UNIVERSITY OF SÃO PAULO

SCHOOL OF PHARMACEUTICAL SCIENCES

Post-Graduation Program in Food Science

Area of Bromatology

Impact of the maternal diet and the intervention with fructooligosaccharide on the human milk microbiota

Marina Padilha

Thesis presented for the degree of

Doctor in Sciences

Advisor:

Full Prof. Susana Marta Isay Saad

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Prof. Dr. Carla Taddei de Castro Neves

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Marina Padilha

Impact of the maternal diet and the intervention with fructooligosaccharide on the human milk microbiota

Commission of Thesis for the degree of Doctor in Sciences

Prof. Susana Marta Isay Saad
Advisor/president

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1st Examiner

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2nd Examiner

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3rd Examiner

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4th Examiner

São Paulo, _____, 2018.

DEDICATION

To my little baby, the most beloved and exciting project of my life.

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There is a driving force more powerful than steam, electricity and atomic energy: the will.

- Albert Einstein

RESUMO

PADILHA, M. **Impacto da dieta materna e da intervenção com fruto-oligossacarídeo sobre a microbiota do leite humano**. 2018. 148 p. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2018.

O leite humano é, reconhecidamente, o principal componente para o crescimento e o desenvolvimento metabólico e imunológico de lactentes. Adicionalmente, durante a lactação, o leite humano consiste em uma importante fonte de micro-organismos para a formação da microbiota intestinal de neonatos. Fatores relacionados à mãe têm sido associados à composição da microbiota do leite humano. Entretanto, poucos estudos avaliaram a dieta materna como componente modulador da microbiota do leite humano. Os objetivos deste estudo foram investigar o impacto da dieta materna sobre a composição da microbiota do leite humano de mães saudáveis e, posteriormente, avaliar a influência da intervenção com fruto-oligossacarídeo na microbiota do leite humano, durante 20 dias de lactação. O estudo foi dividido em duas partes; a primeira parte consistiu de um estudo transversal, com 94 lactantes atendidas no Hospital Universitário da Universidade de São Paulo (HU/USP), a fim de investigar a associação entre o consumo materno de nutrientes durante a gestação e durante o primeiro mês de lactação e a microbiota do leite humano. A segunda parte consistiu em um ensaio clínico, aleatorizado, placebo-controlado, com 53 lactantes, classificadas em grupo FOS, que recebeu 4.5 g de fruto-oligossacarídeo + 2 g de maltodextrina (n = 28) ou grupo placebo, que recebeu 2 g de maltodextrina (n = 25), suplementados por 20 dias. O DNA das amostras de leite foi isolado e utilizado como molde para amplificação e sequenciamento em Illumina MiSeq® System. Em geral, a dieta materna durante a lactação (consumo a curto prazo) apresentou influência pontual sobre diversos grupos de micro-organismos, incluindo correlações positivas entre ácidos graxos poli-insaturados/linoleico e o gênero *Bifidobacterium*. No entanto, somente a dieta materna durante a gestação (consumo a longo prazo) foi estatisticamente significante ($p = 0.02$) para as análises de agrupamento das amostras (análises de estrutura de comunidade), sendo o maior teor de vitamina C consumido durante a gestação relacionado ao agrupamento 2, direcionado por maiores populações do gênero *Staphylococcus*. Após o período de intervenção na dieta materna, não foram encontradas diferenças entre a abundância relativa de gêneros entre os grupos placebo e FOS. No entanto, as distâncias do percurso das amostras do início até o final da suplementação foram maiores para o grupo FOS ($p = 0.0007$). De acordo com os resultados, a idade materna influencia essa resposta à suplementação por FOS ($p = 0.02$), embora, não tenham sido encontrados padrões nítidos nas diferenças de abundância relativa entre os grupos. Os resultados obtidos sugerem que a dieta materna consiste em um fator de modulação da microbiota do leite humano, sendo a dieta durante a gestação um fator mais intenso sobre a estrutura da comunidade bacteriana do leite humano. No entanto, o consumo a curto prazo ou a intervenção alimentar com prebiótico sobre a dieta materna apresentou influência pontual sobre a dinâmica da microbiota do leite, ainda que mudanças observadas sejam indivíduo-dependentes e influenciadas pela idade materna, como no caso do estudo de intervenção.

Palavras – chave: leite materno; microbiota; prebiótico; lactação; dieta materna; colonização intestinal.

ABSTRACT

PADILHA, M. **Impact of the maternal diet and the intervention with fructooligosaccharide on the human milk microbiota**. 2018. 148 p. Thesis (PhD) – School of Pharmaceutical Sciences, University of São Paulo, São Paulo, 2018.

Human milk is recognized as the main component for growth, metabolism, and immune development in infants. Furthermore, during lactation, human milk is an important source of microorganisms for the intestinal colonization of newborns. Mother-related factors have been associated with the human milk microbiota composition. Nevertheless, apparently, there has not been any study in which the maternal diet was evaluated as a modulator of the human milk microbiota. Therefore, the aim of this study was to investigate the impact of the maternal diet on the human milk microbiota composition of healthy women, and subsequently, to evaluate the effect of fructooligosaccharides supplementation on the human milk microbiota. This study consisted of two parts; the first was a cross-sectional study, including 94 lactating women recruited at the University Hospital of the University of São Paulo (HU/USP), to investigate the association between the maternal nutrient intake during pregnancy and lactation over the first month and the human milk microbiota. The second part consisted of a randomized, placebo-controlled clinical trial with 53 lactating, classified as FOS group (n = 28), which received 4.5 g of fructooligosaccharides + 2 g of maltodextrin or placebo group (n = 25), which received 2 g of maltodextrin, over a period of 20 days. The DNA was isolated and used as template for amplification and sequencing by the Illumina MiSeq® System. Overall, the maternal diet during lactation ("short-term" food intake) influenced specific bacterial groups, including positive correlations between polyunsaturated fatty acids/linoleic fatty acids and *Bifidobacterium*. However, only the maternal diet during pregnancy ("long-term" food intake) was statistically significant ($p = 0.02$) for the clustering analyzes (community structure analyzes), in which higher levels of vitamin C intake during pregnancy was related to cluster 2, driven by the *Staphylococcus* genus. After the intervention period on the maternal diet, no differences were found for relative abundance of genera between the placebo and the FOS groups. However, the distances of the trajectories covered by the samples from the beginning to the end of the supplementation was higher for the FOS group ($p = 0.0007$). According to our results, the maternal age affects the response for FOS supplementation ($p = 0.02$), though no patterns in the differences of relative abundances were found between the groups. Our results suggest that the maternal diet may influence the human milk microbiota, and the diet during pregnancy is a stronger factor over the bacterial community structure. Minor changes were found by the maternal short-term food intake or the maternal intervention with the prebiotic, and the changes seem to be individual-dependent and influenced by the maternal age, particularly in the intervention study.

Key words: Breast milk; microbiota; prebiotic; lactation; maternal diet; gut colonization

ABBREVIATIONS

24-HR - 24-hour food recall

ANVISA - Brazilian Health Surveillance Agency (in Portuguese, *Agência Nacional de Vigilância Sanitária*)

BMI - Body mass index

CLA - Conjugated Linoleic Acid

CLnA - Conjugated Linolenic Acid

CNCD - Chronic Non-Communicable Diseases

DHA - Docosahexaenoic Acid

EPA - Eicosapentaenoic Acid

FOS - Fructooligosaccharides

GIT - Gastrointestinal Tract

GOS - Galactooligosaccharides

IBD - Inflammatory Bowel Diseases

JSD - Jensen-Shannon Distance

MUFA - Monounsaturated Fatty Acids

OTU - Operational Taxonomic Unit

PCoA - Principal Coordinate Analysis

PCR - Polymerase Chain Reaction

PUFA - Polyunsaturated Fatty Acids

QFFQ - Quantitative Food Frequency Questionnaire

qPCR - Quantitative Polymerase Chain Reaction

rRNA - 16S ribosomal RNA

SCFA - Short-Chain Fatty Acids

SFA - Saturated Fatty Acids

WICF - Written Informed Consent Form

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SUMMARY

PRESENTATION

The first part of the current thesis is composed by **LITERATURE REVIEW**, **JUSTIFICATION**, and **OBJECTIVES**. The second part is composed by the methods, results and discussions, presented as 2 **CHAPTERS**, which are versions of the 2 scientific papers to be submitted for publication. Finally, the third part is composed by the **GENERAL CONCLUSIONS**. Other documents, and relevant information are presented as **ATTACHMENTS** and **ADDITIONAL FILES**.

PART I

LITERATURE REVIEW

The human microbiota

In the course of their life, human beings share their living space with a wide variety of microorganisms. Although this condition increases the susceptibility to pathogens, in most cases the contact with microorganisms shows to be innocuous and plays an essential role in health (POSSEMIERS et al., 2011; FAUST et al., 2012; INSTITUTE OF MEDICINE, 2013; MORGAN, SEGATA & HUTTENHOWER, 2013).

The significance of the relationship between humans and microorganisms becomes evident by the approximately 10^{14} cells that compose the human microbiota, ten times as many as human cells. These microorganisms comprise bacteria, fungi, viruses, and archaea, and are collectively known as our microbiota (or microbiome when genetic elements are also considered (LEY, PETTERSON & GORDON, 2006; GIBSON et al., 2017; SHEN, 2017).

Given the importance of the microbial community in the human body, several studies have investigated the human microbiome in the context of human health and disease. Worth mentioning are the projects *International Human Microbiome Consortium* (IHMC), *European Commission - Metagenomics of the Human Intestinal Tract* (MetaHIT), *United States National Institutes of Health's Human Microbiome* and the *Canadian Microbiome Initiative* (CMI). These projects have helped to characterize and study the genetic potential of the metabolic activities and interactions between microorganisms and hosts in different body sites (BÄCKHED et al, 2012).

Different microbial abundance and diversity patterns were observed, depending on the *habitat* they occupy, such as oral cavity, gut, skin, and vagina (HUMAN MICROBIOME PROJECT CONSORTIUM, 2012).

The genera *Streptococcus* and *Lactobacillus* are more abundant in oral cavity and vagina samples, respectively. The oral microbial community presents a greater species diversity, while the vaginal microbiome consists of a community of a smaller spectrum (HUMAN MICROBIOME PROJECT CONSORTIUM, 2012).

Over 90% of the bacteria in the intestinal microbiome are from phyla *Bacteroidetes* and *Firmicutes*, while *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia* and *Cyanobacteria* are represented to a lesser extent. Methanogenic archaea (mainly *Methanobrevibacter smithii*),

eukaryotes (mainly yeasts), and virus (mainly phages) may also be present (LOZUPONE et al., 2012).

Members of the genus *Bacteroides*, phylum *Bacteroidetes*, are predominant in the gut microbiota, although members of the genera *Prevotella*, *Capnocytophaga*, *Bergeyella*, *Porphyromonas,* and *Tannerella* can also be found (THOMAS et al., 2011). The *Firmicutes* phylum is mainly represented by the genera *Ruminococcus*, *Aerococcus*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Carnobacterium*, *Tetragenococcus*, *Vagococcus* and *Weissella* (STOLAKI et al., 2012).

In this context, ARUMUGAM et al. (2011) suggested that the gut microbiome of individuals is categorized into one of three enterotypes based on their dominant genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2), or *Ruminococcus* (enterotype 3).

Although it has been suggested that most individuals share specific bacterial phyla or genera, there is a huge range of variation of species among individuals (INSTITUTE OF MEDICINE, 2013). Some authors have suggested that the characterization of the microbiome of healthy individuals should be the initial approach. However, considering the huge microbial diversity, the gene expression profile has also been studied, because it represents a metabolic profile. It seems to be a more stable pattern among individuals (ARUMUGAM et al., 2011; HUMAN MICROBIOME PROJECT CONSORTIUM, 2012).

On the other hand, studies on the characterization of microbiome composition have found diversity to be relevant. TURNBAUGH et al. (2009) and QIN et al. (2010) observed that a smaller diversity of microorganisms in feces is directly related to obesity and inflammatory bowel disease, whereas FREDRICKS et al. (2005) observed that a great diversity of genital microorganisms is associated to bacterial vaginosis.

In this sense, human health or the characterization of a healthy microbiome depends on achieving and maintaining a complex homeostasis. When this balance is disturbed, negative effects occur, leading to changes in metabolic activities and/or in the bacterial dynamics, causing diseases (BÄCKHED et al., 2012; KUNDU et al., 2017).

Despite the recent developments in the human microbiome, its complexity and inter-individual variations are still not completely clear. Therefore, additional studies are required to understand the microbiota's structure, composition, as well as its determining factors (INSTITUTE OF MEDICINE, 2013).

The development of the human microbiome: impacts on human health and disease

The development of the human microbiome is a complex process, which is influenced by interactions between microorganisms and their host (FANARO et al., 2003). Physical factors, such as oxygen, moisture and pH levels, as well as immunological factors, genetic characteristics, nutrient availability and microbial interactions significantly influence the local microbiota composition (FAUST et al., 2012; GOODRICH et al., 2014; HILLMAN et al., 2017).

The scientific literature suggests that the interaction between microorganisms and their hosts starts at birth (VAISHAMPAYAN et al., 2010). Nevertheless, some recent studies have suggested the hypothesis that microbial colonization starts even before birth, since DNA of bacterial communities has been isolated from placenta, amniotic fluid, and meconium from healthy pregnancies (BEARFIELD et al., 2002; JIMÉNEZ et al., 2008; RAUTAVA et al., 2012; GOSALBES et al., 2013). However, it is during the delivery and postpartum period that microorganisms from the mother and the environment play an important role in the microbial colonization of the newborn's gastrointestinal tract (GIT) (SCHWIERTZ et al., 2003). In the first phases of colonization, which occurs within the first week after birth, facultative anaerobes that belong to *Enterobacterium*, *Enterococcus*, and *Streptococcus* genera are predominant. Later, strict anaerobes, such as *Bifidobacterium, Bacteroides*, and *Clostridium,* become predominant when compared to facultative anaerobes (WEBER & POLANCO, 2012).

The delivery mode (vaginal or Cesarean section) seems to be one important factor in the development of the microbiota composition. The GIT of vaginally born infants is colonized by bacteria from the maternal genital and gastrointestinal tracts, such as *Lactobacillus*, *Bifidobacterium*, *Streptococcus, Prevotella*, and *Enterobacter*. On the other hand, C-section infants are first exposed to hospital environment and skin bacteria of their mother, including the genera *Staphylococccus*, *Corynebacterium*, and *Propionibacterium* (SCHWIERTZ et al., 2003; SALMINEN et al., 2004; BIASUCCI et al., 2008; YOUNES et al., 2018).

In addition, GRÖLUND et al. (1999) observed a delay in gut colonization by *Lactobacillus* and *Bifidobacterium* in C-section infants compared to vaginally born infants. The authors also suggest that differences in microbiota composition may persist up to the sixth month of life. Comparing modes of delivery, PENDERS et al. (2006) also observed that infants born by Cesarean delivery are more frequently colonized by *Clostridium difficile*, while

vaginally delivered neonates show a microbial profile that is predominantly characterized by *Bifidobacterium longum* and *Bifidobacterium catenulatum* (BIASUCCI et al., 2008).

Other factors may also influence the composition of the gut microbiota, such as gestational age at birth, hygiene conditions, use of medication and diet (MSHVILDADZE & NEU, 2010).

Regarding the gestational age, preterm infants have a particularly sensitive intestinal mucosal surface due to immature intestinal epithelial cells and may present exaggerated inflammatory responses to stimulation from commensal bacteria or pathogens (CLAUD & WALKER, 2001). Therefore, the interaction of preterm newborns with microorganisms is delicate: it can establish a stable microbiota or an imbalanced and abnormal situation (MAI et al., 2011).

An important and well-documented abnormality of the intestinal microbial composition and that offers a high risk to preterm infants is necrotizing enterocolitis (MOROWITZ et al., 2010). MAI et al. (2011) showed the correlation between gut microbiota and this disease. Prior to the necrotizing enterocolitis diagnosis, they observed a decrease of *Proteobacteria* in the stool samples of preterm neonates compared to the control group. On the other hand, they observed a surge of *Proteobacteria* after the occurrence of necrotizing enterocolitis. In this study, MAI et al. (2011) suggested that the low exposure or colonization by *Proteobacteria* in the first week of life could compromise the adaptive immune response modulation in case of a subsequent increase of this population.

In addition, the use of antibiotics represents a risk factor for the occurrence of necrotizing enterocolitis, because antibiotics may interfere with the composition of the gut microbiota and in children may compromise the intestinal barrier function against pathogens (MSHVILDADZE & NEU, 2010).

Studies with term neonates reinforce the significant impact antibiotics have on gut microbiota. BRANDT et al. (2012) observed a reduction in anaerobic bacteria and *Escherichia* and an increase of *Klebsiella* in a neonate that received treatment for 10 days, compared to a group of neonates who did not receive the treatment. Nevertheless, TANAKA et al. (2009) suggested that the microbiota tends to be restored after the treatment with antibiotics, although there is a possibility of some changes becoming permanent.

The use of antibiotics and better hygiene and sanitation, as well as nutrition, especially in the Western world, has contributed to reducing child mortality and increasing life expectancy. However, these conditions come at a cost: the progressive reduction of important bacteria

groups, which are essential to the development and strengthening of the immune system (PROKOPAKIS et al., 2013).

In 1989, David Strachan formulated the "Hygiene Hypothesis", according to which infections in early childhood could reduce the risk of allergic diseases. The reduced exposure to microorganisms resulting from Western "antiseptic" conditions was to blame for the increased incidence of allergic and autoimmune disorders (PROKOPAKIS et al., 2013).

Since exposure to microorganisms is reduced, especially in childhood, the immune system is not appropriately stimulated, which encourages the onset of inflammatory bowel diseases and allergies (PROKOPAKIS et al., 2013). Although there is no consensus as to the correlation between gut microbiota and the etiology of immune diseases, studies have observed that individuals that manifest these diseases present a peculiar gut microbiota (NEUMAN & NANAU, 2012; D'ARGENIO et al., 2013).

PENDERS et al. (2007) studied the fecal microbiota of 957 one-month-old breast-fed infants and observed a positive association between the presence of *Escherichia coli* and the risk of developing atopic eczema. The same authors observed that the colonization by *Clostridium difficile* also presented a higher risk of eczema, allergic sensitization, and atopic dermatitis.

Studies of inflammatory bowel diseases (IBD), which include ulcerative colitis and Crohn's disease, have shown that IBD subjects microbiome fluctuates more than those of healthy individuals, based on deviation from a newly defined healthy plane (HALFVARSON et al., 2017), and significant differences in gut microbial composition of diagnosed patients (WRIGHT et al., 2015). The main observed differences in the composition of gut microbiota of IBD patients is low colonization by *Clostridium leptum* and *Akkermansia muciniphila*, and the presence of some unknown species (MANICHANH et al., 2006; NEUMAN & NANAU, 2012). Specifically, in Crohn's disease, *E. coli* is enriched, while *Faecalibacterium prausnitzii* is found at lower abundance (WRIGHT et al., 2015). Interestingly, in the same individual, inflammatory and noninflammatory mucosal sites also present differences in terms of microbial community structure (WALKER et al., 2011).

Several studies have suggested diet therapy as an attempt to control the microbiota dysbiosis that occurs in IBD and successfully recover the balance of microbial composition (DAY et al., 2008, D'ARGENIO et al., 2013).

The study by D'ARGENIO et al. (2013) shows the modulation of the gut microbiota through therapeutic polymeric enteral nutrition consisting of proteins, antioxidants, and lipids

with anti-inflammatory properties. After eight weeks of treatment, the authors observed a microbiota profile in the individual with Crohn's disease that was similar to an individual that did not have the disease.

In fact, several studies have suggested the importance of nutrition for the intestinal microbial composition (WU et al., 2011; XIAO et al., 2014). The influence of nutrition on the composition of the gut microbiota can already be observed in early childhood, depending on the infant's diet.

According to PENDERS et al. (2006), exclusively formula-fed neonates show a higher incidence of *E. coli*, *Clostridium difficile*, *Bacteroides*, and *Lactobacillus* in their stool than their breastfed counterparts do. On the other hand, exclusively breastfed infants tend to have a more beneficial microbiota, predominantly groups of *Bifidobacterium* and *Lactobacillus*, and smaller populations of *Bacteroides*, *C. difficile*, *Clostridium coccoides*, *Staphylococcus*, *Enterobacteriaceae*, and *E. coli* (HARMSEN et al., 2000; PENDERS, 2006; SOLÍS et al., 2010).

After weaning, the gut microbiota continues to develop until the infant is approximately two years of age. At this point, children reach a relative stability of the gut microbiota and resemble the microbiota of an adult (KOENIG et al., 2011).

Several studies have suggested the importance of microbial colonization in childhood, leading to repercussions in the early life or in the adulthood (AJSLEV et al., 2011; KAPLAN & WALKER, 2012). In addition, studies have suggested a correlation between gut microbiota and chronic non-communicable diseases (CNCD), such as overweight/obesity (TURNBAUGH et al., 2006; 2009), type 1 diabetes (WEN et al., 2008), metabolic syndrome (VIJAY-KUMAR et al., 2010), and inflammatory bowel diseases (D'ARGENIO et al., 2013).

Differences in colonization acquired during childhood may have consequences to the individual's health or the development of CNCD (GOULET, 2015). When KALLIOMÄKI et al. (2008) studied the fecal microbiota of infants in early childhood (6 to 12 months), they found an inverse correlation between the presence of *Bifidobacterium* and overweight or obesity at 7 years of age. Some species of microorganisms have been linked to changes in energy metabolism and weight gain (TURNBAUGH et al., 2006; KAPLAN & WALKER, 2012).

Since the gut microbiota is characterized by its large diversity, studying its composition, particularly the factors that influence this composition, may offer ways of modulating the microbiota, when necessary, in order to maintain health and reduce the risk of diseases (MSHVILDADZE & NEU, 2010; KOENIG et al, 2011).

The influence of diet on the composition of the gut microbiota

Diet is one of the most important factor in modulating the composition and metabolic activity of the human gut microbiota. The main dietary nutrients, particularly macronutrients (carbohydrates, proteins, and fats), their amounts, types, and ratios have a great impact on the gut microbiota. Diet can indirectly influence the intestinal transit time and luminal pH, which are closely linked to the composition of the gut microbiota (SCOTT et al., 2013).

The nutrients that are not absorbed after food is digested remain in the intestinal lumen to be used by gut microorganisms. Particularly *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* have an enzymatic complex that allows them to degrade and metabolize a wide variety of substrates from the digestive process (SCOTT et al., 2013).

Intestinal bacteria mainly rely on fermentation to obtain energy. Under anaerobic conditions, the main products of carbohydrate fermentation are gases (CO_2, H_2, and CH_4) and short-chain fatty acids (SCFA); acetic acid (acetate), propionic acid (propionate), and butyric acid (butyrate) are the most abundant in a molar ratio of 3:1:1. The presence of SCFA reduces the luminal pH and is an important source of energy for enterocytes (SCOTT et al., 2013).

Particularly butyrate is the main source of energy for enterocytes. Propionate and acetate are transported to the liver, where they play important roles as substrates in hepatic gluconeogenesis and lipogenesis, respectively (IBRAHIM & ANISHETTY, 2012).

Since the quality and quantity of consumed nutrients vary from one individual to another, the amount of SCFA that is produced, and the composition of the GIT microbiota also differ, proving the close relation between diet and intestinal microbiome (MUSSO, GAMBINO & CASSADER, 2011).

LEY et al. (2006) investigated how diet influenced the composition of the gut microbiota in obese subjects and observed a lower ratio of *Bacteroidetes* compared to their lean counterparts. Interestingly, after putting these obese individuals on a carbohydrate and fat restricted diet, the authors observed a significant increase in the *Bacteroidetes* ratio and a microbiota profile that is more characteristic of lean individuals.

Similarly, changes in the gut microbiota were observed in individuals on a high protein and low carbohydrate diet. Their *Eubacterium*, *Roseburia* spp., and *Bifidobacterium* population and fecal butyrate levels were reduced (SANZ, SANTACRUZ & PALMA, 2008).

Protein fermentation by proteolytic bacteria, mainly represented by species of *Bacteroides*, results in a more diversified metabolite profile compared to carbohydrate

fermentation. In addition to SCFA, protein fermentation produces ammonia and branched-chain fatty acids, amines, and hydrogen sulfide. Some of the protein fermentation metabolites, such as ammonia and amines, may be toxic to the intestinal tissue and act as carcinogenic promoters (SCOTT et al., 2013). On the other hand, the ingestion of carbohydrates, including prebiotic carbohydrates, may reduce protein fermentation and the use of peptides by intestinal bacteria, and thus avoid the production of unwanted metabolites (PRETER et al., 2007).

In addition to protein, dietary fats may also be an important factor to change the gut microbiota (SCOTT et al., 2013). However, very few studies have investigated the effect of fat ingestion on the gut microbiota.

According to BRINKWORTH et al. (2009), a high-fat diet significantly reduced the production of SCFA and bifidobacteria population, compared to a low-fat diet. However, in that study the low-fat diet had to be complemented with carbohydrates to be adjusted for energy requirements. This made it difficult to come to conclusive results.

WU et al. (2011) also observed evidence of an association between food intake and the gut microbiota. The authors found a positive correlation between the ingestion of animal protein and fat and the prevalence of the genus *Bacteroides*, while the ingestion of carbohydrates was linked to the genus *Prevotella*. In addition, they observed that short-term changes to the diet did not cause significant alterations in the gut microbiota, and thus attributed microbial modulation to long-term dietary changes.

Concurrently, other studies have observed that specific foods/nutrients influence microbial dynamics. MASSOT-CLADERA et al. (2012) found a significant reduction in *Bacteroides*, *Clostridium*, and *Staphylococcus* species in the feces of rats that were on a standard diet enriched by 10% with cocoa compared to the control group that was only on the standard diet. Another study investigated the impact of polyphenols from black tea or red wine/grape juice in an *in vitro* simulator of the human intestinal microbiota. In both interventions, the authors reported a shift in the *Firmicutes*: *Bacteroidetes* ratio, and an increase in *Klebsiella* and *Akkermansia* in comparison with *Bifidobacterium*, *Blautia coccoides*, and *Anaeroglobus* (KEMPERMAN et al., 2013).

In fact, the diet plays a central role in the maintenance of health and prevention of diseases and seems to be directly linked to intestinal health. According to SCOTT et al. (2013), the maintenance of a healthy microbiota is linked to a diet high in non-digestible carbohydrates and a restricted protein and fat intake.

Therefore, studies that focus on clarifying how nutrients influence the modulation of the gut microbiota are essential, since they may offer a new approach for future nutritional interventions in order to reduce the risk of certain diseases and to maintain health (INSTITUTE OF MEDICINE, 2013).

The effects of prebiotics on health and modulation of the gut microbiota

In the last years, food has been valued not only for their nutritional and sensory properties but also for their health benefits. In this context, prebiotic ingredients have received special attention (SAAD, 2006; MCGILL, 2009).

The more recent definition of prebiotic is "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (GIBSON et al., 2017). By this definition, three criteria were required for a prebiotic: to be resistant to human enzymes and gastric acid, be fermented in the intestinal microbiota, and selectively stimulating the growth and/or activity of bacteria associated with health. The health effects of prebiotics include not only benefits to the gastrointestinal tract (e.g., inhibition of pathogens, immune stimulation), but also cardiometabolism (e.g, reduction in blood lipid levels, effects upon insulin resistance), mental health (e.g., metabolites that influence brain function, energy, and cognition), bone (e.g., mineral bioavailability), and beyond.

Oligosaccharides are the primary prebiotics, and according to ROBERFROID (2007), inulin and fructooligosaccharides (FOS) are among the main prebiotic oligosaccharides. In addition to inulin and FOS, the European Union also includes galactooligosaccharides (GOS) and lactulose in the prebiotic concept (KOLIDA & GIBSON, 2011).

Inulin occurs naturally in plants, such as chicory, onion, garlic, Jerusalem artichoke, tomato and banana. Oligofructose is found in wheat, honey, leek, banana and onion. Commercially available inulin and oligofructose are mainly produced from chicory and beet sugar (ROBERFROID, 2007).

The Brazilian Health Surveillance Agency ANVISA (in Portuguese, *Agência Nacional de Vigilância Sanitária*) has recognized the prebiotic properties of inulin and FOS (ANVISA, 2008). Until December 2016, the legislation determined solid foods to contain a minimum required quantity of 3 grams and liquid foods, 1.5 grams. From 2017, the legislation determined a minimum of 5 grams of FOS/inulin should be recommended for daily intake, not exceeding

the maximum of 30 grams in daily consumption to receive the prebiotic health claim (BRASIL, 2016).

As long as they are not fermented, prebiotics exert an osmotic effect in the intestinal lumen. As soon as fermentation by the endogenous microbiota starts, especially in the colon, the production of gas and SCFA increases. In some cases, individuals with irritable bowel syndrome may not tolerate prebiotics. At low doses, however, they are generally well tolerated (SAAD, 2006).

Prebiotics increase bifidobacteria and SCFA levels. They protect against pathogens, reduce diarrhea, increase the absorption of nutrients and stimulate the immune system (MORO et al., 2006; LAVANDA et al., 2011; WHELAN, 2013).

Human breast milk is our first source of prebiotics. Several studies have attributed the main differences between the intestinal microbial composition of exclusively breastfed neonates and their formula-fed counterparts to the presence of oligosaccharides in breast milk (CHAMP & HOELBER, 2009). Human milk oligosaccharides promote the growth of bifidobacteria, protecting against potential pathogens and thus reducing the risk of several diseases (HINDE & GERMAN, 2012).

Given the importance of these compounds, studies have focused their investigation on the effects of prebiotic supplements in infant formulas and pregnant women (CHAMP & HOEBLER, 2009; CEAPA et al., 2013). In formula-fed infants, the supplementation with a mixture of GOS/FOS (9:1 ratio; 8g/L concentration) reduced the incidence of infections (ARSLANOGLU, MORO & BOEHM, 2007). In infants at high risk for developing atopic dermatitis, on the other hand, the administration of GOS/FOS-supplemented hydrolyzed formula resulted in protection against developing this condition (MORO et al., 2006).

In pregnant women, the supplementation with a daily dose of 9 grams of GOS/FOS (9:1 ratio) in the last trimester of pregnancy promoted the increase of bifidobacteria in maternal stool samples, although this increase was not observed in neonatal stool samples (SHADID et al., 2007). In addition, CHAMP e HOEBLER (2009) highlighted that the administration of prebiotic supplementation during pregnancy is a tool to reduce the risk of gestational diabetes and excessive weight gain. The authors also suggested that supplementing the maternal diet with prebiotics is a dietary strategy for the primary prevention of CNCD for the new generation.

The evidences support the claim that the administration of oligosaccharides is beneficial to human health and reduce the risk of diseases, both in newborn infants and in pregnant women, and demonstrate the correlation between these compounds and microbial colonization.

The presence of oligosaccharides in human milk reinforces their importance in the early life on the health of infants (CEAPA et al., 2013).

Human milk: from nutrition to modulation of the infant's intestinal microbiome

Breastfeeding is considered the gold standard method of nourishment for infants. Except in very rare situations, breastfeeding should be encouraged, since it has numerous indisputable health, psychological, social, and economic benefits (WORLD HEALTH ORGANIZATION, 2000).

The World Health Organization recommends exclusive breastfeeding, i.e. without any solid or liquid foods, except for medication and nutritional supplements, for the first 6 months of life and continued breastfeeding with complementary foods up to 2 years of age or beyond (WORLD HEALTH ORGANIZATION, 2000).

Human milk is a complex biological fluid with a species-specific composition, which meets all nutritional requirements and promotes optimal infant growth (FERNÁNDEZ et al., 2012).

The first fluid produced in the first few days postpartum is colostrum. It is secreted in small amounts. Colostrum contains low concentrations of lactose, but is rich in protein and in immune components, including immunoglobulin A (IgA), lactoferrin, leukocytes, and developmental factors, such as the epidermal growth factor (EGF), indicating its function to be immunologic and trophic (BALLARD & MORROW, 2013).

Transitional milk typically occurs from 5 days to two weeks postpartum. It shares some of the characteristics of colostrum, but is higher in carbohydrates and fat. By four to six weeks postpartum, human milk is considered fully mature, is rich in carbohydrates and fat and remains relatively stable in composition over the course of lactation (BALLARD & MORROW, 2013).

The nutrients of human milk originate by synthesis in the lactocyte, from maternal stores or diet. The nutritional quality of human milk is conserved, but the maternal diet is an important factor for vitamins and the fatty acid composition of human milk (VALENTINE & WAGNER, 2013).

The relationship between maternal diet and human milk composition became clear in ALLEN (2012), who analyzed studies, which showed that maternal supplementation of vitamins, such as thiamine (vitamin B1) and pyridoxine (vitamin B6), during lactation, was effective to increase their human milk concentrations. JENSEN et al. (2000) observed that the

supplementation of docosahexaenoic acid (DHA) promoted higher concentrations of this essential fatty acid in the breast milk of supplemented women compared to the control group. Similarly, NISHIMURA et al. (2014) reported that the maternal dietary DHA and eicosapentaenoic acid (EPA) content during late pregnancy may affect the fatty acid composition of mature breast milk. The study also shows that the maternal dietary intake of ω-3 to ω-6 fatty acid ratio, during late pregnancy and the postpartum period, can affect the polyunsaturated fatty acid composition of breast milk.

In addition to offering excellent nutritional value, human milk also plays an essential role in the development of the neonatal gut microbiome, mainly due to the oligosaccharides and microorganisms that naturally occur in human milk (BODE, 2012; FERNÁNDEZ et al., 2012).

Human milk oligosaccharides, remotely known as "*bifidus* factor", are the most studied compounds as to the modulation of the neonatal gut microbiota (BARILE & RASTALL, 2013). They result from the addition of monosaccharides to lactose in the mammary gland by glycosyltransferases (BALLARD & MORROW, 2013).

Proportionally, human milk oligosaccharides constitute the third most abundant solid compound of human milk. Over 200 different structures have been defined for human milk oligosaccharides. Since they are not digested by human enzymes, they are used as energy substrate for intestinal bacteria (WARD et al., 2006; BALLARD & MORROW, 2013; BARILE & RASTALL, 2013).

Human milk oligosaccharides contain fucose and sialic acid and share common structural patterns with the glycans present on the infant's intestinal epithelia, which are known to be receptors for pathogens (BARILE & RASTALL, 2013). These oligosaccharides provide a defensive strategy: they resemble glycans and therefore prevent binding of pathogens to epithelial cells (MORROW et al., 2005).

Interestingly, there are differences in the human milk oligosaccharides composition along the lactation period, and among the lactating women. According to BODE (2012), the major concentration is found in the colostrum, while the mature milk has lower concentrations. Besides, genetic differences in the activities of the Secretor and Lewis blood group system genes lead to differences in the fucosylation of the human milk oligosaccharides, influencing the presence of specific structures.

Several studies have also shown the selective properties of human milk oligosaccharides (SELA et al., 2008). WARD et al. (2006) observed that *Bifidobacterium infantis* used human

milk oligosaccharides as sole source of carbon, while *L. gasseri* was not able to use this carbohydrate as energy substrate.

In fact, the presence of *Bifidobacterium* in fecal samples of exclusively breast-fed infants has been extensively discussed in literature and linked to health benefits and to the reduction of risks of developing diseases in the short- and long-term (BISGAARD et al., 2011; RINGEL-KULKA et al., 2013).

In addition to oligosaccharides, microorganisms that naturally occur in human milk are believed to participate directly in the composition of the infant's intestinal microbiota. In the last couple of years, the identification of nonpathogenic microorganisms in human milk samples has received increasing attention, considering human milk as a continuous resource of commensal, symbiotic or potentially probiotic bacteria for the infant gut (MARTÍN et al., 2003; MARTÍN et al., 2004; FERNÁNDEZ et al., 2012).

The relevance of microorganisms found in human milk becomes clear, when we consider that an infant consuming approximately 800 mL/day of milk would ingest between 10^5 and 10^7 microorganisms daily (MARTÍN et al., 2004). Bacterial species that have been isolated from human milk by cultured and uncultured methods include *Lactobacillus gasseri*, *L. rhamnosus*, *L. plantarum*, *L. fermentum*, *Enterococcus faecium*, *Bifidobacterium breve*, *B. adolescentis*, *B. bifidum*, *B. longum*, and *B. dentium* (MARTÍN et al. 2007; MARTÍN et al., 2009; MARQUES et al., 2010).

In 2011, the first study was published that focused on the characterization of the human milk microbiome through DNA pyrosequencing and that offered a global overview of commonly found genera (HUNT et al., 2011). This study identified a high complexity and inter-individual variability, although they shared the following groups *Streptococcus*, *Staphylococcus*, *Serratia*, *Pseudomonas*, *Corynebacteria*, *Ralstonia*, *Propionibacterium*, *Sphingomonas*, and *Bradyrhizobiaceae* (HUNT et al., 2011).

Other studies observed high proportions of *Weisella* and *Leuconostoc* populations in colostrum samples, followed by *Staphylococcus*, *Streptococcus*, and *Lactococcus* (CABRERA-RUBIO et al., 2012). In addition, human milk samples taken from healthy women at days 3 - 6, 9 -14 and 25 - 30 postpartum identified the genera *Bifidobacterium*, *Bacteroides*, and *Blautia*, which are strict anaerobes commonly found in the intestinal microbiota (JOST et al., 2013).

Although several studies have demonstrated the presence of microorganisms naturally occurring in human milk, the mechanisms by which these microorganisms reach human milk is not entirely clear (FERNÁNDEZ et al., 2012).

One hypothesis to explain the presence of bacteria in human milk is that these microorganisms would come from the skin or oral cavity microbiota of the infant, since these species, which are often isolated from milk, are sometimes found on these sites (BIAGI et al., 2017).

Although many of the isolated species are found on human skin and in human milk, they do not always share the same genotypic traits. The presence in human milk of strictly anaerobic species, such as the *Bifidobacterium*, diverges from the traditional assumption of contamination through the skin or oral cavity of the infant (MARTÍN et al., 2003; FERNÁNDEZ et al., 2012; JOST et al., 2013a).

A hypothesis to the origin of human milk bacteria assumes that milk microbiota also originates from the mother's gut (JEURINK et al., 2013). According to this hypothesis, bacteria would reach the mammary gland via an endogenous route, the entero-mammary pathway. This mechanism would involve dendritic cells, which would penetrate the gut epithelium and be able to take up commensal bacteria directly from the maternal gut lumen (JEURINK et al., 2013).

Once the gut bacteria are in the dendritic cells, they can reach different locations through the circulatory system from the gut-associated lymphoid tissue (FERNÁNDEZ et al., 2012). This mechanism was firstly suggested in the study of RESCIGNO et al. (2001), in which a strain of *Salmonella typhimurium* with no invasive genes was isolated from the spleen of mice, after oral administration.

In fact, ALBESHARAT et al. (2011) and JOST et al., (2013a) reported that some species, particularly those of the genera *Bifidobacterium* and *Lactobacillus*, may be present in maternal fecal, breast milk or infant fecal samples. These studies suggest a vertical transfer of microorganisms from the maternal gut to the breast milk and from there to the infant gut.

In this line, a recent study identified bacteria living "free" (in "planktonic" state) and associated to human immune cells, observed by SEM microscopy and fluorescence microscopy. The results reinforce the hypothesis of a translocation of bacteria to the mammary gland through blood and/or lymph stream by its association to human immune cells (BOIX-AMORÓS et al., 2016).

Interestingly, MACPHERSON e UHR (2004) also observed that dendritic cells are able to take up commensal microorganisms from the gut lumen and, contrary to what happens when macrophages are involved in the response, allow some commensal microorganisms to remain alive for several days. This mechanism could be responsible for allowing viable bacteria to reach the mammary glands (THUM et al., 2012).

On the other hand, some studies, which analyzed the human milk microbiome, found low proportions of *Bifidobacterium* and *Lactobacillus* (HUNT et al., 2011; CABRERA-RUBIO et al., 2012), contrary to what had been previously observed (MARTÍN et al., 2007; MARTÍN et al., 2009). Although differences in the methodological approach for bacteria identification among the studies might be one reason for differences in those results, the authors attribute these differences to genetic, cultural, environmental or dietary factors affecting the studies' participants (HUNT et al., 2011).

Indeed, CABRERA-RUBIO et al. (2012) observed that differences in the composition of the human milk microbiota were related to the stage of breast milk (colostrum, transitional milk and mature milk), the delivery mode (vaginal or Cesarean section), and maternal factors, such as the pre-pregnancy body mass index (BMI) and pregnancy weight gain.

The above-mentioned studies suggest a huge variability in terms of human milk bacterial community among individuals, as well as in terms of nutrients, immunological and oligosaccharides composition. Given that the human milk microorganisms are important elements for the development of the infant gut microbiota at the early life, to identify the factors that can influence the human milk microbiota is essential, since they may indirectly influence the infant colonization (FERNÁNDEZ et al., 2013).

JUSTIFICATION

Human milk is known to be the most important component for the infant's growth and metabolic and immune development (CABRERA-RUBIO et al., 2012). In addition, the microorganisms that naturally occur in breast milk are among the main factors responsible for the infant gut microbiota composition during lactation (COLLADO et al., 2015).

Several studies have discussed how maternal factors influence the nutritional composition and bioactive compounds of human milk (ALLEN, 2012; BALLARD & MORROW, 2013). However, so far, very few studies have assessed whether maternal factors may influence the composition of the human milk microbiome (HINDE & GERMAN, 2012; CABRERA-RUBIO et al., 2012; BALLARD & MORROW, 2013). The maternal diet therefore deserves special attention.

Studies suggest that diet may play an important role in the composition and metabolic activity of the gut microbiota (WU et al., 2011, SCOTT et al., 2013), as well as, in the nutrients composition of the human milk in lactating women. Considering that the maternal gut, and the

milk nutrients composition may influence the establishment of commensal bacteria in the human milk, it is very important to assess the impact the maternal diet may have on the composition of the human milk microbiome (FERNÁNDEZ et al., 2013; JEURINK et al., 2013, JOST et al., 2013b; BOIX-AMORÓS et al., 2016).

It also seems that no study has so far evaluated the effect of the maternal diet during pregnancy, and the maternal diet supplementation with FOS on the human milk microbiota. Only one recent study has evaluated the effect of the maternal diet during lactation in modulating the human milk microbiota (WILLIAMS et al., 2017). However, the reduced number of participants and the presence of confounding factors in that study further substantiates the relevance of this research study.

OBJECTIVES

General

- To investigate the correlation between the maternal diet and the human milk microbiota profile. In addition, to assess the impact of maternal diet supplementation with prebiotics (fructooligosaccharides) on the human milk microbiota during lactation.

Specific

- To investigate the correlation between the maternal diet during pregnancy ("long-term" food intake) and the first month of lactation ("short-term" food intake) with the human milk microbiota profile.
- To assess the influence of the maternal diet supplementation with prebiotics (fructooligosaccharides) on the dynamics of the *Bifidobacterium* and *Lactobacillus* population in human milk.

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PART II

CHAPTERS

The current thesis is organized in the format of two scientific articles (Chapter 1 and Chapter 2), which are inside the scope of this thesis, as follows:

a. Study I: to investigate the correlation between the maternal diet during pregnancy ("long-term" food intake) and the first month of lactation ("short-term" food intake) with the human milk microbiota (**Chapter 1: Maternal dietary patterns in pregnancy drive the human milk microbiota profile, whereas minor changes are evidenced by short-term diet during lactation**).
b. Study II: to assess the influence of the maternal diet supplementation with prebiotics (fructooligosaccharides) on the dynamics of the *Bifidobacterium* and *Lactobacillus* populations in human milk **(Chapter 2: Response of the human milk microbiota to a maternal prebiotic intervention is individual-dependent and influenced by the maternal age).**

The first study was a cross-sectional study, and the second study was a clinical trial. Both studies were conducted according to Figure 1 and Figure 2.

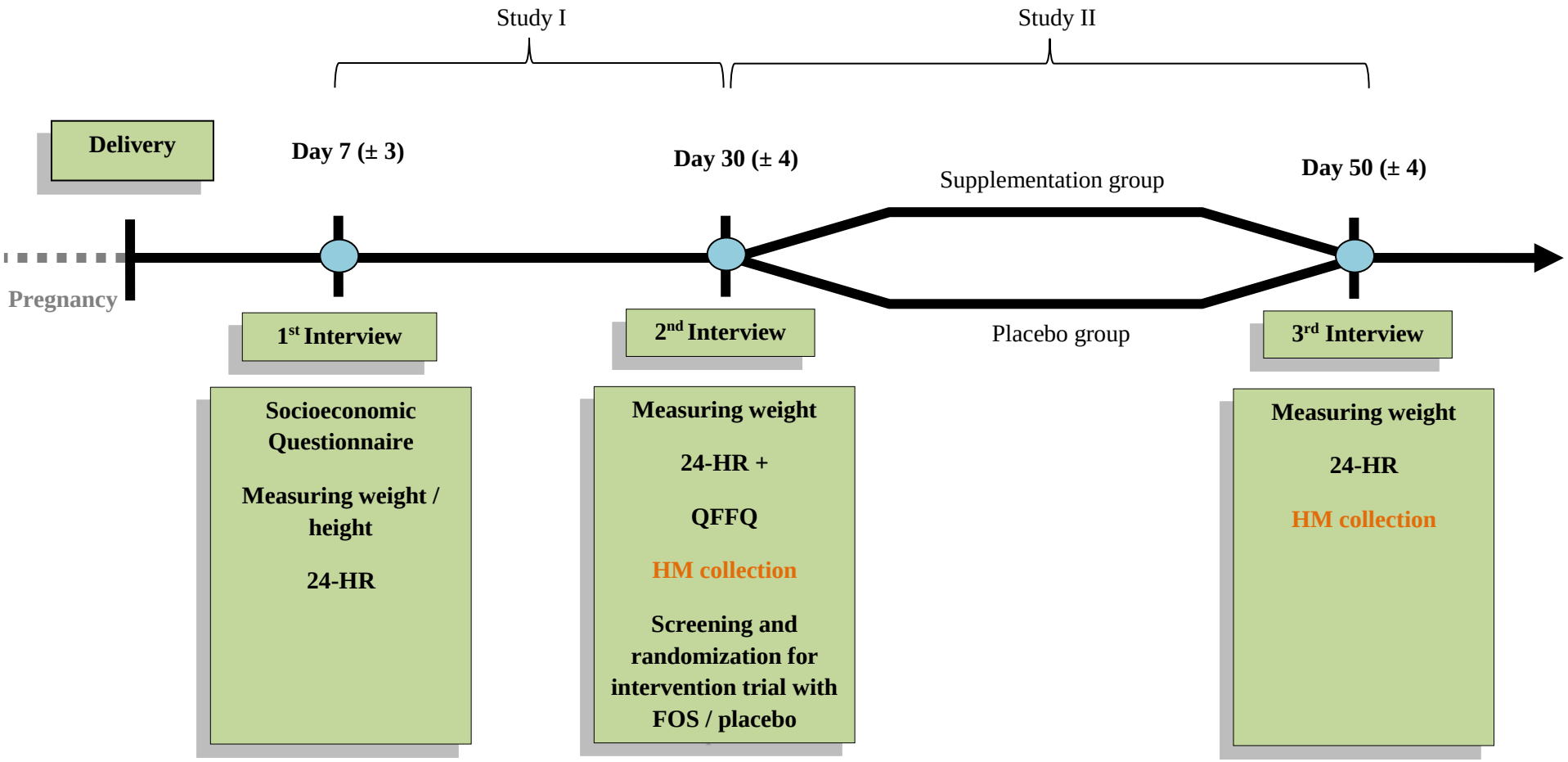

Figure 1. Study designs for data and milk samples collection.

24-HR: 24-hour food recall; HM: Human milk; QFFQ: Quantitative Food Frequency Questionnaire; FOS: Fructooligosaccharides.
Supplementation group: 4.5g of Fructooligosaccharides (FOS) + 2g of Maltodextrin; Placebo group: 2g of Maltodextrin.
Study I: Maternal dietary patterns in pregnancy drive the human milk microbiota profile, whereas minor changes are evidenced by short-term diet during lactation
Study II: Response of the human milk microbiota to a maternal prebiotic intervention is individual-dependent and influenced by the maternal age

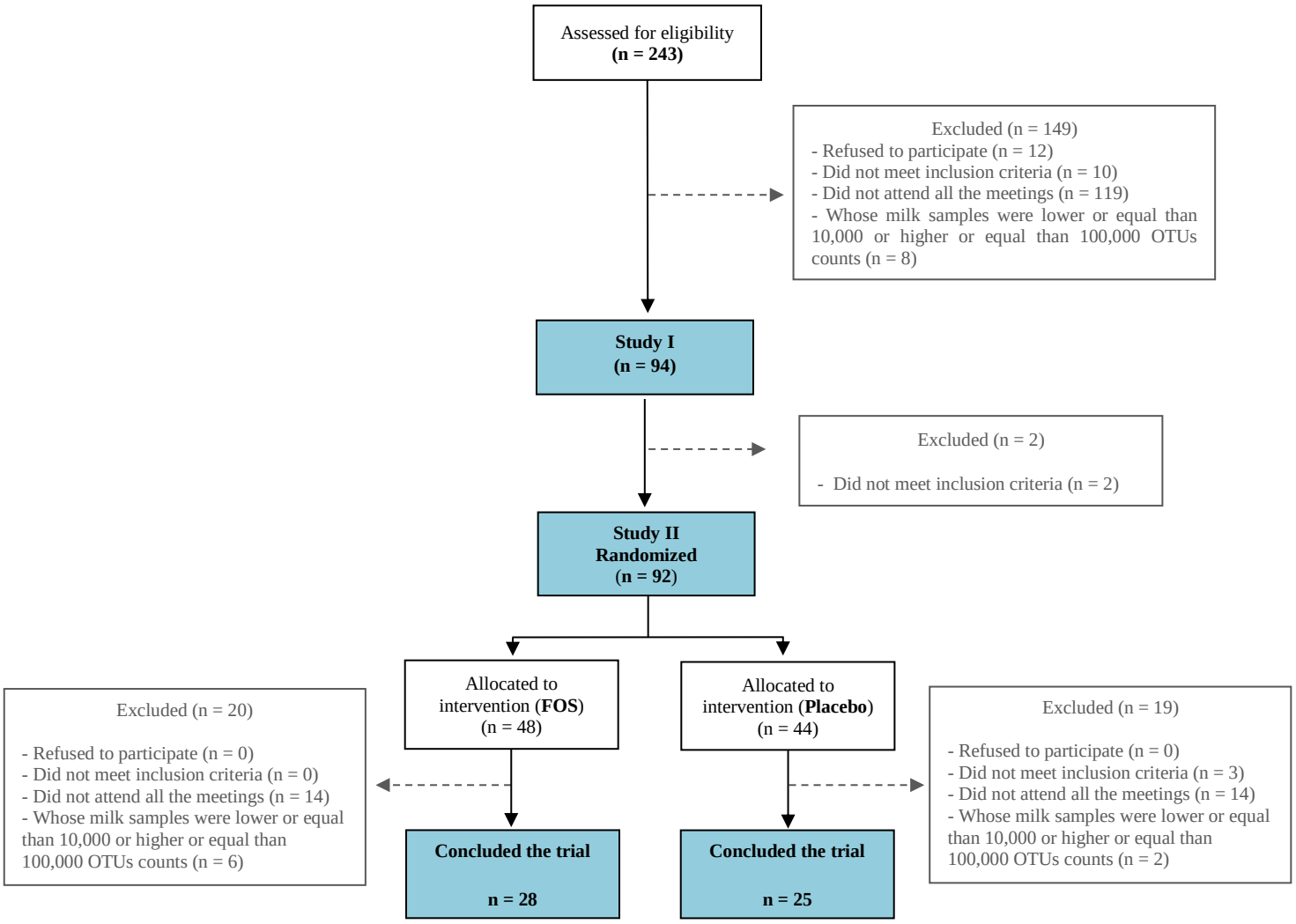

Figure 2. The flow diagram of participant recruitment, for each Study.

Study I: Maternal dietary patterns in pregnancy drive the human milk microbiota profile, whereas minor changes are evidenced by short-term diet during lactation
Study II: Response of the human milk microbiota to a maternal prebiotic intervention is individual-dependent and influenced by the maternal age

FOS: Fructooligosaccharide
OTU: Operational Taxonomic Unit

Chapter 1

Chapter 1. Maternal dietary patterns in pregnancy drive the human milk microbiota profile, whereas minor changes are evidenced by short-term diet during lactation

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Abstract

Background: microorganisms introduced through the human milk are important contributors for the development of the infants' immune system and are the pioneers in shaping the gut microbiota. To understand the factors that could influence the human milk microbiota profiles, especially the role of the maternal diet, is required. Here, we evaluated the effect of the maternal diet during pregnancy ("long-term" food intake) and during the first month of the lactation period ("short-term" food intake) on the human milk microbiota. **Methods:** the human milk microbiotas from 94 healthy women at their first month of lactation were analyzed by *16S rRNA* sequencing in Illumina MiSeq. The Maternal dietary data related to pregnancy and to the first month of lactation were collected by Quantitative Food Frequency Questionnaire and by 24-hours food recall, respectively. **Results:** three genera were present in all samples: *Streptococcus*, *Staphylococcus*, and *Corynebacterium*, with means of relative abundance of 42%, 22%, and 7%, respectively. Several other genera, including *Bifidobacterium* and *Lactobacillus* were identified at lower levels. Clustering analysis based on weighted UniFrac distance and prediction strength revealed 2 clusters, driven by *Streptococcus* (cluster 1) and *Staphylococcus* (cluster 2) genera. We found statistically significant Spearman's correlations between several maternal nutrient intake and bacterial taxa in both the pregnancy and the lactation period, underlining the positive correlation between polyunsaturated fatty acids/linoleic fatty acids intake during the lactation period and *Bifidobacterium* (coefficient = 0.29 and 0.27, respectively; $p < 0.01$). However, only maternal intake during pregnancy was statistically significant for the cluster analyzes, with higher intakes of vitamin C in the cluster 2 (cluster 1 = 175 mg/d, cluster 2 = 232 mg/d; $p = 0.02$). We also found trends towards higher levels of pectin and lycopene intake during pregnancy in the cluster 2, although this trend was not statistically significant ($p \geq 0.05$). **Conclusion:** according to our results, we identified that maternal long-term dietary habits, represented by pregnancy, play a crucial role in the human milk microbiota structures. Maternal short-term diet, represented by the lactation period, can influence the prevalence and abundance of specific bacteria in the human milk, however it was not sufficient to drive the community structure.

Keywords: maternal diet; microbiome; microorganism; breast milk; gut colonization; breastfeeding

Background

The gut microbiota has been extensively studied in the recent years and has become a relevant aspect of human health and disease (POSSEMIERS et al., 2011; FAUST et al., 2012; MILANI et al., 2017). The composition of the gut microbiota starts to develop especially during the early life, and converges toward the characteristic microbiota of an adult by 2-5 years of life (RODRIGUEZ et al., 2015).

The bacteria colonizing the infant gut during the first days of life are originated mainly from the mother and the environment (PENDERS et al. 2006 DOMINGUEZ-BELLO et al., 2010, BÄCKHED et al. 2015). Infants born by C-section are first exposed to the hospital environment and to their mother's skin bacteria, such as *Staphylococccus*, *Corynebacterium*, and *Propionibacterium*. In contrast, vaginally delivered infants are reported as enriched in genera like *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, and *Prevotella* (SCHWIERTZ et al., 2003; SALMINEN et al., 2004; BIASUCCI et al., 2008; YOUNES et al., 2018). These differences acquired in early childhood seem to persist in a long-term period and could be related to an increased risk for asthma (KERO et al., 2002; RODUIT et al., 2009), type 1 diabetes (ALGERT et al., 2009), and obesity (KALLIOMÄKI et al., 2008; AJSLEV et al., 2011) in individuals born by C-section.

It also well-documented that breast-fed babies develop distinct gut microbiota patterns, more enriched in *Bifidobacterium* and *Lactobacillus* compared to formula-fed babies, which are enriched in species belonging to *Clostridia* (BÄCKHED et al., 2015). According to KALLIOMÄKI et al. (2008), there is an inverse correlation between *Bifidobacterium* in fecal microbiota composition at early life and the incidence of overweight at age 7.

Particularly the human milk is an important source of potentially probiotic bacteria for the infant gut colonization, which have potential implications on the health of women and their infants (FUNKHOUSER and BORDENSTEIN 2013; MURPHY et al. 2017). Species already isolated from milk include *Lactobacillus gasseri*, *L. rhamnosus*, *L. plantarum*, *L. fermentum*, *Enterococcus faecium*, *Bifidobacterium breve*, *B. adolescentis*, *B. bifidum*, *B. longum*, and *B. dentium* (MARQUES et al. 2010; MARTÍN et al. 2007, 2009).

In addition, studies employing high throughput sequencing have shown that human milk contains a large diversity, including more than 207 bacterial genera (MURPHY et al. 2017). Among these genera, *Staphylococcus* and *Streptococcus* are the predominant bacterial groups (MURPHY et al. 2017; FITZSTEVENS et al., 2017). According to MURPHY et al. (2017), a

"core" of 12 genera, which were present in 90% of the milk samples evaluated by the authors, represented 81% of the microbiota relative abundance in milk samples at the first month after delivery. However, the large diversity among the samples suggests individual variation in the microbial composition (HUNT et al. 2011).

In this context, the pre-gestational Body Mass Index (BMI) (CABRERA-RUBIO et al., 2012; WILLIAMS et al., 2017), lactation time (CABRERA-RUBIO et al., 2012), mode of delivery (CABRERA-RUBIO et al., 2012, KUMAR et al., 2016) or even the geographic location (KUMAR et al., 2016) have been related to the differences on the human milk microbiota composition.

In addition, the maternal diet (WILLIAMS et al., 2017), and the composition of nutrients in human milk (KUMAR et al., 2016, BOIX-AMORÓS et al., 2016) were recently proposed as factors that may influence the microbiota composition in human milk. At first, the hypothesis that the mother's diet may help in shaping the human milk microbiota could be based on previous studies which reported that specific nutrients from the maternal intake affect the nutrients composition of the human milk (ALLEN et al., 2012; NISHIMURA et al., 2014).

Another hypothesis has suggested an entero-mammary pathway as a route for bacteria to reach the human milk, enabling the transfer of bacteria from the maternal gastrointestinal tract to the mammary gland (FERNÁNDEZ et al., 2013; BOIX-AMORÓS et al., 2016). Since the diet might influence the gut microbiota (DAVID et al. 2014; WU et al. 2011), including during the perinatal period (CARROTHERS et al., 2015), consequently the maternal diet could indirectly influence the human milk microbiota. This theory helps to explain the origin of bacteria that are not found in the maternal skin or the infant's mouth, which are described as the potential bacterial sources for the human milk microbiota (FERNÁNDEZ et al., 2013; BIAGI et al., 2017; PANNARAJ et al., 2017).

Since a vertical transfer of bacteria via breastfeeding has been suggested as an important contributor for the initial establishment of the microbiota in the developing infant gut (MURPHY et al., 2017), it is necessary to understand the factors that could influence the human milk microbiota, especially the role of maternal diet, for which limited information is available. Moreover, to our knowledge, the relation between the maternal diet during pregnancy and the milk microbiota has not yet been explored. In this paper, we evaluated the effect of the maternal diet during pregnancy ("long-term" food intake) and during the first month of the lactation period ("short-term" food intake) on the human milk microbiota profile.

Methods

Subjects and study design

This was a cross-sectional investigation involving healthy volunteers lactating women with ages ranging from 18 to 37, with uncomplicated pregnancy, and who had vaginal deliveries at the University Hospital of the University of São Paulo, in São Paulo city, and were assessed for eligibility to the study. To be eligible, participants were required to be free of any chronic gastrointestinal disease, genetic disease, cardiac disease, kidney disease, hypertension, diabetes mellitus or immunodeficiency diseases, eclampsia or gestational diabetes during pregnancy, mastitis during lactation period, besides having babies born between 37 and 42 weeks, with adequate weight for gestational age, having normal bowel frequency (minimum once every 2 days, maximum 3 times per day), and in practice of breastfeeding. Participants could not have taken proton pump inhibitors, H2 receptor antagonists, antidepressants, narcotics, anticholinergic medications, laxatives, or anti diarrhea medications within 30 days prior to collection the milk samples, and a regular consumption of commercially available prebiotic- or probiotic-supplemented products.

The project was approved by the Research Ethics Committee of the School of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil and by the Research Ethics Committee of the University Hospital of the University of São Paulo, São Paulo, Brazil - CAAE: 27247614.6.0000.0067 (ATTACHMENT 1 and 2). The study was conducted between September 2014 and June 2016.

The volunteers were screened through medical records, and from information obtained from them. The selected women were invited to participate in the study and enrolled after agreeing with the Written Informed Consent Form (WICF) (ATTACHMENT 3).

After the screening, two meetings were scheduled during the first month of lactation, at days 7 (± 3) and 30 (± 4) after delivery (Figure 1). During the first meeting, at day 7 (± 3) after delivery, a structured questionnaire was applied (ATTACHMENT 4) to collect data on age, familiar income, number of children, use of supplements/medicines during pregnancy/lactation, bowel frequency, alcohol consumption, smoking, pre-gestational and perinatal anthropometric data (weight and height) and information about the newborn. In addition, the first 24-hour food recall (24-HR) for the maternal diet record was performed.

On the 30th (± 4) day after delivery, the second meeting was held. An additional 24-HR was performed to reduce the intra-personal variability in food consumption assessment and a Quantitative Food Frequency Questionnaire (QFFQ) (ATTACHMENT 5) relative to the pregnancy period was applied. Samples of human milk were obtained from the participants during the second meeting at the University Hospital.

The QFFQ, as well as the collection of human milk were obtained in the second meeting (on the 30th ± 4 day after delivery) due to the longer available time of the volunteers, since in the first meeting the volunteers had a medical appointment, besides the research meeting.

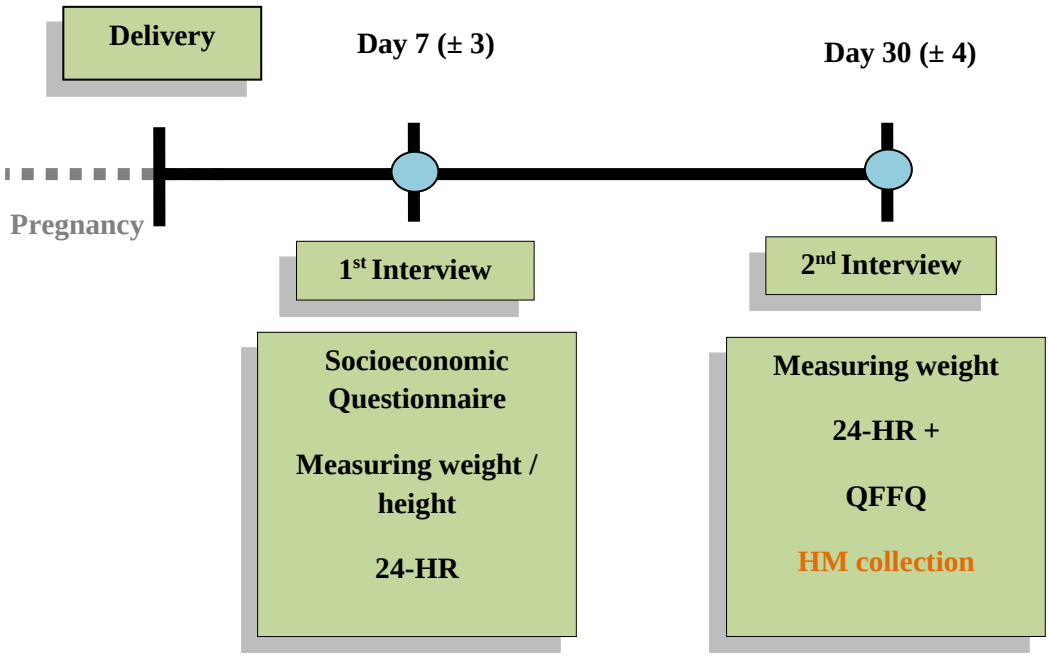


Figure 1. Study design for data and milk samples collection.

24-HR: 24-hour food recall; HM: Human milk; QFFQ: Quantitative Food Frequency Questionnaire.

Maternal diet records

The maternal diet records during pregnancy were estimated by QFFQ, developed (OLIVEIRA et al., 2010) and validated (BARBIERI et al., 2013) for pregnant women in Brazil, encompassing the entire period of gestation. The QFFA included 85 items of usual foods and recipes for this population, the consumption frequency (daily, weekly, monthly or during the gestational period), the number of times the participant consumes that food, the median portion - in home measures and in g/mL - and the size of the portion of each participant. The maternal diet records during the lactation period was estimated by two 24-HR, performed at day 7 (± 3)

and 30 (± 4) days after delivery. The 24-HR consists in obtaining information about the food intake of the last 24 hours prior to the interview, data on foods and beverages currently consumed, including preparation, and information on weight and portion size in grams, milliliters or home measures. The 24-HR were performed through the Multiple-Pass Method, in which the respondent is guided through five steps (quick list, forgotten foods list, time and occasion, detail and review, final probe) in a standardized process, which helps to maintain the individual interested and engaged in the interview, and helps them to remember all the items consumed (MOSHFEGH et al., 2008). The food surveys were conducted by trained nutritionists.

It is noteworthy that differences in the methods used in the present study to investigate the usual intake during pregnancy (QFFQ) and during lactation (24-HR) is due to the study design, which was planned to start after delivery. This was due to the inclusion criteria, which included only women who had vaginal delivery to avoid adding more variables in the study. Therefore, since we could not predict the mode of delivery of the volunteer, it would be unfeasible to collect 24-HR from pregnancy.

The Nutrition Data System for Research (NDSR, version 2007, Center for Nutritional Coordination, University of Minnesota, Minneapolis, USA) was used for the analysis of nutrients intake during pregnancy and lactation. Since this software uses the American food composition database developed by the United States Department of Agriculture (USDA), the adequacy of nutritional values was checked using the Brazilian Table of Food Composition (NEPA, 2011). In addition, folate and iron values were corrected considering the mandatory fortification of prevailing wheat and corn flours in Brazil since 2004. In case of use of nutritional supplements by the volunteers, these were added to the usual intake.

The nutrient data from 24-HR were processed in the *Multiple Source Method* program, in order to estimate the usual dietary intake of the volunteer (HARTTIG et al. 2011) during the lactation period. Afterwards, the nutrient intake was normalized using the residual method to standardize for caloric intake.

Human milk samples collection

Human milk samples were taken from volunteers at 30 (± 4) days after delivery, at the University Hospital of the University of São Paulo, according to MARTÍN et al. (2009) and JOST et al. (2013). Then, the women were oriented to clean their nipple and surrounding area with chlorhexidine 1% to reduce the presence of skin bacteria. Next, the first drops (approximately 200 uL) were discarded and the milk samples were collected in a sterile tube

by manual pressure using sterile gloves. All the samples were kept on ice for up to 4 hours; the samples were then aliquoted and stored at -80 °C for later DNA extraction.

DNA extraction

Total DNA was extracted with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's protocol for Gram-positive bacteria, with slight adaptations. Briefly, 1.5 mL of human milk sample was centrifuged at 15,700 g for 15 min to pellet prokaryotic cells. The supernatant was discarded, and the pellet washed in 1000 µL of Tris EDTA (10 mM Tris-HCl [pH 7.5], 1 mM EDTA [pH 7.6]) buffer. The suspension was centrifuged at 15,700 g for 15 min. The samples were lysed in 200 µL of TELS (20 mg/ml lysozyme:1 M Tris-HCl [pH 7.5], 0.5 M EDTA [pH 8.0], 20% sucrose) buffer; next, it was incubated for 60 min at 37 ºC. The next steps followed the manufacturer's instructions.

DNA quality and concentrations were determined using a Nanodrop ND-1000 (Thermo Fisher Scientific, Waltham, USA).

Amplicon sequencing

The PCR-based library formation was performed by a nested PCR. The primer pair 1 for the first round of amplifications was 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACHVGGGTWTCTAAT), published by YU et al. (2005). For the second round, the primer pair 2 targeting the *16S rRNA* gene's variable region (V4) was as follows:

tagged 515F (AATGATACGGCGACCACCGAGATCTACAC NNNNNNNN GT GTGCCAGCMGCCGCGGTAA)

tagged 806R (CAAGCAGAAGACGGCATACGAGAT NNNNNNNNNNNN AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT)

where "N" indicates the nucleotides of the barcode sequence. Both primers with Illumina adaptor sequences in the 5' end (CAPORASO et al., 2012).

The PCR reactions were carried out in a 25 µL mixture (final volume), containing 500 nM (for the first round) or 200 nM (for the second round) of each primer pair, 0.2 mM dNTPs (Thermo Fisher Scientific), 0.5 units Phusion high fidelity DNA polymerase (Thermo Fisher Scientific), 1 x Phusion Green HF buffer (Thermo Fisher Scientific), and 10 µL of the DNA

sample (for the first round) or 2 µL of PCR product from the first amplification (for the second round). Cycling conditions were: 98 °C for 30 s followed by "X" cycles of 98 °C for 5 s, 56 ºC for 20 s, and 72°C for 20 s, wherein "X" was 40 or 15 for the first and second round of amplification, respectively.

The PCR products were purified using the Agencourt AMPure XP purification system (Beckman Coulter, Danvers, USA). The amplicon concentration was quantified using the PicoGreen kit (Thermo Fisher Scientific) and normalized to 1.2 ng/µl. Subsequently, samples were pooled (3 µl of each sample) and sequenced using an Illumina MiSeq V2 PE500 cartridge (500 cycles) on an Illumina MiSeq (Illumina®, San Diego, USA).

16S rRNA Gene Sequence Processing

For *16S rRNA* gene data analysis, generated sequences were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) v1.9.1 with default settings. Chimera checking was performed using UCHIME69 and de novo Operational Taxonomic Units (OTU)-picking was performed using UCLUST70 with 97% sequence similarity. Representative sequences were assigned taxonomy against the Greengenes database v13_871 using the RDP-classifier (WANG et al. 2007). Subsequent analyses were performed with the R version 3.4.3 using the metagenomeSeq (PAULSON et al. 2013), phyloSeq (MCMURDIE & HOLMES 2014), vegan (OKSANEN et al. 2016), and ggplot2 (WICKHAM 2009) packages. Data were filtered for low-abundance of OTUs by removal of OTUs present in fewer than 3 of all the samples and with a relative abundance higher than 0.5% across all OTUs. Analysis in R were performed with sequences per sample after filtering. Statistical analysis was performed on data filtered based on effective sample sizes, where samples were not included if they had fewer than 10,000 or more than 100,000 OTUs.

Statistical Analysis

All statistical analyses were performed using the statistical computing language R. For differences in the human milk microbiota composition, "Adonis" function (PERMANOVA) was performed using weighted and unweighted UniFrac distances, for each variable, using 999 permutations (vegan package).

Associations between nutrient intake and microbial taxa were conducted with a Spearman rank correlation test. Heatmaps of Spearman rank-order correlation coefficients were constructed with the use of the "pheatmap" package in R.

Wilcoxon signed ranks test was used to compare the mean of nutrients between samples which are present or absent for each OTU.

For the clustering analysis, samples were clustered based on relative genus abundances, and using weighted UniFrac distance via partitioning around medoid (PAM) clustering algorithm in the R package "cluster" (MAECHLER et al., 2012). Optimal number of clusters was estimated using the prediction strength (PS) (TIBSHIRANI & WALTHER, 2005). Alpha-diversity analyses were performed after applying rarefactions (10,000 sequences/sample) to standardize sequence counts (vegan package). After checking for normality, t-test or Wilcoxon signed ranks test was used for parametric or non-parametric data to compare clusters and variables.

A false discovery rate p value ≤ 0.01 was considered as significant for any analyses with multiple comparisons. A standard p value < 0.05 was considered as significant for all other analyses.

Results

Two hundred forty-three lactating women were assessed for eligibility. Of this group, 12 refused to participate, 10 did not meet the inclusion criteria, 119 did not attend all the meetings, and 8 volunteers were excluded by the filter criteria of OTUs counts in their milk samples (milk samples with lower or equal than 10,000 and higher or equal than 100,000 OTUs counts were excluded). Thus, 94 volunteers were included in the analysis of this work. The clinical and demographic characteristics of these volunteers are shown in Table 1.

Regarding the sequences generated by sequencing in the Illumina platform, 3,122,436 *16S rRNA* gene sequences were analyzed, after quality filtering, with an average number of high-quality sequences of 33,217 per sample. Reads were classified into 334 OTUs at 97% sequence similarity. Figure 2 presents the rarefaction curves obtained for all samples, comparing the sequencing effect with an estimate of the number of bacteria species, as inferred by the number of OTUs. Based on these rarefaction curves, we can assume that the sequencing was deep enough, covering all the bacterial diversity in human milk, represented by saturated curves in the graph.

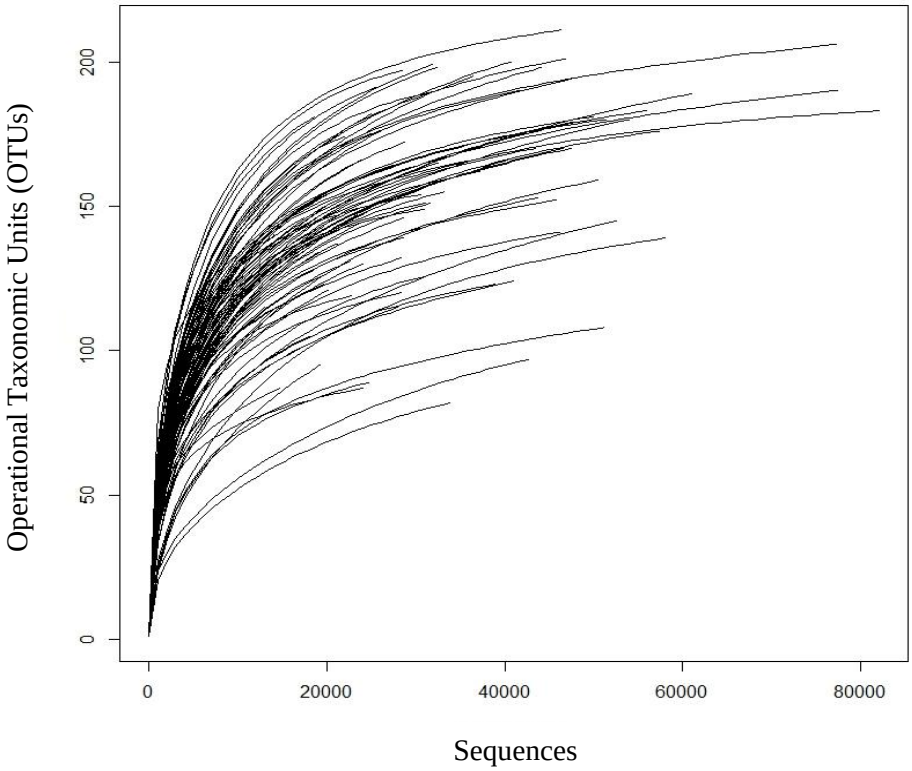


Figure 2. Rarefaction curves of all human milk samples, comparing the sequencing effect with an estimate of the number of bacteria species, as inferred by the number of Operational Taxonomic Units (OTUs).

Considering that human milk samples might have a low microbial load, a no template PCR control and a DNA extraction kit reagent control were sequenced alongside the samples. According to Figure 3, we observed that the genera abundances in the controls were uncorrelated with the abundances in the milk samples ($p = 0.001$ by PERMANOVA test comparing controls *vs* milk samples). Thus, we conclude that the controls had different profiles than those of the milk samples.

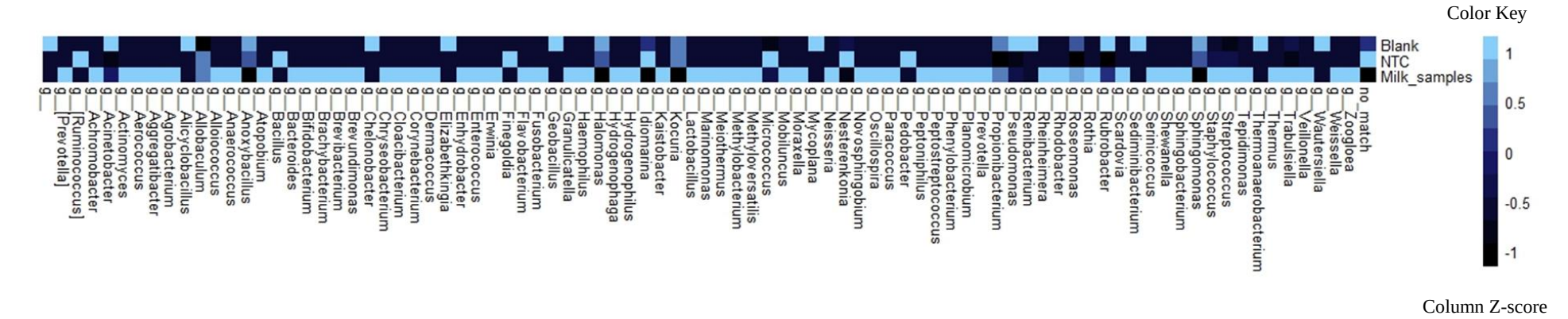


Figure 3. Comparison of bacterial profile between human milk samples and controls.

To verify the microbiota from reagents in either the DNA extraction kit or from PCR, we extracted the DNA of an empty tube containing the reagents from the DNA extraction kit ("Blank") and a no template control ("NTC") for PCR alongside the milk samples. Data presented as genera relative abundance normalized by using z-score in columns, for ease of visualization of the differences between controls and samples of human milk. As observed, the bacterial profile from milk samples differs from those of controls ($p = 0.001$ by PERMANOVA test comparing controls *vs* milk samples).

Apart from the diverse parameters analyzed in this study, the characterization of the milk microbiota in the 94 volunteers showed that *Firmicutes* was the most abundant phylum identified with an average of 70%, followed by *Actinobacteria* (14.5%), *Proteobacteria* (14%), and *Bacteroidetes* (1%). The 85 genera identified in human milk samples revealed a large diversity of the human milk microbiota. However, three genera were present in all samples: *Streptococcus*, *Staphylococcus*, and *Corynebacterium*, with means of relative abundance of, respectively, 42%, 22%, and 7%. Several other genera, such as *Rothia*, *Haemophilus*, *Rubrobacter*, *Trabulsiella*, *Veillonela*, *Achromobacter*, *Acinetobacter*, *Actinomyces*, *Halomonas*, *Pseudomonas* were identified at lower levels. *Bifidobacterium* and *Lactobacillus*, which are important genus related to infant's health, were present in 1% and 0.3%, respectively. A heatmap with the relative abundances of the main genera found in the human milk samples is shown in the Figure 4.

We evaluated the effect of the maternal diet by one food frequency questionnaire relative to the pregnancy period and two 24-hours dietary recalls to estimate the nutrients intake during the first month of lactation. To identify associations between nutrients and human milk bacteria, PERMANOVA tests were performed using UniFrac (weighted and unweighted) distances for each nutrient. Significant differences were found, based on intake levels of vitamin C ($p = 0.029$) for weighted UniFrac distance, during pregnancy, and based on intake levels of total fat ($p = 0.043$), insoluble fiber ($p = 0.047$), added sugars ($p = 0.039$), vegetable proteins (0.020), and magnesium ($p = 0.040$), for unweighted UniFrac distance, during the lactation period. There were no significant differences in human milk bacteria regarding maternal ethnicity (race), age, socioeconomic level, number of children, duration of pregnancy, antibiotic use during pregnancy/delivery, BMI before pregnancy, weight gain over pregnancy, anesthesia at delivery, BMI at day 30 after delivery, sporadic offering of infant formula or infant weight gain over 30 days after birth, using PERMANOVA tests for both weighted and unweighted UniFrac distances (Table 2).

We also used Spearman's correlations test to identify associations between each nutrient and bacterial genus. The Spearman correlations are presented as heatmap, for nutrients intake related to the pregnancy (Figure 5) and the lactation period (Figure 6). Since we found significant differences for some nutrients intake, during the lactation period, by using unweighted UniFrac, we compared the means of these nutrients intake in women who were "present" or "absent" for each OTU. The differences between these means are shown in Figure 7 for OTUs which had statistical significance for multiple comparisons ($p \leq 0.01$).

Table 1. Clinical and demographic characteristics of the volunteers included in the analysis (n= 94).

	Values
Maternal age (years)	27 (22.3 - 29)
Race	
Black/Brown	41 (44)
White	53 (56)
Family income estimated (USD/month) *	462.5 (462.5 - 770.39)
Number of children	
1	32 (34)
2 or more	62 (66)
Duration of pregnancy (weeks)	39 (38 - 40)
Maternal antibiotic treatment	
During pregnancy	26 (28)
During delivery	40 (42)
Alcohol drinking during pregnancy	5 (5.3)
Smoking during pregnancy	9 (9.5)
BMI before pregnancy (kg/m^2)	23.0 (21.1 - 24.6)
Maternal weight gain over pregnancy (kg)	11.4 (8.7 – 14.0)
Anesthesia	
No anesthesia	63 (67.1)
Pudendal block	3 (3.1)
Epidural	5 (5.3)
Spinal	23 (24.5)
BMI at day 30 after delivery	23.8 (22.1 - 26.7)
Infant diet at day 30 after delivery	
Exclusively breast milk	79 (83)
Breast milk + formula **	16 (17)
Infant weight gain over 30 days after birth (g)	1,160 (890 – 1,470)

Data presented as median (interquartile range) or n (%). BMI: Body Mass Index (kg/m^2)
* Family income estimated by Brazilian Economic Classification Criteria (Brazilian Criteria) 2016.
** frequency of offering formula ≤ 3 times/day.

Table 2. P values obtained by using PERMANOVA tests for weighted and unweighted UniFrac distances for each variable.

Variable	p values	
	Weighted UniFrac	**Unweighted UniFrac**
Clinical and demographic characteristics		
Maternal age	0.312	0.07
Race	0.490	0.110
Socioeconomic level	0.231	0.873
Number of children	0.389	0.102
Duration of pregnancy	0.364	0.184
Maternal antibiotic treatment (pregnancy)	0.115	0.790
Maternal antibiotic treatment (delivery)	0.666	0.340
Alcohol drinking during pregnancy	0.278	0.852
Smoking during pregnancy	0.872	0.795
BMI before pregnancy	0.214	0.501
Maternal weight gain over pregnancy	0.367	0.500
Anesthesia	0.318	0.383
BMI at day 30 after delivery	0.563	0.819
Infant diet at day 30 after delivery	0.474	0.596
Infant weight gain over 30 days after birth	0.555	0.398
Nutrients intake, during pregnancy		
Energy	0.945	0.938
Total carbohydrates	0.962	0.906
Added sugars	0.523	0.681
Total proteins	0.777	0.720
Total fat	0.623	0.541
Total saturated fatty acid	0.369	0.341
Total monounsaturated fatty acid	0.698	0.622
Total polyunsaturated fatty acid	0.727	0.595
Linoleic fatty acid (18:2 n-6)	0.770	0.848
Linolenic fatty acid (18:3 n-3)	0.640	0.715

continuation

Total trans fatty acid	0.623	0.957
Animal protein	0.404	0.305
Vegetable protein	0.723	0.246
Cholesterol	0.777	0.088
Total dietary fiber	0.449	0.636
Soluble fiber	0.473	0.454
Insoluble fiber	0.698	0.487
Pectin	0.295	0.084
Vitamin A	0.782	0.909
Vitamin D	0.464	0.655
Vitamin E	0.792	0.986
Vitamin K	0.716	0.738
Vitamin C	0.029	0.194
Vitamin B1 (thiamin)	0.761	0.794
Vitamin B2 (riboflavin)	0.432	0.596
Vitamin B3 (niacin)	0.807	0.459
Vitamin B5 (pantothenic acid)	0.592	0.798
Vitamin B6 (pyridoxin)	0.403	0.502
Vitamin B9 (folate)	0.821	0.884
Vitamin B12 (cyanocobalamin)	0.201	0.475
Calcium	0.369	0.605
Iron	0.802	0.689
Phosphorus	0.611	0.837
Magnesium	0.624	0.932
Manganese	0.812	0.891
Copper	0.639	0.965
Selenium	0.844	0.622
Sodium	0.877	0.872
Potassium	0.187	0.883
Zinc	0.787	0.409
Lutein – Zeaxanthin	0.688	0.328

continuation

Lycopene	0.654	0.797
Nutrients intake, during lactation		
Energy	0.229	0.657
Total carbohydrates	0.794	0.461
Added sugars	0.659	0.039
Total proteins	0.842	0.906
Total fat	0.320	0.043
Total saturated fatty acid	0.618	0.068
Total monounsaturated fatty acid	0.310	0.086
Total polyunsaturated fatty acid	0.121	0.096
Linoleic fatty acid (18:2 n-6)	0.104	0.068
Linolenic fatty acid (18:3 n-3)	0.287	0.334
Total trans fatty acid	0.283	0.488
Animal protein	0.757	0.910
Vegetable protein	0.206	0.020
Cholesterol	0.508	0.479
Total dietary fiber	0.257	0.061
Soluble fiber	0.419	0.203
Insoluble fiber	0.279	0.047
Pectin	0.305	0.146
Vitamin A	0.920	0.823
Vitamin D	0.689	0.817
Vitamin E	0.624	0.685
Vitamin K	0.860	0.931
Vitamin C	0.837	0.273
Vitamin B1 (thiamin)	0.618	0.428
Vitamin B2 (riboflavin)	0.742	0.570
Vitamin B3 (niacin)	0.375	0.777
Vitamin B5 (pantothenic acid)	0.671	0.557
Vitamin B6 (pyridoxin)	0.572	0.785
Vitamin B9 (folate)	0.737	0.428

continuation

Vitamin B12 (cyanocobalamin)	0.677	0.777
Calcium	0.759	0.547
Iron	0.545	0.237
Phosphorus	0.214	0.695
Magnesium	0.360	0.040
Manganese	0.963	0.510
Copper	0.976	0.620
Selenium	0.677	0.934
Sodium	0.710	0.304
Potassium	0.126	0.189
Zinc	0.389	0.705
Lutein – Zeaxanthin	0.282	0.547
Lycopene	0.400	0.094

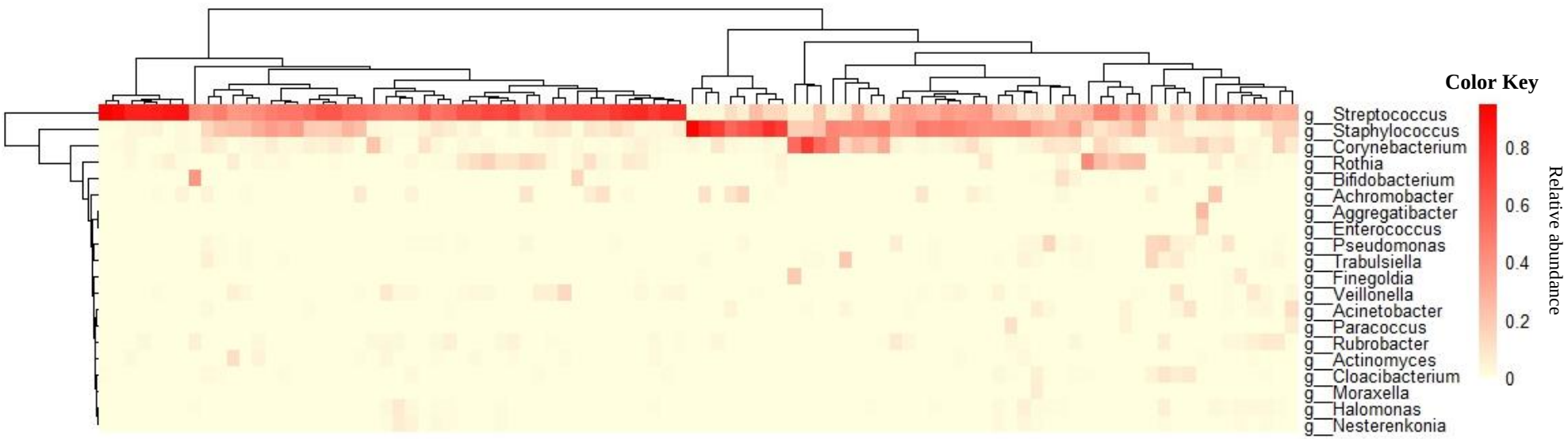


Figure 4. Relative abundance heatmap of the most abundant bacterial genera identified in human milk samples.

The rows present the genera with maximum relative abundance higher than 0.05. Each column represents the milk sample taken from volunteers at 30 (± 4) days after delivery.

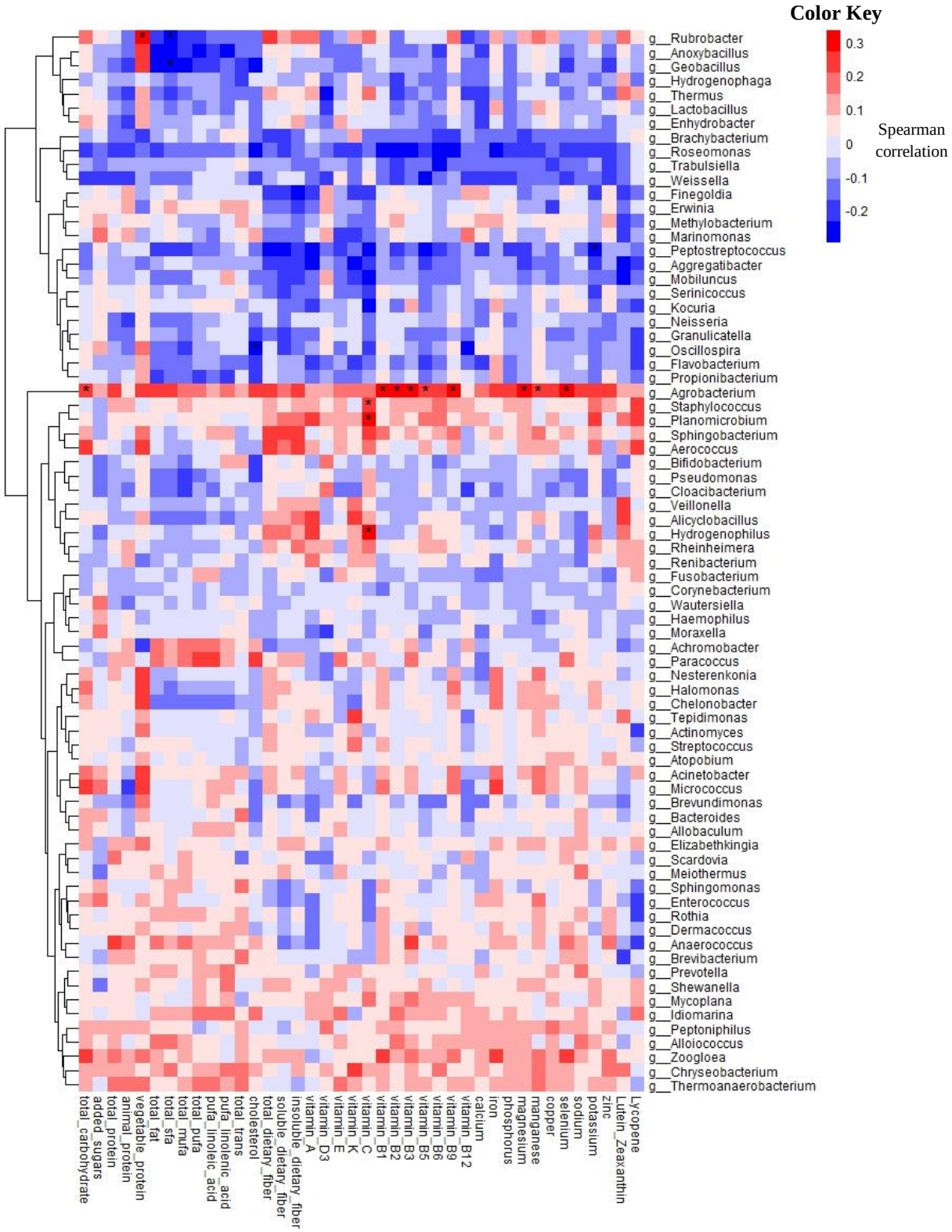


Figure 5. Correlation of maternal diet during pregnancy and human milk bacterial genera.

Columns correspond to estimated nutrient intake measured by a Quantitative Food Frequency Questionnaire; rows correspond to bacterial genus. Red and blue denote positive and negative association, respectively. The intensity of the colors represents the degree of association between the genus abundance and nutrients as measured by Spearman's correlations. Asterisks indicate the associations that are significant for multiple comparisons ($p \leq 0.01$). Rows are clustered by Euclidean distance.

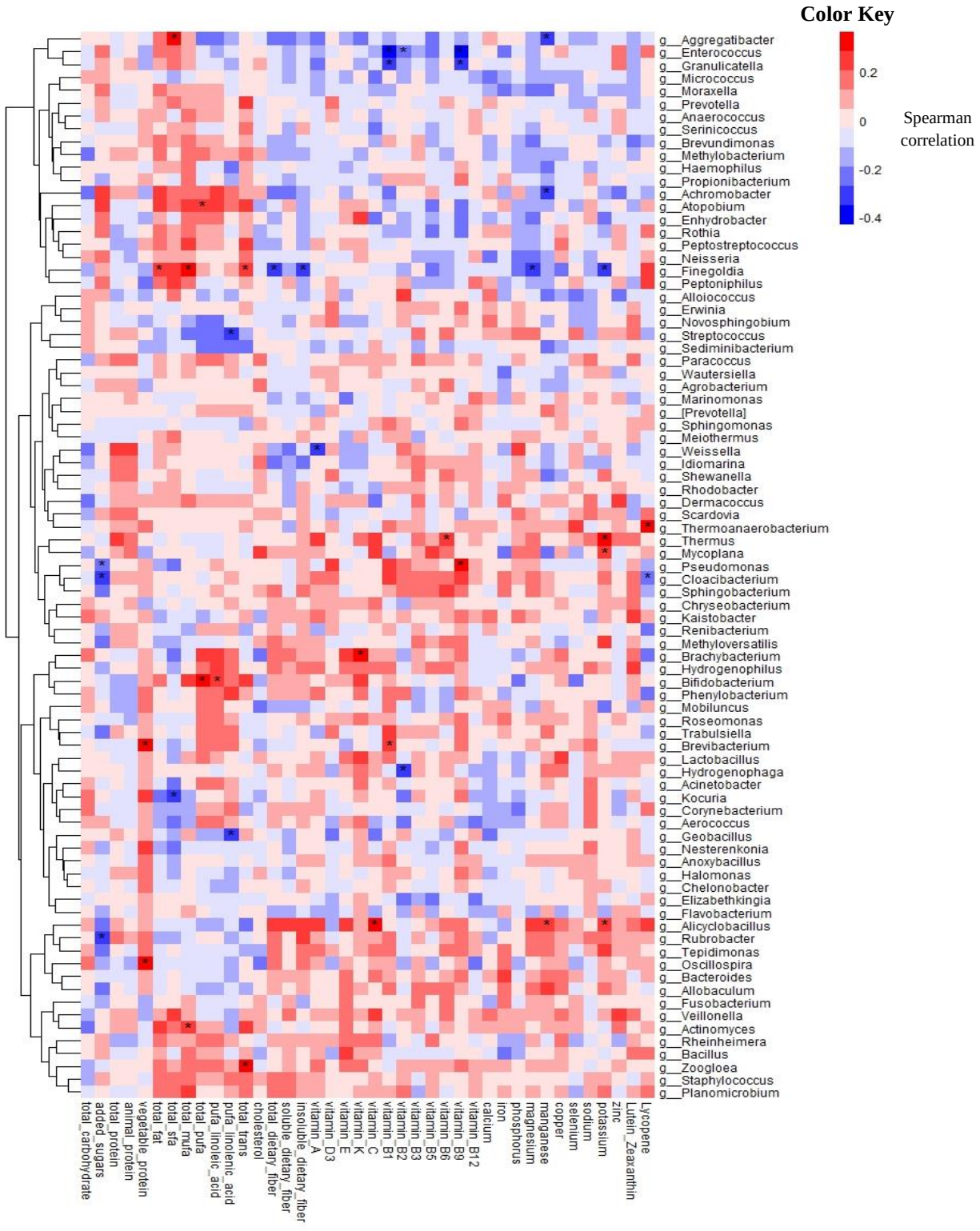


Figure 6. Correlation of maternal diet during lactation and human milk bacterial genera.

Columns correspond to estimated nutrient intake measured by 2 24-hour food recalls; rows correspond to bacterial genus. Red and blue denote positive and negative association, respectively. The intensity of the colors represents the degree of association between the genus abundance and nutrients as measured by Spearman's correlations. Asterisks indicate the associations that are significant for multiple comparisons ($p \leq 0.01$). Rows are clustered by Euclidean distance.

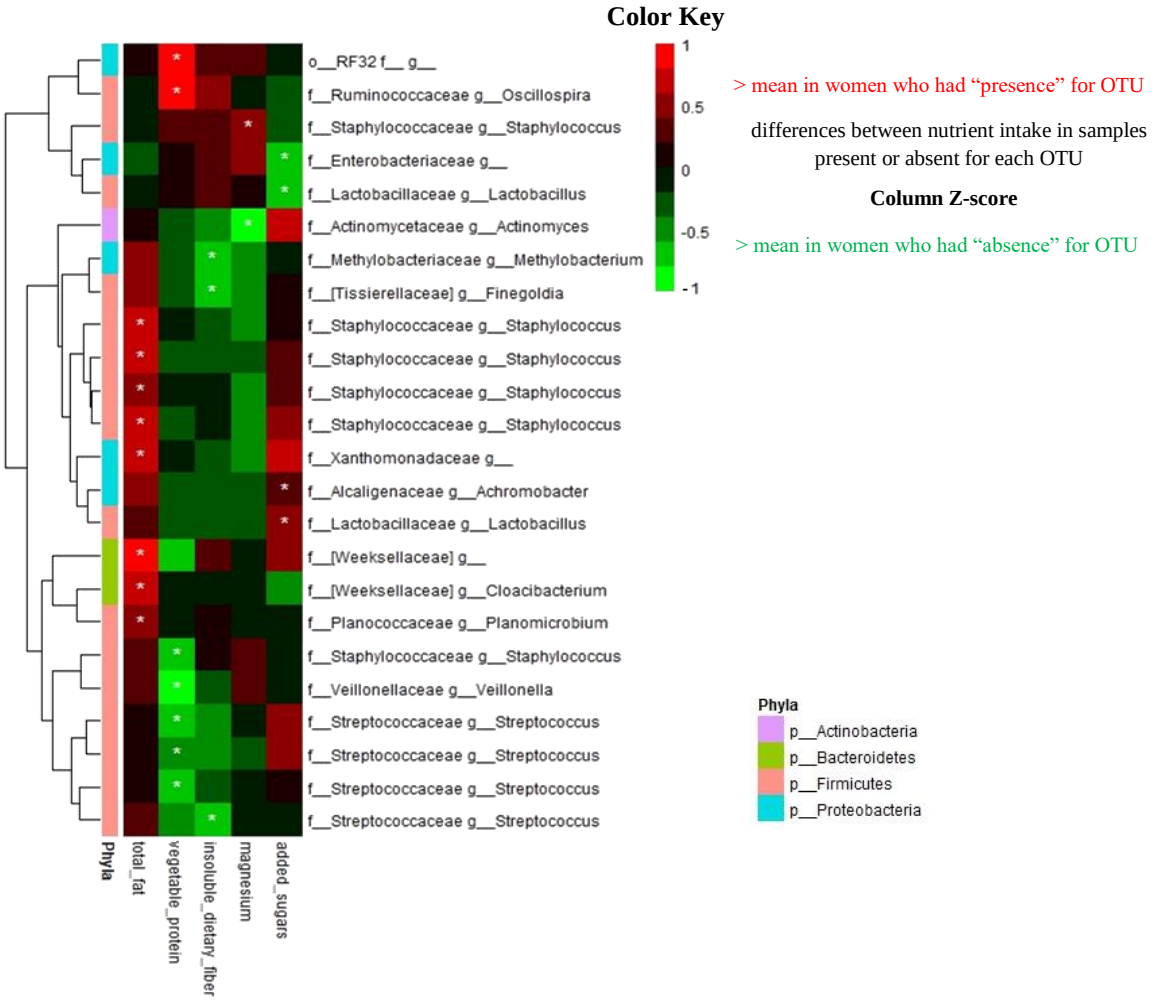


Figure 7. Heatmap for the differences between the means of estimated nutrient intake from the lactation period, selected by PERMANOVA tests using unweighted UniFrac, in samples present or absent for each OTU.

Columns correspond to differences between the means of estimated nutrient intake measured during the lactation period in samples which were present and means of estimated nutrient intake in samples which were absent for each bacterial Operational Taxonomic Unit (OTU) (Rows). The differences were calculated by [(mean of estimated nutrient intake in women who have human milk samples present for certain OTU) – (mean of estimated nutrient intake in women who have human milk samples absent for certain OTU)], therefore, positive values (Red) denote higher nutrient amount in samples which were present for certain OTU and negative values (Green) denote higher nutrient amount in samples which were absent for certain OTU. The intensity of the colors represents the degree of the differences between the means. The values were z-score transformed for ease of visualization of the differences. Only OTUs and nutrients which have significant p values, using Mann-Whitney tests ($p \leq 0.01$ for multiple comparisons), are shown. The asterisks indicate the differences that are significant. Rows are clustered by Euclidean distance.

Overall, during pregnancy, we found a positive association between vitamin C intake and the genera *Planomicrobium* (coefficient = 0.33, p = 0.0009), *Hydrogenophilus* (coefficient = 0.30, p = 0.003), and *Staphylococcus* (coefficient = 0.25, p = 0.01). We also found positive associations between other nutrients and genera present in minor relative abundance in the human milk samples analyzed, such as total carbohydrate, complex B vitamins, Magnesium, Manganese, and Selenium intake associated with the *Agrobacterium* genus. Vegetable protein had a positive association with the relative abundance of *Rubrobacter*, whereas this genus and *Geobacillus* were negatively associated with total saturated fatty acids.

The maternal diet estimated during the lactation period, representing a more recent intake, seems to have a distinct pattern of associations with the bacterial genera compared to the patterns found for pregnancy. Overall, the nutrients seem to present higher number of associations, although most of the associated genera were present in minor relative abundance in the human milk samples. In terms of lipid fractions, negative associations were found for saturated fatty acids (SFA) and *Kocuria* and for linolenic fatty acids with *Streptococcus* and *Geobacillus*. Positive associations were found for SFA and *Aggregatibacter*; polyunsaturated fatty acids (PUFA) with *Atopobium*; monounsaturated fatty acids (MUFA) and *Actinomyces*, and total fat, MUFA, and trans fatty acids with *Finegoldia*. We also found positive associations between *Bifidobacterium* and PUFA (coefficient = 0.29, p = 0.005) and linoleic fatty acid (coefficient = 0.27, p = 0.007).

In addition, negative associations were found for total dietary fibers and insoluble fibers with *Finegoldia*, as well as magnesium and potassium with this genus. Added sugars with *Pseudomonas*, *Cloacibacterium*, and *Rubrobacter*; vitamin A with *Weissela*; vitamin B1 and B9 with *Granulicatella*; vitamin B2 with *Hydrogenophaga*, manganese with *Achromobacter* and *Aggregatibacter*, besides lycopene with *Cloacibacterium* were all negatively associated. The complex B vitamins, particularly B1, B2, and B9 were also negatively correlated with *Enterococcus*. Positive correlations were found between vegetable protein and vitamin B1 with *Brevibacterium*; vitamin B9 with *Pseudomonas*, vitamin B6 and potassium with *Thermus*; manganese and potassium with *Alicyclobacillus*; vitamin K with *Brachybacterium*; vitamin C with *Alicyclobacillus*, and lycopene with *Thermoanaerobacterium*.

Regarding the influence of the presence or absence of taxa on the bacterial community of the human milk microbiota, we found that OTUs belonged to *Staphylococcus* and *Streptococcus* genera were the most representative among the significant OTUs identified in this analysis (Figure 7). In general, women who reported a higher total fat intake during the

lactation period also often presented OTUs belonging to *Staphylococcus* genus in their milk samples. On the other hand, women who reported a higher vegetable protein intake during the lactation period were frequently absent for OTUs belonging to the *Streptococcus* genus.

To get an overview of the human milk samples, and reduce the variability of the data, we grouped the samples in clusters, based on their profile similarities. The best statistical support for the optimal number of clusters was obtained by using the reduction strength and the weighted UniFrac metric (prediction strength = 0.78 for 2 clusters). The analysis revealed two clusters from the milk samples (Figure 8).

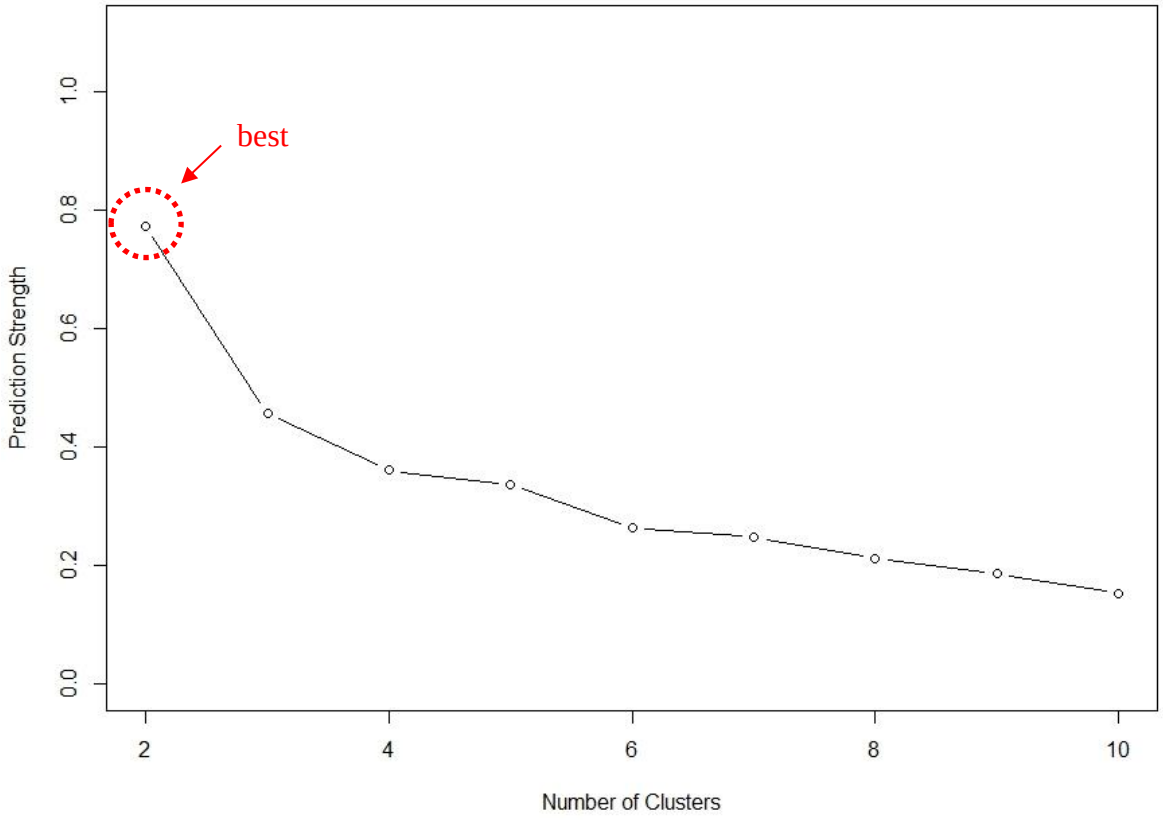


Figure 8. Optimal number of clusters displayed using the prediction strength measure and the weighted UniFrac distance. The panel shows that the data are most naturally separated into two clusters by the partitioning around medoid (PAM) method.

A Principal Coordinate Analysis (PCoA) indicates that each of these two clusters are driven by the variation in the levels of one of two genera: *Streptococcus* (cluster 1) and *Staphylococcus* (cluster 2) (Figure 9A and 9B).

A. **B.**

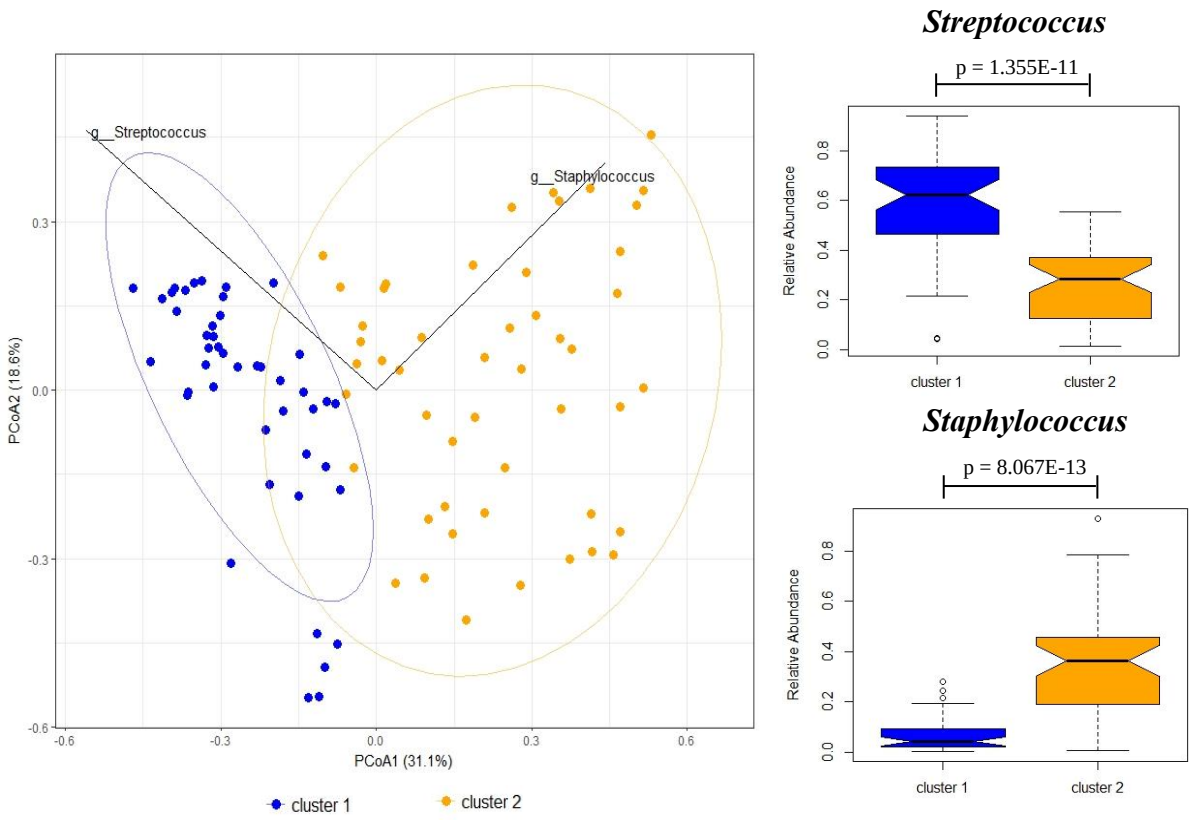


Figure 9. Clusters identified in human milk samples. (A) Principal Coordinate Analysis (PCoA) of clustering human milk samples driven by *Streptococcus* (cluster 1) and *Staphylococcus* (cluster 2). (B) Relative abundance of bacterial taxa characteristic of each cluster. Box-plot representing the interquartile range (IQR) and the line inside represents the median. Small circles denote outliers.

Mann-Whitney test was performed to compare the relative abundance between the clusters.

No differences were found between alpha-diversity measured by Chao1, Shannon and Observed indexes, and the clusters (Figure 10). In addition, there were no significant differences between clusters and maternal ethnicity, age, socioeconomic level, number of children, duration of pregnancy, antibiotic use during pregnancy/delivery, BMI before pregnancy, weight gain over pregnancy, anesthesia at delivery, BMI at day 30 after delivery, sporadic offering of infant formula or infant weight gain over 30 days after birth (Table 3).

We found differences in terms of vitamin C intake during pregnancy and the clusters (median in cluster 1 = 175 mg/d, cluster 2 = 232 mg/d, p = 0.0249; Figure 11A). The vitamin

C intake was higher in the cluster 2, driven by *Staphylococcus* genus. We also found trends towards higher levels of pectin (Figure 11B) and lycopene (Figure 11C) intake during pregnancy in cluster 2, even though this trend was not statistically significant.

In terms of nutrient intake during the first month of lactation, no statistical differences were found between these nutrients and the clusters (Table 3).

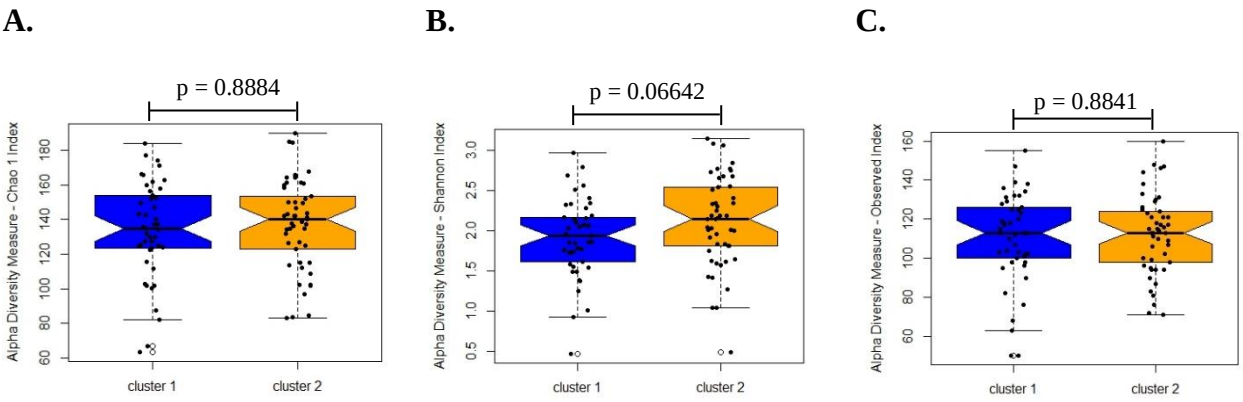


Figure 30. Alpha diversity values of milk samples, by clusters. The Alpha diversity is measured for Chao1 (**A**), Shannon (**B**), and Observed (**C**) indexes.

Operational Taxonomic Unit table for each measure was rarefied to 10,000 sequences. The box-plot is representing the interquartile range (IQR) and the line inside represents the median. Black-filled small circles denote the distribution of measures for each sample; white-filled small circles denote outliers. Mann-Whitney test was performed to compare the values between the clusters.

Table 3. P values obtained by using t tests (parametric) or Mann-Whitney test (non-parametric) for comparisons between clusters and continuous data or Chi-square test for categorical data.

Variable	p values
Clinical and demographic characteristics	
Maternal age	0.385
Race	0.697
Socioeconomic level	0.346
Number of children	0.159
Duration of pregnancy	0.09
Maternal antibiotic treatment (pregnancy)	0.061
Maternal antibiotic treatment (delivery)	1.000
Alcohol drinking during pregnancy	0.411
Smoking during pregnancy	0.893
BMI before pregnancy	0.764
Maternal weight gain over pregnancy	0.946
Anesthesia	0.881
BMI at day 30 after delivery	0.324
Infant diet at day 30 after delivery	0.475
Infant weight gain over 30 days after birth	0.141
Nutrients intake, during pregnancy	
Energy	0.792
Total carbohydrates	0.874
Added sugars	0.640
Total proteins	0.547
Total fat	0.976
Total saturated fatty acid	0.757
Total monounsaturated fatty acid	0.940
Total polyunsaturated fatty acid	0.587
Linoleic fatty acid (18:2 n-6)	0.424
Linolenic fatty acid (18:3 n-3)	0.507
Total trans fatty acid	0.874

continuation

Animal protein	0.460
Vegetable protein	0.958
Cholesterol	0.757
Total dietary fiber	0.587
Soluble fiber	0.116
Insoluble fiber	0.512
Pectin	0.053
Vitamin A	0.021
Vitamin D	0.845
Vitamin E	0.908
Vitamin K	0.657
Vitamin C	0.025
Vitamin B1 (thiamin)	0.821
Vitamin B2 (riboflavin)	0.833
Vitamin B3 (niacin)	0.668
Vitamin B5 (pantothenic acid)	0.552
Vitamin B6 (pyridoxin)	0.507
Vitamin B9 (folate)	0.465
Vitamin B12 (cyanocobalamin)	0.845
Calcium	0.746
Iron	1.000
Phosphorus	0.803
Magnesium	0.469
Manganese	0.483
Copper	0.934
Selenium	0.833
Sodium	0.982
Potassium	0.145
Zinc	0.951
Lutein – Zeaxanthin	0.239
Lycopene	0.058

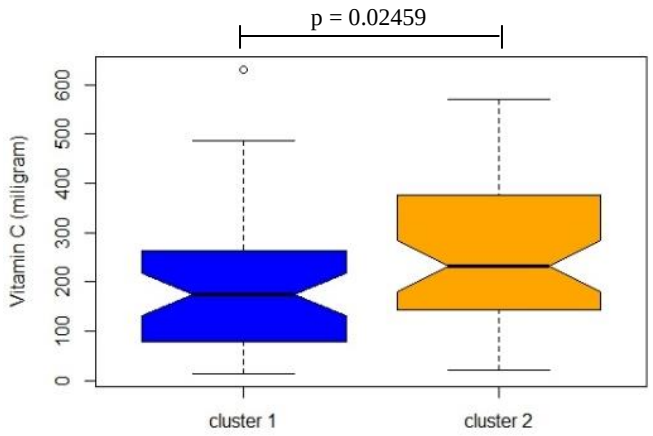
continuation

Nutrients intake, during lactation	
Energy	0.260
Total carbohydrates	0.519
Added sugars	0.620
Total proteins	0.530
Total fat	0.953
Total saturated fatty acid	0.931
Total monounsaturated fatty acid	0.707
Total polyunsaturated fatty acid	0.760
Linoleic fatty acid (18:2 n-6)	0.714
Linolenic fatty acid (18:3 n-3)	0.820
Total trans fatty acid	0.406
Animal protein	0.496
Vegetable protein	0.513
Cholesterol	0.864
Total dietary fiber	0.066
Soluble fiber	0.092
Insoluble fiber	0.132
Pectin	0.231
Vitamin A	0.323
Vitamin D	0.388
Vitamin E	0.158
Vitamin K	0.688
Vitamin C	0.173
Vitamin B1 (thiamin)	0.396
Vitamin B2 (riboflavin)	0.702
Vitamin B3 (niacin)	0.161
Vitamin B5 (pantothenic acid)	0.520
Vitamin B6 (pyridoxin)	0.261
Vitamin B9 (folate)	0.639
Vitamin B12 (cyanocobalamin)	0.285

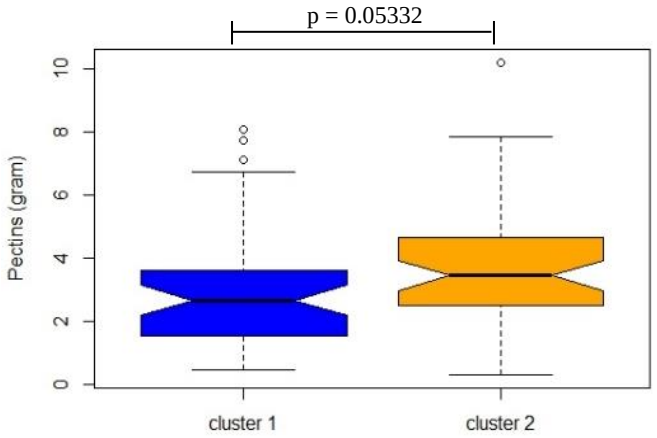
continuation

Calcium	0.781
Iron	0.217
Phosphorus	0.890
Magnesium	0.752
Manganese	0.452
Copper	0.692
Selenium	0.088
Sodium	0.425
Potassium	0.078
Zinc	0.153
Lutein – Zeaxanthin	0.638
Lycopene	0.618

A.



B.



C.

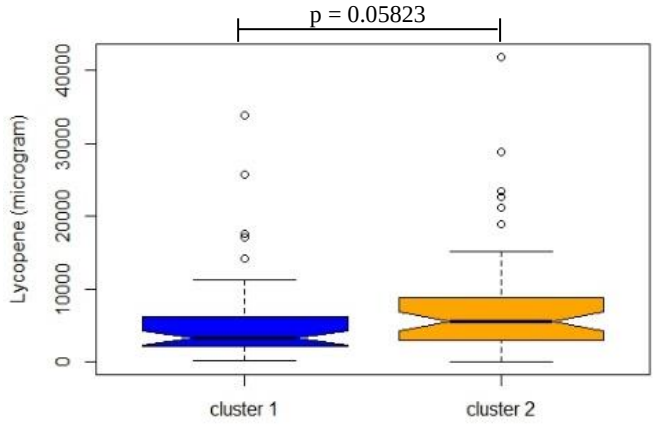


Figure 41. Distribution of nutrients intake from pregnancy, by clusters identified in human milk samples. Vitamin C (**A**), pectins (**B**), and lycopene (**C**) intake estimated by a Quantitative Food Frequency Questionnaire (QFFQ) for pregnancy, in each cluster.

The box-plot is representing the interquartile range (IQR) and the line inside represents the median. Small circles denote outliers. Mann-Whitney test was performed to compare the values between the clusters.

Discussion

Microbiota profile

The importance of the human milk on the health of an individual is well documented. In the last years, studies have been focused on the potential role of human milk strains in the development of the immune system (CABINIAN et al. 2016), as well as on modulating the infant gut microbiota (ARRIETA et al., 2014; GOLDSMITH et al., 2015). Given that the human milk microbiota plays an important role on the infant's health, the factors that could influence the milk bacterial composition require to be elucidated. Our study shows how the long and short-term diet of mothers can affect the array of microorganisms in the human milk.

Our results revealed 4 phyla - *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes* prevailing in the human milk microbiota on day 30 after delivery. *Firmicutes* and *Proteobacteria* were the most abundant phyla, in line with previous studies (MURPHY et al., 2017; URBANIAK et al., 2016). At genus level, *Streptococcus* and *Staphylococcus* were identified as the most important genera, followed by *Corynebacterium*. The presence of more than 80 genera found by sequencing the samples reinforces the complexity of the microbiota composition of the human milk with a much greater diversity than previously identified by culture-dependent methods or even using fingerprinting techniques.

Several studies have also identified *Staphylococcus* and *Streptococcus* as the most abundant genus in human milk (HUNT et al. 2011; JIMENEZ et al. 2015). Since these genera are typical inhabitants of the skin and oral cavity, our findings are consistent with the hypothesis of a retrograde flux of bacteria present in the mother's skin/infant's oral cavity to the human milk, enabling these bacteria to contribute with the establishment of the human milk microbiota (JEURINK et al. 2013).

In contrast with other authors, we identified the genus *Corynebacterium* in the core (presence in every sample) of the human milk microbiota, representing around 8% of the relative abundance. Although this genus has been reported by other studies, the relative abundances were found to be lower than 2% (CABRERA-RUBIO et al. 2012; JOST et al. 2013; MURPHY et al. 2017). The results published by HUNT et al. (2011) identified higher relative abundances of *Corynebacterium* only for a few samples of human milk.

According to CHU et al. (2017), *Corynebacterium* was more related with the infant's and the mother's nares. However, these authors also reported the presence of the *Corynebacterium* genus in the mother's skin at lower proportions. Possibly, the mother's skin

explains the origin of *Corynebacterium* in the human milk, still supporting the retrograde flux hypothesis. The presence of *Corynebacterium* also explains the higher relative abundances of *Actinobacteria* in our study, compared to other studies (MURPHY et al. 2017; URBANIAK et al. 2016).

We were expecting to find higher relative abundances of *Bifidobacterium* and *Lactobacillus*, since these genera are substantially present in the gut microbiota of breastfeeding infants. However, in our study, the average of relative abundances ranged from 0.1 to 1% for *Bifidobacterium* and from 0.1 to 0.3% for *Lactobacillus*. Our findings revealed lower proportions of these genera compared to the study of URBANIAK et al. (2016), who reported around 3% of *Lactobacillus*, and MURPHY et al. (2017), who reported around 2% of *Bifidobacterium* and *Lactobacillus* in the first weeks after delivery.

Different outcomes from sequencing analyses may be due to individual characteristics of the volunteers who donated the samples, as well as to differences in methodological factors, including DNA extraction procedures, PCR bias, use of different primer sets assessing different *16S rRNA* gene regions, as well as depth of sequencing of the study (JOST et al. 2013).

In our study, we searched for factors that could be potentially related to differences in microbial profiles of human milk samples. In terms of the maternal characteristics analyzed, no differences were found, which included ethnicity, age, socioeconomic level, number of children, duration of pregnancy, antibiotic use during pregnancy/delivery, BMI before pregnancy, weight gain over pregnancy, anesthesia at delivery, BMI at day 30 after delivery, sporadic offering of infant formula or infant weight gain over 30 days after birth. Previous studies have reported the influence of duration of pregnancy (KHODAYAR-PARDO et al., 2014), ethnicity (KUMAR et al., 2016), and pre-pregnancy (CABRERA-RUBIO et al., 2012) or postpartum BMI (WILLIAMS et al., 2017); however, the population included in this study were very homogeneous concerning these terms, since our focus were the effects of the maternal diet. Thus, we did not include preterm delivery or volunteers from different countries, as the previous studies, whose objectives were to investigate the effect of the duration of pregnancy or geographic location on the milk microbiota. Our purpose with these analyses were just to verify if it would be necessary to adjust the model of analysis to any other variable.

Concerning the role of the maternal diet on the human milk microbiota, previous studies have associated fermented food (ALBESHARAT et al. 2011) or probiotic consumption with the milk microbiota (JIMÉNEZ et al. 2008). In addition, one recently published study, which enrolled 21 US volunteers, reported that the maternal diet may play a key role in determining

the bacterial community in milk. To our knowledge, this is the first study to deal with the influence of maternal long-term (pregnancy) and short-term (1st month after delivery) food intake on the human milk microbiota. Our results revealed that the maternal diet has a significant influence on the human milk microbiota, but the effects seem to be different for diet during pregnancy and during the lactation period. Below we discuss our findings with major relevance in the clinical practice, with regard to the infant's health.

Correlations between the maternal macronutrients intake and the human milk microbiota

Our results suggest that higher intake of total fat from the lactation period was statistically related with the presence of some OTUs belonging to the *Staphylococcus* genus. Although not confirmed by statistical tests, we also observed a tendency towards positive correlations by Spearman's correlation tests between lipid fractions and the *Staphylococcus* genus, while a negative correlation was statistically confirmed between linolenic fatty acids and *Streptococcus*.

Several studies have suggested the direct effect of lipid fractions from the maternal diet on the lipid fraction of the human milk. In this context, XIANG, HARBIGE and ZETTERSTRÖM (2005), comparing 23 Swedish and 19 Chinese mothers, identified a significantly higher linoleic fatty acid content in the Chinese diet (15.9g vs 11.7g), which was correlated with a higher linoleic fatty acid content in the Chinese mother's milk (26.0g/100g vs 14.1g/100g). Likewise, NISHIMURA et al. (2014) reported a positive association between maternal dietary ω-3 to ω-6 ratio in the late pregnancy, as well as in the postpartum period, and the polyunsaturated fatty acid milk composition. Recently, KIM et al. (2017) reported significant positive associations between mothers' daily intake of eicosapentaenoic acid, docosahexaenoic, total polyunsaturated fatty acids, linoleic fatty acids, linolenic fatty acids, saturated fatty acids, and total fat and the correspondent fatty acids concentrations in human milk. Changes in the milk composition lead to different availability of nutrients for bacteria energy supply. While certain bacteria are able to metabolize different substrates, other species have specific preferences, which could lead to changes in the microbiota (SCOTT et al. 2013).

Curiously, the concentrations of *Staphylococcus* were shown to be significantly higher in the gut of infants whose mothers were overweight, during the first 6 months of life, possibly related with a maternal higher fat diet (COLLADO et al., 2010), although this was not

confirmed by a further study (CHU et al., 2016). We speculate that the relation between a higher maternal fat intake and the higher presence of *Staphylococcus* species in their milk may be related to the ability of members of the *Lactobacillales* order, including *Staphylococcus* genus, to incorporate extracellular fatty acids into membrane lipids. This, in turn, would confer a competitive advantage for this genus in face of increased availability of fats in the human milk (YAO & ROCK 2015; NGUYEN et al., 2016). Despite being a member of the *Lactobacillales* order, some authors suggest that *Streptococcus* can use the same basic pathway to incorporate fatty acids into their membrane, but they may have a distinct system (YAO & ROCK, 2015). Although infrequent studies have reported the effects of linolenic fatty acid over the *Streptococcus* genus, a previous study found that linolenic fatty acid in a concentration of 5 µg/mL in a culture media was associated to inhibitory activity over the *Streptococcus agalactiae* specie (WILLETT & MORSE, 1966), in agreement with the negative correlation between linolenic fatty acids and *Streptococcus* found in our study.

Interestingly, the total PUFA and the linoleic fatty acid were positively correlated to *Bifidobacterium,* an important genus for the infant's health (PENDERS et al., 2006; MILANI et al., 2017). The effect of polyunsaturated fat on the milk microbiota seems to be consistent with other studies, which have reported a direct relation between the polyunsaturated fat intake and the human milk composition (NISHIMURA et al., 2014; KIM et al., 2017). Recently, the new consensus statement on the definition and scope of prebiotics proposed prebiotic properties to PUFA (GIBSON et al., 2017). According to GIBSON et al. (2017) the mechanisms involved in the prebiotic property of PUFA is related to the conversion to the health-promoting conjugated linoleic acid (CLA) and conjugated linolenic acid (CLnA). Several species of *Bifidobacterium* have been considered as great producers of CLA and CLnA from PUFA (COAKLEY et al., 2003; GORISSEN et al., 2010). The metabolism of PUFA, to convert PUFA to CLAs, is followed by the growth of *Bifidobacterium* (COAKLEY et al., 2003), in line with the positive correlations between PUFA/linoleic fatty acid and *Bifidobacterium* found in this study.

Higher insoluble fibers and vegetable proteins intake during the lactation period were found to be related with a lower frequency of presence of some *Streptococcus* OTUs. Indeed, a previous study reported that *Streptococcus* were less prevalent in the intestine of subjects with higher dietary fiber patterns (CHEN et al., 2013). The sequencing analysis did not allow us to reach the species level of the OTUs identified, but a possible reason for the lower presence of these OTUs in volunteers with higher insoluble fiber and vegetable protein intake may be

related to some species of *Streptococcus* which are stricter, with regard the spectrum of substrates to obtain energy (NEIJSSEL et al., 1997), even though *Streptococcus* genus are known to be able to use amino acids as a source to obtain energy (LAW, 1978).

We found an inverse correlation between added sugars and *Pseudomonas*. In addition, a higher added sugar intake was significantly related with a lower frequency of an OTU belonging to the *Enterobacteriaceae* family. Among the human milk carbohydrates, lactose is the main one, followed by glucose and fructose (GORAN et al., 2017). Most of the studies reported that lactose contents in milk appears to be remarkably similar among women, and there is no convincing evidence that human milk lactose content can be influenced by the maternal diet (INSTITUTE OF MEDICINE, 1991). However, recent findings suggest that the maternal intake of fructose might be related to the fructose content in breast milk (GORAN et al., 2017).

The *Pseudomonas* genus, as well as the *Enterobacteriaceae* family, are Gram negative bacteria, that include along with many harmless symbionts, a group of familiar opportunistic pathogens, including *Pseudomonas aeruginosa* (*Pseudomonas* genus) and *Salmonella*, *Klebsiella pneumoniae*, *Escherichia coli*, and the specie *Enterobacter sakazakii* (*Enterobacteriaceae* family) involved with bacteremia, necrotizing enterocolitis, and infant meningitis in neonates and infants (IVERSEN & FORSYTHE, 2003). However, in healthy conditions, positive effects of the colonization of non-pathogenic *E. coli* strains have been described, including the reduced risk of allergies in infants, since it may affect the levels of several cytokines and specific anti-*E. coli* antibodies (LODINOVÁ-ŽÁDNÍKOVÁ et al., 2010).

Although *Pseudomonas* has been largely identified in human milk samples, it is poorly found in the infant's gut (MURPHY et al., 2017), and has been more associated with an unbalanced gut due to antibiotic treatment of preterm infants (ZHU et al., 2017). However, the relative abundance of this genus has been reported to be higher in subacute and acute mastitis (PATEL et al., 2017).

The inverse relation between added sugars and *Pseudomonas* is expected, indeed, since previous studies demonstrated *Pseudomonas* preference for amino and organic acids, rather than sugars (NELSON et al., 2013). However, the carbohydrate metabolism seems to be different for members of the *Enterobacteriaceae*, depending on the strain and the carbohydrate source (HOEFLINGER et al., 2015).

Our findings suggested a relation between several macronutrients and human milk bacteria, considering the influence of the maternal diet on the human milk nutrients

composition. However, further *in vitro* studies with bacteria isolated from human milk in media containing different proportions of the interested nutrients are required to confirm these relations herein described.

Correlations between maternal vitamins and minerals intake and the human milk microbiota

A major factor influencing the vitamin content of human milk is the mother's vitamin status (INSTITUTE OF MEDICINE, 1991). In general, low maternal intakes of a vitamin lead to lower levels of that vitamin in breast milk. As maternal intakes increase, the levels in milk also increase, but for many vitamins a plateau is reached, and levels do not respond further to supplementation (INSTITUTE OF MEDICINE, 1991).

In our study, we found a positive correlation between the maternal folate intake during lactation and *Pseudomonas*, which could be explained by the ability of this genus to degrade folate (RAPPOLD & BACHER, 1974). Conversely, we found negative correlations between complex B vitamins intakes during the lactation period, particularly thiamin, riboflavin, and folate and the *Enterococcus* genus. The complex B vitamins are hydro soluble and are extremely important during pregnancy and during the lactation period. Cases of deficiencies during pregnancy might cause poor pregnancy outcomes and, during the lactation period, the low consumption of these vitamins may result in infants consuming substantially less than the recommended amounts (ALLEN, 2005). The *Enterococcus* genus was found in human milk samples in previous studies (KUMAR et al., 2016, MURPHY et al., 2016), as well as in infant guts (BÄCKHED et al., 2015). These microorganisms are considered as commensal bacteria, among the first colonizers of the infant gut. In the last years, *E. faecium* and *E. faecalis* have become important pathogens worldwide, especially due to hospital-acquired infections (PINHOLT et al., 2014). On the other hand, infants with atopic eczema seem to have less Gram-positive bacteria, including *Enterococcus*, among their gut microbiota, when compared to healthy infants exclusively on breast-feeding (KIRJAVAINEN et al., 2001). However, these findings are not conclusive (BJÖRKSTÉN et al., 2001).

Most *Enterococci* require complex B vitamins, such as riboflavin, pyridoxin, cyanocobalamin, and sometimes folate, as coenzymes in their metabolism, for maximal growth (RAMSEY et al., 2014). Extending this literature, we would expect positive correlations between these vitamins and *Enterococcus*, contrary to our findings. Since vitamins are usually

bound to other compounds, predominantly proteins, it has been suggested that maternal factors regulating protein secretion are more likely to affect milk levels of these vitamins, than the fluctuations of the maternal intake. Thus, further studies are required to confirm the correlation between maternal intake of complex B vitamins and *Enterococcus*.

Regarding the maternal intake during pregnancy, we found positive correlation between vitamin C and *Staphylococcus* in human milk. Likewise, the vitamin C levels in human milk are associated to the maternal intake of this vitamin (INSTITUTE OF MEDICINE, 1991). In contrast to our findings, vitamin C has been reported to inhibit the *Staphylococcus* growth (KALLIO et al., 2012). However, most of the studies investigated the impact of high concentrations of this vitamin (more than 1 g/L), which is above the physiologic concentrations found in human milk samples (ca. 0.04 g/L) (INSTITUTE OF MEDICINE, 1991; KALLIO et al., 2012). On the other hand, other studies performed with citrus fruit juice, which are rich in vitamin C, showed no inhibitory effect over *Staphylococcus* (HINDI & CHABUCK, 2013; BENOY et al., 2016). Since very few studies reported on the role of vitamins in *Staphylococcus* metabolism, the positive relation between vitamin C and *Staphylococcus* metabolism remains unclear.

Regarding the minerals, few correlations with major importance in the clinical practice were found among minerals or bioactive compounds analyzed and the milk microbiota. However, we found a negative correlation between *Aggregatibacter* and the maternal manganese intake during the lactation period. *Aggregatibacter* is a Gram-negative coccobacillus and has been found to make part on the infant's gut microbiota (RUBIN, 2012; BÄCKHED et al., 2015). In fact, the concentrations of minerals, such as calcium, phosphorus, and magnesium, in the maternal serum are tightly regulated. Consequently, it is not at all expected that the maternal diet would influence these minerals content in the human milk (INSTITUTE OF MEDICINE, 1991). Nevertheless, a positive correlation was observed by a previous study between maternal manganese intake and the manganese content of human milk (VUORI et al., 1980). Manganese is a cofactor for numerous enzymes and regulators in metabolic pathways (RAMSEY et al., 2014). In human milk, it is bound to proteins (CHAN et al., 1982), but the mechanisms by which the manganese content would negatively affect the abundance of *Aggregatibacter* is still unknown, since very few studies reported the physiology and metabolism of this genus.

Our findings with regard the correlations between maternal vitamins/minerals intake could not be completely elucidated, partially due to the lack of reports on the role of these

nutrients in the metabolism of different bacteria and to the limited knowledge regarding the availability of vitamins and minerals in the human milk for the bacterial use. Thus, we reinforce that *in vitro* studies with bacteria isolated from human milk inoculated in human milk/culture media containing different concentrations of vitamins and mineral are required to confirm our results.

Influence of the maternal nutrients intake on clustering structures

The microbiota from all samples was classified into two clusters, driven by *Streptococcus* (median of relative abundance in Cluster 1: 62%) or *Staphylococcus* (Cluster 2: 36%), in line with previous studies, which reported higher relative abundance of these genera in human milk samples (HUNT et al., 2011; KUMAR et al., 2016; URBANIAK et al., 2016). Indeed, a recently published systematic review of the human milk microbiota identified the predominance of *Streptococcus* and *Staphylococcus* over 12 analyzed studies, suggesting that these genera may be universally predominant in human milk, regardless of differences in geographic location or analytic methods (FITZSTEVENS et al., 2017).

In addition, LI et al. (2017) reported 3 clusters based on family profiles of human milk microbiota in samples collected from Taiwan and six regions of mainland China. The clusters were driven by the *Streptococcaceae*, *Staphylococcaceae* or *Pseudomonadaceae* families, suggesting a human milk microbiota pattern closely similar to the one found in our study.

The benefits of *Staphylococcus* and *Streptococcus* on the infant's health is not completely clear, but it is known that these genera are present in the infant's gut, especially during the first weeks (AVILA et al., 2009; BÄCKHED et al., 2015; RODRÍGUEZ et al., 2015). Studies suggest that *Staphylococcus* and *Streptococcus*, as well as other facultative aerobic species are the initial gut colonizers. These species contribute to the consumption of oxygen from the infant's gut, providing a suitable environment for the colonization of strict anaerobic species, such as *Bifidobacterium*, *Bacteroides*, and *Clostridium* (WEBER & POLANCO, 2012).

In our study, we did not find any statistically significant differences among maternal socioeconomic/clinical information or nutrients intake from the lactation period and the clusters. However, we found significant differences in the intake levels of vitamin C during pregnancy and the clusters, which were higher in the cluster 2, driven by the *Staphylococcus* genus. This finding is in accordance with the results obtained by the Spearman's correlation

test, which indicated a positive correlation between vitamin C from pregnancy and the *Staphylococcus* genus.

Although the role of vitamin C in the *Staphylococcus* metabolism is not elucidated, as previously mentioned, it seems that the effect of vitamin C and *Staphylococcus* on the infants' health have been linked to a common outcome: the development of the immune system. According to HOPPU et al. (2005), higher vitamin C levels in breast milk was associated to reduced risk of atopy in high-risk infants (HOPPU et al., 2005). At the same time, non-allergic children were shown to have higher counts of *Staphylococcus* in feces, when compared to allergic counterparts (LUNDEL et al., 2007; SUZUKI et al., 2008). In this context, SALMINEN et al. (2016) suggested that *Staphylococcus aureus*, traditionally considered as harmful, but frequently isolated from infant feces, may aid in educating the coevolving immune system.

It is noteworthy to mention that we have also observed a tendency of higher maternal intakes of pectin and lycopene during pregnancy in cluster 2. Vitamin C, pectin, and lycopene are compounds typically found altogether in citrus fruits. In this context, higher maternal consumption of citrus fruit, as well as vegetables and β-carotene during pregnancy were protective against the development of eczema in the offspring, in line with the positive effects of vitamin C and *Staphylococcus* on the immune system (MIYAKE et al., 2010).

Curiously, fruits and fruit juices have been reported as craved foods in pregnancy by several studies (PATIL et al., 2012). The fluctuations of hormones produced during pregnancy and the instinct to protect the embryo from toxic substances and pathogens have been hypothesized to be the reason for food cravings, and/or aversions (PATIL et al., 2012; BUSTOS et al., 2017). We speculate that this behavior commonly found in pregnant women could be, not only related to the physiology of pregnancy, but also a contributor for the bacterial community structure in the human milk.

In a previous study, WU et al. (2011) demonstrated the role of the long-term dietary patterns associated to certain gut enterotypes; particularly protein and animal fat were associated to *Bacteroides* and carbohydrates to the *Prevotella* enterotype. Our results again highlight the most relevant effects of long-term dietary habits, herein represented by the maternal intake during pregnancy, over the bacterial structure. Although the effect of short-term diet, represented by the maternal intake during the first month of the lactation period, was verified for specifics genus and OTUs, it was not strong enough to be associated to the structure of the bacterial community in human milk.

Study limitations

Our study describes for the first time a comprehensive investigation of the role of the maternal diet evaluated in a long-term (pregnancy) and short-term (first month of lactation) approach on the human milk microbiota. Differently from previous studies, we collected human milk samples from vaginally term delivery, in a unique lactation stage (ca. 30 days after delivery), and evaluated several maternal factors in the analysis.

However, this study had limitations with regard to the distinct methods performed to collect the dietary data (Quantitative Food Frequency Questionnaire for pregnancy and 24-hour food recall for the lactation period). As previously mentioned, different methods were selected based on the study design, which started after delivery. Despite the fact that previous reports have extensively shown that maternal nutrient intake influences the nutrients profile in the human milk directly, further analyzes are required to confirm the correlations found between the maternal diet and the microbiota profile. Lastly, we did not investigate the effect of long-term dietary habits throughout the lactation period, which could be relevant to clarify if it could drive the clusters.

Conclusion

In our study, we investigated the effects of the maternal diet during pregnancy and during the first month of the lactation period on the human milk bacterial microbial community. Our results suggest that the maternal diet may influence the human milk microbiota, and the diet during pregnancy is a stronger factor over the bacterial community structure, particularly driven by the vitamin C intake. Minor changes were found by the maternal short-term food intake, represented by the lactation period, which can influence the abundance of specific bacteria in the human milk, underlining the positive correlation between polyunsaturated fatty acids/linoleic fatty acids intake and the *Bifidobacterium* genus in human milk. However, it was not sufficient to drive the community structure.

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Chapter 2

Chapter 2. Response of the human milk microbiota to a maternal prebiotic intervention is individual-dependent and influenced by maternal age

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Abstract

Background: recent studies have suggested that maternal bacteria are shared with their infant via breastfeeding. The maternal gut has been proposed to provide part of these human milk bacteria, by an entero-mammary pathway. Since prebiotics are recognized as modulating the gut microbiota, promoting health benefits to infants and pregnant/lactating women, we investigated if the maternal diet supplementation with prebiotics (fructooligosaccharides) could influence the dynamics of the human milk microbiota. **Methods**: this study was performed as a single-blind randomized placebo controlled trial. Twenty-eight lactating women received 4.5g of fructooligosaccharides + 2g of maltodextrin (FOS group) and twenty-five received 2g of maltodextrin, for 20 days (placebo group). Breast-milk samples were taken before and after the intervention. The DNA from samples was used as template for amplification and sequencing by Illumina MiSeq®. **Results:** no statistical differences were found among the genera relative abundance between the groups, after the intervention. However, the distances of the trajectories covered by the samples from the beginning to the end of the supplementation was higher for the FOS group ($p = 0.0007$). Linear regression models, using Jensen-Shannon distance between "before" and "after" the supplementation, by group revealed that the maternal age seems to affect the response for FOS supplementation ($p = 0.02$), suggesting that younger mothers were more influenced by the FOS supplementation. However, no patterns in the differences of relative abundances were found for younger mothers from the FOS group. **Conclusion:** we identified that maternal age can affect the response by the supplementation with FOS. However, the pattern of changes seems to be different for each subject. Further studies are required to deep understanding if other factors, such as the individual gut microbiota could be related to the differentiated responses by FOS supplementation.

Keywords: fructooligosaccharides; gut colonization; breast milk; breastfeeding; infant; clinical intervention.

Background

The gut microbiota has been shown to play an important role in human health (CLEMENTE et al., 2012; ASNICAR et al., 2017). The benefits of the interaction with microbes are particularly related to the development of the immune system, prevention against pathogens, and protection against diseases, such as allergy, type 1 diabetes and obesity (JOHANSSON et al., 2011; ALGERT et al., 2009; KALLIOMÄKI et al., 2008; AJSLEV et al., 2011).

The human gut colonization by microorganisms begins immediately at birth. As soon as the baby is born, it is exposed to a complex microbial community, which are the first microorganisms to shape the newborn's gut microbiota (SHEN et al., 2017). Thereafter, practices, including formula or breast feeding and use of antibiotics have been linked to the assembly of the gut microbiota structure (WEN & DUFFY, 2017)

The maternal microbiota seems to be a relevant contributor to the early gut colonization. Maternal-neonate transfers have been suggested as an evolutionary phenomenon preserved among several species of the animal kingdom (FUNKHOUSE & BORDENSTEIN, 2013), and can occur during delivery, by the birth canal (BÄCKHED et al., 2015), and via breastfeeding (GREGORY et al., 2016).

Indeed, studies have demonstrated that babies born by C-section have distinct gut microbiota profiles than infants vaginally delivered. According to BÄCKHED et al. (2015), newborns vaginally delivered have a most similar gut bacterial profile to their mothers' gut, compared to newborns C-section delivered. In addition, ASNICAR et al. (2017) identified that specific strains, including *Bifidobacterium bifidum*, *Coprococcus comes*, and *Ruminococcus bromii*, were present in breast milk and infant feces from the same mother-infant pair, while being distinct from those carried by other pairs, indicating a vertical transmission via breastfeeding.

The maternal gut has been proposed as providing part of the microbial community transferred to vaginally delivered newborns, possibly due to the proximity of the maternal gut and the birth canal. Similarly, the human milk seems to be, at least partially, composed by bacteria from the maternal gut (BOIX-AMORÓS et al., 2016; MCGUIRE & MCGUIRE, 2017). Particularly for the human milk, the main mechanism involves an entero-mammary pathway, in which dendritic cells would take up commensal bacteria directly from the maternal gut lumen, via the gut epithelium, and reach the mammary gland through the circulatory system

from the gut-associated lymphoid tissue (FERNÁNDEZ et al., 2013). Interestingly, PEREZ et al. (2007) investigated the intracellular transport of bacteria from the maternal gut to the mammary glands through the circulatory system in healthy lactating women. The authors found common bacterial DNA signatures maternal peripheral blood mononuclear cells and in milk, as well as in maternal and infant feces, supporting that some bacteria are transferred from the mother's gut to the milk, and then to the infant.

Certain gut bacteria, in particular those of *Bifidobacterium* and *Lactobacillus* species are strongly related to benefits in infant and adult health (CARLSON & SLAVIN, 2016). It has been shown that the growth of these bacteria in the gut is stimulated by the presence of specific compounds, namely prebiotics.

By definition, prebiotic is "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (GIBSON et al., 2017). The most studied prebiotics in infant's, pregnant and lactation women's health are fructooligosaccharides (FOS) and galactooligosaccharides (GOS) (CHAMP & HOEBLER, 2009; KOLIDA & GIBSON, 2011). These oligosaccharides are non-digestible by humans, and can be used as substrates by specific bacteria in the gut, to obtain energy (CHARBONNEAU et al., 2016; MILANI et al., 2017).

Given that prebiotics are recognized as modulating the gut microbiota, promoting benefits to the human's health, and considering the maternal gut as a "seeding" for the infant's gut colonization via breastfeeding, we investigated if the maternal diet supplementation with prebiotics (fructooligosaccharides) could influence the dynamics of the human milk microbiota. To our knowledge, this is the first work addressed to this issue.

Methods

Subject and study design

Ninety-two healthy pregnant women aged 18–37 years with uncomplicated pregnancies, and who had vaginal deliveries at the University Hospital of the University of São Paulo, in São Paulo city, were enrolled in the study.

Inclusion criteria were having no chronic gastrointestinal disease, genetic disease, cardiac disease, kidney disease, hypertension, diabetes mellitus or immunodeficiency diseases, eclampsia or gestational diabetes during pregnancy, mastitis during the lactation period, besides having term babies (born between 37 and 42 weeks), with adequate weight for gestational age,

having normal bowel frequency (minimum once every 2 days, maximum 3 times per day), and in practice of breastfeeding. Participants could not have taken proton pump inhibitors, H2 receptor antagonists, antidepressants, narcotics, anticholinergic medications, laxatives, or anti diarrhea medications within 30 days prior to collection the milk samples, and a regular consumption of commercially available, prebiotic- or probiotic-supplemented product. The volunteers were screened through medical records, and from information obtained from them.

The study was performed as a single-blind randomized placebo controlled trial. After randomization, the FOS group participants received 20 sachets containing 4.5g of the prebiotic fructooligosaccharides (FOS, Beneo P95, Orafti, Oreye, Belgium) + 2g of maltodextrin (Nidex, Nestlé, Araçatuba, Brazil). The placebo group participants received 20 sachets containing only 2g of maltodextrin (PLACEBO group) (Figure 1). The volunteers were instructed to dissolve all the powder content of one sachet in approximately 200 mL of water, once a day, for 20 days, starting at the day after the first meeting and ending at the day before the second meeting. Detailed instructions were described on the label of each sachet. The volunteers were also asked to maintain their habitual diet throughout the intervention period. Written informed consent was obtained from all participants (ATTACHMENT 3). The study protocol was approved by the Research Ethics Committee of the School of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil and by the Research Ethics Committee of the University Hospital of the University of São Paulo, São Paulo, Brazil - CAAE: 27247614.6.0000.0067 (ATTACHMENT 1 and 2). The study was conducted between September 2014 and June 2016.

Figure 1. Packages delivered to the volunteers with 20 sachets containing 2 g of maltodextrin (placebo group), on the left or 4.5 g of fructooligosaccharides + 2 g of maltodextrin (FOS group), on the right side. Each page of the block had one sachet and was identified with the week day for consumption.

Data collection

Information on pregnancy, familiar income, bowel frequency, alcohol consumption, smoking, besides pre-gestational and perinatal anthropometric data (weight and height) and information about the newborn were obtained by a structured questionnaire applied in a face-to-face interview, before the clinical trial (ATTACHMENT 4). Two 24-hour food recalls (24-HR) were performed, at day 30 (± 4) after delivery, and 21 days later, immediately after the consumption of the last sachet of the intervention, to monitor the volunteer diet before and after the trial. Dietary data were analyzed by Dietpro® software (Dietpro, Viçosa, Brazil), to obtain the estimated nutrient intake. Data on complaints about bowel behavior (e.g. abdominal pain, distension, flatulence) were registered before and after the intervention.

In addition, the volunteers were contacted by telephone at day 10 (in the middle of the intervention period), to promote compliance and to assess the occurrence of side effects and complaints. We considered the adherence for women who have consumed at least 90% of

sachets (18 sachets), which was checked asking the number of returned sachets at the end of the study.

Milk samples collection

Milk samples were collected by manual pressure from volunteers before [at 30 (± 4) days after delivery] and immediately after the intervention, into a sterile tube, according to MARTÍN et al. (2009) and JOST et al. (2013). Previously, the nipple and surrounding area were cleaned with chlorhexidine 1% to reduce the presence of skin bacteria. The first drops (approximately 200 uL) were discarded. All the samples were kept on ice within 4 hours; the samples were aliquoted and stored at -80 °C for later DNA extraction.

DNA isolation

Human milk samples (1.5 mL) were centrifugated at 15,700 g for 15 minutes to pellet prokaryotic cells. Pellets were resuspended in 1000 µL of Tris EDTA (10 mM Tris-HCl [pH 7.5], 1 mM EDTA [pH 7.6]) buffer. The suspension was centrifuged at 15,700 g for 15 min. The samples were lysed in 200 µL of TELS (20 mg/ml lysozyme:1 M Tris-HCl [pH 7.5], 0.5 M EDTA [pH 8.0], 20% sucrose) buffer; next, it was incubated for 60 min at 37 ºC. The DNA isolation proceeded using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's protocol for Gram-positive bacteria.

DNA quality and concentrations were determined using a Nanodrop ND-1000 (Thermo Fisher Scientific, Waltham, USA).

PCR amplification for Sequencing

The genomic DNA isolated from the clinical samples was amplified by a nested PCR, For the first round of amplifications, the primers 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACHVGGGTWTCTAAT), published by YU et al. (2005), were used. For the second round, barcoded primers that amplify the V4 hypervariable region of the 16S ribosomal RNA (*16S rRNA*) were used. The primers pair 2 are described as follows:

tagged 515F (AATGATACGGCGACCACCGAGATCTACAC NNNNNNNN GT GTGCCAGCMGCCGCGGTAA)

tagged 806R (CAAGCAGAAGACGGCATACGAGAT NNNNNNNNNNNN AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT)

where "N" indicates the nucleotides of the barcode sequence. Both primers with Illumina adaptor sequences in the 5' end (CAPORASO et al., 2012).

The PCR reactions were carried out in a 25 µL mixture (final volume), containing 500 nM (for the first round) or 200 nM (for the second round) of each primer pair, 0.2 mM dNTPs (Thermo Fisher Scientific), 0.5 units Phusion high fidelity DNA polymerase (Thermo Fisher Scientific), 1 x Phusion Green HF buffer (Thermo Fisher Scientific), and 10 µL of the DNA sample (for the first round) or 2 µL of PCR product from the first amplification (for the second round). Thermal cycling was carried out in a Veriti® Thermal cycler (Applied Biosystem, Thermo Fisher Scientific) under the following conditions: 98 °C for 30 s followed by "X" cycles of 98 °C for 5 s, 56 ºC for 20 s, and 72°C for 20 s, wherein "X" was 40 or 15 for the first and second round of amplification, respectively.

After amplification, the PCR products were purified using the Agencourt AMPure XP purification system (Beckman Coulter, Danvers, USA). The amplicon concentration was measured with the PicoGreen kit (Thermo Fisher Scientific). Equimolar amounts of each PCR product were then pooled together and sequenced using an Illumina MiSeq V2 PE500 cartridge (500 cycles) on an Illumina MiSeq (Illumina®, San Diego, USA).

Sequence Processing

Initial analysis was carried out using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline v1.9.1 with default settings. Chimera checking was performed using UCHIME69 and de novo OTU-picking was performed using UCLUST70 with 97% sequence similarity. Representative sequences were assigned taxonomy against the Greengenes database v13_871 using the RDP-classifier (WANG et al. 2007). Subsequent analyses were performed with the R version 3.4.3 using the metagenomeSeq (PAULSON et al. 2013), phyloSeq (MCMURDIE & HOLMES 2014), vegan (OKSANEN et al. 2016), and ggplot2 (WICKHAM 2009) packages. Data were filtered for low-abundance of Operational Taxonomic Units (OTUs) by removal of OTUs present in fewer than 3 of all the samples and with a relative abundance

higher than 0.5% across all OTUs. Analysis in R were performed with sequences per sample after filtering. Statistical analysis was performed on data filtered based on effective sample sizes, where samples were not included if they had fewer than 10,000 or more than 100,000 OTUs.

Quantitative PCR

The quantitative PCR (qPCR) technique was employed for the quantification of *Bifidobacterium* spp. and *Lactobacillus* spp. from human milk samples collected before and after 20 days of supplementation. The qPCR was performed using Taqman (ThermoFisher Scientific) or SYBR green (ThermoFisher Scientific) for *Bifidobacterium* spp. and *Lactobacillus* spp., respectively.

For *Bifidobacterium* spp., the amplifications were carried out in a 25 µL mixture (final volume) containing 12.5 µL of TaqMan® Universal PCR 2X Master Mix (ThermoFisher Scientific), 200 nM of each primer (F_Bifid 09c CGG GTG AGT AAT GCG TGA CC, R_Bifid 06 TGA TAG GAC GCG ACC CCA; FURET et al., 2009), and 250 nM of probe (P_Bifid 6FAM-CTC CTG GAA ACG GGT G; FURET et al., 2009), and 5 µL of the DNA template. Reactions were performed under the following conditions: 1 cycle at 95 ºC for 10 minutes, followed by 40 cycles at 95 ºC for 30 seconds, and at 60 ºC for 1 minute.

For *Lactobacillus* spp., 12.5 µL of SYBR Green® PCR 2X Master Mix (ThermoFisher Scientific), 500 nM of each primer (Lac-F 5'-AGCAGTAGGGAATCTTCCA-3'; Lac-R 5'-CACCGCTACACATGGAG-3'; RINTTILÄ et al. 2004), and 5 µL of the DNA template. The PCR conditions were: 1 cycle at 95 ºC for 5 minutes, followed by 40 cycles at 95 ºC for 15 seconds, 58ºC for 20 seconds, 72ºC for 30 seconds and at 80 ºC for 30 seconds.

All qPCR were performed using an ABI-PRISM 7500 sequencing detection system (Applied Biosystems, Bridge-water, NJ, USA).

For the construction of standard curves, 10-fold dilution series between 10^5 and 10^1 copies numbers of genomic DNA from known quantities of genomic DNA extracted from a pure culture of target species were applied for qPCRs. Negative besides "blanks" - controls from the DNA extraction kit controls were included in the PCR runs. All amplification reactions were performed in triplicates.

The coefficients for reaction efficiency, calculated as $10^{(-1/slope)}$ -1, ranged from 98% to 102%, and the correlation coefficients R^2 obtained for the standard curve were between 0,98 and 0,99.

The Ct (cycle threshold) from each sample was compared with the Ct from the standard curve in order to get the number of copies of the *16S rRNA* gene in the samples. The minimum limit of detection of the qPCR technique was 1.4 log equivalent cells/mL of human milk. Below that, quantities were considered as not detected.

Statistical Analysis

All statistical analyses were performed using the statistical computing language R. For differences in the human milk microbiota composition, "adonis" function (PERMANOVA) was performed using weighted and unweighted UniFrac distances, to compare the groups (FOS and placebo) by day (before and after the intervention), using 999 permutations (vegan package). In addition, to compare the abundance of individual taxa before and after the intervention trial, ANCOM (MANDAL et al. 2015) was applied, for repeated measures.

Wilcoxon signed ranks test for paired samples was used to compare the means of differences in relative abundances between before and after the intervention. Alpha-diversity analyses were performed after applying rarefactions (10,000 sequences/sample) to standardize sequence counts (vegan package). Mann-Whitney test or Wilcoxon signed ranks test were used to compare the alpha-diversity of independent or dependent samples, respectively.

Jensen-Shannon distance (JSD) was used to calculate the distribution of the distance between "before" and "after" paired samples, by each group (placebo or FOS group). Mann-Whitney test was used to compare the distributions.

Mann-Whitney test or t-test were used to compare *Bifidobacterium* spp. and *Lactobacillus* spp. counts from qPCR between the interventional groups. Wilcoxon signed ranks test for paired samples was used to compare *Bifidobacterium* spp. and *Lactobacillus* spp. counts from qPCR between "before" and "after" supplementation.

Linear regression models were carried out employing the distances between "before" and "after" supplementation as the dependent variable and supplemented group interacting with maternal variables as independent variables. Afterwards, ANOVA was performed to identify statistically significant variables which could explain the variability in those distances. In order to identify the OTUs that could be influencing the distances, linear mixed-effects models were used employing the relative abundance of OTUs in human milk as the dependent variable and supplemented group, time as factors, adjusted by subjects. ANOVA was performed to compare

the models including or not the interaction effect of the maternal variables. For this propose, the “lmer” function (lm4 package) was performed.

A false discovery rate p value ≤ 0.01 was considered as significant for analyses with multiple comparisons. A standard p value < 0.05 was considered as significant for the analyses.

Results

Twenty-eight volunteers from the FOS group and twenty-five volunteers from the placebo group concluded the clinical trial (Figure 2). The clinical characteristics and the estimated nutrient intakes “before” and “after” the supplementation of these volunteers are shown in Tables 1 and 2, respectively. No differences were found between the placebo and the FOS groups with regard to the clinical characteristics of the volunteers (Table 1). No differences were found for nutrient intakes before the intervention, comparing the groups ($p > 0.05$, data not shown). After the intervention period, the carbohydrate intake was higher in the placebo group ($p = 0.006$; Table 2). However, the difference was around 22 g, which would not imply in a great variation in terms of nutrients consumed.

Overall tolerance of the FOS and the placebo (maltodextrin) supplements was good. Complaints about bloating were reported by 5 volunteers in the FOS group. No major side effects were observed.

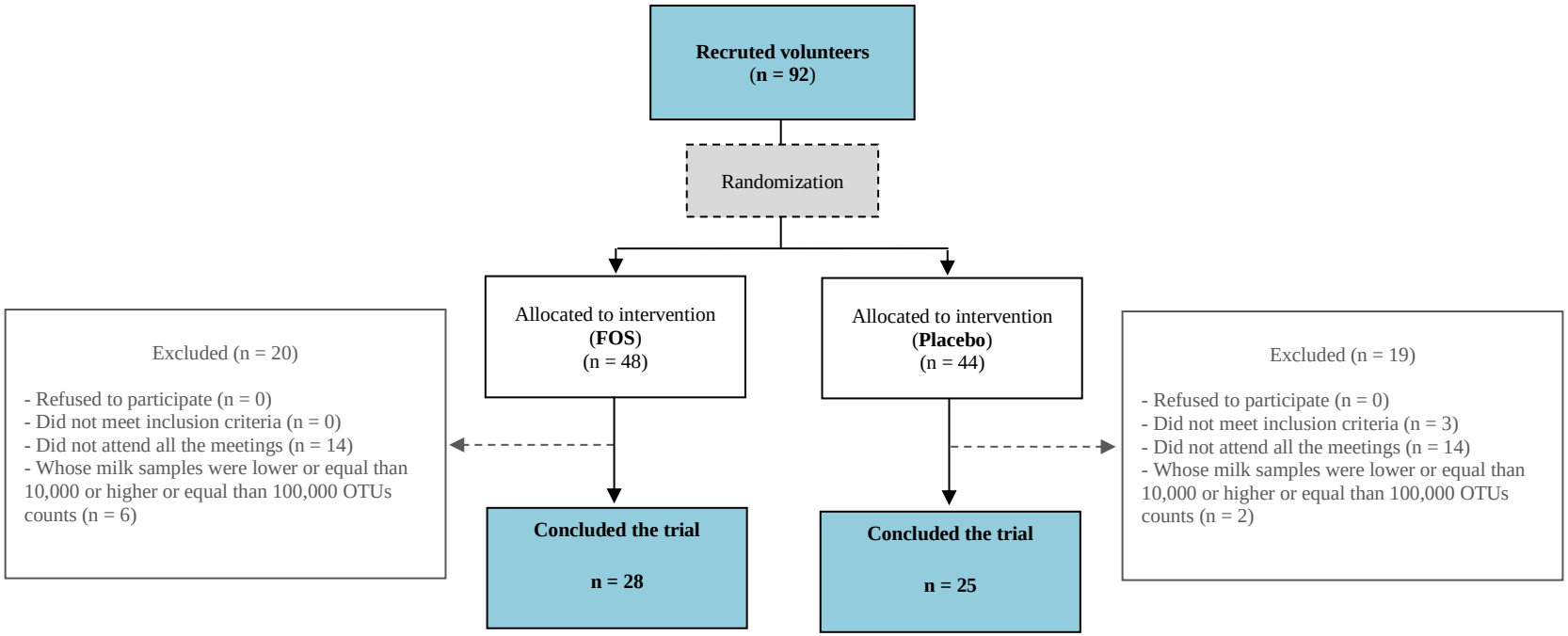


Figure 2. Flow diagram of participant recruitment followed for the study.

FOS: Fructooligosaccharide
OTU: Operational Taxonomic Unit

Table 1. Clinical and demographic characteristics of the volunteers who concluded the clinical trial, according to the groups (n= 53).

Variable	Groups		p value**
	FOS	Placebo	
	n = 28	n = 25	
Maternal age (years)	28 (23 - 31)	28 (21 - 31)	0.469
Race			0.597
Black/Brown	12 (42.9)	12 (48)	
White	16 (57.1)	13 (52)	
Family income estimated (USD/month) *	770.39 (462.5 - 770.39)	462.5 (462.5 - 770.39)	0.458
Duration of pregnancy (weeks)	39 (38 - 40)	39 (38 - 40)	0.611
Maternal antibiotic treatment			
during pregnancy	6 (21.4)	7 (28)	0.814
during delivery	11 (39.3)	10 (40)	1.000
Alcohol drinking during pregnancy	3 (10.7)	1 (4)	0.687
Smoking during pregnancy	2 (7.1)	1 (4)	1.000
BMI before pregnancy (kg/m^2)	23.0 (20.7 - 25.2)	22.6 (20.9 - 24.5)	0.929
Maternal weight gain over pregnancy (kg)	11.2 (8 - 14.1)	11.9 (8.3 - 16.6)	0.497
Anesthesia			1.000
No anesthesia	7 (25)	3 (12)	
Local	13 (46.4)	14 (56)	
Pudendal block	0	0	
Epidural	0	4 (16)	
Spinal	8 (28.6)	3 (12)	
BMI at day 30 after delivery (before the supplementation)	23.6 (21.7 - 27.4)	24.5 (22.3 - 26.9)	0.879
BMI at day 50 after delivery (after the supplementation)	24.1 (21.8 - 27.7)	24.6 (22.1 - 26.5)	0.973
Baby gender			0.940
Male	17 (60.7)	14 (56)	
Female	11 (39.3)	11 (44)	
Birth weight (g)	3205 (2933.7 - 3563.7)	3210 (2977.5 - 3425)	0.979
Birth lenght (cm)	48.5 (47.1 - 50)	49 (47 - 50)	1.000

continuation

Infant diet before the supplementation			0.145
Exclusively breast milk	23 (82.1)	24 (96)	
Breast milk + formula	5 (17.8)	1 (4)	
Infant diet at day 50 after delivery after the supplementation			0.145
Exclusively breast milk	21 (75)	21 (84)	
Breast milk + formula	7 (25)	4 (16)	
Infant Δ weight over the intervention period (g)	655.0 (458.7 - 832.5)	792.5 (578.7 - 900.0)	0.212

Data presented as median (interquartile range) or n (%).
* Family income estimated by Brazilian Economic Classification Criteria (Brazilian Criteria) 2016.
** p values were calculated to compare FOS and Placebo groups, using the Mann-Whitney test for continuous data and the chi-square test for categorical data.
BMI: Body Mass Index (kg/m^2)
NA: not applicable

Table 2. Estimated nutrients intake of "before" and "after" the supplement intervention, by groups.

	PLACEBO			FOS		
	before	after	*p values**	before	after	*p values**
Energy (kcal)	2,091.1 (1949.9 - 2327.6)	2,295.3 (1956.5 - 2607.9)	0.691	2,266 (2104.8 - 2502.7)	2,161.0 (1959.9 - 2338.4)	0.418
Carbohydrates (g)	298.8 (255.7 - 340.9)	272.0 (256.9 - 319.1)	0.731	355.2 (296.1 - 398.5)	249.6 (226.7 - 338.4)	0.006
Proteins (g)	75.8 (67.3 - 97.2)	91.4 (68.2 - 109.8)	0.560	80.4 (67.1 - 90.8)	84.1 (73.8 - 98.1)	0.866
Fat (g)	71.5 (54.8 - 82.9)	71.8 (61.9 - 82.1)	0.874	71.8 (63.0 - 81.2)	76.1 (70.4 - 83.6)	0.797
Saturated fatty acids (g)	23.4 (19.7 - 30.5)	15.6 (10.9 - 17.6)	0.691	26.1 (21.3 - 28.6)	28.8 (21.3 - 31.1)	0.797
Monounsaturated fatty acids (g)	19.9 (15.8 - 25.9)	19.3 (17.5 - 25.6)	0.560	22.5 (17.1 - 25.1)	24.9 (20.7 - 28.8)	0.884
Polyunsaturated fatty acids (g)	11.3 (8.7 - 13.4)	28.0 (21.6 - 33.3)	0.300	14.8 (12.6 - 17.9)	16.1 (12.8 - 18.2)	0.537
Total dietary fibers (g)	21.8 (19.2 - 22.6)	22.2 (18.1 - 25.0)	0.596	25.7 (20.7 - 35.5)	21.9 (16.8 - 25.7)	0.099

* p values were calculated by Wilcoxon signed ranks test for paired samples to compare the nutrients intake between the days for each group.

Regarding the sequences generated by sequencing in the Illumina platform, 3,488,486 *16S rRNA* gene sequences were analyzed, after quality filtering, with an average number of high-quality sequences of 32,910 per sample. Reads were classified into 334 OTUs at 97% sequence similarity.

Considering that human milk samples might have a low microbial load, a no template PCR control and a DNA extraction kit reagent control were sequenced alongside the samples.

According to Figure 3, we observed that the genera abundances in the controls are different from the abundances in the milk samples ($p = 0.001$ by the PERMANOVA test comparing controls *vs* milk samples). Thus, we conclude that the controls had different profiles than those of the milk samples.

At the beginning of the trial, all the milk samples were clustered together (Figure 4), independently of the group FOS or placebo, indicating no statistical differences between the groups in the human milk microbiota before the intervention ($p = 0.477$ and 0.626 for weighted and for unweighted UniFrac, respectively).

Overall, no statistically significant changes were found in terms of alpha diversity (Figure 5) or relative abundance of genera or OTUs, comparing "before" and "after" supplementation ($p > 0.05$ by ANCOM analysis) in both groups. Figure 6 shows the differences (delta) of the relative abundances between "after" and "before" supplementation of the main genera identified in the human milk samples for each volunteer. The means of the differences (delta) found for the main genera of microorganisms after the supplementation period, for each group supplemented FOS and Placebo) is shown in Figure 7. Although we found trends towards an accentuated reduction of *Staphylococcus* and a higher increase of the *Actinomyces* genera in the FOS group, it was not confirmed by statistical tests.

In contrast with what was expected, no significant differences were found with regard to the effect of the prebiotic supplementation in increasing the *Bifidobacterium* and *Lactobacillus* populations in human milk. These findings inferred from sequencing were later confirmed by qPCR using genera-specific primers, which are more precise for quantifying these genera (Figure 8).

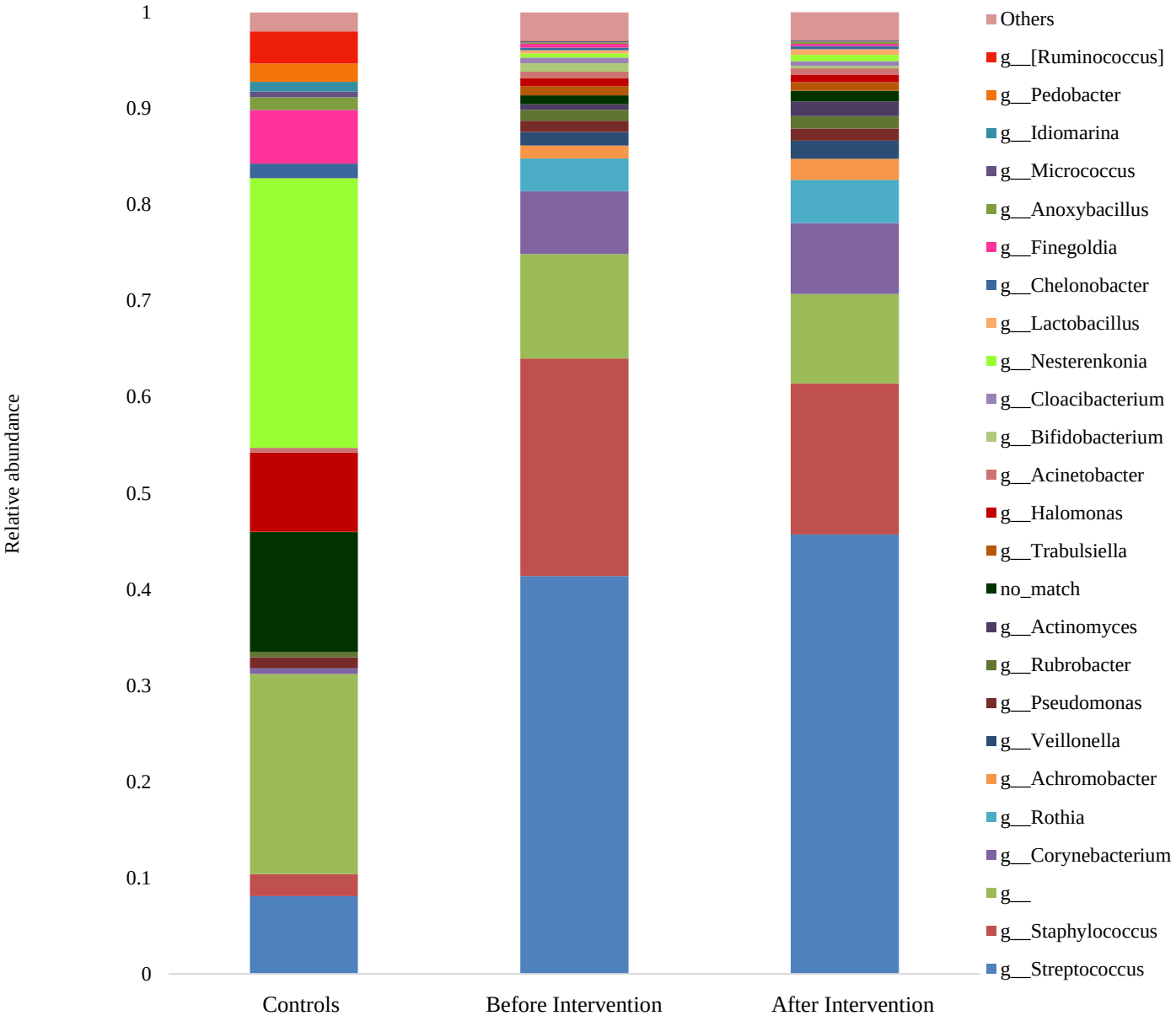


Figure 3. Comparison of bacterial profiles between controls and all human milk samples collected before and after the clinical intervention.

To verify the microbiota from reagents in either the DNA extraction kit or from PCR, we extracted the DNA of an empty tube containing the reagents from the DNA extraction kit and a no template control ("controls") for PCR alongside the milk samples, before and after the intervention. Data presented as genera relative abundance. As observed, the bacterial profile from milk samples differs from controls ($p = 0.001$ by the PERMANOVA test comparing controls *vs* milk samples before and after the intervention).

* Taxa present in less than 0.5 % abundance are displayed as "Others.

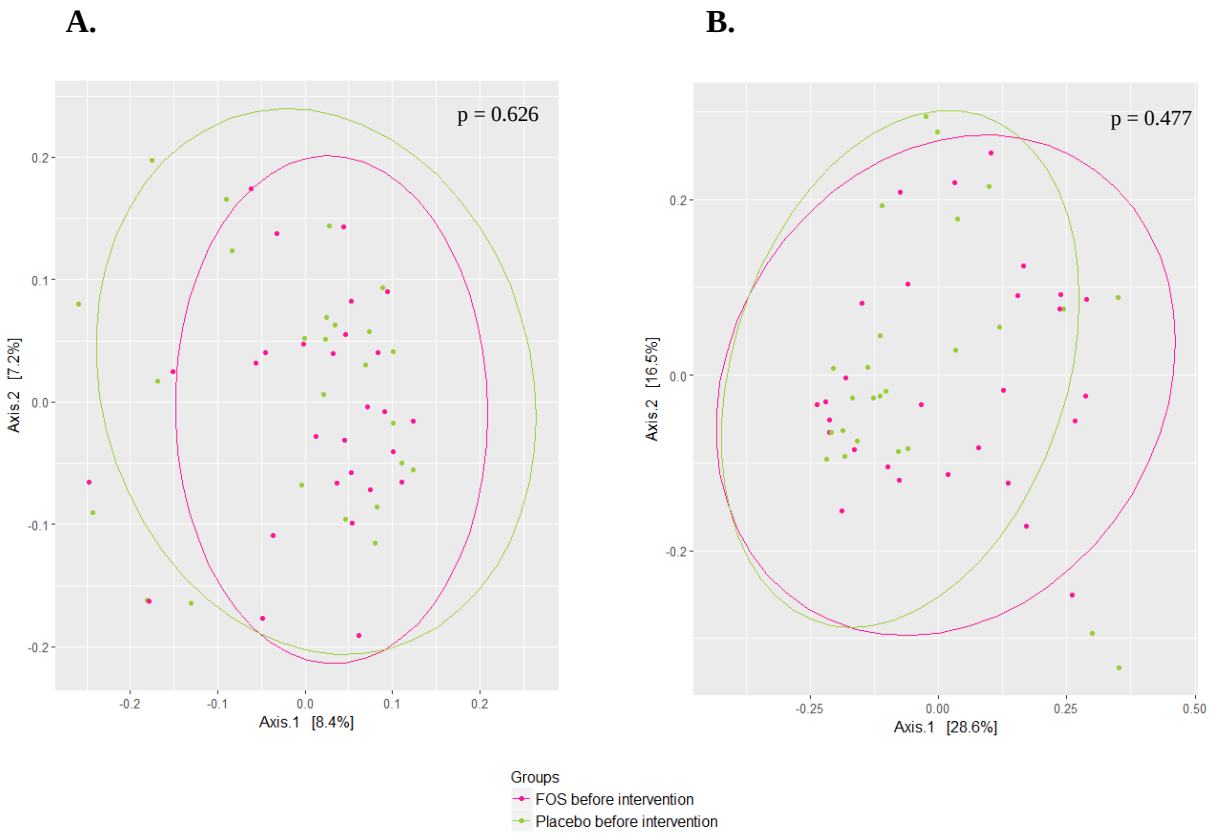
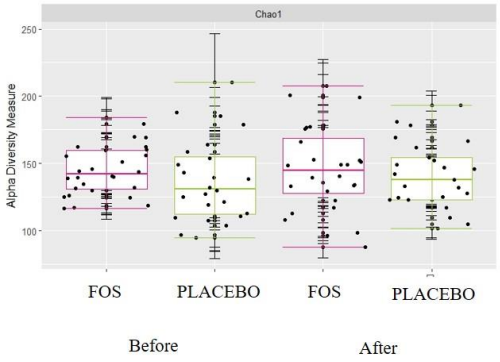
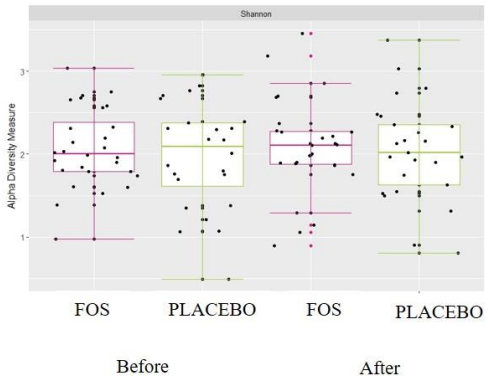


Figure 4. Principal Coordinate Analysis (PCoA) of the human milk microbiota of samples at the day before the beginning of the clinical trial for each group (FOS or placebo). Distances measured by unweighted (**A**) or weighted (**B**) UniFrac. No statistical differences were found between the groups by the PERMANOVA test.

A.



B.



C.

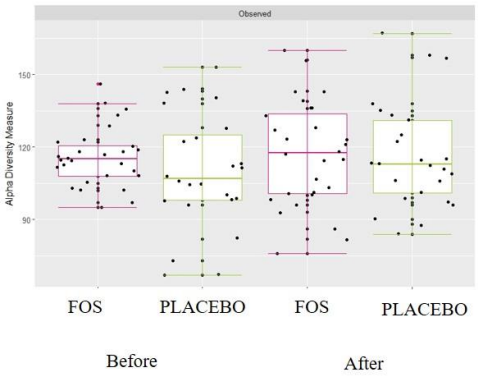


Figure 5. Alpha diversity values of milk samples, by supplemented groups, for each day. The Alpha diversity is measured by for Chao1 (**A**), Shannon (**B**), and Observed (**C**) indexes.

Operational Taxonomic Unit table for each measure was rarefied to 10,000 sequences. The box-plot is representing the interquartile range (IQR) and the line inside represents the median. Black-filled small circles denote the distribution of measures for each sample. Mann-Whitney test or Wilcoxon signed ranks test for paired samples was performed to compare the values between the groups for each day or between the days for each group, respectively. No statistical differences were found ($p > 0.05$).

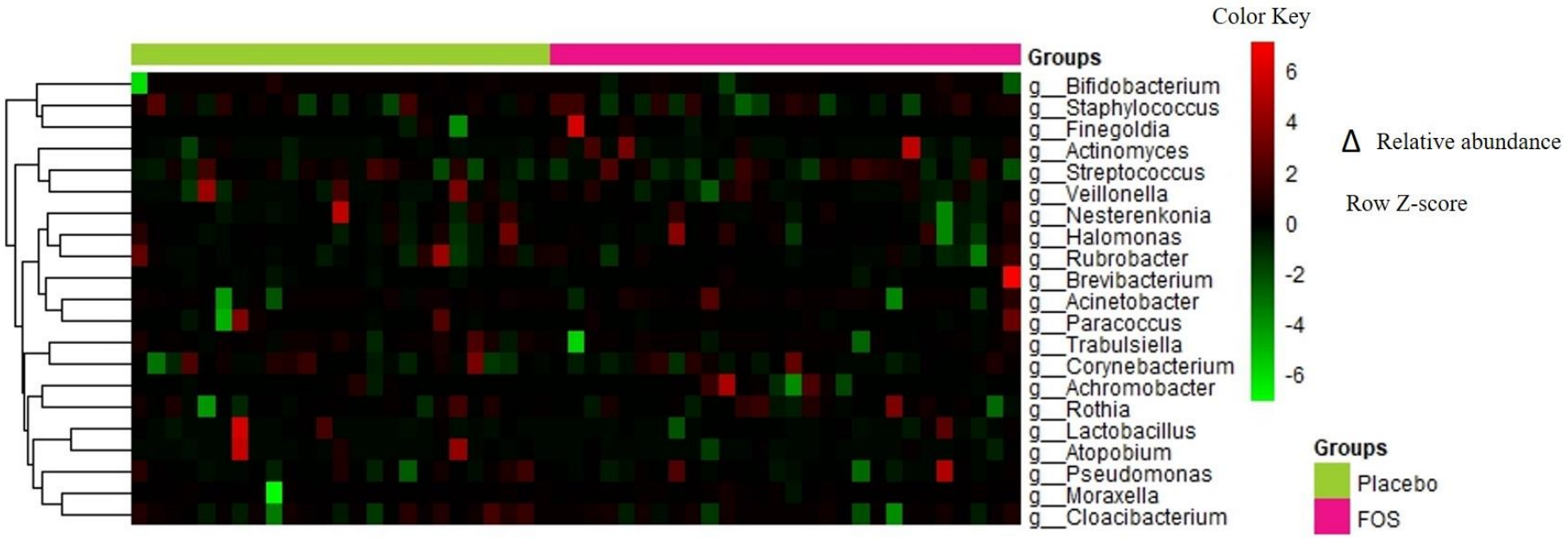


Figure 6. Delta of relative abundances of genera between the day after the intervention period and the day before the intervention, for volunteers from Placebo group or FOS group.

Columns correspond to differences between relative abundances for each volunteer. Rows correspond to genera with maximum abundance higher than 0.05. Delta was calculated by [(relative abundance of genus at day after supplementation) – (relative abundance of genus at day before supplementation)], therefore, positive values (red) denote an increase of the relative abundance of the taxa along the intervention period, while negative values (green) denote a decrease of the relative abundance of the taxa in the milk samples. The intensity of the colors represents the degree of the differences between the means. The values were z-score transformed by row for ease of visualization of the differences. Rows are clustered by Euclidean distance.

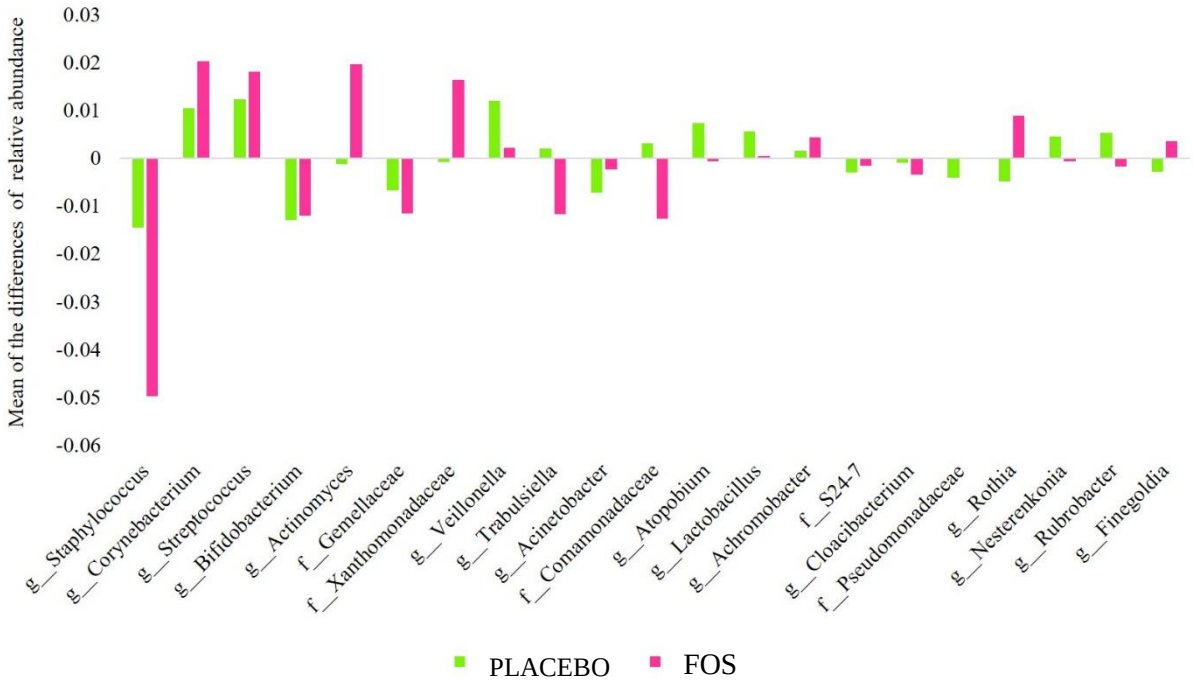
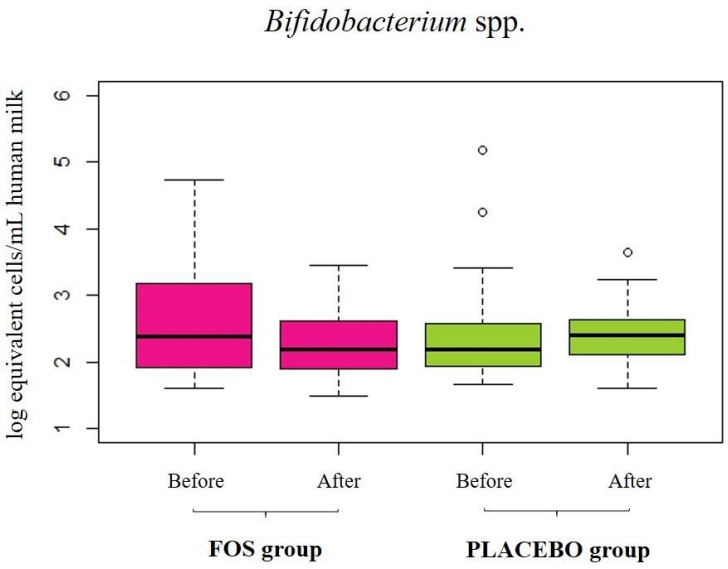


Figure 7. Mean of the differences (delta) of taxa relative abundances between "after" and "before" the supplementation period, for each group (FOS and PLACEBO).

Only genera with differences higher or equal 1% between the groups are shown. Mann-Whitney tests were performed to compare the mean of the differences for each genus, between the supplemented groups. No statistically significant differences were detected ($p > 0.05$).

A.



B.

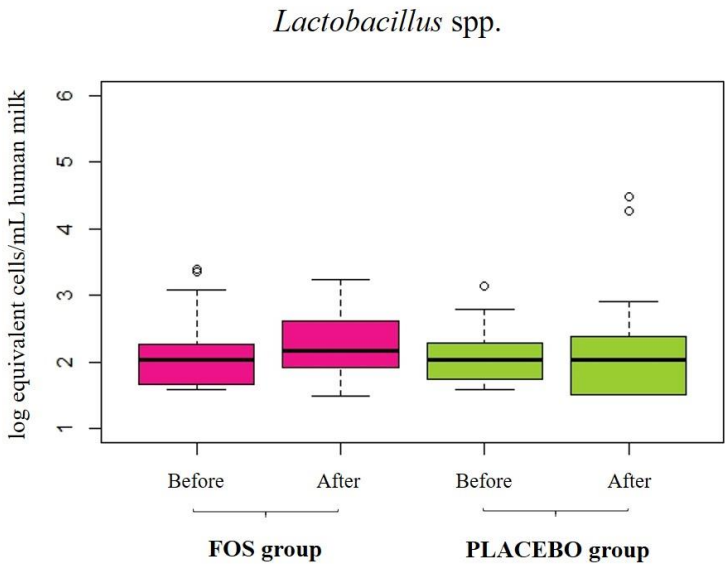


Figure 8. Box plot of the distribution of data obtained by quantitative PCR (qPCR) before and after 20 days of supplementation with fructooligosaccharide (FOS group) or maltodextrin (PLACEBO group). (A) *Bifidobacterium* spp. levels in human milk samples collected before and after supplementation for FOS group (pink) or PLACEBO group (green). (**B**) *Lactobacillus* spp. levels in human milk samples collected before and after supplementation for FOS group (pink) or PLACEBO group (green).

The medians are indicated in each box. The box plot ranges from the 25th to the 75th percentile, while whiskers indicate the minimum and maximum values that were obtained.

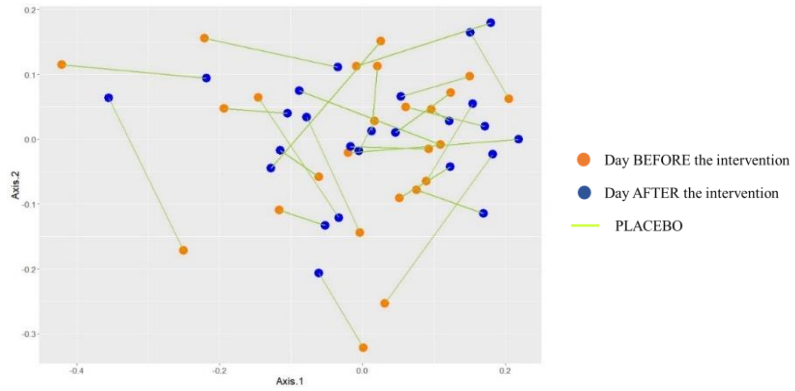
No significant differences were found between the groups for each day (analyses using t-test or Mann-Whitney test, for normal or non-normal data distribution, respectively) or between the days for each group (analyses using Wilcoxon signed ranks test for paired samples).

At the same time, we explored the trajectory of the human milk microbiota from the starting point (before the supplementation) to the ending point (one day after the end of the supplementation), in the PCoA, by the supplemented groups (Figure 9A and 9B). According to Figure 9C, there is a statistically significant difference between the trajectories of the groups ($p = 0.0007$).

Additionally, analysis of linear regression models using Jensen-Shannon distance between "before" and "after" the supplementation by group revealed that the maternal age seems to affect the response for FOS supplementation ($p = 0.02$). Our results indicate that younger mothers were more influenced by the FOS supplementation, whereas older mothers seem to have similar response than the placebo group (Figure 10). No statistically differences were found for other variables tested in the model, including pre-pregnancy BMI ($p = 0.8528$), BMI at 1st month ($p = 0.4705$), antibiotic treatment during pregnancy ($p = 0.5896$), antibiotic treatment during delivery ($p = 0.7130$), ethnicity ($p = 0.2004$), number of children ($p = 0.3381$), baby gender ($p = 0.1832$), and baby feeding at first month ($p = 0.7154$).

Subsequent analyses found statistically significant OTUs belonging to *Bacteroides*, *Shewanella*, *Rheineimera*, *Idiomarina*, *Staphylococcus*, *Streptococcus*, and *Hymenobacter* genera; *Gemellaceae*, *S24-7* family, and *Bacillales* order, when models with or without the maternal age as an interaction factor were compared. However, the differences between the relative abundances of "after" and "before" the supplementation for these OTUs had not a pattern related to younger mothers from the FOS group, as our previous analysis suggested (Figure 11). Accordingly, the FOS supplementation influenced the human milk microbiota of younger mothers, compared to the placebo group. However, the changes found seem to be different for each subject.

A.



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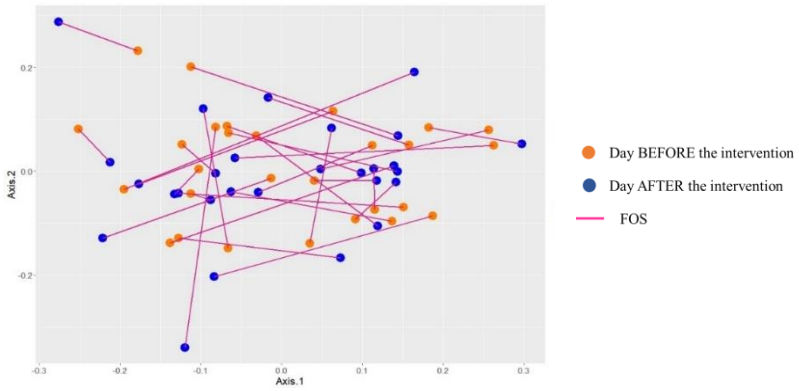

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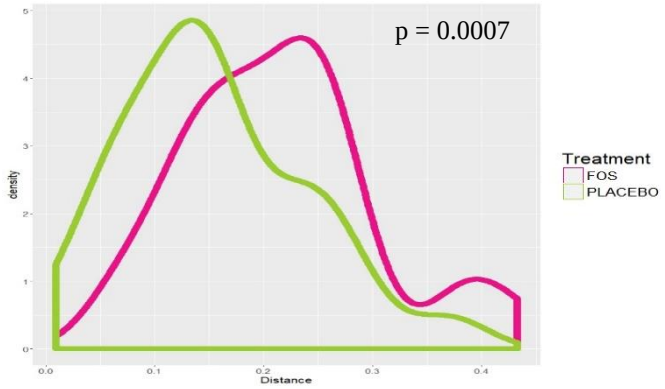

Figure 9. Effects of the supplementation with placebo or fructooligosaccharide (FOS) on the human milk microbiota of each subject. (**A** and **B**) PCoA plots of Jensen-Shannon distance (JSD) shows the effects of the maternal supplementation with placebo (**A**) or FOS (**B**) on the phylogenetic structures of the human milk microbiota. (**C**) Distribution of the distances (JSD) between "before" and "after" supplementation for each subject, by group shows statistically significant differences between the placebo and the FOS group (Mann-Whitney test).

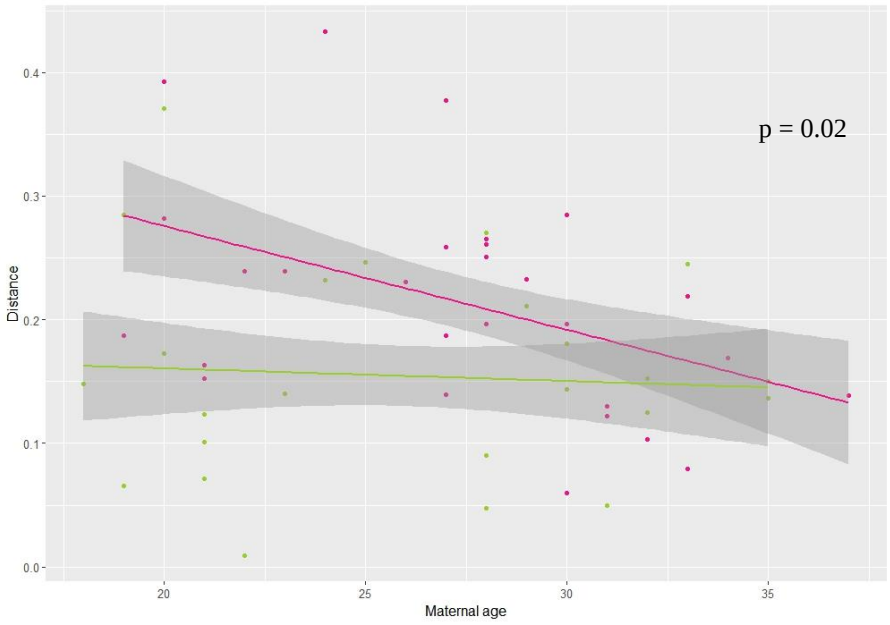

Figure 10. Jensen-Shannon distance between "before" and "after" the supplementation by FOS (pink) or placebo (green) groups, according to the maternal age. Least-squares means was used to compare the slopes of the regression of distance vs maternal age between FOS and placebo groups.

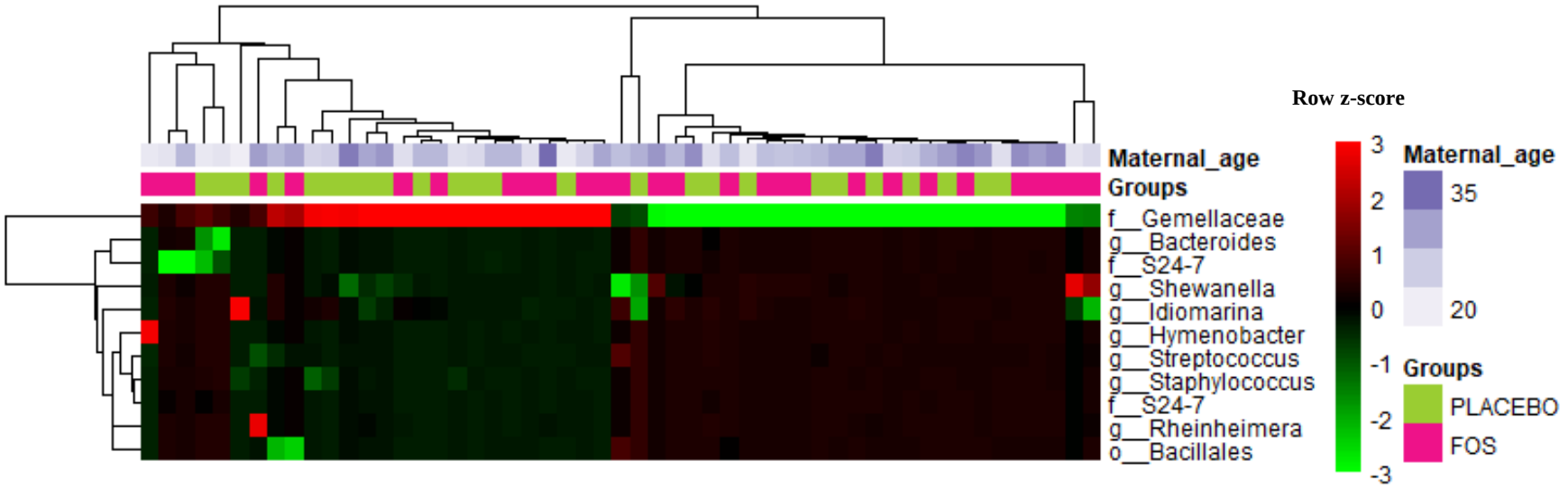

Figure 11. Heatmap for differences between the relative abundances of "after" and "before" the intervention with placebo (green) or FOS (pink). Only statistically significant OTUs are displayed, according to ANOVA test for two linear mixed-effects models; one considering the relative abundance of OTUs in human milk as the dependent variable and supplement group and time as the factors and other including the interaction effect of the maternal age.

Columns correspond to differences between the relative abundance of "after" and "before" the intervention for each sample and Operational Taxonomic Unit (OTU) (Rows). Positive values (red) and negative values (green) denote increase or decrease of bacterial taxa after the intervention period, respectively. The intensity of the colors represents the degree of the differences between the means. The values were row z-score transformed for ease of visualization of the differences. Rows and columns are clustered by Euclidean distance.

Discussion

In order to investigate the role of the maternal diet supplementation with prebiotics (fructooligosaccharides), during lactation on the human milk microbiota, we performed a randomized, placebo-controlled clinical trial with prebiotic (FOS) or placebo (maltodextrin) supplementation in the maternal diet.

The beneficial effects of fibers, particularly prebiotics, on the human health are well established (CHAMP & HOEBLER, 2009; YASMIN et al., 2015). Especially during pregnancy and during the lactation period, the role of prebiotics draws attention to reduce the risk for postpartum weight retention, contributing towards a lower risk for long-term obesity, and consequently, reducing the risk for non-transmissible chronic diseases (CHAMP and HOEBLER, 2009). Additionally, prebiotics are known by their ability to stimulate beneficial microorganisms in the human gut (YASMIN et al., 2015). In this context, some studies suggest that gut bacteria could be transferred to the neonate via breastfeeding, since identical strains have been isolated in paired-samples of maternal feces and breast milk (JOST et al., 2014; MARTIN et al., 2012), supporting the hypothesis of an entero-mammary-pathway (FERNÁNDEZ et al., 2013).

We expected higher relative abundances of *Bifidobacterium* and *Lactobacillus* in the FOS-supplemented group, since these genera are recognized FOS consumers and could be increased in the mother's gut (POKUSAEVA; FITZGERALD; and VAN SINDEREN, 2011). However, no differences were observed for these genera between the groups, analyzed by either Illumina MiSeq® or qPCR using genera-specific primers.

In this line, SHADID et al. (2007) investigated the effect of the supplementation with galactooligosaccharide and long chain fructooligosaccharide in women in the last trimester of pregnancy in a randomized, double-blind placebo-controlled study on the maternal and neonate gut microbiota. The authors reported significantly higher *Bifidobacterium* populations in the mother's feces from the supplemented group than in those from the placebo group. However, no differences were found in the neonate gut microbiota between the placebo or the supplemented group, suggesting that the bifidogenic effect on the maternal gut microbiota were not transferred to the neonates. In our study, the short period of supplementation could be a factor for no statistical differences between the groups. Our recent study identified that long-term maternal dietary habits are more likely to influence the structure of the human milk microbial community, whereas short-term maternal intake induce minor changes in the microbiota composition (unpublished data).

We did not find any differences for changes in the relative abundance of the human milk microbiota, comparing the prebiotic-supplemented and the placebo group, in our initial analyzes. Nevertheless, further analyzes suggested that the distances in the PCoA trajectory performed by the FOS group from the initial to the end of the supplementation was longer than those of the placebo group, and maternal age seems to affect this behavior. According to these results, younger mothers seem to be influenced by the FOS supplementation, since they have major changes in their microbiota profile after supplementation, whereas older mothers have similar distances than the placebo group, indicating no differences between the groups for older mothers. In addition, our results suggest that this influence of the FOS supplementation was different for each subject, since no patterns of changes at the end of the intervention were found.

To our knowledge, no previous study has reported the effect of the maternal age on the human milk microbiota; however, the role of age is well established in terms of modulating the gut microbiota. Metabolic and physiologic patterns of each stage of life are described as the main responsible for changings in the gut microbiota (ODAMAKI et al. 2016). Nonetheless, the age range of the volunteers in this study was from 18 to 37 years old, which would not characterize substantial physiologic changes. It is possible that differences in dietary or lifestyle patterns could be related to the differential response to FOS supplementation, since the dietary intake may vary substantially across the life-course (PARSONS et al., 2005; MISHRA et al., 2006).

In this context, PARSONS et al., (2005) identified marked diet changes in most subjects evaluated over 8 years (from age 33 to 42). Greater proportion of cohort members reduced their chips consumption and increased their fruit and salad consumption. Similarly, MISHRA et al. (2006) investigated longitudinal changes in dietary patterns during adult life (at age 36 to 53). In their study, meat, potatoes and sweet foods pattern in women recorded a decline.

Consequences of different dietary patterns throughout life may be related to influences on the gut microbiota (WU et al., 2011), which have been extensively related to inter-individual variations in response to dietary interventions (WALKER et al., 2011; GRIFFIN et al., 2017).

Marked inter-individual variation in the gut microbiota of individuals who received non-digestible carbohydrates was reported by WALKER et al. (2011). The authors findings suggested that differential responses were depend on the initial composition of an individual's gut microbiota. In addition, GRIFFIN et al. (2017) suggested that prior dietary practices, based on chronic calorie restriction with adequate nutrition or without dietary restrictions, might impair responses to dietary interventions, which would require the introduction of diet-

responsive bacterial lineages present in other individuals. Similarly, an *in vitro* study evaluated the effect of a prebiotic mixture in two different fermentation systems, varying in their nutritional availability. The authors reported differential responses of the gut microbiota to the same prebiotic formula, including some OTUs within the same genus, which responded to the prebiotic in opposite ways (LONG et al., 2015).

Besides differences in the gut microbiota composition, immunological effects of diet interventions could also be associated with the presence of individual taxa. According to MARTÍNEZ et al. (2013), a short-term intake of whole grains induced compositional alterations of the gut microbiota. However, subjects with greater improvements in plasm Interleukin-6 levels harbored significantly higher proportions of *Dialister* and lower abundance of *Coriobacteriaceae*. In this context, immunological changes resulting from prebiotic supplementation seem to be transferred to the human milk (NIKNIAZ et al., 2013; KUBOTA et al., 2014), which could influence the local microbiota (COLLADO et al., 2012).

In our study, we describe for the first time the influence of the prebiotic supplementation on the human milk microbiota. However, we could not obtain samples of maternal feces to investigate whether the maternal gut microbiota would be related to the differential microbial changes observed in milk for the FOS supplementation. In addition, we did not evaluate the immunological compounds present in the human milk, which would be interesting to explore if changes in immunological factors could be also associated to changes in the milk microbiota composition.

The factors that influence the human milk microbiota, particularly concerning the maternal diet, are still largely unknown, and further investigations are required to better understand the possible benefits of maternal diet intervention on maternal and infant health.

Conclusion

In spite of not having found differences in the genera relative abundance between the groups, after the intervention, we identified that the maternal age can affect the response by the supplementation with FOS. However, the pattern of changes seems to be individual-dependent. Further studies are required for a deeper understanding whether other factors, especially the individual gut microbiota could be related to the differentiated responses towards FOS supplementation.

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PART III

GENERAL CONCLUSIONS

In this study, we described for the first time the effects of the maternal diet during pregnancy and during the first month of the lactation period on the human milk bacterial microbial community. In addition, we investigated the impact of maternal diet supplementation with prebiotics (fructooligosaccharides) on the human milk microbiota during lactation.

Our results suggested that the maternal diet may influence the human milk microbiota, and the diet during pregnancy is a stronger factor over the bacterial community structure. Minor changes were found by the maternal short-term food intake or the maternal intervention with the prebiotic, and the changes seem to be individual-dependent and influenced by the maternal age, particularly in the intervention study.

ATTACHMENTS

Attachment 1. Approval issued by the Research Ethics Committee of the School of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil.

FACULDADE DE CIÊNCIAS FARMACÊUTICAS DA UNIVERSIDADE DE SÃO

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Impacto da dieta materna e da intervenção com fruto-oligossacarídeos sobre a microbiota do leite humano

Pesquisador: Susana Marta Isay Saad

Área Temática:

Versão: 2

CAAE: 27247614.6.0000.0067

Instituição Proponente: Faculdade de Ciências Farmacêuticas da Universidade de São Paulo

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 663.131

Data da Relatoria: 23/06/2014

Apresentação do Projeto:

O objetivo geral do projeto é investigar a associação entre a dieta materna e a microbiota do leite humano. Adicionalmente, avaliar o impacto da suplementação da dieta materna com um ingrediente prebiótico (fruto-oligossacarídeo) sobre a microbiota do leite humano, durante o período de lactação. O projeto encontra-se bem descrito, estruturado e organizado.

Objetivo da Pesquisa:

O objetivo principal foi mencionado no item anterior. Os objetivos específicos são:

Investigar a associação da dieta materna na gestação (consumo alimentar pregresso) e na lactação(consumo alimentar atual) com a microbiota do leite humano. Avaliar a influência da intervenção com prebiótico (fruto-oligossacarídeo) na dieta materna, sobre a dinâmica de populações de Bifidobacterium e Lactobacillus no leite humano. Avaliar a influência da intervenção com prebiótico (fruto-oligossacarídeo) na dieta materna, sobre a microbiota intestinal materna. Avaliar a influência da intervenção com prebióticos (fruto-oligossacarídeo) na dieta materna, sobre a microbiota intestinal do lactente.

Avaliação dos Riscos e Benefícios:

Riscos: os riscos são mínimos. Não foram encontradas evidências de riscos ou desconforto relacionado ao consumo da fibra prebiótica na quantidade e período de consumo propostos nesta

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pesquisa. Embora geralmente sejam bem tolerados por indivíduos saudáveis, em alguns casos pode haver leve desconforto abdominal, devido a produção de gás decorrente do processo de fermentação da fibra prebiótica.

Benefícios: Não haverá um benefício direto, porém as participantes estarão contribuindo, de forma voluntária, para o desenvolvimento de uma pesquisa que contribuirá para o esclarecimento sobre a influência da alimentação da mãe, sobre os micro-organismos naturalmente presentes no leite materno. Uma vez que o leite materno é um alimento direcionado ao bebê, os resultados poderão esclarecer sobre a influência da alimentação materna na saúde e o desenvolvimento intestinal do bebê que recebe este leite.

Comentários e Considerações sobre a Pesquisa:

O projeto é relevante e de importância. É apresentado de forma clara e com justificativas pertinentes e buscando resultados inovadores.

Considerações sobre os Termos de apresentação obrigatória:

Os pesquisadores contemplaram de forma apropriada todas as questões levantadas no parecer anterior.

Recomendações:

Recomenda-se a aprovação do projeto na forma em que se encontra.

Conclusões ou Pendências e Lista de Inadequações:

Recomenda-se a aprovação do projeto na forma em que se encontra.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Tendo em vista as considerações acima, este CEP entende que o projeto pode ser aprovado.

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SAO PAULO, 27 de Maio de 2014

Assinado por:
Mauricio Yonamine
(Coordenador)

Endereço: Av. Prof. Lineu Prestes, 580, Bloco 13A, sala 112
Bairro: Butantã **CEP:** 05.508-000
UF: SP **Município:** SAO PAULO
Telefone: (11)3091-3622 **Fax:** (11)3031-8986 **E-mail:** cepfcf@usp.br

Attachment 2. Approval issued by the Research Ethics Committee of the University Hospital of the University of São Paulo, São Paulo, Brazil.

São Paulo, 07 de julho de 2014.

II^mo(a). S^r(a).
Profa. Dra. Susana Marta Isay Saad
Departamento de Tecnologia Bioquímico-Farmacêutica
Faculdade de Ciências Farmacêuticas
UNIVERSIDADE DE SÃO PAULO

REFERENTE: **Projeto de Pesquisa** "Impacto da dieta materna e da intervenção com fruto-oligossacarídeos sobre a microbiota do leite humano"
Pesquisador(a) responsável: Profa. Dra. Susana Marta Isay Saad
Equipe de Pesquisa: Carla Taddei de Castro Neves, Edna Maria de Albuquerque Diniz, Marina Padilha, Silvia maria Ibidi
CAAE: 27247614.6.3001.0076
Registro CEP-HU/USP: 1370/14

Prezado(a) Senhor(a)

O Comitê de Ética em Pesquisa do Hospital Universitário da Universidade de São Paulo, em reunião ordinária realizada no dia 27 de junho de 2014, analisou o Projeto de Pesquisa acima citado, considerando-o como **APROVADO**, bem como o seu **Termo de Consentimento Livre e Esclarecido**.

Lembramos que cabe ao pesquisador elaborar e apresentar a este Comitê, relatórios parciais e final, de acordo com a Resolução nº 466/2012 do Conselho Nacional de Saúde, inciso XI.2, letra "d".

O primeiro relatório está previsto para 27 de dezembro de 2014.

Atenciosamente,

Dr. Mauricio Seckler
Coordenador do Comitê de Ética em Pesquisa
Hospital Universitário da USP

COMITÊ DE ÉTICA EM PESQUISA DO HOSPITAL UNIVERSITÁRIO DA USP
Avenida Professor Lineu Prestes, 2565 – Cidade Universitária – 05508-000 São Paulo – SP
Tel.: (11) 3091-9457 – E-mail: cep@hu.usp.br

Attachment 3. Written Informed Consent Form (WICF)

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – TCLE

1. Informações da Participante da Pesquisa

Nome:			
Documento de Identidade nº:			Sexo: ( ) M ( )F
Data de Nascimento: / /			
Endereço:	Nº	Complemento:	
Bairro:	Cidade:		Estado:
CEP:	Telefones:		

Título do Projeto de Pesquisa: "Impacto da dieta materna e da intervenção com fruto-oligossacarídeos sobre a microbiota do leite humano".
2. Duração da Pesquisa: 3 anos
3. Nome do pesquisador responsável: Profª Drª Susana Marta Isay Saad
Cargo/ Função: Professora Associada
Instituição: Faculdade de Ciências Farmacêuticas/USP

Você está sendo convidada a participar da pesquisa: "Impacto da dieta materna e da intervenção com fruto-oligossacarídeos sobre a microbiota do leite humano". O projeto é de responsabilidade da Profª Drª Susana Marta Isay Saad e conta com a colaboração da doutoranda Marina Padilha, ambas pertencentes à Faculdade de Ciências Farmacêuticas/USP. Como colaboradoras da pesquisa, participam a Drª Silvia Maria Ibidi, médica pediatra responsável pela Neonatologia e Profª Drª Edna Maria de Albuquerque Diniz, responsável técnica e coordenadora de Ensino e Pesquisa da Neonatologia do Hospital Universitário da Universidade de São Paulo (HU/USP).

A amamentação, ou aleitamento materno, é mundialmente considerada o método ideal de alimentação e nutrição do bebê, uma vez que o leite materno é totalmente constituído para atender as necessidades nutricionais e promover o crescimento adequado do bebê. Com exceção de raras situações, a amamentação deve ser incentivada, pois são inúmeras as vantagens na saúde e no contexto emocional, social e econômico.

O leite materno contém, naturalmente, alguns micro-organismos (por exemplo, bactérias) que podem trazer benefícios à saúde do bebê. Estes micro-organismos, naturalmente presentes, podem participar, principalmente, do adequado desenvolvimento intestinal e imunológico (sistema de defesa), além de proteger contra micro-organismos prejudiciais à saúde do bebê.

Diversos estudos indicam que a alimentação pode influenciar na composição de micro-organismos presentes em algumas regiões do corpo humano. Portanto, a referida pesquisa tem por objetivo avaliar se a alimentação da mãe pode apresentar efeitos na composição de micro-organismos presentes no leite materno.

Rubrica do pesquisador responsável
(Susana Marta Isay Saad)

Rubrica do participante da pesquisa

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – TCLE

Como forma de atingir os objetivos propostos, serão realizados 2 encontros presenciais para coleta de informações pessoais, medidas de peso e altura e história da alimentação materna, por meio de recordatórios de 24 horas (descrição da alimentação no dia anterior à entrevista) e questionário de frequência alimentar (questionário referente à alimentação, durante a gravidez). No segundo encontro será coletada uma amostra de leite materno. Eventualmente, será necessário obter informações do prontuário médico do Hospital Universitário.

A duração desta parte da pesquisa será de 23 dias, e os encontros estão previstos para serem realizados no 7º e 30º dias após o parto, conforme agendados com o pesquisador. Serão selecionadas 150 participantes saudáveis, com idades entre 19 e 35 anos, preferencialmente atendidas no Hospital Universitário (HU) na cidade de São Paulo – SP - Brasil, que queiram participar da pesquisa.

As mães selecionadas deverão apresentar as seguintes características:
1) Ter apresentado ganho de peso adequado durante a gestação; 2) Ter apresentado parto a termo - entre 37 e 42 semanas de gestação; 3) Com recém-nascidos de peso adequado para a idade gestacional ao nascimento; 4) Em prática de aleitamento materno exclusivo - sem suplementação de líquidos ou sólidos, exceto medicamentos ou suplementos nutricionais; 5) Com funcionamento intestinal normal – mínimo de duas evacuações a cada dois dias e máximo de três evacuações/dia.

Além disso, não serão selecionadas participantes que apresentarem doenças como diabetes, cardiopatias (doenças no coração), renais (doenças dos rins), imunes (níveis muito baixos ou muito elevados de células de defesa), doenças genéticas, hipertensão (pressão alta), ter apresentado diabetes gestacional (diabetes durante a gestação), apresentar inflamação das mamas (mastite) e estar em uso ou ter utilizado alguns tipos de medicamentos ou tóxicos que poderão interferir nos resultados da pesquisa.

As participantes serão orientadas a manter a alimentação habitual, durante todo o período de estudo. Não será solicitado às participantes seguir qualquer dieta específica.

Acerca da coleta do leite materno, esta será realizada manualmente, em ambiente limpo e confortável, por técnicos treinados. Todo material utilizado será descartável e estéril. Antes da coleta, as mamas e os mamilos serão higienizados com sabonete antisséptico e água e o primeiro fluxo de leite será desprezado. Serão coletados, aproximadamente, 25 mL (volume equivalente a meio copinho de café) de leite materno. Todo o procedimento será supervisionado pela doutoranda Marina Padilha.

As amostras serão mantidas em gelo, por até 4 horas e, posteriormente, as análises do material coletado serão realizadas nos laboratórios da Faculdade de Ciências Farmacêuticas da USP, por métodos qualitativos e/ou quantitativos apropriados para investigar a composição do leite.

Rubrica do pesquisador responsável
(Susana Marta Isay Saad)

Rubrica do participante da pesquisa

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – TCLE

Informações às participantes:

- ✓ Os riscos de sua participação nesta pesquisa são mínimos, embora possa ocorrer algum constrangimento ou desconforto durante a aplicação de questionários;
- ✓ Quaisquer danos resultantes da pesquisa serão indenizados;
- ✓ Você poderá recusar ou desistir da pesquisa a qualquer momento, sem prejudicar o acompanhamento médico realizado pela equipe do HU/USP. A participação ou a desistência não interfere no atendimento oferecido pelo Hospital Universitário.
- ✓ A qualquer momento você poderá solicitar que os seus dados sejam excluídos da pesquisa;
- ✓ Você poderá solicitar explicações todas as vezes que achar necessário sobre a pesquisa que estará participando;
- ✓ Todas as amostras de leite materno coletadas serão descartadas após as análises laboratoriais;
- ✓ Todas as participantes serão identificadas por um código para evitar que o seu nome seja relacionado aos resultados obtidos e quando os resultados desta pesquisa forem publicados em eventos e revistas científicas especializadas, os nomes não serão divulgados;
- ✓ As participantes terão ressarcimento com os custos de transporte, caso elas tenham que se deslocar até o Hospital Universitário para os procedimentos relativos a esta pesquisa.

Benefícios:

- ✓ Não haverá um benefício direto, porém as participantes estarão contribuindo, de forma voluntária, para o maior conhecimento sobre a influência da alimentação da mãe, sobre os micro-organismos naturalmente presentes no leite materno. Uma vez que o leite materno é um alimento direcionado ao bebê, os resultados poderão esclarecer sobre a influência da alimentação materna na saúde e o desenvolvimento intestinal do bebê que recebe este leite.

Rubrica do pesquisador responsável
(Susana Marta Isay Saad)

Rubrica do participante da pesquisa

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – TCLE

Em caso de dúvidas, intercorrências clínicas ou reações adversas, o participante da pesquisa será encaminhado pela equipe do projeto de pesquisa ao HU/USP. Entrar em contato com o pesquisador responsável:

Dra. Susana Marta Isay Saad, tel (011) 3091-2378.
Endereço: Depto de Tecnologia Bioquímico-Farmacêutica da FCF/USP
Av. Prof. Lineu Prestes, 580 Bloco 16, CEP: 05508-900 São Paulo - SP
Telefone: (011) 3091-2378 e-mail: susaad@usp.br.
Ou com a pesquisadora colaboradora: Marina Padilha, tel: (11) 3091-2691 ou e-mail: marina.padilha@usp.br

Uma via deverá será entregue a você e outra via ficará com o pesquisador responsável, arquivado pelo período de 5 (cinco) anos.

Consentimento Pós-Esclarecido:

Declaro que, após convenientemente esclarecido pelo pesquisador e ter entendido o que me foi explicado, consinto em participar do presente Protocolo de Pesquisa.

São Paulo, de de .

Assinatura da participante de pesquisa	**Assinatura do pesquisador responsável (Susana Marta Isay Saad)**

Em caso de dúvida, esclarecimento ou reclamação sobre aspectos éticos dessa pesquisa, favor entrar em contato:

- Comitê de Ética em Pesquisas da Faculdade de Ciências Farmacêuticas da USP – Av. Prof. Lineu Prestes, 580 - Bloco 13A – Cidade Universitária – CEP: 05508-900 – São Paulo/SP. Fone: (11) 3091-3622, Fone-Fax: 3091-3677 – E-mail: cepfcf@usp.br

- Comitê de Ética em Pesquisa do Hospital Universitário da USP - Av. Prof. Lineu Prestes, 2565 – Cidade Universitária - CEP: 05508-000 – São Paulo/SP - Fone: (11) 3091-9457, Fax: (11) 3091-9479 E-mail: cep@hu.usp.br

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – TCLE

1. Informações do Participante da Pesquisa

Nome:			
Documento de Identidade nº:			Sexo: ( ) M ( )F
Data de Nascimento: / /			
Endereço:		Nº	Complemento:
Bairro:	Cidade:		Estado:
CEP:	Telefones:		

Título do Projeto de Pesquisa: "Impacto da dieta materna e da intervenção com fruto-oligossacarídeos sobre a microbiota do leite humano".
2. Duração da Pesquisa: 3 anos
3. Nome do pesquisador responsável: Profª Drª Susana Marta Isay Saad
Cargo/ Função: Professora Associada
Instituição: Faculdade de Ciências Farmacêuticas/USP

Você está sendo convidada a participar da pesquisa: "Impacto da dieta materna e da intervenção com fruto-oligossacarídeos sobre a microbiota do leite humano". O projeto é de responsabilidade da Profª Drª Susana Marta Isay Saad e conta com a colaboração da doutoranda Marina Padilha, ambas pertencentes à Faculdade de Ciências Farmacêuticas/USP. Como colaboradoras da pesquisa, participam a Drª Silvia Maria Ibidi, médica pediatra responsável pela Neonatologia e Profª Drª Edna Maria de Albuquerque Diniz, responsável técnica e coordenadora de Ensino e Pesquisa da Neonatologia do Hospital Universitário da Universidade de São Paulo (HU/USP).

A amamentação, ou aleitamento materno, é mundialmente considerada o método ideal de alimentação e nutrição do bebê, uma vez que o leite materno é totalmente constituído para atender as necessidades nutricionais e promover o crescimento adequado do bebê. Com exceção de raras situações, a amamentação deve ser incentivada, pois são inúmeras as vantagens na saúde e no contexto emocional, social e econômico.

O leite materno contém, naturalmente, alguns micro-organismos (por exemplo, bactérias) que podem trazer benefícios à saúde do bebê. Estes micro-organismos, naturalmente presentes, podem participar, principalmente, do adequado desenvolvimento intestinal e imunológico (sistema de defesa), além de proteger contra micro-organismos prejudiciais à saúde do bebê.

Diversos estudos científicos indicam que a alimentação pode influenciar na composição de micro-organismos presentes em algumas regiões do corpo humano. Particularmente algumas fibras alimentares (inulina e fruto-oligossacarídeos) apresentam a propriedade de estimular no intestino, a multiplicação de micro-organismos benéficos à saúde humana. Comercialmente, essas fibras podem ser apresentadas na forma em pó, de cor branca, inodoro, com sabor levemente adocicado.

Rubrica do pesquisador responsável
(Susana Marta Isay Saad)

Rubrica do participante da pesquisa

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – TCLE

A referida pesquisa tem por objetivo avaliar se o consumo de uma fibra alimentar (fruto-oligossacarídeo) pela mãe influencia na composição de micro-organismos no leite humano.

Como forma de atingir os objetivos propostos, serão realizados 2 encontros presenciais para coleta de informações pessoais, medidas de peso e altura e história da alimentação materna, por meio de recordatórios de 24 horas (descrição da alimentação no dia anterior à entrevista). Eventualmente, será necessário obter informações do prontuário médico do Hospital Universitário. Além disso, durante os encontros serão coletadas amostras de leite materno e será solicitado, anteriormente, que a participante traga amostras de fezes, coletadas conforme orientação do pesquisador.

Esta parte da pesquisa terá duração de 20 dias e os encontros estão previstos para serem realizados no 30º e 50º dias após o parto, conforme agendados com o pesquisador. Serão selecionadas 60 participantes saudáveis, com idades entre 19 e 35 anos, preferencialmente atendidas no Hospital Universitário (HU) na cidade de São Paulo – SP - Brasil, que queiram participar da pesquisa.

As mães selecionadas deverão apresentar as seguintes características:
1) Ter apresentado ganho de peso adequado durante a gestação; 2) Ter apresentado parto a termo - entre 37 e 42 semanas de gestação; 3) Com recém-nascidos de peso adequado para a idade gestacional ao nascimento; 4) Em prática de aleitamento materno exclusivo - sem suplementação de líquidos ou sólidos, exceto medicamentos ou suplementos nutricionais; 5) Com funcionamento intestinal normal – mínimo de duas evacuações a cada dois dias e máximo de três evacuações/dia.

Além disso, não serão selecionadas participantes que apresentarem doenças como diabetes, cardiopatias (doenças no coração), renais (doenças dos rins), imunes (níveis muito baixos ou muito elevados de células de defesa), doenças genéticas, hipertensão (pressão alta), ter apresentado diabetes gestacional (diabetes durante a gestação), apresentar inflamação das mamas (mastite) e estar em uso ou ter utilizado alguns tipos de medicamentos ou tóxicos que poderão interferir nos resultados da pesquisa.

Nesta parte da pesquisa, as participantes serão divididas em dois grupos: um grupo receberá a fibra alimentar (fruto-oligossacarídeo) e outro grupo receberá um produto similar (maltodextrina), porém sem a propriedade de influenciar a multiplicação de micro-organismos benéficos. A divisão dos grupos será realizada sem o conhecimento do pesquisador e da participante, a fim de não interferir nos resultados finais. Tanto o fruto-oligossacarídeo quanto o produto similar são apresentados na forma de pó de cor branca, sem odor, de sabor levemente adocicado e não deverão apresentar efeitos colaterais, durante o período de consumo. Somente a mãe participante deverá consumir o produto da pesquisa. **SEU BEBÊ NÃO DEVE CONSUMIR O PRODUTO**.

No primeiro encontro, será entregue um informativo para cada participante sobre a forma como o produto deverá ser consumido e armazenado, além de informações adicionais.

As participantes serão orientadas a manter a alimentação habitual, durante todo o período de estudo. Não será solicitado às participantes seguir qualquer dieta específica, exceto pelo consumo do produto da pesquisa.

Rubrica do pesquisador responsável
(Susana Marta Isay Saad)

Rubrica do participante da pesquisa

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – TCLE

Acerca da coleta do leite materno, esta será realizada manualmente, em ambiente limpo e confortável, por técnicos treinados. Todo material utilizado será descartável e estéril. Antes da coleta, as mamas e os mamilos serão higienizados com sabonete antisséptico e água e o primeiro fluxo de leite será desprezado. Serão coletados, aproximadamente, 25 mL (volume equivalente a meio copinho de café) de leite materno. Todo o procedimento será supervisionado pela doutoranda Marina Padilha.

As fezes deverão ser coletadas pela participante e armazenadas em recipientes estéreis, em refrigerador, e transportadas com gelo reciclável até entrega ao pesquisador. Os recipientes e o gelo reciclável serão fornecidos pelo pesquisador.

As amostras de leite materno e fezes serão mantidas em gelo, por até 4 horas e, posteriormente, as análises do material coletado serão realizadas nos laboratórios da Faculdade de Ciências Farmacêuticas da USP, por métodos qualitativos e/ou quantitativos apropriados para investigar a composição do leite.

Informações às participantes:

- ✓ Os riscos de sua participação nesta pesquisa são mínimos. Não foram encontradas evidências de risco ou desconforto relacionado ao consumo da fibra prebiótica na quantidade e período de consumo propostos nesta pesquisa. Embora geralmente sejam bem tolerados por indivíduos saudáveis, em alguns casos pode haver leve desconforto abdominal, devido à produção de gases, decorrente do processo de fermentação da fibra prebiótica.
- ✓ Quaisquer danos resultantes da pesquisa serão indenizados. Se houver algum benefício detectado durante o período de intervenção com a fibra alimentar às participantes, não nos comprometeremos a fornecer o suplemento, após o término da pesquisa;
- ✓ Você poderá recusar ou desistir da pesquisa a qualquer momento, sem prejudicar o acompanhamento médico realizado pela equipe do HU/USP. A participação ou a desistência não interfere no atendimento oferecido pelo Hospital Universitário.
- ✓ A qualquer momento você poderá solicitar que os seus dados sejam excluídos da pesquisa;
- ✓ Você poderá solicitar explicações todas as vezes que achar necessário sobre a pesquisa que estará participando;
- ✓ Todas as amostras de leite materno e fezes coletadas serão descartadas após as análises laboratoriais;
- ✓ Todas as participantes serão identificadas por um código para evitar que o seu nome seja relacionado aos resultados obtidos e quando os resultados desta pesquisa forem publicados em eventos e revistas científicas especializadas, os nomes não serão divulgados;
- ✓ As participantes terão ressarcimento com os custos de transporte, caso elas tenham que se deslocar até o Hospital Universitário para os procedimentos relativos a esta pesquisa.

Rubrica do pesquisador responsável
(Susana Marta Isay Saad)

Rubrica do participante da pesquisa

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – TCLE

Benefícios:

- ✓ Não haverá um benefício direto, porém as participantes estarão contribuindo, de forma voluntária, para o maior conhecimento sobre a influência da alimentação da mãe, sobre os micro-organismos naturalmente presentes no leite materno. Uma vez que o leite materno é um alimento direcionado ao bebê, os resultados poderão esclarecer sobre a influência da alimentação materna na saúde e o desenvolvimento intestinal do bebê que recebe este leite.

Em caso de dúvidas, intercorrências clínicas ou reações adversas, o participante da pesquisa será encaminhado pela equipe do projeto de pesquisa ao HU/USP. Entrar em contato com o pesquisador responsável:

Dra. Susana Marta Isay Saad, tel (011) 3091-2378.
Endereço: Depto de Tecnologia Bioquímico-Farmacêutica da FCF/USP
Av. Prof. Lineu Prestes, 580 Bloco 16, CEP: 05508-900 São Paulo - SP
Telefone: (011) 3091-2378 e-mail: susaad@usp.br.
Ou com a pesquisadora colaboradora: Marina Padilha, tel: (11) 3091-2691 ou e-mail: marina.padilha@usp.br

Uma via deverá será entregue a você e outra via ficará com o pesquisador responsável, arquivado pelo período de 5 (cinco) anos.

Consentimento Pós-Esclarecido:

Declaro que, após convenientemente esclarecido pelo pesquisador e ter entendido o que me foi explicado, consinto em participar do presente Protocolo de Pesquisa.

São Paulo, de de .

_____	_____
Assinatura da participante de pesquisa	**Assinatura do pesquisador responsável (Susana Marta Isay Saad)**

Em caso de dúvida, esclarecimento ou reclamação sobre aspectos éticos dessa pesquisa, favor entrar em contato:

- Comitê de Ética em Pesquisas da Faculdade de Ciências Farmacêuticas da USP – Av. Prof. Lineu Prestes, 580 - Bloco 13A – Cidade Universitária – CEP: 05508-900 – São Paulo/SP. Fone: (11) 3091-3622, Fone-Fax: 3091-3677 – E-mail: cepfcf@usp.br

- Comitê de Ética em Pesquisa do Hospital Universitário da USP - Av. Prof. Lineu Prestes, 2565 – Cidade Universitária - CEP: 05508-000 – São Paulo/SP - Fone: (11) 3091-9457, Fax: (11) 3091-9479 E-mail: cep@hu.usp.br

Attachment 4. Structured questionnaire applied in the study.

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas

Hospital Universitário USP

FICHA DE CADASTRO

Pesquisa: "Impacto da dieta materna e da intervenção com fruto-oligossacarídeo sobre a microbiota do leite humano".

Pesquisadora Responsável: Profª Drª Susana Marta Isay Saad.

Data da 1ª entrevista: _____/_____/_____

INFORMAÇÕES MATERNAS (LACTANTE)

1. Informações Pessoais **CÓDIGO:____ ____ ____**

Nome Completo:
Documento de Identidade nº: ( ) RG ( ) CPF
Data de Nascimento:_____/_____/_____ **Idade**: _____ anos Cor: ( ) B ( ) P ( ) N ( ) A
Estado Civil: ( ) Solteira ( ) Casada ( ) Divorciada ( ) Viúva ( ) Outro: _____
Profissão:
Pratica alguma religião: ( ) Sim ( ) Não **Se sim, qual:** _____
Mora com quem (marido/pais/etc):
Quantas pessoas moram em sua casa:
Telefone residencial: ( ) **Telefone celular**: ( )
Escolaridade:

( ) Nenhuma escolaridade;
( ) Ensino fundamental incompleto (até a 4ª série do primeiro grau);
( ) Ensino fundamental completo (até a 8ª série do primeiro grau);
( ) Ensino médio (segundo grau) incompleto;
( ) Ensino médio (segundo grau) completo;
( ) Superior incompleto;
( ) Superior completo;
( ) Pós-graduação/MBA/especialização;
( ) Mestrado/doutorado.

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FICHA DE CADASTRO

Renda Familiar

Itens	***Quantidade de Itens***				
	0	**1**	**2**	**3**	**4 ou +**
Banheiros	0	3	7	10	14
Empregados domésticos	0	3	7	10	13
Automóvel	0	3	5	8	11
Computador	0	3	6	8	11
Lava louça	0	3	6	6	6
Geladeira	0	2	3	5	5
Freezer (da geladeira ou independente)	0	2	4	6	6
Lava roupas	0	2	4	6	6
DVD	0	1	3	4	6
Micro-ondas	0	2	4	4	4
Motocicleta	0	1	3	3	3
Secadora de roupa	0	2	2	2	2

Grau de instrução do chefe de família (pessoa de referência)

Analfabeto/ Fundamental I incompleto	0	Serviços públicos		
Fundamental I completo/ Fundamental II incompleto	1		**Não**	**Sim**
Fundamental II completo/ Médio incompleto	2	Água encanada	0	4
Médio completo/ Superior incompleto	4	Rua pavimentada	0	2
Superior completo	7	**Soma de pontos =**		

*Critério Brasil 2014-2015

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FICHA DE CADASTRO

História de doença atual: ( ) Nenhuma ( ) Diabetes mellitus ( ) Dislipidemia ( ) Cardiopatia ( ) Nefropatia
( ) Imunodeficiência ( ) Doença inflamatória ( ) Doença genética ( ) Hipertensão arterial
História de doença pregressa:
Apresentou alguma doença durante a gestação? ( ) Diabetes ( ) Pré eclampsia/ Eclâmpsia ( ) Outra **qual(is)**: _____
Presença de Mastite: ( ) Sim ( ) Não
Utilizou medicamento durante a gestação? ( ) Sim ( ) Não **Se sim, qual (is):** _____
Suplementação nutricional na gestação? ( ) Sim ( ) Não **Se sim, qual (is):** _____
Utilizou antibiótico durante a gestação? ( ) Sim ( ) Não **Se sim, há quanto tempo?** ( ) há 4 meses ou + ( ) há menos de 4 meses
Em uso de algum medicamento? ( ) Sim ( ) Não **Se sim, quais:** _____ **Se sim, há quanto tempo?** ( ) há 1 mês ou + ( ) há menos de 1 mês
Hábito intestinal: ( ) > ou = 3 x/dia ( ) 1-2 x/ dia ( ) 1 x a cada 2 dias ( ) 1 x a cada 3 dias ou +
Com que frequência consumiu bebida alcoólica no último mês? ( ) Nenhuma ( ) 1-4 x/mês ( ) 1-7 x/semana
Companheiro fuma? ( ) Sim ( ) Não
A senhora fuma? ( ) Sim ( ) Não **Se sim, quantos maços/dia?**
A senhora fumou durante a gestação? ( ) Sim ( ) Não **Se sim, quantos maços/dia?**
Companheiro utiliza alguma outra substância? ( ) Sim ( ) Não **Se sim, saberia relatar o que?**
O companheiro já incentivou a senhora a usar alguma substância? ( ) Sim ( ) Não **Se sim, saberia relatar o que?**
Se sim, com que frequência utilizou alguma substância tóxica? ( ) Nenhuma ( ) 1-4 x/mês ( ) 1-7 x/semana

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Hospital Universitário HU USP

FICHA DE CADASTRO

2. Dados Antropométricos

Peso habitual: _____ kg	( ) relatado ( ) prontuário
Peso antes da gestação: _____ kg	( ) relatado ( ) prontuário
Índice de massa corporal pré-gestacional: _____ kg/m^2	
Ganho de peso na gestação: _____ kg	( ) relatado ( ) prontuário
Altura: _____ m	( ) aferido ( ) prontuário

3. Informações sobre o Parto

Idade gestacional: _____ semanas	( ) relatado ( ) prontuário
Bolsa: ( ) Rota espontânea ( ) Íntegra	( ) relatado ( ) prontuário
Tipo de parto: ( ) cesárea ( ) vaginal ( ) fórceps	( ) relatado ( ) prontuário
Anestesia: ( ) Sim ( ) Não **Se sim, qual (is): _____**	( ) relatado ( ) prontuário
Antibiótico no parto: ( ) Sim ( ) Não **Se sim, qual (is):** _____	( ) relatado ( ) prontuário
Intercorrências no parto: ( ) Sim ( ) Não **Se sim, qual (is):** _____	( ) relatado ( ) prontuário
Realização de Enema? ( ) Sim ( ) Não	( ) relatado ( ) prontuário

4. Informações sobre Aleitamento Materno

Amamenta? ( ) Sim ( ) Não
Iniciou amamentação no hospital? ( ) Sim ( ) Não
Apresentou algum destes problemas para amamentar? ( ) Fissura/Rachaduras ( ) Ingurgitamento ( ) Mastite ( ) Nódulos ( ) Abscesso mamário ( ) Outros:_____
O bebê recebe/recebeu leite materno de outras fontes? ( ) Sim ( ) Não
O bebê recebe/recebeu fórmula infantil? ( ) Sim ( ) Não

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FICHA DE CADASTRO

Pesquisa: "Impacto da dieta materna e da intervenção com fruto-oligossacarídeo sobre a microbiota do leite humano".

Pesquisadora Responsável: Profª Drª Susana Marta Isay Saad.

Data da 1ª entrevista: _____/_____/_____

INFORMAÇÕES DO BEBÊ (LACTENTE)

1. Informações Pessoais **CÓDIGO: ____ ____ ____ - ____**

Nome Completo:
Nome do Pai:
Nome da Mãe:
Local de Nascimento (Hospital, Cidade, Estado): **Sexo:** ( )F ( )M
Data de Nascimento:_____/_____/_____ **Idade**: _____ dias
Intercorrências desde o nascimento: ( ) Sim ( ) Não **Se sim, qual (is):** _____
Utilizou algum medicamento desde o nascimento ( ) Sim ( ) Não **Se sim, qual (is):** _____
Utilizou antibiótico desde o nascimento ( ) Sim ( ) Não **Se sim, quando:** _____
Apresenta alguma doença: ( ) Sim ( ) Não **Se sim, qual(is):** _____
Em uso de medicamentos: ( ) Sim ( ) Não **Se sim, qual(is):** _____
Suplemento alimentar: ( ) Sim ( ) Não **Se sim, qual (is):** _____ **Se sim, há quanto tempo** _____
Funcionamento intestinal: _____ x/dia ( ) fezes líquidas ( ) fezes amolecidas ( ) fezes endurecidas

FICHA DE CADASTRO

2. Dados antropométricos do lactente

Peso ao nascer: _____ g	( ) aferido ( ) prontuário
Comprimento ao nascer: _____ cm	( ) aferido ( ) prontuário
Percentil na curva de Alexander (ao nascer)	( ) < p10 ( ) p10 < p90 ( ) > p90
Peso atual: _____ g	( ) aferido ( ) prontuário

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas

FICHA DE ACOMPANHAMENTO

Pesquisa: "Impacto da dieta materna e da intervenção com fruto-oligossacarídeo sobre a microbiota do leite humano".

Pesquisadora Responsável: Profª Drª Susana Marta Isay Saad.

Data da 2ª entrevista: _____/_____/_____

INFORMAÇÕES DE ACOMPANHAMENTO MATERNO (LACTANTE)

CÓDIGO: ____ ____ ____

Peso atual: _____ g	( ) aferido ( ) relatado
Altura: _____ m	( ) aferido ( ) relatado
Índice de massa corporal atual: _____ kg/m^2	
Intercorrências desde a última entrevista? ( ) Sim ( ) Não **Se sim, qual (is):** _____	
Utilizou algum medicamento: ( ) Sim ( ) Não **Se sim, qual(is):** _____	
Suplemento alimentar: ( ) Sim ( ) Não **Se sim, qual (is):** _____ **Se sim, há quanto tempo** _____	
Funcionamento intestinal: _____ x/dia ( ) fezes líquidas ( ) fezes amolecidas ( ) fezes endurecidas	

INFORMAÇÕES DE ACOMPANHAMENTO DO BEBÊ (LACTENTE)

CÓDIGO: ____ ____ ____ - ____

Peso atual: _____ g	( ) aferido ( ) relatado
Comprimento: _____ m	( ) aferido ( ) relatado
Intercorrências desde a última entrevista? ( ) Sim ( ) Não **Se sim, qual (is):** _____	
Utilizou algum medicamento: ( ) Sim ( ) Não **Se sim, qual(is):** _____	
Suplemento alimentar: ( ) Sim ( ) Não **Se sim, qual (is):** _____ **Se sim, há quanto tempo** _____	
Funcionamento intestinal: _____ x/dia ( ) fezes líquidas ( ) fezes amolecidas ( ) fezes endurecidas	

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas

FICHA DE ACOMPANHAMENTO

Informações sobre Aleitamento Materno

Apresentou algum destes problemas para amamentar desde a última entrevista? ( ) Fissura/Rachaduras ( ) Ingurgitamento ( ) Mastite ( ) Nódulos ( ) Abscesso mamário ( ) Outros:_____
O bebê recebe leite materno de outras fontes? ( ) Sim ( ) Não
Já ofereceu alguma vez água, chá ou suco para o bebê? ( ) Sim ( ) Não
Já ofereceu alguma vez mel, açúcar ou algum outro alimento? ( ) Sim ( ) Não
O bebê usa chupeta? ( ) Sim ( ) Não **O bebê usa mamadeira?** ( ) Sim ( ) Não

Attachment 5. Quantitative Food Frequency Questionnaire (QFFQ)

Questionário Quantitativo de Freqüência Alimentar para Gestantes

Oliveira et al. Cad. Saúde Pública, Rio de Janeiro, 26(12):2296-2306, dez, 2010

As questões seguintes relacionam-se ao seu hábito alimentar usual DURANTE A GESTAÇÃO. Responda, por favor, a freqüência que melhor descreva QUANTAS VEZES a senhora costuma comer cada item e a respectiva UNIDADE DE TEMPO (se por dia, por semana, por mês ou desde que engravidou). Depois, responda qual a quantidade consumida.

* D: diário, S: semanal, M: no último mês, G: durante esta gestação
**P: pequena, M: média, G: grande, EG: extra-grande.

GRUPOS DE ALIMENTOS	QUANTAS VEZES VOCÊ COME	FREQUÊNCIA*	PORÇÃO MÉDIA	SUA PORÇÃO**	CODIFICAÇÃO
Pão francês, pão de fôrma.	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 unidade (50g)	P M G EG	___ ___ ___ ___
Rosca doce ou sonho	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 un P (60g)	P M G EG	
Bolo	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 ft G (100g)	P M G EG	___ ___ ___ ___
Pão integral	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 fatias (50g)	P M G EG	
Torrada, bolacha salgada ou biscoito de polvilho	N 1 2 3 4 5 6 7 8 9 10	D S M G	5 unidades (33g)	P M G EG	___ ___ ___ ___
Bolacha doce sem recheio (Maisena, cookies simples, amanteigada, mel e aveia)	N 1 2 3 4 5 6 7 8 9 10	D S M G	10 unidades (50g)	P M G EG	
Bolacha doce com recheio (bolachas recheadas, com goiabada ou wafer)	N 1 2 3 4 5 6 7 8 9 10	D S M G	7 unidades (87,5g)	P M G EG	___ ___ ___ ___
Geléia, mel ou melado	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 col sob (10g)	P M G EG	
Manteiga	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 pt faca (5g)	P M G EG	___ ___ ___ ___
Margarina ( )comum ( ) light	N 1 2 3 4 5 6 7 8 9 10 N 1 2 3 4 5 6 7 8 9 10	D S M G D S M G	1 pt faca (5g)	P M G EG P M G EG	
Requeijão	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 pt faca CH (10g)	P M G EG	___ ___ ___ ___
Queijo branco (fresco, ricota, cottage)	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 pdçs M (50g)	P M G EG	
Queijos amarelos (parmesão, mussarela, provolone, prato)	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 ft M (30g)	P M G EG	___ ___ ___ ___
Mortadela, salame, presunto, peito de peru ou salsicha	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 ft M (40g)	P M G EG	___ ___ ___ ___
Leite ( )integral ( )desnatado	N 1 2 3 4 5 6 7 8 9 10 N 1 2 3 4 5 6 7 8 9 10	D S M G D S M G	1 cp req CH (250g)	P M G EG P M G EG	___ ___ ___ ___ ___ ___ ___ ___
Achocolatado ou cappuccino (pó)	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 cols sob (22g)	P M G EG	
Vitamina de fruta com leite	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 cp G CH (300g)	P M G EG	___ ___ ___ ___

Mingau	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 prato fundo raso (200g)	P M G EG	
Iogurte integral (Coalhada, iogurte natural ou iogurte de frutas)	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 cp Req r (200g)	P M G EG	___ ___ ___ ___
Iogurte desnatado	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 copo P (150g)	P M G EG	
Suco de laranja natural	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 copo de Req CH (250g)	P M G EG	___ ___ ___ ___
Suco de outras frutas (natural)	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 copo de Req CH (250g)	P M G EG	
Suco artificial ou refrigerante	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 copo de Req CH (250g)	P M G EG	___ ___ ___ ___
Café	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 xícara de café (50g)	P M G EG	
Abacaxi	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 fatias médias (200g)	P M G EG	___ ___ ___ ___
Banana	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 unidade média (80g)	P M G EG	
Mexerica, laranja	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 unidade média (160g)	P M G EG	___ ___ ___ ___
Goiaba	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 unidades médias (340g)	P M G EG	
Manga, caqui	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 unidade média (180g)	P M G EG	___ ___ ___ ___
Maçã, pêra	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 unidade média (93g)	P M G EG	
Melancia, melão	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 fatia média (200g)	P M G EG	___ ___ ___ ___
Mamão	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 fatia média (170g)	P M G EG	
Morango	N 1 2 3 4 5 6 7 8 9 10	D S M G	9 unidades grandes (108g)	P M G EG	___ ___ ___ ___
Pêssego	N 1 2 3 4 5 6 7 8 9 10	D S M G	3 unidades médias (300g)	P M G EG	
Abacate ou abacatada	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 fatia média (147,5g)	P M G EG	___ ___ ___ ___
Uva	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 cacho pequeno (170g)	P M G EG	
Acelga, alface, repolho (cru ou cozido)	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 prato de sobremesa	P M G EG	___ ___ ___ ___

			(36g)		
Agrião, almeirão, rúcula, couve	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 pt CH (50g)	P M G EG	
Beterraba	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 ft G (52g)	P M G EG	___ ___ ___ ___
Cenoura	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 col S CH (30g)	P M G EG	
Pepino	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 pires CH (120g)	P M G EG	___ ___ ___ ___
Tomate	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 un M (90g)	P M G EG	
Abóbora	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 pires (135g)	P M G EG	___ ___ ___ ___
Abobrinha	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 un P (72g)	P M G EG	
Mandioca, batata ou purê de batata ou mandioquinha ( ) Frita ( ) Cozida	N 1 2 3 4 5 6 7 8 9 10 N 1 2 3 4 5 6 7 8 9 10	D S M G D S M G	1 esc M r (95g) 1 esc M r (95g)	P M G EG P M G EG	___ ___ ___ ___
Brócolis	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 ramo M (30g)	P M G EG	
Vagem, chuchu, couve-flor	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 esc M CH (90g)	P M G EG	___ ___ ___ ___
Milho verde	N 1 2 3 4 5 6 7 8 9 10	D S M G	4 col Sp CH ou 1 espiga (100g)	P M G EG	
Arroz branco	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 esc M CH (170g)	P M G EG	___ ___ ___ ___
Risoto, arroz carreteiro ou arroz à grega, canja	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 col A CH (134g)	P M G EG	
Arroz integral	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 col A CH (134g)	P M G EG	___ ___ ___ ___
Feijão cozido	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 co M (156g)	P M G EG	
Feijoada, feijão com lingüiça ou bacon	N 1 2 3 4 5 6 7 8 9 10	D S M G	3 e ½ co M (273g)	P M G EG	___ ___ ___ ___
Miojo	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 pacote (80g)	P M G EG	___ ___ ___ ___
Lasanha ou massas recheadas com carne	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 esc G r ou 1 pedaço P (122,5)	P M G EG	___ ___ ___ ___
Macarrão, outras massas	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 esc M CH (220g)	P M G EG	___ ___ ___ ___
Quando consome massa, qual o tipo de molho adicionado?					
( ) Branco ( ) À Bolonhesa ou de frango ( ) Ao sugo ( ) Alho e óleo	N 1 2 3 4 5 6 7 8 9 10 N 1 2 3 4 5 6 7 8 9 10 N 1 2 3 4 5 6 7 8 9 10 N 1 2 3 4 5 6 7 8 9 10	D S M G D S M G D S M G D S M G		P M G EG P M G EG P M G EG P M G EG	___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___
Carne bovina frita, carne de panela	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 filé M ou 3 pedaços M (100g)	P M G EG	
Bife grelhado	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 filé M	P M G EG	___ ___ ___ ___

Carne moída	N 1 2 3 4 5 6 7 8 9 10	D S M G	(100g) 4 cols Sp CH (120g)	P M G EG	___ ___ ___ ___
Estrogonofe de carne, bife à role, carne com legumes	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 colhs A CH (80g)	P M G EG	
Frango frito	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 filé M (180g)	P M G EG	___ ___ ___ ___
Frango assado	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 filé M (180g)	P M G EG	
Frango xadrez, estrogonofe de frango ou fricassê	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 colhs Sp CH (120g)	P M G EG	___ ___ ___ ___
Pernil ou lombo Lingüiça	N 1 2 3 4 5 6 7 8 9 10 N 1 2 3 4 5 6 7 8 9 10	D S M G D S M G	1,5 Filé P (150g) 1 gomo (60g)	P M G EG P M G EG	
Bacon ou torresmo	N 1 2 3 4 5 6 7 8 9 10	D S M G	6 ft (600g)	P M G EG	___ ___ ___ ___
Peixe cozido	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 Filé M (100g)	P M G EG	
Peixe frito	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 Filé M (100g)	P M G EG	___ ___ ___ ___
Atum	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 Col SP Ch (32g)	P M G EG	
Sardinha	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 unidades (82g)	P M G EG	___ ___ ___ ___
Ovo ( ) Cozido ( ) Frito ( ) Omelete	N 1 2 3 4 5 6 7 8 9 10 N 1 2 3 4 5 6 7 8 9 10 N 1 2 3 4 5 6 7 8 9 10	D S M G D S M G D S M G	1 unidade (50g)	P M G EG P M G EG P M G EG	
Fígado ou moela	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 un M (30g)	P M G EG	___ ___ ___ ___
Dobradinha	N 1 2 3 4 5 6 7 8 9 10	D S M G	3 colhs Sp CH (97,5g)	P M G EG	
Frutos do mar	N 1 2 3 4 5 6 7 8 9 10	D S M G	5 colhs Sp CH (100g)	P M G EG	___ ___ ___ ___
Castanhas, nozes, amendoim.	N 1 2 3 4 5 6 7 8 9 10	D S M G	8 unidades (20g)	P M G EG	
Sopa de legumes	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 prato fundo CH (310g)	P M G EG	___ ___ ___ ___
Doces com frutas ou picolé de frutas	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 colhs Sp CH (80g) 1 picolé ou 1 fatia M (60g)	P M G EG	
Doces com leite	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 fatia M (69g)	P M G EG	___ ___ ___ ___
Sorvete (massa)	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 bola G (100g)	P M G EG	
Chocolate	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 bombom ou 1 filete (30g)	P M G EG	___ ___ ___ ___
Paçoca, pé de moleque	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 unidades (60g)	P M G EG	
Salgado frito	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 unidade G (100g)	P M G EG	___ ___ ___ ___
Salgado assado	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 unidade M (80g)	P M G EG	

Salgadinho tipo “Chips” ou pipoca	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 pct (96g) ou 1 saco M de pipoca (20g)	P M G EG	___ ___ ___ ___
Lanches, cachorro quente, hambúrguer	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 unidade (125g)	P M G EG	___ ___ ___ ___
Pizza	N 1 2 3 4 5 6 7 8 9 10	D S M G	2 fatias M (210g)	P M G EG	___ ___ ___ ___
Açúcar (adicionado em bebidas)	N 1 2 3 4 5 6 7 8 9 10	D S M G	1 col sob (16g)	P M G EG	___ ___ ___ ___

Com qual freqüência a senhora consome vegetais e quantas porções?

FREQUÊNCIA	QUANTAS VEZES VOCÊ COME
D S M G	N 1 2 3 4 5 6 7 8 9 10

Com qual freqüência a senhora consome frutas e quantas porções?

FREQUÊNCIA	QUANTAS VEZES VOCÊ COME
D S M G	N 1 2 3 4 5 6 7 8 9 10

Quando consome frango você retira a pele? (1) Sim (2) Ás vezes (3) Não __
Quando consome carne bovina você retira a gordura aparente? (1) Sim (2)às vezes (3)Não __
Quando a senhora consome atum é em água ou em óleo? (1) Óleo (2) Água __
Como a senhora tempera a salada?
(1) Azeite extra-virgem (2) Óleo vegetal (3) Molho industrializado (4) sal __ __
Que tipo de gordura a senhora usa para preparar as refeições?
Óleo vegetal: (1) soja (2) milho (3)girassol (4) canola (5) composto
(6) Margarina (7) Manteiga (8) Banha (9) Azeite __ __

Há algum alimento que você consome pelo menos 1x/semana que não foi citado?

ALIMENTO	FREQÜÊNCIA POR SEMANA	QUANTIDADE CONSUMIDA	COD

ADDITIONAL FILES

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial
FICHA DO ALUNO

9131 - 5400304/1 - Marina Padilha

Email:	marina.padilha@usp.br
Data de Nascimento:	18/07/1986
Cédula de Identidade:	RG - 44.325.685-8 - SP
Local de Nascimento:	Estado de São Paulo
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Graduação:	Nutricionista - Faculdade de Medicina de Ribeirão Preto - Universidade de São Paulo - São Paulo - Brasil - 2009
Mestrado:	Mestra em Ciências - Área: Tecnologia de Alimentos - Faculdade de Ciências Farmacêuticas - Universidade de São Paulo - São Paulo - Brasil - 2013

Curso:	Doutorado
Programa:	Ciência dos Alimentos
Área:	Bromatologia
Data de Matrícula:	31/10/2013
Início da Contagem de Prazo:	31/10/2013
Data Limite para o Depósito:	28/02/2018
Orientador Acadêmico:	Prof(a). Dr(a). Bernadette Dora Gombossy de Melo Franco - 31/10/2013 até 17/02/2014. Email: bfranco@usp.br
Orientador:	Prof(a). Dr(a). Susana Marta Isay Saad - 18/02/2014 até o presente. Email: susaad@usp.br
Co-orientador:	Prof(a). Dr(a). Carla Taddei de Castro Neves - 08/04/2015 até o presente. Email: crtaddei@usp.br
Proficiência em Línguas:	Inglês, Aprovado em 31/10/2013
Prorrogação(ões):	120 dias Período de 31/10/2017 até 28/02/2018
Data de Aprovação no Exame de Qualificação:	Aprovado em 14/12/2015
Data do Depósito do Trabalho:	
Título do Trabalho:	
Data Máxima para Aprovação da Banca:	
Data de Aprovação da Banca:	
Data Máxima para Defesa:	
Data da Defesa:	
Resultado da Defesa:	
Histórico de Ocorrências:	Primeira Matrícula em 31/10/2013 Prorrogação em 28/08/2017

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 5473 em vigor de 18/09/2008 até 19/04/2013).
Última ocorrência: Matrícula de Acompanhamento em 05/02/2018
Impresso em: 23/02/2018 12:34:02

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial
FICHA DO ALUNO

9131 - 5400304/1 - Marina Padilha

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
FBA5752-1/1	Probióticos em Alimentos e Suas Implicações na Saúde Humana	05/11/2013	16/12/2013	60	4	100	A	N	Concluída
EDM5791-6/1	Metodologia do Ensino Superior (Faculdade de Educação - Universidade de São Paulo)	11/03/2014	03/06/2014	120	8	83	A	N	Concluída
FBT5700-3/1	Preparo de Artigos Científicos na Área de Tecnologia Bioquímico-Farmacêutica	03/04/2014	04/06/2014	90	6	85	A	N	Concluída
MPE5746-2/1	Imunologia da Relação Mãe-Filho (Faculdade de Medicina - Universidade de São Paulo)	05/05/2014	25/05/2014	60	4	100	A	N	Concluída
BIE5782-4/2	Uso da Linguagem R para Análise de Dados em Ecologia (Instituto de Biociências - Universidade de São Paulo)	08/05/2017	28/05/2017	60	0	-	-	N	Matrícula cancelada

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito de tese	
Disciplinas:	0	20	22
Estágios:			
Total:	0	20	22

Créditos Atribuídos à Tese: 167

Conceito a partir de 02/01/1997:

A - Excelente, com direito a crédito; B - Bom, com direito a crédito; C - Regular, com direito a crédito; R - Reprovado; T - Transferência.

Um(1) crédito equivale a 15 horas de atividade programada.

Última ocorrência: Matrícula de Acompanhamento em 05/02/2018
Impresso em: 23/02/2018 12:34:02