figure 2d, e) revealed no observable alterations associated with synbiotic supplementation.

**Figure 2.** Impact of synbiotic supplementation on the adipose tissue. (a) Relative weights of different sections of adipose tissue; (b) Representative histological sections of WAT (epididymal or parametric, for males or females, respectively, were chosen as representative of the WAT); (c) Analysis of WAT: adipocyte area and number of adipocytes; (d) Representative histological sections of BAT; (e) Analysis of BAT: number of nuclei and area of lipid droplets (LDs). Histological sections (b, d) were stained with hematoxylin-eosin. Images were taken at 40x and 20x magnification, respectively. Data (a, c, e) are expressed as mean ± SEM. Statistical differences: $*p < 0.05$ vs. REF (n=9–11). WAT, white adipose tissue; BAT, brown adipose tissue; E, epididymal; P, parametric; R, retroperitoneal; D, dorsal; I, Inguinal.

### 3.5. *Intestinal morphology and maturation*

Intestinal maturation was examined at both biochemical and microscopic levels (Figure 3). *FcRn* and *Blimp1*, recognized as reliable indicators of intestinal maturation [35], displayed reduced gene expression after synbiotic supplementation, thus indicating a higher level of maturation. In terms of morphological changes resulting from supplementation, the SYN pups exhibited an increase in villi width and area, greater crypt depth, and a higher ratio of goblet cells per villi.

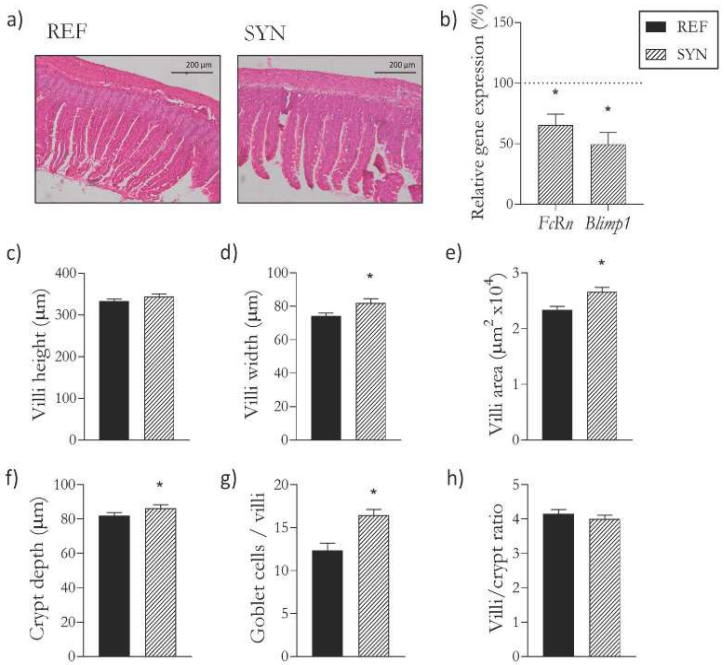



**Figure 3**. Influence of SYN supplementation on the pups' intestine. (a) Representative sections of the small intestine stained with hematoxylin and eosin, 10x; (b) Relative gene expression analysis in the small intestine of immunological proteins; (c) Villi height; (d) Villi width; (e) Villi area.; (f) Crypt depth; (g) Ratio of villi/crypt; (h) Ratio of goblet cells/villi. Relative gene expression (b) was calculated with respect to REF, which corresponded to 100% of transcription (represented with a horizontal dotted line). Results (c–h) are expressed as mean ± SEM. Statistical differences: $^{*}p < 0.05$ vs. REF (n=9–11). *Blimp1*, B lymphocyte-induced maturation protein-1; *FcRn*, neonatal Fc receptor.

### 3.6. Intestinal expression of barrier and crosstalk genes

Supplementation increased the tight (TJ) protein *Zo-1* mRNA levels, whereas it did not modify *Cldn2*, *Clnd4* and *Ocln* (Figure 4a). The genes associated with mucus production, specifically *Muc2* and *Muc3*, remained unaffected. (Figure 4b). Among the multiple evaluated TLRs, only *Tlr9* exhibited a gene expression increase after the supplementation (Figure 4c).

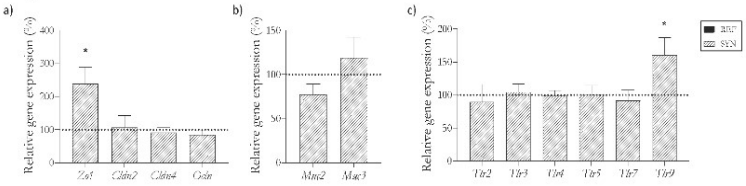



**Figure 4**. Impact of SYN supplementation on the small intestine gene expression. (a) Relative gene expression of (a) tight junction proteins; (b) mucins; (c) toll-like receptors.

Relative gene expression was calculated with respect to REF, which corresponded to 100% of transcription (represented with a horizontal dotted line). Statistical differences: $*p < 0.05$ vs. REF. (n=9–11). *Zo1*, zonula occludens-1; *Cldn2*, claudin 2; *Cldn4*, claudin 4; *Ocln*, occludin; *Muc2*, mucin2; *Muc3*, mucin3; *Tlr*, toll-like receptor.

### 3.7. Gastrointestinal Ig profile

The effects of synbiotic supplementation were analyzed regarding the Ig profile in the gastrointestinal tract (Figure 5). In the GW, the sIgA was quantified at gene and protein level. The IgA mRNA levels were reduced after the SYN supplementation (Figure 5a). However, the total sIgA protein remained unaffected (Figure 5b). The IgM levels were also not modified after the synbiotic supplementation (Figure 5c). To further understand the impact of the supplementation on sIgA functionality, the analysis of the sIgA content was conducted in the cecum. A reduction in the total levels of sIgA after the supplementation was observed (Figure 5d). Thus, cecal sIgA plays a crucial role in binding to cecal bacteria to neutralize pathogenic bacteria and maintain intestinal homeostasis [36,37]. The proportion of IgA-CB was also evaluated and, our results indicated that maternal and early life infant synbiotic intervention led to an increase in the total cecal bacteria and in the Ig-CB proportion, suggesting that the lower levels of the free Ig are due to the higher presence of coating bacteria (Figure 5f–h).

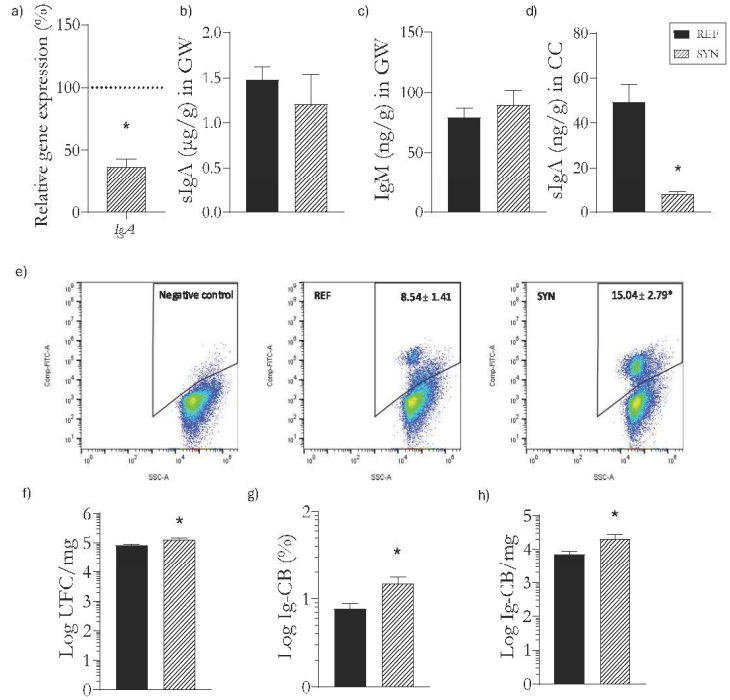



**Figure 5.** Impact of synbiotic supplementation at day 28 of life on the gastrointestinal tract. (a) IgA expression levels in the GW; (b) Protein sIgA levels in the GW; (c) Protein IgM levels in the GW; (d) Protein sIgA levels in the cecum; (e) Representative dot-plots of the proportion of Ig-CB in the cecum; (f) Counts of total bacteria of the cecum; (g) Proportion

of Ig-CB of the cecum; (h) Total counts of Ig-CB in the cecum. Relative gene expression (a) was calculated with respect to REF, which corresponded to 100% of transcription (represented with a horizontal dotted line). Data (b–h) are expressed as mean ± SEM. Statistical differences: $^{*}p < 0.05$ vs. REF (n=9–11). sIgA, secretory immunoglobulin *A*; GW, gut wash; CC, cecal content; Ig-CB, immunoglobulin-coated bacteria.

### 3.8. Microbiota

Differences in the microbiota composition were observed between the REF and SYN groups, and also between sample type (IC and CC) (Figure 6 and Figure 7).

Beta-diversity and alpha-diversity analysis showed significant differences depending on the sample type (intestine vs. cecum) and on the intervention (REF vs. SYN). In detail, regarding beta-diversity, two general distinct microbial clusters depending on sample-type CC vs. IC were found, accounting for 62.8% of the total variation (PERMANOVA test F-value: 18.98; R-squared: 0.60; p-value: 0.001, Figure 6a). In addition, when groups were compared considering the intervention, we observed a significant impact of the SYN intervention on the cecum (PERMANOVA test F-value: 4.80; R-squared: 0.201; $p = 0.001$) but not on the microbiota from the small IC (PERMANOVA F-value: 1.68; R-squared: 0.0813; $p = 0.125$) (Supplementary Figure 1a,b). DESEq tests showed that in CC an enrichment of *Bifidobacterium* (FDR $p = 0.002$) and *Lactobacillus* sp. oral clone HT002 (FDR $p < 0.0001$) was found in addition to a significant reduction of the genus *Rikenellaceae*_RC9_gut_group (FDR $p = 0.002$), *Prevotellaceae*_UCG_001 (FDR $p = 0.021$), other *Lachnospiraceae* (FDR $p = 0.007$) and also *Intestinimonas* (FDR $p = 0.1541$) (Supplementary Figure 1c). In IC, the SYN intervention increased the presence of *Turicibacter* (FDR $p = 0.005$), *Hungatella* (FDR $p = 0.007$), *Lachnoclostridium* (FDR $p = 0.085$), *Erysipelatoclostridium* (FDR $p = 0.192$), and decreased the presence of on *Escherichia_Shigella* (FDR $p < 0.0001$), *Lactococcus* (FDR $p = 0.019$) as well as *Enterobacter* (FDR $p = 0.027$) (Figure 6b and Supplementary Table 3). The LEfSe test also demonstrated the role of these microbial genera depending on the SYN intervention in both CC and IC.

Regarding alpha-diversity metrics, SYN intervention did not influence either the microbial richness (measured by Chao1 index) or the microbial diversity (measured by Shannon Index) in the cecum (CC, $p = 0.943$ and $p = 0.149$, respectively) and in the intestine (IC, $p = 0.654$ and $p = 0.149$, respectively) (Figure 6c–d). Moreover, it should be mentioned that a higher significant microbial diversity and richness were observed in CC compared to IC.

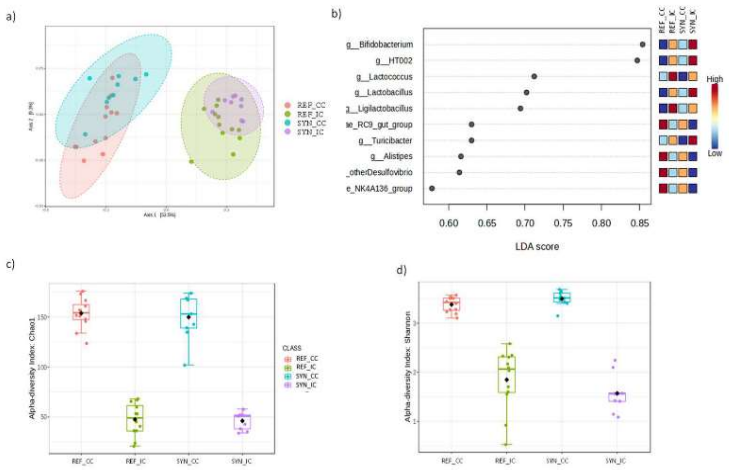


**Figure 6.** (a) Beta-diversity in IC and CC microbiota depending on SYN intervention; (b) Linear discriminant analysis (LDA) effect size (LEfSe) plot of taxonomic genera identified in CC and IC; Alpha-diversity indexes (c) (Shannon index) and (d) richness (Chao1 index) for CC and IC. Statistical testing was performed by PERMANOVA using Bray–Curtis distances, and the Mann–Whitney test was used for alpha-diversity indexes (n=9–11). Intestinal content, IC; cecal content, CC.

Taking into account the different bacterial proportions, in the IC, the proportion of Actinobacteria phylum (mainly *Bifidobacterium* genus) was significantly increased after the SYN supplementation (Figure 7a). The *Bifidobacteria* family was significantly increased in the SYN group (Figure 7c). And, at the genus level, *Turicibacter* and *Bidifobacterium* members were increased while *Ligilactobacillus* was reduced in the SYN pups (Figure 7e).

In the CC, SYN intervention increased the relative abundance of members from the Firmicutes and Actinobacteria phylum and reduced the relative abundance of members from Bacteroidota phyla (Figure 7b). At the family levels, a significative reduction in the abundance of *Rikenecellaceae* members and an increase in the *Lactobacillaceae* members were observed in the pups in the SYN group (Figure 7d). In the minority families (< 1 %), a significant increase of the *Bifidobacteriaceae* and a reduction of *Staphylococaceae* were also found in the SYN group. Regarding genera proportions, changes in the minority populations (0.1–1 %) were found (Figure 9f). After SYN supplementation, *Bifidobacterium*, *Blautia*, *Faecalibaculum*, and *Lactobacillus* proportions were significantly increased. In addition, *B. breve* M-16V was detected by PCR in cecum samples in the SYN group after one week of supplementation at 2-3 *$10^9$ UFC/mg in 100% of the animals. These results are in line with the increase of *Bifidobacterium* proportions found.

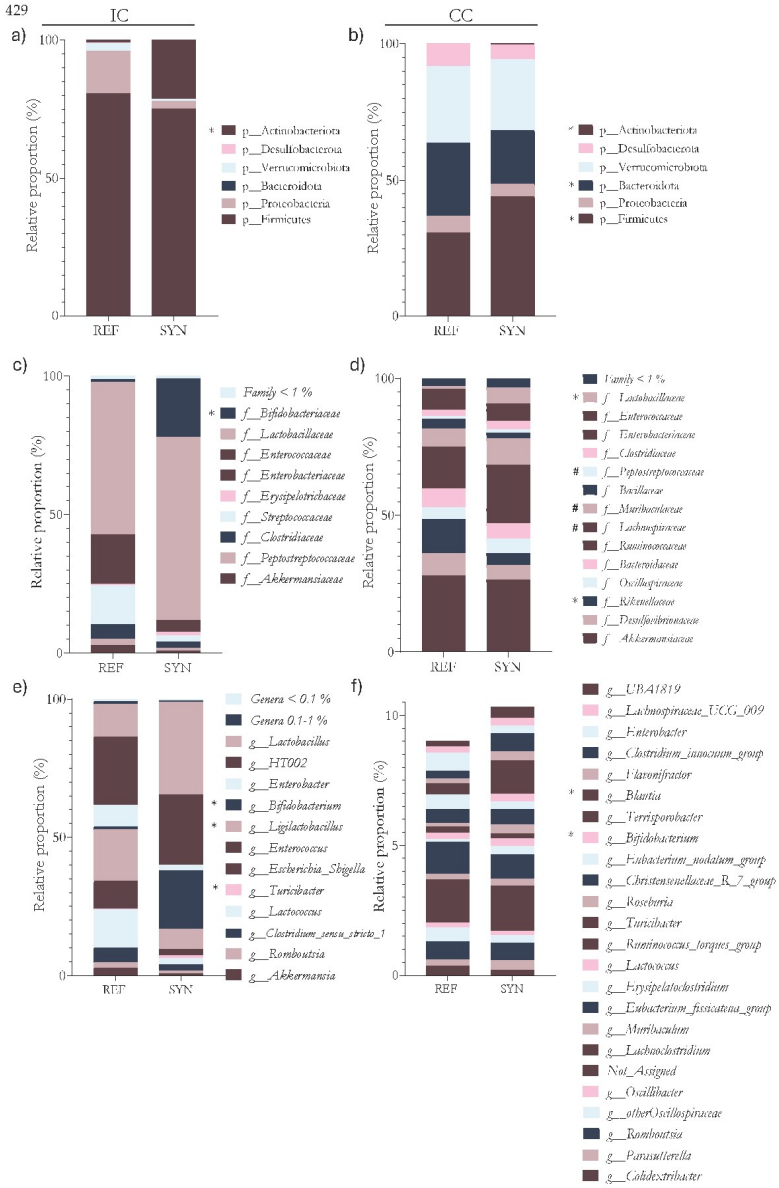



**Figure 7.** Microbiota composition in the intestinal content (IC) and cecal content (CC). Relative abundance of phylum in (a) IC and (b) CC; Relative family abundance in (c) IC and (d) CC; Relative genus abundance in (e) IC and (f) CC. Results are expressed as relative proportions of population. Statistical differences: *$p < 0.05$ vs. REF; #$p < 0.1$ (n=9–11).

*3.9. SCFA profile*

The SCFAs at both the intestinal and cecal levels was examined to establish a relationship with the microbiota findings in both compartments (Figure 8). In the IC, a decrease in SCFA production was found, specifically in acetic, propanoic, isobutanoic, butanonic, iso-valeric, valeric, and hexanoic acids (Figure 8a). Conversely, in the CC, the overall SCFA production exhibited an increase, attributed to higher levels of all the aforementioned acids (Figure 8b).

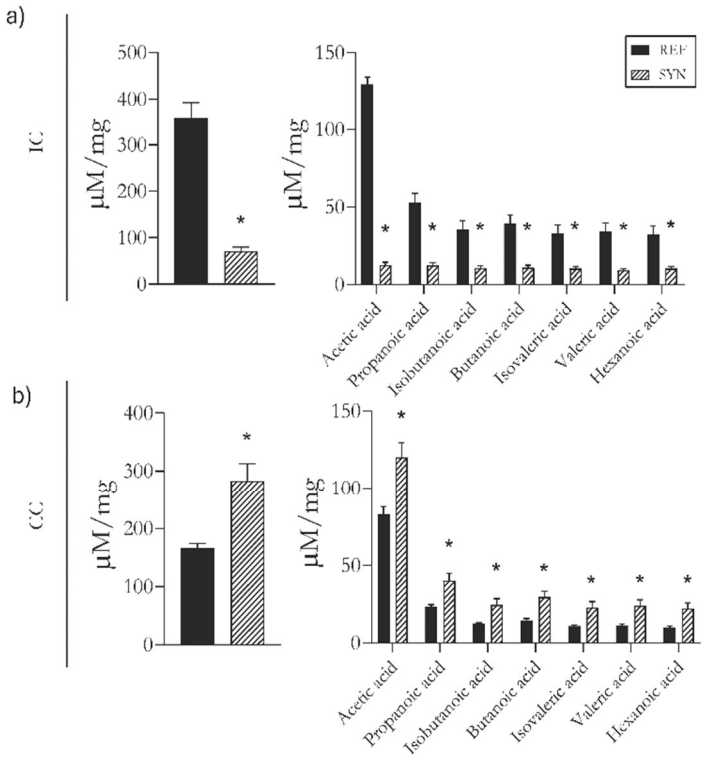



**Figure 8.** Intestinal and cecal short-chain fatty acid (SCFA) levels at day 28 of life. (a) SCFAs in intestine content (IC); (b) SCFAs in cecal content (CC). Results are expressed as mean ± SEM. Statistical differences: *$p < 0.05$ (n=9–11).

*3.10. Lymphocyte subset characterization*

The impact of the synbiotic supplementation was also assessed on lymphocytic populations both at systemic level, in the spleen, and at the mucosal level, in the MLN. In addition to the main lymphocyte populations, markers related to intestinal migration (αE and CD62L) were also measured, as well as the levels of the cellular activation marker CD25. Furthermore, the expression of the CD8 receptor on T lymphocytes was also assessed (Table 3).

In the spleen, the percentage of B lymphocytes was higher after the SYN supplementation, whereas activated B cell (CD25+) proportion was not altered. The analysis of the T cell subsets showed no changes in the total T cells, including in the TCRαβ+ and the

TCRgδ+ subpopulations. However, a reduction of the total T CD4+ and the activated T CD4+ cells was observed after the nutritional intervention. The SYN supplementation reduced the surface cell expression migratory markers in the spleen after the supplementation. Evaluation of lymphocyte populations at the mucosal level did not significantly affect the B or T cell populations.

**Table 3.** Lymphocyte subsets phenotype in the spleen and MLN at day 28 of life

| | Spleen | | MLN | |
|---|---|---|---|---|
| | **REF** | **SYN** | **REF** | **SYN** |
| B cells (CD45RA+) | 25.44 ± 2.26 | 35.14 ± 0.64 * | 17.05 ± 1.58 | 17.85 ± 1.17 |
| % CD25+ | 2.51 ± 0.43 | 1.40 ± 0.26 | 2.86 ± 0.19 | 2.10 ± 0.32 |
| % CD62L+ | 46.15 ± 2.69 | 39.52 ± 2.78 | 65.84 ± 5.24 | 70.23 ± 2.32 |
| % αE+ | 2.95 ± 0.58 | 4.04 ± 0.84 | 0.09 ± 0.03 | 0.06 ± 0.01 |
| T cells (TCRαβ+NK- and TCRgδ) | 54.01 ± 6.12 | 36.94 ± 1.21 | 79.17 ± 1.86 | 78.77 ± 1.04 |
| TCRαβ+ NK- | 50.60 ± 6.33 | 33.20 ± 1.06 | 75.03 ± 1.49 | 75.86 ± 1.07 |
| % CD8 | 24.24 ± 0.75 | 24.51 ± 0.49 | 27.24 ± 1.20 | 28.78 ± 0.69 |
| TCRgδ+ | 3.40 ± 0.31 | 3.74 ± 0.31 | 4.14 ± 1.08 | 2.90 ± 0.15 |
| % CD8 | 55.50 ± 4.29 | 59.96 ± 3.62 | 57.57 ± 1.65 | 54.54 ± 3.07 |
| CD4+ CD8- | 43.18 ± 3.62 | 29.96 ± 0.91* | 58.81 ± 1.40 | 57.84 ± 1.02 |
| % CD25+ | 7.68 ± 0.40 | 5.33 ± 0.44* | 7.67 ± 0.32 | 8.56 ± 0.39 |
| % CD62L+ | 80.69 ± 0.91 | 77.31 ± 1.53 | 61.70 ± 4.59 | 49.18 ± 9.02 |
| % αE+ | 1.30 ± 0.15 | 1.49 ± 0.26 | 3.51 ± 1.65 | 11.46 ± 3.55 |
| CD8+ CD4- | 17.59 ± 0.52 | 16.20 ± 0.55 | 19.82 ± 0.82 | 20.12 ± 0.53 |
| % CD25+ | 4.29 ± 0.31 | 3.56 ± 0.47 | 3.77 ± 0.38 | 4.03 ± 0.40 |
| % CD62L+ | 66.59 ± 2.62 | 65.13 ± 1.65 | 68.40 ± 1.55 | 67.75 ± 3.99 |
| % αE+ | 0.89 ± 0.11 | 0.85 ± 0.14 | 1.27 ± 0.21 | 0.99 ± 0.25 |
| CD4+ CD8+ | 1.55 ± 0.67 | 1.42 ± 0.13 | 1.61 ± 0.06 | 1.59 ± 0.09 |
| % CD25+ | 73.51 ± 6.20 | 90.4 ± 3.63 | 53.86 ± 5.27 | 45.68 ± 3.79 |
| NK (TCRαβ- NK+) | 5.31 ± 1.36 | 6.42 ± 0.71 | 1.09 ± 0.11 | 1.10 ± 0.07 |
| % CD8 | 7.82 ± 1.78 | 6.28 ± 0.75 | 10.95 ± 1.19 | 13.50 ± 0.06 |
| NKT (TCRαβ+ NK+) | 2.59 ± 0.40 | 3.11 ± 0.30 | 1.87 ± 0.11 | 1.63 ± 0.10 |
| % CD8 | 45.83 ± 1.81 | 48.71 ± 2.04 | 61.59 ± 3.29 | 70.12 ± 1.02 |
| αE+ | 1.17 ± 0.11 | 1.65 ± 0.20 | 0.66 ± 0.10 | 0.60 ± 0.14 |
| CD62L+ | 62.72 ± 3.08 | 54.44 ± 1.24* | 72.16 ± 1.82 | 70.73 ± 2.07 |

Data are expressed as mean ± SEM. Statistical differences: *$p < 0.05$ vs REF (n=9–11)

### *3.11. Systemic and mucosal immunoglobulin profiles*

The impact of the synbiotic supplementation on the immunological profiles at both the systemic and mucosal levels was evaluated at day 28 of rats' life (Figure 9). In all compartments, the most abundant Ig is IgG, followed by IgM, with IgA being the less abundant. The synbiotic nutritional intervention did not result in any significant modifications in the total amount of IgG, IgM, and IgA in plasma and MLN. However, the SYN group exhibited a reduction in IgA levels in the SG (Figure 9a–c). In rats, there are four subtypes

of IgG (Ig1b, Ig2a, Ig2b, and Ig2c), and the relative proportions of each one were analyzed in each compartment (Figure 9d–f). In plasma, the IgG2b is the major Ig while in the mucosal compartments (SG and MLN) the predominant Ig is IgG2c. In the MLN of the SYN group, a reduction in the relative proportion of IgG2c and an increase in IgG2a were observed. The Th1/Th2 ratio was evaluated by measuring the levels of IgG2b and IgG2c (representing Th1) and IgG1 and IgG2a (representing Th2) (Figure 9g–i). The nutritional intervention resulted in a reduction of the Th1/Th2 ratio only in the MLN after one week of administration. To provide a comprehensive analysis of the Ig profiles, NMDS graphs were plotted (Figure 9j–l). After the synbiotic supplementation, there was no significantly different distribution of the clusters in any of the evaluated compartments.

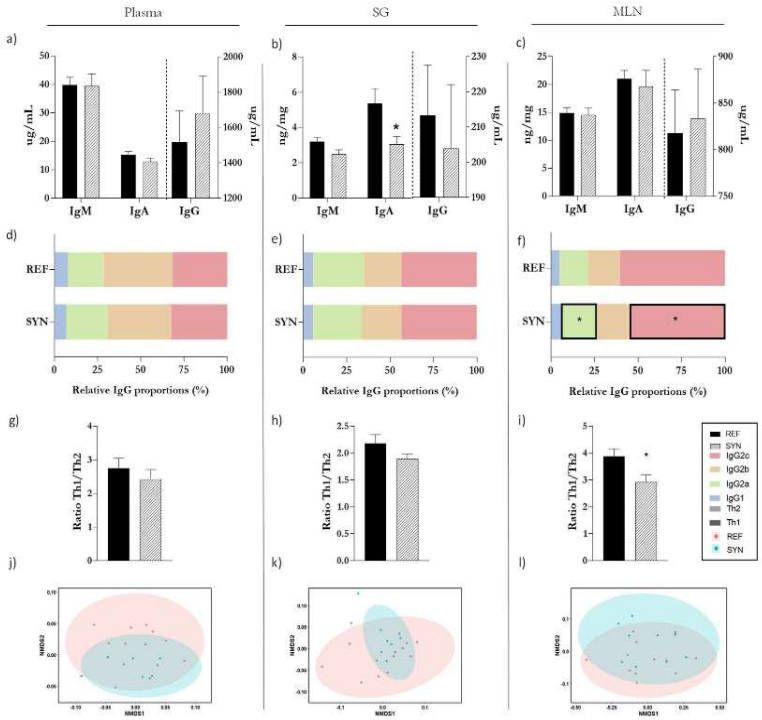



**Figure 9**. Immunoglobulin profile assessment in plasma, SG, and MLN at day 28 of life. Total levels of IgM, IgA, and IgG in plasma (a), SG (b), MLN (c); Relative proportion of IgG subtypes in (d) plasma, (e) SG, and (f) MLN; Analysis of the Th1/Th2 ratio in plasma (g), SG (h), and MLN (i). Analysis of non-parametric multidimensional scaling (NMDS) for the Ig profiles based on the Bray–Curtis distance in plasma (j), SG (k), and MLN (l). Results (a–i) are expressed as mean ± SEM. In NMDS plots (j–l), each point represents an animal by ANOSIM test $*p < 0.05$ vs. REF (n=9–11). SG, salivary gland; MLN, mesenteric lymph nodes; Ig, immunoglobulin.

## 4. Discussion

The present study confirms that synbiotics in early life contribute to a healthy development of the immune system, mainly affecting intestinal maturation and microbiota colonization.

Early life development is highly influenced by environmental factors. During the first 6 months of life, the World Health Organization (WHO) recommends exclusively breastfeeding for proper neonate maturation [38]. BM composition is enriched in bioactive compounds that contribute to nourishing the infant while conferring passive immunity. After weaning, the immune system continues its maturation process and nutritional habits contribute to boost the infant development [39]. In recent years, the introduction of pre-, pro-, and synbiotics in the infant diet has been further studied as an effective strategy to reinforce the immune system [40].

The gastrointestinal tract (GIT) is one of the principal channels of infections due to its constant exposure to external antigens. The reduction of the incidence of intestinal infections after boosting the functionality of the GIT with pro-, pre-, and synbiotics has gained interest in the last decade. Special focus has been given to the interaction between biotics supplementation and the intestinal microbiota. The modulation of the microbiota functionality through biotics contributes to the maintenance of a healthy status and reduces the development of asthma [41], necrotizing enterocolitis (NEC) [42], eczema [43] and inflammatory bowel diseases [44]. *Bifidobacterium* species have been widely studied since they modulate intestinal microbiota. To date, *B. breve* M-16V is a probiotic strain isolated from the infant gut that prevents NEC and reduces the incidence of infectious and atopic diseases when administered in early life [45,46]. Thus, *B. breve* M-16V metabolizes the human milk oligosaccharides (HMOs), which act as prebiotic substrates for the neonatal microbiota of the GIT [47].

Apart from the HMOs, *Bifidobacterium* species also metabolize non-milk oligosaccharides such as GOS, FOS, inulin, lactulose, or their combination [48]. Among the most studied probiotic substances that are metabolized by *B. breve* M-16V are scGOS/lcFOS, which contribute to the growth of the probiotic strain [49,50]. Due to the attributed benefits of the synbiotic mixture of *B. breve* and scGOS/lcFOS, their combination is used for infant formulas to mimic BM [25,51]. Considering the above, we hypothesized that supplementation with *B. breve* M-16V and scGOS/lcFOS during gestation and lactation to dams and continuing to provide it to the pups' rats after weaning may improve the maturation of the immune system. This period in rats is equivalent to the first 1000 days in humans' early life, which has been defined as relevant for infant development [19].

In the present study, animal body weight and diet intake were monitored daily. From birth, similar growth patterns were observed in both the REF and SYN groups, suggesting that the maternal and infant supplementation with the synbiotic did not affect overall growth. Food intake as well as organs' weight were similar in both groups. Other studies based on the administration of synbiotics showed that the growth pattern was not modulated [52–55]. The lack of impact on the infants' body weight after the maternal intervention, nor the continuation of the synbiotic administration into early childhood, raises any question about the safety of this nutritional intervention [54,56,57].

Regarding adiposity, the supplementation with the synbiotic mixture did not affect the weight of WAT but increased the weight of BAT. However, histological analysis of both tissues revealed no significant morphological changes. In metabolic pathologies such as obesity or diabetes, administration of synbiotics have improved impaired lipid parameters through the modulation of gut microbiota [58,59]. However, fewer studies have used synbiotics administration under healthy conditions. The study by Hosseinifard et al. evaluated the impact of a synbiotic (composed of a *Lactobacillus* strain and inulin) in healthy rats and concluded that the synbiotic contributed to the amelioration and reduction of oxidative parameters [57]. Further studies are necessary to evaluate the impact of the combination of B. *breve* M-16V and scGOS/lcFOS on the molecular pathways of BAT metabolism.

The observed trophic effect on the SI due to the synbiotic supplementation led to the analysis of the microscopic structures and the maturation stage of this organ. The HE staining showed an increase in the width and area of the villi, an increase in the crypt depth and a higher number of goblet cells per villi. Additionally, the gene expression of two inverse maturation markers, *FcRn* and *Blimp1*, was reduced after the supplementation. In the poultry industry, supplementation with synbiotics has been widely introduced to increase production and animal performance [60]. One of the most desired effects of the supplementation of the diet is the reduction of gastrointestinal infections by boosting the intestinal microstructures [60]. In addition, an increase in the villi height suggests a more effective absorption of the nutrients due to the higher surface area [61]. In the crypts, the stem cells are involved in the renewal of the epithelial cells, and deeper crypts are associated with increased tissue turnover, essential for maintaining the health and functionality of the SI, as a quicker turnover helps it to replace the damaged cells and to respond better to inflammation [62]. Moreover, *Blimp1*, is expressed during fetal development and is involved in promoting the adaptive and innate immune cell differentiation [63]. After birth, it is downregulated until reaching a low adult profile [64]. Additionally, it has recently been recognized that *FcRn*, responsible for transporting IgG across the placenta during fetal development and milk IgG during lactation, provides humoral immunity to newborns [65]. After weaning, *FcRn* expression is reduced as an indicator that there is no need for more milk IgG caption. So, despite the crucial roles that these markers play in early life, their expression diminishes after weaning, when maturation is achieved [64–66]. Consequently, the observed morphological changes and the reduction in these inverse maturation markers following supplementation indicates that synbiotics enhance intestinal maturation.

Additionally, genes associated with the intestinal barrier functionality were also studied. The synbiotic administration induced an increase in the *Zo1* gene and in the *Tlr9* after one week of supplementation. These molecules are implicated in intestinal immunity, and their modulation through diet has been proven to reduce pathogenic challenges [64]. The TJ proteins contribute to the maintenance of the physiological barrier modulating the transport of nutrients, water, and ions through the intestinal epithelium [67]. Probiotics and prebiotics supplementation has been linked to the modulation of the expression of TJ proteins by preventing its disruption under pathogenic conditions [68,69]. Additionally, weaning is a stressful situation for animals, during which the intestinal barrier suffers an imbalance. In this regard, supplementing weaned pigs with probiotics enhanced the intestinal barrier function, increasing the expression of *Zo1* and favoring the epithelial barrier integrity [70]. TLRs are involved in the recognition of microbial components, activating immunological responses [71]. The effect of *B. breve* in the modulation of the TLR signaling pathway by increasing the *Tlr9* and inducing an anti-inflammatory status has been widely described [72,73]. Taken together, our results suggest that the synbiotic mixture used maintains intestinal barrier functionality while stimulating TLR immunological responses.

In the intestinal compartment, the IgA interacts with the pathogenic bacteria, neutralizes them, and prevents infections. The effect of probiotics, prebiotics, and synbiotics on the intestinal sIgA under pathogenic and healthy conditions has been studied [74,75]. However, under healthy conditions it has been less well analyzed [76]. Our results suggest that the present synbiotic supplementation reduced the IgA mRNA even though this result was not reflected in terms of protein levels. This phenomenon suggests that different transcriptional modifications occur, but that they do not modify the protein levels in the intestine [77].

In the cecum, the IgA binds to the cecal bacteria, neutralizes them and facilitates their elimination [78]. Our synbiotic supplementation induced a reduction in the cecal free sIg but an increase in the bacteria and bound to IgA is seen. This result suggests that free sIgA was reduced due to IgA binding to bacteria after the synbiotic supplementation.

Although there was a positive impact on the Ig profile in the gastrointestinal tract, less impact was observed on the overall Ig profile. Only a punctual reduction of the sIgA in the SG and IgG2c in the MLN was observed, along with, an increase of IgG2a in the MLN. Despite these changes, the maturation ratio measured with the Th1/Th2 ratio responses was not affected in the plasma or in the SG. However, in the intestinal mucosal compartment, in the MLN, the Th1/Th2 ratio was reduced, indicating that the synbiotic supplementation did not shift toward a Th1 response. At birth, the immune system is biased toward a Th2 response; as it matures, the immune response switches towards a Th1 response [79]. Although the nutritional intervention did not promote the intestinal maturation regarding the Ig profile, the maturation markers evaluated at gene expression level indicated a higher maturation status in the SI.

Supplementation did not affect the overall lymphocyte subsets in the MLN or in the spleen. Different studies have demonstrated an impact on the lymphocyte markers after synbiotic supplementation [80,81]. However, it needs to be considered that the synbiotics effects are dose- and strain-specific and that the duration of these nutritional studies was longer than a week and have not been evaluated when the supplement was administered to the mother. Further studies considering long-term supplementation could elucidate the same results after nutritional interventions.

Regarding the main changes in the microbiota composition in the intestine, an increase of *Bifidobacterium* and *Turicibacter* and a reduction in *Ligilactobacillus* was observed after the synbiotic supplementation. It is widely described that breastfeeding contributes to *Bifidobacterium* colonization of the infant gut compared to that of the formula-fed infant. However, in this case, the higher levels of *Bifidobacterium* may be derived, at least partly, from the direct pup supplementation after weaning. Yin et al. supplemented weaned pig with oligosaccharides and found an increase of the *Turicibacter*, which may mediate host metabolism and physiological functions [70,82]. Although the data of the intestinal microbiota changes after synbiotic supplementation is limited, our results confirm that synbiotic supplementation modulates the intestinal microbiota, favoring its colonization and improving its functionality.

In the cecum, the *Bifidobacteriaceae* abundance was increased and a reduction of that of *Staphylococcaceae* was found after the synbiotic intervention. This result is in line with other studies showing that some synbiotics contribute to reducing the proportion of *Staphylococcaceae* [83,84]. The genera *Bifidobacterium*, *Blautia*, *Faecalibaculum*, and *Lactobacillus* were increased after the supplementation. The cessation of breastfeeding is linked with the increase of these genera [85]. *Bifidobacterium* and *Lactobacillus* are well known as probiotics for their potential to produce SCFAs and bacteriocins. However, the importance of *Blautia* has been of particular interest lately due to its ability to alleviate inflammatory and metabolic diseases and for its antibacterial activity [86,87]. The increase of the *Bifidobacterium*, *Blautia*, *Faecalibaculum*, and *Lactobacillus* genera has been reported previously by Qi Zhang et al., when a synbiotic (*Bifidobacterium* and FOS) was added to an *in vitro* fermentation model [88]. All these data, joined with the presence of the administered probiotic strain in the CC, corroborate the fact that the supplementation contributes to the colonization of the cecal microbiota after one week of supplementation.

The functionality of the microbiota is highly linked to the fermentation of the unfermented fibers, including the fermentation of the GOS/FOS. The fermentation of the undigestible carbohydrates and the release of the SCFAs occurs throughout the entire GIT [89]. However, the cecum has been described as the most active place for the production of SCFAs [90,91]. According to our results, the reduction of the SCFAs in the IC could be associated with the lower bacteria diversity compared to the CC and its rapid absorption by the intestinal cells. However, a more in-depth investigation into the mechanism of SCFAs production and their functionality in the intestine is needed to complete the results.

In the cecum, the overall increase in the SFCA production after the supplementation may be attributed to an increase of the most abundant acids – acetic and propionic – which

are produced in large quantities by *Bifidobacterium* populations [49,92]. Also, the SCFAs increase could be linked to *Faecalibaculum*, which has been recently described as a SCFA producer [93]. Additionally, other SCFA such as valeric, isovaleric, isobutyric, and hexanoic, are linked to *Clostridium difficile* strains [94]. However, we did not find any differences in the proportions of *Clostridium* genera after the supplementation.

Apart from the positive results of the synbiotic intervention, some limitations were found within the study. In general, preclinical studies are really useful to understand the biological mechanisms and interactions. However, the translational value of in vivo/preclinical and human clinical results is not always clear – for example, the microbiota colonization process and some biochemical pathways differ among species. Additionally, clarifying which period (gestation, lactation, or early childhood) is the most relevant period in the offspring's development and which is the most appropriate in which to perform synbiotic nutritional intervention has to be analyzed.

## 5. Conclusions

This study provides valuable insights into the potential of *B. breve* M-16V and scGOS/lcFOS intervention during gestation, lactation, and early life. It positively shapes the maturation and functionality of the intestinal barrier and microbiota, ultimately contributing to enhanced gastrointestinal immunity in the offspring. The influence of the maternal diet during pregnancy and lactation also contributes to the maturation of the infant, which is highly important for the first 1000 days of life. Future investigations are needed to explore the long-term consequences of maternal synbiotic supplementation for offspring health into childhood.

**Supplementary Materials**

**Supplementary Table 1.**

**Supplementary Table 1.** Description of the specific TaqMan primers AB.

| Gene | Reference |
|---|---|
| *Tlr2* | Rn02133647_s1, I |
| *Tlr3* | Rn01488472_g1, I |
| *Tlr4* | Rn00569848_m1, I |
| *Tlr5* | Rn04219239_s1, I |
| *Tlr7* | Rn01771083_s1, I |
| *Tlr9* | Rn01640054_m1, I |
| *Muc2* | Rn01498206_m1, I |
| *Muc3* | Rn01481134_m1, I |
| *Ocln* | Rn00580064_m1, I |
| *Cldn2* | Rn02063575_s1, I |
| *Cldn4* | Rn01196224_s1, I |
| *ZO1* | Rn02116071_s1, I |
| *IgA* | 331943, made to order |
| *Blimp1* | Rn03416161_m1, I |
| *Fcgrt* | Rn00566655_m1, I, encoding for FcRn |
| *Gusb* | Rn00566655_m1, I |

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**Supplementary Table 2.**

**Supplementary Table 2.** Lower limits of detection of Procartaplex

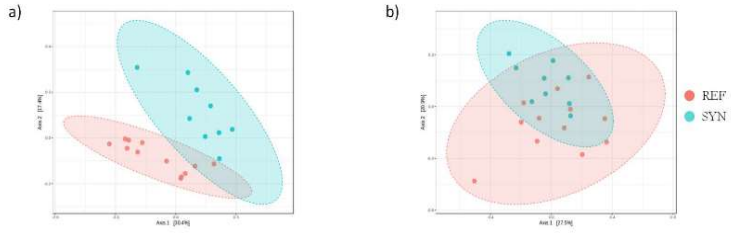
| Immunoglobulin | Lower limits of detection |
|---|---|
| IgA | 0.58 ng/mL |
| IgM | 0.2 ng /mL |
| IgG1 | 1.70 ng/mL |
| IgG2a | 1.73 ng/mL |
| IgG2b | 2.67 ng/mL |
| IgG2c | 3.67 ng/mL |

**Supplementary Table 3.**

**Supplementary Table3.** DESQ2 test in cecal content (CC) and intestinal content (IC).

| | | log2FC | lfcSE | Pvalues | FDR |
|---|---|---|---|---|---|
| CC | *g_Eisenbergiella* | 86.576 | 10.195 | 2,04E-17 | 1,53E-12 |
| | *g_Candidatus_Stoquefichus* | -94.724 | 18.487 | 3,00E-07 | 1,12E-01 |
| | *g_HT002* | 40.062 | 0.92113 | 1,37E-05 | 3,42E+00 |
| | *g_Faecalibaculum* | 52.232 | 13.412 | 9,84E-05 | 0.0018447 |
| | *g_NK4A214_group* | -37.307 | 0.98073 | 1,42E-04 | 0.0021355 |
| | *g_Bifidobacterium* | 42.101 | 11.336 | 2,04E-04 | 0.0025496 |
| | *g_Rikenellaceae_RC9_gut_group* | -17.872 | 0.48891 | 2,57E-04 | 0.0027506 |
| | *g_UCG_009* | -3.335 | 0.94976 | 4,46E-04 | 0.0041791 |
| | *g_Marvinbryantia* | 40.049 | 12.165 | 9,94E-04 | 0.0077699 |
| | *g_otherLachnospiraceae* | -14.485 | 0.44153 | 0.001036 | 0.0077699 |
| | *g_Ruminococcus_gauvreauii_group* | 19.864 | 0.6609 | 0.00265 | 0.018068 |
| | *g_Family_XIII_AD3011_group* | -24.779 | 0.9138 | 0.0066948 | 0.041843 |
| | *g_Blautia* | 26.647 | 11.007 | 0.015482 | 0.082941 |
| | *g_Tuzzerella* | -37.756 | 17.751 | 0.033421 | 0.15419 |
| | *g_Ligilactobacillus* | 12.115 | 0.57356 | 0.034666 | 0.15419 |
| | *g_Intestinimonas* | -0.96904 | 0.46461 | 0.037006 | 0.15419 |
| | *g_Lactobacillus* | 43.247 | 21.596 | 0.045227 | 0.17853 |
| IC | *g_Escherichia_Shigella* | -3.237 | 0.74934 | 1,56E-02 | 5,78E+00 |
| | *g_Turicibacter* | 24.926 | 0.68571 | 2,78E+00 | 0.0051416 |
| | *g_Hungatella* | 54.855 | 1.607 | 6,41E-01 | 0.0079106 |
| | *g_Lactococcus* | -19.655 | 0.63821 | 0.0020723 | 0.019169 |
| | *g_Enterobacter* | -33.948 | 11.707 | 0.0037344 | 0.027635 |
| | *g_Lachnoclostridium* | 25.312 | 10.294 | 0.013935 | 0.085934 |
| | *g_Ligilactobacillus* | -14.179 | 0.60834 | 0.019768 | 0.10449 |
| | *g_Lachnospiraceae_NK4A136_group* | -24.662 | 12.206 | 0.043325 | 0.1924 |
| | *g_Erysipelatoclostridium* | 2.342 | 1.178 | 0.046799 | 0.1924 |
| | *g_otherDesulfovibrio* | -33.991 | 18.096 | 0.060331 | 0.22323 |

**Supplementary Figure 1.**




**Supplementary Figure 1.** Analysis of non-parametric multidimensional scaling (NMDS) for the microbiota profiles based on the Bray–Curtis distance in (a) IC and (b) CC. Statistical testing was performed by PERMANOVA using Bray–Curtis distances and the Mann–Whitney test was used for alpha-diversity indexes. Each point in NMDS represents an animal using the ANOSIM test. Statistical differences: $^{*}p < 0.05$ vs. REF (n=9-11).

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# DISCUSSION

The first 1000 days of life represents the initial period of life, since the intrauterine growth of the fetus until the second year of life when solid food has already been included in infant nourishment[274]. Maternal nutrition during pregnancy influences the maternal health status reducing pregnancy complications and contributing to the fetal development[122]. Later, during breastfeeding the maternal nutrition impacts the breast milk (BM) composition[84,92].

BM is the most suitable nutrition for newborns, ensuring proper infant development. It is rich in various bioactive compounds, including prebiotic substances like human milk oligosaccharides (HMOs) and beneficial bacteria. These components collectively contribute to infant maturation and reduce the incidence of early-life infections[92]. Even though breastfeeding is beneficial for both mother and infants, in many occasions there are complications that led the mother to decide to feed the newborn with infant formulas[275]. To reduce the developmental gap between breast-fed infants and formula-fed infants, the Food and Drug Administration (FDA) decided to add different compounds to infant formulas. The most common included compounds are probiotics, prebiotics and combinations (synbiotics) to mimic the BM composition[92].

Probiotics, prebiotics and synbiotics have been identified as therapeutic strategies to modulate the composition and metabolic activity of the gut microbiota. The combination of probiotics and prebiotics pretend to maximize the effects of both supplements. Among them, supporting the probiotic bacteria's survival and promoting the production of beneficial metabolites such as short chain fatty acids (SCFAs) are highlighted[276].

The benefits of probiotics are strain-specific while the benefits of prebiotics are type dependent[178,277]. According to this, the benefits of synbiotics depend on the probiotic and prebiotic selected and the proportion of each component. The most common combinations of synbiotics are made of probiotics from the genus *Lactobacillus*, *Bifidobacterium,* or *Streptococcus* and prebiotics like oligosaccharides, inulin, or fibre[212.]

Synbiotics have been used in both healthy conditions to prevent infections and in pathological conditions to ameliorate some aspects of the disease. For example, they have been used to treat metabolic disorders such as obesity, overweight and metabolic syndrome, inflammation, irritable bowel syndrome and atopic dermatitis, among others[212].

Recent advances have shown that modulating the maternal diet during pregnancy and lactation can influence the BM composition and, consequently, the infant maturation[84,132,133]. Incorporating synbiotics into the maternal diet during these periods has been part of a strategy to boost the maternal immune system, to reduce adverse outcomes and to support the fetal and infant development prior to birth[278–281]. These approaches finally potentiate the development of the immature newborn reducing their risk of infections.

Herein, we have chosen a synbiotic composed of *Bifidobacterium breve* M-16V and short chain galacto-oligosaccharides (scGOS) and long chain oligosaccharides (lcFOS) in a 9:1 ratio.

*Bifidobacterium breve* M-16V is one of the most used probiotics in early life. It was firstly isolated from the infant gut[282]. *B. breve* M-16V specific effects are based on its anti-inflammatory effects, mucosal regulation, induction of oral tolerance and IgA expression, reduction of gut tissue injury, attenuation of allergic reactions and modulation of the microbiota composition in early life[283–286].

Synthetic prebiotics such as scGOS and lcFOS have demonstrated also beneficial effects similar those observed due to the prebiotic compounds of the BM. Additionally, the combination of GOS and FOS in a 9:1 ratio have demonstrated the most comparable results to breast-fed infants[287].

Lastly, the combination of *Bifidobacterium breve* M-16V and scGOS/lcFOs in a 9:1 ratio has reported lower incidence of viral infections, dermatitis, allergy and asthma outcomes in early life[217,235,267,288].

During gestation and lactation, an active communication is stablished between the mother and the newborn. The transference of maternal bioactive compounds during pregnancy and lactation provides passive immunization and contributes to the early life programming which reduces the incidence of short- and long-term health outcomes[117,289]. The maternal passive immune protection englobes the transference of immunoglobulins (Igs), cytokines (CKs), hormones, immune cells, microbiota and metabolites through the placenta and the BM. All these compounds contribute to the development of the fetus prior to being born and during the first months of life of the newborn to complete his/her maturation and reducing infection susceptibility[289].

In early life, newborns remain immature being susceptible to infections. Rotaviruses (RVs) are one of the main infectious agents causing acute gastroenteritis among children under 5 years old[166]. RVs infections lead to high morbidity and mortality, especially in developing countries. In the recent years the introduction of immunization programs has notably reduced the infant mortality[166]. Despite this progress, once the infant suffers the RV infection and develops diarrhea, the only available treatment is oral rehydration. However, on many occasions, the diarrhea is severe and leads on infant hospitalization[290].To prevent infant hospitalization, some strategies target the reduction of the incidence and severity of the infection. They are mainly based on breastfeeding and supplementation with probiotics and prebiotics[237,291,292].

As mentioned before, one strategy to enhance infant development before birth is through potentiating maternal health during pregnancy and continuing in lactation. Due to ethical reasons, there is limited research focus on the physiological and immunological changes

that occur during pregnancy and lactation in humans. Animal models are frequently used to overcome this gap by simplifying the process of collecting certain invasive samples. In this study, Lewis rats were chosen because they are suitable for nutritional studies[293,294]. Additionally, Lewis rats have demonstrated to be a good model for RV infections because they are more susceptible to RV infection compared with other models like Wistar rats[295]. In particular, RV infection in Lewis rats resulted in a mild diarrhea without affecting their growth pattern while Wistar rats are resistant to RV infection due to its capacity to avoid the natural entry of the virus into the epithelial cells[295].

In the present study, the RVs SA-11 heterologous group A was chosen[174]. This mild infection and diarrhea induced allows the effective evaluation of the maternal synbiotic supplementation impact to boost the infant immune system and its ameliorating effect on the RV diarrhea. As mentioned before, Lewis rats has been widely used in our group as model for RV infections in early life[235,237,292,296,297]. To counteract the RV induced diarrhea, several strategies based on infant supplementation with probiotics (*B. breve* M-16V, *L. acidophilus* NCFM, *L. hevelticus* R0052), prebiotics (GOS, FOS, pectin-derived acidic oligosaccharides) and HMOs (2'-FL) have been performed. These studies generally showed positive effects, reducing the severity and incidence of viral infection in early life[235,237,292,296,297]. Recently, an additionally strategy to enhance infant maturation and its ability to counteract infections has been established. This approach is based on the modulation of the maternal nutrition during pregnancy and lactation. Specifically, we aim to supplement the maternal diet with prebiotics and probiotics to observe the transference of their beneficial effects to the offspring.

This thesis examines the potential effect of the maternal synbiotic (*Bifidobacterium breve* M-16V and scGOS/lcFOS) supplementation during pregnancy and lactation on the maternal and offspring health status. Firstly, it analyzes the impact of this synbiotic supplementation during pregnancy and lactation on the maternal immune system and milk composition. Secondly, it evaluates the effect of the RV infection in early life and the potential benefits of maternal intake of probiotics and prebiotics to counteract their infant's infection. Thirdly, it explores the maternal synbiotic supplementation during pregnancy and lactation on the offspring at the end of suckling. Lastly, it analyzes the beneficial effects of the synbiotic exposure during the rat period that mimics the first 1000 of life by the administration of the synbiotic during pregnancy and lactation to the mother and to the infant the first week-post weaning. The principal findings observed throughout the study are summarized in Figure 17.


**Figure 17. Principal effects of the maternal synbiotic supplementation observed throughout the study.** *The induced changes were analyzed on the mother, on the breast milk and on the offspring at day (d) 8 of life during RV infection, at day 21 of life and at day 28 of life. All comparisons are made with respect to the reference (REF). "↑" represents a statistically higher value than that in the REF; "↓" represents a statistically lower value than that in the REF; Blue arrows represent a tendency (0.05<p<0.1); "=" indicates no change. Empty spaces indicate that the variable was not assessed in that samples.*

*Immunoglobulin (Ig), Mucin (Muc), Toll-like receptors (TRLs); ocludin (Ocln); Brown adipose tissue (BAT); Mesenteric lymph nodes (MLN); Salivary gland (SG); Short chain fatty acids (SCFAs); Neonatal receptor Fc (FcRn); B-lymphocyte-induced maturation protein 1 (Blimp1); Rotavirus (RV); Ig-coated bacteria (Ig-CB). *The protein quantification of the IgA in the gut wash revealed no changes of the total IgA protein levels. However, the quantification of the IgA gene expression in the same intestinal simples showed lower levels of IgA mRNA.*

Probiotic bacteria primarily exert their effects in the gut. They travel through the digestive tract, arrive to the intestine and can then be detected in the last part of the gut or in feces[89]. To review the tracking of the administered bacteria (Figure 18) we checked first its presence in the maternal feces during pregnancy and lactation. As expected, in the supplemented group (SYN) the presence of *B. breve* M-16V was increased 1000 times in feces with respect to the non-supplemented dams (REF) both at the end of pregnancy and at the end of the lactation period (**Article 1**). During lactation, certain bacteria can be transported to the mammary gland via specific entero-mammary pathway. Selected bacteria are internalized by mononuclear cells in the gut and migrate to the mammary gland. As a result, they are transferred to the BM and to the neonate and contribute to the colonization of the infant[298]. The recognized species in humans that enter in the entero-mammary gland and are detected in both maternal BM and infant gut are *B. breve, B. longum, E. faecalis, L. fermentum, L. gasseri, L. salivarius* and *S. epidermidis* [299–302]. In this study, after the synbiotic supplementation, the probiotic *B. breve* M-16V was not detected either in BM or in the mammary gland, at least in the unique sample was obtained from the animal (**Article 1**). However, we cannot discard, that in another sampling, the bacteria could be present. In addition, we have to consider the low levels of bacteria in BM, thus the bacteria could have been present but under our limit of detection.

Anyway, although *B. breve* M-16V was not detected in the BM, we checked if it has been transferred somehow to the offspring at different stages of the infancy. The analysis performed at day 8 of life, during strict breastfeeding, revealed that the *B. breve* M-16V was detected in the 50% of the fecal samples of the neonatal rats (**Article 3**). This result observed in first half of lactation suggested that the supplemented probiotic to the mother during pregnancy and breastfeeding, reaches infant's gut. Consequently, the bacteria somehow is transferred to the infant, potentially influencing the gut microbiota colonization in early life.

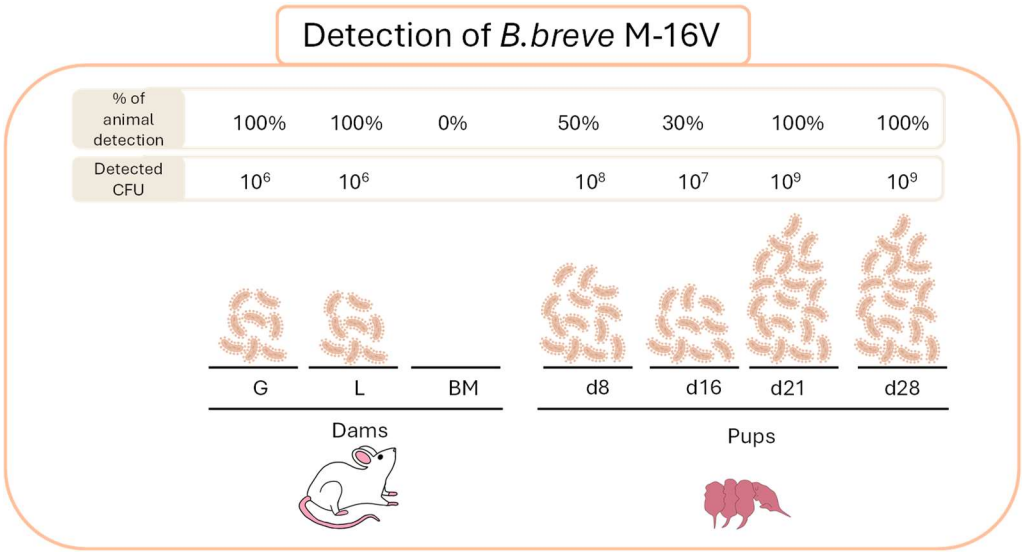


**Figure 18**. Detection of *Bifidobacterium breve* M-16V throughout the study. *Detailed information of the percentage of detection in the supplemented group and the detected colony-forming units (CFU). G, gestation; L, lactation; BM, breast milk; d, day of pups' life.*

Later, the tracking of the probiotic strain was performed during the second half of the lactation and at the end of suckling (**Article 4**). The results showed that in the middle of lactation, the probiotic was detected in only 30% of the pups in the SYN group. However, by the end of suckling, the administered probiotic was detected in 100% of the SYN pups. The last high percentage found can be justified due to the coprophagic behavior of the rats. Pups begin to chew by day 16 of life, a few days before weaning. Consequently, the SYN pups may chew mother's excrements to obtain the probiotic that was given to them. Therefore, probiotic bacteria also reached some offspring's gut during breastfeeding (**Article 3**). And, may be due to their coprophagic behavior, all pups acquired the administered probiotic at the end of suckling.

Finally, the probiotic bacteria at day 28, after one week of direct synbiotic supplementation, was detected in 100% of the feces from the SYN pups (**Article 5**). In addition, the amount of these bacteria in feces was higher in these animals than in the maternal feces. This can be due because the probiotic strain comes from an infant intestine and could be more adapted to this young intestine environment. It can be also due to the

differential dose administered between mothers and infants. These results agree with the maternal data when the probiotic was directly administered (**Article 1**).

It's important to note that detecting probiotic bacteria that enter into the entero-mammary route can be challenging. Previous studies have concluded that probiotic strains such as

*Lactobacillus* CECT5716 and *Bifidobacterium longum subsp longum* enter in the entero-mammary route. These strains have been detected in BM, in the mammary gland or in the infant intestine after maternal supplementation[89,299,301]. However, other authors indicated that after supplementation of *L. fermentum* CECT5716 to pregnant rats, only some milk samples and some cecal samples of the offspring were positive for the administered probiotic[89].

*To sum up, the findings indicate that direct probiotic supplementation with B. breve M-16V leads to colonization of both maternal and infant gut. Also, although we have not been able to demonstrate the probiotic transference through the entero-mammary pathway due to their absence in both BM and mammary gland, we can confirm that the probiotic reach somehow the offspring during breastfeeding. As a result, the probiotic supplementation during pregnancy and breastfeeding influences the offspring gut colonization in early life. Further investigations are needed to understand the mechanism of transference of the probiotic to the offspring.*

**Immunological impact of synbiotic supplementation during pregnancy and lactation on the maternal immune system, adipose tissue and microbiota**

The first specific objective was assessed in the **Article 1**. Herein, it is discussed the potential benefits of the synbiotic supplementation to improve the maternal immune system during the pregnancy and lactation (Figure 19).

Pregnancy is a well-established period of changes and adaptations. The maternal immune system suffers certain suppression of the immune system modulating the Th1/Th2 balance towards a Th2 phenotype to avoid the reaction against the fetus, but then leading to a higher susceptibility to infections[4]. In agreement with this immunological adaptation, the lipid metabolism, the gastrointestinal system, the immune phenotypes and the microbiota, undergo to different changes.

One of the most characteristic features of the pregnancy is the increase in the body weight. Several studies have evaluated that the body weight of the mother before pregnancy may affect the infant health outcomes[303]. The maternal weight gain during pregnancy is an indicator of the prediction of morbidity and mortality in infants and mothers[304]. For example, an excessive body gain weight during pregnancy increases the risk of child overweight in early life[305]. We checked if the synbiotic supplementation affected the maternal body weight changes during pregnancy and lactation. The supplemented (SYN) and non-supplemented (REF) dams followed the same body gain pattern during pregnancy and breastfeeding. Thus, it can be confirmed that the synbiotic intake during these periods did not alter the regular body weight changes, and together with the lack of affectation on the hematological parameters and organs weights, can be considered as a safe approach and therefore not increasing outcome risks for the infant and the mother.

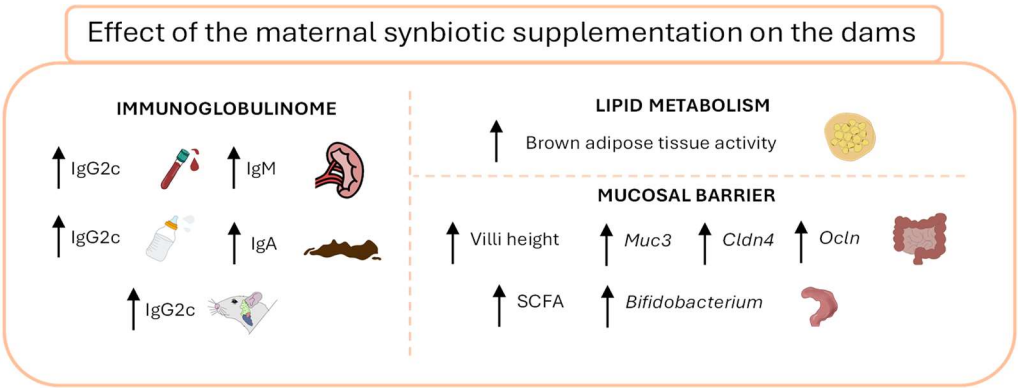


**Figure 19.** Summary of the principal effects of the maternal synbiotic supplementation on the mother. *The maternal nutritional intervention modulates the immunoglobulin (Ig) profile of different fluids and compartments, such as blood, milk, mesenteric lymph nodes, feces and salivary gland. Also, modulates the activity of the brown adipose tissue, improves the mucosal barrier enhancing the expression of different genes and by modulating the cecal microbiota and metabolome of the cecum. Muc, mucin; Cldn, claudin, Ocln, occludin; "↑" increase; "↓" decrease.*

During pregnancy the fatty acid metabolism changes to support the fetal growth and development[306]. These metabolism changes are reflected in the lipid profile. Indeed, the fat reservoirs increase during first and second trimester[306]. Although the increase of the adipose tissue  a common change in this period, the overexpansion of the adipose tissue during this period may increase the risk of alterations in both the mother and the infant[307]. Considering the importance of the maternal diet during pregnancy, its modulation towards a healthy diet including the supplementation with prebiotics and probiotics may contribute to control the accumulation of the adipose tissue and the metabolism of lipids[308].

The brown adipose tissue (BAT) is involved in the maintenance of the body temperature, and it is diminished in obese individuals. Its activity can be measured checking its morphology (the number of nuclei and the area of the lipid droplets (LDs))[309] and the expression of certain genes[310,311]. An increase in the number of nuclei and a reduction in the area of the LDs is associated with a higher activity of the BAT[312]. In the present study the synbiotic supplementation during pregnancy and lactation contributed to enhance the activity of the BAT, modulating its morphology towards an active structure and increasing the expression of *uncoupling protein* 1 *(Ucp1)* (**Article 1**). The *Ucp1* is an important marker of the BAT activity and its upregulation is linked to the thermogenic activity of the BAT. In fact, nutritional studies have demonstrated the potential of the diet modulation to enhance its expression[313,314]. All these contribute to conclude that synbiotic supplementation modulates the adipose tissue metabolism towards a higher activation contributing to reduce the risk of fat accumulation.

The maternal humoral and cellular responses adapt their functionality during pregnancy to avoid reactivity against the fetus while transfer immunity passively but protecting the mother against infections during conception[315]. Igs are the key components of the humoral immunity, and the levels of each Ig isotypes depend on their functionality[316]. The IgG levels during pregnancy have been discussed in the last 30 years, some authors suggested that total IgG levels remain stable[317,318] while some others demonstrated a decrease in late pregnancy[319,320]. In spite of the controversial data about the IgG levels during pregnancy, their plasma and milk concentrations during breastfeeding are less studied. IgG is the unique Ig that is transferred across the placenta via the neonatal receptor Fc (FcRn) providing passive immunity to the fetus. The IgG's affinity for the receptor and therefore to be transferred depends on the IgG subtype. In humans, IgG1 has the highest affinity for FcRn[40]. Comparing humans' and rats' Igs is quite challenging. One of the most accepted approaches divide the IgG isotypes using their association to either the Th1 or the Th2 responses. In rats the IgG2b and IgG2c are Th1-associated isotypes and IgG1 and IgG2a are Th2-associated isotypes while in humans IgG1, IgG2, and IgG3 are Th1-associated IgG isotypes and IgG4 is Th2-associated[79,321]. Herein, the Ig profile was checked at the end of lactation. The results indicated that synbiotic supplementation tends to increase the plasmatic IgG levels. This increase is highly linked to the increase in the proportion of the IgG2c (**Article 1**). The functionality of the IgG2c in rats is not acutely described, however, its analog in mice, the IgG3 is involved in regulatory responses in the neonate's intestine and in long term immunity[322]. The fact that the nutritional intervention enhances the production of this IgG could be linked to a higher transference to the fetus through the placenta boosting the passive immunization. The effect of certain bifidobacteria on the humoral immunity enhancing the IgA, IgM and IgG levels in plasma has been already described[323]. However, it has not been analyzed either during pregnancy or supplemented as a synbiotic instead of alone.

Regarding the cellular immunity, the spleen and the mesenteric lymph nodes are valuable sites for the understanding of the immunological responses at systemic and mucosal levels, respectively[324,325]. In the present study, a low impact was observed in the cellular immunity with the supplementation during pregnancy and lactation without any particular effect on each of the immune lineages. Previous studies have demonstrated the potential effects of prebiotics and probiotics on the immunological cells but without analyzing them in pregnancy[326–330].Punctually, a study supplementing GOS and inulin during late pregnancy in mice demonstrated changes in the B cell activation in the placenta and uterus[331].

In pregnancy, the maternal intestine adapts its structure to meet the higher nutrients demands[332]. The nutrients absorption occurs along the gastrointestinal tract but specially in the small intestine. One of the mechanisms to optimize the nutrients absorption is maximizing the surface area available for this function[333]. The intestinal epithelium layer is formed by villi that contain the enterocytes that absorb nutrients[334]. In the base of the

villi, invaginations are found forming the crypts. The intestinal crypts are composed of goblet and Paneth cells, which secrete mucus and antimicrobial peptides[335]. Diet modulation can contribute to enhance the activity of intestinal cells, increasing nutrient absorption and their secretory functions[336–338]. Apart from the diet modulation, the supplementation with probiotics and prebiotics can also potentiate the functionality of the intestinal cells favoring the nutrient absorption[339]. In the present study when *B. breve* M-16V and scGOS/lcFOS was administered during pregnancy and breastfeeding the villi height and the crypts depth were increased when compared to those from REF animals. These results confirmed that maternal synbiotic supplementation boost the maternal gut enhancing nutrients uptake to better achieve the nutritional demands.

In the small intestine apart from nutrients absorption, the intestinal epithelium acts a physic barrier to protect the host from invasive pathogens[340].To maintain the functionality of the integrity of the intestinal barrier and homeostasis, different proteins are involved. Claudins and occludins are the most relevant tight junction (TJ) proteins involved in maintenance of the physical barrier of the intestine. Upon luminal stimulation, the TJ proteins allow the pass between intestinal cells, leading to paracellular flux of ions, solutes and water from the intestinal lumen, through the lamina propia to the blood[341]. Apart from the TJ proteins, mucins form the intestinal mucus layer are also involved in protecting the intestinal cells[342]. Nutritional interventions, including diet modifications and biotics administration have demonstrated a positive impact on the intestinal integrity by stimulating the intestinal immune system and reducing infections incidence[343]. In this study, the maternal synbiotic administration during pregnancy and lactation demonstrated the potential effects of *B. breve* M-16V and scGOS/lcFOS to improve the integrity and homeostasis of the small intestine increasing the gene expression of mucins (*Muc3*) and TJ (*Clnd4* and *Ocln)* (**Article 1**). It would be necessary though, to confirm these observations at protein level, either quantifying their protein levels in those which are secreted or their localization in an intestinal histological study. The positive effects of these two biotic compounds have been studied separately, and similar results have been observed, restoring or increasing the presence of the TJ proteins or mucins in the intestine[344,345]. According to this, enhancing the function of the gastrointestinal system during pregnancy by introducing a synbiotics into the mother's diet may help lower the incidence of intestinal infections in the mother.

Mucosal immunity is highly mediated by the sIgA which is continuously exposed to antigens, food and microorganisms. sIgA protects the host by neutralizing toxins and viruses, blocking colonization and penetration of pathogenic bacteria[346]. IgA interacts with the pathogenic bacteria, coats them and enhances their elimination[347]. During pregnancy, the IgA is not transferred to the fetus through the placenta, however, it plays an important role after birth, as it can be transferred to the newborn through the BM. Data of the present study indicated that the synbiotic supplementation during pregnancy and lactation increased the amount of IgA in the intestine and also enhanced its neutralizing capacity

by the results observed in the Ig-coated bacteria (Ig-CB) analysis (**Article 1**). This functionality of probiotics, prebiotics and synbiotics has been previously demonstrated in human and animal models[348–350]. Particularly, to potentiate the production of IgA, the supplementation with probiotics and prebiotics like *Bifidobacterium spp* and fructo-oligosaccharides seem to be good strategies[351,352]. These results suggest that the synbiotic supplementation during these periods enhance the maternal gastrointestinal immunity which will contribute to the maternal protection.

Probiotics and prebiotics modulate intestinal microbiota composition due to their ability to colonize the gut and to be available substrates that ensure the growth and survival of the host bacteria and the administered probiotics, respectively[353]. Pregnancy is linked to an imbalanced gut microbiota reducing the alpha and increasing the beta diversity[354,355]. Additionally, it is known that maternal microbiota alterations impact on the offspring microbiota colonization[356]. According to these statements, promoting beneficial bacteria in the mother could contribute to the colonization of beneficial bacteria in the offspring. One of the mechanisms to attenuate the maternal dysbiosis during pregnancy is through the enrichment of the diet with fiber or by the intake of supplements such as probiotics and prebiotics during this period[281,357–359].In this study, the supplemented dams showed higher abundance of cecal *Bifidobacterium* and *Faecalibaculum* at the end of lactation (**Article 1**). One of the attributed features of prebiotics are the growth promotion of certain microbiota groups, particularly, they induce the proliferation of *Bifidobacterium spp*. This phenomenon is denominated as bifidogenic effect[183,360]. *Bifidobacterium spp* have been described as probiotic species and are believed to exert positive health benefits on their host[361]. Considering all above, the supplementation with *B. breve* M-16V and scGOS/lcFOS during pregnancy and lactation contributes to the modulation of the maternal microbiota towards a more beneficial microbiota profile, which will impact on the offspring colonization.

SCFAs are the principal metabolites produced by the intestinal microbiota after the fermentation of non-digestible fibers. SCFAs provide immunostimulatory signals that activate innate and immune responses, are involved in energy metabolism, maintenance of the intestinal structure and integrity and participate in the immune regulation of anti-inflammatory activities[362,363]. Apart from the inner benefits of the SCFAs, during pregnancy they are able to cross the placental barrier collaborating in fetal development[19]. This research shows that the synbiotic supplementation during pregnancy and lactation increases the overall levels of cecal SCFAs, specifically with a rise in acetic, propanoic, butanoic and isovaleric acids (**Article 1**). The increase in these acids could be attributed to the observed changes in the microbiota populations. To date, *Faecalibaculum* has been described as a producer of butanoic acid and *Bifidobacterium* as acetic and propanoic producer[364,365]. Other strategies based on probiotic, prebiotics and synbiotics supplementation have demonstrated similar results by increasing the proportion of SCFAs in the gut, which impact positively on the intestinal health[366,367].

*Joining all these, the present study confirmed that a synbiotic supplementation during pregnancy and lactation is safe and impacts positively the maternal immunity, the intestinal and microbial functions, and even the adipose tissue activity.*

**Impact of synbiotic supplementation on breast milk composition**

Besides the vertical transference of nutrients and bioactive compounds during pregnancy, after birth, breastfeeding is the suitable option for newborn nourishment. The bioactive composition of the BM contributes to the maturation of the neonate immune system and the microbiota colonization[368]. Furthermore, maternal diet and habits influence the BM composition. Therefore, the maternal supplementation with probiotics and prebiotics during lactation is a possible option to enrich its composition[369]. In this study, it was evaluated the effect of the maternal synbiotic supplementation during pregnancy and lactation on BM composition (Figure 19).

One of the most important bioactive components of the BM are the Igs. They contribute to the passive immunization to the infant by supplying these compounds. In humans, the most abundant Ig in BM is IgA while in rats is IgG[75,79]. The analysis of the Ig profile of the BM revealed that maternal synbiotic supplementation increased the levels of IgG, specifically, the IgG2c subtype (**Article 1**). It is important to remember that this Ig was also increased in the maternal plasma (**Article 1**), suggesting that the maternal synbiotic supplementation not only modulates the maternal immune system, but also affects the BM composition, thus enhancing the infant passive immunization. As mentioned, the functionality of the IgG2c has not been highly analyzed, but it is considered as important in long-term immunity[71].

One of the latest compounds to be identified in the BM is the bacteria population. Initially, the BM was considered sterile, however, in the XXI century, many studies have concluded that BM is full of bacteria which contribute to the infant gut colonization[370–372]. As mentioned above, some bacteria from the maternal intestine arrive to the mammary gland and BM and then having the opportunity of influencing the infant microbiota[299]. In this case, although the presence of the administered probiotic in the BM has not been proved, the microbiota composition of the BM was clearly modulated. In particular, in the milk of the supplemented dams, the *Bifidobacterium* proportion was more abundant (**Article 1**). Combining the microbiota results of the maternal gut and the BM, it can be concluded that the synbiotic supplementation modulates the intestinal microbiota composition, which is reflected in the BM microbiota composition, in particular, by the higher abundance of the *Bifidobacterium* proportions in both compartments. The bifidogenic effect of prebiotics such as GOS and FOS alone or in combination has been widely analyzed[373,374]. It would be interesting to analyze other type of components in breast milk and ascertain whether they are also being modulated by the synbiotic supplementation.

To study in depth the association of the effects of the maternal synbiotic supplementation on the maternal immune system and on the BM composition some correlations between variables have been performed. Among the most relevant data some interesting correlations between the overall increase of the IgG2c and *Bifidobacterium* in both plasma and milk and the increase in SCFAs appeared. The correlations indicated that the synbiotic impacts on both fluids modulating their composition in the same way. Specifically, the increase of IgG2c in milk and plasma is positively correlated with the higher abundance of *Bifidobacterium* in the cecum and with the higher levels of acetic, formic and propanoic acids. Altogether confirmed that maternal synbiotic supplementation improves the maternal immune status and the BM composition through the microbiota modulatory actions, suggesting then a possible effect in the development of the offspring.

*All these reported data point out that maternal synbiotic supplementation with B. breve M-16V and scGOS/lcFOS modules the maternal immunological profile, metabolic activity and intestinal barrier and microbiota and milk composition.*

**Impact of maternal synbiotic supplementation during pregnancy and lactation on the offspring under RV infection**

To follow with the second objective, a RV (SA-11) infection was performed in early life pups. The evaluation of the induced-diarrhea was performed until the infection was solved, at day 13 of life. Firstly, the underlying mechanisms altered during the RV infection has been studied, particularly, the research was focused on the small intestine gene expression changes. Additionally, it was studied whether these gene expression changes could be counteracted by a nutritional intervention on the pup, in this case with the supplementation with 2´-Fucosylactose (2´-FL) (**Article 2**). Secondly, we aimed to explore the efficacy in RV prevention, but not acting on the pup, but on the mother. For that, the study consisted on supplementing the mothers with the synbiotic *B. breve* M-16V and scGOS/lcFOS during gestation and lactation and to study the preventive effect on RV infection of the offspring during suckling (**Article 3**).

At birth the infant immune system remains immature, leading to a higher susceptibility to suffer viral and bacterial infections[375]. Breastfeeding during early life reduce the incidence of these infections because the bioactive compounds of the BM provide more protection than those found in formula-fed infants[291]. Thus, the maternal synbiotic supplementation, could influence the fetal development or/and to shape the milk composition, both in terms of Ig profile, microbiota and SCFAs, contributing all these together to the promotion of the infant maturation and may be to a better defensive action against pathogens (**Article 1**).

During early life, the RVs agents are the main responsible for the acute gastroenteritis in children until 5 years of age. The entrance of the viral particles in the enterocytes induces

their death leading to a deregulation of liquid transporter proteins and the disruption of TJs, as consequence the principal clinical symptom is a watery diarrhea[166,167].

The 2´-FL is the most abundant human milk oligosaccharide (HMO), its functionality has been widely studied to evaluate its impact on offspring development and its potential to reduce infections during early life[376,377]. To date, 2´-FL inhibits the attachment of different bacteria like *Campylobacter jejuni*, enteropathogenic *Escherichia coli*, *Salmonella enterica*, *Pseudomonas aeruginosa* to the intestinal epithelia[378]. Additionally, it has demonstrated potential effects to attenuate viral infections of RV and norovirus[68,379,380]. Due to the attributed benefits of the 2'-FL, it has been included in infant formulas to simulate BM composition and improve and modulate the infant's immune system and gut microbiota [381,382].

Previous studies have been focused on the effect of 2´-FL to ameliorate the RV infection. They have reported a reduction in the severity, incidence and in the duration of the diarrhea in a suckling rat model[237]. Following this research line, this research focused on deciphering the gene expression modifications induced by the RV and the potential of the 2´-FL to attenuate the changes induced by the RV.

In the present work and previous studies, the 2´-FL supplementation to suckling rats during a RV infection has demonstrated to attenuate the incidence and severity of the induced diarrhea[237]. Using array and qPCR techniques, it was studied in depth the upregulated and downregulated genes during the RV infection. As expected, the majority of the up-regulated genes were host antiviral genes such as *Oas1a*, *Irf7*, *Ifi44*, *Isg15* and the main down-regulated genes were the ones involved in absorptive processes and intestinal maturation like *Onecut2*, and *Ccl19* (**Article 2**). Additionally, the gene expression patterns between the supplemented and non-supplemented pups were compared to understand the potential changes counteracted by the 2´-FL. In general, the gene expression patterns were similar in both groups, however, some genes involved in immunity and maturation such as *Ccl12, Rpl39l* and *Afp* were differentially expressed (**Article 2**). *Ccl12* participates in the first line of defense and collaborates in immune cell recruitment[383]. However, the functionality of *Rpl39l* and *Apf* has not been linked immune responses[384,385]. *Afp* gene expression is important during fetal growth and contributes to an appropriate gastrointestinal development[385]. Although little information is known about the relationship between *Rpl39l*, *Afp,* and RV infections, we suggest that the mechanism of action of 2'-FL under RV conditions to reduce diarrhea is linked to intestinal development. In summary, this study revealed critical genes involved in RV infection and diarrhea, and identified that some of them could be modulated by direct bioactive supplementation in pups.

Once that the RV gene modifications were analyzed and the possibility to be modulated were evidenced, the potential of another strategy was also checked: using a maternal synbiotic supplementation. In this case, part of the RV infected pups from the litters were

euthanized at the peak of the viral process to evaluate the immunological impact of the RV infection.

RV SA-11 infection induces a mild diarrhea in rats that in general is not accompanied by a body weight loss[174,237]. Similar to the literature, the infected pups did not suffer a body weight loss during the infection (**Article 2** and **3**). The most interesting result was that pups whose mothers were supplemented with the synbiotic showed a lower incidence and severity of the diarrhea (Figure 20). Additionally, the fecal weight was evaluated as an objective variable of the watery content of the feces. Both groups showed similar fecal weight in the pre-diarrhea and diarrhea periods. However, at the end of the infection, the SYN pups showed lower fecal weight compared to the REF ones (**Article 3**). This data, joined with the associated diarrhea parameters such as the diarrhea period and the days with diarrhea confirmed that maternal synbiotic supplementation reduces the RV infection in early life, reducing its incidence and severity and the duration of the infectious process (**Article 3**). It must be noted that the positive effects of the *B. breve* and the scGOS/lcFOS to ameliorate the RV induced diarrhea have been previously described in pups[235]. In that case, the nutritional intervention was performed to the pups, so the supplementation acts directly on the infected pup. However, in the present study, the supplementation was given to the mother, suggesting that this positive impact observed in the pups comes indirectly from the mother through the benefits induced during pregnancy and lactation. Overall, a new strategy to prevent infections in early life can be suggested.

Viral infection stimulates both innate and adaptive immune system to counteract the pathogen and to promote the immunological memory, leading to specific antibody (Ab) production to neutralize further infections[236,386,387]. In this study, it was analyzed if maternal synbiotic supplementation induced the production of specific anti-RV Ab during the infection and thereafter on both pups and dams (**Article 3**, **4**, **5** and **1**, respectively). Herein, we couldn't prove that synbiotic supplementation to the dams during pregnancy and lactation stimulates the pup's immune system to produce higher levels of specific anti-RV Abs in any of the analyzed samples, during or after infection (**Article 1**, **3**, **4** and **5**). *Rigo Adrover et al*. demonstrated that pup supplementation with probiotics and prebiotics stimulates the production of specific anti-RV Ab. In particular, when the same synbiotic was administered directly to the pup in this same model, the production of specific anti-RV IgM was increased[236]. Again, in this case, the supplementation was done directly to the pup under infection conditions, suggesting that direct exposure to the supplements is effective to stimulate the production of Ab but the indirect exposure through the mother seem not to be enough and other mechanisms should be involved.

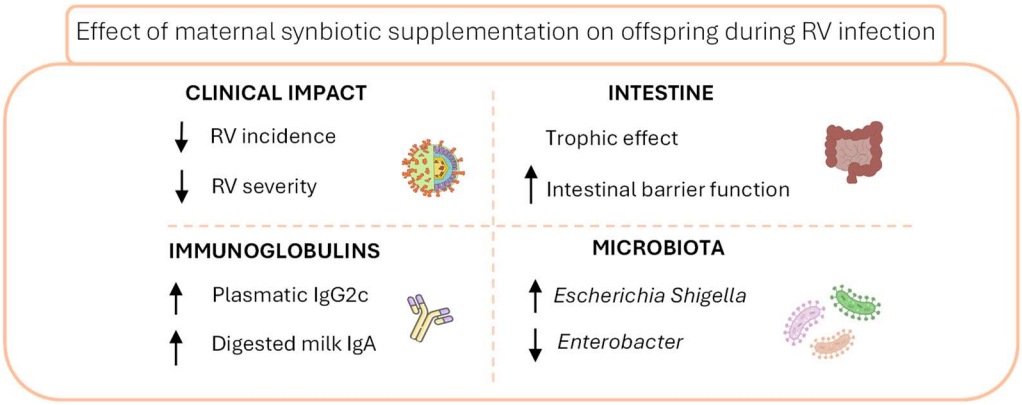


**Figure 20**. Principal changes observed in the offspring after maternal synbiotic supplementation during RV infection. *Maternal synbiotic supplementation reduced the incidence and severity of the viral infection. Moreover, the maternal nutritional intervention modulates the immunoglobulin profile of the pups, enhances the intestinal barrier functions and modulates the microbiota colonization. RV, rotavirus; Ig, immunoglobulin, "↑" increase; "↓" decrease.*

The RV primarily targets the intestinal cells, particularly the enterocytes which are disrupted by the virus. The RV encodes for multiple viral proteins which generate a damage to the intestinal epithelia and modulate the intestinal gene expression to facilitate the viral replication[165]. RV infections are associated with an increase in the expression of TLR, including *Tlr2* and *Tlr7*[165]. Additionally, the infectious process is linked with a disruption to the TJ and alteration in mucins[165]. Herein, maternal synbiotic supplementation increased the expression of *Tlr2* and *Tlr7*, TJ protein genes such as *Ocln*, and some mucosal barrier-associated genes *Muc2* and *Muc3* (**Article 3**). TLRs contribute equally to host defense, however, the ligand that recognize, their location and the immune response that triggers are different for each one. These differences explain their different gene expression levels during viral or healthy conditions[388,389]. TJ proteins, as mentioned in the previous section, such as occludin, along with intestinal mucins genes such as *Muc2* and *Muc3*, play crucial roles in innate defense mechanisms by preserving the integrity of the intestinal barrier and counteracting pathogens such as RV[390]. Probiotic and prebiotics, specifically, some *Bifidobacterium* strains and scGOs/lcFOS have demonstrated a positive effect on the defense and integrity of the epithelium, increasing the expression of both receptors *Tlr2* and *Tlr7* and improving the TJ proteins and mucus secretion after their supplementation[391]. In this work, some of these genes, such as *Ocln* were also increased in the maternal gut after the synbiotic supplementation (**Article 1**). According to this, the maternal synbiotic supplementation improves the defense functions of the intestinal barrier, favoring the recognition of the viral particles to trigger a more effective immune response.

Apart from the biomolecular changes induced by the RV, the small intestine architecture is also altered, for example, the villi height is reduced due to the infection[392]. The reduction

of the intestinal structures can be linked to a reduction in the absorption of nutrients and water, leading to watery diarrhea[393]. Herein, the maternal synbiotic supplementation reduced the damaged caused by the RV in the pup's intestine. In particular, in the pups whose mother received the synbiotic, the height and area of the villi were higher and there was a tendency to increase the width (**Article 3**). Thus, these changes in the morphological structures of the intestinal barrier restrict the passage of the RV, protecting the host[393]. This beneficial effect has been found also in previous studies with 2'-FL and scGOS/lcFOS, but this is the first time that it is observed when the supplementation is produced at maternal level[237].

Gastrointestinal infections also induce alterations in the intestinal microbiota. The interest of the intestinal microbiota to maintain a healthy status has been raising in the last decade. In fact, the disruption of the microbiota in the early stages of life is linked to future adult alterations[394]. In early infancy, the microbiota diversity is reduced with respect to adulthood. In the present work, at the peak of infection Proteobacteria and Firmicutes were the dominant phyla, and these were not modified with the maternal synbiotic supplementation (**Article 3**). However, at the family and genera levels, some changes were observed with the maternal synbiotic supplementation. Notably, the proportion of the *Enterococcaceae* family and the *Enterococcus* genus were lower, whereas the *Escherichia Shigella* proportion was higher in the SYN than in the REF animals (**Article 3**). The increase of *Escherichia Shigella* during RV infection has already been documented[395]. In general, probiotics and prebiotics have been described as effective treatments to counteract this increase[396]. In the present study, the maternal synbiotic supplementation was not able to reduce the abundance of *Escherichia Shigella*. However, the SYN pups showed a higher proportion of certain beneficial bacteria such as *Ligilactobacillus* which has been used as anti-infective probiotic due to its microbial activity[397]. Overall, this study confirms that the maternal synbiotic supplementation is able to modulate the infant gut colonization in early life, enhancing the presence of beneficial bacteria and reducing pathogenic ones, which probably participates in the amelioration of the infectious process.

Rat pups only ingest BM in an exclusively manner until day 14-16 of life when they start to chew, and then to combine both types of feeding. Consequently, by day 8 of life pups only ingest BM. Therefore, the stomach content is exclusively composed of the digested milk. The BM is a dynamic fluid that changes throughout the lactation depending on the infant requirements and needs[398,399]. The analysis of the milk immunoglobulinome at this stage revealed that the maternal synbiotic supplementation increased the levels of sIgA when the pup undergoes an infectious process (**Article 3**). This data confirmed that although the BM immune profile at the end of lactation was similar in both groups (**Article 1**), the earlier milk during pups' infection was enriched in sIgA to confer passive immunization to the offspring to counteract the viral infection. It is plausible to think that the higher sIgA levels

in BM in this period could lead to higher blockage of the virus, thus avoiding its entrance to the pup's intestine.

Regarding the stimulation of the pups' humoral response, the Ig profile was checked at systemic and mucosal levels on day 8 of life. In the intestinal mucosa, the maternal supplementation did not affect the pup's sIgA but tended to increase the total IgM (**Article 3**). sIgA and IgM play an important role in the intestinal epithelium. sIgA is the most abundant in the mucosa and neutralizes pathogens while IgM contributes to tissue homeostasis and collaborates in the humoral defense[400,401]. At systemic level, the immunoglobulin profile remains almost similar in both groups, however, an increase on the proportion of the IgG2c was found (**Article 3**). The IgG2c, analog in mice and humans of the IgG3, has been associated with regulatory response in the neonate intestine may help the pup to counteract the viral infection[322]. Additionally, the IgG2c increase was also observed in the maternal plasma and milk of the supplemented dams (**Article 1**). This result clearly indicates a vertical transmission between the mother and the offspring in early life. Although we did not check the plasma immunoglobulin profile during gestation, it could be that the synbiotic supplementation had increased the IgG2c levels during that period and therefore could have been transferred to the pup before birth. This boost in addition to the one received by BM higher levels, could together, be enough to counteract pup RV infection. It could be of interest to ascertain which period is more effective in this transfer.

*To sum up, it can be confirmed that maternal synbiotic supplementation during pregnancy and lactation contributes to counteract the RV infection in their offspring and to modulate their microbiota colonization*.

**Maternal-infant pair communication during early life: impact of synbiotic supplementation until one week after weaning.**

The third objective aims to assess the effect of the indirect maternal synbiotic exposure until weaning and until one week after by direct administration (**Article 4** and **Article 5**, respectively) (Figure 21).

The first 1000 days of life include different stages (embryonic stage, perinatal period, lactation and until 2 years of life). During all these periods the offspring is exposed to several vulnerabilities that threat the development and growth of the infant[402]. In rats, suckling is considered to last until day 21 of life and then, until day 28 of life it could be considered as the equivalent to the first 1000 days of human's life.

In early life the maternal health influences the infant development, indeed, alterations in the maternal body weight during preconception and pregnancy may contribute to alterations in the offspring weight. Firstly, it was evaluated the potential effect of the maternal synbiotic supplementation during pregnancy in the pups' weight at birth. As

mentioned before, the synbiotic supplementation during pregnancy and lactation did not affect the maternal body gain during pregnancy (**Article 1**). According to this, we expected that the weight at birth of both groups was similar. However, the body weight of the pups from the supplemented dams was lower than the ones from the non-supplemented mothers (**Article 3**, **4** and **5**). In spite of this, when the growth pattern was analyzed throughout the study both groups showed similar body weight gains since birth until the end of the study (**Article 5**). The lower body weight observed at birth in the SYN group seemed to be linked to a bigger size of the litters. However, this observation did not reach statistical significance and complicates the interpretation. Anyway, the impact on dam fertility and litter size requires further investigation. Independently of that, daily pup's body weight gain analysis showed no significant differences, indicating that synbiotic supplementation did not affect the overall growth of the animals despite the low body weight at birth.

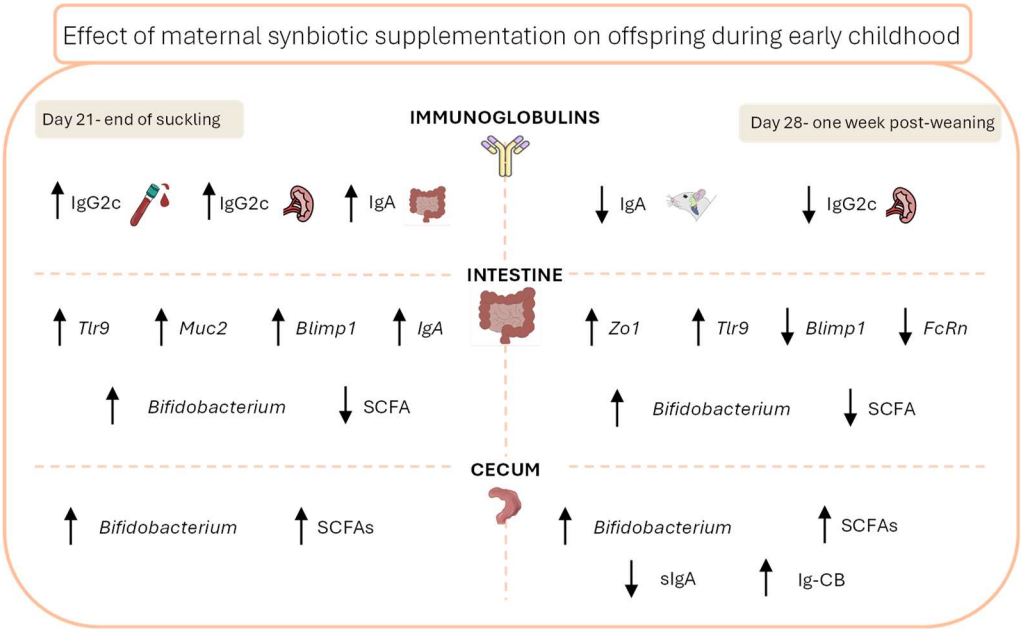


**Figure 21.** Summary of the principal effects of the synbiotic supplementation at the end of the suckling period (day 21 of pups' life) and after one week of weaning (day 28 of pups' life). *The observed changes are focus on the immunoglobulin (Ig) profile of different samples and compartments, on the small intestine and on the cecum. SG, salivary gland, MLN, mesenteric lymph nodes, Tlr, toll-like receptor; Muc, mucin; Blimp1, B-lymphocyte-induced maturation protein 1; Zo, zonula occluden; FcRn, Neonatal receptor Fc; SCFAs, Short-chain fatty acids; Ig-CB, immunoglobulin-coated bacteria; "↑" increase; "↓" decrease.*

Synbiotics act firstly in the gastrointestinal tract, and some trophic effects have been observed after their administration[403]. The macroscopic analysis of the small intestine was performed at the end of the lactation period (**Article 4**) and after one week of direct synbiotic supplementation (**Article 5**). The length and weight of the small intestine was increased in the animals from the supplemented group at the two analyzed points. Similar results were found in the maternal intestine at the end of lactation (**Article 1**). However, at day 8 of life during the RV infection this trophic effect was not detected (**Article 3**). Altogether suggests that synbiotic supplementation, in particular *B. breve* M-16V and scGOS/lcFOS, has a positive impact when administered directly (dams and pups at day 28 of life) and indirectly at weaning (day 21 of life). Thus, to observe the trophic effect on the pups' intestine the maternal supplementation should last more than gestation and part of lactation, as it was not observed at day 8 of life (**Article 3**). Further studies are needed to clarify this interesting effect.

Apart from the observed trophic effects, probiotics, prebiotics, and synbiotics, have the ability to modulate the intestinal morphology and to enhance the functionality of the gut. The results of this study indicated that the trophic effect observed in the small intestine at the end of lactation was not associated with prominent changes in the intestinal villi and crypts morphology (**Article 4**). However, the results observed in **Article 5**, when the synbiotic was also administered for one additional week indicated that the trophic effect was associated with some changes in the intestinal architecture such as an increase in the villi area and width and the abundance of goblet cells (**Article 5**), which participate in the intestinal homeostasis and barrier defense[333,404]. Many studies have analyzed the impact of biotics on the intestinal morphology, which normally increase the villi height. However, most of these studies are focused on the direct administration of the pro-, pre- or synbiotics[405–407] and, the impact of maternal supplementation on the offspring's gut has been less analyzed. Only a few authors have analyzed the impact of maternal synbiotic supplementation on the offspring intestinal structures and observed an increase in the height of the villi[174,408]. They used a synbiotic (*Lactiplantibacillus Plantarum*, *Saccharomyces cerevisiae* and xylo-oligosaccharides) in a model of pregnant sows[409], suggesting that the specificity of the synbiotic composition could be involved in the modulation of the intestinal architecture.

After birth, the immune system, in particular, the intestinal immune system, remains immature and during early life achieves the complete maturation by antigen exposure. *FcRn* and *Blimp1* are two intestinal makers that can be used to measure intestinal maturation. *FcRn* is an import gene involved in the transport of IgG across the placenta during fetal development. Also, FcRn participates in the intestinal IgG translocation during breastfeeding, consequently, it is highly linked to the passive immunity transferred from the mother[410]. *Blimp1* is a maturation marker gene associated with the promotion of the adaptive and innate immune cell differentiation and is expressed during fetal and early life. After weaning, it is expected that these two maturation markers diminish their

expression, when the maturation is achieved[411,412]. In this study, the levels of these intestinal maturation markers were checked at weaning (**Article 4**) and one week after weaning (**Article 5**). At day 21 of life, the end of lactation, the pups from the supplemented group did not show changes in the *FcRn* expression, probably due to the exposure to the maternal milk, and the absorption of the IgG of the maternal milk (**Article 4**). However, after weaning, the direct synbiotic supplementation induced a reduction of this intestinal marker, suggesting that the synbiotic contributes to intestinal maturation (**Article 5**). Regarding *Blimp1*, its levels were increased at weaning (**Article 4**) and, then one week after weaning the gene expression levels were reduced in the supplemented animals (**Article 5**). Overall, it can be concluded that synbiotic supplementation contributes to the intestinal maturation in early life as the effect on these key genes is in line with a more mature status.

The functionality of the intestinal barrier was also reinforced. Data showed that *Tlr9* gene expression was upregulated at the end of the suckling period (**Article 4**) and one week after weaning (**Article 5**). The interaction of the epithelial cell surface receptor TLR9 and *B. breve* has already been described *in vitro*[413]. Talking these results together it can be suggested a better immune response against components from both bacterial or viral origin. Regarding the mucins and TJ proteins, herein, the synbiotic supplementation enhances the expression of some mucins and TJ proteins, such as *Muc2* and *Zo1*, after the maternal supplementation during pregnancy and lactation and after one week of synbiotic supplementation (**Article 4** and **5**, respectively). Previous studies in animal models have demonstrated that probiotics influences the expression of TJ proteins and mucins favoring intestinal integrity[414,415]. Overall, after analyzing different parameters of the intestinal epithelium, this study confirmed that maternal synbiotic supplementation and direct supplementation during early life positively influences the rat intestinal barrier function development, that could be assigned to the first 1000 days of a human infant.

As reported above, one of the most important features in early life development is the microbiota colonization which is highly influenced by maternal diet, breastfeeding, and early life infections, among others[104,416]. To evaluate the maternal diet and breastfeeding contribution to the infant colonization and direct synbiotic supplementation in early childhood, the intestinal and cecal microbiota of the pups was examined. The study revealed that maternal synbiotic and direct pups' synbiotic supplementation influenced the microbiota composition in early life. Specifically, it was observed a higher proportion of some beneficial genera such as *Bifidobacterium* and *Faecalibaculum* at the end of lactation (**Article 4**) and one week after weaning (**Article 5**). It is commonly known that, in contrast to infant formula feeding, lactation promotes *Bifidobacterium* colonization of the infant's intestine, probably due to its ability to metabolize BM carbohydrates like the milk oligosaccharides[104]. *Bifidobacterium* species such as *B. breve*, *B. bifidum*, and *B. longum subsp. infantis* predominate in early life and have a positive impact on infant development[360,417,418]. Apart from the mentioned changes, *Faecalibaculum* proportion was

also increased in the SYN group in both at the end of suckling (**Article 4**) and one week after weaning (**Article 5**). The relevance of this genera has been less studied in early life. However, it has recently been identified as higher SCFAs producer in weaned animals[419]. Additionally, in this study, the vertical microbiota transference from the mother to the offspring was confirmed, a higher proportion of maternal *Bifidobacterium* in the cecum and in the BM (**Article 1**) is linked with a higher proportion of this genera in the offspring (**Article 3**, **4** and **5**). Similar results were observed when *Faecalibaculum* proportions were assessed, the higher proportion observed in the maternal cecum (**Article 1**) is also observed at the end of the suckling period (**Article 4**) and one week after weaning (**Article 5**). Additionally, other beneficial genera like *Blautia* and *Lactobacillus* were increased one week after weaning (**Article 5**). Both genera were also increased in the maternal gut (**Article 1**), suggesting that the direct synbiotic supplementation enhances the colonization of both genera. To date, the potential effect of a synbiotic (*Bifidobacterium* and FOS) on promoting *Blautia* and *Lactobacillus* proportions was previously identified by an *in vitro* fermentation model[420]. Taking together all these microbiota results from dams and offspring, it can be concluded that the synbiotic supplementation seem to be involved in a higher vertical transmission of some genera such as *Bifidobacterium* and *Faecalibaculum* which contribute to the infant intestinal colonization.

As mentioned above, SCFAs are the link between the microbiota and the host and provide useful information about the microbiota composition and its activity. The principal site of SCFAs production is the cecum, however, in the small intestine lower levels are also produced. The data of the study showed that SCFAs in the small intestine were lower in SYN animals than in REF ones at weaning (**Article 4**) and one week after weaning (**Article 5**). However, the cecal levels were higher after the synbiotic supplementation than in REF in both end points, at the end of the suckling (**Article 4**) and one week after weaning (**Article 5**). To be precise, butyrate, acetate, and propionate were the most notable increased acids, being their production linked to *Bifidobacterium* and Firmicutes[365,421] which were also increased in the cecum at the end of suckling and after weaning (**Article 4** and **5**). Additionally, the increase of *Faecalibaculum* proportion probably contributed to the increase of the amount of SCFAs, due to its recent functionally identification[419]. Comparing the offspring results with the maternal data, it can be confirmed that the supplementation to the dams impacts on the maternal and offspring cecum SCFAs profile inducing a similar pattern (**Article 1**). Although, whether the SCFAs in the dams are transferred to the offspring remains unclear, the literature suggests that cecal SCFAs of the dam are transferred to the BM and this probably influences the abundance of SCFAs on the offspring's gut[422]. It remains to evaluate the differential levels of SCFAs in BM in this study. However, based on those observations it can be hypothesized that maternal synbiotic supplementation could not only positively modulate the infant cecal microbiota, favoring also the production of SCFAs, but also to provide some of these components through BM. These modulatory effects of probiotics and

prebiotics on SCFAs have already been described under multiple health and disease conditions[353,423–427]. It has to be mentioned that these studies were focused on the direct supplementation with the probiotics and prebiotics. Less attention has been paid to the impact of the maternal supplementation during pregnancy and breastfeeding on the SCFAs. However, a recent study evaluated the effect of GOS/FOS supplementation during pregnancy and lactation. This research concluded that the maternal microbiome and the SCFA profile of both mother and infant were improved [246].

As mentioned before, synbiotics also exert immunomodulatory effects on the humoral immunity, specifically, exist many studies demonstrating positive effects boosting the humoral responses after vaccination[428]. Although the data showed herein is not focused on vaccination response, synbiotic supplementation to the mother and its influence during breastfeeding and its direct administration in early life, modulates the Ig profile at systemic and mucosal levels (**Article 3** and **4**). Additionally, the observed changes are mostly in line with the previous results discussed in the dams and in pups during RV infection (**Article 1** and **3**). These changes are mainly observed in the IgG isotypes, specifically, in the IgG2c. Similar to the reported microbiota results, it can be confirmed a higher vertical transmission of the maternal humoral immunity to the offspring through BM. Thus, this synbiotic in early life seem to be involved in promoting immune maturation at humoral level.

In the present work, the intestinal sIgA was assessed at the end of suckling at protein and gene level (**Article 4**). Results showed higher protein levels of sIgA and higher gene expression levels of the *IgA* in the intestinal compartment of the SYN group. This data can be compared with the IgA amount of the BM obtained at the end of suckling (**Article 1**), but the milk IgA was not increased with the maternal synbiotic supplementation. Thus, considering the intestinal sIgA results, it can be suggested that maternal synbiotic supplementation also stimulates intestinal Ig production of pups at the end of suckling.

The intestinal sIgA was also assessed one week after weaning (**Article 5**). The results indicated a reduction of the intestinal *IgA* gene, however, the protein levels in the gut wash of the sIgA remained stable (**Article 5**). This finding indicates that the protein levels in this compartment were not modified may be due to some transcriptional changes that took place[429]. However, after weaning, we observed an overall reduction in the sIgA in other compartments such as the salivary gland and cecum. These results could be connected with the transition from weaning to solid food which it is known to have an impact on the IgA[430]. Nevertheless, this drop in IgA deserves to be further studied and could be related to its lack of detection by ELISA if it is coating bacteria.

The cecal IgA interacts with the cecal bacteria for neutralizing them and favors their elimination[347]. The observed increase in the intestinal IgA at the end of suckling was not accompanied with an increase of the Ig-CB (**Article 4**), suggesting that even though maternal synbiotic supplementation was able to modulate the proportion of Ig-CB

(**Article 1**), this effect was not vertically transferred to the offspring during gestation and lactation. However, at day 28 of pups' life, the free cecal IgA was reduced in the synbiotic group but the IgA-CB was increased (**Article 5**). This data suggested that the decrease in free sIgA observed after synbiotic supplementation may be attributed to its binding to the bacteria, as suggested before, thereby enhancing its efficacy to neutralize and eliminate them. The strengthening of the IgA for coating bacteria to facilitate its elimination was previously described in the maternal samples (**Article 1**), reinforcing the hypothesis that synbiotic supplementation modulate gastrointestinal functions.

To finish with the potential effect of the synbiotic to modulate the immune system activity, we checked the changes in the cellular components. In the present notorious changes in the lymphocyte subsets after the maternal synbiotic supplementation until weaning (**Article 4**) or after its continued direct supplementation to the offspring (**Article 5**) were not found. These results were expected as we did not find relevant changes in the subset proportions on the maternal samples after the synbiotic supplementation (**Article 1**). Prebiotics, specifically HMOs, and probiotics have been described as potential modulators of the cellular immunity[431–434]. Most of these studies were performed to enhance the lymphocyte populations during disease or vaccination conditions, and only some of them were focused on healthy conditions. Additionally, they used different synbiotic components like *Lactobacillus spp* and other oligosaccharides and none of them were performed in early life. For example, supplementation with *Bacillus polyfermenticus* improves the functionality of immune cell population such as CD4+ and CD8+ T cells and NK cells[433]. In future studies, in order to better know the impact on the intervention on the cellular immune response, functional associated functions could be planned in addition to just the phenotype. It can be evaluated some phagocytic or cytotoxic activity, or the capacity to proliferate upon polyclonal stimulus.

Lastly, the potential effect of the synbiotic on offspring adipose tissue was evaluated at the end of suckling (**Article 4**) and after one week of weaning (**Article 5**). The importance of the maternal diet during pregnancy on the offspring adipose tissue programming has been gathering interest[435,436]. The intake of synbiotics has been linked to a reduction in metabolic disorders such as diabetes or obesity[437,438]. For example, a cocktail of *Lactobacillus rhamnosus* CGMCC1.3724 and inulin contributes to the weight loss of obese women[213]. In the present study, there were no associated metabolic disorders, and no significant changes were observed in adipose tissue either at day 21 of life (**Article 4**) or at day 28 of life (**Article 5**). Generally, there is a research gap concerning the influence of the maternal synbiotic supplementation on offspring adipose tissue. Some authors have evaluated the impact of the maternal diet during pregnancy on offspring adipose tissue programming, but they do not analyze biotic supplementation[439]. Some others examined the impact of a synbiotic supplementation (*Lactobacillus plantarum* and inulin) on the metabolic parameters, but in both case the supplementation was done directly to the host, and the maternal effect was not studied[440]. Thus, further investigations to

understand the effect of biotics on the offspring adipose tissue and their metabolic programming are required.

*Overall, the maternal synbiotic supplementation during pregnancy and lactation and, then the direct exposure during one extra-week contributes significantly to the maturation of the offspring. The most relevant findings are found at immune level and in the gastrointestinal tract, where the supplementation enhances intestinal barrier functionality and participates in the microbiota colonization.*

# CONCLUSIONS

The results obtained in the current thesis led us to conclude that:

**Conclusion 1**: Maternal supplementation with *Bifidobacterium bre*ve M-16V and scGOS/lcFOS (9:1) during pregnancy and lactation modulates the maternal immune system and the milk composition.

Particularly, the synbiotic supplementation during gestation and lactation modulates:

- **Maternal metabolic activity**: Stimulates the metabolic activity of the maternal adipose tissue.
- **Humoral immune response**: Changes the plasmatic and fecal immunoglobulin profile, increasing the abundance of IgG2c and sIgA, respectively. Consequently, promoting the Th1 responses and the intestinal defense.
- **Intestinal immunity**: Exerts a trophic effect on the small intestine, increasing the available surface to uptake nutrients and boosting the functionality, integrity and defense of the intestinal epithelium by modulating some aspects of the tight junction, mucus and toll-like receptors gene expression.
- **Microbiota composition**: Influences the microbiota composition of the small intestine and cecum, increasing the proportion of beneficial bacteria such as *Bifidobacterium* and inducing a differential SCFA profile
- **Milk composition**: Modulates the abundance of beneficial bacteria and shapes the immunoglobulin profile to reinforce the neonatal development.

**Conclusion 2**: 2'-Fucosylactose (2'-FL) and *Bifidobacterium bre*ve M-16V and scGOS/lcFOS (9:1) reduce the RV infection incidence and severity in early life.

RV infection modulates the gene expression patterns in early life. Specially, the infection upregulates the host antiviral genes and downregulate the genes involved in absorptive processes and intestinal maturation.

The direct supplementation with the 2'-FL reduces the severity of the viral infection, participating in the modulation of the gene expression patterns of some involved genes such as *Afp*, *Rpl39* and *Ccl12*.

Maternal supplementation with the synbiotic during gestation and lactation ameliorates the rotavirus infection, enhancing the antiviral mechanism and boosts the immune system. Specifically, modulates:

- **Rotavirus impact**: Ameliorates the severity and incidence of the RV-induced diarrhea.
- **Immunoglobulin profile**: Boosts the immunoglobulin profile of the infant through the passive immunization provided by the bioactive components of the breast milk.

- **Intestinal immunity**: Potentiates the intestinal immune system of the offspring, participating in the microbiota colonization, enhancing the intestinal epithelial barrier and, upregulating the expression of genes involved in the maintenance of the intestinal defense.

**Conclusion 3:** Synbiotic supplementation to the mothers with *Bifidobacterium bre*ve M-16V and scGOS/lcFOS (9:1) during gestation and lactation and both to the mothers and directly to the offspring for one week after weaning exert similar changes. Specifically, modulates:

- **Humoral immune system**: the maternal supplementation affects the plasmatic and mucosal immunoglobulin profiles at weaning, increasing the IgG2c and the sIgA. However, this effect does not persist after weaning.
- **Intestinal barrier**: the direct synbiotic supplementation after weaning contributes to the intestinal maturation and the enhancement of the intestinal barrier. This improvement of the epithelial barrier is also observed after the maternal supplementation until the end of lactation.
- **Microbiota composition**: the direct and the maternal synbiotic supplementation shapes the colonization of the intestinal microbiota with the growth of beneficial bacteria.

*Overall, the synbiotic supplementation during gestation and lactation impacts positively on the maternal immune system. These benefits extend to the offspring contributing to the microbiota colonization, the immune system development and providing protection against RV infection, emphasizing the importance of maternal health and nutrition in shaping the infant health.*

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