TURUN YLIOPISTO
UNIVERSITY OF TURKU

HUMAN MILK: MICROBIOTA COMPOSITION AND IMPACT OF PROCESSING ON PROBIOTIC PROPERTIES

Anastasia Mantziari

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA – SER. D OSA – TOM. 1788 | MEDICA – ODONTOLOGICA | TURKU 2024

TURUN YLIOPISTO
UNIVERSITY OF TURKU

HUMAN MILK: MICROBIOTA COMPOSITION AND IMPACT OF PROCESSING ON PROBIOTIC PROPERTIES

Anastasia Mantziari

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA – SER. D OSA – TOM. 1788 | MEDICA – ODONTOLOGICA | TURKU 2024

University of Turku

Faculty of Medicine
Department of Clinical Medicine
Paediatrics
Doctoral Programme in Clinical Research

Supervised by

Professor Seppo Salminen
Functional Foods Forum
University of Turku
Turku, Finland

Associate Professor Samuli Rautava
Children's Hospital
University of Helsinki
Helsinki, Finland

Reviewed by

Professor Sonia González-Solares
Department of Functional Biology
University of Oviedo
Oviedo, Spain

Docent Sampo Lahtinen
Department of Life Technologies
University of Turku
Turku, Finland

Opponent

Professor Hani El-Nezamy
Institute of Public Health and Clinical Nutrition
University of Eastern Finland
Kuopio, Finland

The originality of this publication has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

Cover Image: This illustration has been generated with the help of DALL-E 3.

ISBN 978-951-29-9674-2 (PRINT)
ISBN 978-951-29-9675-9 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama, Turku, Finland 2024

Last but not least, I wanna thank me
I wanna thank me for believing in me
-Snoop Dogg

UNIVERSITY OF TURKU
Faculty of Medicine, Department of Clinical Medicine, Paediatrics
ANASTASIA MANTZIARI: Human milk: microbiota composition and impact of processing on probiotic properties
Doctoral Dissertation, 187 pp.
Doctoral Programme in Clinical Research
March 2024

ABSTRACT

Preterm birth complications represent a leading cause of mortality in children under five years old. To mitigate these complications, strategies such as the use of donor human milk, fortification with human milk fortifiers (protein supplements), and probiotic administration are employed. This thesis aimed to thoroughly understand the microbiota composition of preterm human milk and the influence of various factors on specific probiotics' properties.

The study began with analyzing human milk microbiota from healthy Argentinian women across different gestational ages, focusing on changes in microbiota from birth to term equivalent age. It was found that term milk exhibited greater microbial diversity than preterm milk, and by term equivalent age, the microbiota composition of human milk closely resembled that of term milk, suggesting breastfeeding's role in promoting microbial maturity. The research further assessed the stability of probiotics, specifically *Lacticaseibacillus rhamnosus* GG and *Bifidobacterium animalis* ssp. *lactis* Bb12, during cold storage of human milk. Results indicated that these probiotics maintained their viability for up to 72 hours. Additionally, this thesis examined the effect of liquid or powdered human milk fortifiers on the adhesion properties of these probiotics in donor human milk, finding that fortification did not adversely affect probiotic adhesion. The final study of the thesis explored the role of polyamines, in modulating the adhesion of probiotics and pathogens with intestinal mucus in infants. The presence of the polyamine spermidine significantly enhanced the adhesion of Bb12 in infant mucus under six months, whereas the polyamine spermine reduced the adhesion of *Cronobacter sakazakii*.

In conclusion, the thesis showed that the microbiota profiles of human milk significantly vary with gestational age. A key finding is that the microbiota composition of preterm human milk undergoes a transformation, eventually resembling that of term milk as the infant reaches term equivalent age. It was also found that probiotic adhesion properties are unaffected by cold storage or the fortification of donor human milk. However, the influence of polyamines on bacterial adhesion to mucus varies based on the bacterial strain and the mucus donor's age employed. These insights should be considered for developing optimized gut colonization strategies for infants, particularly for those born preterm.

KEYWORDS: Human milk microbiota, donor human milk, fortification, probiotics, adhesion, polyamines, storage

TURUN YLIOPISTO
Lääketieteellinen tiedekunta, Kliininen laitos, Lastentautioppi
ANASTASIA MANTZIARI: Äidinmaito: mikrobiston koostumus ja prosessoinnin vaikutus sen probioottisiin ominaisuuksiin
Väitöskirja, 187 s.
Turun kliininen tohtoriohjelma
maaliskuu 2024

TIIVISTELMÄ

Ennenaikaisen synnytyksen komplikaatiot ovat yleisin alle viisivuotiaiden lasten kuolinsyy. Luovutettua äidinmaitoa, äidinmaidon täydentämistä proteiinilisällä sekä probioottien antamista käytetään näiden komplikaatioiden ehkäisemiseksi. Tämän opinnäytetyön tavoitteena oli selvittää ennenaikaisesti synnyttäneiden äitien rintamaidon mikrobistokoostumusta ja eri tekijöiden vaikutusta tiettyjen probioottien ominaisuuksiin.

Tutkimuksessa analysoitiin terveiden argentiinalaisten naisten äidinmaidon mikrobikoostumusta keskittyen mikrobiston muutoksiin ennenaikaisesta synnytyksestä laskettuun aikaan. Huomattiin, että täysiaikaisesti synnyttäneiden naisten äidinmaidon mikrobiston monimuotoisuus oli suurempaa kuin ennenaikaisesti synnyttäille naisilla. Laskettuna aikana ennenaikasesti synnyttäneiden äidinmaidon mikrobikoostumus muistutti läheisesti täysiaikaisen maidon mikrobistoa, mikä viittaa siihen, että imetyksellä on tärkeä rooli mikrobiston kypsymisen edistämisessä. Tutkimuksessa, jossa arvioitiin *Lacticaseibacillus rhamnosus* GG:n ja *Bifidobacterium animalis* ssp. *lactis* Bb12 -probioottien stabiiliutta äidinmaidossa kylmävarastoinnin aikana, nämä probiootit säilyttivät elinkykynsä jopa 72 tuntia. Lisäksi havaittiin, että äidinmaidon vahvistaminen nestemäisellä tai jauhemaisella vahvikkeella ei vaikuttanut haitallisesti probioottien tarttumisominaisuuksiin luovutetussa äidinmaidossa. Opinnäytetyön viimeisessä tutkimuksessa selvitettiin polyamiinien roolia bakteerien tarttumisen säätelyssä imäveisen suolistolimassa. Polyamiini spermidiinin läsnäolo paransi merkittävästi Bb12:n kiinnittymistä alle kuuden kuukauden ikäisen imeväisten limaan, kun taas spermiini vähensi *Cronobacter sakazakii*-bakteerin tarttumista.

Opinnäytetyö osoitti, että äidinmaidon mikrobistoprofiilit vaihtelevat merkittävästi raskauden keston mukaan. Ennenaikaisen synnytyksen jälkeen äidinmaidon mikrobistokoostumus muuttuu ja alkaa lopulta muistuttaa täysiaikaisen synnytyksen äidinmaidon mikrobistokoostumusta vauvan saavuttaessa taysiaikaista vastaavan iän. Lisäksi havaittiin, että kylmävarastointi tai luovutetun äidinmaidon vahvistaminen eivät vaikuttaneet probioottien tarttumisominaisuuksiin. Polyamiinien vaikutus bakteerien tarttumiseen limaan vaihtelee bakteerikannan ja limanluovuttajan iän mukaan. Tulokset auttavat kehittämään optimoituja suoliston kolonisaatiostrategioita vastasyntyneille ja erityisesti keskosille.

AVAINSANAT: äidinmaidon mikrobiota, luovuttajamaito, väkevöinti, probiootit, adheesio, polyamiinit, varastointi

Table of Contents

Abbreviations ... 9

List of Original Publications ... 11

1 Introduction ... 12

2 Review of the Literature ... 14
2.1 Common Problems related to Preterm Birth ... 14
2.1.1 The Neonatal Intestinal Dysbiosis ... 15
2.2 Potential Clinical Intervention Strategies ... 16
2.2.1 Human Milk ... 16
2.2.1.1 Protective Functions ... 16
2.2.1.2 Composition ... 18
2.2.1.2.1 Nutrients ... 19
2.2.1.2.2 Bioactive Factors and Human Cells ... 21
2.2.1.2.3 The Human Milk Microbiota ... 22
2.2.1.3 Human Milk Processes ... 33
2.2.1.3.1 Pasteurization and Cold Storage ... 33
2.2.1.3.2 Human Milk Fortification ... 35
2.2.2 Probiotics ... 36
2.2.2.1 Normalization of Intestinal Microbiota Composition and Protective Functions ... 36
2.2.2.2 Mechanisms of action ... 38
2.2.2.3 Quality assurance criteria for probiotics ... 41
2.2.2.3.1 Adhesion Ability to Intestinal Surfaces ... 41
2.2.2.3.2 Factors Affecting Probiotic Stability in Milk ... 42
2.2.3 Polyamines ... 46

3 Aims ... 49

4 Materials and Methods ... 50
4.1 Subjects, sample collection and preparation ... 50
4.2 Pasteurization, fortification, and storage of Human Milk ... 51
4.3 Culturing ... 51
4.3.1 Bacterial strains and growth conditions ... 51
4.3.2 Plate counts ... 52
4.4 DNA extraction (I-III) ... 52

4.4.1 DNA extraction using MasterPure™ kit (I) 52
4.4.2 DNA extraction using KingFisher (II)........................... 52
4.4.3 DNA extraction from colonies (II and III)...................... 53
4.5 Confirmation by colony polymerase chain reaction (cPCR) (II and III).. 53
4.6 Identification of human milk isolates by Sanger Sequencing (II) 53
4.7 Quantitative polymerase chain reaction (qPCR) analysis (II) 54
4.8 16S rRNA gene sequencing analysis of human milk microbiota (I) 55
4.9 *In vitro* adhesion assays with radiolabeled bacteria (III and IV) 55
4.9.1 Bacterial radiolabeling 55
4.9.2 Preparation of bacteria prior to *in vitro* adhesion assays 56
4.9.3 Isolation of human intestinal mucus from feces 56
4.9.4 *In vitro* adhesion assay.. 56
4.10 Statistical analyses... 57
4.10.1 Microbiota composition of human milk samples 16S rRNA gene sequencing (I) 57
4.10.1.1 Bioinformatics 57
4.10.1.2 Statistical analysis 58
4.10.2 Viability Assays (II and III) 59
4.10.3 Adhesion Assays (III and IV) 59

5 Results 60
5.1 **HMM composition affected by prematurity (I)** 60
5.1.1 Clinical Data Profile and Human Milk Microbiota Profile.. 60
5.1.2 Overall Human Milk Microbiota Composition 61
5.1.3 Evolution of the HMM Composition from Preterm Birth to TEA (P versus PT) 62
5.1.4 Differences In Human Milk Microbiota Profiles Between Mothers of Preterm Infants at TEA And Full-Term Infants (PT versus T).................................. 65
5.1.5 Differences In Human Milk Microbiota Profiles Between Mothers of Preterm And Full-Term Infants (P versus T).. 66
5.1.6 Human Milk Microbiota and its Association with Delivery Mode, Antenatal Corticoids, Antibiotics, Gestational and Term Equivalent Ages 67
5.2 Storage of Human Milk and its Effect on Microbial Stability (II and III).. 69
5.3 Probiotic adhesion affected by donor human milk (III) 71
5.4 Probiotic and pathogen adhesion modulated by exogenous polyamines (IV) 75

6 Discussion 78
6.1 Human Milk Microbiota Composition is impacted by Gestational Age and alters with time (**I**).................................. 78

6.2 Fortification and Storage of Human Milk and their Impact on Microbial Stability (**II** and **III**) ... 82
6.3 Adhesion Properties of Probiotics (**III** and **IV**) ... 84
6.3.1 Strain dependence of the probiotic adhesion properties (**III**) ... 84
6.3.2 The role of fortification and cold storage of DHM on probiotic adhesion (**III**) ... 85
6.3.3 Age dependency of probiotic, pathogen, and commensal adhesion ability to intestinal mucus (**III** and **IV**) ... 88
6.3.4 Adhesion Properties of Probiotics Mediated by Exogenous Polyamines (**IV**) ... 89

7 Limitations and Suggestions for Future Research ... 92

8 Summary/Conclusions ... 96

Acknowledgements ... 98

References ... 102

Original Publications ... 139

Abbreviations

Bb12	*Bifidobacterium animalis* subsp. *lactis* Bb12
16S rRNA	16S ribosomal RNA gene
ANOVA	Analysis of Variance
BMI	Body Mass index
CFU	Colony Forming Units
cPCR	colony Polymerase Chain Reaction
DC	Dendritic Cell
DHM	Donor Human Milk
DNA	Deoxyribonucleic Acid
EGF	Epidermal Growth Factor
EV	Extracellular Vesicles
FDHM	Fortified Donor Human Milk
GAM	Gifu Anaerobic Medium
GI	Gastrointestinal
GM	Gut Microbiota
HH	HEPES-Hanks Buffer
HM	Human Milk
HMF	Human Milk Fortifier
HMM	Human Milk Microbiota
HMO	Human Milk Oligosaccharides
Ig	Immunoglobulin
IL	Interleukin
LGG	*Lacticaseibacillus rhamnosus* GG
lHMF	liquid HMF
LOS	Late Onset Sepsis
LPS	Lipopolysaccharides
MFGM	Milk Fat Globule Membrane
MixHigh	Polyamine Mix in High Concentration
MixLow	Polyamine Mix in Low Concentration
MOM	Mother’s Own Milk
MRS	de Man, Rogosa and Sharpe

NEC	Necrotizing Enterocolitis
NGS	Next Generation Sequencing
NICU	Neonatal Intensive Care Unit
NMDS	Non-Metric Multidimensional Scaling
NTCs	no-template controls
PAs	Polyamines
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
pHMF	powdered HMF
PUFAs	Polyunsaturated Fatty Acids
PUT	Putrescine
qPCR	quantitative Polymerase Chain Reaction
RCT	Randomized Control Trials
RT	Room Temperature
SCFAs	Short-Chain Fatty Acids
SDS	Sodium Dodecyl Sulphate
SPD	Spermidine
SPM	Spermine
UV	Ultraviolet Light
VBNC	Viable but Not Culturable
VLBW	Very-Low Birth Weight

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Oddi S*, **Mantziari A***, Huber P, Binetti A, Salminen S, Collado MC, Vinderola G. Human Milk Microbiota Profile Affected by Prematurity in Argentinian Lactating Women. *Microorganisms*. 2023; 11(4):1090.

II **Mantziari A**, Aakko J, Kumar H, Tölkkö S, du Toit E, Salminen S, Isolauri E, and Rautava S. The Impact of Storage Conditions on the Stability of *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* Bb12 in Human Milk. *Breastfeeding Medicine*. 2017; 12(9): 566-569.

III **Mantziari A**, Tölkkö S, Ouwehand AC, Löyttyniemi E, Isolauri E, Salminen S, Rautava S. The Effect of Donor Human Milk Fortification on The Adhesion of Probiotics In Vitro. *Nutrients*. 2020; 12(1):182.

IV **Mantziari A**, Mannila E, Collado MC, Salminen S, Gómez-Gallego C. Exogenous Polyamines Influence In Vitro Microbial Adhesion to Human Mucus According to the Age of Mucus Donor. *Microorganisms*. 2021; 9(6):1239.

*Equal contribution.

The original publications have been reproduced with the permission of the copyright holders. Articles I, III, and IV are licensed under a Creative Commons license, which permits reproduction. In addition, unpublished data not included in the original publications are presented.

1 Introduction

Preterm birth is the leading cause of neonatal mortality worldwide. Preterm neonates are hospitalized for longer periods of time due to low birth weight and immaturity of several organ systems, which increases the risk of developing infections and necrotizing enterocolitis (NEC) and imposes a significant financial burden on society (Jiang et al., 2020; S. W. Kim et al., 2021). Although the pathogenesis of NEC remains poorly understood, it is believed that gut microbes and intestinal dysbiosis play a significant role in its development.

After delivery, preterm infants harbor distinct gut microbiota compared to term infants, which possibly contributes to the development of NEC (M. C. Collado, Cernada, et al., 2012). Thus, different microbiota modulation interventions, including the use of human milk (HM) are employed to establish a normal commensal gut microbiome. Although the mechanism of protection is not yet clear, numerous reports suggest that the human milk microbiota (HMM) plays a critical role in directing the initial colonization pattern of the preterm gut and for this reason it is important to characterize it. When a mother's own milk is not available, preterm newborns typically receive pooled milk from donors who delivered at full term which is pasteurized and stored. Although the viability of beneficial bacteria is diminished with pasteurization of HM, evidence shows that the protective properties of HM in reducing NEC are maintained (Yang et al., 2020).

Probiotic therapy, i.e., administration of beneficial microbes, is another way to modulate the neonatal gut microbiota. Studies on very low birth weight infants argue that the use of HM supplemented with specific probiotics appears to confer better protection against NEC than non-supplemented HM (Bin-Nun et al., 2005). Preterm infants are also at high risk of experiencing growth failure as a result of inadequate nutrient intake. Despite its beneficial composition, HM alone cannot meet the protein requirements of preterm neonates. As a result, protein supplements, known as HM fortifiers, are routinely used to improve the nutritional properties of both mother's own and donor HM. Alternatively, bioactive compounds known as polyamines may guide the intestinal maturation of newborns and therefore could be investigated in combination with donor HM as an alternative feeding strategy when the mother's own milk is not available. (Plaza-Zamora et al., 2013).

Thus, this project aims to understand the influence of specific perinatal factors, including gestational age and term equivalent age on the HMM configuration of Argentinian mothers. In addition, we aim to assess the impact of cold storage, and fortification of donor HM, and the presence of polyamines on the adhesion properties of specific probiotics.

2 Review of the Literature

2.1 Common Problems related to Preterm Birth

Preterm birth is divided into three categories based on gestational age at birth: extremely preterm (<28 weeks), very preterm (28-<32 weeks) and moderate to late preterm (32-37 weeks) groups (Ohuma et al., 2023). Neonates in these gestational age categories are characterized by a varying degree of immaturity in several organ systems including respiratory, central nervous system, gastrointestinal tract, and immunologic function. The level of physiological immaturity is reflected in disease risk and need for postnatal medical and nutritional care. Neonates born less than 1500 g are defined as very low birth weight (VLBW) infants, and the vast majority of them are born prematurely.

Prematurity can have profound long-term health consequences and may contribute to the development of childhood and adolescent obesity. A recent meta-analysis of four studies involving 156,439 infants confirmed this hypothesis, indicating that preterm birth increases the risk of childhood obesity in these infants between the ages of six and sixteen when compared to those born full term (Ou-Yang et al., 2020). Prematurity, but also accelerated weight gain in preterm neonates, may increase the risk of obesity later in life. Indeed, a recent animal study found that preterm newborn pigs gained weight faster than their term counterparts (Adjerid et al., 2021). Research also shows that preterm infants who survive the neonatal period have a higher risk of respiratory and digestive issues, sight and hearing problems, as well as learning difficulties (M. C. Sullivan et al., 2012). Health issues linked to preterm birth or VLBW are also extending into adulthood. Indeed, prematurity combined with VLBW can predispose newborns to noncommunicable diseases such as cardiovascular disease and asthma later in life (Arroyas et al., 2020; Crump et al., 2019; Lewandowski et al., 2020). A recent cohort study including 6 million individuals from four Nordic nations (Finland, Sweden, Norway, and Denmark) assessed the effect of preterm birth in premature adult death caused by non-communicable diseases (Risnes et al., 2021). Data analysis showed that young adults who were born preterm had an increased risk of dying from cardiovascular diseases and diabetes.

However, not all prematurely born infants make it through the neonatal period (the first 28 days of life). Although the mortality rate for children under the age of

five has decreased dramatically over the last three decades, significant progress remains to be made (Perin et al., 2021). In fact, an estimated 5 million children under the age of five lost their lives in 2020, with nearly half (47%) dying during the neonatal period (UN IGME, 2021). The risk of death during the neonatal period is significantly higher in low-income countries than in high-income countries (UN IGME, 2022). Newborns born in Sub-Saharan Africa and Southern Asia, in particular, have a lower chance of surviving past the first month following birth. Preterm birth complications, neonatal sepsis, and lower respiratory infections are among the leading causes of neonatal deaths (UN IGME, 2019).

2.1.1 The Neonatal Intestinal Dysbiosis

Neonatal intestinal dysbiosis in preterm infants refers to an imbalance in the composition of the gut microbiota (GM), where there is a disruption in the normal, healthy balance of microbial species in the intestine. This condition is particularly concerning in preterm infants due to their underdeveloped immune system and gastrointestinal tract, which make them more susceptible to adverse health outcomes.

The metabolic barrier integrity is one of the functions directly influenced by the composition and diversity of the GM (Butel, 2014). In healthy infants, the GM comprises a diverse array of microorganisms that play critical roles in digestion and regulate the intestinal immune system. The composition of the human GM is primarily characterized by the presence of four phyla: *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* (Bilen et al., 2018).

However, in preterm infants, this balance can be disrupted, leading to an overgrowth of potentially harmful bacteria and a decrease in beneficial bacteria. This imbalance in the GM can have several consequences for preterm infants, such as an increased risk of necrotizing enterocolitis (NEC), a severe and potentially life-threatening intestinal disease (Neu, 2020). It can also contribute to the development of late-onset sepsis, a significant infection that occurs after the first week of life. Additionally, there is growing evidence that dysbiosis may impact long-term health outcomes, including the development of allergies, asthma, and other immune-related conditions (Hufnagl et al., 2020; Mezouar et al., 2018).

Dysbiosis in preterm infants is characterized by low bacterial diversity and an increased relative abundance of *Enterobacteriaceae*, which precedes the development of NEC and postnatal growth failure (Pammi et al., 2017). Indeed, the GM of preterm infants is characterised by a higher abundance of Enterobacteriaceae, enterococci, and staphylococci, and a lower abundance of bifidobacteria and lactobacilli (Xiang et al., 2023). This could mean that dysbiosis in preterm neonates is associated with lower prevalence of beneficial bacteria and higher prevalence of potentially pathogenic bacteria. Factors contributing to dysbiosis in preterm infants

include birth before reaching term gestational age, the underdevelopment of the neonatal gut and immune system, the use of antibiotics, and the type of feeding (human milk versus formula) (Arboleya et al., 2022).

2.2 Potential Clinical Intervention Strategies

Low birth weight and infections prolong the hospitalization of preterm neonates, imposing a significant financial burden on society. In fact, annual spending on preterm neonates in Finland reaches EUR 30 million while the United States spends $26.2 billion per year (Behrman et al., 2007; Terveyden Ja Hyvinvainnin Laitos, 2018). The costs associated with preterm birth are inversely related to gestational age. According to a cohort study including 11 European countries, children born at <28 weeks of gestation have significantly higher care costs than preterm neonates born later, even up to 5 years of age (S. W. Kim et al., 2021). Nonetheless, negative outcomes associated with premature birth could be addressed through infant nutrition, which is a critical piece of the infant mortality puzzle. Nutritional interventions for preterm neonates, in particular, aim to achieve postnatal growth comparable to that of the infant while still in utero. These include exclusive breastfeeding, probiotic administration, and other promising strategies discussed in detail in the following chapters.

2.2.1 Human Milk

2.2.1.1 Protective Functions

Multiple studies have proposed HM as a key mediator for reducing the incidence of NEC in preterm neonates (Gomart et al., 2021; Lapidaire et al., 2021; Savarino et al., 2021). When it comes to which type of infant feeding is optimal, evidence from clinical trials clearly favors the use of HM over infant formula in extremely preterm newborns. Indeed, preterm neonates following an infant formula diet develop NEC and/or late onset sepsis (LOS) significantly more often than those exclusively fed with HM (Schanler et al., 2005; S. Sullivan et al., 2010). Complementary to that, a recent systematic review and meta-analysis of six randomized trials (1472 neonates) and 43 observational studies (14950 neonates) showed that the provision of HM to preterm and/or VLBW neonates results in a lower incidence of NEC and LOS compared to formula feeding (Miller et al., 2018). Later, Altobelli et al. conducted a meta-analysis of six randomized controlled trials (1626 neonates) and 18 observational studies (6405 neonates) to understand the occurrence of NEC among breastfed and formula-fed neonates, confirming Miller and colleagues' previous findings (Altobelli et al., 2020). Moreover, in their recent work Morais and

colleagues demonstrated a significant increase in fecal alkaline phosphatase activity of very preterm neonates fed with HM compared to those fed with formula (Morais et al., 2021). Higher activity of this particular enzyme was previously reported to protect against NEC in mouse models (Rader, 2017).

Preterm infants are known to have an immature immune system, leaving them vulnerable to potential microbial infections. So, providing HM could be beneficial for infants born preterm from an immunological perspective. For example, immunoglobulins (Igs) present in HM are more stable in the preterm stomach than the term stomach. By surviving digestion, Igs could enhance the immature immune function of the preterm infant and provide additional protection against pathogens (Demers-Mathieu et al., 2018). In fact, recent evidence does not only underline the role of HM IgA in protecting against NEC development, but also proves that this bioactive component plays a crucial part in modulating the GM composition in preterm neonates (Gopalakrishna et al., 2019). Although the development of the infant GM is affected by multiple determinants, mentioned in the Neonatal Intestinal Dysbiosis chapter (2.1.1.), infant diet is singled out as the greatest contributor (Galazzo et al., 2020). Therefore, HM may also play a significant role in the microbial colonization of the infant gut. To support this hypothesis, Pärnänen and colleagues investigated the impact of HM and infant formula feeding on the infant GM configuration (K. M. M. Pärnänen et al., 2021). Findings from this study indicate that formula feeding does not only result in a higher load of opportunistic pathogens in the gut, but also contributes to increased amounts of antibiotic resistance genes. Increased resistance to antibiotics could be detrimental for infants with severe infections. Conversely, the load of beneficial bacteria including *Bifidobacterium* and *Akkermansia muciniphila* is higher in the gut of neonates fed with HM indicating that formula feeding delays intestinal bacterial colonization and possibly intestinal maturation (K. M. M. Pärnänen et al., 2021; Rader, 2017). Altogether, it is evident that HM, besides protecting against NEC and LOS, contributes to the maturation of the immune system and the establishment of a beneficial infant GM and ensures an optimal infant neurodevelopment (Boquien, 2018; Chiurazzi et al., 2021).

Due to all these protective and biological functions, many scientists have collectively released statements recommending HM as the optimal feeding for all premature and VLBW neonates (Arslanoglu et al., 2019; Committee on Nutrition et al., 2017; Moro et al., 2015). Although it is not yet clear what confers this protection, it is likely that multiple compounds present in HM could simultaneously contribute to these beneficial effects.

2.2.1.2 Composition

The milk composition is unique and distinct across mammalian species, differs substantially between mothers and changes according to the lactation stage (Boquien, 2018). It is hypothesized that the temporal variations in the HM composition occur to adjust to the growing infant's nutritional needs. As lactation progresses the HM composition converts from an immune-rich to a nutrient-rich source (S. Y. Kim & Yi, 2020). Therefore, HM is divided in three lactation stages according to its composition: colostrum (0-5 days postpartum), transitional milk (6-15 days postpartum), and mature milk (>16 days postpartum) (Floris et al., 2020).

Colostrum, is rich in antibodies, immune factors, and growth hormones, compounds that could protect against infections and assist in the postnatal development of the newborn (Godhia & Patel, 2013). Transitional milk on the other hand, is characterized by reduced levels of antibodies compared to colostrum, has higher fat content and is more caloric dense than colostrum (Samuel et al., 2020). Finally, mature milk is having an altered but stable composition compared to the one of colostrum and transitional milk (Ballard & Morrow, 2013). Besides lactation stages, the composition of HM can vary even during a single feeding. Indeed, marked differences have been observed between foremilk (start of feeding) and hindmilk (end of feeding), with the former having lower fat and vitamins A and E content than the later (Nielsen et al., 2017). The different constituents of HM, from nutrients to bioactive compounds and microbes, are discussed in the following subchapters and are summarized in Figure 1.

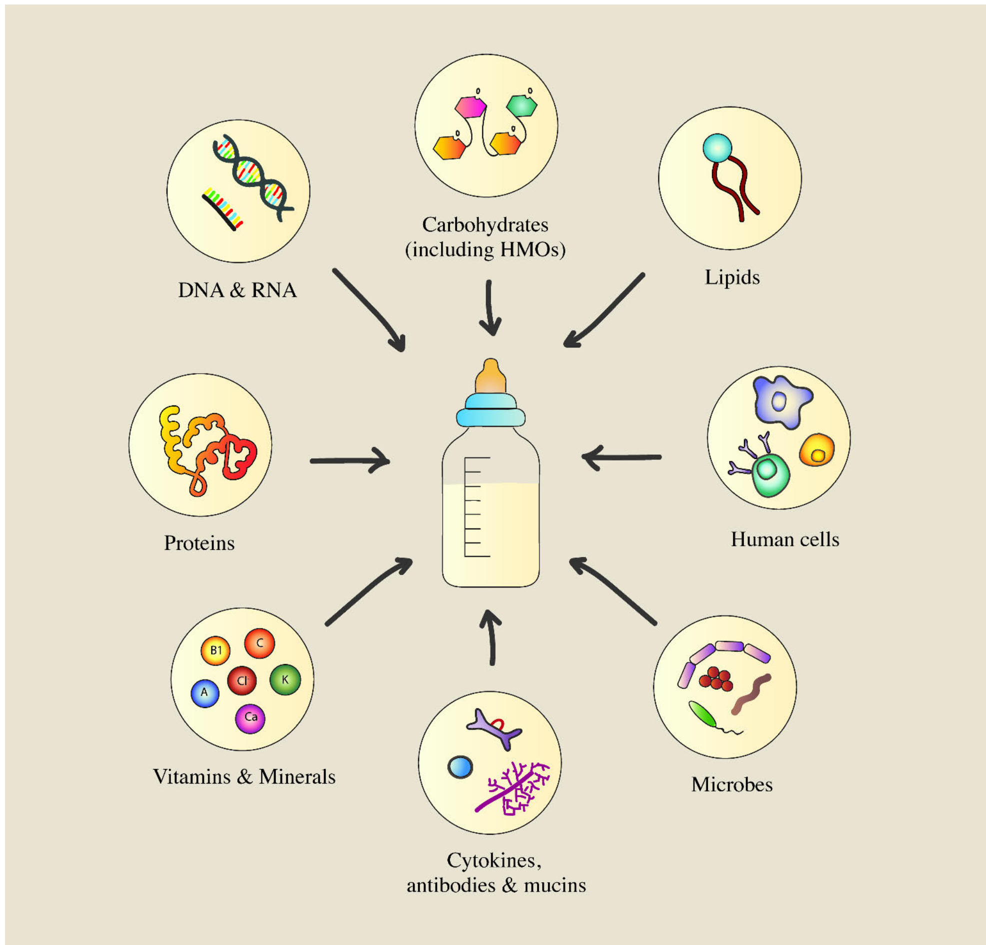


Figure 1. Nutrients, bioactive factors, and cells of human milk. Own drawing, adapted from (Mantziari, 2016).

2.2.1.2.1 Nutrients

In HM, the primary energy sources for the newborn infant are fat, followed by carbohydrates. The milk fat content varies over the day, changes throughout lactation and is affected by maternal genetic factors, diet, and gestational age (Italianer et al., 2020; H. Lee et al., 2018). In fact, changes in HM fatty acid composition can be achieved within a short period in time by changing dietary fat content (Nasser et al., 2010). Fat in HM is comprised of a complex mixture of different lipids encapsulated in a milk fat globule membrane (MFGM). The MFGM has received much attention in recent years due to its ability to impact on infant metabolism and offer protection from pathogenic bacteria (Brink & Lönnerdal, 2020). The milk fat globule is also rich in polyunsaturated fatty acids (PUFAs), including docosahexaenoic acid,

required for optimal brain growth and development (Wei et al., 2019). The proportion of docosahexaenoic acid in HM can be modified by changing the diet. For example, docosahexaenoic acid was about 2.5 times higher in the milk of mothers following a low fat diet compared to those following a high fat diet (Nasser et al., 2010).

The second major energy source for the infant is carbohydrates in the form of lactose. Lactose represents approximately 40% of HM's energy content, and its concentration correlates positively with milk yield (Nommsen et al., 1991). Like lactose, human milk oligosaccharides (HMOs) consist of glucose and galactose but also contain fucose, sialic acid, and N-acetylglucosamine (Bode et al., 2016). However, although the HMO composition is relatively stable throughout the lactation period, it is unique to each mother, depends on secretor status, and varies depending on maternal pre-pregnancy body mass index (BMI) (Lagström et al., 2020). Interestingly, these compounds are also included as added ingredients in infant formulas due to the number of biological functions that they offer. Among them is their ability to prevent pathogen adhesion to the intestinal epithelium or suppress their growth (Ackerman et al., 2017; Monteagudo-Mera et al., 2019). In addition, HMOs can shape the infant GM through their metabolism by specific bacteria in the gut that in turn produce short-chain fatty acids (SCFAs), thus favoring the growth of beneficial bacteria against pathogens (M. Li et al., 2014). A recent study has also suggested that a specific HMO could be a potential biomarker to predict NEC in preterm neonates (Masi et al., 2020).

HM is rich in a diverse mixture of proteins that differ in composition and structure from those of other species' milk. Moreover, their concentration depends not only on gestational age (preterm milk has a higher protein content than term milk) but also changes during the course of lactation to reflect the developmental needs of the growing infant (Gidrewicz & Fenton, 2014). Caseins and whey are the main HM proteins known to offer a variety of bioactive functions. Caseins may protect the infant against pathogen infection and bind minerals, thus enhancing their bioavailability, while whey proteins like α-Lactalbumin demonstrate antimicrobial activity and may regulate the immune system after their digestion (Layman et al., 2018; Pellegrini et al., 1999).

Vitamins and minerals are also present in HM and play an essential role in infant development. For example, B vitamins facilitate the maintenance of nervous system functions and play an essential role in glucose, amino acid, and fatty acid metabolism (Calderón-Ospina & Nava-Mesa, 2019). On the other hand, vitamin A is associated with rapid postnatal growth (Soares et al., 2019). Finally, vitamin D is essential for bone development as it regulates the absorption of two minerals also present in HM: calcium and phosphorus (Rigo et al., 2018). However, calcium and phosphorus should be included as a supplement in preterm infant nutrition to avoid the

development of rickets and supplementation with vitamin D is necessary for all infants (Abrams, 2020). Other essential minerals of HM include iron and zinc that are involved in various biological functions. Iron, for instance, is an essential element of several proteins, including hemoglobin which is involved in oxygen transport, and its deficiency has been associated with anemia (Tong & Vichinsky, 2021). Similarly, zinc is known for its role in the metabolism of nutrients, and its deficiency is linked with delayed growth and immune system dysfunction (S.-G. Park et al., 2017).

2.2.1.2.2 Bioactive Factors and Human Cells

The benefits of HM go beyond just providing essential nutrients to the newborn and extend to immunomodulatory and antimicrobial functions specifically relevant for the preterm neonate who is characterized by a naïve immune system. Among the most important immunological and functional compounds are Igs, growth factors, cytokines, mucins, polyamines, and milk extracellular vesicles (EVs). Immunoglobulins like IgA, IgG, IgM, and their isotypes can block the adhesion of pathogens to intestinal epithelial cells and modulate the function of dendritic cells (DC) (Corthesy, 2013). Epidermal growth factor (EGF) is a major growth factor of milk and can stimulate the growth of the gastrointestinal (GI) tract, as illustrated by Sullivan et al., who found that EGF intravenous administration in premature neonates with severe NEC resulted in faster mucosal repair (P. B. Sullivan et al., 2007). Interestingly, greater concentrations of EGF were detected in the milk of mothers who delivered preterm versus term neonates (Jakaitis & Denning, 2014; Oguchi et al., 1997).

Cytokines are proteins involved in cell signaling, interacting with cells influencing the infant's immune responses by either decreasing or increasing inflammation. The most prevalent cytokines in HM are interleukins (IL) IL-1β, IL-6, IL-8, IL-10, and tumor necrosis factor-α (Ballard & Morrow, 2013). Mucins are glycosylated proteins with many functions, including bacterial and viral infection prevention (Linden et al., 2008). On the other hand, polyamines are non-protein nitrogen compounds secreted in HM known for their immunomodulatory effects and significance in neonatal gut tissue development (Ojo-Okunola et al., 2020). Milk EVs are protein-, lipid-, and nucleic acid-containing structures secreted by cells (Matei et al., 2019). Milk EVs are the current focus of HM bioactives research due to their promising functional effects (Y. He et al., 2021; Yi & Kim, 2021). Most importantly, recent evidence from preclinical studies illustrates their potency in reducing the incidence of NEC or reducing its severity (Besner et al., 2020; S. He et al., 2021; Miyake et al., 2020; X. Wang et al., 2019). Lastly, cell-free DNA and microRNA have also been detected in HM and could be involved in cellular growth, and differentiation (Song et al., 2020; Tingö et al., 2021)

Aside from nutrients and bioactive compounds, HM is enriched with human immune, epithelial, and stem cells. Providing immune cells like leukocytes to newborns through milk is essential as neonates have little antigen exposure. Interestingly, when the infant or its mother becomes infected, the concentration of leukocytes in HM increases and returns to initial levels after recovery (Hassiotou et al. 2013). On the other hand, stem cells present in HM may assist in the development of the infant through their migration and differentiation in different organs (Hassiotou et al., 2014).

Preterm infants have immune systems characterized by developmental immaturities. As previously discussed, this predisposes them to severe infections compared to term infants but following a feeding regime of HM instead of infant formula gives them a protective advantage against NEC development. A potential mechanism behind this finding is that some beneficial HM bacteria may colonize the infant gut, assisting in developing the immune system and thus providing protection against infections (Gritz & Bhandari, 2015).

2.2.1.2.3 The Human Milk Microbiota

Potential Origins

Twenty years ago, when HM research was still in its infancy, scientists argued that HM is sterile and instead, the presence of bacteria indicates maternal infection. Indeed, maternal infections like mastitis result in the overall increase of specific bacteria like *Staphylococcus aureus* in HM (Marín et al., 2017). However, as HM research progressed, the view of sterility was challenged. Various studies have since found viable bacteria in the milk of healthy women free from infections, and thus the current most popular opinion is that HM is a source of several hundred microbes (Cabrera-Rubio et al., 2012; M. C. Collado et al., 2009; Gueimonde et al., 2007; Heikkilä & Saris, 2003).

But how did these microbes end up in HM? Research has led to three hypotheses that could also hold true concurrently: the resident mammary gland microbiota, the retrograde inoculation pathway, and the enteromammary pathway. The exact mechanisms, however, are yet to be elucidated (Gueimonde et al., 2007). Several observational studies in non-lactating women have suggested commensal bacteria of the mammary gland as factors that play a role in the HMM composition (Klann et al., 2020; Urbaniak et al., 2014). Additionally, HM and breast tissue of healthy lactating mothers appear to share specific microbes including *Cutibacterium acnes*, *Staphylococcus epidermidis*, and *Streptococcus agalactiae* in their breast tissue (Togo et al., 2019). Nevertheless, more studies are required to elucidate the role of mammary gland bacteria in the microbial composition of the HM ecosystem.

Besides the hypothesis of a commensal mammary microbiota, retrograde inoculation is another logical source of microbes since, during delivery, the infant is exposed to either vaginal or maternal skin microbes (Rodriguez, 2014). These microbes could potentially colonize the infant's mouth, which comes in contact with the nipple through breastfeeding. Additional evidence to substantiate this hypothesis is derived from the observation that microorganisms detected in HM are also identified in maternal skin (*Staphylococcus*, *Corynebacterium*, *Propionibacterium*) and the infant oral cavity (*Streptococcus*, *Veillonella*, *Variovorax*) (J.-E. Lee & Kim, 2019; Murphy et al., 2017; Togo et al., 2019). In a more recent work, Kordy et al. explored the microbial composition of HM employing SourceTracker analysis, a tool used to predict the source of microbes from a given habitat (Kordy et al., 2020). Interestingly, their results indicated that the presence of specific bacteria in HM does not only originate from the maternal areola skin but also the infant's mouth. The microbial composition of HM, maternal, and infant GM from 20 mother-infant dyads was compared in the same study. As a surprise, a specific *Bifidobacterium breve* strain detected in the milk of one mother who delivered by cesarean section was also found in the maternal and infant gut. Similarly, a recent report from China confirmed a co-occurrence of seven bifidobacterial phylotypes, including *Bifidobacterium breve*, across the same three niches in 23 mother-infant pairs (Yan et al., 2021). As bifidobacteria are generally strictly anaerobic (certain species are aerotolerant), this does not only point out a vertical transmission of specific bacteria to the infant's gut through HM but also implies the existence of another HMM seeding pathway beside the mammary microbiota and the retrograde inoculation theories. In fact, complementary findings suggest that bacteria commonly colonizing the oral cavity are preceding delivery, as they were identified in precolostrum samples (Ruiz et al., 2019).

Indeed, scientists have speculated the existence of an enteromammary pathway (Figure 2) where maternal intestinal microbes are trapped inside DCs or macrophages and translocated to the mammary glands (Selvamani et al., 2021). Given the hormonal changes and the increased intestinal permeability of late pregnancy, immune cells may be introducing openings between the tight junctions of the intestinal epithelium (Selma-Royo, García-Mantrana, et al., 2021). There immune cells could sample microbes, carry them through the lymphatic and blood circulation to the mammary ducts and secrete them in the milk (Rodríguez et al., 2021). Current knowledge indicates that besides influencing the infant GM composition after birth, HMOs are detected in amniotic fluid, suggesting a fetal exposure to these compounds already during gestation (Wise et al., 2018). Additional evidence argues that prenatal HMOs are detected in maternal serum during pregnancy (Hirschmugl et al., 2019; Jantscher-Krenn et al., 2019). Assuming the enteromammary pathway hypothesis is true, specific HMOs might reach the

maternal GI tract through the bloodstream and modulate the selection of bacteria to be transferred to the mammary gland.

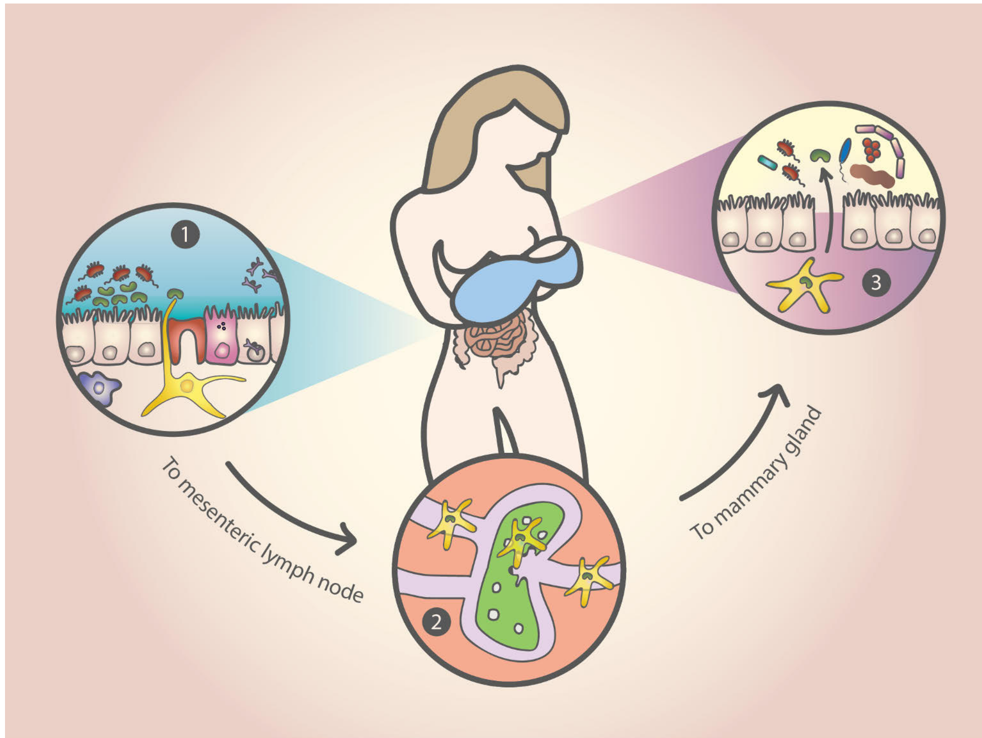


Figure 2. Origins of Human Milk Microbiota: Enteromammary pathway hypothesis. Own drawing, adapted from (Mantziari, 2016).

Composition

Since 2003, many scientists have attempted to identify which microorganisms are present in HM and what function they serve (Fernández et al., 2020). The composition of the HMM was first accessed by culturing HM in appropriate growth media, a method that enables the detection of viable and culturable microorganisms in HM. New molecular technologies, such as polymerase chain reaction (PCR), have been developed and integrated into HMM research over the years. Indeed, since the late 2000s, new culture-independent techniques like quantitative polymerase chain reaction (qPCR) and next-generation sequencing (NGS) have begun to replace traditional culturing methods although culturing gives information on the viability of bacteria in HM. According to culture-independent studies, such methods can detect dormant or unculturable microorganisms (Bodor et al., 2020). Furthermore, they are more sensitive than culture-dependent methods and have made clear that

HMM diversity is greater than previously anticipated. Given the distinct advantages of both methodologies, their simultaneous implementation for the accurate assessment of HMM profiles, seems well-grounded. In this context, a recent systematic review including 9183 lactating women demonstrated that 26% of the detected species were shared between culture-dependent and culture-independent approaches while 62% and 12% were solely identified by molecular methods and traditional culturing, respectively (Togo et al., 2019).

Despite these variations, both analytical approaches suggest that microbes belonging to 7-9 taxa are constituting the "core HMM" (Hunt et al., 2011; Jiménez et al., 2015). The most prevalent and consistently identified genera in HM are *Staphylococcus* and *Streptococcus* (Fernández et al., 2020). However, further investigations revealed that HM is a source of beneficial aerobic and anaerobic microbes including species belonging to *Bifidobacterium* and *Lactobacillus* genera (N. Li et al., 2020; Murphy et al., 2017; Olivares et al., 2015; Urbaniak et al., 2016). As previously discussed in the HMM Potential Origins subchapter, a few genera in HM are also part of the skin microbiota (i.e., *Propionibacterium*, *Cutibacterium*, and *Corynebacterium*) or the GM (i.e., *Acinetobacter*, *Bacteroides*, *Blautia*, *Clostridium*, *Dorea*, *Enterococcus*, *Escherichia*, *Mucispirillum*, *Pseudomonas*, *Rothia*, Salmonella, *Serratia*, *Shigella*, and *Veillonella*) (Togo et al., 2019; Zimmermann & Curtis, 2020). However, bacteria are not the only microbial agents in HM. In fact, evidence from observational studies in healthy mothers showed a great abundance of viruses (Myoviridae, Podoviridae, Siphoviridae, Phycodnaviridae, Iridoviridae, and Imiviridae), fungi (Malassezia, Candida, Saccharomyces, Rhodotorula, Davidiella, Sistotrema, and Penicillium), archaea (Haloarcula, Halorhabdus, and Halomicrobium), and protozoa (Giardia and Toxoplasma) in their milk (Boix-Amorós et al., 2017, 2019; Jiménez et al., 2015; Pannaraj et al., 2018). Finally, the microbial content of HM is extremely variable from one mother to another and is influenced by several factors discussed in detail in the following chapters.

Functions

HM is a biological fluid with lower bacterial biomass (between 10^3-10^5 CFU/mL) than other human sites. However, microorganisms present in HM could have beneficial implications for infant health. Some argue that the protective effect of HM against the incidence of NEC and LOS may be due to a combination of factors, as it contains a variety of bioactive compounds like HMOs and microbes (Murphy et al., 2021). This implies that the HMM could be another explanation as to why HM can protect VLBW neonates from NEC. In fact, preterm infants fed with mother's own milk (MOM) rather than infant formula, which lacks the diverse bacterial communities found in HM, are less likely to develop NEC (T. J. Johnson et al.,

2015). Moreover, Altobelli and colleagues found that only the combination of mother's own milk and pasteurized donor HM (DHM) significantly reduces the risk of NEC in preterm neonates (Altobelli et al., 2020). DHM refers to HM from different lactation stages, expressed by mothers who delivered at term and produced HM in excess, which was pooled, pasteurized, and frozen. This decrease could be attributed to the restoration of microbial content in pasteurized DHM following supplementation with the mother's own preterm milk (Mallardi et al., 2021).

Overall, the protection against NEC may be mediated by the altered GM profiles and increased GM richness and diversity observed in preterm infants who breastfed compared to those who received formula (Cong et al., 2017; Z. Wang et al., 2020; Zanella et al., 2019). More specifically, breastfeeding has been linked to a higher abundance of beneficial bacteria like *Bifidobacterium* and *Lactobacillus* in the infant gut compared to formula feeding (Harmsen et al., 2000). Moreover, the relative abundance of these genera is lower in the feces of infants suffering from NEC than in their healthy counterparts (Ellaby, 2018; Stewart et al., 2016). Consequently, it is possible that the HMM plays an important role in seeding the infant gut, providing protection against infections (Gritz & Bhandari, 2015; Pannaraj et al., 2017). For instance, preliminary evidence from *in vitro* experiments suggests that specific *Lactobacillus* strains isolated from HM can suppress the growth of gastrointestinal nosocomial pathogens (Jara et al., 2011)

HM is indeed recognized as a major selective factor in shaping the composition of the infant GM as many microbes found in HM are also detected in infant feces (Corona-Cervantes et al., 2020; K. Pärnänen et al., 2018). This was also demonstrated in a recent study on healthy mothers and their respective infants, which reported a significant association between specific microbial clusters of HM and infant GM, suggesting that microbes in HM may play an essential role in infant GM composition (Pace et al., 2021). In addition, HM bacteria like *Staphylococcus*, *Faecalibacterium*, *Akkermansia, Bifidobacterium*, and *Lactobacillus* have also been isolated from infant feces. In fact, infants who breastfeed have a higher prevalence of *Staphylococcus* in their feces than in formula-fed ones (Balmer & Wharton, 1989). Members of this genus utilize oxygen while growing in the gut. This is necessary for the proliferation of beneficial members of the HMM and infant GM like *Faecalibacterium*, *Akkermansia, Bifidobacterium*, and *Lactobacillus* in the gut who are known to generate short-chain fatty acids as an end product of their metabolic activity (LeBlanc et al., 2017).

The subsequent production of short-chain fatty acids in the gut could reduce the risk of dysbiosis-related diseases like NEC. For instance, *Faecalibacterium prausnitzii* produces butyrate and thus could modulate inflammation while *Akkermansia muciniphila* and *Bifidobacterium* produce acetate, which may help prevent pathogenic infection (Belzer & de Vos, 2012; Martín et al., 2018). Since

the relative abundance of *Akkermansia*, *Bifidobacterium* and *Lactobacillus* is lower in the feces of NEC infants than in their healthy counterparts (Ellaby, 2018; Stewart et al., 2016; Tarracchini et al., 2021), HM, rather than formula, could be a source of these bacteria for this vulnerable population. Indeed, breastfeeding has been associated with a higher abundance of *Bifidobacterium* and *Lactobacillus* in the infant gut (Boudry et al., 2021). However, not all studies have identified bifidobacteria in HM while their relative abundance is low (Yan et al., 2021) and this may also impact their prevalence in the infant gut. In fact, recent evidence suggests that certain species of *Akkermansia*, *Bifidobacterium* and *Lactobacillus* can utilize HMOs, which could improve their survival and enhance their persistence in the neonatal gut (Kostopoulos et al., 2020; Thongaram et al., 2017a). By colonizing the gut, HM-related bacteria can protect the host by interfering with pathogen adhesion to the intestinal mucus and therefore suppress or inhibit their growth (García-Ricobaraza et al., 2021).

Finally, in addition to their beneficial role in infant health, several members of the *Bifidobacterium* and *Lactobacillus* genera in HM are significantly associated with the absence of breast infections in lactating mothers suggesting a protective effect against mastitis (Togo et al., 2019; Urbaniak et al., 2016). Nonetheless, the microbial content of HM varies greatly from one mother to another (Asbury et al., 2020) and is influenced by a number of factors (Figure 3), which are discussed in detail in the following chapter.

What Defines the Human Milk Microbiota Composition?

Figure 3. Factors driving the Human Milk Composition. Own drawing, adapted from (Mantziari & Rautava, 2021)

Maternal Health Status

Gestational prehypertensive status and maternal obesity contribute to lower microbial diversity and changes in the configuration of the HMM. Gestational prehypertensive status is responsible for a lower relative abundance of *Lactobacillus* in HM (Wan et al., 2020). *Staphylococcus*, *Corynebacterium*, and *Brevundimonas* are elevated in the milk of obese mothers compared to overweight or healthy-weight mothers (LeMay-Nedjelski, Butcher, et al., 2020). In addition, overweight and obese mothers produce milk with higher levels of *Staphylococcus*, *Akkermansia*, and *Granulicatella* and lower *Bifidobacterium* counts than normal-weight women (Cabrera-Rubio et al., 2012; M. C. Collado, Laitinen, et al., 2012; Williams, Carrothers, et al., 2017). Similarly, a lower abundance of *Bifidobacterium* was found in the milk of mothers with allergy or celiac disease than in healthy mothers (Grönlund et al., 2007; Olivares et al., 2015).

Maternal postnatal distress can also contribute to a less diverse HMM and is associated with its altered composition (Browne et al., 2019). Indeed, high

psychosocial distress leads to a significant decrease in the relative abundance of *Staphylococcus* in HM, while low psychosocial distress significantly increases the relative abundance of *Lactobacillus*, *Acinetobacter*, and *Flavobacterium*. Doing the month is a 20–30-day postpartum confinement program typical in Asian cultures. This practice uniquely combines dietary and herbal therapies and is associated with fewer postpartum physical and depressive symptoms in mothers. Adhesion to this program led to a higher prevalence of *Lactobacillus*, *Bifidobacterium*, and Archaea in the HM (Chen et al., 2020; Chien et al., 2006).

Maternal infections, including lactational mastitis and breast abscess (which may be developed as a complication of mastitis), are significant modulators of the HMM. Both conditions have been associated with an overall lower bacterial diversity, the dominance of *Staphylococcus aureus* (often the cause of mastitis), and a complete absence of *Bifidobacterium breve* in the milk of infected mothers (Jiménez et al., 2015; Togo et al., 2019). Most importantly, the absence of both mastitis and breast abscess correlates with several bifidobacteria and lactobacilli in HM. Lower diversity, increased bacterial loads, and altered composition were also confirmed in HM of mothers with sub-acute mastitis (Boix-Amorós et al., 2020). Viral infections have also been linked to changes in HMM composition (Almand et al., 2017; Domínguez-Díaz et al., 2019). However, conversely to mastitis infected mothers, HIV-infected mothers were found to have a more diverse HMM characterized by higher counts of *Lactobacillus* and decreased abundance of *Staphylococcus* (González et al., 2013).

Antibiotics are a common strategy against maternal infections which may occur during pregnancy, perinatal period, or lactation. However, numerous reports indicate that the prophylactic use of antibiotics has significant effects on several HMM parameters. For instance, a recent study focused on the preterm mother's milk microbiota showed that mothers who received antibiotics perinatally had a less diverse HMM composition when compared to those never exposed to them (Asbury et al., 2020). Previous work in term milk has also shown that mothers who received antibiotics during delivery have an altered HMM composition but an increased bacterial diversity. At the same time, *Bifidobacterium* is only detected in the milk of those mothers who did not receive intrapartum antibiotics (Hermansson et al., 2019). Other groups have also confirmed a lower relative abundance of *Bifidobacterium* and *Lactobacillus* in the milk of mothers who received antibiotics during pregnancy or lactation (Padilha, Iaucci, et al., 2019; Soto et al., 2014).

Infant Sex, Delivery Mode, Feeding Mode, and Geographic Location

Contrasting findings have been presented for infant sex and mode of delivery as determining factors of the HMM composition. Previous studies reported a higher prevalence of *Rothia* (Moossavi, Sepehri, et al., 2019) and *Staphylococcus*

(Williams, Carrothers, et al., 2017) in the milk of mothers of female infants and a significant association between infant sex and HMM composition (M. R. Simpson et al., 2018a). Regarding delivery mode, the milk of mothers who delivered by cesarean section is less diverse and characterized by a lower relative abundance of *Bifidobacterium*, *Lactobacillus,* and *Leuconostocaceae* than the milk of mothers who delivered vaginally (Cabrera-Rubio et al., 2012; Hermansson et al., 2019; Khodayar-Pardo et al., 2014). Contrary to these findings, Urbaniak and colleagues did not observe any differences in the HMM profiles based on the sex of the infant and mode of delivery (Urbaniak et al., 2016). Different study designs in terms of sample size, collection, and analytical methods could cause the inconsistencies between the studies mentioned above.

A recent study led by Holdsworth and colleagues, has shown that breastfeeding patterns, specifically the total duration and frequency (breastfeeding sessions separated by >30 seconds) of breastfeeding, as well as the frequency of non-maternal physical contact, have an influence on the diversity and/or composition of the HMM (Holdsworth et al., 2023). In further detail, the study revealed a negative correlation between the frequency of breastfeeding sessions and non-maternal physical contact, and the richness of HMM. The group of mothers who breastfed for a shorter time exhibited the lowest abundance levels of *Bifidobacterium*.

It has also been shown that each mother harbors a unique microbial pattern in each breast (Tušar et al., 2014). However, not all mothers resolve to feed their newborn through direct breastfeeding, and those who decided to express their milk using a breast pump had a lower abundance of *Bifidobacterium* and a higher abundance of *Enterobacteriaceae* and *Pseudomonadaceae* (Moossavi, Sepehri, et al., 2019). Conversely, mothers who fed their infants through direct breastfeeding produced milk enriched in *Gemellaceae*, *Vogesella*, and *Nocardioides*. Moreover, using a breast pump instead of manual expression by hand results in a lower abundance of cultivable staphylococci and a higher content of bacterial DNA in milk (Treven et al., 2019).

The impact of geographic location on the HMM composition has been evidenced in multiple studies (Kumar et al., 2016; Lackey et al., 2019). *Bacteroidetes* dominated the milk of vaginally-delivering mothers from Spain compared to their Finnish, Chinese, and South African counterparts who delivered vaginally (Kumar et al., 2016). On the other hand, *Actinobacteria* were the most predominant phylum in milk produced by Chinese mothers who delivered by cesarean section compared to their European or African counterparts. On the genus level, *Bifidobacterium*, *Propionibacterium*, *Veillonella*, and *Serratia* were present in lower levels in the milk of Central African Republic mothers (Meehan et al., 2018) compared to US or Swiss mothers (Hunt et al., 2011; Jost et al., 2013). Similarly, HMM differences can be found among populations in rural and urban areas. Generally, residing in rural areas

results in a more diverse HMM composition (Järvinen, 2018) and may contribute to higher levels of *Lactobacillus* in the milk (Taghizadeh et al., 2015). These differences could stem from the distinct environmental exposures and dietary patterns of rural communities.

Lactation Stage and Gestational Age

Current evidence suggests that the HM composition changes with time according to the infant's immediate physiological needs and therefore is divided into three distinct stages: colostrum, transitional milk, and mature milk. The first stage, colostrum, is characterized by increased bacterial diversity with predominant bacteria belonging to the *Weisella*, *Leuconostoc*, *Staphylococcus*, *Streptococcus,* and *Lactococcus* genus (Cabrera-Rubio et al., 2012). As the lactation period progresses, total bacterial concentration increases while bacterial diversity decreases. The relative abundance of *Bifidobacterium*, *Enterococcus*, *Veillonella*, *Leptotrichia*, *Prevotella*, *Lactobacillus*, and *Staphylococcus* was increased in transitional and mature milk when compared to colostrum (Khodayar-Pardo et al., 2014). This variation across the lactation period can be partly explained by the retrograde inoculation pathway since an increased abundance of typical oral bacteria has been reported in milk from the later stages. Indeed, levels of oral bacteria in milk (mainly *Streptococcus* and *Rothia*) are on the rise after each breastfeeding session, accompanied by an increased bacterial diversity (Biagi et al., 2018).

Limited evidence shows conflicting results regarding gestational age and compositional changes of the HMM. Khodayar-Pardo et al. conducted an observational study, assessing the HMM composition of Spanish mothers with preterm and term gestations. The authors reported higher levels of *Enterococcus* and lower levels of *Bifidobacterium* in the milk of preterm-delivering mothers compared to those who delivered at term (Khodayar-Pardo et al., 2014). Moreover, understanding how the different subcategories of prematurity affect the HMM configuration is the first step for understanding whether the current practice, namely term DHM, is the best solution for feeding preterm infants in the Neonatal Intensive Care Unit (NICU). Interestingly, when examining the different degrees of prematurity, Khodayar-Pardo et al. detected an overall lower bacterial load in the milk of mothers of extreme preterm compared with those of late preterm infants in contrast to a later study which found no differences (Urbaniak et al., 2016). A more recent study by Moossavi and coworkers agreed with the findings of Urbaniak et al. (Moossavi, Sepehri, et al., 2019). However, the authors recruited mothers delivering at >35 weeks of gestation, thus excluding extremely preterm pregnancies from their study. The significant discrepancies between these reports underline the importance of controlling for other confounding factors which could affect the HMM configuration, including the nutritional content of HM.

Maternal Diet and HM nutrients

Generally, the effect of maternal diet on the nutritional content of HM has been scarcely explored (Rodríguez et al., 2021). Similarly, few research groups have investigated the impact of maternal diet on the HMM composition. Most recently, Seferovic and coworkers did not report any significant changes in the HMM profiles; however, maternal diet was found to profoundly influence the HMM function through changes in the HMO composition (Seferovic et al., 2020). HMOs, in turn, can induce changes in the HMM composition while their content in milk is dependent on the maternal secretor status. This was demonstrated by a group of Spanish scientists who found significant associations between specific HMOs and HMM guided by maternal secretor status (Cabrera-Rubio et al., 2019). In addition, *Bifidobacterium* was more frequently detected in the milk of secretor mothers. Significant associations between certain HMOs and the relative abundance of *Bifidobacterium* in milk have also been documented (Moossavi, Atakora, et al., 2019). Moreover, HMO-degrading bifidobacteria can enhance the growth of non-HMO-degrading bifidobacteria, at least *in vitro* (Lawson et al., 2020). This observation could potentially lead to their dominance in HM, affecting its microbial composition. Altogether, these findings could justify the higher bifidobacterial abundance observed in the gut of infants of secretor mothers (Lewis et al., 2015).

The impact of specific nutrient intake by the mother on her respective HMM profiles has been explored. A Brazilian study found a positive correlation between Vitamin C intake during pregnancy and *Staphylococcus* in the HM of full-term delivering mothers (Padilha, Danneskiold-Samsøe, et al., 2019). In the same study, the relative abundance of *Pseudomonas* in HM was increased after high Vitamin B9 intake and decreased after high sugar intake during the lactation period. *Bifidobacterium* and specific fatty acid (PUFAs and linoleic acid) intake during lactation were also positively associated. Interestingly, another study indicated that saturated fatty acid intake is negatively associated with *Corynebacterium* in the milk (Williams, Carrothers, et al., 2017). Dietary intake of specific nutrients affects their concentration in HM (Keikha et al., 2017). This finding could directly impact the HMM composition as many nutrients in HM could serve as growth substrates for bacteria. Indeed, saturated fatty acids in HM were negatively associated with *Streptococcus* and *Acinetobacter,* while the presence of *Bifidobacterium* and *Lactobacillus* was inversely related to monounsaturated fatty acids and n-3 PUFA milk phospholipids (Kumar et al., 2016). Greater protein concentration in HM was related to an increase in the levels of *Bacillus*, *Peptoniphilus*, and *Anaerococcus* (Boix-Amorós et al., 2016) and a decrease in the relative abundance of *Novosphingobium* (Williams, Price, et al., 2017). However, none of these studies looked for differences in the lactose, protein, and fat content of preterm HM or whether these relate to changes in the preterm HMM composition. Indeed, none of

the studies mentioned above provided gestational age information besides Padhila and coworkers who recruited mothers of term infants. Nevertheless, this information is essential for developing nutritional strategies specifically targeting the different categories of prematurity.

Accordingly, other milk constituents could modulate the bacterial taxonomic composition of HM. For example, concentrations of the polyamine putrescine in HM are correlated with the levels of *Gammaproteobacteria* and have a strongly negative correlation with other *Proteobacteria*, *Clostridia*, and *Actinobacteria* (Gómez-Gallego et al., 2017). The presence of other nutritional substances like nitrite and nitrate in HM has received little attention. These anions can be provided through diet or synthesized endogenously and are involved in various physiological processes, including stimulation of mucus secretion (Jansson et al., 2007) and protection against hypoxia and ischemia (L. Ma et al., 2018) among others. Moreover, preliminary evidence in mice shows that nitrite, a precursor of nitric oxide, could be considered as a preventive strategy against NEC (Yazji et al., 2013). Despite that, no reports have been available on the impact of nitrites/nitrates on the HMM composition itself.

Probiotics

External factors like probiotics (described in detail in the Probiotics chapter, 2.2.2) may have an impact on the HMM composition in HM. However, conflicting evidence has been presented. According to a few studies, while prenatal and postnatal maternal administration of specific probiotics can result in their presence in HM, it has no effect on the diversity and composition of HMM (Hermansson et al., 2019; M. R. Simpson et al., 2018b). Other probiotic strains, on the other hand, have been shown to effectively reduce the numbers of *S. aureus* in the milk of mothers with mastitis or increase the relative abundance of the lactobacilli and bifidobacteria (Jiménez et al., 2008; Mastromarino et al., 2015). This difference could be due to the ability of HM isolated probiotics to metabolize HMOs directly or due to other factors such as mode of delivery. Indeed, maternal probiotic supplementation in the late stages of pregnancy increased the concentration of certain HMOs in colostrum (Seppo et al., 2019). This modulation is likely to affect not only the HMM configuration, but also which bacteria are transferred from mother to infant and established in the newborn infant gut.

2.2.1.3 Human Milk Processes

2.2.1.3.1 Pasteurization and Cold Storage

Preterm neonates or infants born with a health condition that needs special care are admitted to the NICU. However, feeding infants in the NICU is quite different from

feeding healthy infants and remains an ongoing challenge. This is likely because preterm neonates have a weak suckling capability or their digestive system is still underdeveloped and immature (C. Lau, 2015). As discussed at the beginning of this chapter, HM is the best nutrition for the preterm infant; however, in some cases, MOM is not available, or insufficient to meet the nutritional needs of the newborn (A. L. Patel et al., 2020). This could occur because the mother is either receiving specific medication, fighting certain viral infections, physically far from the NICU, or producing low HM volumes (Davanzo, 2018). To overcome this challenge, many NICUs offer DHM either as the sole source of feeding or as a complement to MOM. The use of DHM in the NICU results in significantly lower hospital treatment costs for prematurity-related conditions (Carroll & Herrmann, 2013; Gephart & Newnam, 2019).

The collection, processing, and distribution of DHM is the responsibility of the HM bank, which then provides it to the NICUs. Most European HM banks follow the recommended thermal treatment for HM, which is Holder pasteurization (62.5°C for 30 minutes) to eliminate potential pathogens. The same applies to storage after pasteurization, where DHM in most HM banks is stored at temperatures ≤-18°C for up to 3-6 months. Nevertheless, a small percentage of European HM banks still store pasteurized DHM in the fridge (+4°C) for 24-72h (Kontopodi et al., 2021). Both processes have been found to profoundly impact the composition of specific HM nutrients and bioactives while others are preserved or even increased. For instance, the concentration and bioactivity of lactoferrin and lysozyme are both reduced to a certain extent in DHM (Binte Abu Bakar et al., 2021; Paulaviciene et al., 2020).

On the other hand, HMOs, some cytokines, and growth factors remain stable while free fatty acid, IL7, and IL-8 concentrations increase (Daniels et al., 2017; Peila et al., 2016; Riskin, 2020). Similarly, the storage of DHM may affect its composition. According to a study by Meng and coworkers, storing DHM at room temperature (RT) for 12h can significantly decrease the total protein content and secretory IgA activity (Meng et al., 2016). Alternatively, storage at 4°C for up to 7 days preserves the total protein content, lysozyme, and secretory IgA activity in DHM. A similar effect was observed for vitamin C in DHM that is stable during 6-day refrigeration (4°C) but significantly decreases when DHM is stored for a month at -20°C or -80°C (Abramovich et al., 2013). A significant decrease in total IgM has been reported after pasteurization and storage of HM at -20°C for an hour, although other immunoglobulins (secretory IgA, total IgA, and IgG) withstood these treatments (Demers-Mathieu et al., 2019). Finally, a recent study has shown that the use of fresh HM has an impact on the diversity of the infant GM composition and is associated with higher levels of *Lactobacillus* and *Bifidobacterium* than frozen HM (Merter & Altay, 2024). Despite the adverse effects of thermal process and cold storage, many studies present favorable results towards using DHM over formula in

the NICU (L. Fang et al., 2021). Indeed, a recent meta-analysis including high-quality randomized controlled trials showed incidence of NEC is lower in VLBW neonates receiving DHM as compared to those receiving preterm infant formula (Yang et al., 2020).

As pasteurization and cold storage might impact the composition of DHM, it would make sense to supplement it with specific probiotics that would partly restore its microbial content. Indeed, many NICUs will administer specific probiotics to preterm neonates due to their role in reducing NEC development (discussed in detail in chapter 2.2.2.1). Interestingly, evidence from an Australian NICU suggests that simultaneous introduction of DHM and probiotics in very preterm neonates can significantly reduce mortality and, although not significantly due to the small sample size but clinically relevant, reduce the incidence of NEC (Sharpe et al., 2018). In addition, a recent meta-analysis indicated that probiotics combined with an exclusive HM diet (either MOM or DHM) have resulted in a significant reduction of LOS in preterm neonates (Aceti et al., 2017). Probiotic powdered preparations are first mixed with MOM or DHM in the NICU milk kitchen before administration through a nasogastric tube or mouth (Robertson et al., 2020; Watkins et al., 2018). However, the suitability of DHM as a matrix candidate for the delivery of probiotics in preterm infants is not yet clear. This is relevant information when developing NICU nutritional regimes as the delivery matrix of probiotics can have a profound impact on probiotic characteristics, like viability and adhesion ability to the intestinal mucus, and therefore affect probiotic functionality and efficacy (Yoha et al. 2021).

2.2.1.3.2 Human Milk Fortification

An infant born prematurely is missing out on the nutrients passed *in utero* during the final weeks of pregnancy. While HM is the best possible feeding alternative, it is not adequate to meet the nutritional needs and growth requirements of these fragile neonates (Schulz & Wagner, 2021). In addition, Holder pasteurization can significantly reduce protein, lipid, and lactose content in DHM (Piemontese et al., 2019). For these reasons, HM is fortified with human milk fortifiers (HMFs) which are essentially protein and mineral supplements. The vast majority of commercially available fortifiers derive from bovine milk, contain different amounts of protein, carbohydrates, and minerals, and may be enriched with fatty acids or vitamins (Arslanoglu et al., 2019). Specifically, protein to energy intake ratio is crucial for the synthesis of new tissue, and supplementing HM with protein has previously led to short-term increases in weight gain, linear and head growth (Kuschel & Harding, 2000). Moreover, increased postnatal protein intake in extremely preterm infants was associated with higher white matter integrity in their brains (Hortensius et al., 2021) which could contribute to better neurodevelopmental outcomes (Feng et al., 2019).

Despite the benefits, some healthcare professionals and scientists have expressed their skepticism regarding the use of fortification in preterm infants due to its potential contribution to childhood obesity and positive association with NEC development (Lucas et al., 2020; Thoene et al., 2016; Villar et al., 2019). However, a recently published ten-year retrospective analysis in preterm infants demonstrated opposite effects (Jordan et al., 2021). Especially extremely premature infants had less risk of developing NEC if they were fed with HMF. While HMFs are available in two forms powdered (pHMF) and liquid (lHMF), the overall growth of VLBW neonates fed with fortified HM seems to be independent of the type of HMF used (Cibulskis & Armbrecht, 2015; Masoli et al., 2021). Nevertheless, liquid HMF is recommended over powder HMF due to safety concerns of pathogen transfer and infection or in those cases when the produced HM volume is limited (Pillai et al., 2018).

Given the different beneficial effects of fortified HM and probiotics, fortifying HM and enriching it with probiotics may be a good strategy for feeding neonates in the NICU, and thus worth exploring. However, there is still no information on the suitability of fortified HM as a carrier matrix for probiotics or whether the type of fortifier can influence specific probiotic properties. This should be defined as it could profoundly impact probiotic survival and extended function.

2.2.2 Probiotics

2.2.2.1 Normalization of Intestinal Microbiota Composition and Protective Functions

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002; Hill et al., 2014). Gram-positive bacteria, particularly strains of the *Lactobacillus* and *Bifidobacterium* genera, are the most commonly used probiotics in preterm neonates (Ayivi et al., 2020). More specifically, *Lacticaseibacillus rhamnosus* GG (LGG; previously known as *Lactobacillus rhamnosus* GG) and *Bifidobacterium animalis* subsp. *lactis* Bb12 (Bb12) are the two most studied probiotic strains in clinical trials (Dronkers et al., 2020).

Lactobacilli and bifidobacteria are common GM constituents of full-term breastfed infants (S. Wang et al., 2020) and their administration in preterm neonates may be able to reverse the altered GM composition (Alcon-Giner et al., 2020; Yousuf et al., 2020) and delayed gut colonization by bifidobacteria which is associated with formula feeding and antibiotic exposure (Senn et al., 2020). Indeed, administration of Bb12 to preterm neonates improved their GM composition as evidenced by the a higher levels of bifidobacteria and a lower levels of potentially pathogenic

enterobacteria in their feces (Mohan et al., 2006). Exposure to HM and *Bifidobacterium longum* subsp. *infantis* EVC001 for a month, resulted in persistent GM changes which lasted up to 1 month post intervention (Frese et al., 2017). A few of these changes include increased numbers of *Bifidobacteriaceae*, and a significant decrease in enteropathogens. A recent follow up study confirmed the increased *Bifidobacteriaceae* load in infants who received the probiotic and extended the effects on the gut colonization to a year (O'Brien et al., 2021). Moreover, *B. infantis* EVC001 supplementation silenced intestinal inflammation in newborn children (Henrick et al., 2020).

Probiotics have also surfaced as promising preventive strategies for preterm neonate complications, with a large number of randomized control trials (RCTs) and meta-analysis studies successfully demonstrating that probiotics can prevent or reduce the risk of NEC, LOS and overall mortality (Bi et al., 2019; Denkel et al., 2016; Dermyshi et al., 2017; Gray et al., 2020; C. S. M. Lau & Chamberlain, 2015). Considering these benefits many NICUs around the world are implementing the use probiotics in preterm infants some routinely and others occasionally. More specifically in 2016, 14% of the NICUs in the USA (Viswanathan et al., 2016) administer probiotics to preterm neonates while preliminary results focusing in European NICUs (mostly from France) report a use of 55% (Blanchetiere, 2021). On the other hand, recognized risk factors associated with probiotics belonging to the *Lactobacillus* and *Bifidobacterium* genera include bacteremia which may lead to neonatal sepsis (Bertelli et al., 2015; Kunz et al., 2004; Zbinden et al., 2015). Nevertheless, a few researchers have argued that it is preferable to administer probiotics proven to be safe and might confer a benefit to these vulnerable newborns than not administering anything at all (Szajewska et al., 2023; Usman et al., 2018).

To overcome safety concerns, researchers investigated which specific probiotic strains or their combination could have the same protective potential against NEC without the previously mentioned risks, resulting in the publication of a position paper (van den Akker et al., 2020). In this work, it became evident that not all probiotic strains are equally effective, and that only thoroughly tested and safe strains should be used. Most importantly only two preparations, LGG and a mix of *Bifidobacterium longum* subsp. *infantis* BB-02, Bb12 and *Streptococcus thermophilus* TH-4 appeared to reduce NEC.

However, there is still no consensus on whether a single strain or combination of probiotics would be more effective in reducing NEC or LOS in preterm infants. In a recent meta-analysis of 25 randomized controlled trials, Aceti at al evaluated the efficacy of probiotics in reducing LOS incidence in breastfeed VLBW preterm neonates. Surprisingly, the incidence of LOS was significantly lower in infants who received a probiotic mix compared to those who received a single strain (Aceti et al., 2017). Hui and colleagues found that preterm infants who received a combination of

LGG and Bb12 starting on the third day after birth had a lower risk of NEC and lower relative abundance of *Klebsiella* in their feces, a microbe whose abundance is found increased before NEC diagnosis (Hui et al., 2021; Olm et al., 2019). The prophylactic administration of this combination was confirmed in a recent network meta-analysis where Bb12+LGG was among the best probiotic mix supplementation options for reducing NEC risk in preterm neonates (Beghetti et al., 2021). Furthermore, recent findings from a UK NICU suggest that routine prophylactic use of *Lactobacillus* and *Bifidobacterium* in preterm neonates results in a significant reduction in NEC, surgical NEC, and LOS (Robertson et al., 2020).

2.2.2.2 Mechanisms of action

The mechanisms through which probiotics influence infant health are diverse, strain specific and not yet fully elucidated. They are, however, primarily organized into four major categories: 1) reduction of intestinal permeability, 2) modulation of mucus production, 3) modulation of the immune system and 4) antagonism against pathogens (Figure 4).

As discussed previously, the preterm gut is characterized by an increased intestinal permeability which may be a risk factor of NEC development (B. Ma et al., 2018). This means that the integrity of the intestinal epithelium, which forms a physical barrier between the lumen and the lamina propria, is compromised, allowing pathogenic microorganisms to pass through and cause infection. The tight junction proteins that keep epithelial cells bound together play an important role in the stability of the intestinal barrier. According to research in various epithelial cell lines, certain probiotics increase the expression of these proteins, ensuring the stability of the intestinal barrier (La Fata et al., 2018). An *in vitro* study, for example, found that pretreatment of epithelial cells with LGG before infection with a pathogenic *Escherichia coli* had a protective effect, preventing the decrease and redistribution of tight junction proteins normally caused by the pathogen (Johnson-Henry et al., 2008). Bb12 displayed similar effects as its fermentation products led to an increased tight junction integrity (Commane et al., 2005). Moreover, specific probiotics have been reported to promote mucus production. For instance, LGG can induce the expression and production of mucin in epithelial cells and mice via its cell wall protein, p40 (L. Wang et al., 2014). This increased mucus production mediated by LGG has been also shown to be responsible for inhibiting pathogen adhesion to the intestinal epithelial cells.

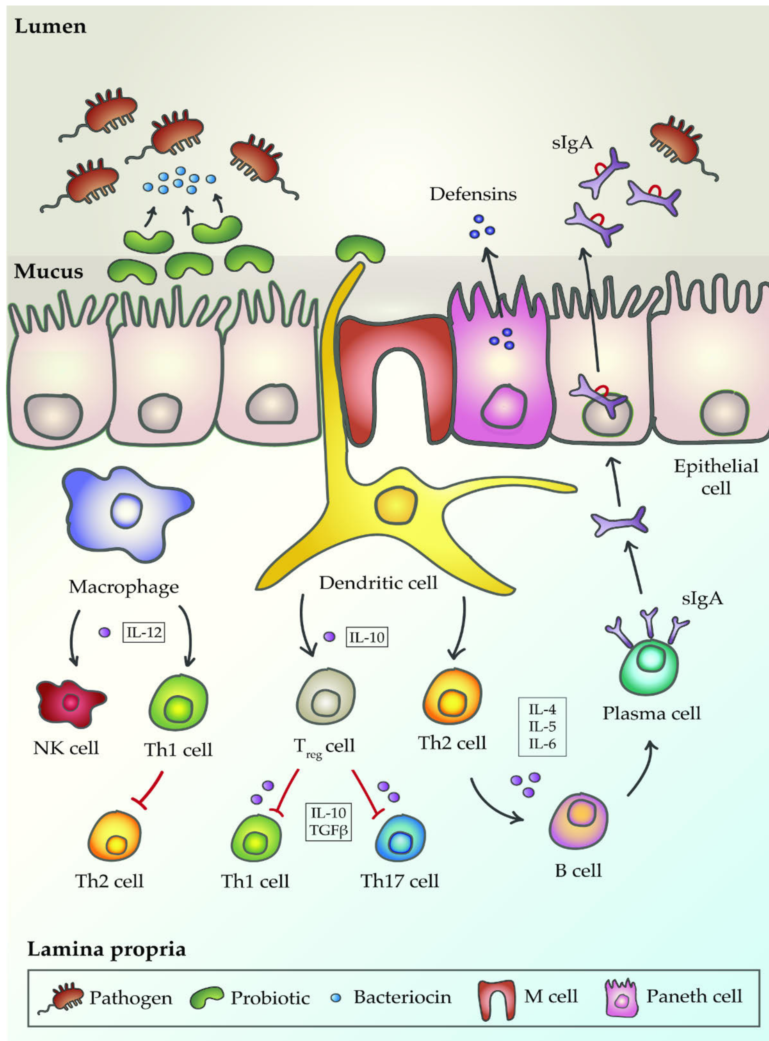


Figure 4. Probiotic mechanisms. Probiotics can produce antimicrobial compounds that act pathogens and prevent their adhesion to the gut epithelium. They are also capable or regulating a various range of immune responses such as activation of immune cells and cytokine and antibody production. Own drawing, adapted from (Mantziari, 2016).

Pathogenic microorganisms can be recognized by host cells via a variety of receptors, each of which is specialized in binding a specific bacterial structure such as lipopolysaccharides (LPS), lipoproteins, lipoteichoic acid, peptidoglycan, flagellin and bacterial RNA and DNA (Warshakoon et al., 2009). In response to pathogen infection, host cells increase the production of signaling molecules known as pro-inflammatory cytokines which attract and activate immune cells (Figure 5). However, excessive pro-inflammatory responses could lead to tissue damage and

eventually to organ failure and death (Delano & Ward, 2016). On the other hand, probiotic bacteria are able to induce anti-inflammatory cytokine production such as interleukin-10 (IL-10) in macrophages and dendritic cells (Kaji et al., 2018). Anti-inflammatory cytokines are essential for achieving homeostatic balance as they can inhibit pro-inflammatory cytokine secretion (de Moreno de LeBlanc et al., 2011).

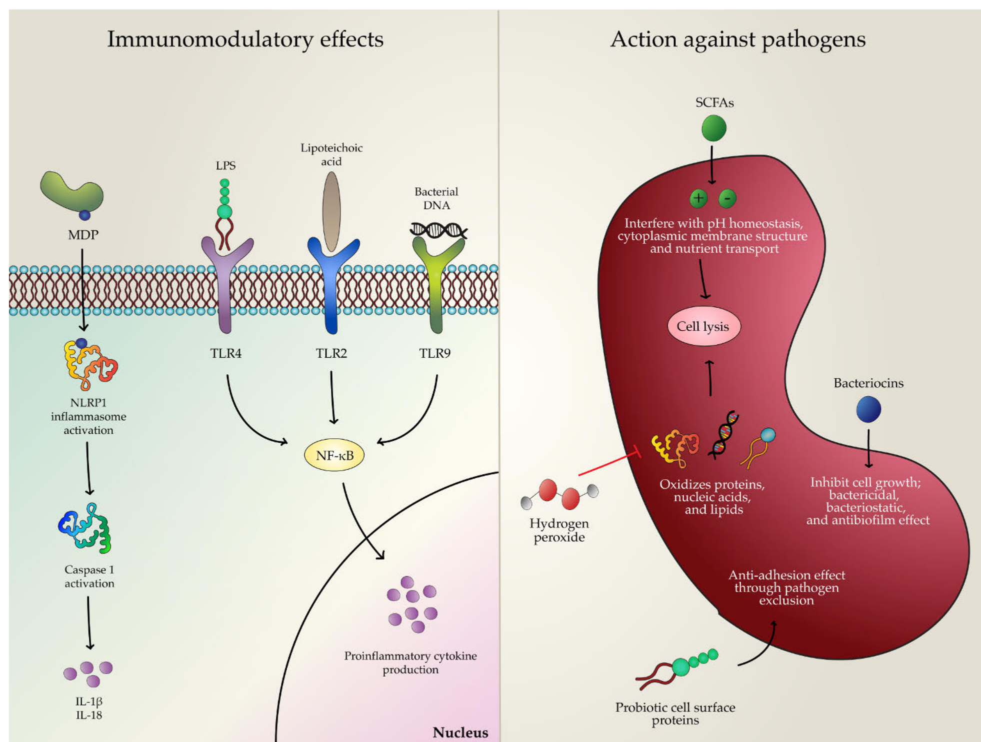


Figure 5. Probiotics modulate the host's immune response and on the pathogen on a specific manner. MDP: muramyl dipeptide; NLRP1: NACHT [NAIP (neuronal inhibitory protein), CIITA (MHC class II transcription activator), HET-E (incompatibility protein from *Podospora anserina*) and TP1 (telomerase-associated protein)] domain-, leucine-rich repeat-, and PYRIN containing protein 1; IL-1β and IL-18: interleukin 1 beta and 18, respectively; LPS: lipopolysaccharide; TLR 4, TLR 2, and TLR 9: toll-like receptor 4, 2, and 9, respectively; NF-κB: nuclear factor kappa B; SCFAs: short-chain fatty acids. Own drawing, adapted from (Mantziari et al., 2020).

Besides their interaction with immune cells, probiotics can directly inhibit gastrointestinal pathogens by either secreting antibacterial metabolites or by competing for common host adhesion sites and nutrients sites (Plaza-Diaz et al., 2019). Antibacterial metabolites commonly produced by probiotic lactic acid bacteria include SCFAs, bacteriocins, and hydrogen peroxide. Apart from modulating the intestinal microbiota composition, SCFAs may protect against

pathogen infection by compromising cellular metabolic reactions, impacting cellular physiology, or regulating virulence gene expression (Sun & O'Riordan, 2013). For instance, propionate produced by LGG (LeBlanc et al., 2017) and acetate produced by Bb12 (Merenstein et al., 2021) may influence pathogen virulence as both of these SCFAs were shown to inhibit the expression of *Salmonella typhimurium* invasion genes (Lawhon et al., 2002). Bacteriocins, on the other hand, interfere with pathogen cell growth and prevent biofilm formation. At the same time, hydrogen peroxide produced by some lactobacilli may disrupt the integrity of bacterial DNA strands by inducing direct breaks, thus leading to transcriptional errors and ultimately to the loss of cell viability (N.-N. Kim et al., 2019; Piekarska-Radzik & Klewicka, 2021). Probiotics may inhibit pathogen adhesion to intestinal surfaces through competitive exclusion. In short, various probiotic surface-bound compounds interact with the host pattern recognition receptors or with mucus glycoproteins, resulting in their adhesion to host cells or intestinal mucus respectively which in turn induces immune responses (do Carmo et al., 2018; Muscariello et al., 2020). Lastly, probiotics can enhance the diversity of the GM. Probiotic administration, has been shown to increase the levels of beneficial bacteria like bifidobacteria in the gut, thus enriching microbial diversity (Krumbeck et al., 2018).

2.2.2.3 Quality assurance criteria for probiotics

2.2.2.3.1 Adhesion Ability to Intestinal Surfaces

One of the main selection criteria and functional characteristic of probiotics required to confer a health benefit is adhesion to the intestinal mucosa. Indeed various *in vitro* and *in vivo* studies of well-known and well-characterized probiotic strains indicate their good adhesion capacity to mucus (Ouwehand & Salminen, 2003). Adhesion to intestinal mucus is also one of the first steps for transient colonization that enables probiotics to modulate GM composition (Korpela et al., 2016; Monteagudo-Mera et al., 2019).

As mentioned before, by adhering to intestinal mucus, probiotics may stimulate the immune system, promote local host defenses, and act against pathogens. Thus, administration of probiotics could effectively shift the microbial pattern of the gut and help prevent diseases by inhibiting pathogen attachment to the gut epithelium. This could also lead to reduced epithelium permeability, by triggering enhanced pro-inflammatory cytokine, mucin, or sIgA production and increasing the barrier integrity (Bermudez-Brito et al., 2012). Competitive exclusion of pathogens has been demonstrated in many studies. For instance, Lee and Puong showed that LGG can compete with *Salmonella* spp. and *E. coli* for the same adhesion sights to Caco-2 intestinal cells presumably by steric hindrance (Y.-K. Lee & Puong, 2002).

Consequently, this probiotic ability interferes with pathogen growth on the mucosal surface and protects against potential infections. It is also important to include that specific combinations of probiotics were reported to be more effective in inhibiting pathogens from adhering to mucosal surfaces than single probiotic strains *in vitro* (M. C. Collado et al., 2006; M. c. Collado et al., 2007; M. C. Collado et al., 2008). The adhesion of probiotics to intestinal surfaces is mediated by cell surface compounds of probiotic bacteria including but not limited to proteins, glycoproteins, lipoproteins, lipoteichoic acids, lipopolysaccharides, adhensins, and flagellins. For instance, the cell wall bound SpaCBA pili of LGG have been well described to mediate its adhesion to the both human intestinal epithelial cells (Caco-2) and human intestinal mucus (Lebeer et al., 2012; Ossowski et al., 2013). More specifically, one of the three pilling subunits SpaC pilin has been proved to be essential for the binding of mucus by this probiotic (Kankainen et al., 2009). In addition, increased ability to adhere to intestinal surfaces may be due to the production of probiotic metabolic products. Evidence from *in vitro* experiments indicates that adhesion of *L. acidophilus* LB to Caco-2 cells was increased when an extracellular protein was present. This protein was present in the spent culture supernatant and mostly a metabolic by-product of probiotic growth (Chauvière et al., 1992).

Models to Study Probiotic Adhesion Ability *In Vitro*

Screening for strongly adherent probiotic candidate strains should be optimally carried out in the host for which the probiotic is intended to be used. However, due to the complexity of host physiology, GM composition and increased ethical concerns of using animal models, this becomes a challenging task. Therefore, various *in vitro* models have been developed over the years giving a more simplified view of what is the situation *in vivo*. Indeed, some of these models appear to have strong correlation with *in vivo* colonization ability of potential probiotic lactic acid bacteria (Sugimura et al., 2011). The most commonly used models to study probiotic adhesion to mucosal surfaces are mainly purified mucins, intestinal mucus, intestinal epithelial cells, host tissues, and biopsies (Ouwehand & Salminen, 2003). Each model serves a different mechanistic purpose: mucus models target mucin glycoprotein-microbe interactions while cell line models study host-microbe interactions (Tassell & Miller, 2011).

2.2.2.3.2 Factors Affecting Probiotic Stability in Milk

As outlined before, besides adhesion ability, another one of the key criteria for a microorganism to qualify as a probiotic is to be alive in the delivery matrix throughout shelf life and during consumption at an efficacious dose (Binda et al.,

2020). Although probiotic doses for specific effects are not yet clearly defined, there are multiple studies suggesting that probiotics have to be viable and in amounts of approximately 10^6 -10^8 CFU/ml or g of the food product per day (A. Mortazavian et al., 2007).

For many years, cell viability has been considered a necessary property for clinically effective probiotics. This stems from the fact that most probiotic efficacy studies have been carried out using viable probiotics. In particular, cytokine and IgA production were higher after administration of viable than non-viable lactobacilli in mice, demonstrating a positive correlation between probiotic viability and effective gut immune stimulation (Galdeano & Perdigon, 2004). On the other hand, recent evidence has shown that inactivated probiotics, their metabolites, or cell wall components can also confer clinical health benefits, indicating that more than one mechanism of action may be responsible for a specific clinical health benefit (Mantziari et al., 2020). However, by definition, probiotics are live microorganisms, and therefore it is critical to enumerate them accurately in every production stage, including their supplementation into the food matrices and subsequent storage. This is because the beneficial effects of probiotics are based on the number of viable organisms present in the beginning and at the end of the recommended shelf-life.

There are numerous factors affecting probiotics' ability to survive during storage, including, but not limited to, delivery matrix, probiotic strain, temperature, and storage time. In the NICU, sachets containing lyophilized probiotic powder are mixed with a diluent (most commonly sterile water, MOM, or DHM) before administration to the infant (Watkins et al., 2018). Interestingly, nature and composition (pH, dissolved oxygen, buffering capacity, the concentration of metabolites) of the diluent may modify the probiotic stability and its tolerance to gastrointestinal conditions, thus compromising or enhancing its beneficial properties and efficacy (Tripathi & Giri, 2014). Sterile water is a universal solvent, but compared to whole milk processed at ultra-high temperature, it appears to be a suboptimal carrier matrix for probiotics (Lo Curto et al., 2011). This difference is probably due to the higher buffering capacity (good resistance to pH change) of milk that offers probiotic protection from the harsh gastrointestinal environment. In general, probiotics in dairy matrices, like fermented milks, can tolerate exposure to simulated gastric juices (Sagheddu et al., 2018). Likewise, many milk components, including casein, whey protein, and lactose, are known as cryoprotectants and are extensively used in the commercial production of freeze-dried probiotics (Reddy et al., 2009). Indeed, glycomacropeptide, a bioactive peptide derived from κ-casein, and whey protein can stimulate the growth of bifidobacteria and/or lactobacilli in milk (Gustaw et al., 2016; Janer et al., 2004). Nevertheless, all these components and their concentration in milk can have a different effect on stability. This depends on each probiotic strain's technological and physiological characteristics. For instance,

LGG maintained its viability in yogurt during storage for 2-3 weeks at 4°C compared to other *Lactobacillus* strains tested in the same conditions (Mani-López et al., 2014; Schillinger, 1999; Vinderola et al., 2011).

Our current knowledge suggests that matrix composition, strain properties, storage temperature and storage time are all crucial in ensuring probiotic survival in milk. An experiment investigating the effect of storage conditions on goat milk fermented by *B. animalis* and *L. acidophilus* showed that both strains were stable for 10 days in 5-7°C (Kongo et al., 2006). In contrast, viability was compromised when the fermented milk was stored at 22°C. Higher storage temperature promotes the growth of lactic acid bacteria and, in extent, their metabolic activity. Besides carbon dioxide and acetic acid, aerobic growth leads to hydrogen peroxide formation, which can be bacteriostatic for lactic acid bacteria thus reducing their stability in the product before consumption (Liptáková et al., 2017; Piard & Desmazeaud, 1992). Moreover, as indicated by a study by Oliveira and coworkers, the viable counts of *L. bulgaricus* LB340, *L. acidophilus* LAC4, *L. rhamnosus* LBA, and *B. lactis* BL 04 in fermented skim milk were retained for 7 days at 4°C (De Souza Oliveira et al., 2011). Likewise, another probiotic strain, *L. casei* BL23, was shown to be stable in fermented skim milk supplemented with whey protein hydrolysate (WPI) for up to 90 days of cold storage at 4°C (Cordeiro et al., 2018). Data also suggest that goat's milk fermented by *L. rhamnosus* CRL1505 and *S. thermophilus* maintained the stability of the former for up to 20 days of cold storage at 4ºC (Salva et al., 2011). Also, one of the world's most studied probiotic, *B. animalis* subsp. *lactis* Bb12, maintained its viable numbers in fermented goat's and cow's milks after storage at the same temperature for 21 days (Martín-Diana et al., 2003). However, this was not the case for *L. acidophilus* LA-5 since storage at the same conditions was found to negatively affect its stability in both milk matrices and goat's milk yogurt (Senaka Ranadheera et al., 2012). Therefore, the effect of storage temperature and time seems to be both strain and matrix dependent.

Methods to Study Probiotic Stability

Based on the current understanding, viability is an essential determinant of probiotic clinical efficacy and required by the definition. Therefore, it is critical to enumerate probiotics accurately in every production stage, including their supplementation into the food matrices and subsequent storage. Various methods have been developed over the years to assess probiotic viability, mainly falling under two categories: traditional culture and molecular methods.

The most popular method to address viability is to plate a proper dilution of the product to be tested on a selective growth medium and count the colonies formed followed by the incubation under appropriate conditions. This method enumerates the

number of bacteria that are metabolically or physiologically active and are able to form visible colonies on specific nutrient media (Ayyash et al., 2016). Although this method is inexpensive and does not require laborious sample preparation, it has a long turnaround time, in most cases, more than 24 hours. Another shortcoming is the need for at least three replicate plates for each dilution due to the difficulty obtaining reproducible counts (Sohrabvandi et al., 2012). Indeed, the colony-forming units on each plate depend on the influence of neighbour cells (which are competing for the same nutrients), growth medium, incubation time, and temperature. This is particularly relevant for cold storage, during which probiotic bacteria are subjected to stress and may be sub lethally damaged (Figure 5). When damaged, cells might become dormant and retain metabolic activity (VBNC, viable but not able to be cultured) or unculturable losing their ability to form colonies and will consequently not be detected (Figure 6) (Lahtinen et al., 2005; L. Li et al., 2014). Indeed, some bacteria have been reported to enter into a VBNC state after pasteurization of milk, something that would lead to their underestimation by traditional culture methods (Gunasekera et al., 2002; Quigley et al., 2013). Thus, conventional culture-dependent methods might lead to underestimation of the total viable probiotics.

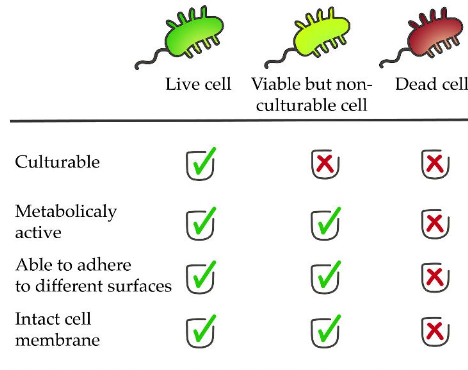


Figure 6. Bacterial states and their characteristics (L. Li et al., 2014). Own drawing, adapted from (Mantziari, 2016)

To overcome this limitation and to enable the accurate enumeration of probiotics, alternative culture-independent methods focused on nucleic acid amplification have been developed (Figure 7). One of the most used cell quantification methods to date is quantitative polymerase chain reaction (qPCR) that can measure bacterial load accurately up to strain level. The benefits of qPCR include increased sensitivity, specificity, and reduced time to obtain results. In short, this method is based on the amplification of bacterial DNA using species-specific primers in the presence of a component that can fluoresce upon binding to double-stranded DNA. In every

amplification cycle, this fluorescent signal is measured and can be used to estimate the amount of DNA in the tested sample by comparing it to a standard curve obtained from dilution series of reference DNA (Carey et al., 2007).

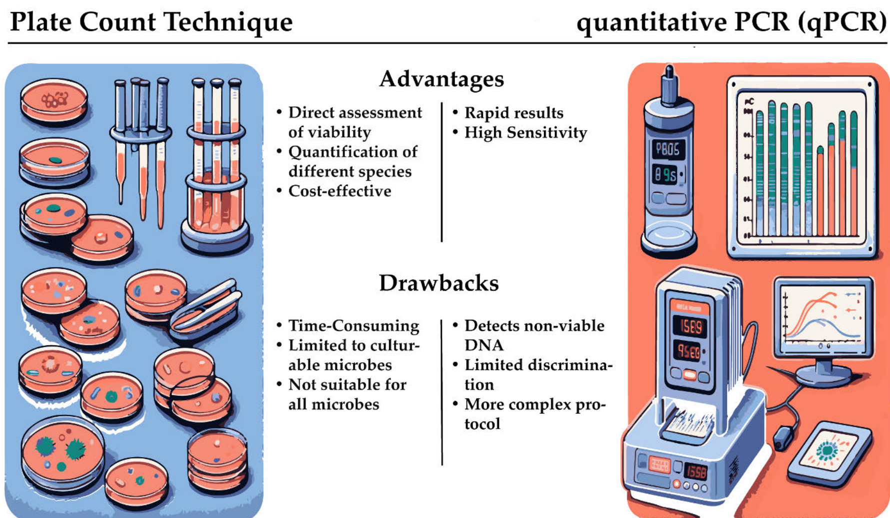


Figure 7. Advantages and disadvantages of plate count technique and qPCR. Own drawing with the assistance of AI.

However, traditional qPCR is mainly used to confirm culturing results because it cannot avoid the identification of multiple copies of the 16S rRNA gene that many bacteria possess nor the DNA amplification from dead bacterial cells (Klumpp & Hwa, 2014; Young et al., 2007). The accurate assessment of probiotic viability by qPCR can also become challenging due to the presence of varying numbers of gene copies in different cell growth stages (Ibal et al., 2019). Consequently, these factors contribute to an overestimation of DNA concentration and thereby to a misleading assumption of the probiotic viability that can be minimized by using a combination of both plating and molecular techniques. Interestingly, Lahtinen and co-workers assessed the stability of bifidobacteria in fermented oatmeal by both methods and found that qPCR results differed significantly from plate counts of both tested bifidobacteria (Lahtinen et al., 2006). Therefore, a combination of methods is essential when determining the total cell count of probiotics in the delivering matrix.

2.2.3 Polyamines

Polyamines (PAs) are primordial polycations mainly known for their essential role in cell proliferation and differentiation (Muñoz-Esparza et al., 2019). Putrescine

(PUT), spermidine (SPD), and spermine (SPM) are the most abundant PAs in the human body. These compounds are required and accumulated in large amounts in rapidly growing tissues like the gastrointestinal epithelium and may provide protection against environmental challenges (Pegg, 2009; Timmons et al., 2012). In addition, PAs due to their positive charge bind to proteins and by changing their conformation may modulate signaling pathways (Schuster & Bernhardt, 2011).

Other PA functions include processes such as metabolite biosynthesis and development of the immune function during the postnatal period (Hesterberg et al., 2018; Muñoz-Esparza et al., 2019). Evidence from animal studies suggests that dietary PAs may play an important role in neonatal and infant development (T. Fang et al., 2016; van Wettere et al., 2016). Indeed, administration of exogenous PAs in suckling rats appeared to protect against decreased small intestinal mucosal weight induced by a PA deficient diet (Löser et al., 1999). This may indicate the implication of PAs in the gastrointestinal tract maturation.

The main sources of PAs are diet and synthesis by the host cells or the GM and diet (Bekebrede et al., 2020). In terms of infant diet, human milk, the optimal source of nutrition for the newborn infant, is rich in PAs. While polyamine concentration in HM varies between mothers and is greatly affected by gestational age, the average amounts are 0.058, 0.580, and 0.825 ppm for PUT, SPD, and SPM (Gómez-Gallego et al., 2016; Plaza-Zamora et al., 2013). Similarly, Gallego and coworkers reported a median concentration of 0.019, 0.512, and 1.03 ppm for PUT, SPD, and SPM, respectively in HM (Gómez-Gallego et al., 2017). In addition, PA concentration is significantly higher in preterm HM compared to term HM while recent studies on neonatal blood metabolome revealed a negative correlation between PAs and gestational age (Ali et al., 2014; Ernst et al., 2020; Muñoz-Esparza et al., 2019; Plaza-Zamora et al., 2013). The higher concentration of PAs in preterm milk may not only reflect the increased nutritional demands of these infants based on their gestational age, but also potentially contribute to an improvement in the integrity of their intestinal barrier. This is particularly relevant for preterm infants as they tend to exhibit higher levels of intestinal permeability compared to their full-term counterparts (Taylor et al., 2009). Most dietary exogenous polyamines are absorbed in the upper digestive tract (Uda et al., 2003). In terms of biosynthesis, endogenous PAs like PUT and SPD are produced by intestinal bacteria present in the lower parts of the intestine, including the colon, and these microbes are able to synthesize PAs by multiple pathways (Hanfrey et al., 2011; Kitada et al., 2018; Matsumoto et al., 2012).

Although the metabolism and function of PAs in eukaryotic cells have been widely studied, their effect on bacteria has not been fully explored (Igarashi & Kashiwagi, 2019). Similar to eukaryotic cells, PUT, SPD, and SPM are the main PAs together with cadaverine, with recent studies highlighting their role in bacterial

activity (Tofalo et al., 2019). More specifically, PAs can influence bacteria–host interactions with various reports describing the mechanism by which PAs may contribute to bacterial pathogenesis (Banerji et al., 2021; Di Martino et al., 2013). For instance, SPD production by *Pseudomonas aeruginosa* protects the pathogen against antibiotics while agmatine (a precursor of SPD and PUT) derived by the same pathogen can reduce the pro-inflammatory response of airway epithelial cells (L. Johnson et al., 2012; McCurtain et al., 2019). At the same time, PAs are involved in pathogen biofilm synthesis and maturation and their transport inside the bacterial cell contributes to pneumococcal virulence (Nanduri & Swiatlo, 2021; Shah et al., 2011). PAs might contribute also to the activity of beneficial microbes since PA transport has been observed in several species while their production by intestinal microbiota could beneficially impact the host (Mikelsaar et al., 2020; Sugiyama et al., 2018).

The impact of probiotics on polyamine production by the intestinal microbiota has been scarcely explored. Recently Singh and coworkers studied the effect of a probiotic mix on the intestinal microbiota composition of mice and its associated metabolic activities (Singh et al., 2021). Interestingly they found that probiotic treatment decreased polyamine biosynthesis-associated genes, suggesting that probiotics may have unbeneficial side effects on the structure of intestinal microbiota. However, this disagrees with earlier studies that used different probiotic strains. Oral administration of *Bifidobacterium animalis* subsp. *lactis* LKM512 or *Lactobacillus acidophilus* NCFM and lactitol modulated the intestinal microbiota composition and increased the concentration of PAs in the feces of mice and healthy adults respectively (Matsumoto et al., 2011a; Ouwehand et al., 2008). Therefore, this effect may be related to the probiotic strain used or the type of predominant bacteria in the gut.

Adhesion to intestinal mucus is the first event in the process by which microbes interact with the intestinal barrier and it could play a critical role during colonization in early life (Harata et al., 2021). However, the role of PAs in bacterial adherence to intestinal tract mucus and epithelium is not known but studies in protozoan parasites suggest that polyamine metabolism and secreted PUT are linked to host cell adherence (Garcia et al., 2005). Considering that probiotic administration may increase and modulate the PA-producing colonic microbiota, (Matsumoto et al., 2011b) it will be interesting to evaluate whether exogenous PAs in similar proportions to those reported in HM can alter the adhesive properties of probiotics to intestinal mucus. Likewise assessing the effects of PAs on commensal and pathogen adhesion capacity might also be of relevance in early life.

3 Aims

The primary goal of this thesis was to investigate two popular infant GM modulating strategies: human milk microbiota and specific probiotics. We hypothesized that gestational age can influence the composition of the HMM whereas other factors such as pasteurization, fortification, and storage of HM and presence of polyamines can modify the viability and adhesion ability of probiotics. To evaluate their extent and impact four studies were designed.

The specific aims were:

1. To characterize the human milk microbiota profiles of healthy Argentinian women who delivered at different gestational ages and to study the changes in the preterm human milk microbiota composition between birth and term equivalent age (**I**)
2. To assess the stability of probiotics in human milk during storage (**II**)
3. To determine the effect of fortified donor human milk on the adhesion properties of probiotics (**III**)
4. To investigate the role of polyamines on probiotic and pathogen interactions with intestinal mucus (**IV**)

4 Materials and Methods

4.1 Subjects, sample collection and preparation

In study **I**, twenty-four healthy breastfeeding mothers from Santa Fe, Argentina, were recruited and grouped as follows: T group (full-term deliveries at ≥ 37 weeks of gestation, n= 12), and P group (preterm deliveries at < 37 weeks of gestation, n=12). Milk samples from both T and P groups were collected during the first postpartum days. In addition, mothers in P group donated a second milk sample (n=12) at term equivalent age (TEA, PT group), which is the age at which the infant would have been born if it had not been born prematurely (37-42 weeks postmenstrual age). Mothers from the T group were recruited spontaneously in Santa Fe and mothers from P (and PT) group were recruited from public (J. B. Iturraspe) and private (San Gerónimo) hospitals in Santa Fe. Written informed consent was obtained from all participants. Approval of the Ethical Committee of Santa Fe (number 640/2017) was obtained. For HM collection, the skin around the nipple was cleaned with soap and water, and the first 500-600 µL were discarded. Less than 5 mL were collected from preterm mothers, while 5-10 mL were collected for the full-term group. Milk was collected in sterile containers, refrigerated, and processed in the laboratory within less than two hours after collection.

For study **II**, a total of eight frozen human milk samples corresponding to eight healthy mothers of term infants from South Africa were included. These samples were used previously in a larger study that was conducted at Functional Foods Forum, University of Turku (Kumar et al., 2016). Before the experiment, samples were thawed overnight in a refrigerator, mixed gently, and pooled in equal parts under a laminar hood. For study **III**, frozen human milk was donated by five healthy mothers of term infants and stored in -80°C until further use.

The fecal samples that were used to isolate the infant intestinal mucus used in studies **III** and **IV**, were obtained from healthy adult subjects from Finland (25–52 years old, n = 14) and from two age groups of healthy Finnish infants: 0–6-month-old infants that were exclusively breastfed (mucus<6; n=5) and from 6–12-month-old infants that were exclusively breastfed and introduced to solid food (mucus>6; n=5).

4.2 Pasteurization, fortification, and storage of Human Milk

To prepare the DHM for pasteurization (studies **II** and **III**), the milk was first pooled and then Holder pasteurized (62.5°C for 30 min) in a thermostatically controlled water bath (model GD100; Grant Instruments Ltd., Cambridgeshire, UK). To ensure that the temperature of the milk was maintained throughout the pasteurization process at 62.5°C, a temperature probe was submerged in a bottle containing Milli-Q water. Following heat processing, the milk samples were rapidly moved onto ice to allow cooling (<4 °C).

For Study **III**, two forms of HMF, liquid and powdered (Enfamil® Human Milk Fortifier Acidified Liquid and Enfamil® Human Milk Fortifier Powder respectively; Mead Johnson, Evansville, IN, USA), were tested and diluted in DHM following the manufacturer's instructions. The fortifiers were provided without cost by Mead Johnson, but the company had no influence on the study design.

Frozen pooled HM (**II**) or fortified DHM (FDHM) (**III**) were room- (23°C), cold- (+4°C) or freeze-stored (-20°C) to assess the influence of the HM matrix on the stability of probiotic bacteria along storage. The storage conditions of milk are summarized in Table 1.

Table 1. Storage conditions of HM to evaluate the stability of probiotics in studies **II** and **III**.

Temperature	Time	Matrix
Room temperature	0 h	probiotic + frozen pooled milk (**II**) probiotic + FDHM (**III**)
	6 h	probiotic + frozen pooled milk (**II**)
+4°C	6 h	probiotic + frozen pooled milk (**II**)
	24 h	probiotic + frozen pooled milk (**II**) probiotic + FDHM (**III**)
	72 h	probiotic + frozen pooled milk (**II**)
-20°C	7 days	probiotic + frozen pooled milk (**II**)

4.3 Culturing

4.3.1 Bacterial strains and growth conditions

The bacterial strains used in this work included two probiotic strains *Lacticaseibacillus rhamnosus* GG (LGG) (ATCC 53013), *Bifidobacterium animalis* subsp. *lactis* Bb12 (DSM15954) (Chr. Hansen, Copenhagen, Denmark), one pathogen (*Cronobacter sakazakii*, ATCC 29544) and one potential opportunistic pathogen (*Escherichia coli* TG1, obtained from C. K. Lim). For both stability and

adhesion studies, the bacterial strains were first reactivated from stocks stored at −70°C in 25% glycerol. LGG and *E. coli* were grown overnight aerobically in de Man, Rogosa and Sharpe (MRS; Merck, Darmstadt, Germany) broth at 37°C while Bb12 and *C. sakazakii* in Gifu Anaerobic Medium (GAM; Nissui Pharmaceutical, Tokyo, Japan) at 37°C for 48 h under anaerobic conditions (10% H2, 10% CO2, and 80% N2; Concept 400 anaerobic chamber, Ruskinn Technology, Leeds, United Kingdom). A growth temperature of 37°C is the best conditions of production of LGG cells with high adhesion ability (Deepika et al., 2012).

4.3.2 Plate counts

To confirm the stability of the probiotics in studies **II** and **III,** milk samples were serially diluted (10^{-4} to 10^{-6}), plated in triplicate on MRS (for LGG) or GAM agar (for Bb12) and incubated at an anaerobic condition at 37°C for 48 and 72 h, respectively. The stability assay was performed three times. Finally, all microbial counts were expressed as $\log_{10}$CFU/mL.

4.4 DNA extraction (I-III)

4.4.1 DNA extraction using MasterPure™ kit (I)

HM samples (1.5 mL) were centrifuged (20000 × g, 20 min, 4ºC) to remove the HM fat fraction and washed twice with 500 µL saline solution. Genomic DNA was extracted using the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre, Madison, Wisconsin) according to the manufacturer's instructions. Subsequently, the concentrations of the extracted DNA were estimated by Qubit 2.0 fluorometer (Life Technology, Carlsbad, CA, USA) and normalized to 5 ng/µL.

4.4.2 DNA extraction using KingFisher (II)

For the qPCR and the 16S rRNA gene sequencing analyses, total DNA was extracted from 1.5 mL of human milk using the InviMag Stool DNA kit (Stratec Molecular, Berlin, Germany) and automated KingFisher magnetic particle system (Thermo Fisher Scientific, Vantaa, Finland). Briefly, human milk samples were mixed with 0,2 g glass beads (1.5mm) and centrifuged at 14,000 rpm for 20 min at 4°C to create a fat layer that was discarded along with the supernatant. Next, the pellet was mixed with lysis buffer P and cells were mechanically lysed by two rounds of bead beating (FastPrep®, FP120-230, Bio 101 ThermoSavant, Holbrook, NY) and heat treatment for 15 min at 95°C. This procedure was repeated twice. The DNA was eluted in 100

μl of Elution Buffer and its quantity and purity were determined using NanoDrop 2000 (Thermo Fisher Scientific) before storage at −70°C.

4.4.3 DNA extraction from colonies (II and III)

Selected colonies were suspended in 50 μL of ultrapure DNase/RNase-Free water and heated at 95°C for 10 minutes. After cooling on ice for 2 minutes, samples were centrifuged at 13,300 rpm for 15 min to precipitate any cellular debris. Finally, the supernatant/lysate containing DNA was stored at −20°C for later analysis. A total of 1 μL lysate was used in the colony PCR.

4.5 Confirmation by colony polymerase chain reaction (cPCR) (II and III)

Colony PCR (cPCR) was performed with strain-specific primers to confirm that the counted colonies in studies **II** and **III** were indeed LGG and Bb12. The primers used were respectively Lrhamn1 (5´-CAATCTGAATGAACAGTTGTC-3´), Lrhamn2 (5´-TATCTTGACCAAACTTGACG-3´), BB12-tuf-F (5´-GTGTCGAGCGCGGCAA-3´), and BB12-tuf-R (5´-CTCGCACTCATCCATCTGCTT-3´). Amplification of the DNA was performed using a PCR iCycler apparatus (Bio-Rad, Espoo, Finland). Initially, PCR reactions were carried out using 1 μL (10 ng/μL) of DNA template, 12.5 μL of Kapa2G Robust HotStart ReadyMix (2X) (Kapa Biosystems, South Africa), 10.5 μL of DNAse/RNAse free H_2O, and 10 pmol/μL of each gene-specific primer in a final volume of 25 μL. The PCR amplification conditions for LGG and Bb12 were respectively as follows: initial denaturation step at 95°C for 3 min; 35 cycles of denaturation at 95°C for 15 s, annealing at 50°C for LGG, 60°C for Bb12, extension at 72°C for 15 s, followed by a final extension step at 72°C for 5 min. PCR products were separated and confirmed by electrophoresis in a 1% (*w*/*v*) agarose gel and visualized with ethidium bromide under ultraviolet (UV) light.

4.6 Identification of human milk isolates by Sanger Sequencing (II)

DNA from the unknown isolated colonies in study **II** was amplified with the same cPCR conditions mentioned above using the PLB16 (5´-AGAGTTTGATCCTGGCTCAG-3´) and MLB16 (5´-GGCTGCTGGCACGTAGTTAG-3´) primer set that targets the amplification of a 470-bp fragment of the 16S ribosomal RNA gene. The annealing temperature used for this primer set was 50°C. The amplicon size was verified by electrophoresis in

1% (*w/v*) agarose gel, and the amplified DNA was then purified with polyethylene glycol 8000/NaCl (PEG 8000; AppliChem, Darmstadt, Germany).

The quantity and purity of the amplicons were determined using NanoDrop 2000 (Thermo Fisher Scientific). Finally, the purified amplified DNA was diluted correctly and sequenced with the ABI Prism-3130XL genetic analyzer (Applied Biosystems, Foster City, CA) by Turku Center for Biotechnology (Turku, Finland) or by the Institute for Molecular Medicine Finland (FIMM, Helsinki, Finland). The resulting sequences were identified using BLAST searches (NCBI) against the GenBank database with ≥ 97 % sequence similarity.

4.7 Quantitative polymerase chain reaction (qPCR) analysis (II)

To measure the number of probiotic cells in human milk in study **II**, quantitative PCR was employed using species-specific primers. Amplification of the DNA was performed with an ABI 7300 Real-Time PCR system (Applied Biosystems). Amplification reactions were performed in 20 µl containing 10 µl of 1x SensiFAST SYBR Hi-ROX kit (Bioline, London, UK), forward and reverse primers at a final concentration of 0.2 µM, and 1 µl template DNA. The amplification program consisted of an initial cycle of 95°C for 3 min and 40 cycles of amplification (95°C for 5 s, annealing at primer-specific temperature for 30s, and extension at 72°C for 30s), followed by a dissociation stage (95°C for 15 s, 60°C for 15 s, 95°C for 15 s). Fluorescence was measured at the end of the extension step of each thermal cycle and a melt curve analysis was carried out at the end of the amplification. Samples were analyzed in duplicate in two independent qPCR runs.

To determine the detection limit of the qPCR assay, standard curves were created using serial 10-fold dilutions of bacterial DNA of different reference strains (Ct value *vs*. CFU/ml). This DNA was obtained from pure cultures with a bacterial population ranging from 2 to 8 $\log_{10}$ CFU/ml estimated by standard plating procedures. CFU/ml in test samples were then determined by interpolation from the standard curve using their respective Ct values. The following reference strains were used to construct the standard curves and to determine the detection limit of the qPCR: *Bifidobacterium animalis* subsp. *lactis* Bb12 (DSM15954) for Bb12 and *Lacticaseibacillus rhamnosus* GG (ATCC 53013) for LGG. The sequences and annealing temperatures of the primers used for qPCR studies I and II are summarized in Table 2.

Table 2. Primers used for the quantification of probiotics in probiotic-supplemented HM by qPCR.

Target group	Primer	Sequence (5' to 3')	Annealing temp.	Reference
Bb12	BB12-tuf-F	GTGTCGAGCGCGGCAA	68°C	(Solano-Aguilar et al., 2008)
	BB12-tuf-R	CTCGCACTCATCCATCTGCTT		
LGG	Lrhamn1	CAATCTGAATGAACAGTTGTC	60°C	(Brandt & Alatossava, 2003)
	Lrhamn2	TATCTTGACCAAACTTGACG		

4.8 16S rRNA gene sequencing analysis of human milk microbiota (I)

The 16S rRNA gene (V3-V4 region) was amplified by PCR and sequenced using the Nextera XT Index Kit. After 16S rRNA gene amplification, the multiplexing step was performed using the Nextera XT Index Kit. Amplicons (1 µL) were quantified using a Bioanalyzer DNA 1000 chip and the libraries were then sequenced using a 2 × 300 bp paired-end run (MiSeq Reagent kit v3) on a MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain). To rule out possible reagent contaminations during DNA extraction and sequencing, we included negative controls from the DNA extraction and no-template controls (NTCs) respectively.

4.9 *In vitro* adhesion assays with radiolabeled bacteria (III and IV)

4.9.1 Bacterial radiolabeling

The bacterial strains tested for their ability to bind to intestinal mucus glycoproteins included two probiotic strains (LGG and Bb12 or their combination), one pathogen (*C. sakazakii*) and one potential opportunistic pathogen (*E. coli*). For adhesion assays, the strains were labelled metabolically, by inoculating 5 µL from each of the reactivated bacteria with 10 µL of [3H]-thymidine (Perkin Elmer NET355001MC, PerkinElmer, Waltham, MA, USA) in 1 mL of the respective media (see bacterial strains and growth conditions section). After growth, the bacteria were harvested by centrifugation (2000× *g*, 5 min), washed twice, and resuspended in HEPES (*N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulphonic acid)-Hanks buffer (HH; 10 mM of HEPES; pH 7.4). The optical density (OD_{600nm}) was adjusted to 0.25 ± 0.01 (~10^8 CFU/mL). The number of bacteria (CFU/mL) was determined from non-labelled bacterial suspensions by plating 10-fold serial dilutions on appropriate agar plates.

4.9.2 Preparation of bacteria prior to *in vitro* adhesion assays

In study **III**, two forms of HMF, liquid and powdered, were tested and diluted in DHM following the manufacturer's instructions. The aim was to compare the probiotic adhesion between DHM and FDHM, considering DHM as control. To prepare DHM + probiotics and FDHM + probiotics, the same volume used to adjust the optical density was added to DHM and FDHM. To test if the adhesion of LGG is influenced by the presence of Bb12 and vice versa, radiolabeled LGG was mixed with an equal amount (~5×10^7 CFU/mL) of non-labeled Bb12 and the other way around. In study **IV**, putrescine (PUT, D13208; Aldrich), spermidine (SPD, 2626; Sigma), spermine (SPM, 85590, Fluka) and their combination in high (MixHigh) and low (MixLow) concentrations were assessed for their effect on the adhesive properties of the above-mentioned bacteria. The concentration levels tested for each polyamine separately were 5 μg/mL. For the low concentration group the levels were 0.5 μg/mL PUT, 10 μg/mL SPD and 20 μg/mL SPM, and for the high concentration group 1 μg/mL PUT, 20 μg/mL SPD and 40 μg/mL SPM. For both polyamine combination groups we chose to use a proportion similar to the one reported in HM (1.6% for PUT, 32.8% for SPD and 65.6% for SPM). (Gómez-Gallego et al., 2012)

4.9.3 Isolation of human intestinal mucus from feces

Human intestinal mucus was prepared from the feces of healthy Finnish infants. In short, 2 g from each fecal sample was suspended in phosphate buffer saline (PBS) supplemented with protease inhibitors and sodium azide and mixed for 1 h at 4°C. The samples were then centrifuged at 13.800 × *g* at 4°C for 30 min. The crude mucus was then isolated by dual ethanol precipitation and further purified by fractionation using an XK26 column packed with Sepharose CL-4B (GE Healthcare, Chicago, IL, USA). In the first step of the purification procedure, the crude mucus (5ml) was applied to the column and fractionated using a peristaltic pump (Alitea XV U1-M; Alitea, Stockholm, Sweden) at a 3.1% rpm flow rate. This process was repeated once more. The void volume was dialyzed against distilled water at 4°C overnight and lyophilized. Equal amounts of lyophilized mucus glycoproteins from each individual were pooled to make a stock suspension of 10 mg/mL in HH that was stored in -20°C.

4.9.4 *In vitro* adhesion assay

For the adhesion assay, mucus glycoproteins were diluted (0.5 mg/mL) in HH, and 100 μL/well of the suspension was immobilized on a polystyrene microtiter plate (Maxisorp, Nunc, Denmark) by overnight incubation at 4°C. Excess mucus was

removed by washing the wells twice with 200 µL of HH. In study **III**, DHM+probiotics and FDHM+probiotics were added to the immobilized mucus. To study whether probiotic adherence to mucus is increased after mucus treatment with FDHM, the wells were incubated at first only with FDHM for an hour at 37°C (INC37).

In contrast, in study **IV**, polyamines were added to the radiolabeled bacterial suspension just before the adhesion assay to avoid degradation by polyamine oxidase. Prior to the adhesion assay, the wells were washed twice with 200 µl of HH to remove excess mucus. To quantify the adhesive ability of the tested bacteria, 100 µL of radioactively labeled strains in HH, DHM, or FDHM were added to the wells and incubated at 37°C for 1.5 h. Non-adherent bacteria were removed by three times washing with 200 µL of HH. The adhered bacteria were released and lysed with 1% sodium dodecyl sulphate (SDS) in 0.1 M NaOH (200 µL per well) at 60°C for 1 h. The lysate was then removed and mixed with the scintillation liquid. The radioactivity of the lysed suspension was measured with a 1450 Microbeta Liquid Scintillation Counter (Wallac Oy, Turku, Finland). The adhesion assay was carried out as three independent experiments, with each experiment performed in triplicate. The adhesion ratio (in %) was calculated by dividing the radioactivity of the bacteria bound (triplicate 100 µL samples) by the radioactivity of the bacteria added to the infant intestinal mucus.

4.10 Statistical analyses

All statistical analyses were carried out using the IBM SPSS statistics 23.0 or 26.0 software (IBM Corp., Armonk, NY, USA).

4.10.1 Microbiota composition of human milk samples 16S rRNA gene sequencing (I)

4.10.1.1 Bioinformatics

All data preprocessing of MiSeq raw sequence reads was performed in USEARCH 10 (Edgar & Flyvbjerg, 2015) following a modified version of the pipeline proposed by Logares (Logares, 2017). Correction of sequencing errors based on the Hamming graph and Bayesian subclustering were performed using the BayesHammer error correction tool in SPAdes v3.5.0 genome assembler software (Nikolenko et al., 2013; Nurk et al., 2013). Then, in USEARCH 10, a quality check was performed using the "fastq_filter" command to remove low quality reads. Using the "UNOISE2" command (Edgar, 2018), the high quality sequences with a minimum abundance of ten reads were denoised and clustered into zero-radius operational taxonomic units

(zOTUs), also known as amplicon sequence variants (ASVs). zOTUs with abundance 10 or less reads were removed from the dataset to ensure that our conclusions are not affected by sequencing errors.

The sequencing yielded a total of 2,299,797 reads (67,641 ± 40,237; sequencing reads per sample) from 34 samples. zOTUs identified in the sequencing controls were considered contaminants and were removed from the dataset. Generation of the zOTU table was done using "otutab" command in USEARCH 10 following the removal of Cyanobacteria- (77 zOTUs) and Archaea- (8 zOTUs) affiliated zOTUs. The final zOTU dataset was assigned to taxonomic identifications based on BLAST search (Altschul et al., 1990), using the SILVA v132 database (SSU Ref NR99) as a reference (Quast et al., 2013). Samples PT1 and P6 were excluded from downstream analyses due to low number of reads (< 10,000) and an irregular distribution of reads and higher abundance in most zOTUs in comparison to the rest of the samples, respectively.

Next, in R software all analyses were performed in R v.3.2.3. to draw rarefaction curves zOTU rarefaction curves were constructed, using vegan v.2.5 to evaluate whether richness was saturated. The rarefaction of each sample was performed to all the following analyses, including the taxonomic diversity and richness determination. The taxonomic diversity and richness of communities were estimated with the Simpson, Inverse Simpson (E. H. Simpson, 1949), and Chao1 (Hughes et al., 2001) indices, respectively, using vegan v.2.5. In adittion, R software 4.1.3 (R Core Team, 2017) was used to create the non-metric multidimensional scaling (NMDS) plot which displayed the group distribution based on the dissimilarity matrix.

4.10.1.2 Statistical analysis

Alpha-diversity indexes, i.e., Richness, Chao1, and Phylogenetic Diversity (PD), were calculated using R.

Using the Paleontological Statistics (PAST) software package (Hammer et al., 2001) permutational multivariate analysis of variance (PERMANOVA) was applied to assess significant differences in the microbiota composition between the different milk sample groups, notably P, PT, and T. The analysis was performed with Bray-Curtis distances using 9999 permutations. The SIMPER (similarity percentage) analysis was then used to determine those zOTUs that contributed the most to the differences across groups. Prior to subsequent analysis, the Ryan-Joiner normality test was applied to determine whether relative abundance at phylum, family, and genus levels followed a normal distribution. Independent groups, pairs P *vs*. T and PT *vs*. T, was carried out by the Student's t-test or Mann-Whitney test according to the data distribution. The pair P *vs*. PT was analyzed with paired t-test or Wilcoxon Rank Sum Test according to the data distribution. The significance level for all statistical analyses was set at $p < 0.05$.

Linear regression was used to assess the associations between the HM microbiota (alpha diversity, taxa abundance at phylum level) and maternal and infant factors. The independent variables were delivery mode, administration of antibiotics and corticoid prenatal. The dependent variables considered were phyla and alpha diversity indices. The continuous variables with no normal distribution were center log-ratio transformed following zero-replacement (Gloor & Reid, 2016). After the individual linear regressions using SSPS software, the β coefficient with $p < 0.05$ was used in the web tool ClustVis to visualize the heatmap (Figure 12) (Metsalu & Vilo, 2015). Then, Spearman's Rank correlation was carried out considering the taxa abundance at phylum and genera levels, alpha diversity indices, gestational age, and TEA by SPSS software. It included *Staphylococcus* and *Streptococcus*, and less abundant genera such as *Bifidobacterium*, *Enterobacter, Pseudomonas*, *Ralstonia*, and *Rothia* (> 1% abundance and showing significant differences among groups).

4.10.2 Viability Assays (II and III)

Collected data from both culturing and qPCR methods were converted from CFU/ml to decadic logarithm of CFU/ml. The bacterial counts are shown as means with standard deviations. Data from study **II** were subjected to repeated measures Analysis of Variance (ANOVA) using the time as a factor with four categories; time points 0 (baseline), 6, 24 and 72 hours for the 4ºC to compare the probiotic counts between baseline and 6, 24 and 72 hours storage at 4ºC. Paired t-test was used for comparison of mean values between baseline and 6 hours at room temperature and baseline and at -20°C for 7 days (baseline with 6 hours at room temperature and baseline with seven days at -20°C). For study **III**, a paired t-test was conducted to examine any significant differences in the viable cell count (CFU/ml) between 0 hours (baseline) and 24 hours of storage at 4°C for each probiotic and type of fortifier separately. Statistical significance was set at 0.05 (two-tailed).

4.10.3 Adhesion Assays (III and IV)

In study **III**, due to the normal distribution of data, a two-sample t-test was used to identify differences between the control and the probiotic combination, presence of DHM, type of HMF, and subject age. A paired t-test was used to determine the statistical difference between the 0 hours (baseline) and 24 hours of storage at 4°C. In study **IV**, normal distribution of data was tested using the Shapiro-Wilk test. Due to normal distribution, one-way ANOVA followed by Tukey's post-hoc test was performed to analyze the differences between means. Statistics with a value of $p<0.05$ were considered significant.

5 Results

5.1 HMM composition affected by prematurity (I)

5.1.1 Clinical Data Profile and Human Milk Microbiota Profile

The characteristics of the mother-infant dyad populations are summarized in Table 3. No differences in clinical data were observed between preterm and term groups, except for the gestational age and weight at birth.

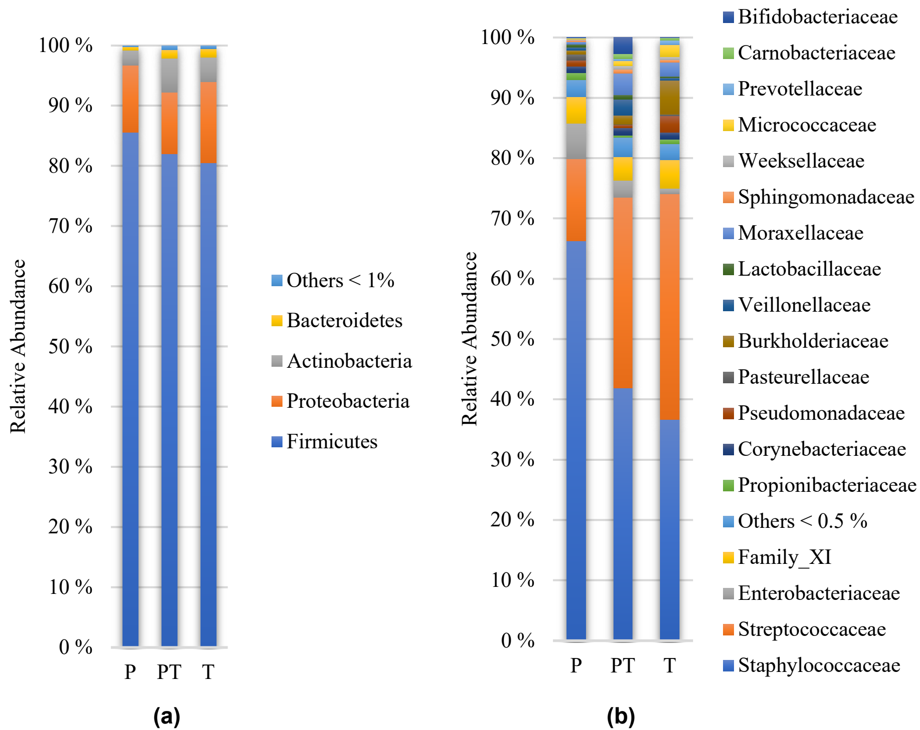
Table 3. Clinical data of the mother-child pair.

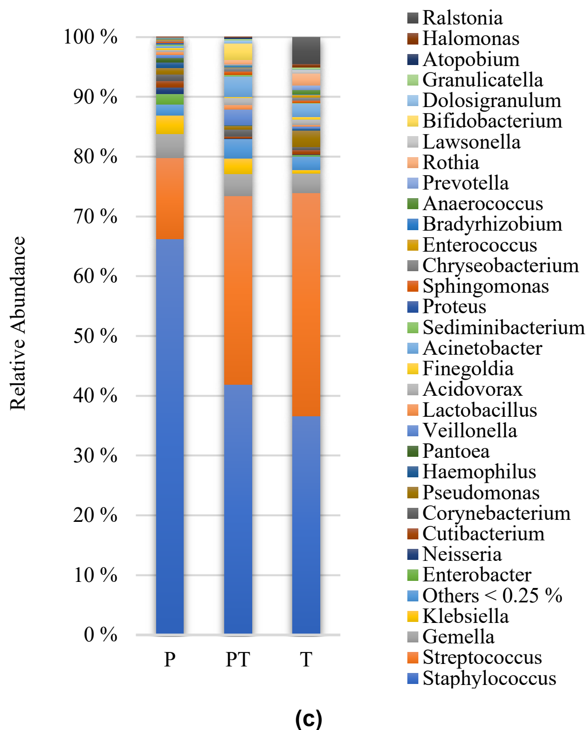
Parameter	Preterm	Term	*p*-value
Number of samples	24	12	Not applicable
Number of mothers	12	12	Not applicable
Days postpartum, 1st sample	8 ± 3	8 ± 3	0.799[2]
Days postpartum, 2nd sample	22 ± 9	Not applicable	Not applicable
Relation vaginal/Caesarean section	3/9	4/8	Not applicable
% Caesarean section	75 (9/12)	67 (8/12)	Not applicable
Gestational age (weeks)	33.4 ± 1,2	38.3 ± 1,2	0.000[1]
Neonatal weight (g) at birth	1968 ± 308	2925 ±484	0.000[1]
Neonate Sex (M/F)	6/6	5/7	Not applicable
Age (years) of mothers[3]	25.7 ± 7.5	29.1 ± 8.2	0.300[1]
Allergies of mothers (Yes/No)	5/7	4/7	Not applicable
Diabetes before pregnancy (Yes/No)	0/12	1/11	Not applicable
Hypo/Hyper Thyroidism (Yes/No)	2/10	1/11	Not applicable
Anemia during pregnancy (Yes/No)	2/10	2/10	Not applicable
Gestational diabetes (Yes/No)	2/10	2/10	Not applicable
Antenatal corticosteroids (Yes/No)	11/1	2/10	Not applicable
Antibiotics (Yes / No)	10/2	7/5	Not applicable
Active smoker during pregnancy (Yes / No)	2/10	2/10	Not applicable
Passive smoker during pregnancy (Yes / No)	5/7	3/9	Not applicable

[1] Student's T-test for independent samples for parametric data
[2] Mann-Whitney U test for non-parametric data
[3] the age of the mothers was measured at partum
* p <0.05, significant

5.1.2 Overall Human Milk Microbiota Composition

The most abundant phylum was *Firmicutes* with relative abundance ranging from 80.5% to 85.6%, followed by *Proteobacteria* (10.2–13.4%), *Actinobacteria* (2.5–5.6%), and *Bacteroidetes* (0.6–1.4%) (Figure 8a). The core genera were *Staphylococcus* (36.13–66.17%) and *Streptococcus* (13.60–36.89%) (Figure 8c). Less abundant genera, such as *Pseudomonas* (0.57-2.67%), *Acinetobacter* (0.30–2.24%), *Corynebacterium* (0.53-1.22%), and *Cutibacterium* (0.33-1.18%) were found in all samples. Nearly all human milk samples contained *Rothia* (0.03-1.93%), except for one from the P group. The relative abundances of *Lactobacillus* (0.26–0.76% abundance) and *Bifidobacterium* (0.01–2.71% abundance) in the samples tested were 85% and 55%, respectively. *Enterobacter*, *Klebsiella*, and *Gemella* were present in at least 85% of the samples.





(c)

Figure 8. Relative abundance in human milk samples by 16S rRNA gene-sequencing at the phylum (**a**); at the family (**b**); and at the genus levels (**c**). Preterm group P comprised of mothers who gave birth before 37 weeks of gestation and HM was collected between 3 to 12 days postpartum. Preterm group PT consisted of the same mothers as the P group, but samples were collected when the infants reached the age of term equivalent (TEA, 37-42 weeks postmenstrual age). Full-term group (T) consisted of mothers who gave birth at or after 37 weeks of gestation.

5.1.3 Evolution of the HMM Composition from Preterm Birth to TEA (P versus PT)

Alpha diversity of the HMM composition was stable over time (Figure 9). In addition, the NMDS plot (Figure 10), indicates that there was greater data dispersion in group PT compared to group P, showing a non-defined pattern while the PERMANOVA analysis did not show changes in the HMM composition over time when comparing groups P and PT.

In more detail, no differences were observed at phylum level between the two timepoints (P *vs.* PT). At family level, *Carnobacteriaceae* relative abundance was found to increase over time from 0.02 % to 0.77 % (p = 0.028). When looking for differences at the genus level, the relative abundance of *Dolosigranulum* and *Rothia* was increased with the passage of time from 0.01 % to 0.52 % (p = 0.043, Table 4) and from 0.03 % to 0.76% (p = 0.021, Table 4), respectively. Likewise, *Lactobacillus*

and *Veillonella* tended to increase from preterm birth towards TEA (0,27 % in P *vs.* 0,74 % in PT, $p = 0{,}090$; and 0,51 in P *vs.* 2,69 % in PT, $p = 0{,}070$; Table 4), but this tendency was not significant. Finally, the relative abundance of *Enterobacter* significantly decreased from 1.80 % to 0.06 % ($p = 0.028$, Table 4) over time.

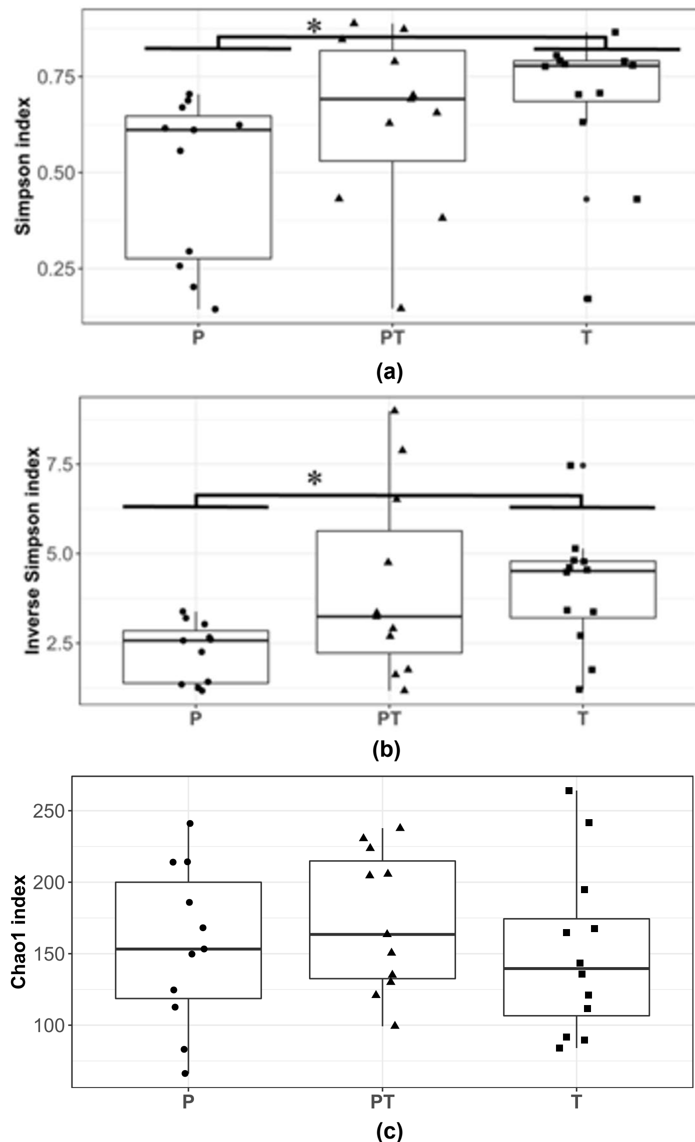


Figure 9. Alpha diversity and richness indices of human milk samples. (**a**) Simpson, (**b**) Inverse Simpson, and (**c**) Chao1 indices. Preterm group P: mothers who gave birth before 37 weeks of gestation (HM collection: 3-12 days postpartum). Preterm group PT: same mothers as the P group (HM collection: when the infants reached 37-42 weeks postmenstrual age). Full-term group T: mothers who gave birth at or after 37 weeks of gestation. Mann–Whitney U test for independent non-parametric samples, * $p < 0.05$.

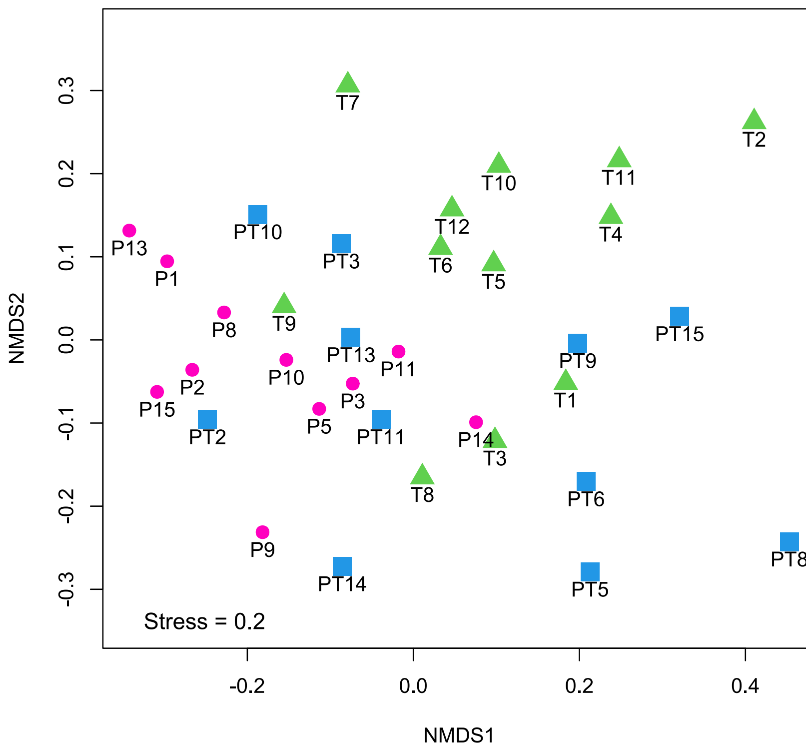


Figure 10. Non-metric multidimensional scaling (NMDS) plot corresponding to the human milk samples. Human milk samples were grouped into three time points according to the postmenstrual age at the time of sampling: preterm (P: <37weeks), term equivalent age TEA (PT: 37-42 weeks), and term (T: 37–41 weeks).

Table 4. Summary of the significant differences between the studied groups at the genus level.

Phylum	Family	Genus	Groups		*p*-value
			P	**PT**	
Firmicutes	*Carnobacteriaceae*	*Dolosigranulum*	0,01	0,52	0,043[2*]
	Veillonellaceae	*Veillonella*	0,51	2,69	0,070[2]
Proteobacteria	*Enterobacteriaceae*	*Enterobacter*	1,80	0,06	0,028[2*]
	Pseudomonadaceae	*Pseudomonas*	1,04	0,56	0,075[1]
Actinobacteria	*Micrococcaceae*	*Rothia*	0,03	0,76	0,021[2*]
			PT	**T**	
Firmicutes	*Veillonellaceae*	*Veillonella*	2,69	0,37	0,051[1]
	Lactobacillaceae	*Lactobacillus*	0,74	0,27	0,091[1]
Proteobacteria	*Beijerinckiaceae*	*Methylobacterium*	0,19	0,06	0,013[1*]
	Burkholderiaceae	*Ralstonia*	0,00	4,49	0,006[1*]
	Pseudomonadaceae	*Pseudomonas*	0,56	2,71	0,037[1*]
Actinobacteria	*Atopobiaceae*	*Atopobium*	0,29	0,00	0,013[1*]
	Propionibacteriaceae	*Cutibacterium*	0,33	0,75	0,016[1*]

Phylum	Family	Genus	Groups		p-value
			P	T	
Firmicutes	*Staphylococcaceae*	*Staphylococcus*	66,26	36,65	0,004[1*]
	Streptococcaceae	*Streptococcus*	13,57	37,33	0,007[1*]
Proteobacteria	*Burkholderiaceae*	*Ralstonia*	0,00	4,49	0,006[1*]
	Xanthomonadaceae	*Stenotrophomonas*	0,00	0,15	0,037[1*]
	Rhodobacteraceae	*Paracoccus*	0,10	0,01	0,044[1*]
Actinobacteria	*Micrococcaceae*	*Rothia*	0,03	1,95	0,000[1*]

[1] Student's T-test for independent samples for parametric data or Mann-Whitney U test for non-parametric data.
[2] Dependent samples T-test for parametric data or Wilcoxon signed rank test for non-parametric data.
* p <0.05, significant

5.1.4 Differences In Human Milk Microbiota Profiles Between Mothers of Preterm Infants at TEA And Full-Term Infants (PT versus T)

There were no differences between term milk and milk at TEA (group T and group PT, respectively) regarding the alpha diversity and richness (Figure 9). Milk at TEA presented a higher data dispersion in comparison to term milk while the PERMANOVA analysis did not show significant differences between the two milk groups.

At the phylum level, no significant differences in the relative abundance were observed between milk at TEA and milk from term pregnancies (Figure 8a). At the family level, HM from TEA had a higher abundance of *Atopobiaceae* (0.29 *vs.* 0.00 %, p = 0.013), *Veillonellaceae* (2.70 *vs.* 0.43 %, p = 0.044) and *Beijerinckiaceae* (0.29 *vs.* 0.09 %, p = 0.016) when compared to term milk (Figure 8b). In contrast, term milk was richer in *Propionibacteriaceae* (0.75% relative abundance compared to 0.33%, p = 0.019) and *Pseudomonadaceae* (2.71 compared to 0.56%, p = 0.037) in comparison to milk at TEA.

At the genus level, term milk was richer in *Ralstonia* (4.49 *vs*. 0.00 %, p = 0.006), *Cutibacterium* (0.75 *vs*. 0.33 %, p = 0.016, Table 4) and *Pseudomonas* (2.71 *vs*. 0.56 %, p = 0.037, Table 4) than milk at TEA, but had lower abundances of *Atopobium* (0.00 *vs*. 0.29 %, p = 0.013, Table 4) than milk at TEA. A trend towards a reduction in *Veillonella* content was observed in term milk compared to milk at TEA, but it was not significant (2.69 *vs.* 0.37 %, p = 0.051, Table 4). Furthermore, *Staphylococcus haemolyticus* was found in lower abundance in term milk compared to milk at TEA (0.00 *vs*. 0.08 %, p = 0.004, Table 4).

5.1.5 Differences In Human Milk Microbiota Profiles Between Mothers of Preterm and Full-Term Infants (P versus T)

Term milk, in comparison to preterm milk had higher alpha diversity indices when the Simpson ($p = 0.004$) and Simpson Inverse ($p = 0.005$) indices were considered. Nevertheless, no significant differences were observed for richness according to the Chao1 index ($p = 0.840$) (Figure 9). The NMDS analysis revealed that the preterm milk samples were positioned towards the left side of the plot, whereas the term milk samples were positioned towards the right side of the plot (Figure 10). When the taxonomic compositions were analyzed by PERMANOVA, the preterm milk samples were significantly different from the samples of term milk ($p = 0.004$). A SIMPER analysis determined the OTUs that were responsible for the observed differences: OTU_1, assigned as *Staphylococcus*, contributed up to 30% of the observed differences, while OTU_2 and OTU_3 contributed up to 54%, both identified as *Streptococcus*.

At the phylum level, no differences were observed between the two different milk groups in terms of relative abundance (Figure 8a). At the family level however, term milk had higher levels of *Streptococcaceae* (37.41 *vs*. 13.59%, $p = 0.006$) and *Micrococcaceae* (2.01 *vs*. 0.14%, $p = 0.001$) than preterm milk (Figure 8b). In contrast *Staphylococcaceae* (66.27 *vs*. 36.65%, $p = 0.004$) and *Rhodobacteraceae* (0.13 *vs*. 0.03%, $p = 0.019$) were in higher abundance in preterm milk than in term milk. Looking closer at the genus level, preterm milk was richer in *Staphylococcus* (66.26 *vs*. 36.65%, $p = 0.004$, Table 4) and *Paracoccus* (0.10 *vs*. 0.01%, $p = 0.044$, Table 4) and had lower abundances of *Streptococcus* (13.57% *vs*. 37.33%, $p = 0.007$, Table 4), *Ralstonia* (0.00% *vs*. 4.49%, $p = 0.006$, Table 4), *Rothia* (0.03% *vs*. 1.95%, $p = 0.000$, Table 4), and *Stenotrophomonas* (0.00% *vs.* 0.15%, $p = 0.037$, Table 4) that term milk.

Considering the abundance and significance of *Staphylococcus* in HM, we decided to identify potential species from this genus. Upon analysis of all collected HM samples, the following *Staphylococcus* species were detected: *Staphylococcus aureus* (OTU_6), *Staphylococcus haemolyticus* (OTU_7, OTU_9, OTU_13, and OTU_23), and a non-cultivable species of *Staphylococcus* (OTU_1 and OTU_4).

When looking into the prevalence of *Staphylococcus* in the two milk groups we found that term milk had significantly higher levels of *S. aureus* compared to preterm milk (10.12% *vs*. 0.30%, $p = 0.000$). In contrast, preterm milk had a higher abundance of *S. haemolyticus* (0.20% *vs*. 0.00%, $p = 0.007$) and non-cultivable species (65.77% *vs*. 26.52%, $p = 0.002$) than term milk.

5.1.6 Human Milk Microbiota and its Association with Delivery Mode, Antenatal Corticoids, Antibiotics, Gestational and Term Equivalent Ages

Gestational age and weight at birth differed among groups (Table 3). The administration of prenatal antibiotics and corticosteroids was higher in the mothers of the preterm group, compared to that of mothers who gave birth at term. In fact the administration of antibiotics to prospective mothers prior to caesarean sections and prenatal corticosteroids to improve neonatal outcomes after preterm birth (Thevathasan et al., 2023). No significant association was observed between HMM composition at phylum level and mode of delivery, prenatal corticoids, or antibiotics (Table 5 and Figure 11).

Table 5. Regression coefficients and significances of clinical data association with milk microbiota at phylum, genera levels, and alpha diversity indices.

Comparison	**β**	***p*-value**
Delivery mode *vs*. *Actinobacteria* phylum	0.124	0.484
Delivery mode *vs*. *Bacteroidetes* phylum	-0.154	0.384
Delivery mode *vs*. *Firmicutes* phylum	0.052	0.771
Delivery mode *vs*. *Proteobacteria* phylum	0.021	0.904
Delivery mode *vs*. Chao1 index	-0.167	0.351
Delivery mode *vs*. Inverse Simpson index	0.057	0.749
Delivery mode *vs*. Shannon index	-0.005	0.976
Antenatal Corticosteroids *vs*. *Actinobacteria* phylum	-0.079	0.659
Antenatal Corticosteroids *vs*. *Bacteroidetes* phylum	0.063	0.663
Antenatal Corticosteroids *vs*. *Firmicutes* phylum	0.137	0.441
Antenatal Corticosteroids *vs*. *Proteobacteria* phylum	0.065	0.716
Antenatal Corticosteroids *vs*. Chao1 index	0.133	0.455
Antenatal Corticosteroids *vs*. Inverse Simpson index	-0.259	0.125
Antenatal Corticosteroids *vs*. Shannon index	-0.064	0.718
Antibiotics *vs*. *Actinobacteria* phylum	-0.004	0.984
Antibiotics *vs*. *Bacteroidetes* phylum	0.171	0.334
Antibiotics *vs*. *Firmicutes* phylum	0.139	0.538
Antibiotics *vs*. *Proteobacteria* phylum	-0.154	0.383
Antibiotics *vs*. Chao1 index	0.152	0.391
Antibiotics *vs*. Inverse Simpson index	-0.262	0.134
Antibiotics *vs*. Shannon index	-0.121	0.495

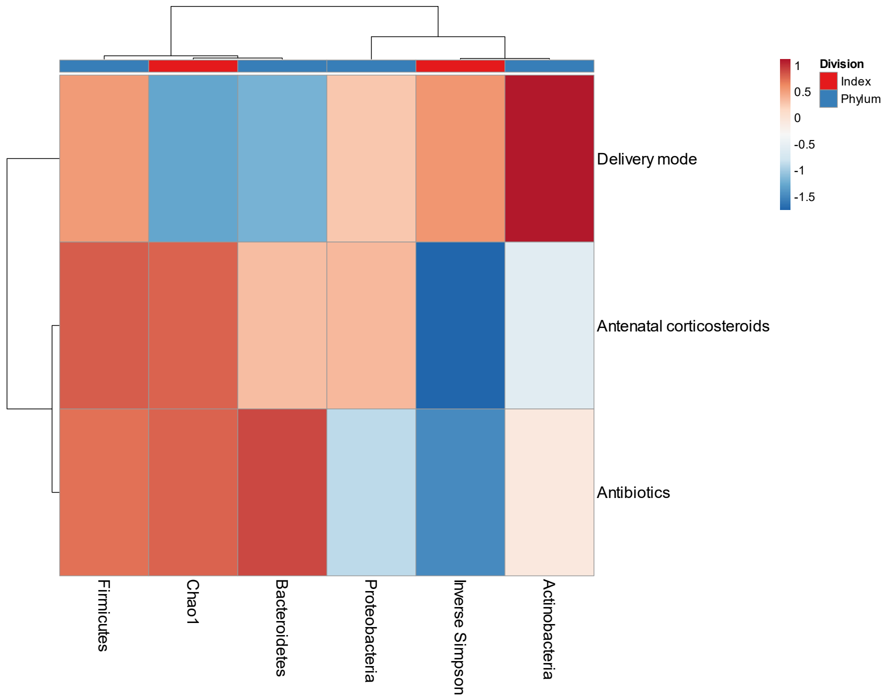


Figure 11. Clinical data association with milk microbiota composition at phylum and alpha diversity indices. Delivery mode: Cesarean section/Vaginal. Prenatal administration of antibiotics and corticoids: Yes/No. β coefficients of univariate associations with linear regression are visualized for taxa abundance and alpha diversity index value. *$p < 0.05$, **$p < 0.001$.

Correlations between HMM and gestational age and HMM and TEA were assessed by Spearman rank correlation. More specifically, higher gestational age was associated with a higher abundance of *Proteobacteria*, *Pseudomonas* and *Cutibacterium*. Finally, higher TEA was positively correlated with *Actinobacteria*, *Cutibacterium*, *Rothia* and *Streptococcus* and negatively correlated with *Staphylococcus* (Figure 12).

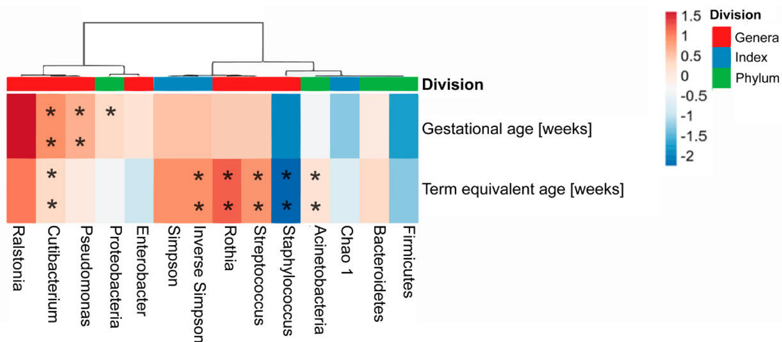


Figure 12. Human milk microbiota associated with gestational, and term equivalent ages (per weeks). Spearman's rank correlation coefficient was performed on the taxa abundance at the phylum and genera (>1%) levels and alpha diversity index values. * $p < 0.05$, ** $p < 0.001$.

5.2 Storage of Human Milk and its Effect on Microbial Stability (II and III)

The second study (**II**) showed that the viability of probiotic cells was not significantly reduced when LGG or Bb12 was mixed in HM and stored at room temperature, 4°C, and -20°C for 6 hours, 72 hours, and 7 days ($p > 0.05$, Figure 13a and 13b).

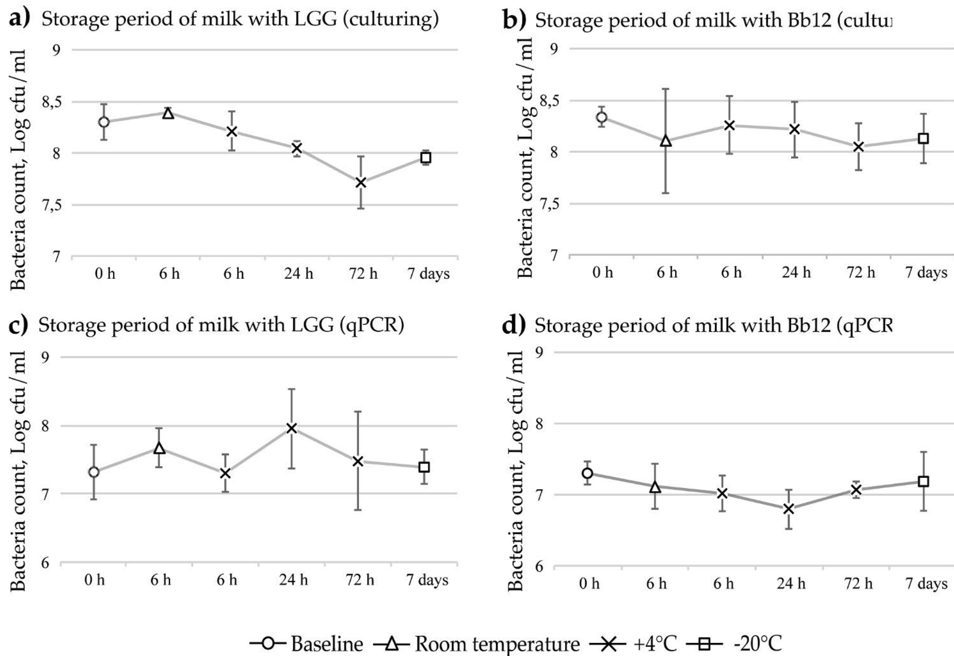


Figure 13. Average bacteria count of probiotic-supplemented human milk samples stored in different conditions. (**a**) Samples of human milk supplemented with LGG evaluated by conventional culturing. (**b**) Samples of human milk supplemented with Bb12 evaluated by conventional culturing. (**c**) Samples of human milk supplemented with LGG evaluated by qPCR. (**d**) Samples of human milk supplemented with Bb12 evaluated by qPCR.

Although not statistically significant, the greatest drop in cell viability for both probiotics occurred after 72 hours at 4°C when accessed by traditional culturing (LGG: 0.6 (SD 0.3); Bb12: 0.3 (SD 0.2)) (Table 6). The qPCR analysis confirmed the culturing results; however, when supplemented in HM and stored at room temperature for 6 hours, the copy number count of LGG increased slightly ($p = 0.046$) (Table 7). Generally, when the stability of both probiotic strains in HM during storage was assessed by qPCR, we found copy number counts that were one log lower than the viable counts detected using the culturing method (Figure 13c and 13d). Slight differences in concentrations detected by the qPCR method between

different time points could be due to DNA loss during the extraction and purification procedures. When the milk used as control was cultured, we were able to isolate only two bacterial species identified as *Staphylococcus epidermidis* (211 CFU/mL) and *Staphylococcus warneri* (61 CFU/mL).

Table 6. Results of conventional plate count analysis for probiotic counts (means log CFU/mL and SD) when added in HM (n=3) and stored under different conditions

	LGG	**Bb12**
0h at Room temperature	8.3 (SD 0.2)	8.3 (SD 0.1)
6h at Room temperature	8.4 (SD 0.1)	8.1(SD 0.5)
6h at +4ºC	8.2 (SD 1.2)	8.3 (SD 0.3)
24h at +4ºC	8.0 (SD 0.1)	8.2 (SD 0.3)
72h at +4ºC	7.7 (SD 0.3)	8.1 (SD 0.2)
7d at -20ºC	8.0 (SD 0.1)	8.1 (SD 0.2)
6h at RT *vs*. baseline (*p*-value)*	0.046	0.447
7d at -20ºC *vs*. baseline (*p*-value)*	0.056	0.296
ANOVA (*p*-value)	0.162	0.263

*paired t-test
SD = standard deviation

Table 7. Results of quantitative PCR analysis for probiotic counts (means log CFU/mL and SD) when added in HM (n=3) and stored under different conditions

	LGG	**Bb12**
0h at Room temperature	7.3 (SD 0.4)	7.3 (SD 0.2)
6h at Room temperature	7.7 (SD 0.3)	7.1 (SD 0.3)
6h at +4ºC	7.3 (SD 0.3)	7.0 (SD 0.3)
24h at +4ºC	8.0 (SD 0.6)	6.8 (SD 0.3)
72h at +4ºC	7.5 (SD 0.7)	7.1 (SD 0.1)
7d at -20ºC	7.4 (SD 0.3)	7.2 (SD 0.4)
6h at RT *vs*. baseline (*p*-value)*	0.549	0.530
7d at -20ºC *vs*. baseline (*p*-value)*	0.789	0.612
ANOVA (*p*-value)	0.443	0.247

*paired t-test
SD = standard deviation

Similar to fresh HM, fortified DHM supplemented with LGG or Bb12 was a good matrix for preserving the viability of both probiotic preparations as colony counts of LGG and Bb12 were maintained during storage at 4°C for 24 hours ($p >$

0.05, Figure 14) (**III**). However, there was a significant difference in the CFU/mL of both bacteria between baselines of liquid and powder FDHM (LGG: 7.9 log_{10} CFU/mL (liquid FDHM), 7.7 log_{10} CFU/mL (powder FDHM), $p < 0.001$; Bb12: 7.8 log_{10} CFU/mL (liquid FDHM), 7.7 log_{10} CFU/mL (powder FDHM), $p < 0.001$).

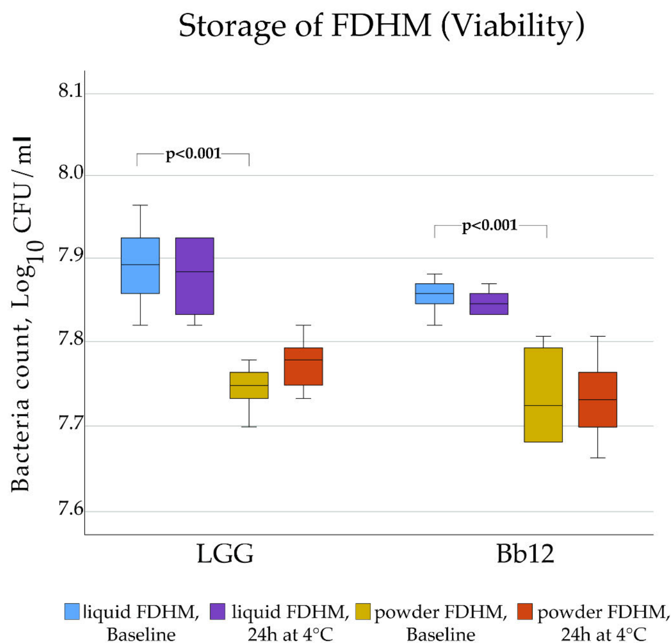


Figure 14. Stability of LGG and Bb12 in fortified donor human milk (FDHM) after storage at 4°C for 24 h is presented with box plots.

5.3 Probiotic adhesion affected by donor human milk (III)

The adhesion percentage varied between LGG and Bb12; nevertheless, both strains demonstrated a tendency for high levels of adherence to infant mucus. The adhesion assay conducted on the control sample, where probiotics were dispersed in HH buffer, revealed that the adhesion levels of LGG were comparable when combined with Bb12 (56.3%) and when LGG was evaluated as a single strain (46.3%, $p = 0.19$; Figure 15a). Similar observations were made for Bb12 where its combination with LGG resulted in an adhesion percentage of 34%, while Bb12 alone resulted in an adhesion of 32.5% ($p = 0.72$; Figure 15b). In contrast to the HH buffer, DHM significantly reduced the adhesion ability of the two probiotics. (LGG: 11.3%, $p < 0.001$; Bb12: 19.1%; $p = 0.012$).

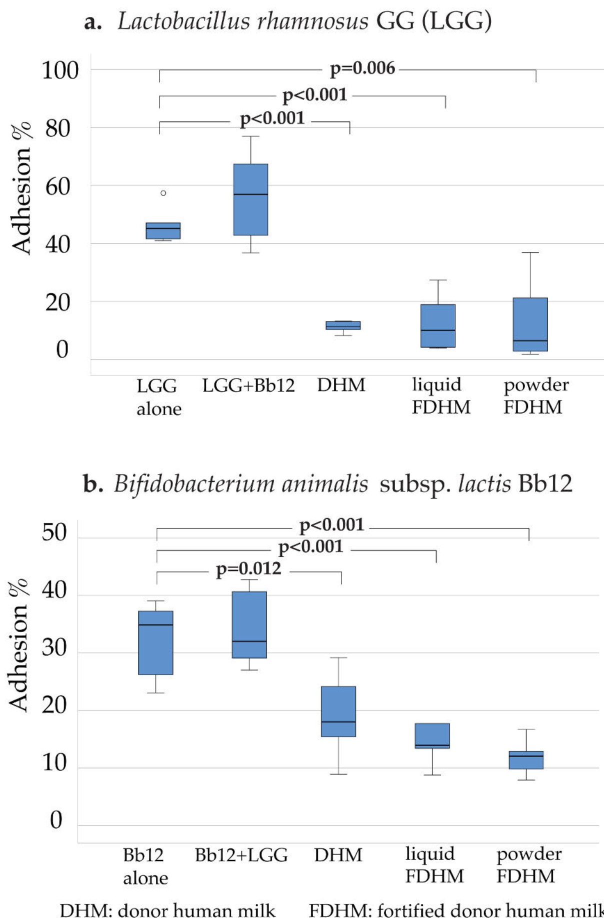


Figure 15. The adherence of LGG (**a**) and Bb12 (**b**) to human intestinal mucus glycoproteins *in vitro* as presented with box plot with outliers marked with a circle (o).

While the type of fortifier had little effect on the adhesion ability of either probiotic, the overall presence of fortifier had a negative impact on the adhesion of LGG and Bb12. In more detail, both liquid FDHM (12.4%, $p < 0.001$) and powdered FDHM (13.8%, $p = 0.006$) lowered the adhesion capacity of LGG to the infant intestinal mucus. Similar findings were observed for Bb12: liquid FDHM: 14.3%, $p < 0.001$; powdered FDHM: 11.9%; $p < 0.001$).

In fact, the next best adhesion levels of Bb12 besides those achieved in the control sample (HH buffer), were obtained when Bb12 was added to the mucus after it had been treated with powder FDHM (21.5%). These adhesion levels were significantly higher than when Bb12 was mixed in powder FDHM and then added to the infant mucus (11.9%, $p = 0.012$; Figure 16b).

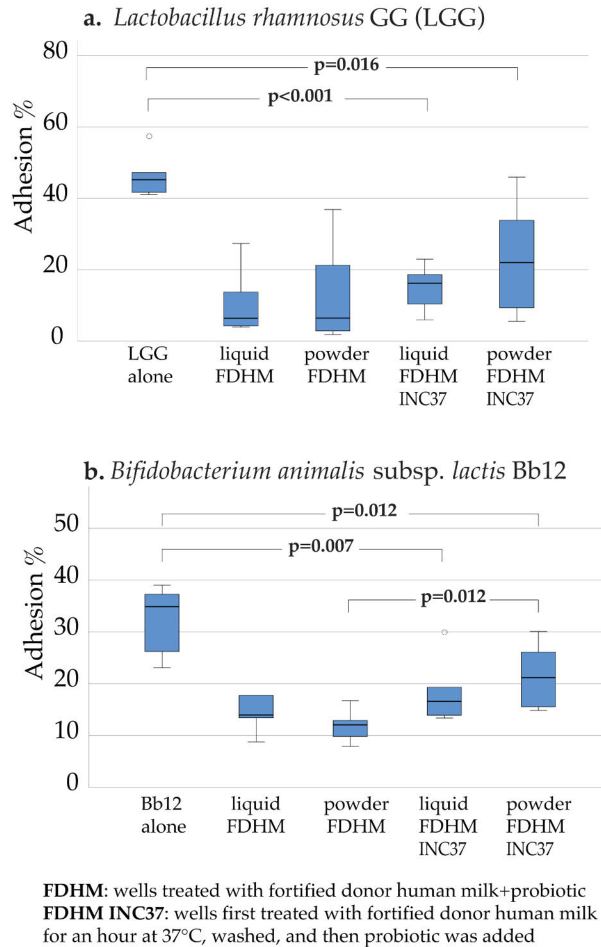


Figure 16. Mucus adhesion of LGG (**a**) and Bb12 (**b**) in fortified donor human milk (FDHM) or after initial mucus treatment with FDHM as presented with box plot with outliers marked with a circle (o).

The adhesion of probiotics to mucus was found to be significantly influenced by the subject age, with the specific strain employed also playing a crucial role. LGG had a higher tendency for adhesion to mucus obtained from infants over 6 months old than to the mucus obtained from infants under 6 months old, although the difference was not found to be statistically significant (43.3%; $p = 0.26$; Figure 17). In contrast, the adhesion of Bb12 to mucus obtained from infants aged less than six months was significantly higher (37.3%; $p = 0.05$) compared to mucus obtained from infants aged over six months (27.8%).

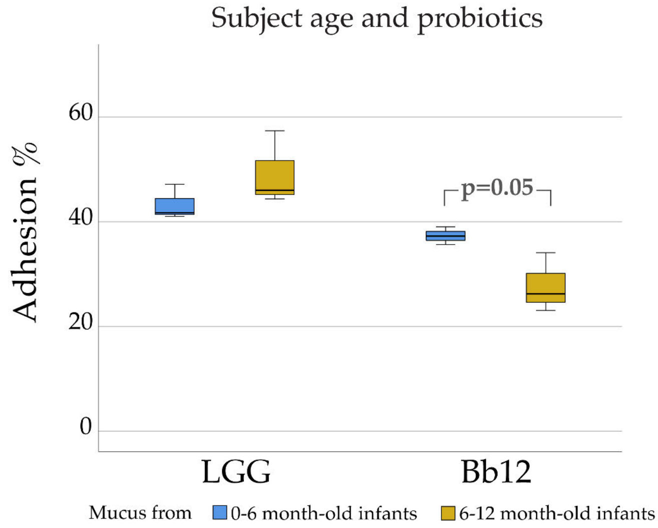


Figure 17. Adhesion of LGG and Bb12 as affected by age of mucus donor. Box-plot graphs represent the median and quartiles of adhesion (%) for the three independent experiments in triplicate.

Finally, storage did not significantly affect probiotic adhesion to intestinal mucus (Figure 18). Interestingly, only Bb12 in liquid FDHM saw a notable decline in its adhesion capacity after storage for 24h at 4°C (Baseline: 14.3%, Storage: 11.9%; $p = 0.034$).

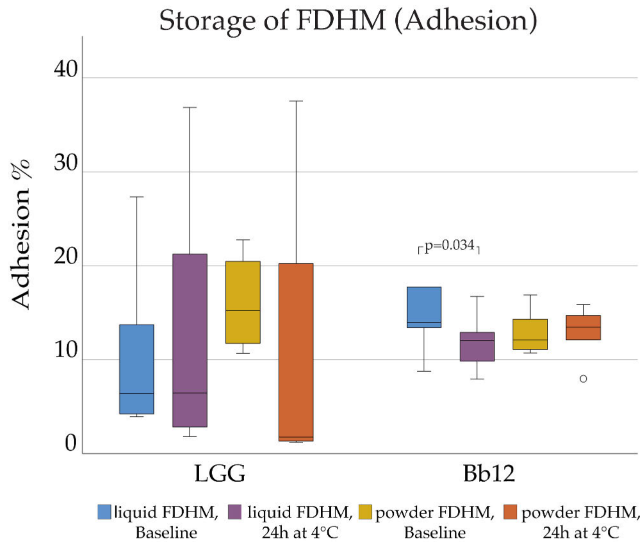


Figure 18. Mucus adhesion of LGG and Bb12 in fortified donor human milk (FDHM) after storage at 4°C for 24 h are presented with box plots. Outliers are marked with a circle (o).

5.4 Probiotic and pathogen adhesion modulated by exogenous polyamines (IV)

Overall, in the absence of polyamines LGG, Bb12, and *C. sakazakii* presented higher adherence to the adult mucus than to the infant mucus (Figure 19). Both probiotic strains examined in the present study exhibited similar adhesive capacity to our mucus model in line with previous studies (Harata et al., 2021; Ouwehand, Niemi, et al., 1999). LGG and Bb12 showed the lowest adherence when added to the mucus of infants aged 0–6 months. However, the presence of PAs resulted in altered adhesion patterns (Figure 19). These changes were observed to be dependent on the specific type of polyamines and their respective concentrations (Figure 20).

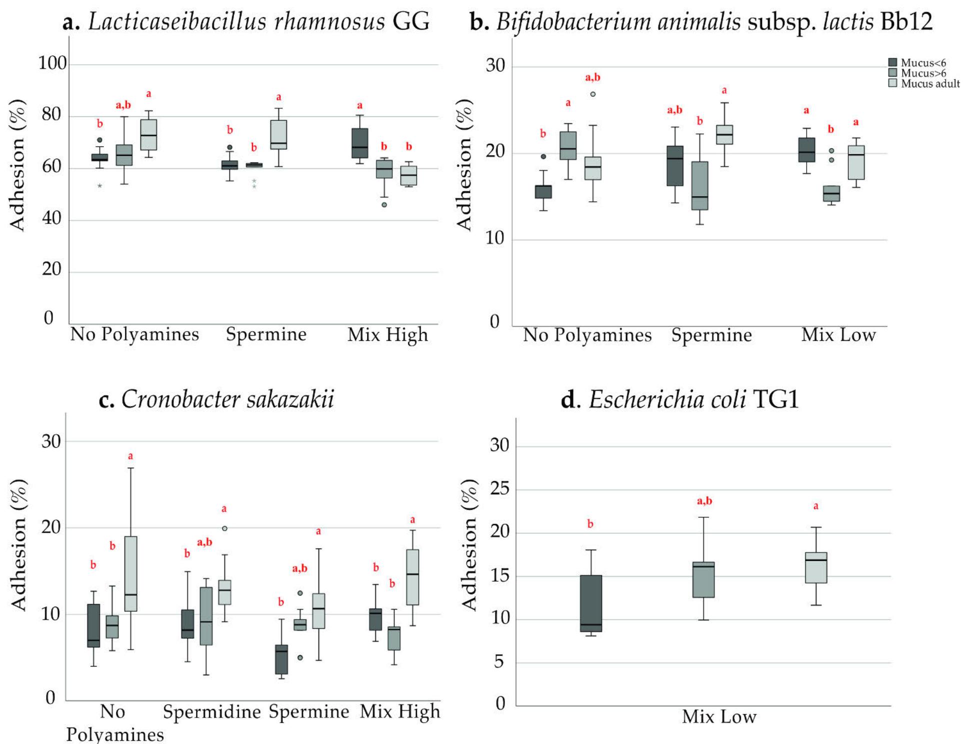


Figure 19. Age-dependent adhesion properties of (a) LGG, (b) Bb12, (c) *Cronobacter sakazakii*, and (d) *E. coli* TG1 in the presence of exogenous polyamines. Box plots represent the median and quartiles of adhesion percentage. The letters are indicating significant differences at $p < 0.05$ when comparing mucus from different age categories.

The adhesion capacity of LGG between the different types of PAs was not significantly different between the two infant groups. Despite that, PUT and SPD (No PAs: 63.5%, PUT: 67.5%, SPD: 68.9%; Figure 20a) appeared to slightly

promote the adhesion of LGG in the younger age group, although not significantly at the concentration tested. The results however for the adult group were different compared to the infant group. In the adult mucus, LGG adhesion was decreased significantly by the high PAs com- bination (No PAs: 73.5%, high PAs: 57.7%; $p < 0.05$; Figure 20a).

Bb12 exhibited better adhesion properties to the mucus of the older infant group (20.8%) than to the mucus originating from the feces of infants aged <6 months (16.1%, $p < 0.05$; Figure 19b). Additionally, Bb12 was able to adhere significantly better in mucus from the youngest group of infants in the presence of SPD (No PAs: 16.1%, SPD: 20.3%; $p = 0.05$) and well in low PAs combination (No PAs: 16,1%, low PAs: 20.2%; $p = 0.055$; Figure 20b).

Regarding *C. sakazakii*, our results show that adhesion to intestinal mucus of adults was higher than to infant mucus (0–6-month-old: 8%, 6–12-month-old: 8.9%, adult: 14.9%; Figure 19c). The adhesion levels of *C. sakazakii* in adult mucus in our study are in line with those previously reported by Collado and coworkers (M. C. Collado et al., 2008). Specifically for the mucus obtained in early infancy (0-6 months), we found that SPM was significantly more effective than PUT and polyamine combinations in high concentrations in reducing the adhesion of *C. sakazakii* ($p < 0.05$; Figure 20c).

The adhesion of *E. coli* TG1 in our study was moderate (10–20%) compared with previously reported for pathogenic *E. coli* NCTC 8603 and was independent of the presence of exogenous polyamines at any age in the concentrations tested (M. C. Collado et al., 2005, 2006). Despite the non-significant effect, worth mentioning is that SPM tended to slightly decrease the adhesion of *E. coli* to the mucus isolated from the younger age group (No PAs: 12.5%, SPM: 11%; Figure 20d) while in the presence of low concentration of polyamines, *E. coli* adhesion was significantly lower in the early infant mucus (11.5%) than in adult mucus (16.1%, $p < 0.05$, Figure 19d).

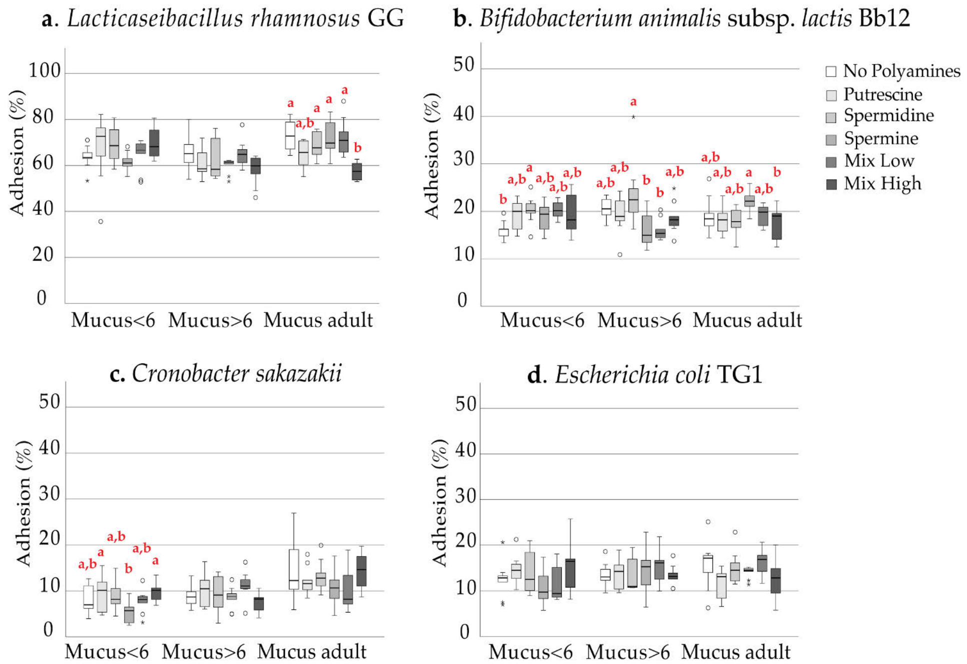


Figure 20. Adhesion to human intestinal mucus of (**a**) LGG, (**b**) Bb12, (**c**) *Cronobacter sakazakii,* and (**d**) *E. coli* TG1 in the presence of exogenous polyamines. Box plots represent the median and quartiles of adhesion percentage. Different letters indicate statistically significant differences at $p < 0.05$ among polyamine exposure for each type of mucus.

6 Discussion

6.1 Human Milk Microbiota Composition is impacted by Gestational Age and alters with time (**I**)

Research indicates that HM does not only offer vital nutrients but also plays a critical role in immune development and the establishment of a healthy GM in infants, influencing their long-term health and development. The microbiota in HM is crucial for forming the infant's GM. Studies highlight that microbes in HM contribute significantly to the infant's GM. For instance, Le Doare and colleagues emphasized the importance of HM in providing most microbes needed for the development of the infant GM (Le Doare et al., 2018). Additionally, it is reported that HM microbes constitute around 25% of the infant's GM (Pannaraj et al., 2017; Selma-Royo, Calvo Lerma, et al., 2021).

As discussed in chapter 2.2.1.2.3 The Human Milk Microbiota, perinatal factors like geographical location shape the HM bioactive components, including the microbial profile of HM. For example, Chinese mothers have higher levels of *Streptococcus* in their milk than mothers from South Africa, Finland, and Spain. On the other hand, the milk of Spanish mothers is richer in *Cutibacterium* and *Pseudomonas* (Kumar et al., 2016). Likewise a recent study in Dubai showed differences between Emirati and UAE-expatriated women in terms of the HMM configuration (Ayoub Moubareck et al., 2020). More specifically it was shown that Dubai-based mothers had significantly lower *Bifidobacterium* and *Lactobacillus* levels in their milk while *Pseudomonas* was detected at a higher abundance compared to the milk of mothers from other countries. Altogether, the aforementioned studies have unveiled the influence of geographic location on the composition of HMM, underscoring the significance of understanding and characterizing the HMM of the Argentinean population. Therefore, in study **I**, we aimed to define the HMM composition of Argentinian mothers and the differences between samples obtained from mothers who delivered preterm and full-term infants. When comparing between our study and the findings of Padilha and colleagues who investigated Brazilian HM, we noted that while the levels of *Proteobacteria* and *Bacteroidetes* remained similar between Argentinian and

Brazilian HM, notable differences were observed in other bacterial phyla (Padilha, Danneskiold-Samsøe, et al., 2019).. In Argentinian HM, the *Firmicutes* phylum accounted for over 80% and the *Actinobacteria* phylum was less than 6%, contrasting with Brazilian HM, where *Firmicutes* and *Actinobacteria* constituted 70% and 14%, respectively. Despite Argentina and Brazil being neighboring countries in Latin America, these variations in HMM highlight the significant influence of geographic location, along with other factors, in determining the composition of HMM.

Overall all genera observed in our study were part of the 10 more frequently found genera in HM identified in the systematic review of Zimmermann and Curtis (Zimmermann & Curtis, 2020). In more detail, our data agreed with previous studies that had identified a predominance of the genera *Staphylococcus* and *Streptococcus* in HM (Fernández et al., 2020; Fitzstevens et al., 2017; Stinson et al., 2021). Specifically two of the most known studies on the HMM composition, the MAMI (MAternal MIcrobes study) and CHILD (Canadian Healthy Infant Longitudinal Development Study) identified a "core genera" of the HMM which primarily includes *Staphylococcus* and *Streptococcus* (Cortes-Macías et al., 2021; Fehr et al., 2020). This observation in our study population could be related to the maternal diet that may modulate specific genera in HM as discussed in chapter 2.2.1.2.3 The Human Milk Microbiota. Recent studies have shown that diets rich in carbohydrates, trans-fat, or saturated fatty acids are correlated with an increased presence of *Staphylococcus* in HM (Azagra-Boronat et al., 2020; LeMay-Nedjelski, Asbury, et al., 2020). Additionally, a study by Tavella and colleagues examining trans and saturated fats in various food items revealed that Argentinian products have higher contents of trans fatty acids compared to similar products from other parts of the world (Tavella et al., 2000). Moreover, half of the female Argentinian population has been classified as overweight or obese, and the typical daily diet includes a high intake of sugars, refined grains, white bread, rice, red meats, and saturated fats (Becaria Coquet et al., 2020; Instituto Nacional de Estadística y Censos (INDEC)—Secretaría de Gobierno de Salud de la Nación, 2019).

Another genus present in HM but less abundant was *Corynebacterium* which is also a commensal of the skin and other mucous membranes (S. Park et al., 2016). There are reports linking *Corynebacterium* isolated from the placenta and vaginal tract of pregnant women with preterm birth outcomes respectively (Onderdonk et al., 2008; Shukla et al., 2003). *Corynebacterium* is also relevant from the preterm neonate perspective due to potential cause of fatal infections and is known as one of the bacteria causing infectious mastitis (Berner et al., 1997; Mediano et al., 2017),

A further objective of this study was to understand whether the HMM profile of preterm milk will resemble the one of term milk when the infant born preterm would reach the TEA (>37-41 weeks). Understanding this point could contribute to novel

approaches to address the nutritional management of preterm neonates. When examining the changes in the preterm HMM from birth to TEA, we observed that Inverse Simpson diversity significantly increased over time. A recent study that looked for changes in the alpha diversity of preterm MOM during post-partum weeks 1–12, reported similar results (Filatava et al., 2023). Consistent with our findings, Filatava and colleagues found that preterm HM was dominated by *Firmicutes* (76.2%), *Proteobacteria* (12.8%), and *Actinobacteria* (9.5%). Another commonality of the two studies is identified at the genus level, where *Streptococcus* and *Rothia* tended to increase with time, while *Staphylococcus* tended to decrease. The same findings regarding *Streptococcus* and Staphylococcus were confirmed by Asbury and co-workers (Asbury et al., 2020). *Streptococcus* and *Rothia* are microorganisms normally present in the oral cavity of infants (Ruiz et al., 2019) which in addition to the present study have been identified in preterm HM after the infants had breastfed (Biagi et al., 2018). These findings support the hypothesis of neonatal oral microbe retrograde transfer to the mammary gland. In turn, the same study showed that the provision of *Streptococcus* and *Rothia* to those infants through breastfeeding was associated with increased levels of *Bifidobacterium* in their feces. Although we can only speculate, it could be possible that the increase in fecal bifidobacteria could be due to interactions between *Streptococcus* and *Rothia* with bifidobacteria present in the HM. However, this needs to be elucidated in future studies focusing on understanding microbe-microbe interactions in HM.

Another genus whose abundance increased over time was *Dolosigranulum* which was previously identified in HM with its abundance being inversely associated with the number of respiratory tract infections experienced (Cheema et al., 2022; Lif Holgerson et al., 2023; Selma-Royo, Calvo Lerma, et al., 2021). In contrast, a reduction in the *Enterobacter* levels was observed from preterm birth to TEA. This genus was also recently identified by Japanese scientist in preterm HM (Miura et al., 2023). The probable origin of *Enterobacter* in HM could be the maternal areolar skin or the offspring's mouth (Kordy et al., 2020). Since *Enterobacter* species are often responsible for NICU infections we consider its reduction in TEA as positive. However, presence of *Enterobacter* in preterm HM could potentially increase the risk for nosocomial infections in NICUs. Indeed, earlier studies have indicated that *Enterobacter* is a predominant bacterium in the gut of preterm infants, and this event has been linked to an increased likelihood of developing NEC (Korpela et al., 2018).

In terms of differences between preterm and term milks, our research indicates that preterm milk collected in the first few days after birth contains a higher level of *Staphylococcus*, particularly *S. hemolyticus*, compared to term milk from the same period. This observation aligns with findings from other studies in this area (Soeorg et al., 2017). Notably, while HM is generally not a common source of *S. haemolyticus*, its elevated presence in preterm HM has been linked to late-onset

sepsis in preterm neonates. Additionally, the gut and skin of preterm neonates are more frequently colonized with *S. haemolyticus* than those of term infants, as reported in further research by Soeorg and colleagues (Soeorg et al., 2019). One factor that could contribute to the higher levels of *Staphylococcus* in preterm than in term HM is maternal BMI but due to lack of data this remains speculative (Daiy et al., 2022; Filatava et al., 2023). Indeed, the relative abundance of *Staphylococcus* was found to be higher in HM from overweight or obese mothers than in milk from mothers with lower BMIs (Asbury et al., 2020). In contrast, *Streptococcus*, *Ralstonia*, *Rothia*, and *Stenotrophomonas* were more abundant in term milk. As mentioned before, *Streptococcus* and *Rothia* are common oral bacteria and their higher abundance in term milk can be attributed to the fact that term infants are breastfed, whereas premature neonates receive HM through enteral feedings. Consequently, there is no physical contact between the mouth of the preterm neonate and the mother's breast. On the other hand, *Stenotrophomonas* is often detected in hospitals, particularly in the water supply and is a cause of infections (Miura et al., 2023). The differences in the abundance of different genera in HM between preterm and term infants can be attributed to various factors, including the relationship between HMM and HMOs. Studies have begun to elucidate this relationship, with findings indicating that certain bacterial genera are associated with specific HMOs with this association being influenced by the secretor status of the mother (Aakko et al., 2017; Cabrera-Rubio et al., 2019). Secretor status is also modulating the concentration of specific HMOs in preterm milk (Thurl et al., 2017). In addition, preterm milk differs from term in regards to its HMO composition (Austin et al., 2019; Chiurazzi et al., 2021). This could possibly be influencing its microbial configuration. Moreover, a recent study found that HMO concentration and composition in the serum of pregnant women was not only shaped by maternal body composition but also by gestational age and secretor status (Jantscher-Krenn et al., 2019). However, because we did not control for secretor status this remains speculative. Regarding alpha diversity, our study identified differences between preterm and term HM, which contrasts with the findings of Urbaniak and colleagues who reported no significant differences in this regard (Urbaniak et al., 2016). However, it's important to note that our study had a limitation in the relatively small number of HM samples analyzed. This limited sample size could potentially introduce bias into the observed differences, suggesting that our results should be interpreted with caution. Further research with a larger sample size would be beneficial to validate and expand upon these findings.

Finally, when comparing between TEA and term milk, *Pseudomonas,* and *Ralstonia*, which were more prevalent in term HM, had also previously been found at high levels in HM from mothers with acute and subacute mastitis (S. H. Patel et al., 2017). Considering that most episodes of mastitis occur in the first six weeks

after delivery, it would have been more likely to find these genera in milk from TEA. Recent studies have also revealed a loss of microbiome diversity in mastitis cases, but this was not observed in term milk of our study nor we controlled for cases of clinical mastitis in our study (Angelopoulou et al., 2018). It is important to note that we found no differences in the bacterial diversity between TEA and term milk and that the relative abundance of *Rothia* was significantly higher in both groups when compared with preterm milk from the first days after birth. These observations could potentially explain why the GM diversity of exclusively breastfed preterm infants increases with age and is similar to that of the term infant at two years of life (Dahl et al., 2018). Preterm neonates typically reach TEA between 38 to 42 weeks postmenstrual age (Huf et al., 2023). The research conducted by Korpela and colleagues is significant in this context, as it demonstrates that preterm infants fed HM may develop a GM similar to that of term infants (Korpela et al., 2018). Furthermore, their study found a correlation between postmenstrual age and GM maturation in these infants. Considering these insights, along with our own findings, TEA could be considered in future studies when trying to understand HMM maturation in preterm births.

6.2 Fortification and Storage of Human Milk and their Impact on Microbial Stability (**II** and **III**)

In studies **II** and **III** we chose to use LGG and Bb12 as both probiotic strains are generally recognized as safe for infant consumption (Luoto et al., 2010; Szajewska et al., 2010). To exert a beneficial effect on the host, probiotic strains should retain their viability and functional activity throughout the storage period. The probiotic concentrations in food should be between $1x10^6$ and $1x10^8$ CFU/mL at the end of the storage period (Ferdousi et al., 2013). For this reason, a concentration of $1x10^8$ CFU/mL for both probiotic strains for both studies was chosen.

The strains isolated from the DHM in study **II**, namely *S. epidermidis* and *S. warneri*, did not have any negative impact on the viability of LGG and Bb12. However, the concentration of these bacteria was lower (10^2 CFU/mL) than the concentration of LGG or Bb12 (10^8 CFU/mL) which may limit their interaction with the added probiotic strains.

Moreover, in study **II** we showed that the viability of LGG and Bb12 was maintained in unfortified HM for 24h at 4°C, when assessed by both culturing and genetic methods. Since some probiotic strains do not grow well in milk, we hypothesized that the probiotic viability would be compromised during storage under the examined conditions. Our hypothesis was based on the fact that LGG lacks the ability to utilize lactose or intact casein and that Bb12 is an anaerobic microorganism (Kankainen et al., 2009). Additionally it is known that both lactobacilli and

bifidobacteria exibit limited growth in cow's milk although the growth rate could be strain dependent (Champagne et al., 2005; Slačanac et al., 2013).

Previous studies confirm our observations. For instance, LGG can grow at a minimal temperature of 2.7°C in ultrahigh temperature-processed milk (Valik et al., 2013) and is able to remain stable during storage at 5°C to 7°C for 28 days in yoghurt and cultured buttermilk (Nighswonger et al., 1996). Likewise, freeze-dried Bb12 in skimmed milk remained viable during storage for 30 days at 4°C and presented moderate oxygen tolerance (P. J. Simpson et al., 2005). Recently an US NICU evaluated the viability of a probiotic mix including Bb12, *Bifidobacterium infantis* Bb-02, and *Streptococcus thermophilus* (TH-4) (Schimmoeller et al., 2021). Results indicated that storage at RT for an hour followed by storage at 4°C for 3h did not impact the viability of probiotics as it was maintained in all the tested matrices including preterm formula and HM. Additionally, they did not observe any changes in viscosity as all samples passed easily through the feeding tube. However, this study assessed the total count of probiotics that survived storage. This does not explain whether one strain reduced and another increased.

Several studies suggest that the food matrix is an important factor that affects the viability of the added probiotics (Loh & Maznah, 1999). The main factors contributing to the decline in probiotic viability have been determined as reduction in the pH of the medium and the presence of hydrogen peroxide and organic acids, among other factors (A. M. Mortazavian et al., 2007). Given that HM has a pH range of approximately 7-7.4 during the 10-month postpartum period, it is reasonable to hypothesize that the alkaline nature of HM may be associated with the findings we have observed (Basdeki et al., 2021). Nevertheless, the lack of DHM pH measurement in our study leaves this hypothesis unexplored.

Multiple other factors may have an impact on the stability of the probiotics during cold storage. For instance, the probable fermentation of HMO constituent monomers or utilization of other constituents of HM by LGG (is able to ferment galactose, L-fucose, and *N*-acetyl-D-glucosamine) and Bb12 (is able to utilize lactose and hydrolyze the milk proteins of bovine milk) could explain why both strains remain stable in this study (Gonzalez-Rodriguez et al., 2013; Liepke et al., 2002; Martinez-Villaluenga & Gómez, 2007; Salli et al., 2021; Thongaram et al., 2017a). If indeed future studies indicate that these specific probiotics reduce the content of important HM constituents, it is crucial to also define at which extent. If the reduction is biologically significant then it would make sense to administer DHM and probiotics separately. At the same time, fermentation of carbohydrates in HM may result in taste change and sourness which in turn may discourage its intake by the infant. These parameters should be therefore considered in future studies.

Fortifying HM has been shown to affect its nutrient composition and osmolality which in turn may have an impact on bacterial growth (Donovan et al., 2017). For

example, increased osmolality can drive water out of the bacterial cell, resulting in plasmolysis (Poolman et al., 2004). Similar to the findings of study **II**, study **III** demonstrated that the viability of both probiotics was maintained after cold storage and was not affected by the type of fortifier, when culturing the probiotic supplemented- liquid and powder FDHM and storing it for 24h at 4°C. In NICUs fortified DHM can be stored at 4°C for up to 24h, making the results clinically relevant for preterm neonates in similar units (Barbero et al., 2020). Similar findings were observed in previous studies, which focused on the HMM viability as affected by the type of fortifier and storage in RT or at 37°C. Despite an increase in milk osmolality after fortification with a powdered HMF, total bacterial colony counts did not significantly differ between fortified and unfortified HM after 24h storage at RT (Jocson et al., 1997). Recently, Codipilly et al. showed similar results for liquid HMF which increased osmolality but did not affect the growth of intrinsic lactobacilli and bifidobacteria in HM after 24h incubation at 37°C (Codipilly et al., 2020). The justification for storing fortified HM at RT in these studies is based on the limited availability of refrigeration for HM in certain regions. The purpose of these studies was to replicate the common practice observed in numerous NICUs in developing nations. In these settings, preterm neonates are often administered fortified HM that is maintained at RT (about 22°C) due to limited resources.

Finally, it is worth noting that differences in the CFU/ml of both probiotics between liquid and powdered FDHM in study **III** could be due to differences in their composition and final osmolality. The two HMFs tested might have different carbohydrate content or different carbohydrate origin. Therefore, the HMF polysaccharides might be broken down by the probiotics to a different degree depending on the type of fortifier.

6.3 Adhesion Properties of Probiotics (**III** and **IV**)

6.3.1 Strain dependence of the probiotic adhesion properties (**III**)

The findings of study **III** are consistent with previous research and indicate that both LGG and Bb12 exhibit strong adhesion properties to the mucus model utilized (Juntunen et al., 2001; Kirjavainen et al., 1998). This phenomenon can be partially explained by the fact that both strains exhibit resistance to acidic conditions (Deepika et al., 2012; Gorbach & Goldin, 1989; Li et al., 2010). According to Collado and coworkers, acid-resistant bifidobacteria show better adhesive properties when compared to their acid-sensitive derivative (M. C. Collado et al., 2006).

Moreover, genomic analysis indicates that LGG presents cell wall-bound pili which are composed of proteins that have been shown to promote mucus adhesion.

More specifically, each pilus tip and backbone structure consists of SpaC pilin that is essential for the mucus binding of LGG (Kankainen et al., 2009). Therefore, it can be speculated that the increased adhesion noted in our study might be partly due to the presence of pili. However, Sybesma and colleagues, have noted that LGG isolated from dairy products was lacking the gene encoding for SpaC pilin compared to the American Type Culture Collection (ATCC) deposited LGG (Sybesma et al., 2013). Thus, the cultivation method that is used for the large-scale production of probiotics is an important element influencing their health properties.

There is increasing evidence that bifidobacteria also encode and express pili on their cell surface. In this regard, Bb12 harbors three proteins that are homologous to proteins documented to participate in sortase-dependent pili formation (Gilad et al., 2011). Additionally, two more extracellular proteins were isolated which are homologous to proteins reported to bind to human mucus.

When LGG and Bb12 were tested directly on infant mucus, we found that Bb12 adhered well (28-37%) but not as well as LGG (43-49%). This could be attributed to the origins of the probiotic strains; LGG was isolated from a healthy human intestine, whereas Bb12 is derived from Chr. Hansen's collection of dairy cultures. When LGG and Bb12 were used together, there was no interference in adhesion, but rather, consistent with previous studies (Ouwehand et al., 2000), LGG adhesion appeared to be slightly, albeit statistically insignificantly, increased. In the presence of LGG, the same was observed for Bb12.

6.3.2 The role of fortification and cold storage of DHM on probiotic adhesion (**III**)

Human milk is a food matrix with a complex composition which contains diverse array of nutrients, bacterial and human cells, immune-modulating components, and human milk oligosaccharides. Although important for its use in the NICU, its potential as an optimal carrier for specific probiotics remains unclear. It is therefore crucial to find out because HM has been shown to play a critical role in the entrapment of probiotics and thus interfere with their adhesion to the infant intestinal mucus. Indeed a previous study has demonstrated that LGG has the ability to adhere to the milk fat globule membrane (MFGM), resulting in a reduction of its adherence to intestinal cells (Guerin et al., 2018).

Interestingly, in study **III** we found that the supplementation of LGG and Bb12 in DHM, liquid FDHM and powder FDHM resulted in a significant decrease in their adhesion. No difference was observed between the two fortifiers. This decrease could be explained by two possible mechanisms: either a milk constituent interacts with mucin glycoproteins, or it is interacting with the probiotics. For example, mannose-binding lectins (MBL) are proteins detected in HM whose binding activity

is not affected by Holder pasteurization (Cossey et al., 2009; Trégoat et al., 2002). Despite being a minor constituent, mannose moieties can be found in the structure of MUC2, the major colonic mucin in humans (Johansson et al., 2011). Depending on the glycosylation and degradation degrees of the intestinal mucus, this could indicate that MBLs present in HM with high affinity for MUC2 mannose receptors could potentially bind to the isolated mucus and interfere with the probiotic adhesion.

In addition, mucin 1 (a constituent of the intestinal mucus), which is thermally stable after pasteurization and rich in mannose structures, has also been identified as one of the major mucins of HM (Liu & Newburg, 2013; Y. Ma et al., 2019; Parry et al., 2006). A recent analysis of the LGG genome has revealed two lectin-like proteins Llp1 and Llp2 with high binding specificity for D-mannose and mannan (Petrova et al., 2016). There is thus a possibility that LGG adheres to mucin 1 or other mannose-containing HM glycoproteins through Llp1 and Llp2. Moreover, both LGG and Bb12 consist of a Gram-positive cell wall rich in peptidoglycans (Gilad et al., 2011; Lahtinen et al., 2004). Soluble toll-like receptor 2 (sTLR2), shown to be present in high concentration in HM, is a molecule responsible for recognizing bacterial peptidoglycan that could bind with both probiotics and prevent their adhesion (LeBouder et al., 2003). Finally, besides mucin 1, HM MBLs and sTLR2, κ-casein, a protein present in HM has a C-terminal whose sequence is similar to that of mucin (Thorn et al., 2015). In fact, the concentration of this protein is increased after Holder pasteurization of HM and could potentially prevent the adhesion of probiotics to the mucus glycoproteins (Y. Ma et al., 2019).

In general, the best adhesion of LGG was achieved when it was tested directly on infant mucus rather than with DHM, fortified or not. One component of DHM that might contribute to this observation is HM macrophages, whose viability is decreased only slightly by the process of pasteurization. (Björkstén et al., 1980; Cress & Jr, 1977; Gibbs et al., 1977). Indeed, a recent study found that the SpaCBA pili of LGG are key molecules for adhering to macrophages (Vargas García et al., 2015). Similarly, analysis of the extracellular proteome of Bb12 revealed that two identified proteins are involved in the binding of manganese, an essential trace element that is also present in HM (Gilad et al., 2011).

Although pasteurization has a negative impact on the concentration/activity of some bioactive constituents of HM, it does not affect the content of HMOs (Bertino et al., 2008). These compounds are structurally very similar to mucus glycoproteins as they are both recognized by the same outer membrane enzyme systems (Marcobal et al., 2011). In addition, LGG contrary to Bb12 has been shown to ferment L-fucose which is a major constituent of both mucus and HMOs (Arnold et al., 2018; Becerra et al., 2015; Thongaram et al., 2017b). HMOs could, therefore, act as decoy receptors for probiotics interfering with their adhesion to the mucus layer.

Polyunsaturated fatty acids (PUFA) are known for their antimicrobial properties and have also been reported in HM. Kankaanpää and colleagues showed that 40 μg/ml of different PUFA (α-linolenic acid, arachidonic acid, and docosahexaenoic acid) suppress the mucus adhesion of LGG but not its viability (Kankaanpää et al., 2001). Supplementary to that, daily HM intakes (around 750 ml/day in the first 4 months of life) contain an average concentration of 157, 134 and 66 μg/ml of α-linolenic acid, arachidonic acid and docosahexaenoic acid respectively (Institute of Medicine (US) Committee on Nutritional Status During Pregnancy and Lactation, 1991; Koletzko, 2016). These concentrations are 1.6-4 times higher than the PUFA concentration tested by Kankaanpää and coworkers indicating the possible interference of these molecules with the adhesion of probiotics.

HM is also rich in antimicrobial proteins and proteolytic enzymes including lysozyme, defensins, and cathelicidins (Baricelli et al., 2015; Murakami et al., 2005). Lysozyme, an enzyme that degrades bacterial cell wall peptidoglycans has been reported to be decreased after Holder pasteurization, however, 60-76% of its activity is still retained (Evans et al., 1978; Viazis et al., 2007; Wills et al., 1982). In addition, Ouwehand and coworkers showed a marked decrease in the mucus binding of LGG after the probiotic was pretreated with lysozyme but observed no effect on Bb12 (Ouwehand et al., 2001). The impact of defensins on probiotic adhesion remains unclear due to conflicting findings in the existing body of literature (De Keersmaecker et al., 2006; Lebeer et al., 2011; X.-F. Wang et al., 2015). Cathelicidins, however, may have a dual adhesion role, as they have the potential to interact with both mucus and probiotics. For instance, cathelicidin LL-37 was found to bind both mucin and negatively charged molecules on the cell wall of Gram-positive bacteria (Felgentreff et al., 2006; Kainulainen et al., 2012). In our study, probiotics were subjected to centrifugation before their supplementation to DHM. Specifically, this step could damage the EPS layer of LGG unmasking its pili that are responsible for its adhesion to intestinal mucus (Bell & Camesano, 2005; Lebeer et al., 2009). At the same time, stripping the EPS from the probiotics before their supplementation to DHM could have negative effects on the probiotic viability. To support this, Lebeer and colleagues showed that the viability of an EPS knock-out LGG mutant was compromised in the presence of LL-37 (Lebeer et al., 2011). However, there are no available data on the effect of LL-37 on Bb12.

Recently, a study has shown that DHM can be safely stored up to 8 months after Holder pasteurization, without compromising its macronutrient and energy content (de Waard et al., 2018). Our observations indicate that the adhesion capacity of both LGG and Bb12 after their supplementation in FDHM was not affected by the subsequent storage at 4°C for 24h nor by the type of fortifier used.

6.3.3 Age dependency of probiotic, pathogen, and commensal adhesion ability to intestinal mucus (**III** and **IV**)

It has been noted that not all probiotic strains can adhere in a similar capacity to mucus from different age groups (Kirjavainen et al., 1998; Ouwehand, Isolauri, et al., 1999). In accordance with the work of Arboleya and coworkers who confirmed that Bb12 adhered significantly better to mucus from 2-month-old infants than to that obtained from 6-month-old infants (Arboleya et al., 2011), in study **III**, Bb12 adhered significantly better to mucus from infants aged less than 6 than to mucus from infants aged more than 6 months. In contrast, in study **IV**, Bb12 appeared to adhere significantly better to mucus of infants aged >6 months than their younger counterparts. At this point, it is necessary to clarify that the results regarding the adhesion properties of Bb12 are not comparable between studies **III** and **IV**. This is because in the analysis of Bb12 adhesion data in study **III**, we incorporated the adhesion of Bb12 when used alongside DHM and FDHM. We chose to do so due to the limited number of replicates (n=3) of Bb12 alone (without any substrate) which would hinder the development of statistically robust and reliable analytical results.

For both studies **III** and **IV**, to prepare the mucus stock for each age group, equal amounts of lyophilized mucus from each individual were pooled to make a stock suspension of 10 mg/ml in HH buffer. From the stock suspension, a 0,5 mg/ml suspension was made in HH for the adhesion assay. Therefore, based on this preparation, the same amount of mucus for each group was guaranteed.

Considering this, we hypothesized that the observed variations in the bacterial adhesion patterns within the mucus samples from different age groups may be attributed to the unique diets of each respective age group. Indeed, diet has been identified as a significant contributor to the glycosylation of intestinal glycoproteins which in turn impacts the susceptibility of mucins to degradation (Fekete & Buret, 2023). The results of study **IV** revealed that mucus isolated from adults allowed for a better adhesion of Bb12 (although not significant) and a significantly better adhesion of LGG, *C. sakazakii* and *E. coli* compared to the mucus isolated from infants aged less than 6 months. The stronger adhesion of Bb12 and LGG to the mucus isolated from adults in study **IV** is in agreement with a recent report by Harata and coworkers (Harata et al., 2021).

Regarding the differences between the two infant age groups in study **IV**, a previous study indicated that the mucus obtained from infants under the age of six months may include a higher concentration of mucin oligosaccharides compared to the one obtained from infants older than six months (Tassell and Miller, 2011). Although this observation may suggest that there are more binding sites available for probiotics to adhere, it is important to note that the composition of the mucus glycoproteins is a major factor in determining the adhesion of specific bacteria.

Specifically for Bb12, we found that it adhered significantly better to the mucus of infants aged more than 6 months. This infant group's diet is characterized by the introduction of foods other than HM (weaning), which can contribute to a transition from high sialylation to high fucosylation terminal in mucins (Gréco et al., 2001; Shub et al., 1983). Indeed, previous research indicates that α1,2-fucosylation is necessary for the adhesion of bifidobacteria and that cell-wall-associated sialidase can promote *B. bifidum* colonization by binding to sialylation conjugations (Lei et al., 2022; Nishiyama et al., 2017). In addition, weaning appears to increase mucus degradation and therefore alters its composition, which could be driven by an accelerated colonization of the infant gut by mucus degrading microbes (Midtvedt et al., 1994). The observed differences in probiotic adhesion patterns between the two age groups could explained by a possible degradation of the mucus obtained from infants aged 6-12 months.

In the context of study **III**, it is worth noting that our analysis considered the adhesion of Bb12 and LGG when combined with DHM and FDHM. In this regard, an alternative explanation for the observed variations in adhesion between the two age groups in this study could be attributed to the level of presence of other glycoproteins, such as lactoferrin, in DHM. These glycoproteins may potentially interact with the studied probiotics, thereby interfering with the ability of probiotics to interact with the intestinal mucins.

6.3.4 Adhesion Properties of Probiotics Mediated by Exogenous Polyamines (**IV**)

As PAs are polycations, their presence at different concentrations can alter mucus secondary structure and viscosity in a way that is directly related to the number of charges (Bansil & Turner, 2018). The number of positive charges is also different for PUT (two), SPD (three), and SPM (four) and it could explain some of the differences in the adhesion of the examined bacteria observed among the different types of PAs and in their mixes (Ioannidis & Kotzabasis, 2007). In addition, different glycosylation patterns related to age and diet may influence how polyamines influence secondary mucus structure and, therefore, bacterial adhesion. However, this remains speculative and should be confirmed in future studies. Another possible explanation would be that bacteria utilize specific PAs to regulate the expression of multiple adhesive surface structures. We could expect that PAs bind to the negatively charged nucleic acids and there by tune the expression of the multiple genes in the studied microorganisms (Orlando et al., 2014). However, considering that PAs were not added in the growth medium of the tested microorganisms, further studies are essential to understand the mechanism of interaction of PAs to the nucleic acids of microorganisms.

PUT and SPD appeared to slightly promote the adhesion of LGG to early infant mucus. However, this increase in the adhesion was not significant at the concentration tested. This observation should be confirmed in future studies with higher concentrations of PAs because the concentration of PAs in the gastrointestinal tract is generally higher than in HM (Matsumoto & Benno, 2007).

The higher adhesion levels of Bb12 when mixed with SPD or with low concentration of PAs mix, observed in study **IV** suggest that exogenous polyamines may influence *Bifidobacterium* adhesion properties in early life. We therefore consider that dietary polyamines via HM or infant formula in combination with specific probiotics may be used to promote a healthy colonization pattern in early life. This could be an important factor to consider when assessing the stepwise colonization of the gastrointestinal tract after delivery. In addition, by adhering to the mucus, specific bifidobacteria may result in altered composition of intestinal bacteria and an increased amount of PAs in the intestine. Indeed, research conducted in aging mice has shown that the intake of *Bifidobacterium animalis* subsp. *lactis* increased the production of PUT in the gut (Kibe et al., 2014). This implies that the administration of specific bifidobacteria could potentially enhance the endogenous production of PAs by bacteria in the gut. This effect holds particular significance for preterm neonates, who require high amounts of protein to fulfill their increased growth demands, with PAs playing a crucial role in facilitating protein synthesis.

Regarding *C. sakazakii*, a decrease in its adhesion to early infant mucus was observed in the presence of SPM, however, this decrease did not reach statistical significance. The presence of SPM may potentially confer a protective effect against *C. sakazakii* infections, particularly in premature neonates with low birth weights who represent a vulnerable population likely susceptible to infections caused by this microorganism (Chandrasekaran et al., 2018). SPM is also one of the most prevalent PA in HM during the first five months of lactation (Muñoz-Esparza et al., 2021). Our findings are also consistent with a report from Collado and colleagues, who demonstrated *C. sakazakii* adhesion rates of 14% in adult mucus. (M. C. Collado et al., 2008). The same study also showed that in the presence of specific probiotics and their combination, the adhesion of *C. sakazakii* was reduced. Altogether and considering the above, future studies could focus on investigating the impact of PAs, either alone or in combination with specific probiotics, on the adhesion of *C. sakazakii* to mucus derived from premature neonates.

Although *E. coli* degrades complex carbohydrates to a small extend and therefore is regarded as non-mucolytic, several studies have demonstrated that it can interact with human mucus through its flagella (Erdem et al., 2007; H. Li et al., 2015). Our findings indicate that the adhesion ability of *E. coli* remained unaffected by exogenous polyamines at any age in the concentrations tested in contrast with the rest of the bacteria used in this study. Interestingly, a previous report indicated that

cadaverine, another PA with a similar structure and the same number of positive charges as PUT, may inhibit *E. coli* adherence (Torres et al., 2005). Based on that report, we would expect that PAs would reduce the adhesion of *E. coli* to intestinal mucus. However, the concentrations of PAs (ranging from 0.025–2 ppm) used in the current study were significantly lower than the concentration of cadaverine used by Torres and colleagues (30.7 and 51 ppm). The lack of effect could also be explained by the fact that *E. coli* uses a different PA biosynthetic pathway than the majority of human GM (Hanfrey et al., 2011). While *E. coli* is a minor microbial component in the adult human intestine, the genus *Escherichia* is predominant and the most abundant in the fecal microbiota composition of healthy infants at 3, 6, and 12 months of life (Nowrouzian et al., 2003; Taddei et al., 2014). At the same time, two cohort studies involving 1-month-old infants showed an association between the presence of *E. coli* in their feces with an increased risk of developing eczema (Penders et al., 2006, 2007). Therefore, it would be interesting to investigate whether *E. coli* adhesion in the gastrointestinal tract can be modulated by other polyamines synthesized by intestinal microbiota—including cadaverine, agmatine, or thermospermine—and understand the mechanism behind this effect.

7 Limitations and Suggestions for Future Research

We recognize that there are certain limitations associated with the research conducted for this thesis and outline a few ideas for further research:

Study I

- To illustrate, one of the weaknesses in Study **I** is the relatively small sample size and the absence of additional longitudinal data points. These limitations may have had an impact on the variability observed in the data. Several previous studies have emphasized the substantial intra- and inter-individual variability in the compositions of HM bioactives and the HMM (Hunt et al., 2011; Samuel et al., 2020). Given the limited number of samples in Study **I**, it is essential to validate our findings in larger study cohorts.
- It's worth noting that carrying out this type of study in Argentina was challenging. This is because some mothers were reluctant in providing multiple milk samples after hospital discharge, especially when multiple samples were requested over time. Having additional sampling points would imply that mothers would have to visit the hospital and this alone was challenging due to the mothers' limited resources or the considerable distance between their homes and the hospital. Additionally, the restricted research budget also posed a constraint that should be considered. However, for being the first of its kind aiming to understand and characterize the HMM of the Argentinean population, this study is valuable and establishes a foundation for future, more comprehensive studies.
- Furthermore, more controlled studies are needed to establish causation as there are multiple factors that modulate the HMM profiles. For example, we found that three bacterial species correlated with gestational age but as previously discussed there are multiple factors that modulate the HMM profiles. Further studies which will include other factors are needed to indeed confirm that these correlations translate to causality.

- Moreover, while our study did not look at live bacteria in HM, a recent report found that 16S studies overestimate both richness and diversity of the HMM (Stinson et al., 2021). As a result, methods assessing the viable microbiota could be incorporated to provide a more accurate view of the HMM. Lastly, certain primer sets appear to favor the enrichment of specific bacterial groups, according to GM studies (Kameoka et al., 2021; Palkova et al., 2021). Consequently, scientists should carefully consider which primers to use based on their research question and objectives.
- Finally, this study did not assess whether the observed differences in the preterm HMM composition between birth and TEA are also reflected in the GM composition of the respective infant. This inclusion could help us understand better the link between HMM and infant GM maturation, and thus could be considered in future studies.

Study II

- Further studies in a larger number of samples are needed to evaluate the stability of added probiotics in HM, as different storage conditions, processing procedures, probiotic strains, and HM samples from different lactation stages could present different results.
- Future studies may also consider isolating new probiotics from HM. Previous work has demonstrated potential in this effort. For example, Argentinian and Iranian scientists were able to isolate HM lactobacilli and bifidobacteria with probiotic potentials inducing IL-10 production by macrophages and restricting the growth of gastrointestinal pathogens (Dallal et al., 2021; Oddi et al., 2020).

Studies III and IV

- The past few years other HM processing techniques are gaining ground, namely UHT and freeze drying. In fact, freeze drying of HM has been shown to conserve protein, carbohydrate, polyphenol, and oxidant levels of HM. However, since we did not investigate whether these processes can influence the adhesion properties of the studied probiotics, we consider this as a potential area for future research.
- Despite the lack of standardization, two major *in vitro* models are used to assess probiotic adhesion: the intestinal mucus model and the cell line model. The results between these two methods vary widely (Laparra & Sanz, 2009). In studies **III-IV**, we chose to use the intestinal mucus model since

the mucus layer is the first interphase for host-probiotic interaction in the gastrointestinal tract. Previous reports have shown that subject age should be taken into consideration when determining probiotic adhesion properties – another reason why we found the mucus model more appropriate than the cell line model. The model has its limitations. Laparra and Sanz have reported that the mucus model cannot properly distinguish hydrophobic binding interactions from mucus binding interactions, making interpretation of results challenging. In addition, isolated mucus alone does not appropriately describe the *in vivo* situation and a combination of both mucus and cell culture models might be appropriate for future work. This could provide more information as it would include both host and mucus interactions.

- Another drawback of the mucus *in vitro* model is the use of a radioactive-based assay that has led to safety, waste disposal, and cost concerns. Vesterlund and coworkers have shown, however, that radioactive labels in adhesion assays are superior to fluorescence tagging or staining especially in the case of bacteria with poor adhesion properties (Vesterlund et al., 2005).
- Another limitation of studies **III-IV** was the omission of the *in vitro* digestion. Based on previous studies, passage through the stomach and small intestine could have a negative impact on the viability and the *in vitro* adhesive capacity of LGG and Bb12 to intestinal mucus (Miettinen et al., 1999; Ouwehand et al., 2001). However, as discussed above, HM may influence the probiotics by either protecting them from digestion or interfering with their adhesion. Therefore, future studies should focus on investigating how the adhesion of probiotics is affected by the digestion of probiotic-supplemented HM. As the nutrient composition of DHM was not characterized before or after Holder pasteurization, we are unable to point out which of the HM components had the strongest influence in the adhesion of the tested probiotics.
- The main limitation of studies **III** and **IV** is the lack of mucus characterization from infants aged <6 months, infants aged >6 months and adults. Therefore, we cannot be certain whether the differences in the adhesion between the two age groups are due to distinct glycosylation patterns of the mucus. In fact, characterizing the mucus would support stronger conclusions. However, when we attempted to previously characterize the mucus, we encountered issues with the protein, mucin, and carbohydrate analyses. It appears that the buffer in which we resuspended the dried mucus (Hepes-Hanks buffer) is interfering with the absorption. This should be taken into consideration for future studies that investigate

intestinal mucus adhesion and perhaps other buffers such as PBS or MilliQ water could be used instead. Another alternative could be characterizing the mucus before the freeze-drying step (at that point suspended in MilliQ). This *in vitro* approach has been previously employed by other authors (Juntunen et al., 2001; Ouwehand, Kirjavainen, et al., 1999) so we consider it a valid attempt to provide similar and comparable results. Considering this limitation, we recognize that that the current observations should be interpreted with caution because host-microbiota interactions in the human gastrointestinal tract are more complex.

- For study **IV**, we experienced difficulties in finding preterm mucus. For this reason, we decided to include mucus from infants of <6months and 6-12 months and therefore cannot extrapolate these findings to preterm infants. Future research could investigate the role of PA fortification in HM and infant formula and how it affects the adhesion of probiotics and relevant infant pathogens on preterm infant mucus. On this note, a previous systematic review indicates that L-arginine, a building block of PAs, appears to be directing the prevention of NEC in preterm infants (Mitchell et al., 2014). If this effect is mediated by the synthesis and action of PAs remains to be elucidated. If this is confirmed, further studies could be conducted to determine whether giving specific PAs to preterm infants can prevent NEC in the NICU setting.
- Finally, in relation to study **IV**, there are numerous additional future research ideas for PAs contained in HM. It will be interesting to demonstrate whether polyamine levels differ between MOM and DHM. As DHM is donated from mothers at term, future research may determine whether the lower PA content in term HM compared to preterm HM might have an impact on the intestinal maturation of preterm infants. It would also be interesting to see if HM PA levels differ depending on maternal factors such as genetics, diet, baseline health, medications or supplements taken, how mothers were fed as infants, mode of infant delivery, and so on.

8 Summary/Conclusions

Considering the important role of gut microbiota (GM) composition for infant health during the neonatal period and beyond, this study set out to examine two commonly employed strategies for modulating infant GM: human milk microbiota (HMM) and specific probiotics. We further hypothesized that certain factors modify the composition of the HMM and the probiotic viability and capacity to adhere to intestinal mucus.

Regarding HMM, the study focused on investigating whether there are differences in HMM composition between preterm and full-term births and whether there are changes in preterm HMM composition from birth to the equivalent term age. To achieve this, a study population from Argentina was chosen, as no previous research had characterized the HMM composition of this particular population. This choice was significant due to known variations in HMM composition between geographic locations. In terms of results, our work revealed that *Firmicutes* and *Proteobacteria* are the most abundant phyla in the HM of the Argentinian population with genera *Staphylococcus* and *Streptococcus* contributing to the observed differences between preterm and term HM. Notably, the presence of the genus *Rothia*, found only in milk samples of term and term equivalent age neonates, may be linked to the delayed GM maturation in preterm infants. Additionally, term HM exhibited greater microbial diversity compared to preterm HM. Variations in taxonomic composition were also observed between these groups. Although preliminary, these findings underscore the need to further investigate the factors influencing changes in HMM and their implications for infant health, reinforcing the importance of promoting breastfeeding.

Regarding probiotics, this study focused on assessing their stability in HM during storage, on determining the effect of FDHM on their adhesion properties, and on investigating the role of polyamines on probiotic and pathogen interactions with intestinal mucus. In NICUs, probiotics are often mixed with MOM or DHM and any excess is discarded. This study assessed whether cold storage could maintain probiotic viability in HM, finding that LGG and Bb12 remain stable for 72 hours at 4°C and 7 days at -20°C. Consequently, HM is a good matrix for probiotic delivery, a finding valuable for milk banks in hospitals. Additionally, we found that adding

liquid or powdered HM fortifiers to DHM does not alter the adhesion of LGG and Bb12 to infant mucus, and further demonstrated that FDHM can be stored at 4°C for up to 24 hours without affecting probiotic adhesion or viability. However, it's important to note that these results may be interpreted with caution and not be extrapolated to preterm infants.

The research also highlighted the age-dependency in probiotic adhesion ability. For instance, spermidine significantly improved the adhesion of Bb12 in the mucus of infants under 6 months, whereas spermine was more effective in reducing the adhesion of *Cronobacter sakazakii* in the same age group. This age-specific response to polyamines suggests that individual age is a crucial factor in determining the effectiveness of probiotics.

Overall, these findings could be considered for developing strategies to optimize GM in infants, particularly in preterm infants. By improving the methods of storing and using donated HM, including maintaining probiotic viability through optimal storage conditions, we can enhance the health benefits for infants while also addressing the significant issue of HM wastage in HMBs. This approach not only supports the health of preterm infants but also contributes to the effective management and utilization of valuable resources in neonatal care.

Acknowledgements

The work for this thesis was carried out at Functional Foods Forum, Faculty of Medicine, University of Turku, Finland and the Institute of Industrial Lactology (INLAIN), National University of Santa Fe Litoral, Argentina.

The Doctoral Programme in Clinical Research of the University of Turku Graduate School, the Turku University Foundation (Turun Yliopistosäätiö), the Finnish Society for Pediatric Research (Lastentautien Tutkimussäätiö), the Päivikki and Sakari Sohlberg Foundation, and the Hospital District of Southwest Finland, are acknowledged for their financial support. I am also grateful for all the travel grants provided by the Turku University Foundation and the University of Turku, which allowed me to participate in numerous conferences around the world and perform the work for the first article in Argentina.

I am deeply grateful to my supervisors, Professor Seppo Salminen and Professor Samuli Rautava for accepting me as a part of their team, and for their excellent guidance and support through the years of my master's and doctoral studies. Your kindness, and trust in me and my skills has been instrumental in seeing this thesis completed. Dear Seppo, a big thank you for trusting me and giving me the opportunity to work on other research projects besides my PhD.

I also want to acknowledge the reviewers of this thesis, Professor Sonia Gonzalez Solares and Dr. Sampo Lahtinen, for accepting to review this work and for their swift constructive criticism and valuable comments on the manuscript. My follow-up committee members Professor Maria Carmen Collado and Professor Erika Isolauri are thanked for their advice and support. MariCarmen thank you for being a bright example of a woman in science for me. Your passion, scientific curiosity and continuous encouragement have been a moving force to finalize this work! I also wish to express my gratitude to all my co-authors involved in the articles of this thesis for their contributions. A special thanks goes to Dr. Himanshu Kumar for his scientific guidance since my first days in Functional Foods Forum, to Dr. Gabriel Vinderola and Dr. Sofia Oddi for the great collaboration, to Dr. Artur Ouwehand for his invaluable guidance related to the intestinal mucus studies, and to Eliisa Löyttyniemi for her advice on statistics. I am also very grateful to my Director, John Bell, for motivating me to finalize this thesis! A big thank you goes to all mothers

that donated a sample of their milk and to all parents that donated the fecal samples of the infant participants. Without these samples the realisation of this work would not have been possible.

I consider myself extremely fortunate to have had the support of so many wonderful people, from whom I have learned a lot. I will be forever grateful to my lab partners in crime! Satu, thank you from the bottom of my heart for always being there for me, celebrating the happy times and comforting me during the hard ones. The support, professional and sentimental, you have been giving me unconditionally all these years played a big role in completing this work and for this reason I dedicate this thesis to you. My 'Suomen Mestarit' lab and skate team, Paulina and Enni, thank you for sharing the good and challenging times with me! Dr. Gabriela Martínez Chacón and Dr. Henni Hiltunen, thank you for being excellent examples of how doctoral research is done! Many thanks also go to my second lab family in Argentina: to Dr. Diego Mercanti, Dr. Lorenzo Siroli, and Desi Llorens for welcoming me in the team and showing me the Argentinian passion for science!

A big, big thank you goes to all the fellow PhD friends from whom I have learnt a great deal! Juhani Aakko, Niko Markkinen, Heikki Aisala, Amruta Kulkarni, and Gabriele Beltrame, all doctors now, you have been an absolute inspiration! Gabriele, a huge thank you for your skillful technical assistance in the freeze-drying of the infant mucus and to you Juhani for providing the finnish translation of the title of this thesis.

I will be forever grateful to all my Turku friends for all the support, laughs and good times together: Nghia Huynh, Diogo Cunha, João Salazar, Flor Zacarias, Martina Angeleri, Jesus Arroyo, Ville Virtanen, Jaakko Räkköläinen. To my friend Dr. Sara Hernandez Perez: amiga, yo también te quiero! Thank you for making not just learning Finnish but also life in Turku more enjoyable! To my GIM master's group, Jane, Sarah, and most importantly Jessica for their endless encouragement and positive attitude! Special thanks go to the 'GreChIndia' group, Sindhu and Xu for their unconditional friendship and countless hangouts over fried chicken. Heidi, I sincerely appreciate you being there for me from the beginning of this adventure, encouraging and helping me through both easy and difficult times! To Anita for sharing the same journey, for the close friendship, and for the many many laughs together! To Hari, who has always been there to listen and to check on me through both my highs and lows! To Sanja, who is a scientist I greatly admire and who never fails to inspire me! To Alena, who joined me halfway through the journey, nevertheless played a big role in helping me push forward and complete this work. To Zoi and Aris for making me feel part of the family, for all the immense support and fun times spent together! To Dr. Wei Thwe, for being a great role model of resilience and perseverance - I have learned so much from you over the years. Wei

Thwe, I consider myself very lucky to have met you and feel honored to call you my friend.

To Azfar with whom our journey started well before the PhD. Azfar, I appreciate all of your support on both a personal and professional level and for being more than just a friend—I would even say family. I will always treasure our thought-provoking conversations, covering anything from the theory of evolution to potential future project collaborations. You have been the most important pillar of support for me since our move to Finland, and for that I will be forever grateful.

To Carlos and Pauliina, I don’t even know how to thank you. I want to say so many things, but I'm worried I'll significantly lower the level of an otherwise well-written segment! So I'll stick to the so-far language level and thank you for the long friendship, for listening to my frustrations, for laughing loudly together, for believing in me, for the endless memories we've created, and, most importantly, for being such amazing people! I am also extremely grateful to you, Pauliina, for providing the Finnish version of the thesis abstract. Carlos I'm also very grateful to you for being my co-author and for addressing the trillion scientific questions that have popped up in my head.

To my brother Nikos and to my sister-in-law (prophetic?) Savvina, for their continuous support in whatever I am doing! Thank you, Niko, for being my biggest supporter in life, for being such a kindhearted person, and for bringing happiness and good memories into my life.

To my partner, Cassiel: Mon ver, thank you for your constant encouragement during the final years of my PhD adventure. Even if you were unfamiliar with the process, you were constantly by my side, trying to motivate me to finalize the thesis using cheap baits like taking me out for dinner. You did that anyways!

And finally, I would like to express my deepest gratitude to my parents. Thank you for raising me to be a person who isn't afraid of hard work, and for always motivating me to do better, not to outperform others, but to become a better version of myself. Thank you for your never-ending love and for always believing in me. Thank you, Mom, for serving as a role model of hard work and responsibility, as well as providing the first example of how research is done. Thank you, Dad, for encouraging my curiosity throughout my life. I hope that one day you will realize you didn’t ‘send’ me to university to play with poop in the lab!!

For my parents in Greek: Και τέλος, θα ήθελα να εκφράσω τη βαθύτατη ευγνωμοσύνη μου στους γονείς μου. Ευχαριστώ που με μεγαλώσατε να γίνω ένα άτομο που δεν φοβάται τη σκληρή δουλειά και που με παρακινούσατε πάντα να κάνω καλύτερη, όχι για να ξεπεράσω τους άλλους, αλλά για να γίνω μια καλύτερη εκδοχή του εαυτού μου. Σας ευχαριστώ για την ατελείωτη αγάπη σας και που πάντα πιστεύετε σε μένα. Ευχαριστώ, μαμά, που λειτούργησες ως πρότυπο σκληρής δουλειάς και υπευθυνότητας, και που ήσουν το πρώτο παράδειγμα για το πώς γίνεται

η έρευνα. Ευχαριστώ, μπαμπά, που ενθάρρυνες την περιέργειά μου σε όλη μου την ζωή. Ελπίζω ότι μια μέρα θα συνειδητοποιήσεις ότι δεν με ‘έστειλες’ στο πανεπιστήμιο για να παίξω με κόπρανα στο εργαστήριο!!

Brussels, 22 March 2024

References

Aakko, J., Kumar, H., Rautava, S., Wise, A., Autran, C., Bode, L., Isolauri, E., & Salminen, S. (2017). Human milk oligosaccharide categories define the microbiota composition in human colostrum. *Beneficial Microbes*, *8*(4), 563–567. https://doi.org/10.3920/BM2016.0185

Abramovich, M., Friel, J. K., & Hossain, Z. (2013). *Polyunsaturated Fatty Acids, Riboflavin and Vitamin C: Effects of Different Storage Conditions of Human Milk. 2*(110). https://doi.org/10.4172/2167-0390.1000110

Abrams, S. A. (2020). Vitamin D in Preterm and Full-Term Infants. *Annals of Nutrition and Metabolism*, *76*(2), 6–14. https://doi.org/10.1159/000508421

Aceti, A., Maggio, L., Beghetti, I., Gori, D., Barone, G., Callegari, M. L., Fantini, M. P., Indrio, F., Meneghin, F., Morelli, L., Zuccotti, G., & Corvaglia, L. (2017). Probiotics Prevent Late-Onset Sepsis in Human Milk-Fed, Very Low Birth Weight Preterm Infants: Systematic Review and Meta-Analysis. *Nutrients*, *9*(8), 904. https://doi.org/10.3390/nu9080904

Ackerman, D. L., Doster, R. S., Weitkamp, J.-H., Aronoff, D. M., Gaddy, J. A., & Townsend, S. D. (2017). Human Milk Oligosaccharides Exhibit Antimicrobial and Antibiofilm Properties against Group B Streptococcus. *ACS Infectious Diseases*, *3*(8), 595–605. https://doi.org/10.1021/acsinfecdis.7b00064

Adjerid, K., Mayerl, C. J., Gould, F. D. H., Edmonds, C. E., Stricklen, B. M., Bond, L. E., & German, R. Z. (2021). Does birth weight affect neonatal body weight, growth, and physiology in an animal model? *PLOS ONE*, *16*(2), e0246954. https://doi.org/10.1371/journal.pone.0246954

Alcon-Giner, C., Dalby, M. J., Caim, S., Ketskemety, J., Shaw, A., Sim, K., Lawson, M. A. E., Kiu, R., Leclaire, C., Chalklen, L., Kujawska, M., Mitra, S., Fardus-Reid, F., Belteki, G., McColl, K., Swann, J. R., Kroll, J. S., Clarke, P., & Hall, L. J. (2020). Microbiota Supplementation with Bifidobacterium and Lactobacillus Modifies the Preterm Infant Gut Microbiota and Metabolome: An Observational Study. *Cell Reports Medicine*, *1*(5), 100077. https://doi.org/10.1016/j.xcrm.2020.100077

Ali, M. A., Strandvik, B., Sabel, K.-G., Kilander, C. P., Strömberg, R., & Yngve, A. (2014). Polyamine levels in breast milk are associated with mothers' dietary intake and are higher in preterm than full-term human milk and formulas. *Journal of Human Nutrition and Dietetics*, *27*(5), 459–467. https://doi.org/10.1111/jhn.12156

Almand, E. A., Moore, M. D., & Jaykus, L.-A. (2017). Virus-Bacteria Interactions: An Emerging Topic in Human Infection. *Viruses*, *9*(3). https://doi.org/10.3390/v9030058

Altobelli, E., Angeletti, P. M., Verrotti, A., & Petrocelli, R. (2020). The Impact of Human Milk on Necrotizing Enterocolitis: A Systematic Review and Meta-Analysis. *Nutrients*, *12*(5), E1322. https://doi.org/10.3390/nu12051322

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Angelopoulou, A., Field, D., Ryan, C. A., Stanton, C., Hill, C., & Ross, R. P. (2018). The microbiology and treatment of human mastitis. *Medical Microbiology and Immunology*, *207*(2), 83–94. https://doi.org/10.1007/s00430-017-0532-z

Arboleya, S., Rios-Covian, D., Maillard, F., Langella, P., Gueimonde, M., & Martín, R. (2022). Preterm Delivery: Microbial Dysbiosis, Gut Inflammation and Hyperpermeability. *Frontiers in Microbiology*, *12*. https://www.frontiersin.org/articles/10.3389/fmicb.2021.806338

Arboleya, S., Ruas-Madiedo, P., Margolles, A., Solís, G., Salminen, S., de los Reyes-Gavilán, C. G., & Gueimonde, M. (2011). Characterization and in vitro properties of potentially probiotic Bifidobacterium strains isolated from breast-milk. *International Journal of Food Microbiology*, *149*(1), 28–36. https://doi.org/10.1016/j.ijfoodmicro.2010.10.036

Arnold, J. W., Simpson, J. B., Roach, J., Bruno-Barcena, J. M., & Azcarate-Peril, M. A. (2018). Prebiotics for Lactose Intolerance: Variability in Galacto-Oligosaccharide Utilization by Intestinal Lactobacillus rhamnosus. *Nutrients*, *10*(10). https://doi.org/10.3390/nu10101517

Arroyas, M., Calvo, C., Rueda, S., Esquivias, M., Gonzalez-Menchen, C., Gonzalez-Carrasco, E., & Garcia-Garcia, M. L. (2020). Asthma prevalence, lung and cardiovascular function in adolescents born preterm. *Scientific Reports*, *10*(1), 19616. https://doi.org/10.1038/s41598-020-76614-0

Arslanoglu, S., Boquien, C.-Y., King, C., Lamireau, D., Tonetto, P., Barnett, D., Bertino, E., Gaya, A., Gebauer, C., Grovslien, A., Moro, G. E., Weaver, G., Wesolowska, A. M., & Picaud, J.-C. (2019). Fortification of Human Milk for Preterm Infants: Update and Recommendations of the European Milk Bank Association (EMBA) Working Group on Human Milk Fortification. *Frontiers in Pediatrics*, *7*, 76. https://doi.org/10.3389/fped.2019.00076

Asbury, M. R., Butcher, J., Copeland, J. K., Unger, S., Bando, N., Comelli, E. M., Forte, V., Kiss, A., LeMay-Nedjelski, L., Sherman, P. M., Stintzi, A., Tomlinson, C., Wang, P. W., & O'Connor, D. L. (2020). Mothers of Preterm Infants Have Individualized Breast Milk Microbiota that Changes Temporally Based on Maternal Characteristics. *Cell Host & Microbe*, *28*(5), 669-682.e4. https://doi.org/10.1016/j.chom.2020.08.001

Austin, S., De Castro, C. A., Sprenger, N., Binia, A., Affolter, M., Garcia-Rodenas, C. L., Beauport, L., Tolsa, J.-F., & Fumeaux, C. J. F. (2019). Human Milk Oligosaccharides in the Milk of Mothers Delivering Term versus Preterm Infants. *Nutrients*, *11*(6), 1282. https://doi.org/10.3390/nu11061282

Ayivi, R. D., Gyawali, R., Krastanov, A., Aljaloud, S. O., Worku, M., Tahergorabi, R., Silva, R. C. da, & Ibrahim, S. A. (2020). Lactic Acid Bacteria: Food Safety and Human Health Applications. *Dairy*, *1*(3), Article 3. https://doi.org/10.3390/dairy1030015

Ayoub Moubareck, C., Lootah, M., Tahlak, M., & Venema, K. (2020). Profiles of Human Milk Oligosaccharides and Their Relations to the Milk Microbiota of Breastfeeding Mothers in Dubai. *Nutrients*, *12*(6), Article 6. https://doi.org/10.3390/nu12061727

Ayyash, S., Wu, W.-I., & Selvaganapathy, P. R. (2016). Fast and Inexpensive Detection of Bacterial Viability and Drug Effectiveness through Metabolic Monitoring. *Sensors (Basel, Switzerland)*, *16*(11). https://doi.org/10.3390/s16111879

Azagra-Boronat, I., Tres, A., Massot-Cladera, M., Franch, À., Castell, M., Guardiola, F., Pérez-Cano, F. J., & Rodríguez-Lagunas, M. J. (2020). Associations of Breast Milk Microbiota, Immune Factors, and Fatty Acids in the Rat Mother–Offspring Pair. *Nutrients*, *12*(2), 319. https://doi.org/10.3390/nu12020319

Ballard, O., & Morrow, A. L. (2013). Human Milk Composition. *Pediatric Clinics of North America*, *60*(1), 49–74. https://doi.org/10.1016/j.pcl.2012.10.002

Balmer, S. E., & Wharton, B. A. (1989). Diet and faecal flora in the newborn: Breast milk and infant formula. *Archives of Disease in Childhood*, *64*(12), 1672–1677. https://doi.org/10.1136/adc.64.12.1672

Banerji, R., Kanojiya, P., Patil, A., & Saroj, S. D. (2021). Polyamines in the virulence of bacterial pathogens of respiratory tract. *Molecular Oral Microbiology*, *36*(1), 1–11. https://doi.org/10.1111/omi.12315

Bansil, R., & Turner, B. S. (2018). The biology of mucus: Composition, synthesis and organization. *Advanced Drug Delivery Reviews*, *124*, 3–15. https://doi.org/10.1016/j.addr.2017.09.023

Barbero, A. H., Rico, N., Oller-Salvia, B., Aldecoa-Bilbao, V., Macías-Muñoz, L., Wijngaard, R., Figueras-Aloy, J., & Salvia-Roigés, M. (2020). Fortifier selection and dosage enables control of breast milk osmolarity. *PLOS ONE*, *15*(6), e0233924. https://doi.org/10.1371/journal.pone.0233924

Baricelli, J., Rocafull, M. A., Vázquez, D., Bastidas, B., Báez-Ramirez, E., & Thomas, L. E. (2015). β-defensin-2 in breast milk displays a broad antimicrobial activity against pathogenic bacteria. *Jornal de Pediatria*, *91*(1), 36–43. https://doi.org/10.1016/j.jped.2014.05.006

Becaria Coquet, J., Caballero, V. R., Camisasso, M. C., González, M. F., Niclis, C., Román, M. D., Muñoz, S. E., Leone, C. M., Procino, F., Osella, A. R., & Aballay, L. R. (2020). Diet Quality, Obesity and Breast Cancer Risk: An Epidemiologic Study in Córdoba, Argentina. *Nutrition and Cancer*, *72*(6), 1026–1035. https://doi.org/10.1080/01635581.2019.1664601

Becerra, J. E., Yebra, M. J., & Monedero, V. (2015). An l-Fucose Operon in the Probiotic Lactobacillus rhamnosus GG Is Involved in Adaptation to Gastrointestinal Conditions. *Applied and Environmental Microbiology*, *81*(11), 3880–3888. https://doi.org/10.1128/AEM.00260-15

Beghetti, I., Panizza, D., Lenzi, J., Gori, D., Martini, S., Corvaglia, L., & Aceti, A. (2021). Probiotics for Preventing Necrotizing Enterocolitis in Preterm Infants: A Network Meta-Analysis. *Nutrients*, *13*(1), 192. https://doi.org/10.3390/nu13010192

Behrman, R. E., Butler, A. S., & Outcomes, I. of M. (US) C. on U. P. B. and A. H. (2007). *Societal Costs of Preterm Birth*. National Academies Press (US). http://www.ncbi.nlm.nih.gov/books/NBK11358/

Bekebrede, A. F., Keijer, J., Gerrits, W. J. J., & Boer, V. C. J. de. (2020). The Molecular and Physiological Effects of Protein-Derived Polyamines in the Intestine. *Nutrients*, *12*(1), Article 1. https://doi.org/10.3390/nu12010197

Bell, C. H., & Camesano, T. A. (2005). *The Effects of Centrifugation and Filtration as Pre-Treatments in Bacterial Retention Studies*. Journal of Young Investigators. https://www.jyi.org/2005-june/2005/6/8/the-effects-of-centrifugation-and-filtration-as-pre-treatments-in-bacterial-retention-studies

Belzer, C., & de Vos, W. M. (2012). Microbes inside—from diversity to function: The case of Akkermansia. *The ISME Journal*, *6*(8), 1449–1458. https://doi.org/10.1038/ismej.2012.6

Bermudez-Brito, M., Plaza-Díaz, J., Muñoz-Quezada, S., Gómez-Llorente, C., & Gil, A. (2012). Probiotic Mechanisms of Action. *Annals of Nutrition and Metabolism*, *61*(2), 160–174. https://doi.org/10.1159/000342079

Berner, R., Pelz, K., Wilhelm, C., Funke, A., Leititis, J. U., & Brandis, M. (1997). Fatal sepsis caused by Corynebacterium amycolatum in a premature infant. *Journal of Clinical Microbiology*, *35*(4), 1011–1012. https://doi.org/10.1128/jcm.35.4.1011-1012.1997

Bertelli, C., Pillonel, T., Torregrossa, A., Prod'hom, G., Fischer, C. J., Greub, G., & Giannoni, E. (2015). Bifidobacterium longum bacteremia in preterm infants receiving probiotics. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *60*(6), 924–927. https://doi.org/10.1093/cid/ciu946

Bertino, E., Coppa, G. V., Giuliani, F., Coscia, A., Gabrielli, O., Sabatino, G., Sgarrella, M., Testa, T., Zampini, L., & Fabris, C. (2008). Effects of Holder Pasteurization on Human Milk Oligosaccharides. *International Journal of Immunopathology and Pharmacology*, *21*(2), 381–385. https://doi.org/10.1177/039463200802100216

Besner, G., Pisano, C., Galley, J., Elbahrawy, M., Wang, Y., & Brigstock, D. (2020). The Effect of Pasteurization on the Ability of Human Breast Milk-Derived Extracellular Vesicles to Protect the

Intestines from Experimental Necrotizing Enterocolitis. *Pediatrics*, *146*(1 MeetingAbstract), 663–664. https://doi.org/10.1542/peds.146.1_MeetingAbstract.663-a

Bi, L.-W., Yan, B.-L., Yang, Q.-Y., Li, M.-M., & Cui, H.-L. (2019). Probiotic strategies to prevent necrotizing enterocolitis in preterm infants: A meta-analysis. *Pediatric Surgery International*, *35*(10), 1143–1162. https://doi.org/10.1007/s00383-019-04547-5

Biagi, E., Aceti, A., Quercia, S., Beghetti, I., Rampelli, S., Turroni, S., Soverini, M., Zambrini, A. V., Faldella, G., Candela, M., Corvaglia, L., & Brigidi, P. (2018). Microbial Community Dynamics in Mother's Milk and Infant's Mouth and Gut in Moderately Preterm Infants. *Frontiers in Microbiology*, *9*. https://doi.org/10.3389/fmicb.2018.02512

Bilen, M., Dufour, J.-C., Lagier, J.-C., Cadoret, F., Daoud, Z., Dubourg, G., & Raoult, D. (2018). The contribution of culturomics to the repertoire of isolated human bacterial and archaeal species. *Microbiome*, *6*(1), 94. https://doi.org/10.1186/s40168-018-0485-5

Binda, S., Hill, C., Johansen, E., Obis, D., Pot, B., Sanders, M. E., Tremblay, A., & Ouwehand, A. C. (2020). Criteria to Qualify Microorganisms as "Probiotic" in Foods and Dietary Supplements. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.01662

Bin-Nun, A., Bromiker, R., Wilschanski, M., Kaplan, M., Rudensky, B., Caplan, M., & Hammerman, C. (2005). Oral Probiotics Prevent Necrotizing Enterocolitis in Very Low Birth Weight Neonates. *The Journal of Pediatrics*, *147*(2), 192–196. https://doi.org/10.1016/j.jpeds.2005.03.054

Binte Abu Bakar, S. Y., Salim, M., Clulow, A. J., Nicholas, K. R., & Boyd, B. J. (2021). Human milk composition and the effects of pasteurisation on the activity of its components. *Trends in Food Science & Technology*, *111*, 166–174. https://doi.org/10.1016/j.tifs.2021.02.055

Björkstén, B., Burman, L. G., De Château, P., Fredrikzon, B., Gothefors, L., & Hernell, O. (1980). Collecting and banking human milk: To heat or not to heat? *British Medical Journal*, *281*(6243), 765–769.

Blanchetiere, A. (2021, July 10). *Probiotics Use in French and European Neonatal Intensive Care Units*. 7th International Congress on Probiotics, Prebiotics in Pediatrics.

Bode, L., Contractor, N., Barile, D., Pohl, N., Prudden, A. R., Boons, G.-J., Jin, Y.-S., & Jennewein, S. (2016). Overcoming the limited availability of human milk oligosaccharides: Challenges and opportunities for research and application. *Nutrition Reviews*, *74*(10), 635–644. https://doi.org/10.1093/nutrit/nuw025

Bodor, A., Bounedjoum, N., Vincze, G. E., Erdeiné Kis, Á., Laczi, K., Bende, G., Szilágyi, Á., Kovács, T., Perei, K., & Rákhely, G. (2020). Challenges of unculturable bacteria: Environmental perspectives. *Reviews in Environmental Science and Bio/Technology*, *19*(1), 1–22. https://doi.org/10.1007/s11157-020-09522-4

Boix-Amorós, A., Collado, M. C., & Mira, A. (2016). Relationship between Milk Microbiota, Bacterial Load, Macronutrients, and Human Cells during Lactation. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.00492

Boix-Amorós, A., Hernández-Aguilar, M. T., Artacho, A., Collado, M. C., & Mira, A. (2020). Human milk microbiota in sub-acute lactational mastitis induces inflammation and undergoes changes in composition, diversity and load. *Scientific Reports*, *10*. https://doi.org/10.1038/s41598-020-74719-0

Boix-Amorós, A., Martinez-Costa, C., Querol, A., Collado, M. C., & Mira, A. (2017). Multiple Approaches Detect the Presence of Fungi in Human Breastmilk Samples from Healthy Mothers. *Scientific Reports*, *7*(1), Article 1. https://doi.org/10.1038/s41598-017-13270-x

Boix-Amorós, A., Puente-Sánchez, F., Toit, E. du, Linderborg, K. M., Zhang, Y., Yang, B., Salminen, S., Isolauri, E., Tamames, J., Mira, A., & Collado, M. C. (2019). Mycobiome Profiles in Breast Milk from

Healthy Women Depend on Mode of Delivery, Geographic Location, and Interaction with Bacteria. *Applied and Environmental Microbiology*, *85*(9). https://doi.org/10.1128/AEM.02994-18

Boquien, C.-Y. (2018). Human Milk: An Ideal Food for Nutrition of Preterm Newborn. *Frontiers in Pediatrics*, *6*, 295. https://doi.org/10.3389/fped.2018.00295

Boudry, G., Charton, E., Le Huerou-Luron, I., Ferret-Bernard, S., Le Gall, S., Even, S., & Blat, S. (2021). The Relationship Between Breast Milk Components and the Infant Gut Microbiota. *Frontiers in Nutrition*, *8*, 629740. https://doi.org/10.3389/fnut.2021.629740

Brandt, K., & Alatossava, T. (2003). Specific identification of certain probiotic Lactobacillus rhamnosus strains with PCR primers based on phage-related sequences. *International Journal of Food Microbiology*, *84*(2), 189–196. https://doi.org/10.1016/S0168-1605(02)00419-1

Brink, L. R., & Lönnerdal, B. (2020). Milk fat globule membrane: The role of its various components in infant health and development. *The Journal of Nutritional Biochemistry*, *85*, 108465. https://doi.org/10.1016/j.jnutbio.2020.108465

Browne, P. D., Aparicio, M., Alba, C., Hechler, C., Beijers, R., Rodríguez, J. M., Fernández, L., & de Weerth, C. (2019). Human Milk Microbiome and Maternal Postnatal Psychosocial Distress. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.02333

Butel, M.-J. (2014). Probiotics, gut microbiota and health. *Médecine et Maladies Infectieuses*, *44*(1), 1–8. https://doi.org/10.1016/j.medmal.2013.10.002

Cabrera-Rubio, R., Collado, M. C., Laitinen, K., Salminen, S., Isolauri, E., & Mira, A. (2012). The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. *The American Journal of Clinical Nutrition*, *96*(3), 544–551. https://doi.org/10.3945/ajcn.112.037382

Cabrera-Rubio, R., Kunz, C., Rudloff, S., García-Mantrana, I., Crehuá-Gaudiza, E., Martínez-Costa, C., & Collado, M. C. (2019). Association of Maternal Secretor Status and Human Milk Oligosaccharides With Milk Microbiota: An Observational Pilot Study. *Journal of Pediatric Gastroenterology and Nutrition*, *68*(2), 256–263. https://doi.org/10.1097/MPG.0000000000002216

Calderón-Ospina, C. A., & Nava-Mesa, M. O. (2019). B Vitamins in the nervous system: Current knowledge of the biochemical modes of action and synergies of thiamine, pyridoxine, and cobalamin. *CNS Neuroscience & Therapeutics*, *26*(1), 5–13. https://doi.org/10.1111/cns.13207

Carey, C. M., Kirk, J. L., Ojha, S., & Kostrzynska, M. (2007). Current and future uses of real-time polymerase chain reaction and microarrays in the study of intestinal microbiota, and probiotic use and effectiveness. *Canadian Journal of Microbiology*, *53*(5), 537–550. https://doi.org/10.1139/W07-039

Carroll, K., & Herrmann, K. R. (2013). The Cost of Using Donor Human Milk in the NICU to Achieve Exclusively Human Milk Feeding Through 32 Weeks Postmenstrual Age. *Breastfeeding Medicine*, *8*(3), 286–290. https://doi.org/10.1089/bfm.2012.0068

Champagne, C. P., Gardner, N. J., & Roy, D. (2005). Challenges in the Addition of Probiotic Cultures to Foods. *Critical Reviews in Food Science and Nutrition*, *45*(1), 61–84. https://doi.org/10.1080/10408690590900144

Chandrasekaran, S., Burnham, C.-A. D., Warner, B. B., Tarr, P. I., & Wylie, T. N. (2018). Carriage of Cronobacter sakazakii in the Very Preterm Infant Gut. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *67*(2), 269–274. https://doi.org/10.1093/cid/ciy062

Chauvière, G., Coconnier, M. H., Kernéis, S., Fourniat, J., & Servin, A. L. (1992). Adhesion of human Lactobacillus acidophilus strain LB to human enterocyte-like Caco-2 cells. *Journal of General Microbiology*, *138 Pt 8*, 1689–1696. https://doi.org/10.1099/00221287-138-8-1689

Cheema, A. S., Trevenen, M. L., Turlach, B. A., Furst, A. J., Roman, A. S., Bode, L., Gridneva, Z., Lai, C. T., Stinson, L. F., Payne, M. S., & Geddes, D. T. (2022). Exclusively Breastfed Infant Microbiota Develops over Time and Is Associated with Human Milk Oligosaccharide Intakes.

International Journal of Molecular Sciences, *23*(5), Article 5. https://doi.org/10.3390/ijms23052804

Chen, P.-W., Kuo, Y.-H., & Lin, Y.-L. (2020). The Impact of the Postpartum "Doing-the-Month" Practice on Human Milk Microbiota: A Pilot Study in Taiwan. *Microorganisms*, *8*(9). https://doi.org/10.3390/microorganisms8091283

Chien, L.-Y., Tai, C.-J., Ko, Y.-L., Huang, C.-H., & Sheu, S.-J. (2006). Adherence to "Doing-the-month" practices is associated with fewer physical and depressive symptoms among postpartum women in Taiwan. *Research in Nursing & Health*, *29*(5), 374–383. https://doi.org/10.1002/nur.20154

Chiurazzi, M., Cozzolino, M., Reinelt, T., Nguyen, T. D., Elke Chie, S., Natalucci, G., & Miletta, M. C. (2021). Human Milk and Brain Development in Infants. *Reproductive Medicine*, *2*(2), Article 2. https://doi.org/10.3390/reprodmed2020011

Cibulskis, C. C., & Armbrecht, E. S. (2015). Association of metabolic acidosis with bovine milk-based human milk fortifiers. *Journal of Perinatology*, *35*(2), 115–119. https://doi.org/10.1038/jp.2014.143

Codipilly, C. N., Koppel, A., Ranasinghe, O., Roffe, S., Ahn, S., Navarathna, M., Abeyweera, N., Coors, C., Purushotham, A., Kamoga, R., & Schanler, R. J. (2020). Effects of human milk fortifier properties on intrinsic probiotic bacteria. *Journal of Perinatal Medicine*, *48*(2), 179–183. https://doi.org/10.1515/jpm-2019-0205

Collado, M. C., Cernada, M., Baüerl, C., Vento, M., & Pérez-Martínez, G. (2012). Microbial ecology and host-microbiota interactions during early life stages. *Gut Microbes*, *3*(4), 352–365. https://doi.org/10.4161/gmic.21215

Collado, M. C., Delgado, S., Maldonado, A., & Rodríguez, J. M. (2009). Assessment of the bacterial diversity of breast milk of healthy women by quantitative real-time PCR. *Letters in Applied Microbiology*, *48*(5), 523–528. https://doi.org/10.1111/j.1472-765X.2009.02567.x

Collado, M. C., Gueimonde, M., Hernández, M., Sanz, Y., & Salminen, S. (2005). Adhesion of selected Bifidobacterium strains to human intestinal mucus and the role of adhesion in enteropathogen exclusion. *Journal of Food Protection*, *68*(12), 2672–2678. https://doi.org/10.4315/0362-028x-68.12.2672

Collado, M. C., Gueimonde, M., Sanz, Y., & Salminen, S. (2006). Adhesion properties and competitive pathogen exclusion ability of bifidobacteria with acquired acid resistance. *Journal of Food Protection*, *69*(7), 1675–1679.

Collado, M. C., Isolauri, E., & Salminen, S. (2008). Specific probiotic strains and their combinations counteract adhesion of Enterobacter sakazakii to intestinal mucus. *FEMS Microbiology Letters*, *285*(1), 58–64. https://doi.org/10.1111/j.1574-6968.2008.01211.x

Collado, M. C., Laitinen, K., Salminen, S., & Isolauri, E. (2012). Maternal weight and excessive weight gain during pregnancy modify the immunomodulatory potential of breast milk. *Pediatric Research*, *72*(1), Article 1. https://doi.org/10.1038/pr.2012.42

Collado, M. c., Meriluoto, J., & Salminen, S. (2007). Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. *Letters in Applied Microbiology*, *45*(4), 454–460. https://doi.org/10.1111/j.1472-765X.2007.02212.x

Commane, D. M., Shortt, C. T., Silvi, S., Cresci, A., Hughes, R. M., & Rowland, I. R. (2005). Effects of Fermentation Products of Pro- and Prebiotics on Trans-Epithelial Electrical Resistance in an In Vitro Model of the Colon. *Nutrition and Cancer*, *51*(1), 102–109. https://doi.org/10.1207/s15327914nc5101_14

Committee on Nutrition, Sectionn on Breastfeeding, Committee on Fetus and Newborn, Daniels, S., Corkins, M., de Ferranti, S., Golden, N. H., Kim, J. H., Magge, S. N., Schwarzenberg, S. J., Meek, J. Y., Johnston, M. G., O'Connor, M. E., Stellwagen, L. M., Thomas, J. P., Ware, J. L., Schanler,

R. J., Watterberg, K. L., Aucott, S. W., … Stewart, D. L. (2017). Donor Human Milk for the High-Risk Infant: Preparation, Safety, and Usage Options in the United States. *Pediatrics*, *139*(1), e20163440. https://doi.org/10.1542/peds.2016-3440

Cong, X., Judge, M., Xu, W., Diallo, A., Janton, S., Brownell, E. A., Maas, K., & Graf, J. (2017). Influence of Infant Feeding Type on Gut Microbiome Development in Hospitalized Preterm Infants. *Nursing Research*, *66*(2), 123–133. https://doi.org/10.1097/NNR.0000000000000208

Cordeiro, B. F., Oliveira, E. R., da Silva, S. H., Savassi, B. M., Acurcio, L. B., Lemos, L., Alves, J. de L., Carvalho Assis, H., Vieira, A. T., Faria, A. M. C., Ferreira, E., Le Loir, Y., Jan, G., Goulart, L. R., Azevedo, V., Carvalho, R. D. de O., & do Carmo, F. L. R. (2018). Whey Protein Isolate-Supplemented Beverage, Fermented by Lactobacillus casei BL23 and Propionibacterium freudenreichii 138, in the Prevention of Mucositis in Mice. *Frontiers in Microbiology*, *9*. https://doi.org/10.3389/fmicb.2018.02035

Corona-Cervantes, K., García-González, I., Villalobos-Flores, L. E., Hernández-Quiroz, F., Piña-Escobedo, A., Hoyo-Vadillo, C., Rangel-Calvillo, M. N., & García-Mena, J. (2020). Human milk microbiota associated with early colonization of the neonatal gut in Mexican newborns. *PeerJ*, *8*, e9205. https://doi.org/10.7717/peerj.9205

Cortes-Macías, E., Selma-Royo, M., García-Mantrana, I., Calatayud, M., González, S., Martínez-Costa, C., & Collado, M. C. (2021). Maternal Diet Shapes the Breast Milk Microbiota Composition and Diversity: Impact of Mode of Delivery and Antibiotic Exposure. *The Journal of Nutrition*, *151*(2), 330–340. https://doi.org/10.1093/jn/nxaa310

Corthesy, B. (2013). Multi-Faceted Functions of Secretory IgA at Mucosal Surfaces. *Frontiers in Immunology*, *4*. https://doi.org/10.3389/fimmu.2013.00185

Cossey, V., Jeurissen, A., Bossuyt, X., & Schuermans, A. (2009). Effect of pasteurisation on the mannose-binding lectin activity and the concentration of soluble CD14 in human milk. *Journal of Hospital Infection*, *73*(1), 96–97. https://doi.org/10.1016/j.jhin.2009.06.021

Cress, C., & Jr, C. L. P. (1977). Breast Milk Macrophages. *Pediatric Research*, *11*(4), 485. https://doi.org/10.1203/00006450-197704000-00693

Crump, C., Howell, E. A., Stroustrup, A., McLaughlin, M. A., Sundquist, J., & Sundquist, K. (2019). Association of Preterm Birth With Risk of Ischemic Heart Disease in Adulthood. *JAMA Pediatrics*, *173*(8), 736–743. https://doi.org/10.1001/jamapediatrics.2019.1327

Dahl, C., Stigum, H., Valeur, J., Iszatt, N., Lenters, V., Peddada, S., Bjørnholt, J. V., Midtvedt, T., Mandal, S., & Eggesbø, M. (2018). Preterm infants have distinct microbiomes not explained by mode of delivery, breastfeeding duration or antibiotic exposure. *International Journal of Epidemiology*, *47*(5), 1658–1669. https://doi.org/10.1093/ije/dyy064

Daiy, K., Harries, V., Nyhan, K., & Marcinkowska, U. M. (2022). Maternal weight status and the composition of the human milk microbiome: A scoping review. *PLoS ONE*, *17*(10), e0274950. https://doi.org/10.1371/journal.pone.0274950

Dallal, M. M. S., Maragheh, N. M., Rajabi, Z., Hosseini, M. B., Papizadeh, M., Hassanpour, G., Yazdi, S. S., Memariani, M., Khajavi, S. H., Langaroodi, S. M. A., Mirzaei, H. M. A., & Mirzababaei, M. (2021). Probiotic properties of Lactobacilli isolated from human milk and their inhibitory effect on gastrointestinal pathogenic bacteria. *African Journal of Microbiology Research*, *15*(6), 318–324. https://doi.org/10.5897/AJMR2021.9528

Daniels, B., Coutsoudis, A., Autran, C., Amundson Mansen, K., Israel-Ballard, K., & Bode, L. (2017). The effect of simulated flash heating pasteurisation and Holder pasteurisation on human milk oligosaccharides. *Paediatrics and International Child Health*, *37*(3), 204–209. https://doi.org/10.1080/20469047.2017.1293869

Davanzo, R. (2018). Controversies in Breastfeeding. *Frontiers in Pediatrics*, *6*, 278. https://doi.org/10.3389/fped.2018.00278

De Keersmaecker, S. C. J., Braeken, K., Verhoeven, T. L. A., Perea Vélez, M., Lebeer, S., Vanderleyden, J., & Hols, P. (2006). Flow Cytometric Testing of Green Fluorescent Protein-Tagged Lactobacillus rhamnosus GG for Response to Defensins. *Applied and Environmental Microbiology*, *72*(7), 4923–4930. https://doi.org/10.1128/AEM.02605-05

de Moreno de LeBlanc, A., del Carmen, S., Zurita-Turk, M., Santos Rocha, C., van de Guchte, M., Azevedo, V., Miyoshi, A., & LeBlanc, J. G. (2011). Importance of IL-10 Modulation by Probiotic Microorganisms in Gastrointestinal Inflammatory Diseases. *ISRN Gastroenterology*, *2011*. https://doi.org/10.5402/2011/892971

De Souza Oliveira, R. P., Perego, P., De Oliveira, M. N., & Converti, A. (2011). Effect of inulin as a prebiotic to improve growth and counts of a probiotic cocktail in fermented skim milk. *LWT - Food Science and Technology*, *44*(2), 520–523. https://doi.org/10.1016/j.lwt.2010.08.024

de Waard, M., Mank, E., van Dijk, K., Schoonderwoerd, A., & van Goudoever, J. B. (2018). Holder-Pasteurized Human Donor Milk: How Long Can It Be Preserved? *Journal of Pediatric Gastroenterology and Nutrition*, *66*(3), 479. https://doi.org/10.1097/MPG.0000000000001782

Deepika, G., Karunakaran, E., Hurley, C. R., Biggs, C. A., & Charalampopoulos, D. (2012). Influence of fermentation conditions on the surface properties and adhesion of Lactobacillus rhamnosus GG. *Microbial Cell Factories*, *11*, 116. https://doi.org/10.1186/1475-2859-11-116

Delano, M. J., & Ward, P. A. (2016). The Immune System's Role in Sepsis Progression, Resolution and Long-Term Outcome. *Immunological Reviews*, *274*(1), 330–353. https://doi.org/10.1111/imr.12499

Demers-Mathieu, V., Huston, R. K., Markell, A. M., McCulley, E. A., Martin, R. L., Spooner, M., & Dallas, D. C. (2019). Differences in Maternal Immunoglobulins within Mother's Own Breast Milk and Donor Breast Milk and across Digestion in Preterm Infants. *Nutrients*, *11*(4). https://doi.org/10.3390/nu11040920

Demers-Mathieu, V., Underwood, M., Beverly, R. L., Nielsen, S. D., & Dallas, D. (2018). Comparison of Human Milk Immunoglobulin Survival during Gastric Digestion between Preterm and Term Infants. *Nutrients*. https://doi.org/10.3390/nu10050631

Denkel, L. A., Schwab, F., Garten, L., Geffers, C., Gastmeier, P., & Piening, B. (2016). Protective Effect of Dual-Strain Probiotics in Preterm Infants: A Multi-Center Time Series Analysis. *PLOS ONE*, *11*(6), e0158136. https://doi.org/10.1371/journal.pone.0158136

Dermyshi, E., Wang, Y., Yan, C., Hong, W., Qiu, G., Gong, X., & Zhang, T. (2017). The "Golden Age" of Probiotics: A Systematic Review and Meta-Analysis of Randomized and Observational Studies in Preterm Infants. *Neonatology*, *112*(1), 9–23. https://doi.org/10.1159/000454668

Di Martino, M. L., Campilongo, R., Casalino, M., Micheli, G., Colonna, B., & Prosseda, G. (2013). Polyamines: Emerging players in bacteria-host interactions. *International Journal of Medical Microbiology: IJMM*, *303*(8), 484–491. https://doi.org/10.1016/j.ijmm.2013.06.008

do Carmo, F. L. R., Rabah, H., De Oliveira Carvalho, R. D., Gaucher, F., Cordeiro, B. F., da Silva, S. H., Le Loir, Y., Azevedo, V., & Jan, G. (2018). Extractable Bacterial Surface Proteins in Probiotic–Host Interaction. *Frontiers in Microbiology*, *9*. https://doi.org/10.3389/fmicb.2018.00645

Domínguez-Díaz, C., García-Orozco, A., Riera-Leal, A., Padilla-Arellano, J. R., & Fafutis-Morris, M. (2019). Microbiota and Its Role on Viral Evasion: Is It With Us or Against Us? *Frontiers in Cellular and Infection Microbiology*, *9*. https://doi.org/10.3389/fcimb.2019.00256

Donovan, R., Kelly, S. G., Prazad, P., Talaty, P. N., Lefaiver, C., Hastings, M. L., & Everly, D. N. (2017). The effects of human milk fortification on nutrients and milk properties. *Journal of Perinatology*, *37*(1), 42–48. https://doi.org/10.1038/jp.2016.166

Dronkers, T. M. G., Ouwehand, A. C., & Rijkers, G. T. (2020). Global analysis of clinical trials with probiotics. *Heliyon*, *6*(7), e04467. https://doi.org/10.1016/j.heliyon.2020.e04467

Edgar, R. C. (2018). Updating the 97% identity threshold for 16S ribosomal RNA OTUs. *Bioinformatics (Oxford, England)*, *34*(14), 2371–2375. https://doi.org/10.1093/bioinformatics/bty113

Edgar, R. C., & Flyvbjerg, H. (2015). Error filtering, pair assembly and error correction for next-generation sequencing reads. *Bioinformatics*, *31*(21), 3476–3482. https://doi.org/10.1093/bioinformatics/btv401

Ellaby, N. (2018). *A Large Scale Metagenomic Analysis of the Faecal Microbiota in Preterm Infants Developing Necrotising Enterocolitis* [Phd, University of Liverpool]. https://livrepository.liverpool.ac.uk/3025353

Erdem, A. L., Avelino, F., Xicohtencatl-Cortes, J., & Girón, J. A. (2007). Host Protein Binding and Adhesive Properties of H6 and H7 Flagella of Attaching and Effacing Escherichia coli. *Journal of Bacteriology*, *189*(20), 7426–7435. https://doi.org/10.1128/JB.00464-07

Ernst, M., Rogers, S., Lausten-Thomsen, U., Björkbom, A., Laursen, S. S., Courraud, J., Børglum, A., Nordentoft, M., Werge, T., Mortensen, P. B., Hougaard, D. M., & S.Cohen, A. (2020). Gestational-age-dependent development of the neonatal metabolome. *medRxiv*, 2020.03.27.20045534. https://doi.org/10.1101/2020.03.27.20045534

Evans, T. J., Ryley, H. C., Neale, L. M., Dodge, J. A., & Lewarne, V. M. (1978). Effect of storage and heat on antimicrobial proteins in human milk. *Archives of Disease in Childhood*, *53*(3), 239–241.

Fang, L., Zhang, M., Wu, L., Wang, R., Lin, B., Yao, J., & Chen, D. (2021). Is preterm donor milk better than preterm formula for very-low-birth-weight infants? *Food & Nutrition Research*, *65*, 10.29219/fnr.v65.5346. https://doi.org/10.29219/fnr.v65.5346

Fang, T., Liu, G., Cao, W., Wu, X., Jia, G., Zhao, H., Chen, X., Wu, C., & Wang, J. (2016). Spermine: New insights into the intestinal development and serum antioxidant status of suckling piglets. *RSC Advances*, *6*(37), 31323–31335. https://doi.org/10.1039/C6RA05361K

FAO/WHO. (2002). *Report of a Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food*. https://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf

Fehr, K., Moossavi, S., Sbihi, H., Boutin, R. C. T., Bode, L., Robertson, B., Yonemitsu, C., Field, C. J., Becker, A. B., Mandhane, P. J., Sears, M. R., Khafipour, E., Moraes, T. J., Subbarao, P., Finlay, B. B., Turvey, S. E., & Azad, M. B. (2020). Breastmilk Feeding Practices Are Associated with the Co-Occurrence of Bacteria in Mothers' Milk and the Infant Gut: The CHILD Cohort Study. *Cell Host & Microbe*, *28*(2), 285-297.e4. https://doi.org/10.1016/j.chom.2020.06.009

Fekete, E., & Buret, A. G. (2023). The role of mucin O-glycans in microbiota dysbiosis, intestinal homeostasis, and host-pathogen interactions. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *324*(6), G452–G465. https://doi.org/10.1152/ajpgi.00261.2022

Felgentreff, K., Beisswenger, C., Griese, M., Gulder, T., Bringmann, G., & Bals, R. (2006). The antimicrobial peptide cathelicidin interacts with airway mucus. *Peptides*, *27*(12), 3100–3106. https://doi.org/10.1016/j.peptides.2006.07.018

Feng, K., Rowell, A. C., Andres, A., Bellando, B. J., Lou, X., Glasier, C. M., Ramakrishnaiah, R. H., Badger, T. M., & Ou, X. (2019). Diffusion Tensor MRI of White Matter of Healthy Full-term Newborns: Relationship to Neurodevelopmental Outcomes. *Radiology*, *292*(1), 179–187. https://doi.org/10.1148/radiol.2019182564

Ferdousi, R., Rouhi, M., Mohammadi, R., Mortazavian, A. M., Khosravi-Darani, K., & Homayouni Rad, A. (2013). Evaluation of Probiotic Survivability in Yogurt Exposed To Cold Chain Interruption. *Iranian Journal of Pharmaceutical Research : IJPR*, *12*(Suppl), 139–144.

Fernández, L., Pannaraj, P. S., Rautava, S., & Rodríguez, J. M. (2020). The Microbiota of the Human Mammary Ecosystem. *Frontiers in Cellular and Infection Microbiology*, *10*, 586667. https://doi.org/10.3389/fcimb.2020.586667

Filatava, E. J., Liu, Z., Xie, J., Tran, D.-B., Chen, K., El Habbal, N., Weinstock, G., Zhou, Y., & Gregory, K. E. (2023). The preterm human milk microbiota fluctuates by postpartum week and is characterized by gestational age and maternal BMI. *mBio*, *14*(6), e02106-23. https://doi.org/10.1128/mbio.02106-23

Fitzstevens, J. L., Smith, K. C., Hagadorn, J. I., Caimano, M. J., Matson, A. P., & Brownell, E. A. (2017). Systematic Review of the Human Milk Microbiota. *Nutrition in Clinical Practice*, *32*(3), 354–364. https://doi.org/10.1177/0884533616670150

Floris, L. M., Stahl, B., Abrahamse-Berkeveld, M., & Teller, I. C. (2020). Human milk fatty acid profile across lactational stages after term and preterm delivery: A pooled data analysis. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *156*, 102023. https://doi.org/10.1016/j.plefa.2019.102023

Frese, S. A., Hutton, A. A., Contreras, L. N., Shaw, C. A., Palumbo, M. C., Casaburi, G., Xu, G., Davis, J. C. C., Lebrilla, C. B., Henrick, B. M., Freeman, S. L., Barile, D., German, J. B., Mills, D. A., Smilowitz, J. T., & Underwood, M. A. (2017). Persistence of Supplemented Bifidobacterium longum subsp. Infantis EVC001 in Breastfed Infants. *mSphere*. https://doi.org/10.1128/mSphere.00501-17

Galazzo, G., Best, N. van, Bervoets, L., Dapaah, I. O., Savelkoul, P. H., Hornef, M. W., Hutton, E. K., Morrison, K., Holloway, A. C., McDonald, H., Ratcliffe, E. M., Stearns, J. C., Schertzer, J. D., Surette, M. G., Thabane, L., Mommers, M., Lau, S., Hamelmann, E., & Penders, J. (2020). Development of the Microbiota and Associations With Birth Mode, Diet, and Atopic Disorders in a Longitudinal Analysis of Stool Samples, Collected From Infancy Through Early Childhood. *Gastroenterology*, *158*(6), 1584–1596. https://doi.org/10.1053/j.gastro.2020.01.024

Galdeano, C. M., & Perdigon, G. (2004). Role of viability of probiotic strains in their persistence in the gut and in mucosal immune stimulation. *Journal of Applied Microbiology*, *97*(4), 673–681. https://doi.org/10.1111/j.1365-2672.2004.02353.x

Garcia, A. F., Benchimol, M., & Alderete, J. F. (2005). Trichomonas vaginalis Polyamine Metabolism Is Linked to Host Cell Adherence and Cytotoxicity. *Infection and Immunity*, *73*(5), 2602–2610. https://doi.org/10.1128/IAI.73.5.2602-2610.2005

García-Ricobaraza, M., García-Santos, J. A., Escudero-Marín, M., Diéguez, E., Cerdó, T., & Campoy, C. (2021). Short- and Long-Term Implications of Human Milk Microbiota on Maternal and Child Health. *International Journal of Molecular Sciences*, *22*(21), Article 21. https://doi.org/10.3390/ijms222111866

Gephart, S. M., & Newnam, K. M. (2019). Closing the Gap Between Recommended and Actual Human Milk Use for Fragile Infants: What Will It Take to Overcome Disparities? *Clinics in Perinatology*, *46*(1), 39–50. https://doi.org/10.1016/j.clp.2018.09.003

Gibbs, J. H., Fisher, C., Bhattacharya, S., Goddard, P., & Baum, J. D. (1977). Drip breast milk: Its composition, collection and pasteurization. *Early Human Development*, *1*(3), 227–245. https://doi.org/10.1016/0378-3782(77)90037-8

Gidrewicz, D. A., & Fenton, T. R. (2014). A systematic review and meta-analysis of the nutrient content of preterm and term breast milk. *BMC Pediatrics*, *14*(1), 216. https://doi.org/10.1186/1471-2431-14-216

Gilad, O., Svensson, B., Viborg, A. H., Stuer-Lauridsen, B., & Jacobsen, S. (2011). The extracellular proteome of Bifidobacterium animalis subsp. Lactis BB-12 reveals proteins with putative roles in probiotic effects. *PROTEOMICS*, *11*(12), 2503–2514. https://doi.org/10.1002/pmic.201000716

Gloor, G. B., & Reid, G. (2016). Compositional analysis: A valid approach to analyze microbiome high-throughput sequencing data. *Canadian Journal of Microbiology*, *62*(8), 692–703. https://doi.org/10.1139/cjm-2015-0821

Godhia, M., & Patel, N. (2013). Colostrum - Its Composition, Benefits As A Nutraceutical: A Review. *Current Research in Nutrition and Food Science Journal*, *1*(1), 37–47. https://doi.org/10.12944/CRNFSJ.1.1.04

Gomart, A., Vallée, A., & Lecarpentier, Y. (2021). Necrotizing Enterocolitis: LPS/TLR4-Induced Crosstalk Between Canonical TGF-β/Wnt/β-Catenin Pathways and PPARγ. *Frontiers in Pediatrics*, *9*, 1098. https://doi.org/10.3389/fped.2021.713344

Gómez-Gallego, C., Collado, M. C., Ilo, T., Jaakkola, U.-M., Bernal, M. J., Periago, M. J., Salminen, S., Ros, G., & Frias, R. (2012). Infant formula supplemented with polyamines alters the intestinal microbiota in neonatal BALB/cOlaHsd mice. *The Journal of Nutritional Biochemistry*, *23*(11), 1508–1513. https://doi.org/10.1016/j.jnutbio.2011.10.003

Gómez-Gallego, C., Kumar, H., García-Mantrana, I., du Toit, E., Suomela, J.-P., Linderborg, K. M., Zhang, Y., Isolauri, E., Yang, B., Salminen, S., & Collado, M. C. (2017). Breast Milk Polyamines and Microbiota Interactions: Impact of Mode of Delivery and Geographical Location. *Annals of Nutrition & Metabolism*, *70*(3), 184–190. https://doi.org/10.1159/000457134

Gómez-Gallego, C., Recio, I., Gómez-Gómez, V., Ortuño, I., Bernal, M. J., Ros, G., & Periago, M. J. (2016). Effect of processing on polyamine content and bioactive peptides released after in vitro gastrointestinal digestion of infant formulas. *Journal of Dairy Science*, *99*(2), 924–932. https://doi.org/10.3168/jds.2015-10030

González, R., Mandomando, I., Fumadó, V., Sacoor, C., Macete, E., Alonso, P. L., & Menendez, C. (2013). Breast Milk and Gut Microbiota in African Mothers and Infants from an Area of High HIV Prevalence. *PLoS ONE*, *8*(11). https://doi.org/10.1371/journal.pone.0080299

Gonzalez-Rodriguez, I., Gaspar, P., Sanchez, B., Gueimonde, M., Margolles, A., & Neves, A. R. (2013). Catabolism of Glucose and Lactose in Bifidobacterium animalis subsp. Lactis, Studied by 13C Nuclear Magnetic Resonance. *Applied and Environmental Microbiology*, *79*(24), 7628–7638. https://doi.org/10.1128/AEM.02529-13

Gopalakrishna, K. P., Macadangdang, B. R., Rogers, M. B., Tometich, J. T., Firek, B. A., Baker, R., Ji, J., Burr, A. H. P., Ma, C., Good, M., Morowitz, M. J., & Hand, T. W. (2019). Maternal IgA protects against the development of necrotizing enterocolitis in preterm infants. *Nature Medicine*, *25*(7), 1110–1115. https://doi.org/10.1038/s41591-019-0480-9

Gorbach, S. L., & Goldin, B. R. (1989). Lactobacillus strains and methods of selection. *United States Patent No. US 4839281 A*.

Gray, K. D., Messina, J. A., Cortina, C., Owens, T., Fowler, M., Foster, M., Gbadegesin, S., Clark, R. H., Benjamin, D. K., Zimmerman, K. O., & Greenberg, R. G. (2020). Probiotic Use and Safety in the Neonatal Intensive Care Unit: A Matched Cohort Study. *The Journal of Pediatrics*, *222*, 59-64.e1. https://doi.org/10.1016/j.jpeds.2020.03.051

Gréco, S., Niepceron, E., Hugueny, I., George, P., Louisot, P., & Biol, M.-C. (2001). Dietary Spermidine and Spermine Participate in the Maturation of Galactosyltransferase Activity and Glycoprotein Galactosylation in Rat Small Intestine. *The Journal of Nutrition*, *131*(7), 1890–1897. https://doi.org/10.1093/jn/131.7.1890

Gritz, E. C., & Bhandari, V. (2015). The Human Neonatal Gut Microbiome: A Brief Review. *Frontiers in Pediatrics*, *3*. https://doi.org/10.3389/fped.2015.00017

Grönlund, M.-M., Gueimonde, M., Laitinen, K., Kociubinski, G., Grönroos, T., Salminen, S., & Isolauri, E. (2007). Maternal breast-milk and intestinal bifidobacteria guide the compositional development of the Bifidobacterium microbiota in infants at risk of allergic disease. *Clinical and Experimental Allergy: Journal of the British Society for Allergy and Clinical Immunology*, *37*(12), 1764–1772. https://doi.org/10.1111/j.1365-2222.2007.02849.x

Gueimonde, M., Laitinen, K., Salminen, S., & Isolauri, E. (2007). Breast milk: A source of bifidobacteria for infant gut development and maturation? *Neonatology*, *92*(1), 64–66. https://doi.org/10.1159/000100088

Guerin, J., Burgain, J., Francius, G., El-Kirat-Chatel, S., Beaussart, A., Scher, J., & Gaiani, C. (2018). Adhesion of Lactobacillus rhamnosus GG surface biomolecules to milk proteins. *Food Hydrocolloids*, *82*, 296–303. https://doi.org/10.1016/j.foodhyd.2018.04.016

Gunasekera, T. S., Sørensen, A., Attfield, P. V., Sørensen, S. J., & Veal, D. A. (2002). Inducible Gene Expression by Nonculturable Bacteria in Milk after Pasteurization. *Applied and Environmental Microbiology*, *68*(4), 1988–1993. https://doi.org/10.1128/AEM.68.4.1988-1993.2002

Gustaw, W., Kozioł, J., Radzki, W., Skrzypczak, K., Michalak-Majewska, M., Sołowiej, B., Sławińska, A., & Jabłońska-Ryś, E. (2016). The effect of addition of selected milk protein preparations on the growth of Lactobacillus acidophilus and physicochemical properties of fermented milk. *Acta Scientiarum Polonorum. Technologia Alimentaria*, *15*(1), 29–36. https://doi.org/10.17306/J.AFS.2016.1.3

Hammer, O., Harper, D. A. T., & Ryan, P. D. (2001). PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontologia Electronica*, *4*, 9.

Hanfrey, C. C., Pearson, B. M., Hazeldine, S., Lee, J., Gaskin, D. J., Woster, P. M., Phillips, M. A., & Michael, A. J. (2011). Alternative Spermidine Biosynthetic Route Is Critical for Growth of Campylobacter jejuni and Is the Dominant Polyamine Pathway in Human Gut Microbiota *. *Journal of Biological Chemistry*, *286*(50), 43301–43312. https://doi.org/10.1074/jbc.M111.307835

Harata, G., Yoda, K., Wang, R., Miyazawa, K., Sato, M., He, F., & Endo, A. (2021). Species- and Age/Generation-Dependent Adherence of Bifidobacterium bifidum to Human Intestinal Mucus In Vitro. *Microorganisms*, *9*(3), Article 3. https://doi.org/10.3390/microorganisms9030542

Harmsen, H. J., Wildeboer-Veloo, A. C., Raangs, G. C., Wagendorp, A. A., Klijn, N., Bindels, J. G., & Welling, G. W. (2000). Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *Journal of Pediatric Gastroenterology and Nutrition*, *30*(1), 61–67.

Hassiotou, F., Heath, B., Ocal, O., Filgueira, L., Geddes, D., Hartmann, P., & Wilkie, T. (2014). Breastmilk stem cell transfer from mother to neonatal organs (216.4). *The FASEB Journal*, *28*(S1), 216.4. https://doi.org/10.1096/fasebj.28.1_supplement.216.4

Hassiotou, F., Hepworth, A. R., Metzger, P., Tat Lai, C., Trengove, N., Hartmann, P. E., & Filgueira, L. (2013). Maternal and infant infections stimulate a rapid leukocyte response in breastmilk. *Clinical & Translational Immunology*, *2*(4), e3. https://doi.org/10.1038/cti.2013.1

He, S., Liu, G., & Zhu, X. (2021). Human breast milk-derived exosomes may help maintain intestinal epithelial barrier integrity. *Pediatric Research*, *90*(2), 366–372. https://doi.org/10.1038/s41390-021-01449-y

He, Y., He, Z., Leone, S., & Liu, S. (2021). Milk Exosomes Transfer Oligosaccharides into Macrophages to Modulate Immunity and Attenuate Adherent-Invasive E. coli (AIEC) Infection. *Nutrients*, *13*(9), 3198. https://doi.org/10.3390/nu13093198

Heikkilä, M. P., & Saris, P. E. J. (2003). Inhibition of Staphylococcus aureus by the commensal bacteria of human milk. *Journal of Applied Microbiology*, *95*(3), 471–478.

Henrick, B. M., Rodriguez, L., Lakshmikanth, T., Pou, C., Henckel, E., Olin, A., Wang, J., Mikes, J., Tan, Z., Chen, Y., Ehrlich, A. M., Bernhardsson, A. K., Mugabo, C. H., Ambrosiani, Y., Gustafsson, A., Chew, S., Brown, H. K., Prambs, J., Bohlin, K., … Brodin, P. (2020). *Bifidobacteria-mediated immune system imprinting early in life* (p. 2020.10.24.353250). https://doi.org/10.1101/2020.10.24.353250

Hermansson, H., Kumar, H., Collado, M. C., Salminen, S., Isolauri, E., & Rautava, S. (2019). Breast Milk Microbiota Is Shaped by Mode of Delivery and Intrapartum Antibiotic Exposure. *Frontiers in Nutrition*, *6*. https://doi.org/10.3389/fnut.2019.00004

Hesterberg, R. S., Cleveland, J. L., & Epling-Burnette, P. K. (2018). Role of Polyamines in Immune Cell Functions. *Medical Sciences*, *6*(1). https://doi.org/10.3390/medsci6010022

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C., & Sanders, M. E. (2014). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology & Hepatology*, *11*(8), 506–514. https://doi.org/10.1038/nrgastro.2014.66

Hirschmugl, B., Brandl, W., Csapo, B., van Poppel, M., Köfeler, H., Desoye, G., Wadsack, C., & Jantscher-Krenn, E. (2019). Evidence of Human Milk Oligosaccharides in Cord Blood and Maternal-to-Fetal Transport across the Placenta. *Nutrients*, *11*(11), 2640. https://doi.org/10.3390/nu11112640

Holdsworth, E. A., Williams, J. E., Pace, R. M., Lane, A. A., Gartstein, M., McGuire, M. A., McGuire, M. K., & Meehan, C. L. (2023). Breastfeeding patterns are associated with human milk microbiome composition: The Mother-Infant Microbiomes, Behavior, and Ecology Study (MIMBES). *PLOS ONE*, *18*(8), e0287839. https://doi.org/10.1371/journal.pone.0287839

Hortensius, L. M., Janson, E., van Beek, P. E., Groenendaal, F., Claessens, N. H. P., Swanenburg de Veye, H. F. N., Eijsermans, M. J. C., Koopman-Esseboom, C., Dudink, J., van Elburg, R. M., Benders, M. J. N. L., Tataranno, M. L., & van der Aa, N. E. (2021). Nutritional Intake, White Matter Integrity, and Neurodevelopment in Extremely Preterm Born Infants. *Nutrients*, *13*(10), Article 10. https://doi.org/10.3390/nu13103409

Huf, I. U., Baque, E., Colditz, P. B., Chatfield, M. D., Ware, R. S., Boyd, R. N., & George, J. M. (2023). Neurological examination at 32-weeks postmenstrual age predicts 12-month cognitive outcomes in very preterm-born infants. *Pediatric Research*, *93*(6), Article 6. https://doi.org/10.1038/s41390-022-02310-6

Hufnagl, K., Pali-Schöll, I., Roth-Walter, F., & Jensen-Jarolim, E. (2020). Dysbiosis of the gut and lung microbiome has a role in asthma. *Seminars in Immunopathology*, *42*(1), 75–93. https://doi.org/10.1007/s00281-019-00775-y

Hughes, J. B., Hellmann, J. J., Ricketts, T. H., & Bohannan, B. J. M. (2001). Counting the Uncountable: Statistical Approaches to Estimating Microbial Diversity. *Applied and Environmental Microbiology*, *67*(10), 4399–4406. https://doi.org/10.1128/AEM.67.10.4399-4406.2001

Hui, Y., Smith, B., Mortensen, M. S., Krych, L., Sørensen, S. J., Greisen, G., Krogfelt, K. A., & Nielsen, D. S. (2021). The effect of early probiotic exposure on the preterm infant gut microbiome development. *Gut Microbes*, *13*(1), 1951113. https://doi.org/10.1080/19490976.2021.1951113

Hunt, K. M., Foster, J. A., Forney, L. J., Schütte, U. M. E., Beck, D. L., Abdo, Z., Fox, L. K., Williams, J. E., McGuire, M. K., & McGuire, M. A. (2011). Characterization of the Diversity and Temporal Stability of Bacterial Communities in Human Milk. *PLOS ONE*, *6*(6), e21313. https://doi.org/10.1371/journal.pone.0021313

Ibal, J. C., Pham, H. Q., Park, C. E., & Shin, J.-H. (2019). Information about variations in multiple copies of bacterial 16S rRNA genes may aid in species identification. *PLoS ONE*, *14*(2). https://doi.org/10.1371/journal.pone.0212090

Igarashi, K., & Kashiwagi, K. (2019). The functional role of polyamines in eukaryotic cells. *The International Journal of Biochemistry & Cell Biology*, *107*, 104–115. https://doi.org/10.1016/j.biocel.2018.12.012

Institute of Medicine (US) Committee on Nutritional Status During Pregnancy and Lactation. (1991). Milk Volume. In *Nutrition During Lactation*. National Academies Press (US). https://www.ncbi.nlm.nih.gov/books/NBK235589/

Instituto Nacional de Estadística y Censos (INDEC)—Secretaría de Gobierno de Salud de la Nación. (2019). *4° Encuesta Nacional de Factores de Riesgo. Resultados definitivos*. https://www.indec.gob.ar/ftp/cuadros/publicaciones/enfr_2018_resultados_definitivos.pdf

Ioannidis, N. E., & Kotzabasis, K. (2007). Effects of polyamines on the functionality of photosynthetic membrane in vivo and in vitro. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1767*(12), 1372–1382. https://doi.org/10.1016/j.bbabio.2007.10.002

Italianer, M. F., Naninck, E. F. G., Roelants, J. A., van der Horst, G. T. J., Reiss, I. K. M., Goudoever, J. B. van, Joosten, K. F. M., Chaves, I., & Vermeulen, M. J. (2020). Circadian Variation in Human Milk Composition, a Systematic Review. *Nutrients*, *12*(8), E2328. https://doi.org/10.3390/nu12082328

Jakaitis, B. M., & Denning, P. W. (2014). Human Breast Milk and the Gastrointestinal Innate Immune System. *Clinics in Perinatology*, *41*(2), 423–435. https://doi.org/10.1016/j.clp.2014.02.011

Janer, C., Peláez, C., & Requena, T. (2004). Caseinomacropeptide and whey protein concentrate enhance Bifidobacterium lactis growth in milk. *Food Chemistry*, *86*(2), 263–267. https://doi.org/10.1016/j.foodchem.2003.09.034

Jansson, E. A., Petersson, J., Reinders, C., Sobko, T., Björne, H., Phillipson, M., Weitzberg, E., Holm, L., & Lundberg, J. O. (2007). Protection from nonsteroidal anti-inflammatory drug (NSAID)-induced gastric ulcers by dietary nitrate. *Free Radical Biology & Medicine*, *42*(4), 510–518. https://doi.org/10.1016/j.freeradbiomed.2006.11.018

Jantscher-Krenn, E., Aigner, J., Reiter, B., Köfeler, H., Csapo, B., Desoye, G., Bode, L., & van Poppel, M. N. M. (2019). Evidence of human milk oligosaccharides in maternal circulation already during pregnancy: A pilot study. *American Journal of Physiology-Endocrinology and Metabolism*, *316*(3), E347–E357. https://doi.org/10.1152/ajpendo.00320.2018

Jara, S., Sánchez, M., Vera, R., Cofré, J., & Castro, E. (2011). The inhibitory activity of Lactobacillus spp. Isolated from breast milk on gastrointestinal pathogenic bacteria of nosocomial origin. *Anaerobe*, *17*(6), 474–477. https://doi.org/10.1016/j.anaerobe.2011.07.008

Järvinen, K. M. (2018). Variations in Human Milk Composition: Impact on Immune Development and Allergic Disease Susceptibility. *Breastfeeding Medicine*, *13*(S1), S-11. https://doi.org/10.1089/bfm.2018.29075.kjs

Jiang, S., Yang, C., Yang, C., Yan, W., Shah, V., Shah, P. S., Lee, S. K., Yang, Y., & Cao, Y. (2020). Epidemiology and microbiology of late-onset sepsis among preterm infants in China, 2015–2018: A cohort study. *International Journal of Infectious Diseases*, *96*, 1–9. https://doi.org/10.1016/j.ijid.2020.03.034

Jiménez, E., de Andrés, J., Manrique, M., Pareja-Tobes, P., Tobes, R., Martínez-Blanch, J. F., Codoñer, F. M., Ramón, D., Fernández, L., & Rodríguez, J. M. (2015). Metagenomic Analysis of Milk of Healthy and Mastitis-Suffering Women. *Journal of Human Lactation*, *31*(3), 406–415. https://doi.org/10.1177/0890334415585078

Jiménez, E., Fernández, L., Maldonado, A., Martín, R., Olivares, M., Xaus, J., & Rodríguez, J. M. (2008). Oral Administration of Lactobacillus Strains Isolated from Breast Milk as an Alternative for the Treatment of Infectious Mastitis during Lactation. *Applied and Environmental Microbiology*, *74*(15), 4650–4655. https://doi.org/10.1128/AEM.02599-07

Jocson, M. A., Mason, E. O., & Schanler, R. J. (1997). The effects of nutrient fortification and varying storage conditions on host defense properties of human milk. *Pediatrics*, *100*(2 Pt 1), 240–243. https://doi.org/10.1542/peds.100.2.240

Johansson, M. E. V., Larsson, J. M. H., & Hansson, G. C. (2011). The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host–microbial interactions. *Proceedings of the National Academy of Sciences*, *108*(Supplement 1), 4659–4665. https://doi.org/10.1073/pnas.1006451107

Johnson, L., Mulcahy, H., Kanevets, U., Shi, Y., & Lewenza, S. (2012). Surface-Localized Spermidine Protects the Pseudomonas aeruginosa Outer Membrane from Antibiotic Treatment and Oxidative Stress. *Journal of Bacteriology*, *194*(4), 813–826. https://doi.org/10.1128/JB.05230-11

Johnson, T. J., Patel, A. L., Bigger, H. R., Engstrom, J. L., & Meier, P. P. (2015). Cost Savings of Human Milk as a Strategy to Reduce the Incidence of Necrotizing Enterocolitis in Very Low Birth Weight Infants. *Neonatology*, *107*(4), 271–276. https://doi.org/10.1159/000370058

Johnson-Henry, K. C., Donato, K. A., Shen-Tu, G., Gordanpour, M., & Sherman, P. M. (2008). Lactobacillus rhamnosus Strain GG Prevents Enterohemorrhagic Escherichia coli O157:H7-Induced Changes in Epithelial Barrier Function. *Infection and Immunity*, *76*(4), 1340–1348. https://doi.org/10.1128/IAI.00778-07

Jordan, K., Rooy, L. D., & Kulkarni, A. (2021). 1379 Exploring the role of breast milk fortifier in neonatal outcomes in preterm neonates, a 10 year retrospective audit. *Archives of Disease in Childhood*, *106*(Suppl 1), A346–A346. https://doi.org/10.1136/archdischild-2021-rcpch.602

Jost, T., Lacroix, C., Braegger, C., & Chassard, C. (2013). Assessment of bacterial diversity in breast milk using culture-dependent and culture-independent approaches. *British Journal of Nutrition*, *110*(7), 1253–1262. https://doi.org/10.1017/S0007114513000597

Juntunen, M., Kirjavainen, P. V., Ouwehand, A. C., Salminen, S. J., & Isolauri, E. (2001). Adherence of Probiotic Bacteria to Human Intestinal Mucus in Healthy Infants and during Rotavirus Infection. *Clinical and Diagnostic Laboratory Immunology*, *8*(2), 293–296. https://doi.org/10.1128/CDLI.8.2.293-296.2001

Kainulainen, V., Loimaranta, V., Pekkala, A., Edelman, S., Antikainen, J., Kylväjä, R., Laaksonen, M., Laakkonen, L., Finne, J., & Korhonen, T. K. (2012). Glutamine Synthetase and Glucose-6-Phosphate Isomerase Are Adhesive Moonlighting Proteins of Lactobacillus crispatus Released by Epithelial Cathelicidin LL-37. *Journal of Bacteriology*, *194*(10), 2509–2519. https://doi.org/10.1128/JB.06704-11

Kaji, R., Kiyoshima-Shibata, J., Tsujibe, S., Nanno, M., & Shida, K. (2018). Short communication: Probiotic induction of interleukin-10 and interleukin-12 production by macrophages is modulated by co-stimulation with microbial components. *Journal of Dairy Science*, *101*(4), 2838–2841. https://doi.org/10.3168/jds.2017-13868

Kameoka, S., Motooka, D., Watanabe, S., Kubo, R., Jung, N., Midorikawa, Y., Shinozaki, N. O., Sawai, Y., Takeda, A. K., & Nakamura, S. (2021). Benchmark of 16S rRNA gene amplicon sequencing using Japanese gut microbiome data from the V1–V2 and V3–V4 primer sets. *BMC Genomics*, *22*(1), 527. https://doi.org/10.1186/s12864-021-07746-4

Kankaanpää, P. E., Salminen, S. J., Isolauri, E., & Lee, Y. K. (2001). The influence of polyunsaturated fatty acids on probiotic growth and adhesion. *FEMS Microbiology Letters*, *194*(2), 149–153. https://doi.org/10.1111/j.1574-6968.2001.tb09460.x

Kankainen, M., Paulin, L., Tynkkynen, S., von Ossowski, I., Reunanen, J., Partanen, P., Satokari, R., Vesterlund, S., Hendrickx, A. P. A., Lebeer, S., De Keersmaecker, S. C. J., Vanderleyden, J., Hämäläinen, T., Laukkanen, S., Salovuori, N., Ritari, J., Alatalo, E., Korpela, R., Mattila-Sandholm, T., … de Vos, W. M. (2009). Comparative genomic analysis of Lactobacillus rhamnosus GG reveals pili containing a human- mucus binding protein. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(40), 17193–17198. https://doi.org/10.1073/pnas.0908876106

Keikha, M., Bahreynian, M., Saleki, M., & Kelishadi, R. (2017). Macro- and Micronutrients of Human Milk Composition: Are They Related to Maternal Diet? A Comprehensive Systematic Review. *Breastfeeding Medicine*, *12*(9), 517–527. https://doi.org/10.1089/bfm.2017.0048

Khodayar-Pardo, P., Mira-Pascual, L., Collado, M. C., & Martínez-Costa, C. (2014). Impact of lactation stage, gestational age and mode of delivery on breast milk microbiota. *Journal of Perinatology*, *34*(8), Article 8. https://doi.org/10.1038/jp.2014.47

Kibe, R., Kurihara, S., Sakai, Y., Suzuki, H., Ooga, T., Sawaki, E., Muramatsu, K., Nakamura, A., Yamashita, A., Kitada, Y., Kakeyama, M., Benno, Y., & Matsumoto, M. (2014). Upregulation of colonic luminal polyamines produced by intestinal microbiota delays senescence in mice. *Scientific Reports*, *4*(1), Article 1. https://doi.org/10.1038/srep04548

Kim, N.-N., Kim, W. J., & Kang, S.-S. (2019). Anti-biofilm effect of crude bacteriocin derived from Lactobacillus brevis DF01 on Escherichia coli and Salmonella Typhimurium. *Food Control*, *98*, 274–280. https://doi.org/10.1016/j.foodcont.2018.11.004

Kim, S. W., Andronis, L., Seppänen, A.-V., Aubert, A. M., Zeitlin, J., Barros, H., Draper, E. S., & Petrou, S. (2021). Economic costs at age five associated with very preterm birth: Multinational European cohort study. *Pediatric Research*, 1–12. https://doi.org/10.1038/s41390-021-01769-z

Kim, S. Y., & Yi, D. Y. (2020). Components of human breast milk: From macronutrient to microbiome and microRNA. *Journal of the Korean Pediatric Society*. https://doi.org/.0.0.0

Kirjavainen, P. V., Ouwehand, A. C., Isolauri, E., & Salminen, S. J. (1998). The ability of probiotic bacteria to bind to human intestinal mucus. *FEMS Microbiology Letters*, *167*(2), 185–189. https://doi.org/10.1111/j.1574-6968.1998.tb13226.x

Kitada, Y., Muramatsu, K., Toju, H., Kibe, R., Benno, Y., Kurihara, S., & Matsumoto, M. (2018). Bioactive polyamine production by a novel hybrid system comprising multiple indigenous gut bacterial strategies. *Science Advances*, *4*(6), eaat0062. https://doi.org/10.1126/sciadv.aat0062

Klann, E., Williamson, J. M., Tagliamonte, M. S., Ukhanova, M., Asirvatham, J. R., Chim, H., Yaghjyan, L., & Mai, V. (2020). Microbiota composition in bilateral healthy breast tissue and breast tumors. *Cancer Causes & Control: CCC*, *31*(11), 1027–1038. https://doi.org/10.1007/s10552-020-01338-5

Klumpp, S., & Hwa, T. (2014). Bacterial growth: Global effects on gene expression, growth feedback and proteome partition. *Current Opinion in Biotechnology*, *0*, 96–102. https://doi.org/10.1016/j.copbio.2014.01.001

Koletzko, B. (2016). Human Milk Lipids. *Annals of Nutrition and Metabolism*, *69*(Suppl. 2), 27–40. https://doi.org/10.1159/000452819

Kongo, J. M., Gomes, A. M., & Malcata, F. X. (2006). Manufacturing of fermented goat milk with a mixed starter culture of Bifidobacterium animalis and Lactobacillus acidophilus in a controlled bioreactor. *Letters in Applied Microbiology*, *42*(6), 595–599. https://doi.org/10.1111/j.1472-765X.2006.01882.x

Kontopodi, E., Arslanoglu, S., Bernatowicz-Lojko, U., Bertino, E., Bettinelli, M. E., Buffin, R., Cassidy, T., Elburg, R. M. van, Gebauer, C., Grovslien, A., Hettinga, K., Ioannou, I., Klotz, D., Mileusnić-Milenović, R., Moro, G. E., Picaud, J.-C., Stahl, B., Weaver, G., Goudoever, J. B. van, & Wesolowska, A. (2021). “Donor milk banking: Improving the future”. A survey on the operation of the European donor human milk banks. *PLOS ONE*, *16*(8), e0256435. https://doi.org/10.1371/journal.pone.0256435

Kordy, K., Gaufin, T., Mwangi, M., Li, F., Cerini, C., Lee, D. J., Adisetiyo, H., Woodward, C., Pannaraj, P. S., Tobin, N. H., & Aldrovandi, G. M. (2020). Contributions to human breast milk microbiome and enteromammary transfer of Bifidobacterium breve. *PLOS ONE*, *15*(1), e0219633. https://doi.org/10.1371/journal.pone.0219633

Korpela, K., Blakstad, E. W., Moltu, S. J., Strømmen, K., Nakstad, B., Rønnestad, A. E., Brække, K., Iversen, P. O., Drevon, C. A., & de Vos, W. (2018). Intestinal microbiota development and gestational age in preterm neonates. *Scientific Reports*, *8*, 2453. https://doi.org/10.1038/s41598-018-20827-x

Korpela, K., Salonen, A., Virta, L. J., Kumpu, M., Kekkonen, R. A., & Vos, W. M. de. (2016). Lactobacillus rhamnosus GG Intake Modifies Preschool Children's Intestinal Microbiota, Alleviates Penicillin-Associated Changes, and Reduces Antibiotic Use. *PLOS ONE*, *11*(4), e0154012. https://doi.org/10.1371/journal.pone.0154012

Kostopoulos, I., Elzinga, J., Ottman, N., Klievink, J. T., Blijenberg, B., Aalvink, S., Boeren, S., Mank, M., Knol, J., de Vos, W. M., & Belzer, C. (2020). Akkermansia muciniphila uses human milk oligosaccharides to thrive in the early life conditions in vitro. *Scientific Reports*, *10*(1), 14330. https://doi.org/10.1038/s41598-020-71113-8

Krumbeck, J. A., Rasmussen, H. E., Hutkins, R. W., Clarke, J., Shawron, K., Keshavarzian, A., & Walter, J. (2018). Probiotic Bifidobacterium strains and galactooligosaccharides improve intestinal barrier function in obese adults but show no synergism when used together as synbiotics. *Microbiome*, *6*, 121. https://doi.org/10.1186/s40168-018-0494-4

Kumar, H., du Toit, E., Kulkarni, A., Aakko, J., Linderborg, K. M., Zhang, Y., Nicol, M. P., Isolauri, E., Yang, B., Collado, M. C., & Salminen, S. (2016). Distinct Patterns in Human Milk Microbiota and Fatty Acid Profiles Across Specific Geographic Locations. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.01619

Kunz, A. N., Noel, J. M., & Fairchok, M. P. (2004). Two Cases of Lactobacillus Bacteremia During Probiotic Treatment of Short Gut Syndrome. *Journal of Pediatric Gastroenterology and Nutrition*, *38*(4), 457–458.

Kuschel, C. A., & Harding, J. E. (2000). Protein supplementation of human milk for promoting growth in preterm infants. *Cochrane Database of Systematic Reviews*, *2*. https://doi.org/10.1002/14651858.CD000433

La Fata, G., Weber, P., & Mohajeri, M. H. (2018). Probiotics and the Gut Immune System: Indirect Regulation. *Probiotics and Antimicrobial Proteins*, *10*(1), 11–21. https://doi.org/10.1007/s12602-017-9322-6

Lackey, K. A., Williams, J. E., Meehan, C. L., Zachek, J. A., Benda, E. D., Price, W. J., Foster, J. A., Sellen, D. W., Kamau-Mbuthia, E. W., Kamundia, E. W., Mbugua, S., Moore, S. E., Prentice, A. M., K., D. G., Kvist, L. J., Otoo, G. E., García-Carral, C., Jiménez, E., Ruiz, L., … McGuire, M. K. (2019). What's Normal? Microbiomes in Human Milk and Infant Feces Are Related to Each Other but Vary Geographically: The INSPIRE Study. *Frontiers in Nutrition*, *6*. https://doi.org/10.3389/fnut.2019.00045

Lagström, H., Rautava, S., Ollila, H., Kaljonen, A., Turta, O., Mäkelä, J., Yonemitsu, C., Gupta, J., & Bode, L. (2020). Associations between human milk oligosaccharides and growth in infancy and early childhood. *The American Journal of Clinical Nutrition*, *111*(4), 769–778. https://doi.org/10.1093/ajcn/nqaa010

Lahtinen, S. J., Gueimonde, M., Ouwehand, A. C., Reinikainen, J. P., & Salminen, S. J. (2005). Probiotic Bacteria May Become Dormant during Storage. *Applied and Environmental Microbiology*, *71*(3), 1662–1663. https://doi.org/10.1128/AEM.71.3.1662-1663.2005

Lahtinen, S. J., Gueimonde, M., Ouwehand, A. C., Reinikainen, J. P., & Salminen, S. J. (2006). Comparison of four methods to enumerate probiotic bifidobacteria in a fermented food product. *Food Microbiology*, *23*(6), 571–577. https://doi.org/10.1016/j.fm.2005.09.001

Lahtinen, S. J., Haskard, C. A., Ouwehand, A. C., Salminen, S. J., & Ahokas, J. T. (2004). Binding of aflatoxin B1 to cell wall components of Lactobacillus rhamnosus strain GG. *Food Additives and Contaminants*, *21*(2), 158–164. https://doi.org/10.1080/02652030310001639521

Laparra, J. m., & Sanz, Y. (2009). Comparison of in vitro models to study bacterial adhesion to the intestinal epithelium. *Letters in Applied Microbiology*, *49*(6), 695–701. https://doi.org/10.1111/j.1472-765X.2009.02729.x

Lapidaire, W., Lucas, A., Clayden, J. D., Clark, C., & Fewtrell, M. S. (2021). Human milk feeding and cognitive outcome in preterm infants: The role of infection and NEC reduction. *Pediatric Research*, 1–8. https://doi.org/10.1038/s41390-021-01367-z

Lau, C. (2015). Development of suck and swallow mechanisms in infants. *Annals of Nutrition & Metabolism*, *66*(0 5), 7–14. https://doi.org/10.1159/000381361

Lau, C. S. M., & Chamberlain, R. S. (2015). Probiotic administration can prevent necrotizing enterocolitis in preterm infants: A meta-analysis. *Journal of Pediatric Surgery*, *50*(8), 1405–1412. https://doi.org/10.1016/j.jpedsurg.2015.05.008

Lawhon, S. D., Maurer, R., Suyemoto, M., & Altier, C. (2002). Intestinal short-chain fatty acids alter Salmonella typhimurium invasion gene expression and virulence through BarA/SirA. *Molecular Microbiology*, *46*(5), 1451–1464. https://doi.org/10.1046/j.1365-2958.2002.03268.x

Lawson, M. A. E., O'Neill, I. J., Kujawska, M., Gowrinadh Javvadi, S., Wijeyesekera, A., Flegg, Z., Chalklen, L., & Hall, L. J. (2020). Breast milk-derived human milk oligosaccharides promote Bifidobacterium interactions within a single ecosystem. *The ISME Journal*, *14*(2), Article 2. https://doi.org/10.1038/s41396-019-0553-2

Layman, D. K., Lönnerdal, B., & Fernstrom, J. D. (2018). Applications for α-lactalbumin in human nutrition. *Nutrition Reviews*, *76*(6), 444–460. https://doi.org/10.1093/nutrit/nuy004

Le Doare, K., Holder, B., Bassett, A., & Pannaraj, P. S. (2018). Mother's Milk: A Purposeful Contribution to the Development of the Infant Microbiota and Immunity. *Frontiers in Immunology*, *9*. https://www.frontiersin.org/articles/10.3389/fimmu.2018.00361

Lebeer, S., Claes, I. J. J., Verhoeven, T. L. A., Vanderleyden, J., & De Keersmaecker, S. C. J. (2011). Exopolysaccharides of Lactobacillus rhamnosus GG form a protective shield against innate immune factors in the intestine. *Microbial Biotechnology*, *4*(3), 368–374. https://doi.org/10.1111/j.1751-7915.2010.00199.x

Lebeer, S., Claes, I., Tytgat, H. L. P., Verhoeven, T. L. A., Marien, E., Ossowski, I. von, Reunanen, J., Palva, A., Vos, W. M. de, Keersmaecker, S. C. J. D., & Vanderleyden, J. (2012). Functional Analysis of Lactobacillus rhamnosus GG Pili in Relation to Adhesion and Immunomodulatory Interactions with Intestinal Epithelial Cells. *Applied and Environmental Microbiology*, *78*(1), 185–193. https://doi.org/10.1128/AEM.06192-11

Lebeer, S., Verhoeven, T. L. A., Francius, G., Schoofs, G., Lambrichts, I., Dufrêne, Y., Vanderleyden, J., & De Keersmaecker, S. C. J. (2009). Identification of a Gene Cluster for the Biosynthesis of a Long, Galactose-Rich Exopolysaccharide in Lactobacillus rhamnosus GG and Functional Analysis of the Priming Glycosyltransferase. *Applied and Environmental Microbiology*, *75*(11), 3554–3563. https://doi.org/10.1128/AEM.02919-08

LeBlanc, J. G., Chain, F., Martín, R., Bermúdez-Humarán, L. G., Courau, S., & Langella, P. (2017). Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins produced by commensal and probiotic bacteria. *Microbial Cell Factories*, *16*. https://doi.org/10.1186/s12934-017-0691-z

LeBouder, E., Rey-Nores, J. E., Rushmere, N. K., Grigorov, M., Lawn, S. D., Affolter, M., Griffin, G. E., Ferrara, P., Schiffrin, E. J., Morgan, B. P., & Labéta, M. O. (2003). Soluble forms of Toll-like receptor (TLR)2 capable of modulating TLR2 signaling are present in human plasma and breast

milk. *Journal of Immunology (Baltimore, Md.: 1950)*, *171*(12), 6680–6689. https://doi.org/10.4049/jimmunol.171.12.6680

Lee, H., Padhi, E., Hasegawa, Y., Larke, J., Parenti, M., Wang, A., Hernell, O., Lönnerdal, B., & Slupsky, C. (2018). Compositional Dynamics of the Milk Fat Globule and Its Role in Infant Development. *Frontiers in Pediatrics*, *6*. https://doi.org/10.3389/fped.2018.00313

Lee, J.-E., & Kim, G.-B. (2019). Human Milk Microbiota: A Review. *Journal of Milk Science and Biotechnology*, *37*(1), 15–26. https://doi.org/10.22424/jmsb.2019.37.1.15

Lee, Y.-K., & Puong, K.-Y. (2002). Competition for adhesion between probiotics and human gastrointestinal pathogens in the presence of carbohydrate. *British Journal of Nutrition*, *88*(S1), S101. https://doi.org/10.1079/BJN2002635

Lei, C., Sun, R., Xu, G., Tan, Y., Feng, W., McClain, C. J., & Deng, Z. (2022). Enteric VIP-producing neurons maintain gut microbiota homeostasis through regulating epithelium fucosylation. *Cell Host & Microbe*, *30*(10), 1417-1434.e8. https://doi.org/10.1016/j.chom.2022.09.001

LeMay-Nedjelski, L., Asbury, M. R., Butcher, J., Ley, S. H., Hanley, A. J., Kiss, A., Unger, S., Copeland, J. K., Wang, P. W., Stintzi, A., & O'Connor, D. L. (2020). Maternal Diet and Infant Feeding Practices Are Associated with Variation in the Human Milk Microbiota at 3 Months Postpartum in a Cohort of Women with High Rates of Gestational Glucose Intolerance. *The Journal of Nutrition*, *151*(2), 320–329. https://doi.org/10.1093/jn/nxaa248

LeMay-Nedjelski, L., Butcher, J., Ley, S. H., Asbury, M. R., Hanley, A. J., Kiss, A., Unger, S., Copeland, J. K., Wang, P. W., Zinman, B., Stintzi, A., & O'Connor, D. L. (2020). Examining the relationship between maternal body size, gestational glucose tolerance status, mode of delivery and ethnicity on human milk microbiota at three months post-partum. *BMC Microbiology*, *20*. https://doi.org/10.1186/s12866-020-01901-9

Lewandowski, A. J., Levy, P. T., Bates, M. L., McNamara, P. J., Nuyt, A. M., & Goss, K. N. (2020). Impact of the Vulnerable Preterm Heart and Circulation on Adult Cardiovascular Disease Risk. *Hypertension*, *76*(4), 1028–1037. https://doi.org/10.1161/HYPERTENSIONAHA.120.15574

Lewis, Z. T., Totten, S. M., Smilowitz, J. T., Popovic, M., Parker, E., Lemay, D. G., Van Tassell, M. L., Miller, M. J., Jin, Y.-S., German, J. B., Lebrilla, C. B., & Mills, D. A. (2015). Maternal fucosyltransferase 2 status affects the gut bifidobacterial communities of breastfed infants. *Microbiome*, *3*. https://doi.org/10.1186/s40168-015-0071-z

Li, H., Limenitakis, J. P., Fuhrer, T., Geuking, M. B., Lawson, M. A., Wyss, M., Brugiroux, S., Keller, I., Macpherson, J. A., Rupp, S., Stolp, B., Stein, J. V., Stecher, B., Sauer, U., McCoy, K. D., & Macpherson, A. J. (2015). The outer mucus layer hosts a distinct intestinal microbial niche. *Nature Communications*, *6*, 8292. https://doi.org/10.1038/ncomms9292

Li, L., Mendis, N., Trigui, H., Oliver, J. D., & Faucher, S. P. (2014). The importance of the viable but non-culturable state in human bacterial pathogens. *Frontiers in Microbiology*, *5*(258). https://doi.org/10.3389/fmicb.2014.00258

Li, M., Monaco, M. H., Wang, M., Comstock, S. S., Kuhlenschmidt, T. B., Fahey Jr, G. C., Miller, M. J., Kuhlenschmidt, M. S., & Donovan, S. M. (2014). Human milk oligosaccharides shorten rotavirus-induced diarrhea and modulate piglet mucosal immunity and colonic microbiota. *The ISME Journal*, *8*(8), 1609–1620. https://doi.org/10.1038/ismej.2014.10

Li, N., Pang, B., Liu, G., Zhao, X., Xu, X., Jiang, C., Yang, B., Liu, Y., & Shi, J. (2020). Lactobacillus rhamnosus from human breast milk shows therapeutic function against foodborne infection by multi-drug resistant Escherichia coli in mice. *Food & Function*, *11*(1), 435–447. https://doi.org/10.1039/c9fo01698h

Li, Q., Chen, Q., Ruan, H., Zhu, D., & He, G. (2010). Isolation and characterisation of an oxygen, acid and bile resistant Bifidobacterium animalis subsp. Lactis Qq08. *Journal of the Science of Food and Agriculture*, *90*(8), 1340–1346. https://doi.org/10.1002/jsfa.3942

Liepke, C., Adermann, K., Raida, M., Mägert, H.-J., Forssmann, W.-G., & Zucht, H.-D. (2002). Human milk provides peptides highly stimulating the growth of bifidobacteria. *European Journal of Biochemistry*, *269*(2), 712–718. https://doi.org/10.1046/j.0014-2956.2001.02712.x

Lif Holgerson, P., Esberg, A., West, C. E., & Johansson, I. (2023). The breast milk and childhood gastrointestinal microbiotas and disease outcomes: A longitudinal study. *Pediatric Research*, *93*(3), Article 3. https://doi.org/10.1038/s41390-022-02328-w

Linden, S. K., Sutton, P., Karlsson, N. G., Korolik, V., & McGuckin, M. A. (2008). Mucins in the mucosal barrier to infection. *Mucosal Immunology*, *1*(3), 183–197. https://doi.org/10.1038/mi.2008.5

Liptáková, D., Matejčeková, Z., & Valík, Ľ. (2017). Lactic Acid Bacteria and Fermentation of Cereals and Pseudocereals. *Fermentation Processes*. https://doi.org/10.5772/65459

Liu, B., & Newburg, D. S. (2013). Human Milk Glycoproteins Protect Infants Against Human Pathogens. *Breastfeeding Medicine*, *8*(4), 354–362. https://doi.org/10.1089/bfm.2013.0016

Lo Curto, A., Pitino, I., Mandalari, G., Dainty, J. R., Faulks, R. M., & John Wickham, M. S. (2011). Survival of probiotic lactobacilli in the upper gastrointestinal tract using an in vitro gastric model of digestion. *Food Microbiology*, *28*(7), 1359–1366. https://doi.org/10.1016/j.fm.2011.06.007

Logares, R. (2017). *ramalok/amplicon_processing: Workflow for Analysing MiSeq Amplicons based on Uparse* [Computer software]. Zenodo. https://doi.org/10.5281/zenodo.259579

Loh, S., & Maznah, I. (1999). The effect of different milks and milk proteins on the growth of Bifidobacterium infantis ATCC 27920 in vitro. *Malaysian Journal of Nutrition*, *5*(1), 61–70.

Löser, C., Eisel, A., Harms, D., & Fölsch, U. R. (1999). Dietary polyamines are essential luminal growth factors for small intestinal and colonic mucosal growth and development. *Gut*, *44*(1), 12–16. https://doi.org/10.1136/gut.44.1.12

Lucas, A., Boscardin, J., & Abrams, S. A. (2020). Preterm Infants Fed Cow's Milk-Derived Fortifier Had Adverse Outcomes Despite a Base Diet of Only Mother's Own Milk. *Breastfeeding Medicine*, *15*(5), 297–303. https://doi.org/10.1089/bfm.2019.0133

Luoto, R., Isolauri, E., & Lehtonen, L. (2010). Safety of Lactobacillus GG Probiotic in Infants with Very Low Birth Weight: Twelve Years of Experience. *Clinical Infectious Diseases*, *50*(9), 1327–1328. https://doi.org/10.1086/651694

Ma, B., McComb, E., Gajer, P., Yang, H., Humphrys, M., Okogbule-Wonodi, A. C., Fasano, A., Ravel, J., & Viscardi, R. M. (2018). Microbial Biomarkers of Intestinal Barrier Maturation in Preterm Infants. *Frontiers in Microbiology*, *9*. https://www.frontiersin.org/article/10.3389/fmicb.2018.02755

Ma, L., Hu, L., Feng, X., & Wang, S. (2018). Nitrate and Nitrite in Health and Disease. *Aging and Disease*, *9*(5), 938–945. https://doi.org/10.14336/AD.2017.1207

Ma, Y., Zhang, L., Wu, Y., & Zhou, P. (2019). Changes in milk fat globule membrane proteome after pasteurization in human, bovine and caprine species. *Food Chemistry*, *279*, 209–215. https://doi.org/10.1016/j.foodchem.2018.12.015

Mallardi, D., Tabasso, C., Piemontese, P., Morandi, S., Silvetti, T., Biscarini, F., Cremonesi, P., Castiglioni, B., Pica, V., Stuknyte, M., De Noni, I., Amato, O., Liotto, N., Mosca, F., & Roggero, P. (2021). Inoculation of mother's own milk could personalize pasteurized donor human milk used for feeding preterm infants. *Journal of Translational Medicine*, *19*(1), 420. https://doi.org/10.1186/s12967-021-03096-7

Mani-López, E., Palou, E., & López-Malo, A. (2014). Probiotic viability and storage stability of yogurts and fermented milks prepared with several mixtures of lactic acid bacteria. *Journal of Dairy Science*, *97*(5), 2578–2590. https://doi.org/10.3168/jds.2013-7551

Mantziari, A. (2016). *The impact of storage conditions on the stability of Lactobacillus rhamnosus GG and Bifidobacterium animalis subsp. Lactis in breast milk* [fi=Turun yliopisto|en=University of Turku|]. https://www.utupub.fi/handle/10024/125246

Mantziari, A., & Rautava, S. (2021). Factors influencing the microbial composition of human milk. *Seminars in Perinatology*, *45*(8), 151507. https://doi.org/10.1016/j.semperi.2021.151507

Mantziari, A., Salminen, S., Szajewska, H., & Malagón-Rojas, J. N. (2020). Postbiotics against Pathogens Commonly Involved in Pediatric Infectious Diseases. *Microorganisms*, *8*(10), Article 10. https://doi.org/10.3390/microorganisms8101510

Marcobal, A., Barboza, M., Sonnenburg, E. D., Pudlo, N., Martens, E. C., Desai, P., Lebrilla, C. B., Weimer, B. C., Mills, D. A., German, J. B., & Sonnenburg, J. L. (2011). Bacteroides in the infant gut consume milk oligosaccharides via mucus-utilization pathways. *Cell Host & Microbe*, *10*(5), 507–514. https://doi.org/10.1016/j.chom.2011.10.007

Marín, M., Arroyo, R., Espinosa-Martos, I., Fernández, L., & Rodríguez, J. M. (2017). Identification of Emerging Human Mastitis Pathogens by MALDI-TOF and Assessment of Their Antibiotic Resistance Patterns. *Frontiers in Microbiology*, *8*, 1258. https://doi.org/10.3389/fmicb.2017.01258

Martín, R., Bermúdez-Humarán, L. G., & Langella, P. (2018). Searching for the Bacterial Effector: The Example of the Multi-Skilled Commensal Bacterium Faecalibacterium prausnitzii. *Frontiers in Microbiology*, *9*. https://doi.org/10.3389/fmicb.2018.00346

Martinez-Villaluenga, C., & Gómez, R. (2007). Characterization of bifidobacteria as starters in fermented milk containing raffinose family of oligosaccharides from lupin as prebiotic. *International Dairy Journal*, *17*(2), 116–122. https://doi.org/10.1016/j.idairyj.2006.02.003

Martín-Diana, A. B., Janer, C., Peláez, C., & Requena, T. (2003). Development of a fermented goat's milk containing probiotic bacteria. *International Dairy Journal*, *13*(10), 827–833. https://doi.org/10.1016/S0958-6946(03)00117-1

Masi, A. C., Embleton, N. D., Lamb, C. A., Young, G., Granger, C. L., Najera, J., Smith, D. P., Hoffman, K. L., Petrosino, J. F., Bode, L., Berrington, J. E., & Stewart, C. J. (2020). Human milk oligosaccharide DSLNT and gut microbiome in preterm infants predicts necrotising enterocolitis. *Gut*. https://doi.org/10.1136/gutjnl-2020-322771

Masoli, D., Mena, P., Dominguez, A., Ramolfo, P., Vernal, P., Pantoja, M. A., Esparza, R., Hübner, M. E., Ríos, A., Faundes, M., Uauy, R., Tapia, J. L., & and the Neocosur Network. (2021). Growth of Very Low Birth Weight Infants Who Received a Liquid Human Milk Fortifier: A Randomized, Controlled Multicenter Trial: A Randomized, Controlled Multicenter Trial. *Journal of Pediatric Gastroenterology and Nutrition*. https://doi.org/10.1097/MPG.0000000000003321

Mastromarino, P., Capobianco, D., Miccheli, A., Praticò, G., Campagna, G., Laforgia, N., Capursi, T., & Baldassarre, M. E. (2015). Administration of a multistrain probiotic product (VSL#3) to women in the perinatal period differentially affects breast milk beneficial microbiota in relation to mode of delivery. *Pharmacological Research*, *95–96*, 63–70. https://doi.org/10.1016/j.phrs.2015.03.013

Matei, A. C., Antounians, L., & Zani, A. (2019). Extracellular Vesicles as a Potential Therapy for Neonatal Conditions: State of the Art and Challenges in Clinical Translation. *Pharmaceutics*, *11*(8), 404. https://doi.org/10.3390/pharmaceutics11080404

Matsumoto, M., & Benno, Y. (2007). The Relationship between Microbiota and Polyamine Concentration in the Human Intestine: A Pilot Study. *Microbiology and Immunology*, *51*(1), 25–35. https://doi.org/10.1111/j.1348-0421.2007.tb03887.x

Matsumoto, M., Kibe, R., Ooga, T., Aiba, Y., Kurihara, S., Sawaki, E., Koga, Y., & Benno, Y. (2012). Impact of Intestinal Microbiota on Intestinal Luminal Metabolome. *Scientific Reports*, *2*, 233. https://doi.org/10.1038/srep00233

Matsumoto, M., Kurihara, S., Kibe, R., Ashida, H., & Benno, Y. (2011a). Longevity in Mice Is Promoted by Probiotic-Induced Suppression of Colonic Senescence Dependent on Upregulation of Gut Bacterial Polyamine Production. *PLOS ONE*, *6*(8), e23652. https://doi.org/10.1371/journal.pone.0023652

Matsumoto, M., Kurihara, S., Kibe, R., Ashida, H., & Benno, Y. (2011b). Longevity in Mice Is Promoted by Probiotic-Induced Suppression of Colonic Senescence Dependent on Upregulation of Gut Bacterial Polyamine Production. *PLOS ONE*, *6*(8), e23652. https://doi.org/10.1371/journal.pone.0023652

McCurtain, J. L., Gilbertsen, A. J., Evert, C., Williams, B. J., & Hunter, R. C. (2019). Agmatine accumulation by Pseudomonas aeruginosa clinical isolates confers antibiotic tolerance and dampens host inflammation. *Journal of Medical Microbiology*, *68*(3), 446–455. https://doi.org/10.1099/jmm.0.000928

Mediano, P., Fernández, L., Jiménez, E., Arroyo, R., Espinosa-Martos, I., Rodríguez, J. M., & Marín, M. (2017). Microbial Diversity in Milk of Women With Mastitis: Potential Role of Coagulase-Negative Staphylococci, Viridans Group Streptococci, and Corynebacteria. *Journal of Human Lactation: Official Journal of International Lactation Consultant Association*, *33*(2), 309–318. https://doi.org/10.1177/0890334417692968

Meehan, C. L., Lackey, K. A., Hagen, E. H., Williams, J. E., Roulette, J., Helfrecht, C., McGuire, M. A., & McGuire, M. K. (2018). Social networks, cooperative breeding, and the human milk microbiome. *American Journal of Human Biology*, *30*(4), e23131. https://doi.org/10.1002/ajhb.23131

Meng, T., Perrin, M. T., Allen, J. C., Osborne, J., Jones, F., & Fogleman, A. D. (2016). Storage of Unfed and Leftover Pasteurized Human Milk. *Breastfeeding Medicine: The Official Journal of the Academy of Breastfeeding Medicine*, *11*, 538–543. https://doi.org/10.1089/bfm.2016.0139

Merenstein, D., Fraser, C. M., Roberts, R. F., Liu, T., Grant-Beurmann, S., Tan, T. P., Smith, K. H., Cronin, T., Martin, O. A., Sanders, M. E., Lucan, S. C., & Kane, M. A. (2021). Bifidobacterium animalis subsp. Lactis BB-12 Protects against Antibiotic-Induced Functional and Compositional Changes in Human Fecal Microbiome. *Nutrients*, *13*(8), Article 8. https://doi.org/10.3390/nu13082814

Merter, Ö. S., & Altay, N. (2024). Effect of Feeding Fresh or Frozen Breast Milk on the Gut Microbiota of Premature Infants: A Prospective Observational Study. *Biological Research For Nursing*, *26*(1), 78–90. https://doi.org/10.1177/10998004231191728

Metsalu, T., & Vilo, J. (2015). ClustVis: A web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. *Nucleic Acids Research*, *43*(W1), W566-570. https://doi.org/10.1093/nar/gkv468

Mezouar, S., Chantran, Y., Michel, J., Fabre, A., Dubus, J.-C., Leone, M., Sereme, Y., Mège, J.-L., Ranque, S., Desnues, B., Chanez, P., & Vitte, J. (2018). Microbiome and the immune system: From a healthy steady-state to allergy associated disruption. *Human Microbiome Journal*, *10*, 11–20. https://doi.org/10.1016/j.humic.2018.10.001

Midtvedt, A.-C., Carlstedt-Duke, B., & Midtvedt, T. (1994). Establishment of a Mucin-Degrading Intestinal Microflora During the First Two Years of Human Life. *Journal of Pediatric Gastroenterology and Nutrition*, *18*(3), 321.

Miettinen, M., Alander, M., Von Wright, A., Vuopio-Varkila, J., Marteau, P., Huis In 't Veld, J., & Mattila-Sandholm, T. (1999). The Survival of and Cytokine Induction by Lactic Acid Bacteria after Passage Through a Gastrointestinal Model. *Microbial Ecology in Health and Disease*, *10*(3–4), 141–147. https://doi.org/10.1080/089106098435223

Mikelsaar, M., Stsepetova, J., Mikelsaar, R.-H., Truusalu, K., Smidt, I., Hütt, P., Rätsep, M., & Songisepp, E. (2020). Polyamines of human strain Lactobacillus plantarum Inducia induce

modulation of innate immune markers. *Journal of Functional Foods*, *72*, 104064. https://doi.org/10.1016/j.jff.2020.104064

Miller, J., Tonkin, E., Damarell, R. A., McPhee, A. J., Suganuma, M., Suganuma, H., Middleton, P. F., Makrides, M., & Collins, C. T. (2018). A Systematic Review and Meta-Analysis of Human Milk Feeding and Morbidity in Very Low Birth Weight Infants. *Nutrients*, *10*(6). https://doi.org/10.3390/nu10060707

Mitchell, K., Lyttle, A., Amin, H., Shaireen, H., Robertson, H. L., & Lodha, A. K. (2014). Arginine supplementation in prevention of necrotizing enterocolitis in the premature infant: An updated systematic review. *BMC Pediatrics*, *14*, 226. https://doi.org/10.1186/1471-2431-14-226

Miura, K., Tanaka, M., Date, M., Ito, M., Mizuno, N., & Mizuno, K. (2023). Comparison of bacterial profiles in human milk from mothers of term and preterm infants. *International Breastfeeding Journal*, *18*(1), 29. https://doi.org/10.1186/s13006-023-00563-3

Miyake, H., Lee, C., Chusilp, S., Bhalla, M., Li, B., Pitino, M., Seo, S., O'Connor, D. L., & Pierro, A. (2020). Human breast milk exosomes attenuate intestinal damage. *Pediatric Surgery International*, *36*(2), 155–163. https://doi.org/10.1007/s00383-019-04599-7

Mohan, R., Koebnick, C., Schildt, J., Schmidt, S., Mueller, M., Possner, M., Radke, M., & Blaut, M. (2006). Effects of Bifidobacterium lactis Bb12 Supplementation on Intestinal Microbiota of Preterm Infants: A Double-Blind, Placebo-Controlled, Randomized Study. *Journal of Clinical Microbiology*, *44*(11), 4025–4031. https://doi.org/10.1128/JCM.00767-06

Monteagudo-Mera, A., Rastall, R. A., Gibson, G. R., Charalampopoulos, D., & Chatzifragkou, A. (2019). Adhesion mechanisms mediated by probiotics and prebiotics and their potential impact on human health. *Applied Microbiology and Biotechnology*, *103*(16), 6463–6472. https://doi.org/10.1007/s00253-019-09978-7

Moossavi, S., Atakora, F., Miliku, K., Sepehri, S., Robertson, B., Duan, Q. L., Becker, A. B., Mandhane, P. J., Turvey, S. E., Moraes, T. J., Lefebvre, D. L., Sears, M. R., Subbarao, P., Field, C. J., Bode, L., Khafipour, E., & Azad, M. B. (2019). Integrated Analysis of Human Milk Microbiota With Oligosaccharides and Fatty Acids in the CHILD Cohort. *Frontiers in Nutrition*, *6*, 58. https://doi.org/10.3389/fnut.2019.00058

Moossavi, S., Sepehri, S., Robertson, B., Bode, L., Goruk, S., Field, C. J., Lix, L. M., de Souza, R. J., Becker, A. B., Mandhane, P. J., Turvey, S. E., Subbarao, P., Moraes, T. J., Lefebvre, D. L., Sears, M. R., Khafipour, E., & Azad, M. B. (2019). Composition and Variation of the Human Milk Microbiota Are Influenced by Maternal and Early-Life Factors. *Cell Host & Microbe*, *25*(2), 324-335.e4. https://doi.org/10.1016/j.chom.2019.01.011

Morais, J., Marques, C., Faria, A., Teixeira, D., Barreiros-Mota, I., Durão, C., Araújo, J., Ismael, S., Brito, S., Cardoso, M., Macedo, I., Pereira, E., Tomé, T., & Calhau, C. (2021). Influence of Human Milk on Very Preterms' Gut Microbiota and Alkaline Phosphatase Activity. *Nutrients*, *13*(5), Article 5. https://doi.org/10.3390/nu13051564

Moro, G. E., Arslanoglu, S., Bertino, E., Corvaglia, L., Montirosso, R., Picaud, J.-C., Polberger, S., Schanler, R. J., Steel, C., van Goudoever, J., Ziegler, E. E., American Academy of Pediatrics, & European Society for Pediatric Gastroenterology, Hepatology, and Nutrition. (2015). XII. Human Milk in Feeding Premature Infants: Consensus Statement. *Journal of Pediatric Gastroenterology and Nutrition*, *61 Suppl 1*, S16-19. https://doi.org/10.1097/01.mpg.0000471460.08792.4d

Mortazavian, A. M., Ehsani, M. R., Mousavi, S. M., Rezaei, K., Sohrabvandi, S., & Reinheimer, J. A. (2007). Effect of refrigerated storage temperature on the viability of probiotic micro-organisms in yogurt. *International Journal of Dairy Technology*, *60*(2), 123–127. https://doi.org/10.1111/j.1471-0307.2007.00306.x

Mortazavian, A., Razavi, S. H., Ehsani, M. R., & Sohrabvandi, S. (2007). Principles and Methods of Microencapsulation of Probiotic Microorganisms. *Iranian Journal of Biotechnology*, *5*(1), 1–18.

Muñoz-Esparza, N. C., Comas-Basté, O., Latorre-Moratalla, M. L., Veciana-Nogués, M. T., & Vidal-Carou, M. C. (2021). Differences in Polyamine Content between Human Milk and Infant Formulas. *Foods (Basel, Switzerland)*, *10*(11), 2866. https://doi.org/10.3390/foods10112866

Muñoz-Esparza, N. C., Latorre-Moratalla, M. L., Comas-Basté, O., Toro-Funes, N., Veciana-Nogués, M. T., & Vidal-Carou, M. C. (2019). Polyamines in Food. *Frontiers in Nutrition*, *6*. https://doi.org/10.3389/fnut.2019.00108

Murakami, M., Dorschner, R. A., Stern, L. J., Lin, K. H., & Gallo, R. L. (2005). Expression and Secretion of Cathelicidin Antimicrobial Peptides in Murine Mammary Glands and Human Milk. *Pediatric Research*, *57*(1), 10. https://doi.org/10.1203/01.PDR.0000148068.32201.50

Murphy, K., Curley, D., O'Callaghan, T. F., O'Shea, C.-A., Dempsey, E. M., O'Toole, P. W., Ross, R. P., Ryan, C. A., & Stanton, C. (2017). The Composition of Human Milk and Infant Faecal Microbiota Over the First Three Months of Life: A Pilot Study. *Scientific Reports*, *7*. https://doi.org/10.1038/srep40597

Murphy, K., Ross, R. P., Ryan, C. A., Dempsey, E. M., & Stanton, C. (2021). Probiotics, Prebiotics, and Synbiotics for the Prevention of Necrotizing Enterocolitis. *Frontiers in Nutrition*, *8*, 600. https://doi.org/10.3389/fnut.2021.667188

Muscariello, L., De Siena, B., & Marasco, R. (2020). Lactobacillus Cell Surface Proteins Involved in Interaction with Mucus and Extracellular Matrix Components. *Current Microbiology*, *77*(12), 3831–3841. https://doi.org/10.1007/s00284-020-02243-5

Nanduri, B., & Swiatlo, E. (2021). The expansive effects of polyamines on the metabolism and virulence of Streptococcus pneumoniae. *Pneumonia*, *13*(1), 4. https://doi.org/10.1186/s41479-021-00082-x

Nasser, R., Stephen, A. M., Goh, Y. K., & Clandinin, M. T. (2010). The effect of a controlled manipulation of maternal dietary fat intake on medium and long chain fatty acids in human breast milk in Saskatoon, Canada. *International Breastfeeding Journal*, *5*(1), 3. https://doi.org/10.1186/1746-4358-5-3

Neu, J. (2020). Necrotizing Enterocolitis: A Multi-omic Approach and the Role of the Microbiome. *Digestive Diseases and Sciences*, *65*(3), 789–796. https://doi.org/10.1007/s10620-020-06104-w

Nielsen, S. D., Beverly, R. L., & Dallas, D. C. (2017). Peptides Released from Foremilk and Hindmilk Proteins by Breast Milk Proteases Are Highly Similar. *Frontiers in Nutrition*, *4*, 54. https://doi.org/10.3389/fnut.2017.00054

Nighswonger, B. D., Brashears, M. M., & Gilliland, S. E. (1996). Viability of Lactobacillus acidophilus and Lactobacillus casei in Fermented Milk Products During Refrigerated Storage. *Journal of Dairy Science*, *79*(2), 212–219. https://doi.org/10.3168/jds.S0022-0302(96)76353-1

Nikolenko, S. I., Korobeynikov, A. I., & Alekseyev, M. A. (2013). BayesHammer: Bayesian clustering for error correction in single-cell sequencing. *BMC Genomics*, *14*(1), S7. https://doi.org/10.1186/1471-2164-14-S1-S7

Nishiyama, K., Yamamoto, Y., Sugiyama, M., Takaki, T., Urashima, T., Fukiya, S., Yokota, A., Okada, N., & Mukai, T. (2017). Bifidobacterium bifidum Extracellular Sialidase Enhances Adhesion to the Mucosal Surface and Supports Carbohydrate Assimilation. *mBio*, *8*(5), 10.1128/mbio.00928-17. https://doi.org/10.1128/mbio.00928-17

Nommsen, L. A., Lovelady, C. A., Heinig, M. J., Lönnerdal, B., & Dewey, K. G. (1991). Determinants of energy, protein, lipid, and lactose concentrations in human milk during the first 12 mo of lactation: The DARLING Study. *The American Journal of Clinical Nutrition*, *53*(2), 457–465.

Nowrouzian, F., Hesselmar, B., Saalman, R., Strannegård, I.-L., Åberg, N., Wold, A. E., & Adlerberth, I. (2003). Escherichia coli in Infants' Intestinal Microflora: Colonization Rate, Strain Turnover, and Virulence Gene Carriage. *Pediatric Research*, *54*(1), Article 1. https://doi.org/10.1203/01.PDR.0000069843.20655.EE

Nurk, S., Bankevich, A., Antipov, D., Gurevich, A. A., Korobeynikov, A., Lapidus, A., Prjibelski, A. D., Pyshkin, A., Sirotkin, A., Sirotkin, Y., Stepanauskas, R., Clingenpeel, S. R., Woyke, T., McLean, J. S., Lasken, R., Tesler, G., Alekseyev, M. A., & Pevzner, P. A. (2013). Assembling single-cell genomes and mini-metagenomes from chimeric MDA products. *Journal of Computational Biology: A Journal of Computational Molecular Cell Biology*, *20*(10), 714–737. https://doi.org/10.1089/cmb.2013.0084

O'Brien, C. E., Meier, A. K., Cernioglo, K., Mitchell, R. D., Casaburi, G., Frese, S. A., Henrick, B. M., Underwood, M. A., & Smilowitz, J. T. (2021). Early probiotic supplementation with B. infantis in breastfed infants leads to persistent colonization at 1 year. *Pediatric Research*, 1–10. https://doi.org/10.1038/s41390-020-01350-0

Oddi, S., Binetti, A., Burns, P., Cuatrin, A., Reinheimer, J., Salminen, S., & Vinderola, G. (2020). Occurrence of bacteria with technological and probiotic potential in Argentinian human breast-milk. *Beneficial Microbes*, *11*(7), 685–702. https://doi.org/10.3920/BM2020.0054

Oguchi, S., Shinohara, K., Yamashiro, Y., Walker, W. A., & Sanderson, I. R. (1997). Growth factors in breast milk and their effect on gastrointestinal development. *Zhonghua Minguo Xiao Er Ke Yi Xue Hui Za Zhi [Journal]. Zhonghua Minguo Xiao Er Ke Yi Xue Hui*, *38*(5), 332–337.

Ohuma, E. O., Moller, A.-B., Bradley, E., Chakwera, S., Hussain-Alkhateeb, L., Lewin, A., Okwaraji, Y. B., Mahanani, W. R., Johansson, E. W., Lavin, T., Fernandez, D. E., Domínguez, G. G., Costa, A. de, Cresswell, J. A., Krasevec, J., Lawn, J. E., Blencowe, H., Requejo, J., & Moran, A. C. (2023). National, regional, and global estimates of preterm birth in 2020, with trends from 2010: A systematic analysis. *The Lancet*, *402*(10409), 1261–1271. https://doi.org/10.1016/S0140-6736(23)00878-4

Ojo-Okunola, A., Cacciatore, S., Nicol, M. P., & du Toit, E. (2020). The Determinants of the Human Milk Metabolome and Its Role in Infant Health. *Metabolites*, *10*(2), 77. https://doi.org/10.3390/metabo10020077

Olivares, M., Albrecht, S., De Palma, G., Ferrer, M. D., Castillejo, G., Schols, H. A., & Sanz, Y. (2015). Human milk composition differs in healthy mothers and mothers with celiac disease. *European Journal of Nutrition*, *54*(1), 119–128. https://doi.org/10.1007/s00394-014-0692-1

Olm, M. R., Bhattacharya, N., Crits-Christoph, A., Firek, B. A., Baker, R., Song, Y. S., Morowitz, M. J., & Banfield, J. F. (2019). Necrotizing enterocolitis is preceded by increased gut bacterial replication, Klebsiella, and fimbriae-encoding bacteria. *Science Advances*, *5*(12), eaax5727. https://doi.org/10.1126/sciadv.aax5727

Onderdonk, A. B., Delaney, M. L., DuBois, A. M., Allred, E. N., Leviton, A., & Extremely Low Gestational Age Newborns (ELGAN) Study Investigators. (2008). Detection of bacteria in placental tissues obtained from extremely low gestational age neonates. *American Journal of Obstetrics and Gynecology*, *198*(1), 110.e1-7. https://doi.org/10.1016/j.ajog.2007.05.044

Orlando, A., Linsalata, M., Notarnicola, M., Tutino, V., & Russo, F. (2014). Lactobacillus GG restoration of the gliadin induced epithelial barrier disruption: The role of cellular polyamines. *BMC Microbiology*, *14*(1), 19. https://doi.org/10.1186/1471-2180-14-19

Ossowski, I. von, Pietilä, T. E., Rintahaka, J., Nummenmaa, E., Mäkinen, V.-M., Reunanen, J., Satokari, R., Vos, W. M. de, Palva, I., & Palva, A. (2013). Using Recombinant Lactococci as an Approach to Dissect the Immunomodulating Capacity of Surface Piliation in Probiotic Lactobacillus rhamnosus GG. *PLOS ONE*, *8*(5), e64416. https://doi.org/10.1371/journal.pone.0064416

Ouwehand, A. C., Isolauri, E., Kirjavainen, P. V., Ölkkö, S. T., & Salminen, S. J. (2000). The mucus binding of Bifidobacterium lactis Bb12 is enhanced in the presence of Lactobacillus GG and Lact. Delbrueckii subsp. Bulgaricus. *Letters in Applied Microbiology*, *30*(1), 10–13. https://doi.org/10.1046/j.1472-765x.2000.00590.x

Ouwehand, A. C., Isolauri, E., Kirjavainen, P. V., & Salminen, S. J. (1999). Adhesion of four Bifidobacterium strains to human intestinal mucus from subjects in different age groups. *FEMS Microbiology Letters*, *172*(1), 61–64. https://doi.org/10.1111/j.1574-6968.1999.tb13450.x

Ouwehand, A. C., Kirjavainen, P. V., Grönlund, M.-M., Isolauri, E., & Salminen, S. J. (1999). Adhesion of probiotic micro-organisms to intestinal mucus. *International Dairy Journal*, *9*(9), 623–630. https://doi.org/10.1016/S0958-6946(99)00132-6

Ouwehand, A. C., Niemi, P., & Salminen, S. J. (1999). The normal faecal microflora does not affect the adhesion of probiotic bacteria in vitro. *FEMS Microbiology Letters*, *177*(1), 35–38. https://doi.org/10.1111/j.1574-6968.1999.tb13710.x

Ouwehand, A. C., & Salminen, S. (2003). In vitro adhesion assays for probiotics and their in vivo relevance: A review. *Microbial Ecology in Health and Disease*, *15*(4), 175–184. https://doi.org/10.1080/08910600310019886

Ouwehand, A. C., Tiihonen, K., Saarinen, M., Putaala, H., & Rautonen, N. (2008). Influence of a combination of Lactobacillus acidophilus NCFM and lactitol on healthy elderly: Intestinal and immune parameters. *British Journal of Nutrition*, *101*(3), 367–375. https://doi.org/10.1017/S0007114508003097

Ouwehand, A. C., Tölkkö, S., & Salminen, S. (2001). The Effect of Digestive Enzymes on the Adhesion of Probiotic Bacteria In Vitro. *Journal of Food Science*, *66*(6), 856–859. https://doi.org/10.1111/j.1365-2621.2001.tb15186.x

Ou-Yang, M.-C., Sun, Y., Liebowitz, M., Chen, C.-C., Fang, M.-L., Dai, W., Chuang, T.-W., & Chen, J.-L. (2020). Accelerated weight gain, prematurity, and the risk of childhood obesity: A meta-analysis and systematic review. *PLoS ONE*, *15*(5), e0232238. https://doi.org/10.1371/journal.pone.0232238

Pace, R. M., Williams, J. E., Robertson, B., Lackey, K. A., Meehan, C. L., Price, W. J., Foster, J. A., Sellen, D. W., Kamau-Mbuthia, E. W., Kamundia, E. W., Mbugua, S., Moore, S. E., Prentice, A. M., Kita, D. G., Kvist, L. J., Otoo, G. E., Ruiz, L., Rodríguez, J. M., Pareja, R. G., … McGuire, M. K. (2021). Variation in Human Milk Composition Is Related to Differences in Milk and Infant Fecal Microbial Communities. *Microorganisms*, *9*(6), Article 6. https://doi.org/10.3390/microorganisms9061153

Padilha, M., Danneskiold-Samsøe, N. B., Brejnrod, A., Hoffmann, C., Cabral, V. P., Iaucci, J. de M., Sales, C. H., Fisberg, R. M., Cortez, R. V., Brix, S., Taddei, C. R., Kristiansen, K., & Saad, S. M. I. (2019). The Human Milk Microbiota is Modulated by Maternal Diet. *Microorganisms*, *7*(11). https://doi.org/10.3390/microorganisms7110502

Padilha, M., Iaucci, J. M., Cabral, V. P., Diniz, E. M. A., Taddei, C. R., & Saad, S. M. I. (2019). Maternal antibiotic prophylaxis affects Bifidobacterium spp. Counts in the human milk, during the first week after delivery. *Beneficial Microbes*, *10*(2), 155–163. https://doi.org/10.3920/BM2018.0046

Palkova, L., Tomova, A., Repiska, G., Babinska, K., Bokor, B., Mikula, I., Minarik, G., Ostatnikova, D., & Soltys, K. (2021). Evaluation of 16S rRNA primer sets for characterisation of microbiota in paediatric patients with autism spectrum disorder. *Scientific Reports*, *11*(1), Article 1. https://doi.org/10.1038/s41598-021-86378-w

Pammi, M., Cope, J., Tarr, P. I., Warner, B. B., Morrow, A. L., Mai, V., Gregory, K. E., Kroll, J. S., McMurtry, V., Ferris, M. J., Engstrand, L., Lilja, H. E., Hollister, E. B., Versalovic, J., & Neu, J.

(2017). Intestinal dysbiosis in preterm infants preceding necrotizing enterocolitis: A systematic review and meta-analysis. *Microbiome*, *5*(1), 31. https://doi.org/10.1186/s40168-017-0248-8

Pannaraj, P. S., Li, F., Cerini, C., Bender, J. M., Yang, S., Rollie, A., Adisetiyo, H., Zabih, S., Lincez, P. J., Bittinger, K., Bailey, A., Bushman, F. D., Sleasman, J. W., & Aldrovandi, G. M. (2017). Association Between Breast Milk Bacterial Communities and Establishment and Development of the Infant Gut Microbiome. *JAMA Pediatrics*, *171*(7), 647–654. https://doi.org/10.1001/jamapediatrics.2017.0378

Pannaraj, P. S., Ly, M., Cerini, C., Saavedra, M., Aldrovandi, G. M., Saboory, A. A., Johnson, K. M., & Pride, D. T. (2018). Shared and Distinct Features of Human Milk and Infant Stool Viromes. *Frontiers in Microbiology*, *9*. https://doi.org/10.3389/fmicb.2018.01162

Park, S., Chung, H.-S., Seo, E. K., Mun, Y. C., & Lee, M. (2016). Two Cases of Medical Device-Related Corynebacterium striatum Infection: A Meningitis and A Sepsis. *Annals of Clinical Microbiology*, *19*(1), 28–31. https://doi.org/10.5145/ACM.2016.19.1.28

Park, S.-G., Choi, H.-N., Yang, H.-R., & Yim, J.-E. (2017). Effects of zinc supplementation on catch-up growth in children with failure to thrive. *Nutrition Research and Practice*, *11*(6), 487–491. https://doi.org/10.4162/nrp.2017.11.6.487

Pärnänen, K., Karkman, A., Hultman, J., Lyra, C., Bengtsson-Palme, J., Larsson, D. G. J., Rautava, S., Isolauri, E., Salminen, S., Kumar, H., Satokari, R., & Virta, M. (2018). Maternal gut and breast milk microbiota affect infant gut antibiotic resistome and mobile genetic elements. *Nature Communications*, *9*(1), 3891. https://doi.org/10.1038/s41467-018-06393-w

Pärnänen, K. M. M., Hultman, J., Markkanen, M., Satokari, R., Rautava, S., Lamendella, R., Wright, J., McLimans, C. J., Kelleher, S. L., & Virta, M. P. (2021). Early-life formula feeding is associated with infant gut microbiota alterations and an increased antibiotic resistance load. *The American Journal of Clinical Nutrition*, nqab353. https://doi.org/10.1093/ajcn/nqab353

Parry, S., Hanisch, F. G., Leir, S.-H., Sutton-Smith, M., Morris, H. R., Dell, A., & Harris, A. (2006). N-Glycosylation of the MUC1 mucin in epithelial cells and secretions. *Glycobiology*, *16*(7), 623–634. https://doi.org/10.1093/glycob/cwj110

Patel, A. L., Meier, P. P., & Canvasser, J. (2020). Strategies to increase the use of mother's own milk for infants at risk of necrotizing enterocolitis. *Pediatric Research*, *88*(1), Article 1. https://doi.org/10.1038/s41390-020-1075-3

Patel, S. H., Vaidya, Y. H., Patel, R. J., Pandit, R. J., Joshi, C. G., & Kunjadiya, A. P. (2017). Culture independent assessment of human milk microbial community in lactational mastitis. *Scientific Reports*, *7*(1), Article 1. https://doi.org/10.1038/s41598-017-08451-7

Paulaviciene, I. J., Liubsys, A., Eidukaite, A., Molyte, A., Tamuliene, L., & Usonis, V. (2020). The Effect of Prolonged Freezing and Holder Pasteurization on the Macronutrient and Bioactive Protein Compositions of Human Milk. *Breastfeeding Medicine*, *15*(9), 583–588. https://doi.org/10.1089/bfm.2020.0219

Pegg, A. E. (2009). Mammalian polyamine metabolism and function. *IUBMB Life*, *61*(9), 880–894. https://doi.org/10.1002/iub.230

Peila, C., Moro, G. E., Bertino, E., Cavallarin, L., Giribaldi, M., Giuliani, F., Cresi, F., & Coscia, A. (2016). The Effect of Holder Pasteurization on Nutrients and Biologically-Active Components in Donor Human Milk: A Review. *Nutrients*, *8*(8). https://doi.org/10.3390/nu8080477

Pellegrini, A., Thomas, U., Bramaz, N., Hunziker, P., & von Fellenberg, R. (1999). Isolation and identification of three bactericidal domains in the bovine α-lactalbumin molecule. *Biochimica et Biophysica Acta (BBA) - General Subjects*, *1426*(3), 439–448. https://doi.org/10.1016/S0304-4165(98)00165-2

Penders, J., Stobberingh, E. E., Thijs, C., Adams, H., Vink, C., Ree, R. V., & Brandt, P. A. V. D. (2006). Molecular fingerprinting of the intestinal microbiota of infants in whom atopic eczema was or was

not developing. *Clinical & Experimental Allergy*, *36*(12), 1602–1608. https://doi.org/10.1111/j.1365-2222.2006.02599.x

Penders, J., Thijs, C., van den Brandt, P. A., Kummeling, I., Snijders, B., Stelma, F., Adams, H., van Ree, R., & Stobberingh, E. E. (2007). Gut microbiota composition and development of atopic manifestations in infancy: The KOALA Birth Cohort Study. *Gut*, *56*(5), 661–667. https://doi.org/10.1136/gut.2006.100164

Perin, J., Mulick, A., Yeung, D., Villavicencio, F., Lopez, G., Strong, K. L., Prieto-Merino, D., Cousens, S., Black, R. E., & Liu, L. (2021). Global, regional, and national causes of under-5 mortality in 2000–19: An updated systematic analysis with implications for the Sustainable Development Goals. *The Lancet Child & Adolescent Health*, *0*(0). https://doi.org/10.1016/S2352-4642(21)00311-4

Petrova, M. I., Imholz, N. C. E., Verhoeven, T. L. A., Balzarini, J., Damme, E. J. M. V., Schols, D., Vanderleyden, J., & Lebeer, S. (2016). Lectin-Like Molecules of Lactobacillus rhamnosus GG Inhibit Pathogenic Escherichia coli and Salmonella Biofilm Formation. *PLOS ONE*, *11*(8), e0161337. https://doi.org/10.1371/journal.pone.0161337

Piard, J. C., & Desmazeaud, M. (1992). Inhibiting factors produced by lactic acid bacteria. 2. Bacteriocins and other antibacterial substances. *Le Lait*, *72*(2), 113–142. https://doi.org/10.1051/lait:199229

Piekarska-Radzik, L., & Klewicka, E. (2021). Mutual influence of polyphenols and Lactobacillus spp. bacteria in food: A review. *European Food Research and Technology*, *247*(1), 9–24. https://doi.org/10.1007/s00217-020-03603-y

Piemontese, P., Mallardi, D., Liotto, N., Tabasso, C., Menis, C., Perrone, M., Roggero, P., & Mosca, F. (2019). Macronutrient content of pooled donor human milk before and after Holder pasteurization. *BMC Pediatrics*, *19*. https://doi.org/10.1186/s12887-019-1427-5

Pillai, A., Albersheim, S., Matheson, J., Lalari, V., Wei, S., Innis, S. M., & Elango, R. (2018). Evaluation of A Concentrated Preterm Formula as a Liquid Human Milk Fortifier in Preterm Babies at Increased Risk of Feed Intolerance. *Nutrients*, *10*(10), 1433. https://doi.org/10.3390/nu10101433

Plaza-Diaz, J., Ruiz-Ojeda, F. J., Gil-Campos, M., & Gil, A. (2019). Mechanisms of Action of Probiotics. *Advances in Nutrition*, *10*(Suppl 1), S49–S66. https://doi.org/10.1093/advances/nmy063

Plaza-Zamora, J., Sabater-Molina, M., Rodríguez-Palmero, M., Rivero, M., Bosch, V., Nadal, J. M., Zamora, S., & Larqué, E. (2013). Polyamines in human breast milk for preterm and term infants. *British Journal of Nutrition*, *110*(3), 524–528. https://doi.org/10.1017/S0007114512005284

Poolman, B., Spitzer, J. J., & Wood, J. M. (2004). Bacterial osmosensing: Roles of membrane structure and electrostatics in lipid–protein and protein–protein interactions. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1666*(1), 88–104. https://doi.org/10.1016/j.bbamem.2004.06.013

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*(D1), D590–D596. https://doi.org/10.1093/nar/gks1219

Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2013). The complex microbiota of raw milk. *FEMS Microbiology Reviews*, *37*(5), 664–698. https://doi.org/10.1111/1574-6976.12030

R Core Team. (2017). R: A Language and Environment for Statistical Computing. *R Foundation for Statistical Computing, Vienna, Austria., Http://Www.R-Project.Org/.*

Rader, B. A. (2017). Alkaline Phosphatase, an Unconventional Immune Protein. *Frontiers in Immunology*, *8*, 897. https://doi.org/10.3389/fimmu.2017.00897

Reddy, K. B. P. K., Awasthi, S. P., Madhu, A. N., & Prapulla, S. G. (2009). Role of Cryoprotectants on the Viability and Functional Properties of Probiotic Lactic Acid Bacteria during Freeze Drying. *Food Biotechnology*, *23*(3), 243–265. https://doi.org/10.1080/08905430903106811

Rigo, J., Pieltain, C., Viellevoye, R., & Bagnoli, F. (2018). Calcium and Phosphorus Homeostasis: Pathophysiology. In G. Buonocore, R. Bracci, & M. Weindling (Eds.), *Neonatology: A Practical Approach to Neonatal Diseases* (pp. 639–668). Springer International Publishing. https://doi.org/10.1007/978-3-319-29489-6_190

Riskin, A. (2020). Immunomodulatory Constituents of Human Donor Milk. *Breastfeeding Medicine: The Official Journal of the Academy of Breastfeeding Medicine*, *15*(9), 563–567. https://doi.org/10.1089/bfm.2020.0192

Risnes, K., Bilsteen, J. F., Brown, P., Pulakka, A., Andersen, A.-M. N., Opdahl, S., Kajantie, E., & Sandin, S. (2021). Mortality Among Young Adults Born Preterm and Early Term in 4 Nordic Nations. *JAMA Network Open*, *4*(1), e2032779. https://doi.org/10.1001/jamanetworkopen.2020.32779

Robertson, C., Savva, G. M., Clapuci, R., Jones, J., Maimouni, H., Brown, E., Minocha, A., Hall, L. J., & Clarke, P. (2020). Incidence of necrotising enterocolitis before and after introducing routine prophylactic Lactobacillus and Bifidobacterium probiotics. *Archives of Disease in Childhood - Fetal and Neonatal Edition*, *105*(4), 380–386. https://doi.org/10.1136/archdischild-2019-317346

Rodriguez, J. M. (2014). The Origin of Human Milk Bacteria: Is There a Bacterial Entero-Mammary Pathway during Late Pregnancy and Lactation? *Advances in Nutrition: An International Review Journal*, *5*(6), 779–784. https://doi.org/10.3945/an.114.007229

Rodríguez, J. M., Fernández, L., & Verhasselt, V. (2021). The Gut–Breast Axis: Programming Health for Life. *Nutrients*, *13*(2), Article 2. https://doi.org/10.3390/nu13020606

Ruiz, L., Bacigalupe, R., García-Carral, C., Boix-Amoros, A., Argüello, H., Silva, C. B., de Los Angeles Checa, M., Mira, A., & Rodríguez, J. M. (2019). Microbiota of human precolostrum and its potential role as a source of bacteria to the infant mouth. *Scientific Reports*, *9*(1), 8435. https://doi.org/10.1038/s41598-019-42514-1

Sagheddu, V., Elli, M., Biolchi, C., Lucido, J., & Morelli, L. (2018). Impact of mode of assumption and food matrix on probiotic viability. *Journal of Food Microbiology*, *0*(0). https://www.alliedacademies.org/abstract/impact-of-mode-of-assumption-and-food-matrix-on-probiotic-viability-11133.html

Salli, K., Hirvonen, J., Siitonen, J., Ahonen, I., Anglenius, H., & Maukonen, J. (2021). Selective Utilization of the Human Milk Oligosaccharides 2′-Fucosyllactose, 3-Fucosyllactose, and Difucosyllactose by Various Probiotic and Pathogenic Bacteria. *Journal of Agricultural and Food Chemistry*, *69*(1), 170–182. https://doi.org/10.1021/acs.jafc.0c06041

Salva, S., Nuñez, M., Villena, J., Ramón, A., Font, G., & Alvarez, S. (2011). Development of a fermented goats' milk containing Lactobacillus rhamnosus: In vivo study of health benefits. *Journal of the Science of Food and Agriculture*, *91*(13), 2355–2362. https://doi.org/10.1002/jsfa.4467

Samuel, T. M., Zhou, Q., Giuffrida, F., Munblit, D., Verhasselt, V., & Thakkar, S. K. (2020). Nutritional and Non-nutritional Composition of Human Milk Is Modulated by Maternal, Infant, and Methodological Factors. *Frontiers in Nutrition*, *7*, 172. https://doi.org/10.3389/fnut.2020.576133

Savarino, G., Carta, M., Cimador, M., Corsello, A., Giuffrè, M., Schierz, I. A. M., Serra, G., & Corsello, G. (2021). Necrotizing enterocolitis in the preterm: Newborns medical and nutritional Management in a Single-Center Study. *Italian Journal of Pediatrics*, *47*(1), 226. https://doi.org/10.1186/s13052-021-01180-8

Schanler, R. J., Lau, C., Hurst, N. M., & Smith, E. O. (2005). Randomized trial of donor human milk versus preterm formula as substitutes for mothers' own milk in the feeding of extremely premature infants. *Pediatrics*, *116*(2), 400–406. https://doi.org/10.1542/peds.2004-1974

Schillinger, U. (1999). Isolation and identification of lactobacilli from novel-type probiotic and mild yoghurts and their stability during refrigerated storage. *International Journal of Food Microbiology*, *47*(1), 79–87. https://doi.org/10.1016/S0168-1605(99)00014-8

Schimmoeller, M., Wulf, K., Walker, C., Barrett-Reis, B., & Vurma, M. (2021). Use of probiotics in the NICU: Evaluating the stability of a three-strain probiotic blend in various media for enteral feeding. *Journal of Neonatal-Perinatal Medicine*, *Preprint*(Preprint), 1–6. https://doi.org/10.3233/NPM-210796

Schulz, E. V., & Wagner, C. L. (2021). Powdered to Liquid Human Milk Fortifiers in the Preterm Infant. *NeoReviews*, *22*(6), e360–e369. https://doi.org/10.1542/neo.22-6-e360

Schuster, I., & Bernhardt, R. (2011). *Interactions of natural polyamines with mammalian proteins*. *2*(1–2), 79–94. https://doi.org/10.1515/bmc.2011.007

Seferovic, M. D., Mohammad, M., Pace, R. M., Engevik, M., Versalovic, J., Bode, L., Haymond, M., & Aagaard, K. M. (2020). Maternal diet alters human milk oligosaccharide composition with implications for the milk metagenome. *Scientific Reports*, *10*(1), 22092. https://doi.org/10.1038/s41598-020-79022-6

Selma-Royo, M., Calvo Lerma, J., Cortés-Macías, E., & Collado, M. C. (2021). Human milk microbiome: From actual knowledge to future perspective. *Seminars in Perinatology*, *45*(6), 151450. https://doi.org/10.1016/j.semperi.2021.151450

Selma-Royo, M., García-Mantrana, I., Calatayud, M., Parra-Llorca, A., Martínez-Costa, C., & Collado, M. C. (2021). Maternal diet during pregnancy and intestinal markers are associated with early gut microbiota. *European Journal of Nutrition*, *60*(3), 1429–1442. https://doi.org/10.1007/s00394-020-02337-7

Selvamani, S., Dailin, D. J., Gupta, V. K., Wahid, M., Keat, H. C., Natasya, K. H., Malek, R. A., Haque, S., Sayyed, R. Z., Abomoelak, B., Sukmawati, D., Varzakas, T., & El Enshasy, H. A. (2021). An Insight into Probiotics Bio-Route: Translocation from the Mother's Gut to the Mammary Gland. *Applied Sciences*, *11*(16), Article 16. https://doi.org/10.3390/app11167247

Senaka Ranadheera, C., Evans, C. A., Adams, M. C., & Baines, S. K. (2012). Probiotic viability and physico-chemical and sensory properties of plain and stirred fruit yogurts made from goat's milk. *Food Chemistry*, *135*(3), 1411–1418. https://doi.org/10.1016/j.foodchem.2012.06.025

Senn, V., Bassler, D., Choudhury, R., Scholkmann, F., Righini-Grunder, F., Vuille-dit-Bille, R. N., & Restin, T. (2020). Microbial Colonization From the Fetus to Early Childhood—A Comprehensive Review. *Frontiers in Cellular and Infection Microbiology*, *10*. https://www.frontiersin.org/article/10.3389/fcimb.2020.573735

Seppo, A. E., Kukkonen, A. K., Kuitunen, M., Savilahti, E., Yonemitsu, C., Bode, L., & Järvinen, Kirsi. M. (2019). Association of Maternal Probiotic Supplementation With Human Milk Oligosaccharide Composition. *JAMA Pediatrics*, *173*(3), 286–288. https://doi.org/10.1001/jamapediatrics.2018.4835

Shah, P., Nanduri, B., Swiatlo, E., Ma, Y., & Pendarvis, K. (2011). Polyamine biosynthesis and transport mechanisms are crucial for fitness and pathogenesis of Streptococcus pneumoniae. *Microbiology (Reading, England)*, *157*(Pt 2), 504–515. https://doi.org/10.1099/mic.0.042564-0

Sharpe, J., Way, M., Koorts, P. J., & Davies, M. W. (2018). The availability of probiotics and donor human milk is associated with improved survival in very preterm infants. *World Journal of Pediatrics*, *14*(5), 492–497. https://doi.org/10.1007/s12519-018-0168-0

Shub, M. D., Pang, K. Y., Swann, D. A., & Walker, W. A. (1983). Age-related changes in chemical composition and physical properties of mucus glycoproteins from rat small intestine. *Biochemical Journal*, *215*(2), 405–411.

Shukla, S. K., Harney, M., Jhaveri, B., Andrews, K., & Reed, K. D. (2003). Is a Black-Pigmented Corynebacterium Species an Opportunistic Pathogen during Pregnancy? Literature Review and Report of 3 New Cases. *Clinical Infectious Diseases*, *37*(6), 834–837. https://doi.org/10.1086/377233

Simpson, E. H. (1949, April 30). *Measurement of Diversity | Nature*. https://www.nature.com/articles/163688a0

Simpson, M. R., Avershina, E., Storrø, O., Johnsen, R., Rudi, K., & Øien, T. (2018a). Breastfeeding-associated microbiota in human milk following supplementation with Lactobacillus rhamnosus GG, Lactobacillus acidophilus La-5, and Bifidobacterium animalis ssp. Lactis Bb-12. *Journal of Dairy Science*, *101*(2), 889–899. https://doi.org/10.3168/jds.2017-13411

Simpson, M. R., Avershina, E., Storrø, O., Johnsen, R., Rudi, K., & Øien, T. (2018b). Breastfeeding-associated microbiota in human milk following supplementation with Lactobacillus rhamnosus GG, Lactobacillus acidophilus La-5, and Bifidobacterium animalis ssp. Lactis Bb-12. *Journal of Dairy Science*, *101*(2), 889–899. https://doi.org/10.3168/jds.2017-13411

Simpson, P. J., Stanton, C., Fitzgerald, G. F., & Ross, R. P. (2005). Intrinsic tolerance of Bifidobacterium species to heat and oxygen and survival following spray drying and storage. *Journal of Applied Microbiology*, *99*(3), 493–501. https://doi.org/10.1111/j.1365-2672.2005.02648.x

Singh, V., Muthuramalingam, K., Kim, Y. M., Park, S., Kim, S. H., Lee, J., Hyun, C., Unno, T., & Cho, M. (2021). Synbiotic supplementation with prebiotic Schizophyllum commune derived β-(1,3/1,6)-glucan and probiotic concoction benefits gut microbiota and its associated metabolic activities. *Applied Biological Chemistry*, *64*(1), 7. https://doi.org/10.1186/s13765-020-00572-4

Slačanac, V., Lučan, M., Hardi, J., Habschied, K., Krstanović, V., & Gagula, G. (2013). Predictive modeling of Bifidobacterium animalis subsp. Lactis Bb-12 growth in cow's, goat's and soy milk. *ResearchGate*, *63*(4), 220–227.

Soares, M. M., Silva, M. A., Garcia, P. P. C., Silva, L. S. da, Costa, G. D. da, Araújo, R. M. A., Cotta, R. M. M., Soares, M. M., Silva, M. A., Garcia, P. P. C., Silva, L. S. da, Costa, G. D. da, Araújo, R. M. A., & Cotta, R. M. M. (2019). Efect of vitamin A suplementation: A systematic review. *Ciência & Saúde Coletiva*, *24*(3), 827–838. https://doi.org/10.1590/1413-81232018243.07112017

Soeorg, H., Metsvaht, H. K., Keränen, E. E., Eelmäe, I., Merila, M., Ilmoja, M.-L., Metsvaht, T., & Lutsar, I. (2019). Genetic Relatedness of Staphylococcus haemolyticus in Gut and Skin of Preterm Neonates and Breast Milk of Their Mothers. *The Pediatric Infectious Disease Journal*, *38*(3), 308. https://doi.org/10.1097/INF.0000000000002056

Soeorg, H., Metsvaht, T., Eelmäe, I., Metsvaht, H. K., Treumuth, S., Merila, M., Ilmoja, M.-L., & Lutsar, I. (2017). Coagulase-Negative Staphylococci in Human Milk From Mothers of Preterm Compared With Term Neonates. *Journal of Human Lactation*, *33*(2), 329–340. https://doi.org/10.1177/0890334417691505

Sohrabvandi, S., Mortazavian, A.-M., Dolatkhahnejad, M.-R., & Monfared, A. B. (2012). Suitability of MRS-bile agar for the selective enumeration of mixed probiotic bacteria in presence of mesophilic lactic acid cultures and yoghurt bacteria. *Iranian Journal of Biotechnology*, *10*(1), 16–21.

Solano-Aguilar, G., Dawson, H., Restrepo, M., Andrews, K., Vinyard, B., & Urban, J. F. (2008). Detection of Bifidobacterium animalis subsp. Lactis (Bb12) in the Intestine after Feeding of Sows

and Their Piglets. *Applied and Environmental Microbiology*, *74*(20), 6338–6347. https://doi.org/10.1128/AEM.00309-08

Song, Q., Zhang, Y., Liu, H., & Du, Y. (2020). Potential of Using Cell-Free DNA and miRNA in Breast Milk to Screen Early Breast Cancer. *BioMed Research International*, *2020*, 8126176. https://doi.org/10.1155/2020/8126176

Soto, A., Martín, V., Jiménez, E., Mader, I., Rodríguez, J. M., & Fernández, L. (2014). Lactobacilli and Bifidobacteria in Human Breast Milk: Influence of Antibiotherapy and Other Host and Clinical Factors. *Journal of Pediatric Gastroenterology and Nutrition*, *59*(1), 78–88. https://doi.org/10.1097/MPG.0000000000000347

Stewart, C. J., Embleton, N. D., Marrs, E. C. L., Smith, D. P., Nelson, A., Abdulkadir, B., Skeath, T., Petrosino, J. F., Perry, J. D., Berrington, J. E., & Cummings, S. P. (2016). Temporal bacterial and metabolic development of the preterm gut reveals specific signatures in health and disease. *Microbiome*, *4*(1), 67. https://doi.org/10.1186/s40168-016-0216-8

Stinson, L. F., Trevenen, M. L., & Geddes, D. T. (2021). The Viable Microbiome of Human Milk Differs from the Metataxonomic Profile. *Nutrients*, *13*(12), Article 12. https://doi.org/10.3390/nu13124445

Sugimura, Y., Hagi, T., & Hoshino, T. (2011). Correlation between in vitro mucus adhesion and the in vivo colonization ability of lactic acid bacteria: Screening of new candidate carp probiotics. *Bioscience, Biotechnology, and Biochemistry*, *75*(3), 511–515. https://doi.org/10.1271/bbb.100732

Sugiyama, Y., Nara, M., Sakanaka, M., Kitakata, A., Okuda, S., & Kurihara, S. (2018). Analysis of polyamine biosynthetic- and transport ability of human indigenous Bifidobacterium. *Bioscience, Biotechnology, and Biochemistry*, *82*(9), 1606–1614. https://doi.org/10.1080/09168451.2018.1475211

Sullivan, M. C., Msall, M. E., & Miller, R. J. (2012). 17-Year Outcome of Preterm Infants with Diverse Neonatal Morbidities: Part 1, Impact on Physical, Neurological, and Psychological Health Status. *Journal for Specialists in Pediatric Nursing*, *17*(3), 226–241. https://doi.org/10.1111/j.1744-6155.2012.00337.x

Sullivan, P. B., Lewindon, P. J., Cheng, C., Lenehan, P. F., Kuo, B.-S., Haskins, J. R., Goodlad, R. A., Wright, N. A., de la Iglesia, F. A., & dela Iglesia, F. A. (2007). Intestinal mucosa remodeling by recombinant human epidermal growth factor(1-48) in neonates with severe necrotizing enterocolitis. *Journal of Pediatric Surgery*, *42*(3), 462–469. https://doi.org/10.1016/j.jpedsurg.2006.10.039

Sullivan, S., Schanler, R. J., Kim, J. H., Patel, A. L., Trawöger, R., Kiechl-Kohlendorfer, U., Chan, G. M., Blanco, C. L., Abrams, S., Cotten, C. M., Laroia, N., Ehrenkranz, R. A., Dudell, G., Cristofalo, E. A., Meier, P., Lee, M. L., Rechtman, D. J., & Lucas, A. (2010). An exclusively human milk-based diet is associated with a lower rate of necrotizing enterocolitis than a diet of human milk and bovine milk-based products. *The Journal of Pediatrics*, *156*(4), 562-567.e1. https://doi.org/10.1016/j.jpeds.2009.10.040

Sun, Y., & O'Riordan, M. X. D. (2013). Regulation of Bacterial Pathogenesis by Intestinal Short-Chain Fatty Acids. *Advances in Applied Microbiology*, *85*, 93–118. https://doi.org/10.1016/B978-0-12-407672-3.00003-4

Sybesma, W., Molenaar, D., van IJcken, W., Venema, K., & Kort, R. (2013). Genome instability in Lactobacillus rhamnosus GG. *Applied and Environmental Microbiology*, *79*(7), 2233–2239. https://doi.org/10.1128/AEM.03566-12

Szajewska, H., Berni Canani, R., Domellöf, M., Guarino, A., Hojsak, I., Indrio, F., Lo Vecchio, A., Mihatsch, W. A., Mosca, A., Orel, R., Salvatore, S., Shamir, R., van den Akker, C. H. P., van Goudoever, J. B., Vandenplas, Y., Weizman, Z., & ESPGHAN Special Interest Group on Gut Microbiota and Modifications. (2023). Probiotics for the Management of Pediatric Gastrointestinal

Disorders: Position Paper of the ESPGHAN Special Interest Group on Gut Microbiota and Modifications. *Journal of Pediatric Gastroenterology and Nutrition*, *76*(2), 232–247. https://doi.org/10.1097/MPG.0000000000003633

Szajewska, H., Guandalini, S., Morelli, L., Van Goudoever, J. B., & Walker, A. (2010). The Effect of Bifidobacterium animalis ssp. lactis Supplementation in Preterm Infants: A Systematic Review of Randomized Controlled Trials. *Journal of Pediatric Gastroenterology and Nutrition*, *51*(2), 203–209. https://doi.org/10.1097/MPG.0b013e3181dc0d93

Taddei, C. R., Oliveira, F. F., Duarte, R. T. D., Talarico, S. T., Takagi, E. H., Ramos Carvalho, I. I., Gomes, F. M. S., Brandt, K., & Martinez, M. B. (2014). High Abundance of Escherichia During the Establishment of Fecal Microbiota in Brazilian Children. *Microbial Ecology*, *67*(3), 624–634. https://doi.org/10.1007/s00248-014-0381-x

Taghizadeh, M., Mirlohi, M., Poursina, F., Madani, G., Khoshhali, M., Bahreini, N., & Safaei, H. G. (2015). The influence of impact delivery mode, lactation time, infant gender, maternal age and rural or urban life on total number of Lactobacillus in breast milk Isfahan—Iran. *Advanced Biomedical Research*, *4*, 141. https://doi.org/10.4103/2277-9175.161546

Tarracchini, C., Milani, C., Longhi, G., Fontana, F., Mancabelli, L., Pintus, R., Lugli, G. A., Alessandri, G., Anzalone, R., Viappiani, A., Turroni, F., Mussap, M., Dessì, A., Cesare Marincola, F., Noto, A., De Magistris, A., Vincent, M., Bernasconi, S., Picaud, J.-C., … Ventura, M. (2021). Unraveling the Microbiome of Necrotizing Enterocolitis: Insights in Novel Microbial and Metabolomic Biomarkers. *Microbiology Spectrum*, *9*(2), e01176-21. https://doi.org/10.1128/Spectrum.01176-21

Tassell, M. L. V., & Miller, M. J. (2011). Lactobacillus Adhesion to Mucus. *Nutrients*, *3*(5), 613–636. https://doi.org/10.3390/nu3050613

Tavella, M., Peterson, G., Espeche, M., Cavallero, E., Cipolla, L., Perego, L., & Caballero, B. (2000). Trans fatty acid content of a selection of foods in Argentina. *Food Chemistry*, *69*(2), 209–213. https://doi.org/10.1016/S0308-8146(99)00257-5

Taylor, S. N., Basile, L. A., Ebeling, M., & Wagner, C. L. (2009). Intestinal Permeability in Preterm Infants by Feeding Type: Mother's Milk Versus Formula. *Breastfeeding Medicine*, *4*(1), 11–15. https://doi.org/10.1089/bfm.2008.0114

Terveyden Ja Hyvinvainnin Laitos. (2018, December 2). *Keskoset*. http://www.thl.fi/fi/tutkimus-ja-asiantuntijatyo/hankkeet-ja-ohjelmat/perfect/osahankkeet/keskoset

Thevathasan, I., Karahalios, A., Unterscheider, J., Leung, L., Walker, S., & Said, J. M. (2023). Neonatal outcomes following antenatal corticosteroid administration prior to elective caesarean delivery in women with pre-gestational diabetes: A retrospective cohort study. *Australian and New Zealand Journal of Obstetrics and Gynaecology*, *63*(1), 93–98. https://doi.org/10.1111/ajo.13586

Thoene, M., Lyden, E., Weishaar, K., Elliott, E., Wu, R., White, K., Timm, H., & Anderson-Berry, A. (2016). Comparison of a Powdered, Acidified Liquid, and Non-Acidified Liquid Human Milk Fortifier on Clinical Outcomes in Premature Infants. *Nutrients*, *8*(8). https://doi.org/10.3390/nu8080451

Thongaram, T., Hoeflinger, J. L., Chow, J., & Miller, M. J. (2017a). Human milk oligosaccharide consumption by probiotic and human-associated bifidobacteria and lactobacilli. *Journal of Dairy Science*, *100*(10), 7825–7833. https://doi.org/10.3168/jds.2017-12753

Thongaram, T., Hoeflinger, J. L., Chow, J., & Miller, M. J. (2017b). Human milk oligosaccharide consumption by probiotic and human-associated bifidobacteria and lactobacilli. *Journal of Dairy Science*, *100*(10), 7825–7833. https://doi.org/10.3168/jds.2017-12753

Thorn, D. C., Ecroyd, H., Carver, J. A., & Holt, C. (2015). Casein structures in the context of unfolded proteins. *International Dairy Journal*, *46*, 2–11. https://doi.org/10.1016/j.idairyj.2014.07.008

Thurl, S., Munzert, M., Boehm, G., Matthews, C., & Stahl, B. (2017). Systematic review of the concentrations of oligosaccharides in human milk. *Nutrition Reviews*, *75*(11), 920–933. https://doi.org/10.1093/nutrit/nux044

Timmons, J., Chang, E. T., Wang, J.-Y., & Rao, J. N. (2012). Polyamines and Gut Mucosal Homeostasis. *Journal of Gastrointestinal & Digestive System*, *2*(Suppl 7), 001.

Tingö, L., Ahlberg, E., Johansson, L., Pedersen, S. A., Chawla, K., Sætrom, P., Cione, E., & Simpson, M. R. (2021). Non-Coding RNAs in Human Breast Milk: A Systematic Review. *Frontiers in Immunology*, *12*, 3522. https://doi.org/10.3389/fimmu.2021.725323

Tofalo, R., Cocchi, S., & Suzzi, G. (2019). Polyamines and Gut Microbiota. *Frontiers in Nutrition*, *6*, 16. https://doi.org/10.3389/fnut.2019.00016

Togo, A., Dufour, J.-C., Lagier, J.-C., Dubourg, G., Raoult, D., & Million, M. (2019). Repertoire of human breast and milk microbiota: A systematic review. *Future Microbiology*, *14*(7), 623–641. https://doi.org/10.2217/fmb-2018-0317

Tong, S., & Vichinsky, E. (2021). Iron Deficiency: Implications Before Anemia. *Pediatrics in Review*, *42*(1), 11–20. https://doi.org/10.1542/pir.2018-0134

Torres, A. G., Vazquez-Juarez, R. C., Tutt, C. B., & Garcia-Gallegos, J. G. (2005). Pathoadaptive Mutation That Mediates Adherence of Shiga Toxin-Producing Escherichia coli O111. *Infection and Immunity*, *73*(8), 4766–4776. https://doi.org/10.1128/IAI.73.8.4766-4776.2005

Trégoat, V., Montagne, P., Béné, M.-C., & Faure, G. (2002). Changes in the mannan binding lectin (MBL) concentration in human milk during lactation. *Journal of Clinical Laboratory Analysis*, *16*(6), 304–307. https://doi.org/10.1002/jcla.10055

Treven, P., Mahnič, A., Rupnik, M., Golob, M., Pirš, T., Matijašić, B. B., & Lorbeg, P. M. (2019). Evaluation of Human Milk Microbiota by 16S rRNA Gene Next-Generation Sequencing (NGS) and Cultivation/MALDI-TOF Mass Spectrometry Identification. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.02612

Tripathi, M. K., & Giri, S. K. (2014). Probiotic functional foods: Survival of probiotics during processing and storage. *Journal of Functional Foods*, *9*, 225–241. https://doi.org/10.1016/j.jff.2014.04.030

Tušar, T., Žerdoner, K., Bogovic Matijašic, B., Paveljšek, D., Benedik, E., Bratanic, B., Fidler, N., & Rogelj, I. (2014). *Cultivable Bacteria from Milk from Slovenian Breastfeeding Mothers*. *52*(2), 242–247.

Uda, K., Tsujikawa, T., Fujiyama, Y., & Bamba, T. (2003). Rapid absorption of luminal polyamines in a rat small intestine ex vivo model. *Journal of Gastroenterology and Hepatology*, *18*(5), 554–559. https://doi.org/10.1046/j.1440-1746.2003.03020.x

UN IGME. (2019). *Levels & Trends in Child Mortality: Report 2019, Estimates Developed by the UN Inter-agency Group for Child Mortality Estimation.* United Nations Children's Fund, New York.

UN IGME. (2021). *Levels & Trends in Child Mortality: Report 2021, Estimates Developed by the UN Inter-agency Group for Child Mortality Estimation.* United Nations Children's Fund, New York.

UN IGME. (2022). *Levels & Trends in Child Mortality: Report 2022, Estimates Developed by the UN Inter-agency Group for Child Mortality Estimation.* United Nations Children's Fund, New York.

Urbaniak, C., Angelini, M., Gloor, G. B., & Reid, G. (2016). Human milk microbiota profiles in relation to birthing method, gestation and infant gender. *Microbiome*, *4*, 1. https://doi.org/10.1186/s40168-015-0145-y

Urbaniak, C., Cummins, J., Brackstone, M., Macklaim, J. M., Gloor, G. B., Baban, C. K., Scott, L., O'Hanlon, D. M., Burton, J. P., Francis, K. P., Tangney, M., & Reid, G. (2014). Microbiota of human breast tissue. *Applied and Environmental Microbiology*, *80*(10), 3007–3014. https://doi.org/10.1128/AEM.00242-14

Usman, S., Zareen, A., Ali, M., Sarwar, H., Azjar, J., & Jamal, S. (2018). Probiotic Prophylaxis in Prevention of Necrotizing Enterocolitis—A Case Control Study. *Pakistan J. Med. Heal. Sci.*, *12*, 1623–1626.

Valik, Ľ., Medveďová, A., Čižniar, M., & Liptáková, D. (2013). Evaluation of temperature effect on growth rate of Lactobacillus rhamnosus GG in milk using secondary models. *Chemical Papers*, *67*(7). https://doi.org/10.2478/s11696-013-0365-1

van den Akker, C. H. P., van Goudoever, J. B., Shamir, R., Domellöf, M., Embleton, N. D., Hojsak, I., Lapillonne, A., Mihatsch, W. A., Berni Canani, R., Bronsky, J., Campoy, C., Fewtrell, M. S., Fidler Mis, N., Guarino, A., Hulst, J. M., Indrio, F., Kolaček, S., Orel, R., Vandenplas, Y., … Szajewska, H. (2020). Probiotics and Preterm Infants: A Position Paper by the European Society for Paediatric Gastroenterology Hepatology and Nutrition Committee on Nutrition and the European Society for Paediatric Gastroenterology Hepatology and Nutrition Working Group for Probiotics and Prebiotics. *Journal of Pediatric Gastroenterology and Nutrition*, *70*(5), 664–680. https://doi.org/10.1097/MPG.0000000000002655

van Wettere, W. H. E. J., Willson, N.-L., Pain, S. J., & Forder, R. E. A. (2016). Effect of oral polyamine supplementation pre-weaning on piglet growth and intestinal characteristics. *Animal*, *10*(10), 1655–1659. https://doi.org/10.1017/S1751731116000446

Vargas García, C. E., Petrova, M., Claes, I. J. J., De Boeck, I., Verhoeven, T. L. A., Dilissen, E., von Ossowski, I., Palva, A., Bullens, D. M., Vanderleyden, J., & Lebeer, S. (2015). Piliation of Lactobacillus rhamnosus GG Promotes Adhesion, Phagocytosis, and Cytokine Modulation in Macrophages. *Applied and Environmental Microbiology*, *81*(6), 2050–2062. https://doi.org/10.1128/AEM.03949-14

Vesterlund, S., Paltta, J., Karp, M., & Ouwehand, A. C. (2005). Measurement of bacterial adhesion—In vitro evaluation of different methods. *Journal of Microbiological Methods*, *60*(2), 225–233. https://doi.org/10.1016/j.mimet.2004.09.013

Viazis, S., Farkas, B. E., & Allen, J. C. (2007). Effects of High-Pressure Processing on Immunoglobulin A and Lysozyme Activity in Human Milk. *Journal of Human Lactation*, *23*(3), 253–261. https://doi.org/10.1177/0890334407303945

Villar, J., Giuliani, F., Figueras-Aloy, J., Barros, F., Bertino, E., Bhutta, Z. A., & Kennedy, S. H. (2019). Growth of preterm infants at the time of global obesity. *Archives of Disease in Childhood*, *104*(8), 725–727. https://doi.org/10.1136/archdischild-2018-315006

Vinderola, G., Binetti, A., Burns, P., & Reinheimer, J. (2011). Cell Viability and Functionality of Probiotic Bacteria in Dairy Products. *Frontiers in Microbiology*, *2*. https://doi.org/10.3389/fmicb.2011.00070

Viswanathan, S., Lau, C., Akbari, H., Hoyen, C., & Walsh, M. C. (2016). Survey and evidence based review of probiotics used in very low birth weight preterm infants within the United States. *Journal of Perinatology: Official Journal of the California Perinatal Association*, *36*(12), 1106–1111. https://doi.org/10.1038/jp.2016.144

Wan, Y., Jiang, J., Lu, M., Tong, W., Zhou, R., Li, J., Yuan, J., Wang, F., & Li, D. (2020). Human milk microbiota development during lactation and its relation to maternal geographic location and gestational hypertensive status. *Gut Microbes*, *11*(5), 1438–1449. https://doi.org/10.1080/19490976.2020.1760711

Wang, L., Cao, H., Liu, L., Wang, B., Walker, W. A., Acra, S. A., & Yan, F. (2014). Activation of epidermal growth factor receptor mediates mucin production stimulated by p40, a Lactobacillus rhamnosus GG-derived protein. *The Journal of Biological Chemistry*, *289*(29), 20234–20244. https://doi.org/10.1074/jbc.M114.553800

Wang, S., Egan, M., Ryan, C. A., Boyaval, P., Dempsey, E. M., Ross, R. P., & Stanton, C. (2020). A good start in life is important-perinatal factors dictate early microbiota development and longer term maturation. *FEMS Microbiology Reviews*, *44*(6), 763–781. https://doi.org/10.1093/femsre/fuaa030

Wang, X., Yan, X., Zhang, L., Cai, J., Zhou, Y., Liu, H., Hu, Y., Chen, W., Xu, S., Liu, P., Chen, T., Zhang, J., Cao, Y., Yu, Z., & Han, S. (2019). Identification and Peptidomic Profiling of Exosomes in Preterm Human Milk: Insights Into Necrotizing Enterocolitis Prevention. *Molecular Nutrition & Food Research*, *63*(13), 1801247. https://doi.org/10.1002/mnfr.201801247

Wang, X.-F., Tian, F., Cao, R.-M., Li, J., Wu, S.-M., Guo, X.-K., & Chen, T.-X. (2015). Antimicrobial activity of human β-defensins against lactic acid bacteria. *Natural Product Research*, *29*(22), 2164–2166. https://doi.org/10.1080/14786419.2014.996754

Wang, Z., Neupane, A., Vo, R., White, J., Wang, X., & Marzano, S.-Y. L. (2020). Comparing Gut Microbiome in Mothers' Own Breast Milk- and Formula-Fed Moderate-Late Preterm Infants. *Frontiers in Microbiology*, *11*, 891. https://doi.org/10.3389/fmicb.2020.00891

Warshakoon, H. J., Hood, J. D., Kimbrell, M. R., Malladi, S., Wu, W. Y., Shukla, N. M., Agnihotri, G., Sil, D., & David, S. A. (2009). Potential adjuvantic properties of innate immune stimuli. *Human Vaccines*, *5*(6), 381–394.

Watkins, C., Murphy, K., Dempsey, E. M., Murphy, B. P., O'Toole, P. W., Paul Ross, R., Stanton, C., & Anthony Ryan, C. (2018). The viability of probiotics in water, breast milk, and infant formula. *European Journal of Pediatrics*, *177*(6), 867–870. https://doi.org/10.1007/s00431-018-3133-y

Wei, W., Jin, Q., & Wang, X. (2019). Human milk fat substitutes: Past achievements and current trends. *Progress in Lipid Research*, *74*, 69–86. https://doi.org/10.1016/j.plipres.2019.02.001

Williams, J. E., Carrothers, J. M., Lackey, K. A., Beatty, N. F., York, M. A., Brooker, S. L., Shafii, B., Price, W. J., Settles, M. L., McGuire, M. A., & McGuire, M. K. (2017). Human Milk Microbial Community Structure Is Relatively Stable and Related to Variations in Macronutrient and Micronutrient Intakes in Healthy Lactating Women. *The Journal of Nutrition*, *147*(9), 1739–1748. https://doi.org/10.3945/jn.117.248864

Williams, J. E., Price, W. J., Shafii, B., Yahvah, K. M., Bode, L., McGuire, M. A., & McGuire, M. K. (2017). Relationships Among Microbial Communities, Maternal Cells, Oligosaccharides, and Macronutrients in Human Milk. *Journal of Human Lactation*, *33*(3), 540–551. https://doi.org/10.1177/0890334417709433

Wills, M. E., Han, V. E., Harris, D. A., & Baum, J. D. (1982). Short-time low-temperature pasteurisation of human milk. *Early Human Development*, *7*(1), 71–80.

Wise, A., Robertson, B., Choudhury, B., Rautava, S., Isolauri, E., Salminen, S., & Bode, L. (2018). Infants Are Exposed to Human Milk Oligosaccharides Already in utero. *Frontiers in Pediatrics*, *6*, 270. https://doi.org/10.3389/fped.2018.00270

Xiang, Q., Yan, X., Shi, W., Li, H., & Zhou, K. (2023). Early gut microbiota intervention in premature infants: Application perspectives. *Journal of Advanced Research*, *51*, 59–72. https://doi.org/10.1016/j.jare.2022.11.004

Yan, W., Luo, B., Zhang, X., Ni, Y., & Tian, F. (2021). Association and Occurrence of Bifidobacterial Phylotypes Between Breast Milk and Fecal Microbiomes in Mother–Infant Dyads During the First 2 Years of Life. *Frontiers in Microbiology*, *12*, 1323. https://doi.org/10.3389/fmicb.2021.669442

Yang, R., Chen, D., Deng, Q., & Xu, X. (2020). The effect of donor human milk on the length of hospital stay in very low birthweight infants: A systematic review and meta-analysis. *International Breastfeeding Journal*, *15*(1), 89. https://doi.org/10.1186/s13006-020-00332-6

Yazji, I., Sodhi, C. P., Lee, E. K., Good, M., Egan, C. E., Afrazi, A., Neal, M. D., Jia, H., Lin, J., Ma, C., Branca, M. F., Prindle, T., Richardson, W. M., Ozolek, J., Billiar, T. R., Binion, D. G., Gladwin,

M. T., & Hackam, D. J. (2013). Endothelial TLR4 activation impairs intestinal microcirculatory perfusion in necrotizing enterocolitis via eNOS–NO–nitrite signaling. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(23), 9451–9456. https://doi.org/10.1073/pnas.1219997110

Yi, D. Y., & Kim, S. Y. (2021). Human Breast Milk Composition and Function in Human Health: From Nutritional Components to Microbiome and MicroRNAs. *Nutrients*, *13*(9), Article 9. https://doi.org/10.3390/nu13093094

Young, G., Turner, S., Davies, J. K., Sundqvist, G., & Figdor, D. (2007). Bacterial DNA Persists for Extended Periods after Cell Death. *Journal of Endodontics*, *33*(12), 1417–1420. https://doi.org/10.1016/j.joen.2007.09.002

Yousuf, E. I., Carvalho, M., Dizzell, S. E., Kim, S., Gunn, E., Twiss, J., Giglia, L., Stuart, C., Hutton, E. K., Morrison, K. M., & Stearns, J. C. (2020). Persistence of Suspected Probiotic Organisms in Preterm Infant Gut Microbiota Weeks After Probiotic Supplementation in the NICU. *Frontiers in Microbiology*, *0*. https://doi.org/10.3389/fmicb.2020.574137

Zanella, A., Silveira, R. C., Roesch, L. F. W., Corso, A. L., Dobbler, P. T., Mai, V., & Procianoy, R. S. (2019). Influence of own mother's milk and different proportions of formula on intestinal microbiota of very preterm newborns. *PLoS ONE*, *14*(5), e0217296. https://doi.org/10.1371/journal.pone.0217296

Zbinden, A., Zbinden, R., Berger, C., & Arlettaz, R. (2015). Case Series of Bifidobacterium longum Bacteremia in Three Preterm Infants on Probiotic Therapy. *Neonatology*, *107*(1), 56–59. https://doi.org/10.1159/000367985

Zimmermann, P., & Curtis, N. (2020). Breast milk microbiota: A review of the factors that influence composition. *Journal of Infection*. https://doi.org/10.1016/j.jinf.2020.01.023

Basdeki, A.-M., Fatouros, D. G., Biliaderis, C. G., & Moschakis, T. (2021). Physicochemical properties of human breast milk during the second year of lactation. *Current Research in Food Science*, *4*, 565–576. https://doi.org/10.1016/j.crfs.2021.08.001

TURUN
YLIOPISTO
UNIVERSITY
OF TURKU

Painosalama, Turku, Finland 2024

ISBN 978-951-29-9674-2 (PRINT)
ISBN 978-951-29-9675-9 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)