PhD thesis

Analysis of the microbiota of human milk and baby faeces in Nigeria, using molecular and culture-based approaches

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Analysis of the microbiota of human milk and baby faeces in Nigeria, using molecular and culture-based approaches

A thesis submitted to Middlesex University in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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Abstract

Human breast milk had been traditionally considered sterile. In recent years, studies have suggested that human milk carries bacteria that may help babies build up a beneficial population of bacteria in their gut, which plays a protective role. This research is the first in Nigeria to explore the bacterial diversity in breast milk and compares it with the gut of breastfed babies in addition to looking at the gut microbiota of formula-fed babies. Nigeria is a developing country and owing to its unique characteristics; it becomes important to investigate the presence of these bacteria in breast milk and have coherent data on the type and diversity of the bacteria in breast milk and babies' guts. This may help to build up more awareness about the importance of breastfeeding and its role in the initiation of infant gut microbiota including its importance in the modulation of the infant immune system in addition to other nutritional benefits.

To achieve this goal, pilot research was undertaken in the UK using milk samples from seven mothers and faeces from their breastfed babies to allow for the optimization of the methodology starting from sample collection. The presence of five bacterial genera, including bifidobacteria, lactobacilli, streptococci, staphylococci, and enterococci, in the breast milk and faeces, was investigated using a traditional culture approach followed by species identification by MALDI-TOF Biotyper, as well as a culture-independent method by extracting total microbial DNA from these samples and then using qPCR. Following that, samples of breast milk from 50 breastfeeding mothers in Nigeria, as well as the faeces of their babies, and the faeces of 8 solely formula-fed babies, were collected and analysed using culture technique and 16S ribosomal ribonucleic acid (16S rDNA) NGS sequencing (MiSeq Illumina).

Human milk has a highly personalised microbiota with a lot of inter-individual variabilities, according to the present study. It was revealed that the milk microbiota of mothers from Nigeria is characterised by the high dominance of phylum Firmicutes (61%) mainly represented by the

orders Lactobacillales and Bacillales next to the phylum Actinobacteria (26%) largely represented by Micrococcales and Corynebacteriales and then Proteobacteria (10.5%) represented by Caulobacterales and Pseudomonadales. In the faeces of breastfed babies, there was high dominance of members of Actinobacteria (62.6%), Proteobacteria (24%), and Firmicutes (11.6%) represented by bifidobacteria, *Escherichia*/*Shigella* as well as streptococci respectively. Within a sample diversity (i.e., alpha diversity) revealed that the milk of Nigerian mothers had higher observed bacterial richness and diversity for a single sample when compared to the gut of breastfed babies. Beta diversity also revealed that human milk and baby faeces had obvious differences, but it is predicted that about 51% of bacteria in baby faeces may have originated from human milk as revealed by source tracker analysis. Furthermore, it was revealed that breastfed babies had lower microbial diversity, but a higher abundance of certain bacteria such as bifidobacteria in their gut compared to formula-fed babies. *Faecalibacterium* was also exclusively found in the gut of formula-fed babies. The delivery mode revealed an association with gut microbiota, while parity revealed an association with mother's milk; for example, babies born by C-section had a higher abundance of *Klebsiella* in their gut compared to babies born naturally via the vagina, while multiparous mothers had a higher abundance of *Brevundimonas* in their milk.

This study, in addition to providing an overview of the microbiota found in mother's milk and babies' faeces in Nigeria, provides evidence that mothers can transmit bacteria to their breastfed babies via breastmilk; that babies' gut microbial composition varied depending on the type of food they received; and that some maternal or baby factors may influence maternal milk or gut microbiota.

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List of Abbreviations

16S rDNA	16S ribosomal deoxyribonucleic acid
BH	Benjamini-Hochberg procedure
BIM-25	*Bifidobacterium* Iodoacetate Medium 25
CFU	Colony Forming Units
CS	Caesarean Section
DNA	Deoxyribonucleic acid
FDR	False Discovery Rate
GE	Genome Equivalents
HCCA	α-Cyano-4-hydroxycinnamic acid
MALDI-TOF MS	Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry
NCBI	National Center for Biotechnology Information
NCIMB	National Collection of Industrial, Food and Marine Bacteria
NCTC	The National Collection of Type Cultures
NGS	Next Generation Sequencing
OTU	Operational Taxonomic Unit
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
QIIME	Quantitative Insights into Microbial Ecology
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
SB	Slanetz and Bartley
SD	Standard Deviation
sIgA	Secretary immunoglobulin A

TE	Tris- ethylenediaminetetraacetic acid

Changes to the *Lactobacillus* Taxonomy

Since March 2020, the genus *Lactobacillus* has changed (Zheng et al., 2020). While some genera still retain their old names, some have acquired new names. The old names of *Lactobacillus* species included in this thesis are summarized below with the old names on the left and new names on the right. A software called lactotax has also been designed by the University of Antwerpen to track the names and can be found at http://www.lactobacillus.uantwerpen.be

L*actobacillus casei* - *Lacticaseibacillus casei*

Lactobacillus paracasei - *Lacticaseibacillus paracasei*

Lactobacillus acidophilus - Unchanged

Lactobacillus fermentum - *Limosilactobacillus fermentum*

Lactobacillus gasseri- Unchanged

Lactobacillus salivarius- *Ligilactobacillus salivarius*

Lactobacillus rhamnosus - *Lacticaseibacillus rhamnosus*

Lactobacillus zeae- *Lacticaseibacillus zeae*

Lactobacillus brevis- *Levilactobacillus brevis*

Lactobacillus plantarum- *Lactiplantibacillus plantarum*

Lactobacillus reuteri- *Limosilactobacillus reuteri*

Lactobacillus delbrueckii subsp. Bulgaricus- Unchanged

Lactobacillus crispatus - Unchanged

Lactobacillus johnsonii- Unchanged

Lactobacillus helveticus - Unchanged

Lactobacillus curvatus- *Latilactobacillus curvatus*

Chapter 1

1.1 Introduction

Human milk is produced from the mammary glands of females and serves as a source of nutrition for the newborn. The production of milk after childbirth is usually influenced by two hormones which are: prolactin (stimulated by the hypothalamus of the brain and triggers the alveoli to make milk) and oxytocin which is responsible for flowing down the milk that has been collected by the alveoli through the duct.

1.2 The production of human milk

The onset of milk secretion also known as lactogenesis involves changes that occur during the early stage of pregnancy, which involves a transformation from an undifferentiated mammary gland to full lactation after birth (Neville et al., 2001; Wagner, 2015). This process is divided into two major stages (Lactogenesis I and II) (Figure 1.1). The first stage normally occurs in the middle of pregnancy, and this is often detected by an increase in the plasma concentration of lactose and α-lactalbumin (Neville et al., 2001; Wagner, 2015). On completion of stage 1, the glands are sufficiently differentiated to secrete, and the resulting product of secretion is what is known as colostrum, which is excreted from the breast of some pregnant women during the late stage of pregnancy (Neville et al., 2001). Stage II of lactogenesis occurs after birth and it involves the production of abundant milk occurring approximately between 32 to 96 hours after delivery (Wagner, 2015).

1.2.1 Colostrum

Colostrum is regarded as the foremilk. It is normally secreted by mothers after birth. Colostrum is rich in antibodies, high in protein and carbohydrate, low in fat and contains more lactalbumin and lactoprotein and it is known for conferring passive immunity in newborn babies (Godhia & Patel, 2013). Due to its laxative effect, it aids in the passing of early stools (meconium) in

newborn and hence aid in the excretion of excess bilirubin that helps in the prevention of jaundice (Godhia & Patel, 2013).

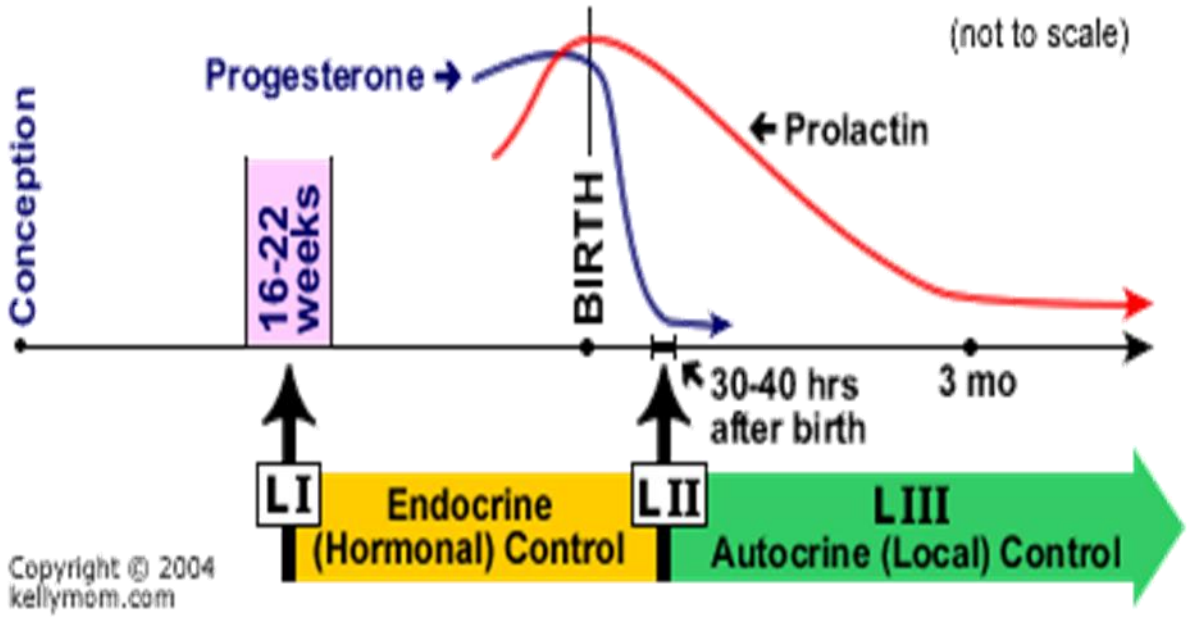


Figure 1.1: Stages of Lactogenesis. LI, LII, LIII-Lactogenesis 1, 2, and 3 respectively Source:(Boyanta, 2018).

1.3 Components of human breast milk

1.3.1 Nutritional components

Proteins

Proteins make up about 75% of the nitrogen compound in breast milk (approximately 0.9 to 1.2 g/dL for term milk). The major proteins of milk are caseins and whey proteins present in a ratio of 40:60 (Ballard & Morrow, 2013; Gao et al., 2012). B-casein is a predominant form of casein which produces a soft and flocculent curd in infants' stomachs, while whey proteins are mostly made up of secretory immunoglobulin A (which is the principal immunoglobulin

present in human breast milk), α-lactalbumin, serum albumin, lysozyme and lactoferrin (Lönnerdal, 2013).

Fats

A high percentage of the fat in breast milk about 98% is in form of triglycerides. The most abundant fatty acid in human breast milk triglyceride is palmitic and oleic acids which are heavily located in position 2 and positions 1 and 3 of the triglyceride respectively. They contain a high amount of linoleic and linolenic fatty acids which are essential fatty acids. The fat content of milk is highly variable, and the last milk of a feed has been suggested to contain about two to three times more than the concentration of milk fat which is found in the initial milk (fore milk) (Ballard & Morrow, 2013; Saarela et al., 2005)

Lactose

Lactose, a disaccharide, is the main sugar in human milk. Lactose concentration in human milk is the least variable macronutrient among mothers, however higher lactose concentrations are found in the milk of women who produce more milk. Other major carbohydrates in human milk are oligosaccharides, which account for around 0.1 g/L in human milk depending on the lactation stage and maternal genetic factors. Other micronutrients such as vitamins A, B1, B2, B12 and iodine are also present in breast milk, and this varies depending on the diet of the mother as well as the body. All these components serve as a source of nutrition for breastfed babies, as well as facilitate the development of their physiologic functions (Ballard & Morrow, 2013).

1.3.2 Immunological components of human breast milk

Human breast milk has also been shown to contain immunological factors such as antibodies, components of the complement system, enzymes and carrier proteins, which serve to protect

neonates against pathogenic organisms (Ruiz et al., 2017). In mucosal surfaces, secretory immunoglobulin A (IgA) is the most common immunoglobulin isotype. Two IgA molecules (dimeric IgA), a joining protein (J chain), and a secretory component make up secretory IgA (Smith et al., 2019). Human breast milk contains secretory Immunoglobulin A (sIgA) which is the most abundant immunoglobulin present in the colostrum. The resistance of immunoglobulin A to pepsin, trypsin and hydrolysis by the gastric acid is a result of the secretory component of sIgA (Araújo et al., 2005).

The concentration of sIgA increases mostly in the first 3-4 postpartum days and declines as the volume of milk increases. This protects neonates against infections caused by some respiratory and gastrointestinal pathogens such as *Vibrio cholerae*, and *Campylobacter (*Le Doare & Kampmann, 2014). IgM and IgG also play important roles. These antibodies can bind complements, thereby opsonise any pathogen which is coated by the antibodies (Brandtzaeg, 2013).

Lactoferrin is another immunological component which is an iron-binding protein. Lactoferrin is bacteriostatic and deprives pathogens of iron. It also blocks the metabolism of carbohydrates in pathogens and attacks their cell wall. Organisms which are commonly inhibited by lactoferrin include those that have high iron requirements, such as yeasts and coliform (Jahani et al., 2015).

Lastly, milk macrophages often synthesize lysozyme, a protein enzyme with physiological and functional features. Lysozyme is also a powerful microbicide, and its concentration rises over time during lactation. Furthermore, lysozyme lyses bacteria's cell walls and, in some cases, works in tandem with lactoferrin and secretory immunoglobulin A to provide antibacterial protection (Primo et al., 2018).

1.3.3 Bacteria in human milk

Human milk has for a long time been considered the best food for neonates and this milk was traditionally considered sterile. However, recent studies have shown that breast milk does not only serve as a source of nutrition for the infant but also contains bacteria that may aid in the establishment of a healthier gut in breastfed babies (Chehab et al., 2021; Corona-Cervantes et al., 2020; Yuan et al., 2022; Wallenborn et al., 2022). Examples of these bacteria are lactic acid bacteria, *Bifidobacterium* spp, *Streptococcus* spp, and *Staphylococcus* spp. (Yuan et al., 2022; Oikonomou et al., 2020; Chen et al., 2018; Murphy et al., 2017; Martín et al., 2016).

1.3.3.1 Prebiotics and Probiotics

Prebiotics are a group of nutrients which are degraded by certain beneficial bacteria in the gut hence improving the health of the host. The presence of prebiotics leads to the stimulation of the growth of these beneficial bacteria and as a result, modifies the function and composition of the gut microbiota (Ali & Nizar, 2018). Prebiotics are present naturally in some foods such as beans, onions, tomatoes, soybeans, cow and human milk to mention a few, but their quantity is low (Davani-Davari et al., 2019). Other prebiotics can also be commercially produced. Examples are compounds such as fructooligosaccharide and galactooligosaccharides. Galactooligosaccharides for example was reported to influence species like *Lactobacillus* and *Bifidobacterium* in the gut (Louise et al., 2016).

Probiotics as defined by FAO/WHO as living bacteria that can confer beneficial properties when adequately administered (Morelli and Capurso, 2012). These bacteria have been reported to help play a role in modulating the immune system as well as maintaining gut homeostasis and preventing the growth of pathogenic bacteria (Behnsen et al., 2013). Examples of probiotic bacteria with health benefits are species of *Lactobacillus* such as *Lactobacillus acidophilus,*

Lactobacillus casei and *Lactobacillus paracasei* (Farahmand et al., 2021) and species of bifidobacteria such as *Bifidobacterium animalis* ssp. lactis (Raeisi et al., 2013).

Several criteria and qualities are evaluated when creating probiotic products, including their ability to withstand harsh environmental conditions, acid and bile salt tolerance for gastrointestinal passage, and the absence of antibiotic-resistance genes or virulence factors to prevent these genes from being transferred to pathogenic bacteria (Kesen and Aiyegoro, 2018). It is also preferable for probiotic bacteria to have a negative impact on enteric microorganisms, possibly through the production of antimicrobial substances such as bacteriocins or through competitive exclusion (Kim et al., 2019; Kesen and Aiyegoro, 2018). Other criteria include the ability to adhere to the intestinal mucosa wall and the ability to produce extracellular enzymes which are important in digestion processes (Kesen and Aiyegoro, 2018).

It has been revealed that colostrum and breast milk may be sources of commensal and probiotic bacteria to the infant's gut (Fernández et al., 2013; Kordy et al., 2020). For example, a human milk strain of *Lactobacillus (Lactobacillus fermentum* CECT5716) was discovered to have probiotic properties (Jiménez et al., 2010) that may help to lower the frequency of illnesses like diarrhoea in breastfed babies (Rodríguez-Sojo and Ruiz-Malagón, 2021).

Moreover, certain factors such as diet (Albesharat et al., 2011; Williams et al., 2017), geographic location (Ding et al., 2019; Drago et al., 2017), and genetic characteristics have been reported to alter the microbiota of human milk (Gomez-Gallego et al., 2016). It is also hypothesized that the current research area's multiple economic and environmental sanitation issues (covered further in section 2.9 of this thesis) may have an impact on the microbiota of mothers' milk as well as the gut microbiota of babies.

1.4 Rationale and justification of the present study

The study of the human milk microbiome is important because human milk plays a major role in the composition of breastfed babies' gut microbiome (Sindi et al., 2021), and indeed the gut microbiome is important because it has been reported to play a role in health and disease, with an unbalanced gut microbiome being associated with various diseases such as allergy in childhood and inflammatory bowel disease in adulthood (Tanaka et al., 2017).

Studies have been carried out in different parts of the world investigating the bacterial diversity in human milk and the faeces of breastfed babies. Among them are in Spain (Maldonado et al., 2012), across Europe (Fallani et al., 2010), the United States (Hunt et al., 2011), the Middle East (Mehanna et al., 2013), and South Africa (Wallenborn et al., 2022). Studies on human milk bacteria in the Western part of Africa are limited and no research has been carried out in Nigeria investigating the bacterial diversity in breast milk and comparing it with that of faeces of breastfed and formula-fed babies up to this moment. Nigeria is a developing country, and owing to its tropical characteristics, socio-economic status, culture and lifestyle of residents; it becomes important to have coherent data on the type and diversity of bacteria in breast milk and gut of Nigerian babies, as some of these factors may influence the microbiome of mothers and babies in Nigeria (Gomez-Gallego et al., 2016; Kashtanova et al., 2016; Ojo-Okunola et al., 2018).

The socioeconomic status of mothers (collected by using a questionnaire) as used in the context of this research carried out on Nigerian mothers are the level of education of mothers, their occupation, their economic status whether low, medium or high as determined by the level of their income, the number of their children and the kind of area they live such as rural or urban area.

In addition, it has been reported that geographic location influences the composition of the milk microbiome (Lackey et al., 2019; Gay et al., 2018), with several studies conducted in various

parts of the world yielding disparate results. It is critical to have reliable data that spans multiple geographic locations to better understand the core microbiome in human milk. As a result, this study is required to add to the data already available on human milk and gut microbiome by analysing samples from Nigerian participants using Next generation sequencing (16S sequencing), which can create a comprehensive picture of the presence of bacteria in breast milk and gut of babies when compared to culture-dependent techniques (Martín et al., 2003) or targeted sequencing which limits population diversity and the number of identified bacteria (Collado et al., 2009; Jost et al., 2013; Martín et al., 2012; Solís et al., 2010).

1.5 Research aims

This research was carried out to investigate the presence of bacteria in the breast milk of Nigerian mothers and the relationship between the mode of feeding and bacteria present in the gut of babies. In addition to this, the effect that diet, age and socio-economic status of participants may have on the microbiota of breast milk and faeces was investigated.

1.6 Objectives

i. To review recent literature in the field of milk microbiome, provide insight into the methodologies used to analyse human milk, including sample collection and microbiome analysis, and make some recommendations.

ii. To select the appropriate experimental groups.

iii. To conduct a pilot study to optimise methods of identification of bacteria from milk and faeces such as culture-dependent methods and culture-independent methods.

iv. To identify bacterial population and diversity in breast milk and faeces using both culture-dependent and culture-independent methods such as qPCR and 16S rDNA sequencing in samples collected in Nigeria.

v. To study the possible relationship between human milk microbiota and gut microbiota of breastfed babies.

vi. To compare the gut microbiome of breastfed babies to that of formula-fed babies.

vii. To investigate the possible relationship between the microbiota of breast milk as well as faeces and the mother's diet, lifestyle, age and socio-economic status using the questionnaire.

1.7 Scheme of work for the study

This study was divided into two parts. The first part was the pilot/preliminary studies carried out in the UK on 7 mothers (breast milk samples) and their babies (faecal samples). Culture and identification by Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry (MALDI-TOF MS) Biotyper was used alongside quantitative polymerase chain reaction to quantify and identify the bacteria present in the samples.

The second part of the study was carried out both in Nigeria and in the UK. Samples were obtained from mothers and babies in Nigeria and a questionnaire was also administered. Analysis of the samples including data interpretation was carried out in the UK. Culture and MALDI-TOF MS Biotyper were used alongside 16S rDNA NGS analysis to identify the bacteria present in the samples. Details of this can be found in Chapter 3.

1.8 Structure of thesis

Chapter 2: Literature Review

This chapter provides a review of the literature on the nature of bacteria in human milk, the types of bacteria which has been reported to be associated with human milk including their possible sources and the impact they have on babies who are breastfed as compared to babies who feed on formula. The factors that may influence breast milk composition as well as methods which have been used to investigate the bacteria in human milk or faeces of babies.

Chapter 3: Methodology

This chapter provides an overview of the methods used in the study. This includes microbiology culture, MALDI-TOF, qPCR (used for the pilot study carried out in the UK to optimise the methods used in the larger study) as well as 16S Next Generation Sequencing.

Chapter 4: Results of Pilot study

This chapter provides an overview of the results of the pilot. It discusses the type of bacteria isolated/detected from breast milk and faeces of mother-baby pairs in the UK. It also compares the microbiology culture method and the molecular qPCR technique that was used to carry out the pilot study

Chapter 5: Results obtained from samples collected from Nigerian participants

This chapter described the results of fieldwork analysis and focuses mainly on the isolation and detection of bacteria in milk and faeces of fed babies in Nigeria using the traditional culture approach and identification by MALDI-TOF MS as well as 16S rDNA Next Generation Sequencing approach. The analysis of the questionnaire to determine the association between the socioeconomic factors and microbiota of mother's milk and baby faeces was also included in this chapter

Chapter 6: Discussion

This chapter discusses the study's main findings. It evaluates the different methods used in the study. The relationship between the bacteria found in the mother's milk and the faeces of babies was also described here, as well as how the microbiota of babies who are fed with breast milk and those that are fed with formula differ. Furthermore, the chapter evaluates the questionnaire in relation to the microbiota of mothers and babies.

Finally, chapter 7 concludes the thesis by summarising the research findings, limitations, recommendations and future reviews.

Chapter 2 : Literature review

2.1 Background on Microbiome Research

The term "microbiota" refers to the collection of live microorganisms found in a specific habitat, while microbiome refers to the collection of the genome of these microbes within their environment (Wang et al., 2017). In recent decades, microbiome research has advanced rapidly and has become a popular topic among scientists and the general public (Berg et al., 2020).

In 2007, the Human Microbiome Project, an international initiative was created. The goal of the study was to use whole-genome sequencing to characterize the microorganisms and discover their roles in the human body. During the first three years of the initiative, nearly 200 microbial species were identified for the first time and since then, more microbiota species and their significance in human health are being identified at an increasing rate, and understanding of microbiota is rapidly expanding (Lu, 2020).

There are trillions of microorganisms in the human body that have direct and indirect effects on health and disease. The presence of microorganisms has also been discovered in the precolostrum as reported in the study of Ruiz et al. (2019) who investigated the microbiota in precolostrum of pregnant women in Spain. Human milk microorganisms have evolved as essential bioactive components. Microorganisms were first discovered in milk during research into the possible transmission of pathogens via breastfeeding (Eidelman & Szilagyi, 1979). Several investigations later revealed the existence of live commensal, mutualistic, or possibly probiotic bacteria in the milk of healthy mothers (Fernández et al., 2013; Ruiz et al., 2019), reigniting interest in the microbiota of human milk and its role in maternal-neonate health.

2.2 Possible origin of bacteria in human milk

To date, the origin of bacteria in human milk is yet to be established. It was thought in the past to be a result of contamination introduced due to the contact between the mother’s breast skin and baby during breastfeeding (West et al., 1979), although some studies have shown that

bacteria isolated from the breast skin of breastfeeding mothers differ from those isolated from the breast milk of same mothers (Ballard & Morrow, 2013; Martín et al., 2009)

Recently, two possible origins of human milk bacteria have been hypothesized (Figure 2.1): the retrograde transfer of bacteria from external sources such as the baby's oral cavity and mother's skin and an entero-mammary pathway of internal bacteria from the mother's gut to the mammary gland (Fernández et al., 2013).

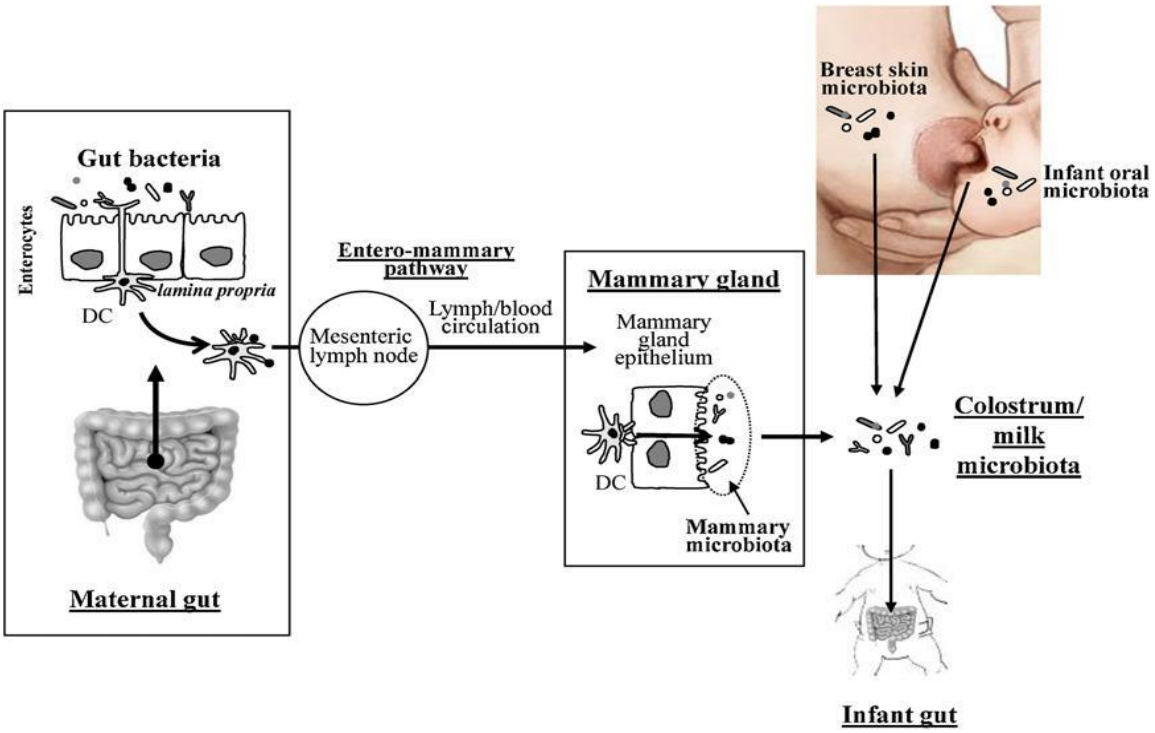


Figure 2.1: Suggested origin of bacteria in human breast milk. Maternal gut, infant gut, infant oral cavity (Source: (Fernández et al., 2013).

The retrograde transfer of bacteria from external sources involves the introduction of bacteria into the mammary gland from the areola skin of mothers as well as the transfer of bacteria from the baby's oral cavity to the mammary gland during suckling. It was reported that during breastfeeding, there could be a flow back of breast milk through the mammalian duct and this could take along some bacteria from the baby's mouth back to the mammary gland (Ramsay et al., 2004), although further studies proved the presence of bacteria in breast milk before the baby starts feeding (Damaceno et al., 2017).

Similarly, to the transmission of bacteria from the baby's oral cavity to the mammary gland during suckling, the possibility of bacteria transferring from the breast pump into the milk duct during pumping has recently been documented (Moossavi & Azad, 2020).

The entero-mammary pathway involves bacteria being transferred from a breastfeeding mother's gut to her mammary gland by immune cells. Dendritic cells and macrophages are immune cells that can carry bacteria and deposit them in lymph nodes throughout the body via the lymphatic system. It has been suggested that during the late stages of pregnancy, dendritic cells and macrophages may take up bacteria from mothers' guts and transport them to the mammary gland via the lymphatic system (Figure 2.1), implying that bacteria in breast milk may have originated from the mothers' gut (Perez et al., 2007; Rodríguez et al., 2015).

More recently, Moossavi and Azad (2020) proposed a slightly modified way for the possible origin of bacteria in human milk which was called ''The oro/entero-mammary pathway'' involving the translocation of both the bacteria from the mother's gut as well as oral cavity to the mammary gland, however, this research is still ongoing and needs further investigation.

2.3 Breast milk as a source of bacteria to breastfed baby's gut

The gastrointestinal tract is a complex system which consists of several different types of microorganisms, mostly bacteria, and they influence the growth as well as the differentiation

of gut epithelial. They also play role in immunological, metabolic and protective functions (Lyons et al., 2020; Fitzsteven et al., 2017).

The intestine of a foetus is normally sterile and contains amniotic fluid (Guaraldi & Salvatori, 2012; Rodríguez et al., 2015). According to Arumugan et al. (2011), the microbiota of the infant's gut is established as a result of a combination of internal and environmental stimuli that begins at birth and continues over time. Yao et al. (2020) investigated the role of microbiota on infant health and reported that internal factors such as breast milk for breastfed babies and mothers' vaginal and intestinal microbiota could influence infant microbiota in early life. Additionally, external factors which could influence infant microbiota include delivery equipment, air, and staff.

Breast milk serves as a source of bacteria to the infant's gut (Kordy et al., 2020; Boudry et al., 2021). Baumann-Dudenhoeffer et al. (2018) in their study of the faecal metagenome of 60 infants found that the makeup of the gut microbiome of breastfed babies is closely tied to the microbiome of their mother's breast milk, while the presence of a more diverse group or organism coincides with the period of weaning.

Numerous studies have shown that there is a mother-to-infant transfer of bacterial strains, and that human breast milk is a source of live bifidobacteria among several hundreds of bacterial phylotypes, to the gut of infants (Kordy et al., 2020; Yuan et al., 2022; Martín et al., 2012).

Some of the benefits of breast milk bacteria to the infant include the promotion of the early establishment of bacteria in the gut of infants and the enhancement of the maturation of the immune system (Toscano et al., 2017). It has also been reported that breast milk bacteria protect the gut of infants against illnesses such as diarrhoea by increasing mucin production while reducing intestinal permeability, thus improving the intestinal barrier function (Martín, et al., 2006; Martín, et al., 2005; Olivares et al., 2006).

Studies have also shown that some of the bacteria present in human breast milk such as some strains of lactic acid bacteria if transferred to the gastrointestinal tract of infants can act by competitive exclusion or by the production of certain antimicrobial compounds (bacteriocins and hydrogen peroxide) and thus inhibit the growth of many pathogenic bacteria (Beasley and Saris, 2004). A study carried out by Maldonado et al. (2012) has shown a reduction in the incidence of gastrointestinal infections and upper respiratory tract infections in infants who were administered a human milk strain of *Lactobacillus* (*Lactobacillus fermentum CECT5716).* Another study reported that *Lactobacillus gasseri* CECT 5714 which is a human milk strain has the potential to reduce the incidence as well as the severity of allergy if passed to the gut of babies via breastfeeding, as it has been shown to reduce the incidence of allergic response in an animal model of cow's milk protein allergy (Olivares et al., 2006). Interestingly, it has also been noted that viridans streptococci, which has been isolated from human milk are associated with the gut of healthy infants in contrast to the gut of infants suffering from atopy (Kirjavainen et al., 2001)

2.4 Overall benefits of breastfeeding

In addition to the benefits an infant derives from the breast milk of the mother, many studies have reported the beneficial effects the act of breastfeeding has on breastfeeding mothers. Some of the benefits include; decreased risk of breast cancer and ovarian cancer (Gaitskell et al., 2015). It may also encourage weight loss after pregnancy and an early return to pre-pregnancy weight (Jarlenski et al., 2014). Chua et al. (1994) also reported that breastfeeding may reduce post-partum bleeding and enhance the rapid return of the uterus to its pre-pregnancy size, which is attributed to the increase in the production of oxytocin. Certain women also use exclusive breastfeeding as a form of contraception to increase child spacing within the first 6 months post-delivery to prevent the return of menstrual blood (Sridhar & Salcedo, 2017).

Some of the social and economic benefits of breastfeeding in society include a decrease in the total annual healthcare cost (Quesada et al., 2020; Weimer, 2001) as it has been shown to reduce the risk of many types of disease and ailments in both the mother and the child. It has also been reported to reduce the burden on the environment because the disposal of formula bottles and cans will be reduced to a minimal level, as well as the reduction in the demand for the production and transport of artificial feeding formulas (Quesada et al., 2020).

It has been found that babies who are breastfed have slightly enhanced cognitive development (neurodevelopment) when compared to those that were not, as analysed in the research of Pereyra-Elías et al. (2022) on 7,855 infants. Furthermore, breastfeeding has for a long time served as a form of analgesia for babies when undergoing a painful procedure as this reduces the pain associated with the procedure possibly due to sucking, skin-to-skin contact, warmth, and the mother's sound and smell (Harrison et al., 2016; Carbajal et al., 2003; Gray et al., 2002).

2.5 Composition of bacteria in the gut of breastfed babies vs gut of babies fed with milk formula

In contrast to babies who are fed with breast milk, studies have shown that infants who are fed with formula supplements tend to show slight variations in the composition of their gut microbiota (Ma et al., 2020; Kashtanova et al., 2016).

Ma et al. (2020) reported a significantly higher number of *Bifidobacterium* and *Bacteroides* and a lower number of *Enterococcus* and *Streptococcus* in breastfed babies as compared to infants who were fed with formula.

It was also revealed in a study carried out by Favier et al. (2002) that the gut microbiota of breastfed babies generally contains a higher amount of bifidobacteria and ruminococci while the gut of babies fed with formula is enriched with bacteria such as *Bacteroides, Klebsiella* and lower levels of bifidobacteria (Kashtanova et al., 2016).

Another study reported that the gut of breastfed babies is dominated by bacterial genera such as *Propionibacterium*, *Staphylococcus*, *Gemella* and *Corynebacterium*, while the gut of infants fed with formula is associated more with bacteria such as *Bifidobacterium dentium*, *Enterococcus* and *Clostridium difficile (*Timmerman et al., 2017).

2.6 Factors that may influence the composition of bacteria in human milk

Factors that have been reported to influence the composition of human milk include nutrition, geographic region, lactation stage and genetic factors (Gomez-Gallego et al., 2016). Similarly, factors which may influence the microbiome of human milk include the lactation period (Hunt et al., 2011), the mother's diet (Albesharat et al., 2011; Williams et al., 2017), Body Mass Index (Moossavi et al., 2019) geographic location (Ding et al., 2019; Drago et al., 2017; Jiménez, et al., 2015; Olivares et al., 2015; Quinn et al., 2012), mode of delivery (Cabrera-Rubio, et al., 2016; Moossavi et al., 2019) and antibiotic usage (Soto et al., 2014).

Recently, breastfeeding practice has been reported to be one of the factors that may influence the composition of human milk. Indirect breastfeeding using a breast pump rather than infant feeding directly from the nipple has been reported to significantly influence the milk microbiota (Moossavi et al., 2019).

As previously noted, diet may influence the microbial composition of human milk (Williams et al., 2017), just as it has been shown that the nutritional component of human milk is influenced by the mother's diet (Gomez-Gallego et al., 2016). For instance, a study carried out in Syria by Albesharat et al. (2011) revealed the presence of shared lactic acid bacterial strains when the composition of lactic acid bacteria in local fermented foods, breast milk of mothers, and faeces of mothers and their babies were phenotypically and genotypically examined.

Furthermore, many studies have revealed that the composition of breast milk varies slightly depending on the stage of lactation (Boix-Amorós et al., 2016; Damaceno et al., 201; Solís et

al., 2010). Some studies have found that colostrum samples produced during the first few days after birth had a higher bacterial load than mature milk (Damaceno et al., 2017; Solís et al., 2010). There have also been documented irregularities in the pattern of breast milk microbial composition as it changes (Solís et al., 2010; Boix-Amorós et al., 2016).

Geographical influences on the microbiota of breast milk have also been reported. For example, Hunt et al. (2011) reported a less common presence of lactobacilli and bifidobacteria in their study carried out in the United States compared to a study carried out in Europe (Collado et al., 2009) in which these two bacterial genera appeared to be common. Similar variations in milk microbiomes have been reported in studies carried out in China and Taiwan (Ding et al., 2019).

Mode of delivery is another factor which has been widely discussed in the literature as stated earlier (Hermansson et al., 2019; Moossavi et al., 2019; Toscano et al., 2017). For mothers who went into labour, there is a possibility of an increased intestinal permeability which may lead to increased bacterial translocation in the maternal gut and, as a result, bacteria transfer to breast milk. Women who also went through caesarean section (CS) are also likely to be exposed to different environmental bacteria. It was reported that women who gave birth vaginally had a higher bacterial richness in their breast milk (Hermansson et al., 2019), while women who gave birth through CS were reported to have a higher abundance of environmental bacteria (Toscano et al., 2017).

Antibiotic usage has also been revealed to have a great impact on the milk microbiome leading to decreased bacterial load (Solís et al., 2010). Additionally, women with high body mass index have been reported to have a reduced bacterial diversity with an abundance of certain genera such as *Akkermansia* (Cabrera-Rubio et al., 2012), *Granulicatella* (Williams et al., 2017) and *Staphylococcus* (Ding et al., 2019) and a lower abundance of *Bacteroides* (Williams et al.,

2017), *Bifidobacterium* (Cabrera-Rubio et al., 2012), *Lactobacillus and Streptococcus (*Ding et al., 2019).

Moreover, because the current study focused on mothers and babies in Nigeria, it's critical to consider the circumstances in the study location and how they may influence the microbiota discovered in mothers' milk or babies' faeces.

2.7 A brief overview of Nigerian culture

Nigeria is in the southeast of the western part of Africa with an area of 923, 768km^2 which is about 4 times the size of the United Kingdom. It has a population of around 192 million people originating from 3 major ethnic groups namely the Hausa, Igbo and Yoruba (Moland, 2015).

Nigeria has a vibrant culture. Family is usually an institution, and they often share a bond. Childbirth and marriage are regarded as pride, and usually, there is a strong bond between mothers and their babies (Falola, 2001; Kirk-Greene, 2022). Great importance is commonly placed on breastfeeding irrespective of the ethnic group (Berde & Yalcin, 2016). The South-western part of Nigeria (where this study was carried out) consists majorly of mothers belonging to the Yoruba ethnic group, who according to their culture, believe that a child born to a healthy mother must be breastfed (Berde & Yalcin, 2016). Midwives organise discussions for each immunisation clinic and speak with mothers about the necessity of breastfeeding to reinforce its benefits (Figure 2.2).

Figure 2.2: Images showing a.) the sampling site (Adeoyo hospital Ibadan), b.) the maternity ward and c.) mothers being addressed at the immunisation clinic at Adeoyo Hospital Ibadan.

2.8 The lifestyle of Nigerian people may have an impact on the microbiota of mothers and babies

In terms of food, Nigeria has its traditional cuisine although they are gradually being replaced by western cuisine comprised mostly of frozen, canned and prepacked foods. For an average Nigerian, these western foods are expensive and so they rely mostly on their traditional foods. However, irrespective of the culture and food preparation techniques, Nigerian meals are comprised mainly of a main food combined with a stew (Muhammad & Amusa, 2005). In the South-western part of Nigeria, crops such as corn, yam and potatoes form the base of their main

diet which is served with an oil-based stew made of red peppers, chilli peppers and onions; combined with beef, goat meat, and sometimes chicken (Oguntona et al., 1999). Common vegetables are okra, melon, and spinach. Fruits such as banana, pawpaw, pineapple, orange and coconut are also common (Oguntona et al., 1999).

2.9 Socio-economic/environmental factors in Nigeria and the influence they may have on milk microbiota and faecal microbiota of babies

In Africa, particularly in Nigeria, there are several economic issues which may also alter the microbiota of the milk or gut (Ayeni et al., 2018). One of them is environmental sanitation which may have an impact on the health of Nigerian residents and may further have an influence on the microbiota of mothers and babies in Nigeria. Improper sewage disposal, poor drainage systems, and disposal of refuse on the streets and in the flowing rivers are some of the sanitary issues faced in many parts of Nigeria (Ezechi et al., 2017; Moruff, 2012). This poses a health hazard to the residents and could also be a breeding site for mosquitoes which transmits the malarial parasite that causes malaria; one of the leading causes of death in Nigeria (Bassey & Izah, 2017). Other related diseases caused by these sanitary issues are respiratory tract infections, and food and water-borne diseases (Ezechi et al., 2017). It is presumed that these health-related factors may generally influence the microbiome of mothers and babies in Nigeria.

In addition to this, there is an issue of inadequate medicines in pharmaceutical industries leading to medicines being run out and residents having to rely on ‘black markets’ which in most cases are expired medicines (Goodman et al., 2007). There are also issues of the unregulated use of antibiotics (Akinyandenu & Akinyandenu, 2014). In Nigeria, antibiotics are available over the counter without a doctor’s prescription which prompts many to self-diagnose and self-medicate (Akinyandenu & Akinyandenu, 2014). All these factors may influence the microbiome of mothers and babies in Nigeria.

Chapter 3 : Methodology

This study was carried out in phases. The first phase is the pilot study (i.e., preliminary studies), which entailed the optimization of methodologies to establish the relevance of various techniques linked to the study as well as the feasibility of the investigation. The second phase was the actual field work analysis, which included sampling at the research site in Nigeria and lastly, further analysis in the United Kingdom (Figure 3.1 and Figure 3.2).

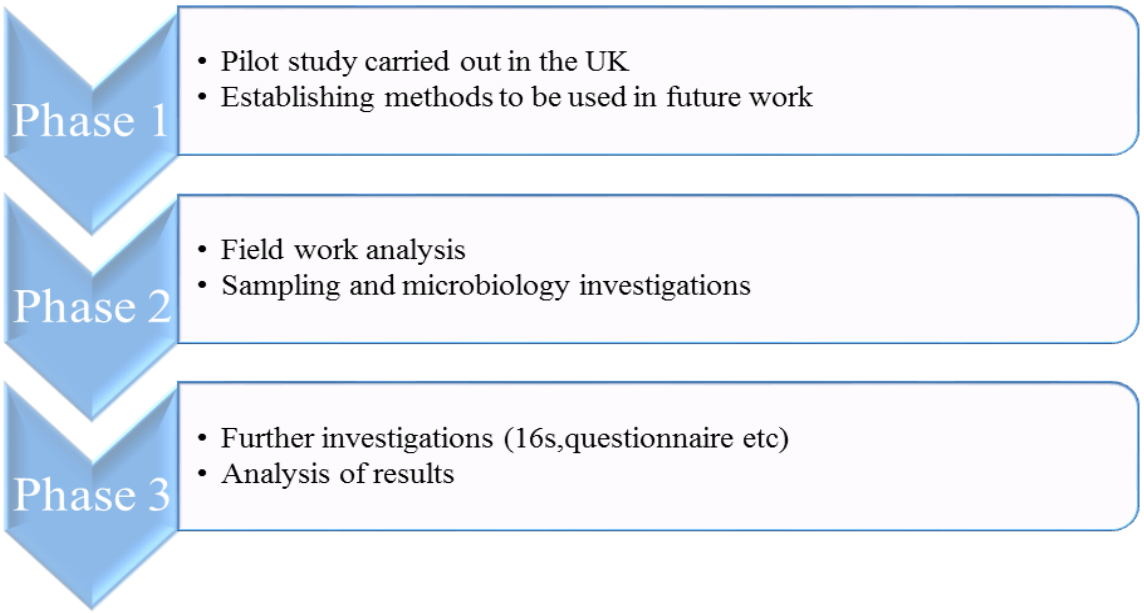


Figure 3.1: Workflow of the project consisting of three main phases

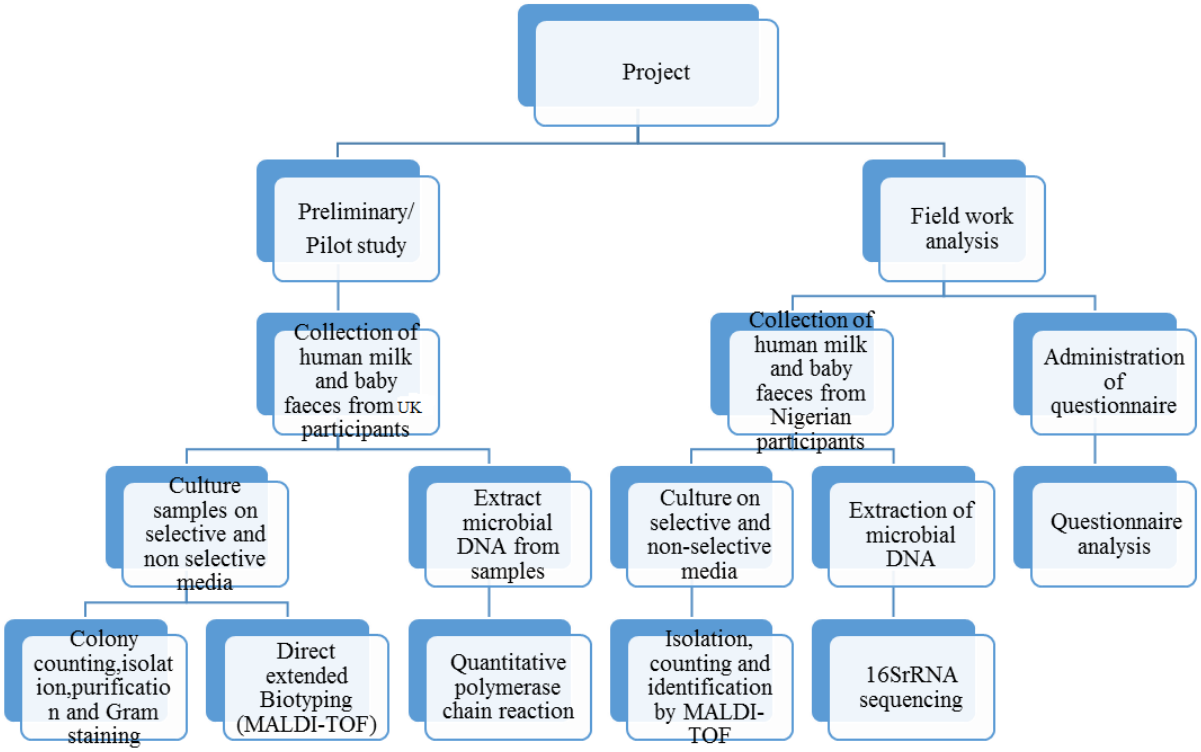


Figure 3.2: Flow diagram showing how the study was planned and carried out. The pilot study is phase one of the research while Field work analysis is phase 2.

3.1 Ethical clearance

The ethical clearance certificate used in the study was approved by the Department of Natural Sciences, Middlesex University ethical sub-committee. Ethical clearance was also obtained from the Department of Planning, Research and Statistics Division, Oyo State Ministry of Health Ibadan, Nigeria (Appendix 2B). The participant information sheet (PIS) explaining the detailed process of sample collection which was given to each participant was provided to the ethical committee as part of the ethical approval process.

3.2 Phase 1 (pilot study)

The pilot study was carried out in England, United Kingdom between January 2018 and January 2019. Breast milk samples were obtained from 7 breastfeeding mothers (represented as P1-P7) within the community and the faecal samples of their babies were obtained from their nappies. Milk and faecal samples from each of participants 1 (P1), 5 (P5) and 6 (P6) were obtained on three occasions, while milk and faecal samples from each of participants 2 (P2), 4 (P4) and 7 (P7) were obtained on two occasions. The breast milk of participant 3 (P3) was collected only on two occasions while the faecal sample was obtained on only one occasion. For swab samples, no breast swab was obtained from participants 1 to 3 while breast swab was obtained from participants 4 to 7.

Selection of participants

For the pilot, included mothers were healthy mothers who were breastfeeding either solely or mixed feeding breast milk with other foods, whereas exclusion criteria include those who have received antimicrobials in the two weeks before the start of the study and those who smoke or consume alcohol. For babies, inclusion criteria include healthy babies who are on breast milk, and exclusion criteria include babies who have received antimicrobials in the two weeks before the start of the study.

Sample collection

Before sample collection, all participants were addressed and given a participant information sheet. Participants washed their hands and used alcohol wipes to wipe their breasts and clean the nipple area. The alcohol was allowed to evaporate for about 1 minute, and a breast skin swab was obtained. Afterwards, little quantity of breast milk was expressed and discarded, before about 5mL was collected into sterile tubes. The faecal samples of their respective babies were obtained from nappies using a sterile spatula and transferred into sterile containers. Both samples were immediately transported to the Microbiology laboratory of Middlesex University

within two hours of collection. On arrival, the samples were immediately cultured on media plates as described in section 3.2.1.4 below. Aliquots (1mL) of milk samples and 0.2g of faeces were also measured into tubes and stored in a -80°C freezer for DNA extraction and to perform qPCR.

3.2.1 Sample analysis by culture

3.2.1.1 Preparation of dilutions

Milk samples were cultured undiluted, whereas 1g of faeces samples were serially diluted 10-fold in Phosphate Buffered Saline (PBS) with cysteine until dilution of 10^{-6}.

3.2.1.2 Preparation of *Bifidobacterium* Iodoacetate Medium 25 (BIM-25*)*

BIM-25 was chosen to isolate bifidobacteria to avoid the cost of commercially available media used to isolate bifidobacteria, and because reinforced clostridial agar which was used as its base was readily available during the study. BIM-25 was prepared according to (Munoa & Pares, 1988). Briefly reinforced clostridia agar (Thermo Scientific Oxoid, Basingstoke) was measured in gram/litre of distilled water according to the manufacturer's instructions and was autoclaved and allowed to cool to about 55°C. 0.02g of 51 nalidixic acid, 0.0085g of polymyxin B sulphate, 0.05g of Kanamycin sulphate, 0.025g of iodoacetic acid, and 0.025g of 2,3,5 triphenyltetrazolium chloride (Sigma Aldrich) were added afterwards and the media were allowed to set.

3.2.1.3 Preparation of Rogosa Agar

Rogosa agar (Thermo Scientific Oxoid, Basingstoke) was prepared according to the manufacturer's instructions with the addition of glacial acetic acid (1.32mL/litre of medium) to adjust the pH of the medium to a low pH of 5.5 (5.4±0.2 as recommended by Oxoid) to allow the growth of lactobacilli (Oxoid.com, 2022).

3.2.1.4 Bacterial culture of milk and faecal samples

A hundred microliters (100µL) of milk sample, as well as 100µL from dilutions 10^{-3} to 10^{-6} of faecal samples, were cultured by spread plate method. Faecal samples were cultured on BIM-25 meant for the isolation and enumeration of bifidobacteria, Rogosa for the isolation and enumeration of lactobacilli and Slanetz and Bartley (SB) (Thermo Scientific Oxoid, Basingstoke) for the isolation and enumeration of enterococci. Milk samples were cultured on BIM-25, Rogosa and blood agar plates (for any other isolates in the milk). Swab specimens of the breast skin areola were streaked directly on blood agar plates and incubated aerobically for 24 hours at 37°C.

3.2.1.5 Incubation and purification of isolates

Inoculated blood agar and SB plates were all incubated aerobically at 37°C for 24-48 hours while Rogosa and BIM-25 were incubated anaerobically at 37°C for 48 to 72 hours. The inoculated plates were incubated in Anaerobic conditions in BD GasPak 150 large anaerobic jars (Thermo Fischer Scientific, UK), each holding about 36 plates in total. Anaerobic conditions were generated by the addition of 3 sachets of BD GasPak EZ Anaerobe System (which contains ascorbic acid and activated carbon that reacts with air to generate carbon dioxide) (Thermo Fischer Scientific, UK) to each jar.

Colony isolation and Subculture

Up to 5 of each type of colony were picked from BIM-25 and subcultured on Reinforced Clostridial Agar [RCA] to support the growth of presumptive bifidobacteria, similarly, 5 of each type of colony were picked from Rogosa and subcultured on de Man, Rogosa and Sharpe [MRS] agar to support the growth of presumptive lactobacilli, and lastly from SB onto blood agar plates to support the growth of presumptive enterococci. Purification was performed 3 times.

Purified isolates were Gram stained and observed microscopically with the aid of a light microscope to differentiate gram-positive and gram-negative bacteria according to standard protocol. Briefly, bacterial smears were made by picking a colony and emulsifying it on a dry clean slide with a drop of sterile distilled water. The smears were air-dried before being exposed to a Bunsen burner flame by moving it circularly over the flame to allow the cells to adhere to the slide (Heat fixed). Smears that had been heat-fixed were covered with crystal violet and left for 1 minute. The crystal violet stain was poured away, and the excess was rinsed with tap water. Thereafter, iodine was used to cover the smears and allowed to stay for 2 minutes before being poured off and excess rinsed with tap water. The smears were thereafter decolourized with a 50:50 mixture of acetone and ethanol, taking care not to over-decolourize it by ceasing the addition of the decolourizer as soon as the solvent was no longer coloured. The smears were washed with water and then counterstained for 30 seconds with safranin. After that, the smears were rinsed and blotted using blotting paper. Finally, the smears were coated with immersion oil and examined under a microscope with x100 oil immersion objective lens. Gram-positive bacteria had a purple appearance, and gram-negative bacteria had a pink or red appearance.

3.2.1.6 Identification of purified isolates by Autoflex speed Matrix Assisted Laser Desorption Ionisation Time of Flight (MALDI-TOF MS) using Bruker MALDI-BioTyper identification method.

MALDI-TOF MS identify isolates by comparing the peptide mass fingerprint of the unknown isolate with that of the organisms in the database and generating a similarity level indicated by a score.

Sample Preparation (Formic acid extraction method)

Fresh cultures of microbial isolates were used for MALDI-TOF analysis. Three hundred microliters of deionised water was pipetted into labelled Eppendorf tubes. A small number of colonies was added into the tubes using a disposable wire loop, and they were mixed thoroughly. Nine hundred microliters of ethanol was added using a pipette and was vortexed.

The mixtures were centrifuged at a maximum speed of 13000 to 15000g for two minutes, and the supernatant was decanted. The mixtures were centrifuged a second time to remove all the residual ethanol by carefully pipetting it off to waste without disturbing the pellet. The ethanol pellet was dried at room temperature for two to three minutes. Seventy per cent formic acid of about 20µL (note: 1 to 80µL depending on the colony size) was added to the pellet with the aid of a pipette and mixed thoroughly by pipetting up and down. Pure acetonitrile solution (Sigma Aldrich) of about 20µL was also added and was mixed carefully (note: equal volume of formic acid and acetonitrile was added). The mixture was centrifuged for 2 minutes at 15000g such that all materials are collected neatly in a pellet. One microliter of the supernatant of each of the samples was spotted on each spot of the 96-spot polished steel MALDI target plate (Bruker Daltonics, GmBH, Germany) and was allowed to dry at room temperature (Figure 3.3).

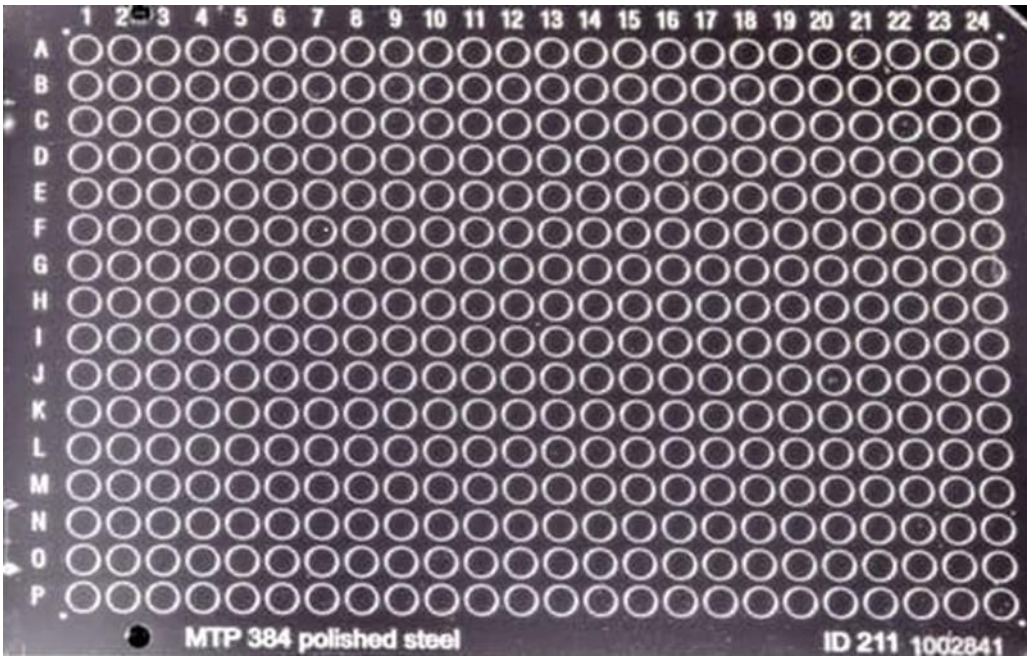


Figure 3.3: MTP 384 polished steel target plate

Each of the samples was spotted at least two times on the plate. The entire spots were covered with 1µL of matrix i.e. α-Cyano-4-hydroxycinnamic acid (HCCA; Bruker Daltonics GmBH, Germany) solution (prepared by dissolving HCCA powder in 250µL of standard solvent

(containing 2 parts acetonitrile, 1 part water and 1% trifluoroacetic acid in a tube at room temperature until it was clear) within an hour and was left to dry at room temperature. One microliter of Bacterial Test Standard (BTS; Bruker Daltonics GmBH, Germany) was also spotted on the 96-spot polished steel MALDI target plate and overlaid with the HCCA solution. This was done to calibrate the machine as well as validate the run. The plate was then inserted into the MALDITOF machine (Figure 3.4).

Figure 3.4: Autoflex speed Matrix Assisted Laser Desorption Ionisation-Time of Flight Mass Spectrometry machine

Measurement of MALDI-TOF mass spectra and Identification of microorganisms

The acquisition and analysis of mass spectra (2000±20000 Da) were performed automatically using the Autoflex III MALDI-TOF mass spectrometer which is integrated with a nitrogen laser and controlled by FlexControl software. The Flex Analysis software (Bruker Daltonics, GmBH, Germany) was used to analyse the spectra. To categorise the strains, the MALDI BioTyper software, version 3.1 (Bruker Daltonics, Bremen, Germany) was used to analyse the raw spectra and compare them. The MALDI BioTyper's automation methodology enables optimal sample gathering usually 300 to 500 high-quality shots from several optimal spots leading to the final identification, by the generation of an identification score. The identification scores of 2.300-3.000 indicated highly probable species identification; a score of 2.000-2.299 indicated secure genus identification, probable species identification; a score of 1.700-1.999 indicated probable genus identification while a score of 0.000-1.699 indicated a non-reliable identification.

3.2.2 Identification of bacteria in milk and faeces of 7 mother-baby pairs by qPCR

3.2.2.1 DNA extraction

DNA was extracted from 2mL of frozen human milk and 0.2g of frozen faecal samples of babies following the recommended protocol by MasterPure DNA/RNA purification kit (Lucigen, USA) with some modifications that included mechanical lysis step and enzymatic lysis step with selected enzymes: lysozyme, mutanolysin and lysostaphin [lysozyme allows for improved extraction and further detection of gram-positive bacteria while mutanolysin and lysostaphin aid in the lysis of other bacterial species that may be resistant to lysozyme (Yuan et al., 2012)] as described below.

Preparation of samples

Milk samples were spun at 14,000g for 20 minutes at 4°C and fats were removed with the aid of transfer pipettes and sterile cotton swab sticks.

To the faecal samples, 1mL of PBS and 0.5mL freshly made 2% beta-mercaptoethanol in PBS were added, vortexed and then subjected to rotation and incubation for 1 hour at room temperature to allow proper mixture and aid the denaturation of unwanted proteins. The sample mixtures were then centrifuged at 14,000g for 10 minutes at room temperature. Supernatants were removed and pellets were re-suspended in 10mL PBS for filtration. Filtration was carried out twice, first by using a 100µm size sterile steriflip centrifuge tube top filter unit (Sigma Aldrich) and then by 40µm size. The filtrate was centrifuged at 6,000g for 20 minutes at room temperature.

Pre-extraction step with enzymes

A cocktail of enzymes lysozyme (10mg/mL), mutanolysin (20U/µL) and lysostaphin (4U/µL) (Sigma Aldrich) were added to the pellets of faeces and milk obtained from the preparation steps above. The mixtures were vortexed and incubated at 37ºC for 1 hour with shaking at 250g. After incubation, 150 µL of 2x tissue and cell lysis buffer and 2 µL of proteinase K (50 µg/µL) from MasterPure Complete DNA and RNA Purification Kit were added, vortexed and incubated at 65ºC for 30 minutes. The next step included a mechanical lysis step that involved cell disruption with 0.2mm acid-washed glass beads (Sigma Aldrich) using a FastPrep machine for 60 seconds at 6.5 m/s. The products were centrifuged at 7,500g for 10 minutes at 4ºC to settle down the beads and to aid the transfer of lysate into a clean Eppendorf DNA LoBind tube (Sigma Aldrich) [DNA LoBind tubes reduce the binding of DNA to the surface of the tube to maximise recovery of DNA]. One microliter of RNase (5 µg/µL) was added to the lysate and the samples were incubated for 15 minutes at 37ºC with shaking. Samples were thereafter cooled on ice for two minutes, and 180 µL of MPC protein precipitation reagent (ammonium acetate) was added and vortexed. The mixtures were subjected to centrifugation at 15,000g for 10 minutes at 4ºC. The supernatants were transferred into a clean DNA LoBind tube and 500 µL of isopropanol was added. The tubes were inverted gently 40 times to allow

for DNA precipitation. The centrifugation step was repeated, and supernatants were discarded. Ethanol (70%) was used to wash the pellets twice and residual ethanol was allowed to dry at room temperature for 5 minutes. Extracted total DNA was suspended in 50 μL Tris-ethylenediaminetetraacetic acid (TE) buffer and stored at -80^{o}C.

3.2.2.2 Measuring DNA quantity and purity

DNA quantity was measured using Qubit 2.0 fluorometers (Thermofisher Scientific Inc.) by following the guidelines of the double-stranded DNA high-sensitivity assay kit and protocol for dsDNA high-sensitivity analysis.

Additionally, the DNA purity was measured using Nanodrop 2000 spectrophotometer (ThermoFisher Scientific Inc) by analysing 1 μL volume of each sample following the standard protocol for assessing A260/280 and A260/230 ratios. The A260/A280 and A260/A230 ratios are absorbance measurements used to determine the purity of DNA. The absorbance A260/A280 ratio is typically accepted to be between 1.8 and 2.0, and if it is much lower, it may indicate a contaminant that absorbs at 280nm or near 280nm e.g., phenol and proteins (Thermo Scientific Nanodrop Spectrophotometer, 2022). Additionally, an absorbance A260/A230 ratio which is lower than the values between 2.0-2.2 may indicate a contaminant which absorbs near 230nm e.g., carbohydrates and EDTA (Thermo Scientific Nanodrop Spectrophotometer, 2022).

3.2.2.3 Detection of bacteria in extracted DNA using qPCR

DNA extracted from milk and faecal samples was used for qPCR assay. Quantification of bacterial cells present in the samples was carried out using universal SYBR Green quantitative PCR protocol. The presence of 5 bacterial genera including bifidobacteria, streptococci, lactobacilli, staphylococci, and enterococci in milk and faecal samples was investigated using genus-specific primers obtained from Eurogentec, Belgium (Table 3.1). These five bacterial

genera were chosen because they are commonly reported in the literature to be commensals in breast milk, and studying all bacteria in milk using qPCR is neither cost-effective nor practical.

Table 3.1: Description of primer sequence used and their conditions

Bacteria and expected product size	Primer sequence	Annealing temperature	Reference
Enterococci (144bp)	Forward: 5'-CCCTTATTGTTAGTTGCCATCATT-3' Reverse: 5'-ACTCGTTGTACTTCCCATTGT-3'	50^0C	(Collado et al., 2009; Khodayar-Pardo et al., 2014)
Lactobacilli (341bp)	Forward: 5′-AGCAGTAGGGAATCTTCCA-3′ Reverse: 5′-CACCGCTACACATGGAG-3′	55^0C	(Khodayar-Pardo et al., 2014; Rinttilä et al., 2004)
Bifidobacteria (243bp)	Forward:5′-TCGCGTC(C/T)GGTGTGAAAG-3′ Reverse: 5′-CCACATCCAGC(A/G)TCCAC-3'	55^0C	(Rinttilä et al., 2004)

Staphylococci (370bp)	Forward: 5'-GGCCGTGTTGAACGTGGTCAAATCA-3' Reverse: 5'-TIACCATTTCAGTACCTTCTGGTAA-3'	50^0C	(Khodayar-Pardo et al., 2014 ; Martineau et al., 2001)
Streptococci (197bp)	Forward: 5'-GTACAGTTGCTTCAGGACGTATC -3' Reverse: 5'-ACGTTCGATTTCATCACGTT-3'	50^0C	(Khodayar-Pardo et al., 2014)

PCR amplification was carried out on a 96-well plate using Light Cycler 96 real-time PCR system (Roche Life Science, United Kingdom). The reaction mixture (20µl) contained 10µl of 2x SYBR GREEN 1 Master (Roche Life Science), 1µL of forward primers with a concentration of 10µM, 1µL of reverse primers with a concentration of 10µM, and a total genomic DNA of 50ng from milk and 100ng from faeces (supplemented with water).

A non-template control which contained 10µl of SYBR GREEN 1 Master (Roche Life Science), 1µL of forward primers with a concentration of 10µM, 1µL of reverse primers with a concentration of 10µM, and 5µL of PCR grade water also ran alongside the reaction mixture

to monitor any form of contamination and the formation of primer dimer which could lead to the production of a false positive result.

The qPCR cycling condition included 3 steps which are the pre-incubation step, amplification step and melting. The specificity of the products was analysed via melting curves of products and on the agarose gel. Tables 3.2 and 3.3 show the qPCR conditions for the primer sets used for the reaction.

Table 3.2: Cycling condition using streptococci, enterococci and staphylococci primers

Steps	Temperature	Duration
Pre-incubation	95^0C	10 minutes
Amplification (45 cycles) Denaturation Annealing Extension	 95^0C **50^0C** 72^0C	 10 seconds 10 seconds 10 seconds
Melting	95^0C 65^0C 97^0C	10 seconds 60 seconds 1 second

Table 3.3: Cycling condition using bifidobacteria and lactobacilli primers

Step	Temperature	Duration
Pre-incubation	95^0C	10 minutes
Amplification (45 cycles)	95^0C **55^0C** 72^0C	10 seconds 10 seconds 10 seconds
Melting	95^0C 65^0C	10 seconds 60 seconds

	97^0C	1 second

3.2.2.4 Absolute quantification of bacteria by qPCR

Absolute quantification was used as a method of quantification by using a standard curve generated for each bacterium and Efficiency of reaction=2. The standard curve was generated using a 10-fold serial dilution of DNA derived from pure reference bacterial strains of *Streptococcus pyogenes* NCTC 889, *Bifidobacterium animalis* subsp *animalis* NCIMB 702242, *Lactobacillus casei* NCIMB 4114, *Enterococcus faecalis* NCTC 775 and *Staphylococcus aureus* NCTC 6571 (Table 3.4) with plate's counts ranging from $8x10^4$ to $9.5x10^8$. Table 3.4 presents the genome size of each reference bacterial strain.

Table 3.4: Bacterial strains used for standards/reference strains and their average genomic size as obtained from National Center for Biotechnology Information database (NCBI)

Bacterial strains	Genome size (Mega bp)
Streptococcus pyogenes NCTC 889	1.85Mbp
Bifidobacterium animalis subsp *animalis* NCIMB 702242	1.93Mbp
Lactobacillus casei NCIMB 4114	2.9Mbp
Enterococcus faecalis NCTC 775	2.8814Mbp
Staphylococcus aureus NCTC 6571	2.82Mbp

The bacterial concentration in each sample was measured as $\log_{10}$ genome DNA equivalent/ µL and one genome was considered to be equivalent to a single cell as previously described by Hoppener-Ogawa et al. (2007).

qPCR products were verified by melting curves and subsequently run on 1 % agarose gel after each reaction to confirm the presence of specific PCR products of expected size or to check for the presence of non-specific PCR products, primer dimers as well as contamination.

3.2.2.5 Generation of cycle quantification values (cq values) of qPCR

A Cycle quantification value is the PCR cycle number at which an amplification curve of a sample intersects with the threshold line (i.e. the point at which the fluorescence of a PCR product can be detected above the background signal) [Figure 3.5].

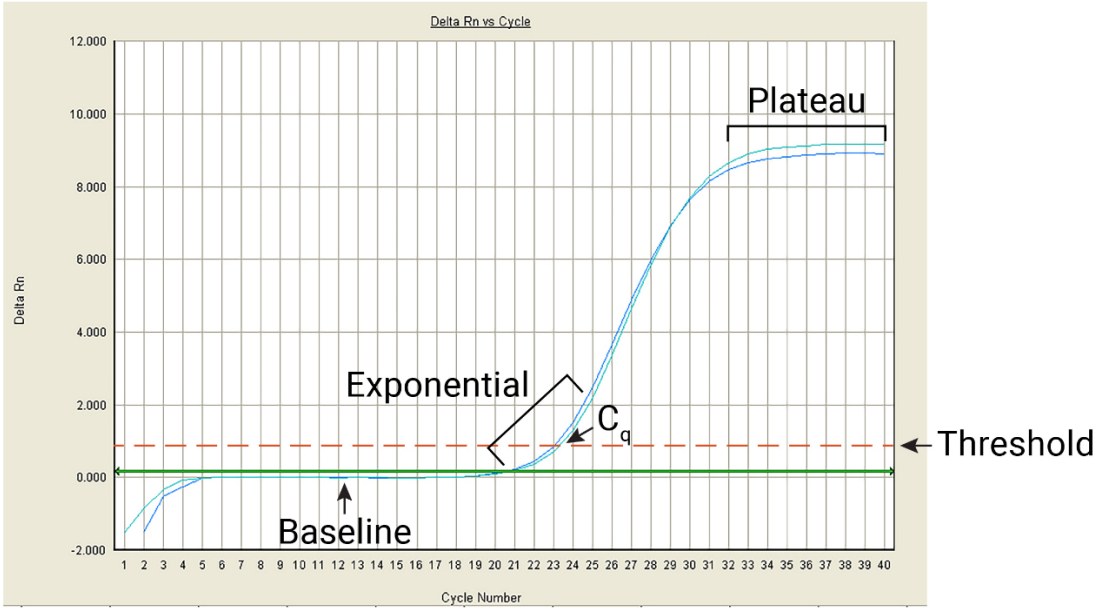


Figure 3.5. Threshold line and Cq value of a qPCR amplification curve (Source: Promega, 2022). Cq value corresponds to the cycle number when a threshold is crossed by the amplification curve.

Cq value relatively measures the concentration of the target of a PCR reaction. It tells how many cycles it takes to detect a real signal from qPCR samples. Each sample will have its amplification curve over time hence many Cq values. Cq values are inversely proportional to the number of target gene copies in a sample, hence the higher the Cq values the lower the number of target gene copies in a sample (Kuang et al., 2018)

The analysis of bacteria in breast milk and faeces by qPCR and 16S NGS are both highly sensitive however while qPCR is effective for a small number of samples, research involving large numbers of samples, such as the current study, will be best conducted utilising NGS.

3.3 Phase 2: (Investigations of human milk and faecal microbiota of babies in Nigeria)

3.3.1 Study site

The fieldwork was carried out in Ibadan Nigeria, the Oyo state capital, which lies between longitude 3° 35' & 4° 42' and latitude 8° 15'&, 9° 00'. Samples were obtained from mothers attending maternity centres at the Adeoyo Maternity Teaching Hospital and the Jericho Specialist Hospital, both of which are located within Ibadan metropolis. To obtain faecal samples from exclusively formula-fed babies, the Cheshire motherless babies' home and the Federation of Muslim Women's Association in Nigeria (FOMWAN) orphanage home were approached, and consent was sought from guardians upon obtaining ethical clearance.

3.3.2 Sample size determination

To learn about the characteristics or attributes of a population, it is not usually feasible to investigate the whole of a population, rather one must settle for a certain sample size. (Roscoe, 1975) sets out some features to determine effective and adequate sample size. Referring to Roscoe, a sample size larger than 30 and less than 500 is appropriate for most research to reduce Type II error i.e., false negative results (Sekaran & Bougie, 2016). In this study, 58 breastfeeding mothers and their babies were recruited to participate, however, eight of the mothers pulled out along the line, leaving samples from 50 mothers and their respective babies to be processed by culture and sequencing. In addition to this, faecal samples were obtained separately from 8 babies fed with formula milk.

3.3.2.1 Samples analysed by culture and sequencing

Fifty milk samples, 50 faecal samples from breastfed babies, and 8 faecal samples from formula-fed babies were eventually analysed by culture as discussed in 3.3.2 above. However, upon sending breast milk and faecal samples for 16S sequencing, two samples each from breast milk (M3, M14) and faeces of breastfed babies (F17, F22) could not be sequenced due to a low amount of DNA leaving 48 breast milk and 48 faecal samples of breastfed babies as well as 8 faecal samples from babies fed with formula milk to be processed for 16S sequencing. Breast milk and faecal samples from each of the participants were obtained and processed on one occasion due to the cost of sequencing analysis. Throughout this document, breast milk samples are represented as M followed by the participant identification number (e.g., M followed by number 58 i.e., M58), and the faecal samples are represented as F followed by the participant identification number (e.g., F followed by number 58 i.e., F58). Faeces of babies fed with formula are also represented as FM followed by an identification number. Mothers and their babies were given similar identification numbers to allow for easy linkage (e.g., if the milk of mother 1 is M1, the faeces of her baby will be F1)

3.3.3 Development of questionnaire

All mothers included in the study were asked to complete a questionnaire written in English Language except for those who were unable to read or write, whose answers were filled in by the researcher. The questionnaire survey contained 30 questions in total, and it was designed to gather information about the demographic details of the participants such as age, ethnicity, level of education, and socio-economic status to assess the level of income and earnings. The last part assessed the diet of mothers, religion, method of feeding babies (breastfed/formula), mode of delivery, and level of education as detailed in Appendix 1.

Participants' data were handled with utmost care and confidentiality. The privacy and confidentiality of every participant were maintained, and the identification of each participant

was done by using number codes. The questionnaire came along with the participant's information sheet detailing what the research was all about.

3.3.4 Selection of participants

Inclusion criteria for mothers include healthy women who were exclusively breastfeeding, whereas exclusion criteria include those who have received antibiotics in the two weeks before the start of the study and those who smoke or consume alcohol.

For babies, inclusion criteria include healthy babies up to 6 months old who are solely on breast milk or formula, while exclusion criteria include babies who have been breastfed by more than one mother, eating other foods asides from breast milk or formula (for formula-fed babies), and babies more than 6 months old.

3.3.5 Administration of questionnaire

Breast milk samples of Nigerian mothers and the faecal samples of their babies were obtained between August 2019 and November 2019, including faecal samples from 8 formula-fed babies. Prior to administering the questionnaire and obtaining samples, all participants were given a participant information sheet (PIS) detailing the purpose of the research. Each participant was then given a questionnaire to fill out. After filling out the questionnaire, participants were addressed before being given a leaflet explaining the detailed process of sample collection. For babies that are fed formula milk, the PIS was given to their guardians to read, and the questionnaire was also filled out by their guardians. Information about the mothers of these formula-fed babies was not collected, however, the babies' demographic details were obtained from their guardians, including the name and brand of the formula the babies consumed using the questionnaire as detailed in section B of the questionnaire (Appendix 1). The formula label was then checked for its composition such as the presence of probiotics and prebiotics.

3.3.6 Sample collection

To obtain each sample, a pair of gloves were worn by the researcher and a cotton pad soaked in 70% alcohol was used to wipe the breast of the mothers to clean the nipple area. The alcohol was allowed to evaporate for about 1 minute. A little quantity of breast milk was expressed first and about 5mL was collected into sterile tubes.

The faecal samples of their respective babies were obtained from nappies using a sterile spatula and transferred into sterile containers. Both samples were immediately put on ice before being transported to the Medical Microbiology Laboratory, College of Medicine, University College Hospital, Ibadan Nigeria within 4 hours of collection.

On arrival, the samples (milk represented as M1-M58, faeces of breastfed babies represented as F1-F58, and faeces of formula-fed babies represented as FM1-FM8) were immediately cultured on selective and non-selective media and incubated as detailed in section 3.2.1. Colonies were counted and picked, and isolates were transferred into sterile glycerol and stored at -20°C. Additionally, aliquots (3mL) of milk samples and 0.2g of faeces were measured into tubes and stored in a -20°C freezer for DNA extraction and sequencing to be carried out in the UK. Contact information of each mother was obtained which facilitated repeated sampling. Samplings were carried out on 2 to 3 occasions from each mother-baby pair. All stored frozen samples were transported on dry ice to the UK for further analysis of 16S rDNA sequencing by following the Material Transfer Agreement for the Supply of Human Tissue Materials (See Appendix 2A).

Disposal of participants' data

All data collected using a paper questionnaire were anonymised by giving each participant her identification number. At the end of the research, all data collected which was saved on only one computer (belonging to Middlesex University) will be erased. Additionally, paper copies

(questionnaires) will be destroyed safely by shredding and disposing of them in the confidential waste bin.

3.3.7 Sample analysis by culture

Frozen culture isolates were retrieved from -80°C and sub-cultured as described in section 3.2.1.5. Identification was also carried out using MALDI-TOF Biotyper as described in sections 3.2.1.6 and 3.2.1.7.

3.3.8 Evaluation of breast milk and faeces microbiota of Nigerian participants by 16S rDNA sequencing

3.3.8.1 DNA Extraction

DNA was extracted from 3mL of frozen human milk (3mL was used for DNA extraction from milk samples obtained from Nigeria to improve DNA yield) and 0.2g of frozen faecal samples of babies following the recommended protocol by MasterPure DNA/RNA purification kit (Lucigen, USA) with some modifications as described in section 3.2.2.1.

3.3.8.2 Measuring DNA quantity and purity

DNA quantity and quality were measured by following the steps described in section 3.2.2.2.

3.3.8.3 Detection of bacteria using 16S rDNA gene Next-Generation Sequencing targeting V3-V4 region

16S rDNA sequencing was carried out using Illumina MiSeq at Eurofins Genomics, Germany. All steps performed have been developed and validated by Eurofins Genomics Europe Sequencing GmbH after consultation, to achieve the research aims of this project. The primer sequence used for the V3-V4 target region is shown in Table 3.5. Briefly, PCR was used to amplify the V3 and V4 regions of the bacterial 16S and PCR products were purified. Illumina protocols were followed to construct the DNA libraries, as illustrated in Figure 3.6. The sequencing was done on an Illumina MiSeq platform.

Table 3.5: The primer sequence used for the target region V3-V4, as selected for this project.

Target	Region	Primer Sequence (5'- 3')	Reference
Bacterial 16S rDNA	16S V3-V4	Fwd: TACGGGAGGCAGCAG	Turner et al., 1999
		Rev: CCAGGGTATCTAATCC	Kisand et al., 2002

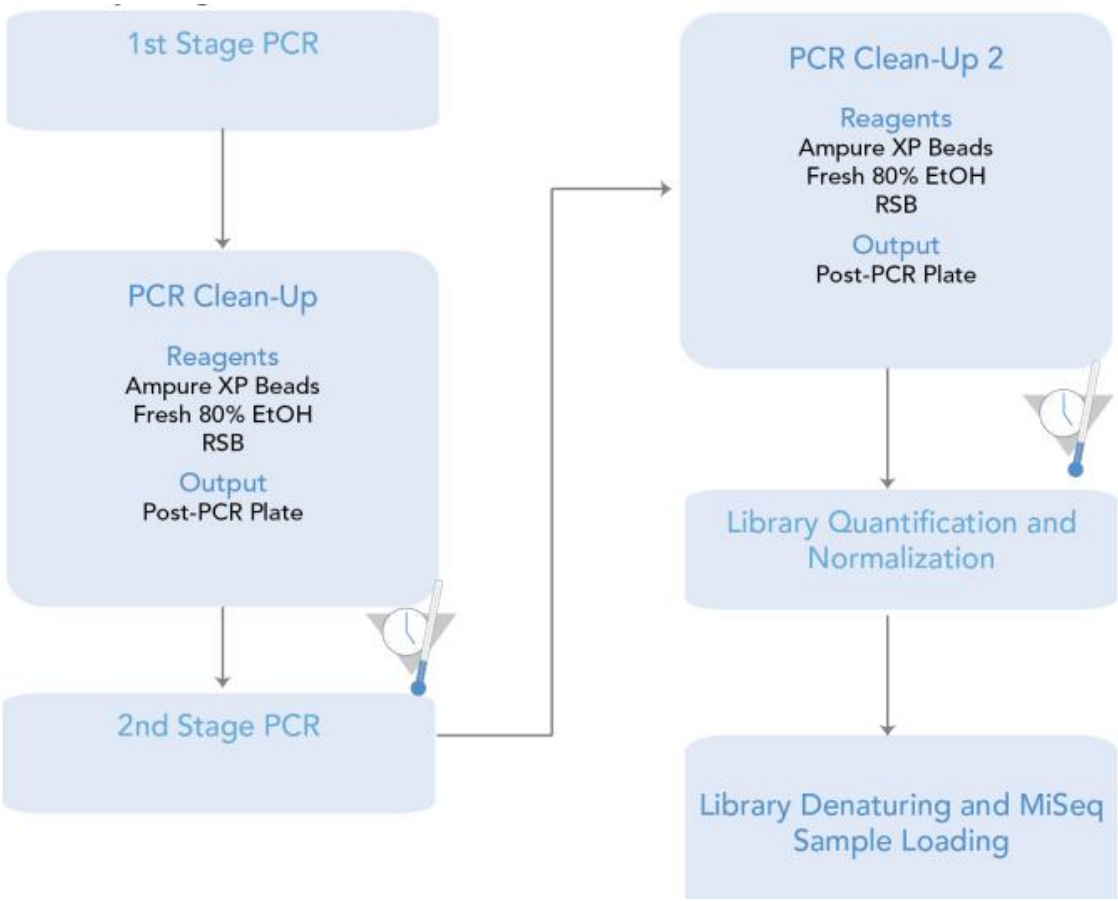


Figure 3.6: 16S Library Preparation workflow. Retrieved from (Illumina.com, 2013). The first stage of PCR involves the amplification of the template out of the DNA sample using primers described in table 3.5. This is followed by a second PCR to amplify pooled amplicons from step 1 using sequence adapters to produce barcoded amplicons ready for MiSeq.

3.3.8.4 Illumina paired-end sequencing protocol

The processing of sequencing reads according to primer sequences was performed with in-house scripts by Eurofins (Germany). All positive reads for amplicons created by forward as well as reverse primer were used for further analysis. To select 'reads', only reads with no mismatches in both primers' sequences were selected. At least 60,000 reads pair were collected per sample for further analysis.

Protocols as provided by Eurofins (Germany) are detailed in sections 3.3.8.5 to section 3.3.8.7 below.

3.3.8.5 Merging of overlapping paired reads

The FLASH programme was used to merge paired-end reads. The FLASH algorithm selects the overlap that yields the lowest proportion of mismatched bases in the overlapped region after taking into account all possible overlaps at or above a minimum length between the reads in a pair. By choosing the base with the higher quality value at each overlapped position, FLASH calculates a consensus sequence in the overlapped region. If the quality values of the two bases are the same, a random choice is made. To reduce false positive merges, pairs were merged with a minimum overlap size of 10bp.

3.3.8.6 Sequencing on Illumina MiSeq platform

Using the 2x300bp sequence mode, sequencing was performed on a MiSeq. The MiSeq system provided on-instrument secondary analysis after samples were loaded by using MiSeq Reporter software (MSR). Using a database of 16S rDNA data, the Metagenomics workflow categorised organisms from the V3-V4 amplicon. This workflow produces read classifications at various taxonomic levels, including kingdom, phylum, class, order, family, genus, and species.

3.3.8.7 Microbial community analysis

Quantitative Insights into Microbial Ecology (QIIME) software version 1.9.1 was used to process the 16S rDNA sequences obtained from the sequencing platform. All reads (Pass-Filter reads) that pass the Illumina chastity filter are demultiplexed in accordance with their index sequences. The raw forward and reverse read starts are examined for the target region-specific forward and reverse primer sequences, and these sequences are then clipped. Read pairs are eliminated in order to keep only high-quality reads if primer sequences could not be perfectly matched.

All reads with ambiguous bases ("N") were eliminated before Operational Taxonomic OTU picking. Minimum entropy decomposition was used to process the remaining set of high-quality reads. Marker gene datasets can be divided into OTUs using the computationally effective technique of minimum entropy decomposition (MED). Each OTU represents a unique cluster with a high degree of sequence divergence from any other cluster. DC-MEGABLAST alignments of cluster representative sequences to the sequence database were carried out in order to assign taxonomic information to each OTU. The set of reference sequences with the best matches was then used to transfer the most specific taxonomic assignment for each OTU. A sequence identity of 70% across at least 80% of the representative sequence was a minimum requirement for considering reference sequences.

3.3.8.8 Data analysis

The microbial abundance of human milk and baby faeces at each taxonomic level (Kingdom, phylum, class, order, family, genus, and species) were determined using QIIME open-source software version 1.9.1 as explained above.

The composition difference of human milk and faeces of breastfed babies given in log2fold change.

To determine any difference between the composition of milk (M) and faeces of breastfed babies (F) or between faeces of breastfed babies (F) and that of babies fed with formula (FM), OTUs reads from each sample were collected; samples with OTU below 10 were excluded for this analysis.

To generate normalized OTU counts, the final table of OTU compositions was used to compute differential OTU compositions using R/Bioconductor DESeq2 package in R software (version 4.1.2), which normalizes the abundance raw read counts to account for observed variance (due to differences in sequencing depths, sample groups and replicates). Statistical test using negative binomial generalized linear models for each OTU to compare the distributions between conditions (e.g., milk vs faeces) was carried out, generating P-values for each OTU. The final P-values were corrected by determining false discovery rates (FDR) using the Benjamini–Hochberg method. Log2fold change >0.5 was set as a threshold/cut-off and FDR corrected P-value of <0.1 was used to determine significantly differential composition.

Calculating the alpha (within-sample diversity) and beta diversity (between-sample diversity)

In order to calculate alpha and beta diversity, the OTU table was rarefied at 10,000 sequences per sample. The 'Chao 1 alpha diversity index' which estimates the microbial richness and the 'Observed species' which considers the number of unique OTUs were calculated. The diversity and dominance were also estimated using 'Shannon diversity index' and 'Simpson index' by using ggplot2 package in R software (version 4.1.2). To determine the compositional dissimilarity (Beta diversity) between human milk (samples M) and baby faeces (samples F), Bray-Curtis dissimilarity was used.

3.3.8.9 Shared OTU between human milk and baby faeces

To determine the taxa which are shared between the human milk (samples M) and faeces of breastfed babies (samples F), all sequences were put together and a closed-reference OTU picking using QIIME (Version 1.9.1) was carried out. The 'Shared_phylotypes.py' script was then run on QIIME to generate the OTU shared between human milk and baby faeces. OTU Venn plot was also generated. Furthermore, microbial source tracker analysis was carried out in R to determine the probable source of bacteria in baby faeces originating from human milk as compared to other unknown sources.

3.3.8.10 Statistical analysis

Demographic data of participants were reported as mean (SD) and percentages. Nonparametric t-tests (Mann-Whitney U test to compare two groups and Kruskal-Wallis to compare two or more groups) were carried out in Statistical Package for Social Sciences (SPSS) version 25.0 to determine the significant difference between alpha diversities and maternal/neonatal characteristics. To find any association between the levels of the top ten most predominant bacterial genera in breast milk or baby faeces and maternal or neonatal characteristics, Mann-Whitney U test was carried out and Benjamini-Hochberg method (BH) was used to estimate the false discovery rate to generate a corrected p-value (q-value), with q-value $q<0.05$ considered statistically significant. Adonis test was also used in R software (Version 4.1.2) to compare milk and faecal microbiota (Beta diversity) and a P-value of $P=0.05$ was used as cut off for statistical significance.

Chapter 4 : Results of Pilot (Preliminary)study

4.1 Microbiology analysis

Pilot studies were carried out on a small group of participants in England. For the purpose of the pilot study, mothers who fed the babies with formula and breast milk and babies up to 1 year of age who have started feeding on other foods apart from breast milk were also considered. Demographic details of the participants are shown in Table 4.1.

Table 4.1: Participant information

Participant	Ethnic Background	Baby's diet	Age
1	African	Breast milk	2 months
2	African	Breast milk	3 months
3	Asian	Breast milk/formula	3 months
4	British	Breast milk	3 months
5	African	Breast milk	5 months
6	African	Breast milk/formula	12 months
7	European	Breast milk	6months

4.1.1 Culture and identification by MALDI-TOF

Cultured plates of milk and faeces from participants 1 to 7 (P1-P7) were observed by using bright light and divided into types according to their shape and colour. Up to five of each colony type were purified and identified by performing MALDI formic acid extraction with the colonies and spotted onto the MALDI plate as described in section 3.2.1.6.

The most frequently isolated bacterial genera from breast milk samples were staphylococci (isolated from all participants on blood agar) and streptococci (isolated from 5 of the 7 participants on blood agar) while the least isolated bacteria from breast milk belong to the genera *Bacillus*, *Serretia*, *Klebsiella*, *Stenotrophomonas*, *Pseudomonas*, and *Sphingobacterium* (Table 4.2 and 4.3). Of the 12 types of bacterial genera isolated by culture, 6 were gram-positive while the remaining 6 were gram-negative. Aerobes accounted for 9 bacterial genera while anaerobes accounted for 3 genera. The most frequently isolated bacterial genera from faecal samples were enterococci while the least isolated were staphylococci (Table 4.2 and 4.3).

Breast milk contained a low level of bacteria with an average count of about $1x10^3$ CFU/mL (apart from one participant with a bacterial count up to about $5.8x10^6$ CFU/mL which was consistent with all three sampling occasions), while faecal samples had a higher count of about 10^9 CFU/g of faeces (Table 4.2 and 4.3). Three mothers and their respective babies also shared similar bacteria at the species level (Table 4.4).

Swab samples of participants 4 to 7 that were obtained prior to the nipple area being cleaned (swab 1) revealed the presence of different bacteria species such as *Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus hominis, Micrococcus luteus Acinetobacter lwoff, Neiseeria perflava, Streptococcus paransanguinis, Corynebacterium mucifaciens* and *Bacillus pumilus.* The swab samples obtained after cleaning with alcohol

wipes (swab 2) revealed the presence of similar species and in some participants, additional species were revealed but generally with a huge reduction in their number. For example, the culture of swab 1 from participant 5 revealed 240 colonies of *Staphylococcus hominis,* while swab 2 only revealed 4 colonies of the same organism. In participant 4, 16 colonies of *Staphylococcus epidermidis* were revealed from the culture of swab 1, while swab 2 revealed no growth (Table 4.5).

Table 4.2: Bacterial count obtained from milk and faecal samples of participants 1 to 4. Colony counts are an average of three plates and three repeats. Identification carried out by MALDI-TOF Biotyper (Scores of identifications are put into brackets).

	P1 (Mean±SD)		P2 (Mean±SD)		P3 (Mean±SD)		P4 (Mean±SD)	
Bacterial types	CFU/mL milk/ (MALDI score)	CFU/g Faeces/ (MALDI score)	CFU/mL milk/ (MALDI score)	CFU/g Faeces/ (MALDI score)	CFU/mL milk/(MALDI score)	CFU/g Faeces/ (MALDI score)	CFU/mL milk/(MALDI score)	CFU/g Faeces/(MALDI score)
Streptococcus salivarius (+ve , A)	$1.7\pm0.26\times10^{2}$ (2.292)	0	$8\pm9.8\times10^{1}$ (2.301)	0	0	0	0	0
Bifidobacterium Breve (+ve, An)	0	0	$4.7\pm0.26\times10^{2}$ (2.234)	$5.9\pm2.9\times10^{8}$ (2.163)	$3.5\pm0.7\times10^{1}$ (2.141)	0	0	$1.3\pm1.7\times10^{8}$ (2.094)
Staphylococcus epidermidis(+ve , A)	$9\pm9.6\times10^{1}$ (2.249)	0	$10.5\pm6\times10^{1}$ (2.291)	0	0	0	$3.5\pm3.3\times10^{2}$ (2.185)	0
Streptococcus vestibularis(+ve , A)	$2.1\pm1.1\times10^{2}$ (2.206)	0	0	0	$2.5\pm2.1\times10^{1}$ (2.133)	0	0	0
Staphylococcus aureus(+ve , A)	$1\pm0.05\times10^{2}$ (2.426)	$4.0\pm2\times10^{7}$ (2.381)	0	0	0	0	0	0
Streptococcus pneumonia(+ve , A)	0	0	$3.6\pm0.4\times10^{2}$ (2.027)	0	0	0	0	0
Streptococcus parasanguinis(+ve , A)	0	0	0	0	0	0	$11.7\pm10.2\times10^{1}$ (2.16)	0
Propionibacterium granulosum(+ve, An)	0	0	$6.6\pm0.4\times10^{2}$ (2.129)	0	0	0	0	0
Bifidobacterium longum (+ve, An)	0	0	0	$8.4\pm1.4\times10^{8}$ (2.251)	0	0	0	0
Staphylococcus hominis(+ve , A)	0	0	0	0	$1\pm2.8\times10^{2}$ (1.904)	0	0	0
Staphylococcus haemolyticus(+ve , A)	0	0	0	0	$11.5\pm9\times10^{1}$ (2.209)	0	0	0
Staphylococcus pasteuri (+ve , A)	0	0	0	0	0	0	$7\pm1.4\times10^{1}$ (2.331)	0
Lactobacillus rhamnosus(+ve, An)	0	0	0	0	0	$3.8\pm0.7\times10^{7}$ (2.347)	0	0
Bacillus pumilus(+ve , A)	0	0	0	0	0	0	$1.9\pm0.2\times10^{2}$ (1.934)	0

Kocuria rhizophila(+ve , A)	0	0	0	0	0	0	$8\pm2.8x\ 10^{1}$ (2.111)	0
Enterococcus faecalis(+ve , A)	0	$7.2\pm8.5\ x10^{8}$ (2.375)	0	$12.6\pm6x10^{8}$ (2.518)	0	$1\pm0x10^{2}$ (2.385)	0	$7\pm10x10^{8}$ (2.219)
Lactobacillus salivarius(+ve, An)	0	$8.9\pm10x10^{8}$ (2.08)	0	0	0	0	0	0

The table above shows the number of bacteria (measured in CFU) that were isolated from the culture of breast milk and faecal samples on two sampling (Participants 2, 3, &4) to three sampling (Participant 1) occasions. Identification was carried out using MALDI-TOF formic acid extraction method with scores for genus/species level identification in the bracket. 0- means the bacterial species was not isolated. P1 to P4- samples from participants 1 to 4. +ve-means gram-positive, -ve –means gram-negative, A-means Aerobic bacterium, An- Anaerobic bacterium

Table 4.3: Bacterial count obtained from milk and faecal samples of participants 5 to 7. Colony counts are an average of three plates and three repeats. Identification carried out by MALDI-TOF Biotyper (Scores of identifications are put into brackets).

	P5 (Mean±SD)		P6 (Mean±SD)		P7 (Mean±SD)	
Bacterial types /MALDI score	CFU/mL milk (MALDI score)	CFU/g Faeces/ MALDI score	CFU/mL milk/(MALDI score)	CFU/g Faeces/(MALDI score)	CFU/mL milk/(MALDI score)	CFU/g Faeces/(MALDI score)
Bifidobacterium Breve(+ve, An)	0	0	0	$7\pm67x10^{7}$ (2.193)	0	$2\pm0.04x10^{9}$ (2.186)
Staphylococcus epidermidis(+ve , A)	0	0	$1.6\pm1.2x10^{1}$ (2.389)	0	$6.6\pm0.5x10^{2}$ (2.099)	0
Streptococcus pneumonia(+ve , A)	0	0	$1.3\pm0.6x10^{1}$ (2.077)	0	0	0
Bifidobacterium longum (+ve, An)	0	0	0	$4\pm60x10^{8}$ (2.152)	0	$1.8\pm0.03x10^{9}$ (2.083)
Staphylococcus hominis(+ve , A)	$2.1\pm0.28x10^{2}$ (2.18)	0	$6\pm1.7x10^{1}$ (2.353)	0	$1.9\pm0.04x10^{3}$ (2.344)	0
Staphylococcus haemolyticus(+ve , A)	0	0	$1\pm0x10^{1}$ (2.308)	0	0	0
Lactobacillus rhamnosus(+ve, An)	0	$8.5\pm5.7x10^{7}$ (2.084)	0	0	0	0
Serretia marcescens(-ve , An)	$3.4\pm0.28x10^{6}$ (2.309)	0	0	0	0	0
Klebsiella oxytocoa(-ve , A)	$1.2\pm0.14x10^{2}$ (2.322)	0	0	0	0	0
Stenotrophomonas maltophila(-ve , A)	$1.5\pm0.7x10^{2}$ (2.307)	0	0	0	0	0
Pseudomonas spp(-ve , A)	$8\pm1.4x10^{1}$ (1.859)	0	0	0	0	0
Sphingobacterium spiritovorum(-ve , A)	$5.8\pm0.28x10^{2}$ (2.196)	0	0	0	0	0
Wautersiella falsenii(-ve , A)	$2\pm0.2x10^{2}$ (2.113)	0	0	0	0	0
Lactobacillus paracasei(+ve, An)	0	0	0	0	$2.5\pm0.3x10^{2}$ (2.359)	$1.3\pm0.04x10^{9}$ (2.417)
Propionibacterium avidum(+ve, An)	0	0	0	0	$3\pm1.5x10^{1}$ (2.131)	0

Lactobacillus gasseri(+ve, An)	0	0	0	$7.7 \pm 103x10^{7}$ (2.35)	0	0
Enterococcus avium (+ve , A)	0	0	0	$2.1 \pm 251x10^{6}$ (2.131)	0	$1 \pm 1412x10^{9}$ (2.266)
Enterococcus Faecium(+ve , A)	0	0	0	$4.5 \pm 65x10^{6}$ (2.43)	0	0
Enterococcus faecalis(+ve , A)	0	$2.2 \pm 0.2x10^{6}$ (2.099)	0	0	0	$5 \pm 0.2x10^{8}$ (2.419)
Lactobacillus zaea(+ve, An)	0	$4.5 \pm 2.1x10^{1}$ (2.034)	0	0	0	0

The table above shows the number of bacteria (measured in CFU) that were isolated from the culture of breast milk and faecal samples on two (Participant 7) to three sampling (Participant 5 & 6) occasions. Identification was carried out using MALDI-TOF formic acid extraction method with scores for genus/species level identification in brackets. 0- means the bacterial species was not isolated. P5 to P7- samples from participants 5 to 7. +ve-means gram-positive, -ve -means gram-negative, A-means Aerobic bacterium, An- Anaerobic bacterium

Table 4.4: Summary of bacteria common to mothers and their respective babies with their numbers

Pair 1		**Pair 2**		**Pair 3**	**Pair 4**	**Pair 5**	**Pair 6**	**Pair 7**	
Mother (Mean±SD)	**Baby (Mean±SD)**	**Mother (Mean±SD)**	**Baby (Mean±SD)**	NC	NC	NC	NC	**Mother (Mean±SD)**	**Baby (Mean±SD)**
Staphylococcus aureus $1 \pm 0.05x10^{2}$CFU/mL	*Staphylococcus aureus* $4.0 \pm 2x10^{7}$CFU/g	*Bifidobacterium breve* $4.7 \pm 0.26x10^{2}$CFU/mL	*Bifidobacterium breve* $5.9 \pm 2.9x10^{8}$CFU/g					*Lactobacillus paracasei* $2.5+0.3x10^{2}$CFU/mL	*Lactobacillus paracasei* $1.3+0x10^{9}$CFU/g

The table above is showing the bacterial species common to both mother and baby including their numbers. NC-No common bacteria found. Pair 1 to pair 7 represents the mother and baby pair from participants 1 to 7. Of all the mother-baby pairs, only pairs of participants 1, 2 and 7 revealed bacterial species common to the mother and respective baby using culture. Values are Mean±SD as extracted from Tables 4.2 and 4.3.

Table 4.5: Comparing the bacterial count and type from milk samples from participants 4 to 7 (P1-P3 not swabbed) to bacteria isolated from breast skin swabs. (Swab 1 was used before cleaning of nipple and areola. Swab 2 was used after cleaning of nipple and areola with 70% alcohol)

	Swab 1 (number of colonies)	Swab 2 (number of colonies)	Milk (CFU/mL)
PARTICIPANT 4			
Staphylococcus epidermidis	16	No growth on swab 2	$3.5\pm3.3x10^{2}$
Staphylococcus pasteuri	0		$7\pm1.4x10^{1}$
Bacillus pumilus	1		$1.9\pm0.2x10^{2}$
Streptococcus parasanguinis	0		$11.7\pm10.2x10^{1}$
Kocuria rhizophila	0		$8\pm2.8x\ 10^{1}$
PARTICIPANT 5			
Staphylococcus hominis	240	4	$2.1\pm0.28x10^{2}$
Streptococcus parasanguinis	44	0	0
Acinetobacter lwoffi	TMTC	0	0
Micrococcus luteus	21	13	0
Acinetobacter pittii	0	14	0
Serretia marcescens	0	0	$3.4\pm0.28x10^{2}$

Klebsiella oxytoca	0	0	$1.2\pm0.14x10^{2}$
Pseudomonas spp	0	0	$8\pm1.4x10^{1}$
Sphingobacterium spiritovorum	0	0	$5.8\pm0.28x10^{2}$
Candida orthopsilosis	0	0	$4\pm1.4x10^{1}$
Stenotrophomonas maltophila	0	0	$1.5\pm0.7x10^{2}$
Wauteriella falsenii	0	0	$2\pm0.2x10^{2}$
PARTICIPANT 6			
Staphylococcus haemolyticus	TMTC	0	$1\pm0x10^{1}$
Neisseria perflava	TMTC	0	0
Corynebacterium pseudodiphtheriticum	TMTC	0	0
Streptococcus parasanguinis	TMTC	1	0
Staphylococcus hominis	0	0	$6\pm1.7x10^{1}$
Staphylococcus epidermidis	0	0	$1.6\pm1.2x10^{1}$
Streptococcus pneumonia	0	0	$1.3\pm0.6x10^{1}$
PARTICIPANT 7			
Staphylococcus haemolyticus	TMTC	0	0

Staphylococcus hominis	TMTC	0	$1.9\pm0.04x10^3$
Corynebacterium mucifaciens	TMTC	0	0
Staphylococcus aureus	0	25	0
Staphylococcus epidermidis	0	108	$6.6\pm0.5x10^2$
Lactobacillus paracasei	0	0	$2.5\pm0.3x10^2$
Propionibacterium avidum	0	0	$3\pm1.5x10^1$

The table above is comparing the number and type of isolated colonies from breast skin swab and milk. TMTC-Too many to count. Swab 1: swab obtained before cleaning; swab 2: swab sample obtained after cleaning. Swab values are microbial colonies as counted on plates. Milk values are Mean±SD. Please note that swab samples were not obtained from participants 1 to 3, hence no data.

Following the completion of the culture-based analysis to investigate the bacteria in milk and faeces, qPCR was also performed to quantify the level of selected bacteria. Numerous studies have demonstrated the use of qPCR in the quantification of bacteria in various samples such as breast milk or faeces (Collado et al., 2009; Martín et al., 2012; Soto et al., 2014).

4.2 Molecular analysis: qPCR

4.2.1 Absolute quantification of bacterial DNA using standard curve

Quantification analysis was performed on all breast milk and faeces of seven participants using qPCR followed by absolute quantification analysis as described in section 3.2.2.4. A standard curve was generated for each bacterium with an Amplification Efficiency of reaction=2. (Which is E=100%) The standard curve was generated using a 10-fold serial dilution of DNA as described in section 3.2.2.4. The bacterial concentration in each sample was measured as $\log_{10}$ genome DNA equivalent/ µL and one genome was considered to be equivalent to a single cell as previously described by (Hoppener-Ogawa et al., 2007)

4.2.2 Calculating the genome equivalent of bacterial strains in each sample

The crossing point values from real-time PCR (Cq values) obtained from the amplification of specific genes of bacteria present in milk and faecal samples were interpolated into a standard calibration curve on Microsoft Excel and this was used to generate the genome equivalents (GE)/number of DNA genome copies in each of the samples.

Briefly, the following formula was used (Hoppener-Ogawa et al., 2007):

$$\text{Genome DNA equivalent/ µL} = X \frac{\text{ng} \times 6.0221 \times 10^{23} \text{molecules/mole}}{(N \times 650\text{g/mole}) \times 1 \times 10^{9} \text{ ng/g}}$$

Where X= amount/concentration of template (ng),

N=length of dsDNA/ genome size

660g/mole= average mass of 1bp of dsDNA. (An online calculator has also been employed for calculations from https://cels.uri.edu/gsc/cndna.html.)

The number of copies of DNA contained in the undiluted DNA extract (stock sample of extracted DNA) of each of the bacterial standards was calculated, and hence the number of copies in each of the diluted series was obtained (Hoppener-Ogawa et al., 2007).

For example, to quantify the level of staphylococci in the breast milk of 7 participants, a standard curve was generated by using 10-fold serial dilutions of genomic DNA isolated from *Staphylococcus aureus* NCTC 6571. The standard curve was generated by plotting the Cq values versus the genome equivalents (see section 4.2.2 for GE calculations) of *S. aureus* NCTC 6571 (Figure 4.1, Appendix 3A and 3B).

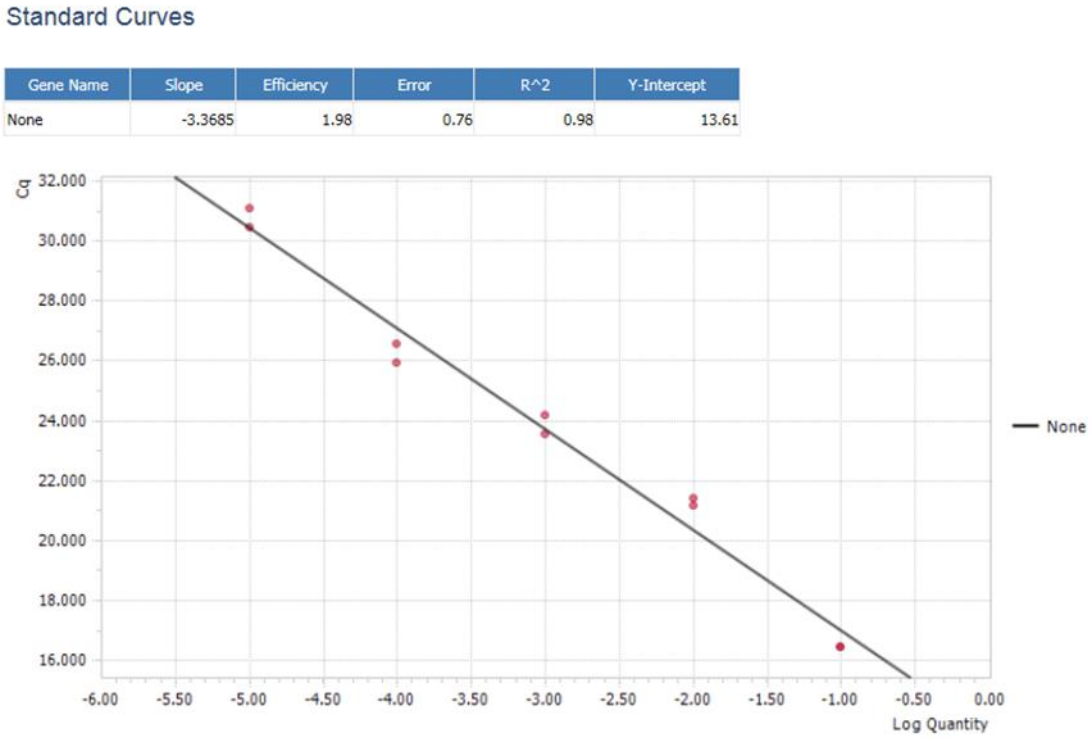


Figure 4.1: Standard curve for the absolute quantification of genus *Staphylococcus* in milk as generated from the qPCR run. Samples were diluted from 10^{-1} to 10^{-5} and was run in duplicates. Efficiency =1.98. The figure shows a graph of Cq values plotted against log quantity of DNA

The undiluted genomic DNA extract of *S. aureus* NCTC 6571 contained 20 ng/ µL genomic DNA, as measured by spectrophotometer. The size of the genomic DNA of *S. aureus* NCTC 6571 has been estimated to be 2.82 Mbp (The National Centre for Biotechnology Information). Considering that the weight of a double-stranded DNA is equivalent to 650 Da, therefore 2.82Mbp is equivalent to $1.8x10^9$Da. Thus, the undiluted DNA extract of *S. aureus* NCTC 6571 contained $6.57x10^6$ genomic DNA equivalents per 1 µL (see section 4.2.2). One genome equivalent was considered to be one cell as described in a protocol used by Hoppener-Ogawa et al., (2007). Log_{10}genome equivalent(cells)/ µL of staphylococci in DNA extract of unknown milk samples of participants 1 to 7 were then determined by using the linear regression line equation from the dilution standard curve (Table 4.6 and Figure 4.2).

Table 4.6: Cq values; concentrations of standards (from *Staphylococcus* genus) and unknown samples from breast milk of participants 1 to 7. Negative control has no Cq value due to expected lack of amplification

Concentration of standard	Sample type	Cq Value	Log_{10}genome equivalent(cells)/ µL
10^{-1}	Standard	16.43	5.82
10^{-2}	Standard	21.28	4.82
10^{-3}	Standard	23.86	3.82
10^{-4}	Standard	26.25	2.82
10^{-5}	Standard	30.79	1.82
Participant 1	Unknown	31.13	1.66
Participant2	Unknown	26.67	2.96
Participant 3	Unknown	28.98	2.28
Participant 4	Unknown	28.03	2.56
Participant 5	Unknown	29.24	2.21
Participant 6	Unknown	27.81	2.63
Participant 7	Unknown	23.85	3.78
Non template control	Negative control	-	-

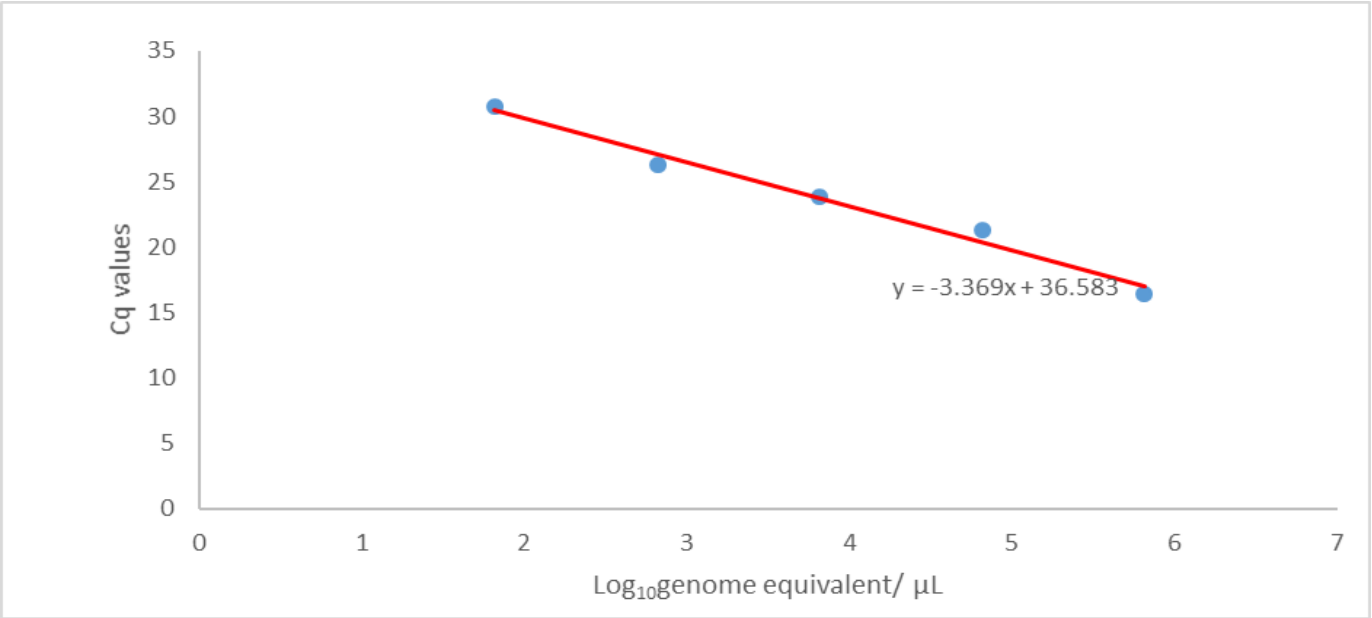


Figure 4.2: Standard curve for the genus *Staphylococcus* generated on excel by plotting Cq values of standards versus Log_{10}genome equivalent(cells) of standards diluted from 10^{-1} to 10^{5}. Efficiency=1.98. Unknown concentrations in breast milk were calculated using the regression line equation.

4.2.3 Melting peak analysis of Staphylococci PCR products

The melting peak analysis from running qPCR shows the presence of specific PCR products (i.e staphylococci) from the breast milk of all 7 participants (Figure 4.3) which were later confirmed by running on 1% agarose gel (Figure 4.4, Appendix 3A and 3B).

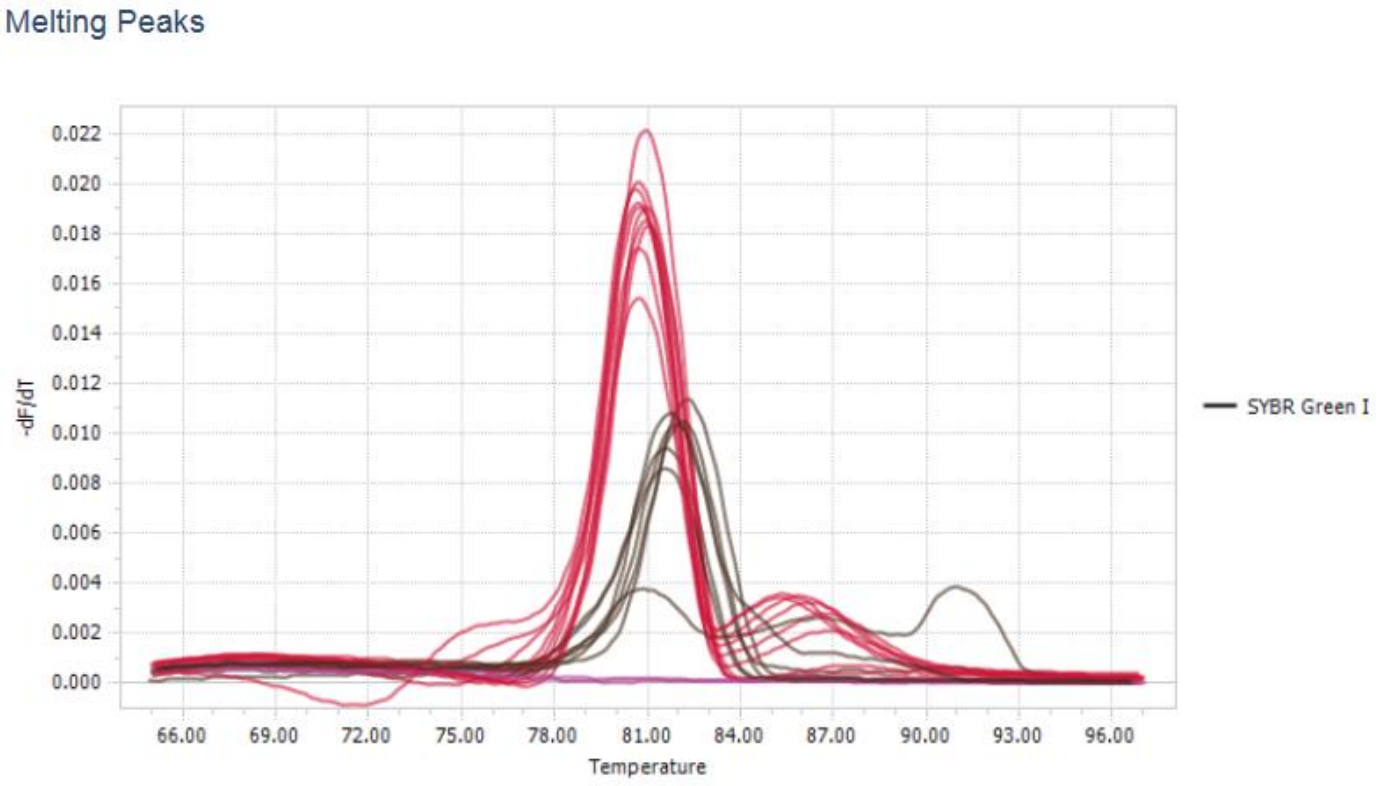


Figure 4.3: Melting peaks analysis of PCR products from standard dilutions of the reference strain of *Staphylococcus aureus* NCTC 6571 and PCR products from breast milk of 7 participants. The melting peak results from a change in fluorescence as the temperature increases (dF/dT, y-axis). On the x-axis is the temperature in °C. The Red colour peaks represent the standards while the brown peaks represent the unknown samples. The flat pink horizontal line represents the non-template control with expected no amplification.

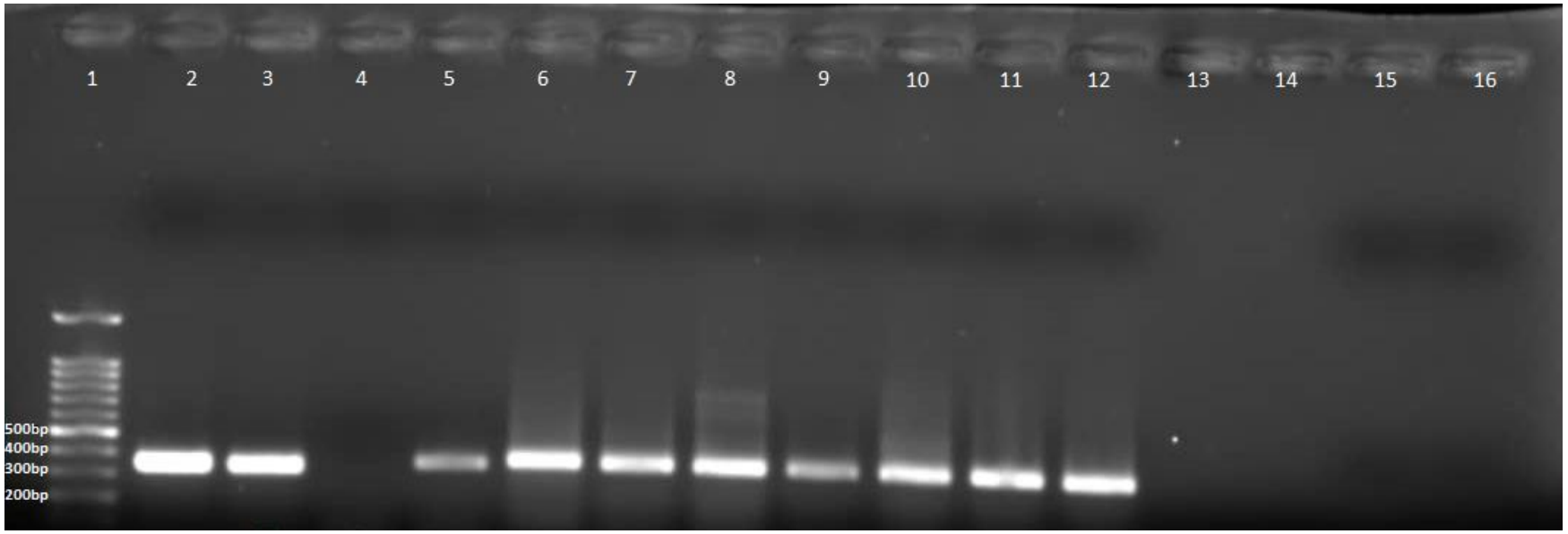


Figure 4.4: Gel image showing PCR products from milk samples of 7 participants using primers specific for *Staphylococcus*. Lanes 6 to 12 represent products from participants 1 to 7 respectively and correspond to 1.66, 2.96, 2.28, 2.56, 2.21, 2.63 and 3.78Log_{10}genome equivalent (cells)/ µL respectively. Lane 1 represents the ladder (1kb DNA ladder, Thermofischer Scientific) while lanes 2, 3 and 5 represent positive controls from standards -4, -5, and -6 respectively. Lanes 15 and 16 are non-template control and appear negative as expected with no amplification. Lanes 4, 13 and 14 are empty. Product size = 370bp

4.2.4 Quantifying the level of staphylococci in one millilitre (1mL) of breast milk (i.e GE(cells)/mL of milk)

It has been discussed previously that DNA was extracted from 2mL of breast milk and dissolved in 50 µL of buffer (section 3.2.2.1). It is also already known that 1 µL of undiluted extracted DNA from milk samples (as obtained from the interpolation of Cq values of the milk samples into the standard curve) contained a specific GE (cells) (Table 4.6). Therefore, 50µL of undiluted DNA extracted from 2ml of milk contains X50 of this.

For example, undiluted DNA extracted from 2mL of the milk sample of participant 1 contained 1.66 Log_{10}genome equivalent(cells) or 45.7GE (cells) of staphylococci per µL and $2x10^3$ GE(cells) in a total of 50 µL. Therefore, the breast milk of participant 1 contained $1.1x10^3$ GE (cells)/mL. All results for tested 7 participants are shown in Tables 4.7 and 4.8.

4.2.5 Level of staphylococci, streptococci, bifidobacteria, lactobacilli and enterococci in one millilitre of milk and one gram of faeces of the participants

The total number of cells (GE) belonging to staphylococci, streptococci, enterococci, bifidobacteria and lactobacilli in 1ml of milk and 1 gram of faeces of all the participants was calculated using the same method described in 4.2.4 (Table 4.7 and 4.8). The result of qPCR analysis using primers specific for staphylococci, streptococci, bifidobacteria, lactobacilli, and enterococci revealed that the breast milk and faeces of all 7 mother-baby pairs contained bacterial DNA belonging to these groups of bacteria. The level of staphylococci and streptococci in breast milk ($1.5x10^5$ genome equivalent (cells)/mL of milk) were the highest as seen in participant 7 and 2 respectively while the level of lactobacilli was the lowest in breast milk samples (8.3 genome equivalent (cells)/ml of milk) [Table 4.7 and 4.8] as observed in participant 4. In faecal samples, the level of *Bifidobacterium* was the highest ($2.1x10^9$ genome equivalent (cells)/g of faeces) while

the level of *Lactobacillus* (1.25 genome equivalents(cells)/g of faeces) was the lowest (Table 4.7 and 4.8).

Table 4.7: Genomic Equivalent (cells)/mL of milk or (cells)/g of faeces in participants 1 to 4 as detected by qPCR absolute quantification

Bacterial Genera	GE (cells)/mL of milk and GE (cells)/g of faeces							
	Participant 1		Participant 2		Participant 3		Participant 4	
	Milk	Faeces	Milk	Faeces	Milk	Faeces	Milk	Faeces
Streptococci	$3.8x10^2$	$3.1\ x10^7$	$1.5\ x10^5$	$9.5\ x10^7$	$6.3\ x10^4$	$2\ x10^8$	$5.6\ x10^3$	$5.5\ x10^6$
Staphylococci	$1.1x\ 10^3$	$2.0\ x10^2$	$2.3\ x10^4$	$9.3\ x10^5$	$4.8\ x10^3$	$1.5\ x10^5$	$9.1\ x10^3$	$8.9\ x10^4$
Enterococci	$2.1\ x10^3$	$1.4\ x10^7$	$3.7\ x10^4$	$1.5\ x10^7$	$1.3\ x10^4$	$1.3\ x10^7$	$1.3\ x10^3$	$9.8\ x10^5$
Lactobacilli	$3.1\ x10^1$	$7.2\ x10^5$	$6.3\ x10^1$	1.25297	$2.2\ x10^1$	$4.1\ x10^7$	8.27828	$4.9\ x10^1$
Bifidobacteria	$1.1\ x10^4$	$4.3\ x10^8$	$5.7\ x10^3$	$9.1\ x10^8$	$1.1\ x10^4$	$2.1\ x10^9$	$1.9\ x10^3$	$8.1\ x10^8$

Table 4.8:Genomic Equivalent (cells)/mL of milk or (cells)/g of faeces in participants 5 to 7 as detected by qPCR absolute quantification

Bacterial genera	GE (cells)/mL of milk and GE (cells)/g of faeces					
	Participant 5		Participant 6		Participant 7	
	Milk	Faeces	Milk	Faeces	Milk	Faeces
Streptococci	$2.2\ x10^4$	$1.3\ x10^7$	$2.7\ x10^4$	$4.1\ x10^6$	$9.7\ x10^3$	$1.8\ x10^7$
Staphylococci	$4.1\ x10^3$	$2.6\ x10^4$	$1.1\ x10^4$	$2.3\ x10^4$	$1.5\ x10^5$	$3.1\ x10^3$
Enterococci	$9.5\ x10^3$	$1\ x10^6$	$3.1\ x10^4$	$5.9\ x10^5$	$8.9\ x10^3$	$1.9\ x10^7$
Lactobacilli	$1.6\ x10^1$	$2.3\ x10^7$	$3.3\ x10^1$	$4.8\ x10^3$	$4.5\ x10^1$	$4.2\ x10^6$
Bifidobacteria	$2.3\ x10^4$	$7.4\ x10^8$	$2.6\ x10^4$	$2\ x10^8$	$1.9\ x10^4$	$1.2\ x10^9$

4.3 Comparison of levels of bacteria in milk and faeces estimated by culture and qPCR.

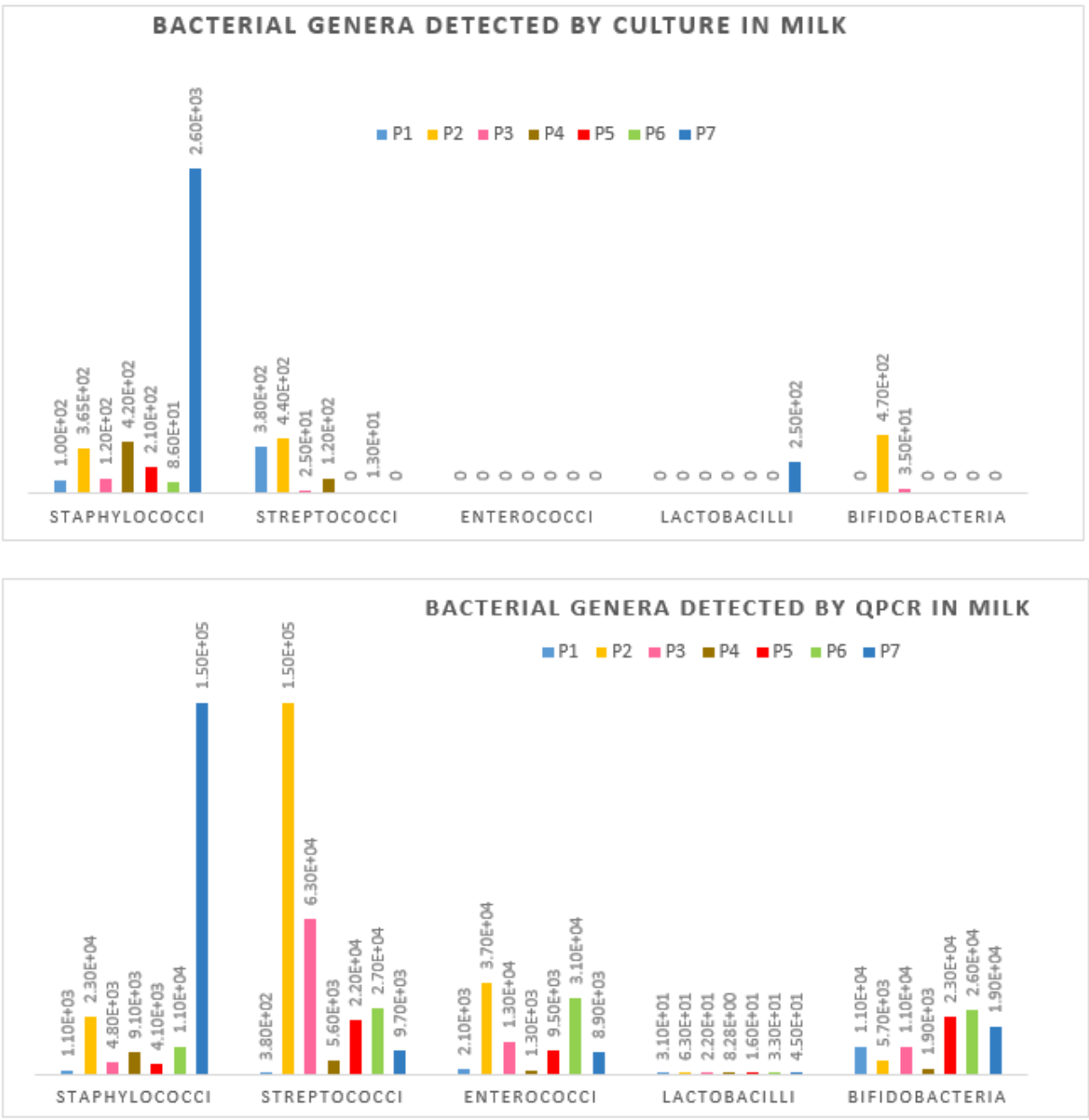


Figure 4.5: Comparing the detection of microbial genera in milk by culture and quantitative PCR. P1 to P7- Participants 1 to 7, each participant with its distinguished colour-coded bars indicating the concentrations of each of the 5 bacterial genera. The upper graph represents the bacterial genera isolated by culture (CFU/mL) in the milk of 7 participants while the lower graph represents the bacterial genera detected and quantified by qPCR (GE(cells)/mL) in the milk of 7 participants

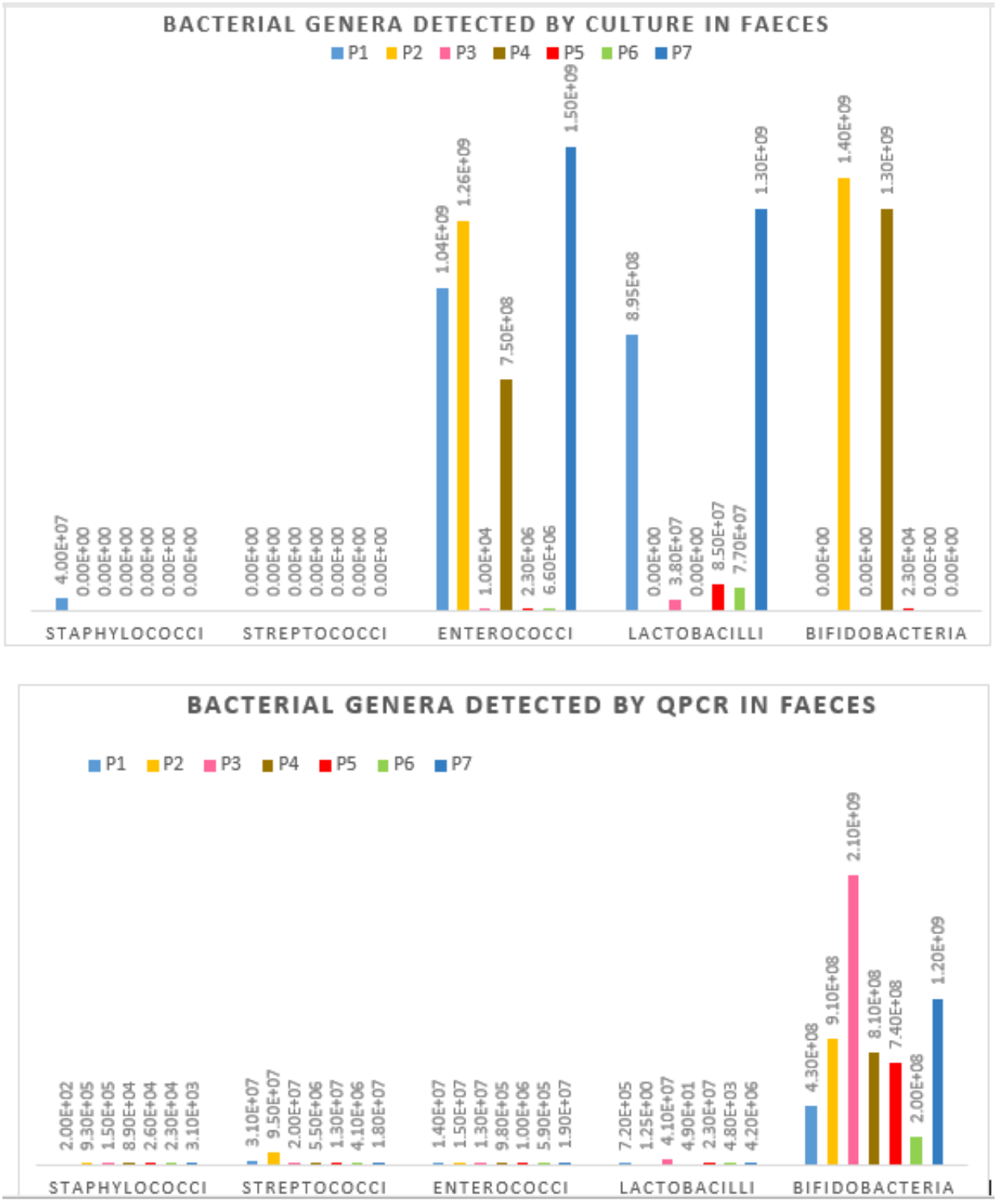


Figure 4.6: Comparing the detection of microbial genera in faeces by culture and quantitative PCR. P1 to P7- Participants 1 to 7, each participant with its distinguished colour-coded bars indicating the concentrations of each of the 5 bacterial genera. The upper graph represents the bacterial genera isolated by culture (CFU/g) in the faeces of 7 participants while the lower graph represents the bacterial genera detected and quantified by qPCR (GE(cells)/g) in the faeces of 7 participants

The qPCR results as shown in figures 4.5 and 4.6 above showed that DNA from all of the analysed bacterial genera could be identified in the milk and faeces of all participants. Some of these are consistent with the result of culture, however, some bacterial genera such as enterococci and streptococci could not be detected in breast milk and faeces respectively by culture only.

By culture, the bacterial genera with the highest frequency in milk is staphylococci with a count ranging from $8x10^1$ to $2x10^3$ CFU/mL, however by qPCR, all 5 selected bacterial genera could be detected but staphylococci and streptococci were the genera with highest concentrations ($1.5x10^5$ GE(cells)/mL). *Lactobacillus* was the genus with the lowest concentrations in milk by both qPCR and culture (Figure 4.5).

In faeces, enterococci are the most detected bacterial genera by culture, detected in all participants with counts ranging from $1x10^4$ to $1x10^9$ CFU/g, followed by lactobacilli which were detected in 3 participants with counts ranging from $3x10^7$ to $1x10^9$CFU/g. By qPCR, all 5 selected bacteria genera were detected in all participants however bifidobacteria were found to be of higher concentration ($2x10^8$ - $2x10^9$GE(cells)/g) (Figure 4.6).

4.4 Discussion (Pilot study)

This preliminary study was carried out to optimise methods used in the larger study. The study investigated the presence of five selected bacterial genera which include bifidobacteria, lactobacilli, streptococci, staphylococci, and enterococci in the breast milk of 7 mothers as well as the faecal samples of their babies using culture-dependent (identification by MALDI-TOF Biotyper) and culture-independent method (extraction of microbial DNA followed by qPCR).

Firstly, to rule out the presence of any skin contaminants in milk, swab samples were obtained before and after cleaning the nipple and areola with alcohol wipes. It is important to note that skin contamination is almost unavoidable when sampling breast milk. As many other studies have

reported, the skin microbiome can affect the composition of breast milk (Gomez-Gallego et al., 2016; Pannaraj et al., 2017). The result of this study has revealed this as well. The number of bacteria present on the swab reduced after cleaning, however for most of the participants, these bacteria were not eliminated. This information can however help to identify the bacteria present on the skin which could come in contact with the breast milk and exclude them from other bacteria. Bacteria commonly isolated from milk samples were mainly staphylococci and streptococci, however common bacteria isolated from swab in this study were more diverse and included *Staphylococcus epidermidis, Micrococcus luteus, Corynebacterium, Bacillus pumillus, Staphylococcus hominis, Streptococcus parasanguinis, Acinetobacter lwoffi and Staphylococcus haemolyticus.* The contribution of breast skin bacteria towards the bacteria present in breast milk cannot be ruled out. That being said, the origin of the breast milk microbiota cannot be linked to the breast skin alone. For example, bifidobacteria which is a strict anaerobe was not isolated from any of the swabs in our pilot study but was isolated in the breast milk of two participants, although in low number (0.3- 4 $x10^2$ CFU/mL) and later confirmed by qPCR.

The most isolated genera from the breast milk of 7 subjects by culture were staphylococci and streptococci. Similar genera were frequently detected across all participants using qPCR. This is consistent with other research such as the study carried out to characterise the diversity of human milk bacteria among South-African women (Ojo-Okunola et al., 2019) as well as studies carried out among breastfeeding mothers in Switzerland (Jost et al., 2013).

Culture dependent technique revealed viridans streptococci including the species salivarius, vestibularis and pneumoniae to be common in the breast milk of these participants. Numerous studies have also reported the abundance of streptococcal species in breast milk (Collado et al., 2009; Jost et al., 2013; Martín et al., 2003; Martín et al., 2016). For example, Martín et al. (2016) used MALDI-TOF and 16S rDNA gene sequencing to identify the streptococcal species present

in breast milk and reported streptococci to be among the core genera of the milk microbiota, which included *Streptococcus salivarius, Streptococcus mitis*, and *Streptococcus parasanguinis*.

Bifidobacteria and lactobacilli are attractive because of their use as probiotics. By culture, *Lactobacillus* was isolated from the breast milk of one of the participants, whereas *Bifidobacterium* was isolated from two participants. However, by qPCR, these two genera were detected in all participants. Similarly, in a study carried out by Murphy et al. (2017) on 10 healthy mothers and their babies on the composition of human breast milk and faeces of babies over the first 3 months of life using culture technique, lactobacilli and bifidobacteria were isolated from only one breast milk and its corresponding faecal sample. Martin et al. (2012), also cultured 66 breast milk samples, and of the 66 cultured samples, bifidobacteria and lactobacilli could be isolated from 7 and 27 samples, respectively. A recent study carried out by Chen et al. (2018) in Taiwan on the profiling of commensal and opportunistic bacteria in the breast milk of 33 mothers using Illumina MiSeq also reported that bifidobacteria accounted for less than 1% of bacterial cells in most milk samples while lactobacilli appeared to be very little and were isolated from 7 samples. The reason behind the infrequent isolation or inability to isolate these bacteria from breast milk could be that there is generally a low abundance of these bacteria in breast milk to be detected by culture or some strains may require fastidious growth requirements (Martín et al., 2009; Murphy et al., 2017).

Bacteria that were less commonly isolated in milk in this study (isolated from one mother only) were *Serretia marcescens, Stenotophomonas maltophilia, Pseudomonas* spp*, Lactobacillis* and *Sphingobacterium spiritovorum.* This is consistent with the culture-independent study on 16 breastfeeding women carried out by Hunt et al. (2011). *Serretia* was among the most abundant genus in breast milk while others such as *Stenotophomonas maltophilia, Pseudomonas* spp and *Sphingobacterium spiritovorum* represent less than 1% across the 16 women. *Klebsiella oxytoca,* and *Wautersiella falsenii* were isolated from 1 of the participants. These species have not been

reported as part of the breast milk microbiome, therefore further investigations may be needed to confirm their origin.

4.4.1 Bacteria isolated from faecal samples

Enterococcus faecalis, Lactobacillus salivarius, Lactobacillus rhamnosus, Lactobacillus zeae Bifidobacterium longum, Bifidobacterium breve and *Staphylococcus aureus* were identified in faecal samples by culture, and the genera staphylococci, bifidobacteria, streptococci, enterococci and lactobacilli were detected by qPCR. Bifidobacteria, enterococci, and lactobacilli were the most abundant bacteria in the seven women by both culture and qPCR, whereas staphylococci were the least abundant. All the isolated and detected genera, however, had previously been documented in research (Martín et al., 2012; Murphy et al., 2017). Martin et al. (2012), for example, found staphylococci, lactobacilli, and bifidobacteria to be among the most frequently isolated taxa in the faeces of 20 Spanish babies using culture and quantitative real-time PCR. Similarly, Murphy et al. (2017) reported the dominance of genera *Bifidobacterium*, *Bacteroides*, *Enterococcus*, *Lactobacillus*, *Clostridium*, *Coprococcus*, and *Escherichia-Shigella* in baby faeces using MiSeq sequencing.

No streptococcal species were isolated in the faeces of babies in the present study by culture, but it was detected by qPCR in the faeces of all participants corresponding to ranges between $1x10^6$ to $1x10^7$ GE (cells)/gram of faeces. This is in contrast with a community-based study undertaken among 121 mothers and infants pairs in Southern Mozambique who identified streptococci (using both culture and molecular techniques) to be one of the most abundant species isolated from the faeces of infants (González et al., 2013). The reason behind the inability to isolate streptococci in faeces in the present study can be linked to our methodology. Selective media for streptococci was not among the selective media used in the cultivation of faecal samples in this study, although SB agar can also support the growth of streptococci

4.4.2 Common bacteria in human milk and faeces identified by culture and qPCR

Bacteria shown to be common to mother and baby pairs using culture-dependent methods are *Staphylococcus aureus*, *Bifidobacterium breve* and *Lactobacillus paracasei*. This is in line with the study carried out by Martin et al. (2012), on the bacterial strains that are shared between mothers' milk and the faeces of their babies. Culturable species of the genera staphylococci, bifidobacteria and lactobacilli were shared among mothers and babies, in which 17 and 11 mother and baby pairs shared *Staphylococcus epidermidis* and *Staphylococcus hominis*, respectively. *Lactobacillus fermentum* and *Lactobacillus gasseri* were reported to be shared among 3 mother-baby pairs, while 3 mother and baby pairs also shared similar species of bifidobacteria.

Various other studies carried out by culture-dependent and molecular techniques have also suggested that breast milk is a potential source of bacteria to the infant's gut, and some have reported the sharing of bacterial strains between mother's milk and baby's faeces (Albesharat et al., 2011; Jiménez et al., 2008; Martín et al., 2012). Similarly, Albesharat et al. (2011) used Randomly amplified polymorphic DNA (RAPD) and MALDI-TOF-MS patterns to compare species of bacteria present in the breast milk of mothers and the faeces of their corresponding babies. He reported identical RAPD genotypes *of L. plantarum, L. fermentum, L. brevis, Enterococcus faecium, Enterococcus faecalis and P. pentosaceus* in the milk of mothers and faeces of their corresponding babies. Although the above studies on the diversity of bacteria in breast milk and infant faeces have reported that some bacterial strains are shared between breast milk of mothers and corresponding baby faeces, the current study does not seem to show the same, however, one needs to put the difference in methodology into consideration. For example, the analytical technique used by Albesharat et al. (2011) allowed strain detection while the current approach did not.

4.4.3 Comparison of traditional culture with qPCR analysis

The result of qPCR revealed that all 5 bacterial genera investigated could be detected in breast milk and faecal samples, which is consistent with the result of the culture. The choice of primers used for qPCR reaction in this pilot study were for the specific detection of bacterial genera and not species, therefore it was not possible to evaluate the similarity of bacteria detected by qPCR with the bacteria isolated by culture (identified by MALDI-TOF) at the species level. In terms of their sensitivity, it has been demonstrated that qPCR is more sensitive in the detection of bacteria than culture in the investigation of bacteria in breast milk and faecal samples of babies coupled with its reduced turn-around time. In terms of bacteria level, qPCR did not only detect more bacterial genera but also in high abundance when compared to culture. For example, while staphylococci ranged from 10^1 to 10^2 CFU/mL of milk in participants 1 to 7 by culture, that of qPCR ranged from 10^3 to 10^5 GE (cells)/ml of milk. This result should, however, be interpreted with caution since qPCR like many other non-culture-based detection methods, cannot distinguish between dead and viable cells which may lead to an overestimation of bacterial levels. Meanwhile, Emerson et al. (2017) investigated how the issue of bacterial viability can be resolved when employing nucleic-acid-based identification methods in a procedure known as ‘‘viability PCR’’. Dyes such as ethidium monoazide or propidium monoazide can penetrate a damaged cell and on exposure to light, irreversibly damage the DNA leaving the DNA of live cells only; to be subsequently affected by extraction steps. This approach of viability assay although promising is laborious and still unable to completely differentiate live cells from dead cells (Codony et al., 2019). Research is still ongoing to improve on the approach and an example is the study of Cechova et al. (2021) that allowed the identification of *Mycobacterium avium* subsp. *Paratuberculosis* with minimal time and increased potential to differentiate dead cells from live cells.

Surprisingly in faeces, the number of bacteria present as detected by qPCR appeared lower than that of culture. For example, the levels of enterococci, bifidobacteria and lactobacilli isolated by culture are higher than that of qPCR. While the number of enterococci as observed by culture method is about 10^9 CFU/g of faeces, the number as detected in similar participants by qPCR is about 10^7cells/g of faeces. This could be due to the presence of inhibitors that could affect qPCR results in faeces because faeces are known to contain many inhibitors which can affect downstream applications (Acharya et al., 2017). Passing DNA extracts from faeces through an inhibitor removal kit may in the future help to improve the results of downstream applications, by removing inhibitors such as phenolic compounds, fats, cellulose, glycogen etc. from impure DNA (Oikarinen et al., 2009). More importantly, comparison between the results of culture and PCR must be carried out with caution as the latter is based on extracted DNA of bacteria while culture is based on viable cells.

In conclusion, the findings of this pilot study revealed the presence of bacteria in breast milk and faeces of babies using both culture and qPCR. In terms of methods employed, it has been shown that qPCR is more sensitive than culture in the detection of bacteria in human milk and faeces of babies, although only culture can confirm the viability of bacteria. It was not possible to make any strong claims regarding the relationship between the microbiota of breast milk and the baby's gut due to the number of participants included in this pilot as well as the methodology employed (i.e., culture technique and qPCR), however, to better understand the microbiome of breast milk and to achieve the overall goal of the study,16S sequencing was employed to study the microbiota of breast milk and gut of babies. This is expected to provide a better picture of bacterial complexity and their quantity in samples when compared to culture and qPCR. Details of the results obtained using this method are discussed in Chapter 5 of this thesis.

Chapter 5 : Results obtained from analysis of the samples collected from Nigerian participants

5.1 Characteristics of lactating mothers and breastfed babies

Samples of human milk and faeces of breastfed babies in Nigeria were obtained, and the microbiota was characterised by both traditional culture and MALDI-TOF as well as 16S rDNA sequencing approach. Table 5.1 summarises the characteristics of the 50 breastfeeding mothers and babies whose samples were included in the study.

Table 5.1: Sociodemographic and clinical characteristics of breastfeeding mothers and breastfed babies

Maternal data	***n*** (%)
Age[a]	28.58±5.8
Birthplace	
Nigeria	50 (100)
Main activity	
Home	17 (34)
Outside the home	33 (66)
Occupation	
Teacher	9 (18)
Banker	1 (2)
Trader	20 (40)
Full housewife	8 (16)
Tailor	5 (10)
NR	2 (4)
Secretary	1 (2)
Student	1 (2)
Health Visitor	1 (2)
Stylist	1 (2)
Nursing	1 (2)

Education level[b]	
Tertiary	36 (72)
Secondary	13 (26)
Primary	1 (2.08)
Parity	
Uniparous	28 (56)
Multiparous	22 (44)
Neonatal mode of delivery	
Vaginal (Natural)	38 (76)
C-Section	12 (24)

Neonatal data

Sex	
Female	24 (48)
Male	26 (52)
Mode of delivery	
Vaginal	38 (76)
C-Section	12 (24)

Notes

Please note that sequencing could not be carried out on samples from 2 mothers and babies due to low concentration of DNA

n-sample number

NR- No response

[a]Expressed as mean ± standard deviation

[b]Equivalent based on Nigeria's Ministry of Education

5.2 Characteristics of formula-fed babies included in the study

Exclusively formula-fed babies living in orphanage homes in Nigeria were enrolled to participate in this study. Faecal samples were initially meant to be obtained from 25 exclusively formula-fed babies, however, due to the widespread breastfeeding culture in Nigeria (Berde & Yalcin, 2016), it was not possible to obtain samples from 25 babies. Therefore, a total of 8 exclusively formula-fed babies were included in the study (Table 5.2). Participants' information sheets were given to their guardians and consent was sought before administering the questionnaire and obtaining the samples as discussed in section 3.3.5.

Table 5.2: Basic demographic details of 8 formula-fed babies included in the study

Participant ID	**Age**	**Gender**	**Mode of delivery**	**Presence of prebiotics in infant formula**	**Presence of probiotics in infant formula**
FM1	4 months	M	Natural	No	*Bifidobacterium lactis*
FM2	6 months	F	Natural	No	*Bifidobacterium lactis*
FM3	5 months	M	Natural	No	*Bifidobacterium lactis*
FM4	5months	F	Natural	No	*Bifidobacterium lactis*
FM5	1 month	M	Natural	No	*Bifidobacterium lactis*
FM6	5 months	F	Natural	No	*Bifidobacterium lactis*
FM7	4 months	M	Natural	No	*Bifidobacterium lactis*
FM8	1 month	F	Natural	No	None

FM1-FM8 represents the 8 formula-fed babies included in the study. The presence of probiotics or prebiotics in the infant formula the 8 babies drank was assessed by using a questionnaire (Appendix 1, Section B).

5.3 Analysis of milk and faecal sample by culture and MALFI-TOF Biotyper

All samples were cultured as previously discussed in section 3.3.7. Upon culture and incubation of plates, visible colonies were observed by using bright light and divided into types according to their size, shape, colour and texture. Up to five of each colony type were purified and identified by MALDI-Biotyper as previously discussed in section 3.2.1.6.

5.3.1 Identification of microorganisms present in the breast milk of 50 mothers by using MALDI-TOF MS Biotyper

The MALDI Biotyper (MBT) was used for the rapid identification of selected colonies. Human milk of Nigerian mothers included in this study generally harboured a low number of bacteria ranging from 1 to 4 log CFU/mL by culture. In total, 7 microbial genera were isolated by culture from milk samples of the participants. Four of them are gram-positive cocci bacteria, while one of them belonged to gram-positive bacilli. No gram-negative bacteria could be isolated by culture from all the samples. In total, 13 microbial species were identified across all the milk samples with scores of scores 2.300 and above (Figure 5.1). *Streptococcus salivarius* and *Staphylococcus epidermidis* were isolated from 25.5% and 23.5% of the milk of breastfeeding mothers using blood agar respectively. *Rothia mucilaginosa* and *Staphylococcus hominis* were isolated each from the milk of 21.5% of the mothers using blood agar. *Wickerhamomyces anomalus* was also detected in 13.7% of mothers' milk on BIM-25, while *Lactobacillus plantarum* and *Staphylococcus aureus* occurred each in 5.8% of the breast milk mothers on blood and rogosa agar plates respectively. Furthermore, *Candida krusei* and *Lactobacillus brevis* occurred each in 3.9% of all mothers and were isolated on rogosa respectively, while *Enterococcus faecium* (retrieved from SB agar), *Candida kefyr* (retrieved from rogosa), *Staphylococcus haemolyticus* and *Streptococcus vestibularis* (retrieved from blood agar) were only isolated from less than 1% of the breastfeeding mothers.

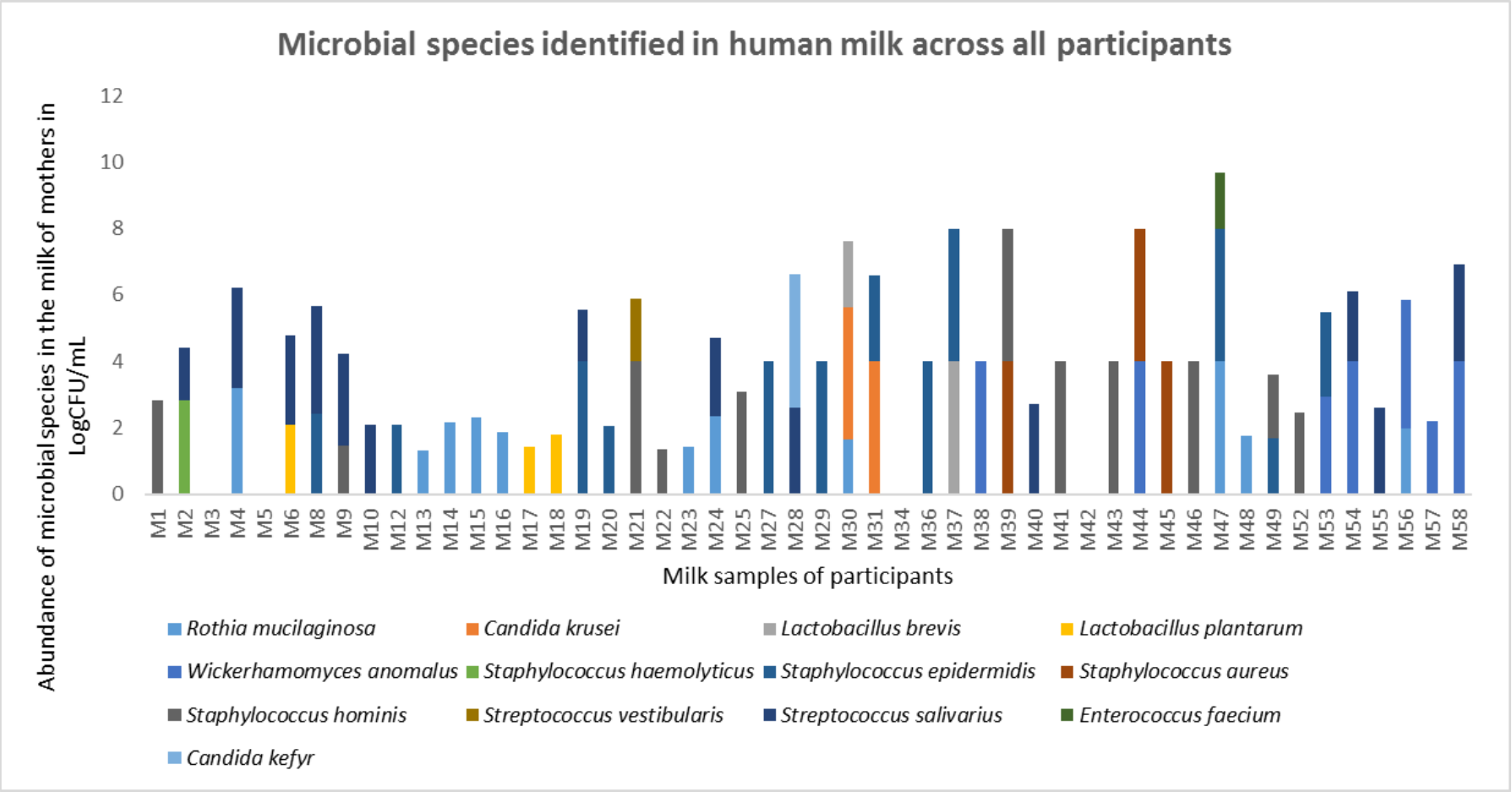


Figure 5.1: Microbial species present in human milk by culture. Identification was carried out by MALDI-TOF MS Biotyper. The relative abundance of species is shown on the y-axis as $\log_{10}$CFU/mL. On the x-axis is each mother's milk represented by 'M' followed by their identification number. The coloured bars represent the microbial species identified. No growth recovered from samples M3, M5, M34 and M42.

Of all the microorganisms isolated from human milk by culture, the most abundant is *Staphylococcus* which accounted for 57% of all the microorganisms identified in human milk, while the least abundant in human milk by culture belonged to Enterococcus (<1%) (Figure 5.2).

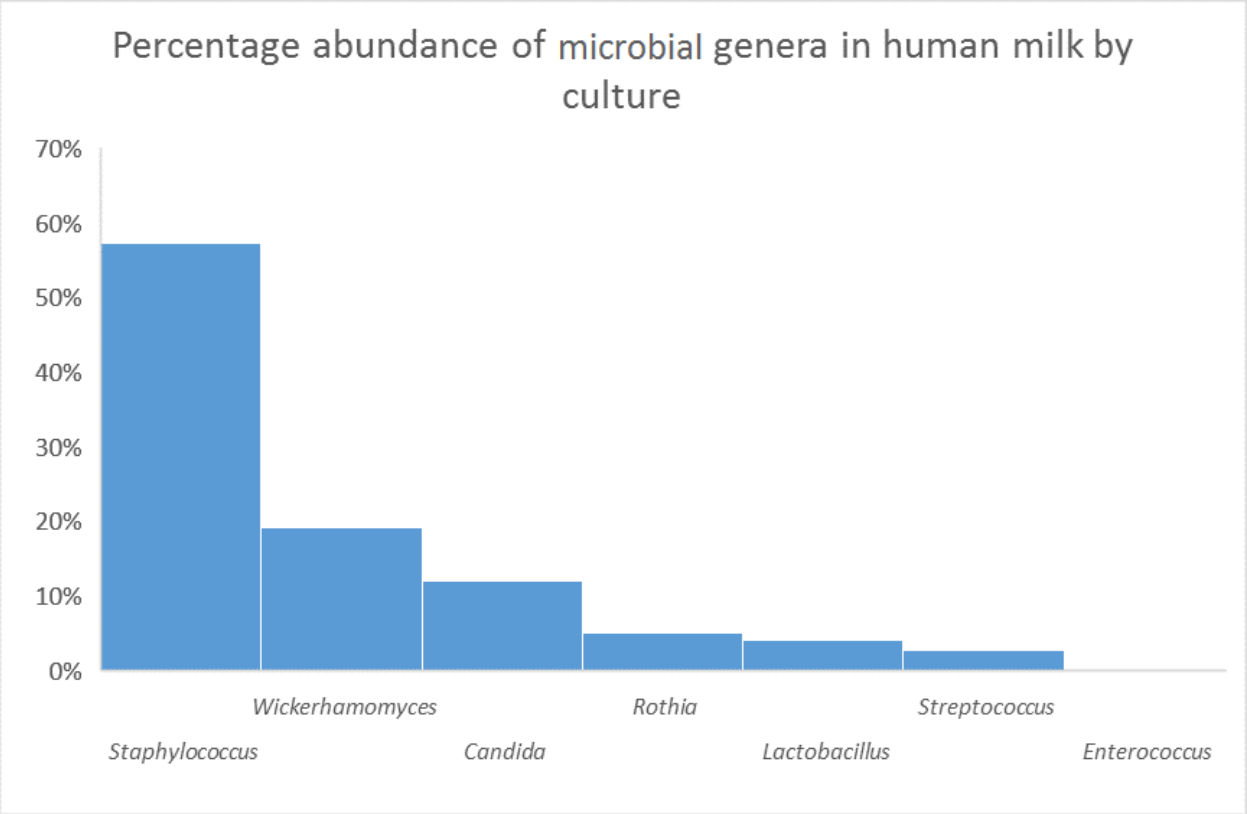


Figure 5.2: Abundance (%) of microbial genera in breast milk across all participants identified by MALDI-TOF Biotyper. The abundance was expressed as percentages with N=50. The bacteria *Staphylococcus* represents the most abundant genus identified in human milk by culture accounting for 57% of all the microorganisms present.

5.3.2 Identification of microorganisms present in faeces of 50 breastfed babies by MALDI-TOF MS Biotyper

In total, 19 microbial species belonging to 7 genera were identified from the faeces of breastfed babies. Microbial counts in faeces of breastfed babies ranged from 4.48 -9.34 log CFU/g. The summary of the results of identified microorganism is shown in figure 5.3. The genus *Enterococcus* (retrieved from SB agar) appeared to be the most predominant microbe across all

samples occurring in 88% of all the participants, followed by the genera lactobacilli (retrieved from rogosa agar) which occurred in 42% of all the participants. Other microbial genera that occurred in 2% of the participants include *Bifidobacterium* (retrieved from BIM-25 agar), *Staphylococcus/ Streptococcus* (SB agar), *Klebsiella /Escherichia* (BIM-25 agar) and *Candida* (rogosa agar).

Of all the microorganisms isolated from faeces of breastfed babies by culture, the most abundant genera were *Enterococcus* (64%), next to *Lactobacillus* (27%) and *Streptococcus* (3%). *Klebsiella* and *Candida* were the least abundant genera and accounted for 1% of the total microorganisms present (Figure 5.4).

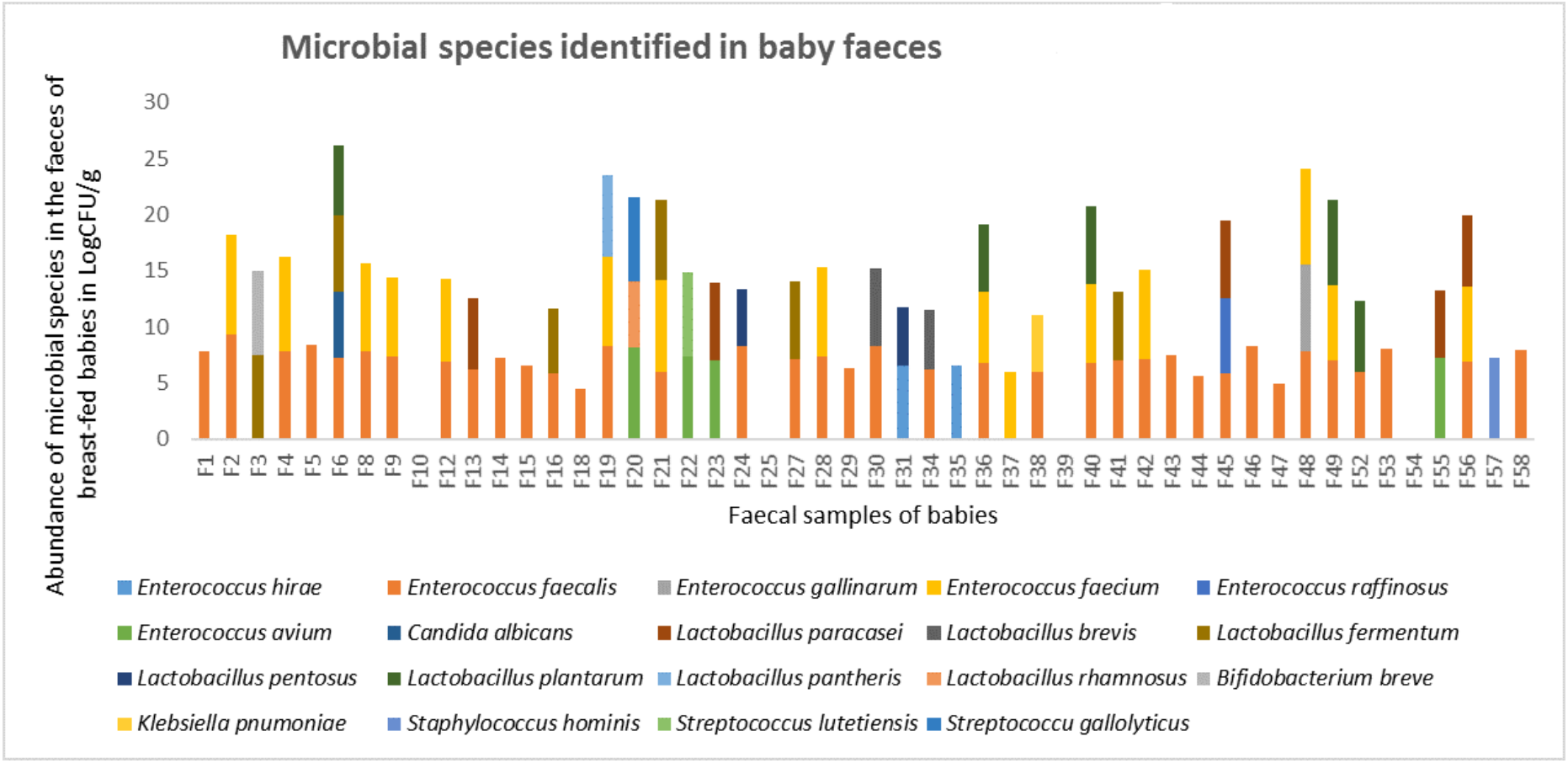


Figure 5.3 Microbial species present in the faeces of breastfed babies by culture. Microbial identification was carried out by MALDI-TOF MS Biotyper. The relative abundance of microbial species is shown on the y-axis as log_{10}CFU/g. On the x-axis are each baby's faeces represented by 'F' followed by their identification number. The coloured bars represent the microbial species identified. No growth recovered from samples F10, F25, F39 and F54.

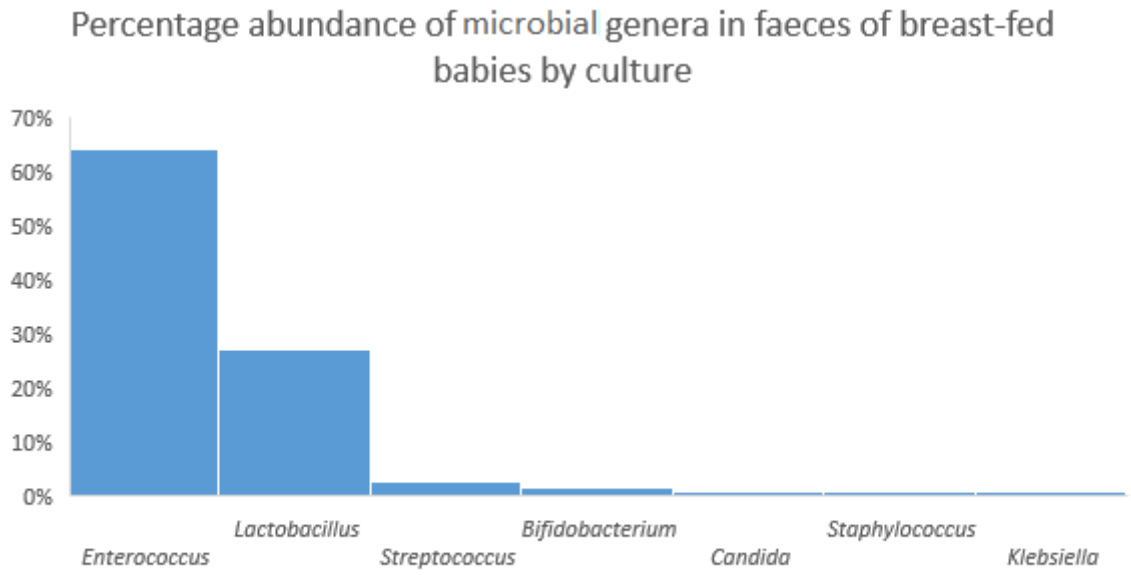


Figure 5.4: Abundance (%) of microbial species in faeces of breastfed babies across all participants identified by MALDI-TOF Biotyper. The abundance was expressed as percentages with N=50. *Enterococcus* represents the most abundant genus identified in the faeces of breastfed babies.

5.3.3 Identification of microorganisms present in the faeces of 8 formula-fed babies by MALDI-TOF MS Biotyper

In total, 6 microbial isolates belonging to 4 genera were identified from the faeces of babies who were exclusively fed with formula with counts ranging from 4.7 -8.4 log CFU/g. The genus *Enterococcus* was the most predominant microorganism across all samples occurring in 7 of the participants, followed by the genera lactobacilli which occurred in 4 of the 8 participants. Other microbial genera are *Escherichia* and *Candida* (Figure 5.5).

Of all the microorganisms isolated, the most abundant is *Enterococcus faecalis* which accounted for 64% of all the microorganisms identified in faeces of breastfed babies cultured on SB agar, next to *Lactobacillus fermentum* (18.3%) and *Enterococcus faecium* (10%). *Escherichia coli* and *Candida kefyr* were the least abundant and accounted for less than 1% of the population and was isolated on BIM-25 agar.

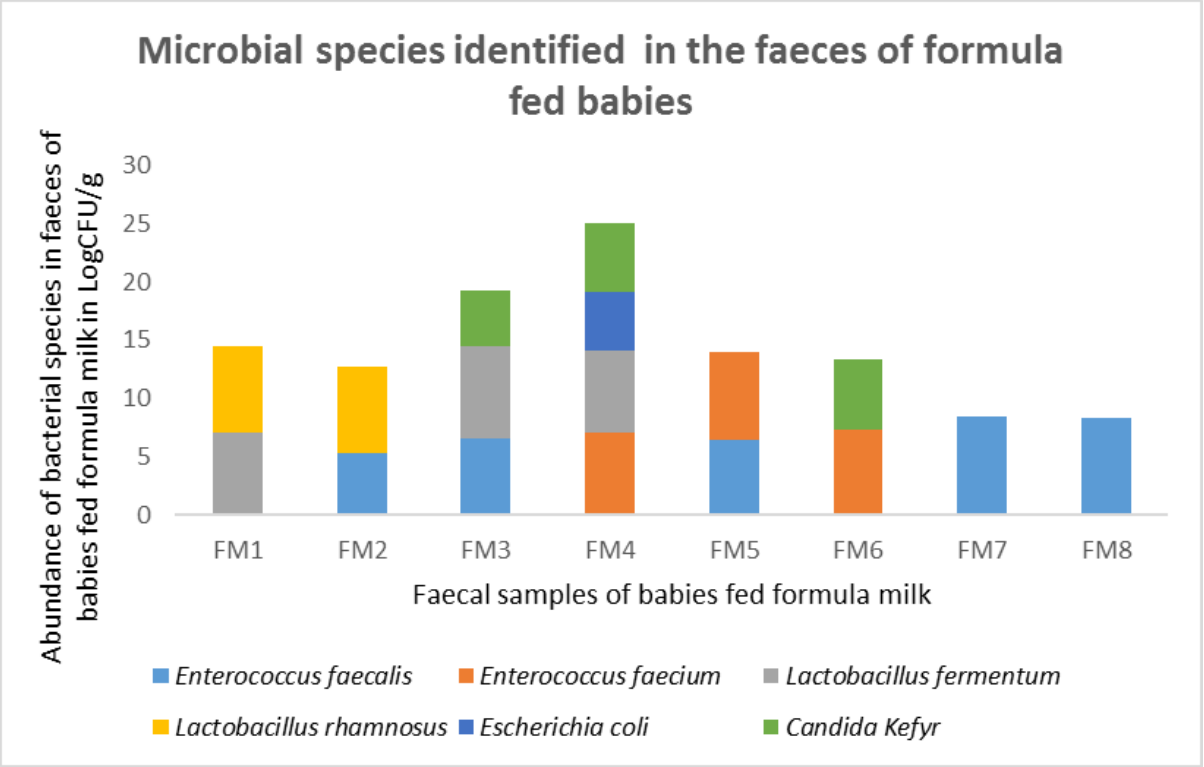


Figure 5.5: Microbial species present in the faeces of formula-fed babies by culture. Microbial identification was carried out by MALDI-TOF MS Biotyper. The relative abundance of microbial species are shown on the y-axis as log_{10}CFU/g. On the x-axis are FM1-FM8 which represents the faecal samples of each of the formula-fed babies. The coloured bars represent the microbial species identified.

5.4 Analysis of milk and faecal samples by using PCR and 16S rDNA amplicon-based sequencing

DNA was isolated from breast milk and faecal samples as described in section 3.2.2.1. DNA quantity and quality were measured with Qubit and Nanodrop respectively and used for target gene 16S rDNA sequencing (at Eurofins Genomics, Germany GmbH).

As explained in section 3.3.2.1, 104 samples were successfully sequenced, including 48 milk samples (48 out of 50) from breastfeeding mothers and 48 (48 out of 50) faecal samples from their respective breastfed babies, as well as 8 faecal samples from babies exclusively fed formula.

5.4.1 Bacterial abundance in human milk and faeces of breastfed babies

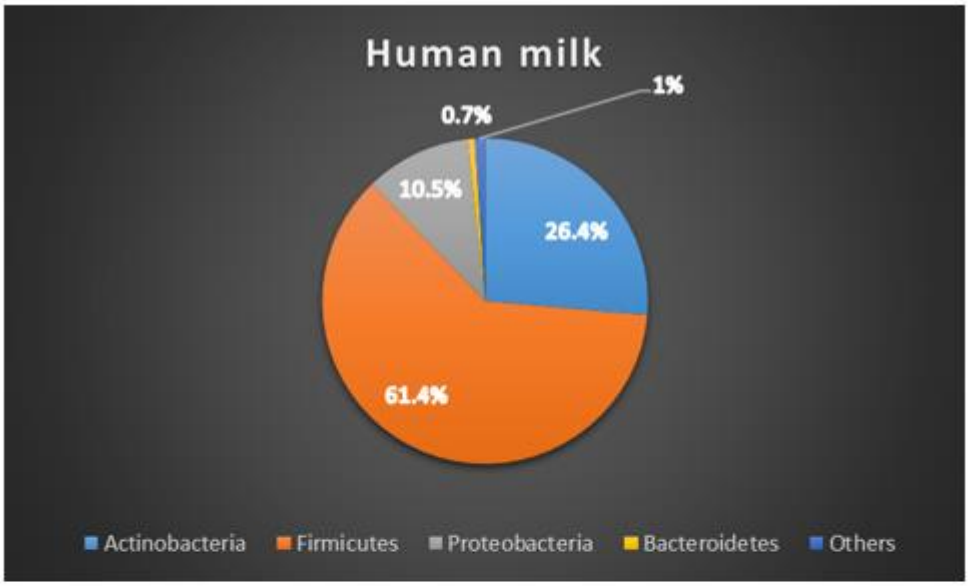
The milk samples of mothers and faecal samples of their respective babies were analysed by sequencing of V3-V4 region 16S rDNA of samples as described in section 3.3.8.3. A total of 13,911,207 read pairs (6,910,238 for human milk and 7,000,969 for breastfed baby faeces) were obtained from milk and faecal samples of breastfed babies, with a mean length of 416bp(±7.1) and a mean phred quality score value of 32.74 (Quality score value 30 (Q30) or above is considered a benchmark for quality of a base calling in DNA Sequencing).

The reads were classified into 2,175 operational taxonomic units (OTUs) for human milk and 1,715 OTUs for baby faeces. OTU corresponding to human milk samples revealed the presence of diverse bacteria as compared to faeces. At phylum level, Firmicutes, Actinobacteria and Proteobacteria accounted for a total of 98.3% and 98.1% of bacterial phyla in human milk and faeces respectively. In human milk, Firmicutes was the most abundant phylum accounting for 61.4% next to Actinobacteria (26.4%) and Proteobacteria (10.5%). In addition to this, the phyla Streptophyta, Bacteroidetes, Deinococcus-thermus, Gemmatimonadetes, and Chordata were classified as others in human milk as they contributed to less than 1% of all the bacteria. In contrast, the most abundant phylum in faeces is Actinobacteria accounting for 62.6%, next to Proteobacteria (24.3%) and Firmicutes (11.6%) (Figure 5.6A and 5.6B).

Proteobacteria was found to be more abundant in the faeces of breastfed babies (11.6%) compared to mother's milk (10.5%) with a statistically significant difference ($P<0.001$) while phylum Firmicutes was more abundant in human milk (61%) than faeces of breast-bed babies (11.6%) ($P=0.003$). Furthermore, Actinobacteria was found to be more abundant in baby faeces (62.6%) compared to human milk (26.4%) ($P<0.001$). However, with no statistically significant difference, the abundance of Bacteroidetes was more in faeces (1.4%) than in milk (0.7%) (Figure 5.6A, Appendix 4 Table 1).

The relative abundance of bacteria was also analysed at the family level. In human milk, four families i.e *Streptococcaceae, Staphylococcaceae, Micrococcaceae and Corynebacteriaceae* were found to predominate, occurring in at least 72% of mothers at a relative abundance of ≥1%, while the families *Bifidobacteriaceae, Enterobacteriaceae* and *Streptococcaceae* were found to predominate faecal samples of babies. An unclassified family belonging to the order Lactobacillales was also found to be abundant in faeces and milk of 25 and 45 of the 48 participants respectively (Appendix 4, Figure 1).

A



B

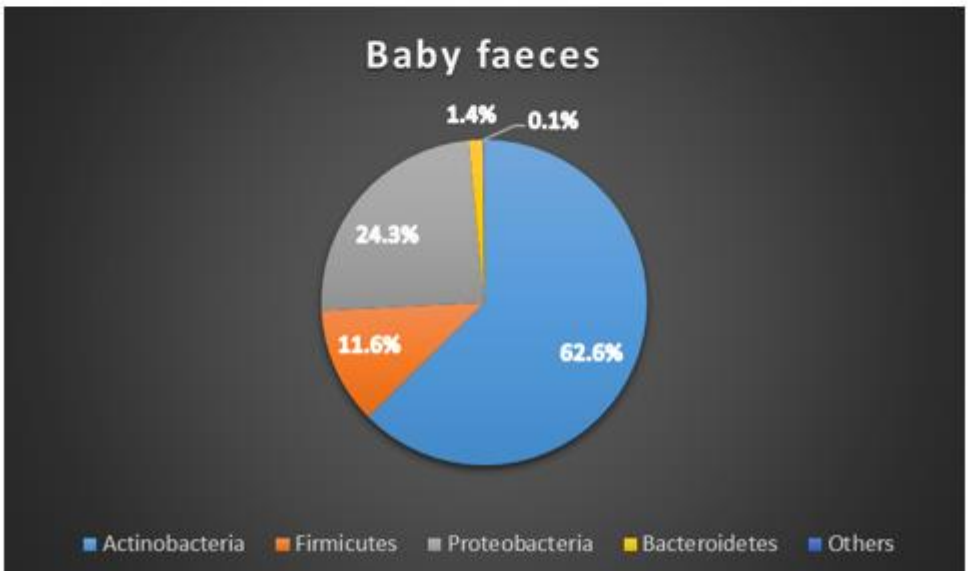


Figure 5.6: Relative abundance of the predominant bacterial phyla in human milk and baby faeces. The abundance of human milk is shown in (A) and baby faeces in (B).

At the genus level, *Streptococcus, Staphylococcus, Rothia* and *Corynebacterium* predominate the human milk samples with an abundance of at least 1%. Their presence occurred in 45,41,41 and 35 participants, respectively. Other genera which predominate human milk and are present in at least 15 mothers are *Pseudomonas, Acinetobacter, Bifidobacterium Actinomyces, Brevundimonas*

and Kocuria. In faeces, the genera *Bifidobacterium* appeared to predominate and was present in 35 participants with at least 1% relative abundance. An unclassified genus belonging to *Enterobacteriaceae* was also present in 39 of the participants. In terms of abundance, 20 OTUs appeared to be abundant in human milk and faeces of breastfed babies (Figure 5.7A and 5.7B).

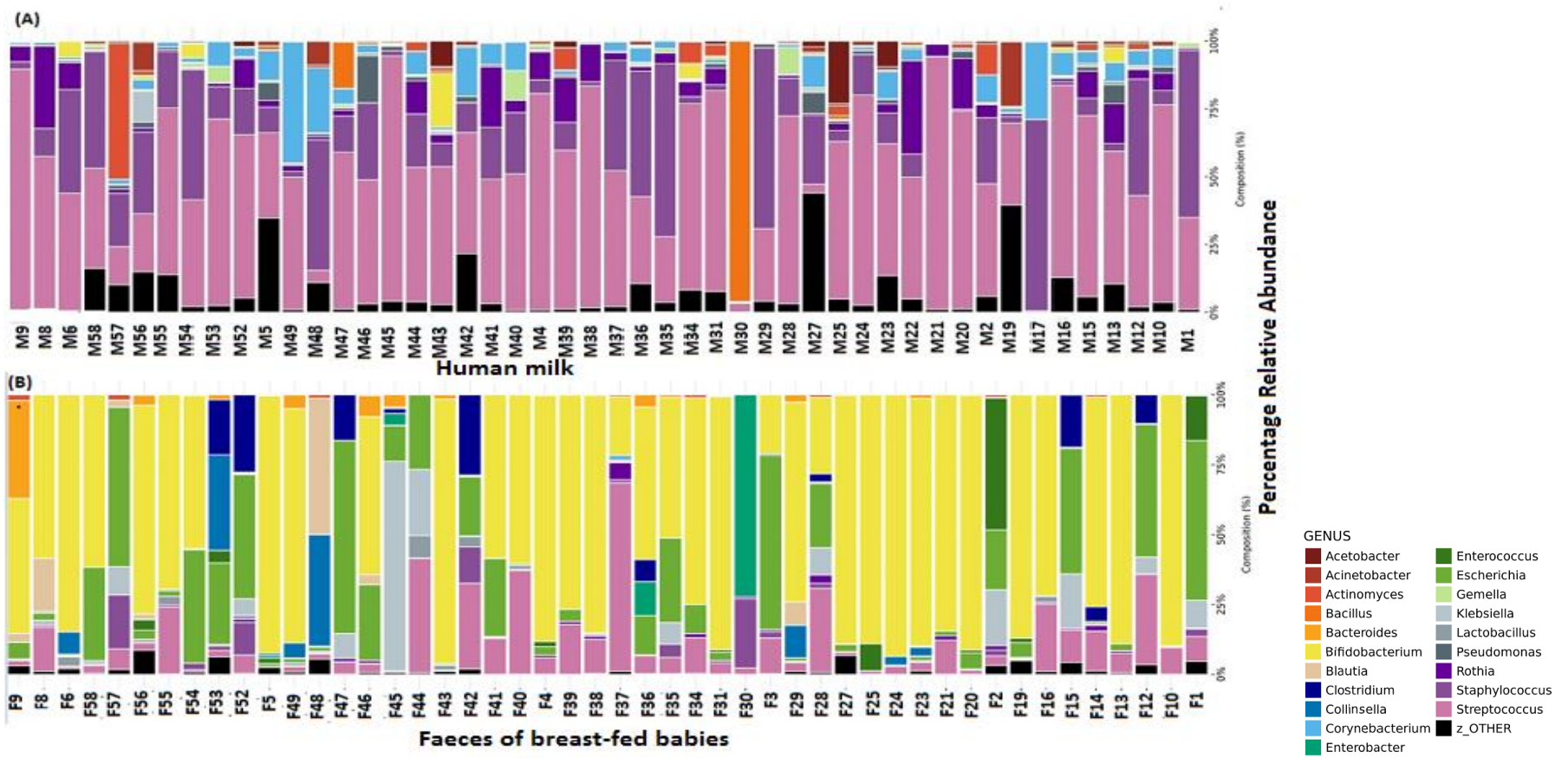


Figure 5.7: The relative abundance of bacterial genera in human milk and baby faeces. Genus-wise OTU distribution shows the top 20 most abundant OTU in human milk (A) and baby faeces (B). The top 20 most abundant OTU are shown in coloured bars while the rest are classified as other. On the y-axis is the percentage abundance, and on the x-axis is the sample label for each participant. M indicates milk and F indicates faeces of breastfed babies. (Figure generated by Eurofins, Germany)

5.4.2 The analysis of the abundance of the bacterial taxa in human milk and faeces of breastfed babies

Statistical tests were performed for each OTU to compare the distributions between conditions (Faeces of breastfed babies (F) vs Milk (M)) generating p-values for each OTU. The final p-values were corrected by determining false discovery rates (FDR) using the Benjamini–Hochberg method. Using an FDR corrected p-value (adjusted p-value) <0.1 and a foldchange of >0.5 as a threshold, 38 genera were identified in baby faeces to be more expressed as compared to milk, while 65 genera were expressed more in breast milk as compared to faeces (Figure 5.8 and Appendix 5, Table 1).

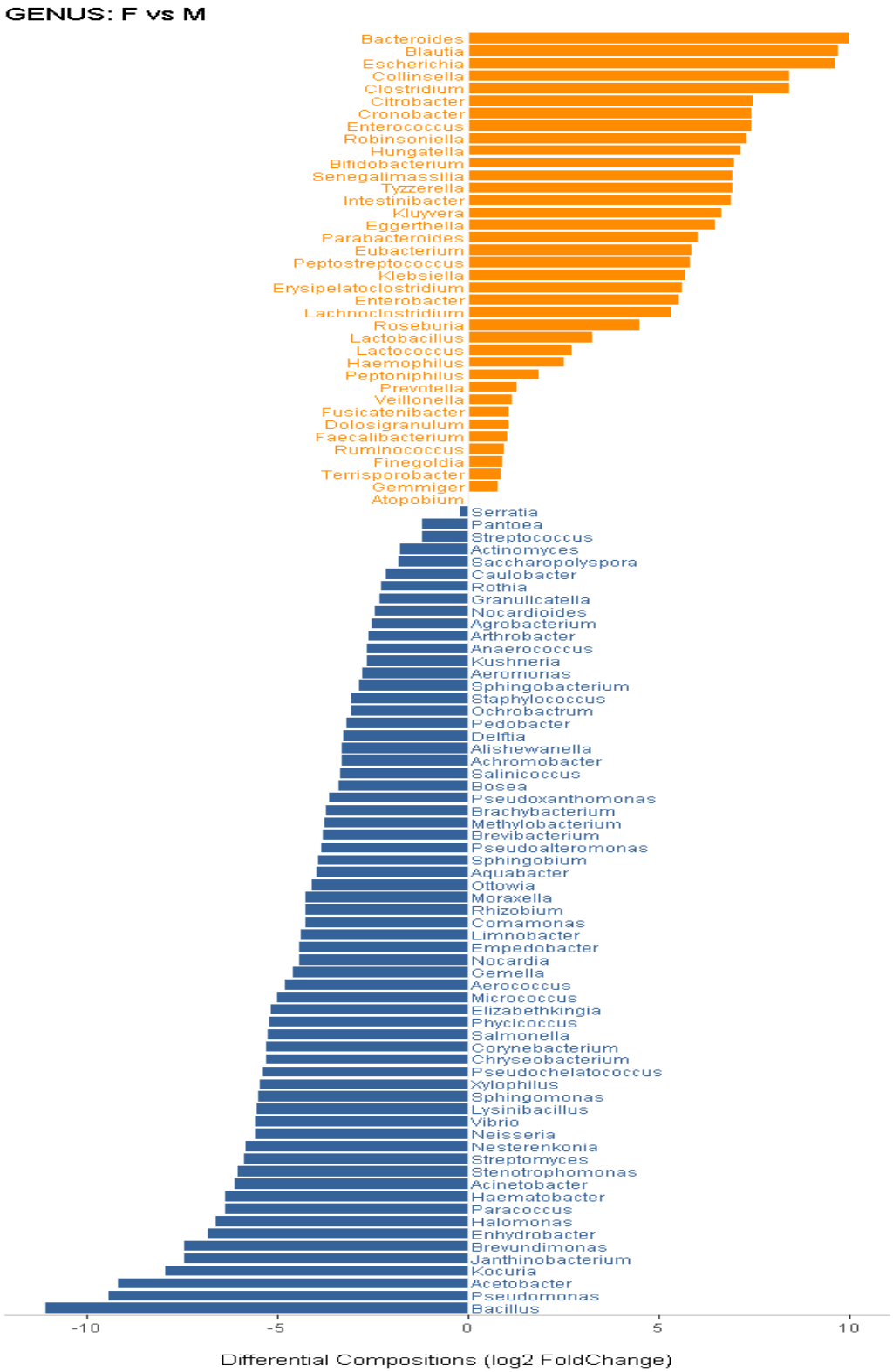


Figure 5.8: Differential Compositions analysis (log2 Fold Change) between milk and faeces. Genus level composition is ordered according to up and downregulated composition differences. Orange bars represent the faeces of breastfed babies (F) while blue bars represent human milk(M). Positive values indicate upregulated OTUs in the time point and negative values indicate

downregulated OTUs in the time point compared to the baseline sample. Differential Compositions at the genus level are measured in Log2 Fold Change between faeces and milk comparison. *Bacteroides* showed Log2 fold change of (10.03) =1045 OTU's more in faeces (F) compared to milk (M), while *Bacillus* showed a Log2 fold change (11.12) =2230 OTU's in milk compared to faeces. FDR corrected p-value (adjusted p-value) of P<0.1 was considered significant. log2foldchange of 0.5 was set as a threshold. (Figure generated by Eurofins, Germany).

5.4.3 Diversity of bacteria in human milk and faecal samples of breastfed babies

To determine if there were differences in the number of bacterial operational taxonomic units (OTUs) present in human milk and baby faeces, the alpha diversity contained in the microbiota of both human milk and breastfed baby faeces was evaluated. The Alpha-diversity of human milk and faecal samples of breastfed babies were estimated and Mann–Whitney U- test was used to find any significant difference between the two groups (Figure 5.9 and Appendix 6, Table 1). The diversity and dominance were estimated using Shannon diversity index and Simpson index, while the microbial richness was estimated by Chao 1 and Observed alpha diversity indexes. Within sample diversity indexes i.e Chao 1 index and Observed number of species (P<0.001) revealed that the human milk microbiota is richer when compared with the microbiota of baby faeces (Figure 5.9). In terms of bacterial diversity and dominance, the Shannon (P<0.001) and Simpson (P<0.001) indexes revealed that human milk has a greater level of bacterial diversity than baby faeces.

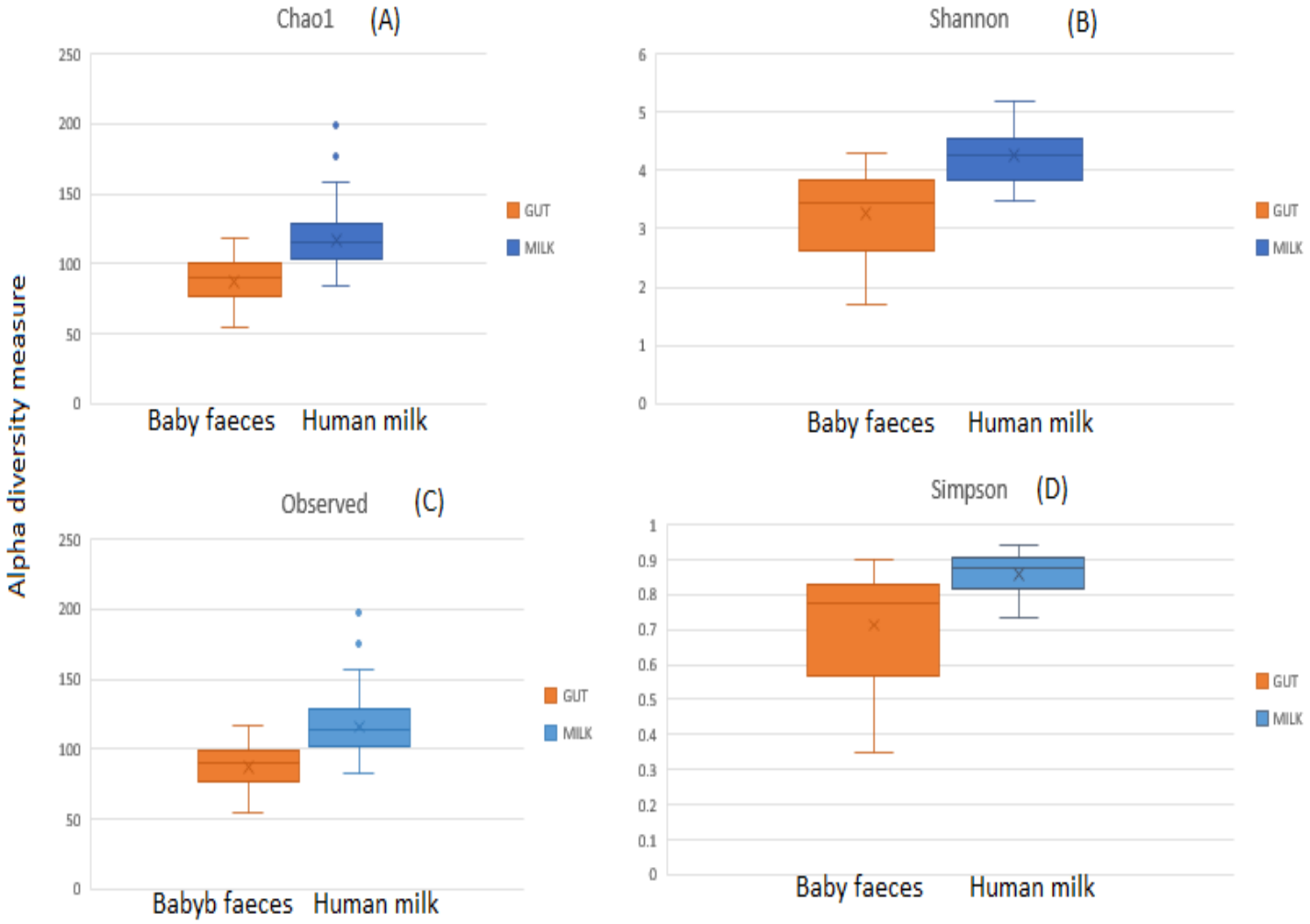


Figure 5.9: Alpha-diversity estimate of the microbiome in human milk and baby faeces. Alpha diversity is based on Simpson Index($P<0.001$), Chao1 ($P<0.001$), Shannon ($P<0.001$) and observed ($P<0.001$). The significant difference was estimated using Mann-Whitney U- test with $P<0.05$ considered statistically significant.

The beta diversity in the microbiota of all mothers and breastfed babies was characterised to see if any trends were specific to the milk samples or faeces of breastfed babies. Beta-diversity based on Bray-Curtis dissimilarity revealed that the microbiota in milk and baby faeces clustered distinctly (Adonis test shows that the two groups significantly differ (R^2=0.231, P=0.001), (Figure 5.10) demonstrating that the milk and baby faecal samples had obvious differences.

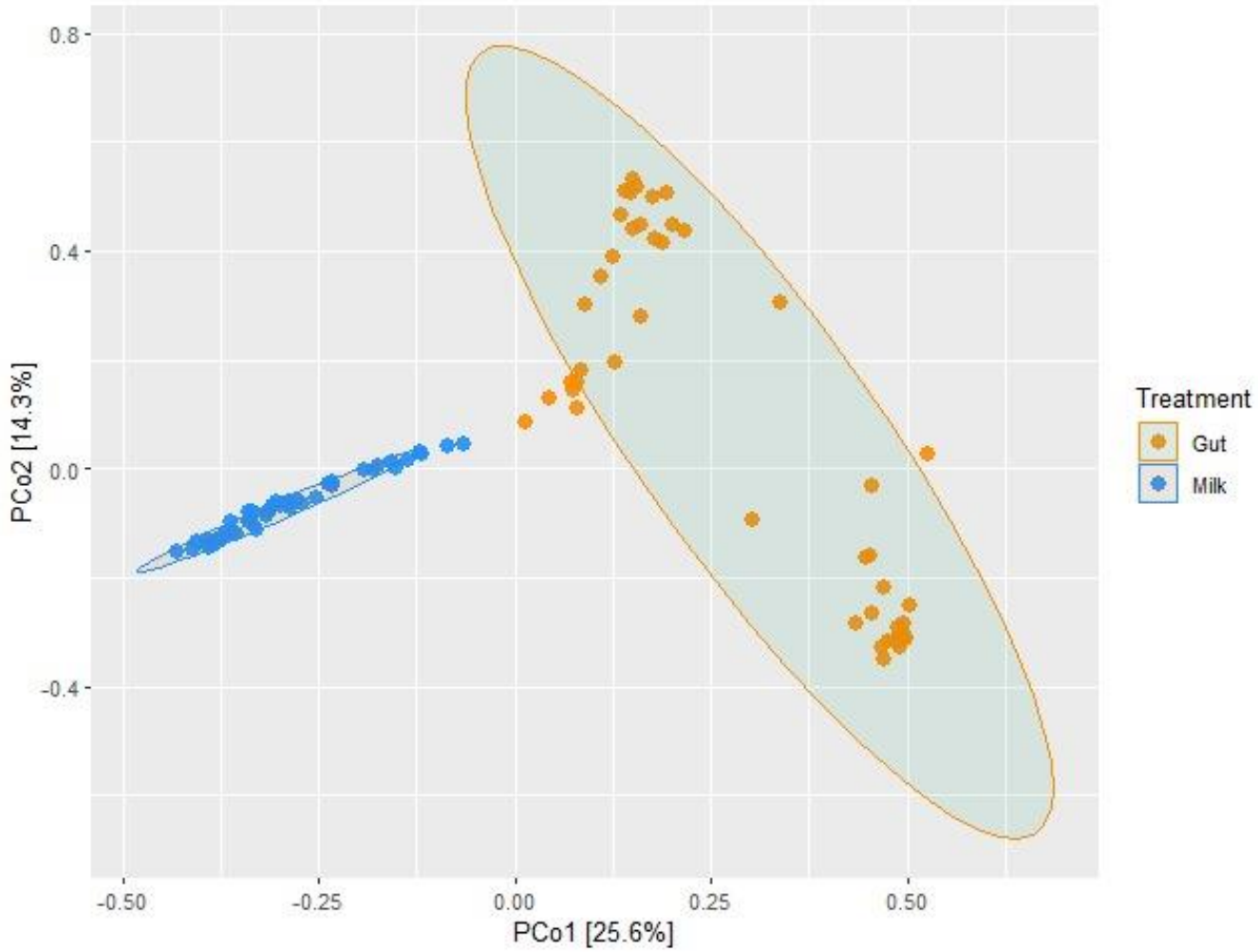


Figure 5.10: Beta-diversity estimate of microbiota in human milk and breastfed babies' faeces. Principal Coordinates Analysis (PCoA) based on Bray-Curtis index of dissimilarity for Beta diversity analysis between microbiome composition in the gut of babies and maternal milk (R^2=0.231, P=0.001). The significant difference was estimated using Adonis test with P<0.05 considered statistically significant.

5.4.4 Bacterial taxa shared between the human milk microbiome and faeces of breastfed babies

After all sequences were put together and a close reference OTU picking using QIIME version 1.9.1 was done, an OTU Venn plot was generated (Figure 5.11A). A total of 882 OTUs were generally shared between human milk (M group) and faeces of breastfed babies (F group).

At the genus level, relevant taxa that were however shared between individual mothers and their babies occurring in 45% or more of the mother-baby pair with at least 1% relative abundance are *Streptococcus* (80%), *Rothia* (60%), *and Bifidobacterium* (48%). Other bacterial genera shared are *Staphylococcus, Corynebacterium, Enterococcus, Klebsiella, Actinomyces* and *Erwinia* (Figure 5.11B).

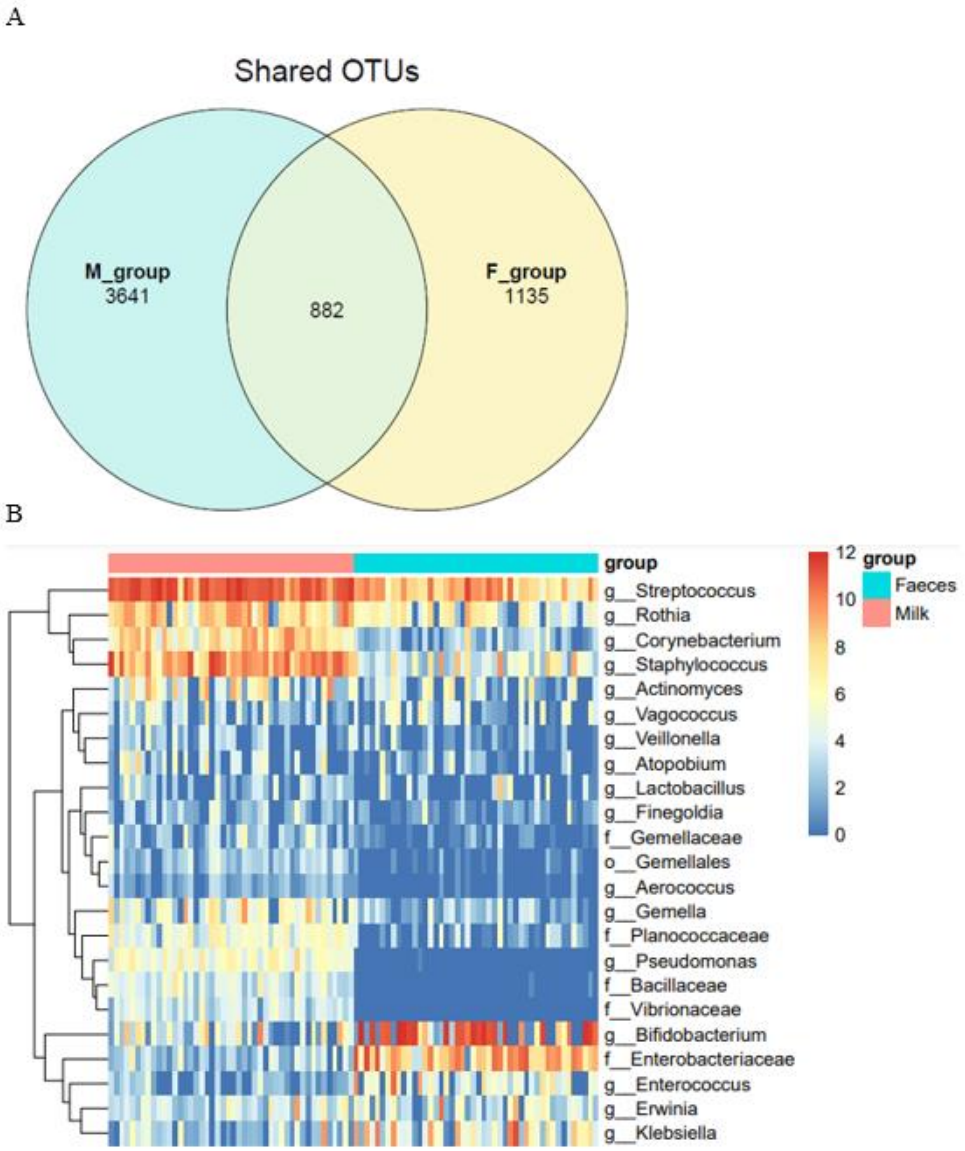


Figure 5.11: Shared OTU between human milk and baby faeces. (A) A Venn diagram showing the OTU unique to the mother's milk and faeces of babies and the OTU shared between the two groups. M_group is mother's milk and F_group is baby faeces (B) Heatmap that shows the OTU present in at least 50% of mother-baby pairs. This was calculated by compute_core_microbiome.py in QIIME software. Heatmap was plotted by taking the log of the counts and the colour code from blue (0) to red (12) shows increasing abundance with blue being the lowest abundance and red being the highest. Milk=Breast milk of mothers and Faeces=Faeces of breastfed babies.

To further determine the fraction of OTU shared and not shared (unshared) between baby faeces and the corresponding mother's milk, an output of 'shared_phylotype' (i.e., shared OTU), which considers what each mother shares with their babies was deducted from the total count of OTU detected in each mother's milk (Figure 5.12 shows shared and unshared variables).

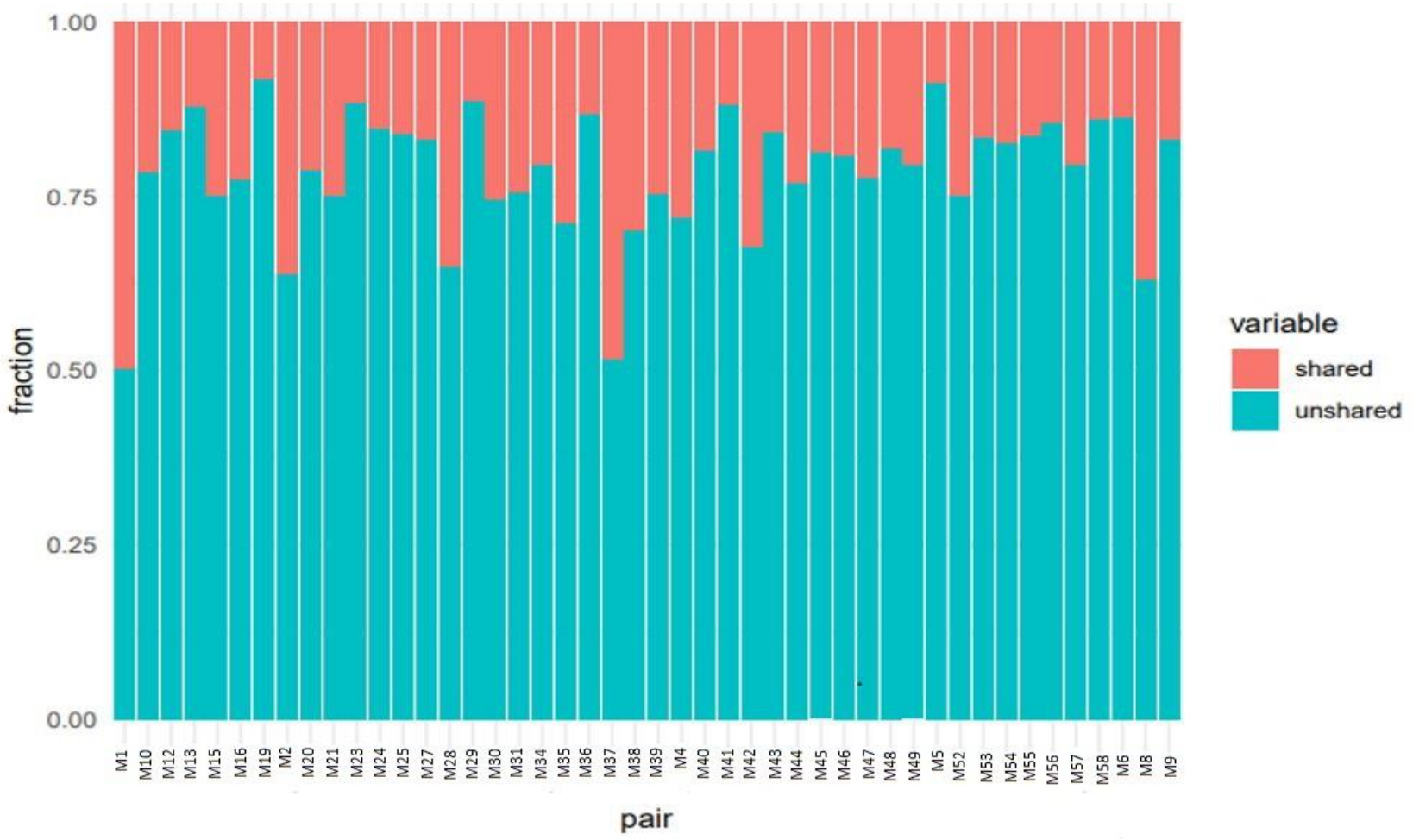


Figure 5.12: The fraction of shared bacteria between the mother's milk and faeces of their respective breastfed babies. The label below the graph shows each mother's milk sample represented by M followed by the identification number. For example, of the total OTU counts detected in the milk of mother 1 (M1), 50% of these are shared with her baby's faeces, remaining 50% are not present in the baby's faeces. M17 and M22 are not included because they have no corresponding baby faeces (i.e no F17 and F22 due to low DNA) to compare the mother's milk with.

Furthermore, microbial source tracker analysis was carried out to predict if the bacterial taxa observed in baby faeces originated from human milk or another source taking all participants into consideration. It was seen that breastfed babies may have generally received about 51% of their gut bacteria from human milk, while the remaining 49% may have come from sources other than breast milk (i.e., Unknown) (Figure 5.13 A and 5.13B). The taxonomic examination of the bacteria in the faecal samples described as the "Unknown Group" revealed a considerable relative abundance of the orders Enterobacteriales followed by Clostridales, and Coriobacteriales, which differed from the abundance reported in the "Milk Source Group".

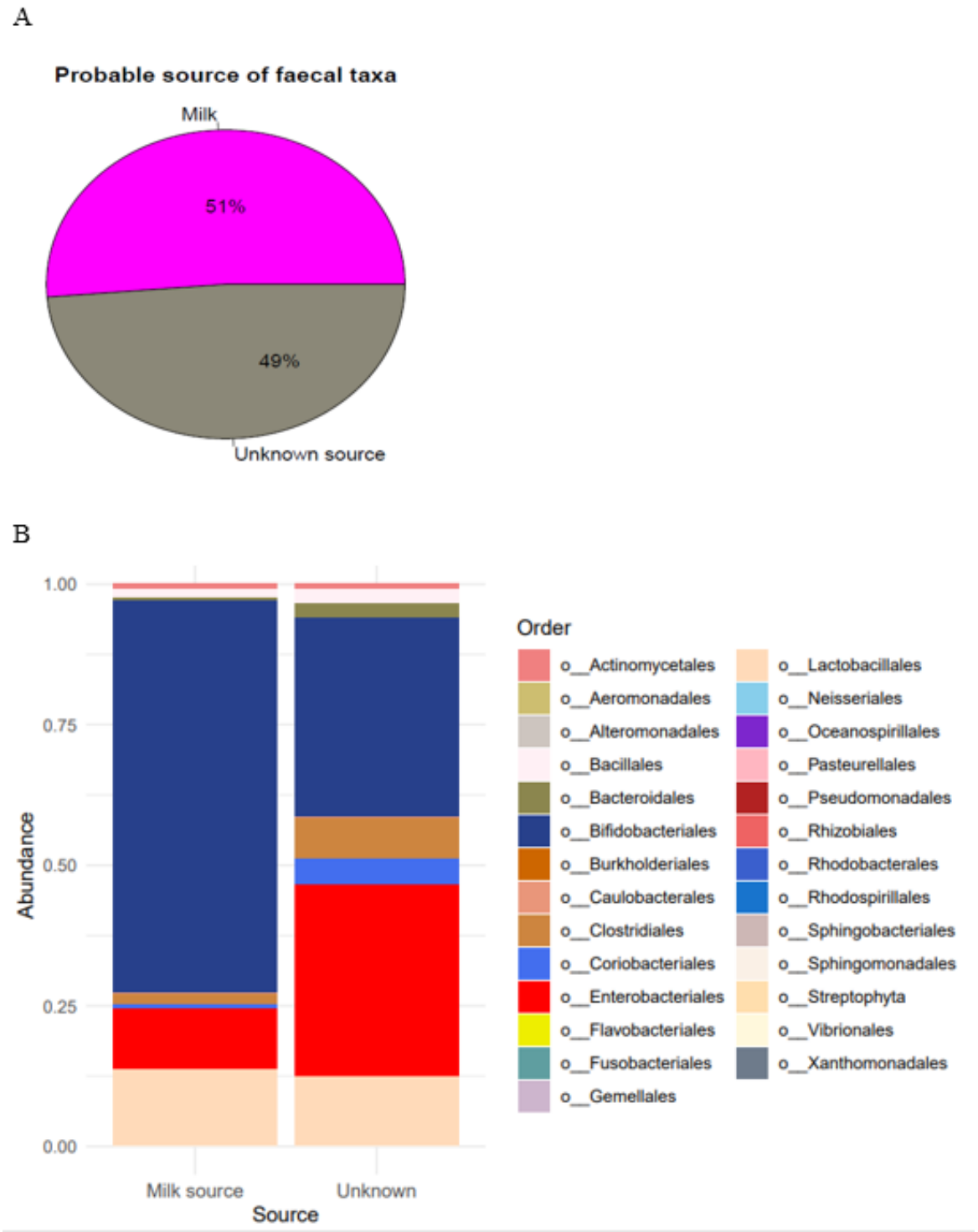


Figure 5.13: Probable source of bacteria in faeces of breastfed babies. (A) The percentage of bacteria arising from human milk and baby faeces was shown by microbial source tracker analysis (P<0.001, Wilcoxon signed-rank test). (B) The relative abundance of bacteria at the Order level, belonging to human milk source and unknown source.

Microbial source tracker analysis also revealed that the probable source of bacteria in the faeces of only 5 breastfed babies (F12, F25, F27, F45 and F5) was completely traced to an unknown source (Figure 5.14).

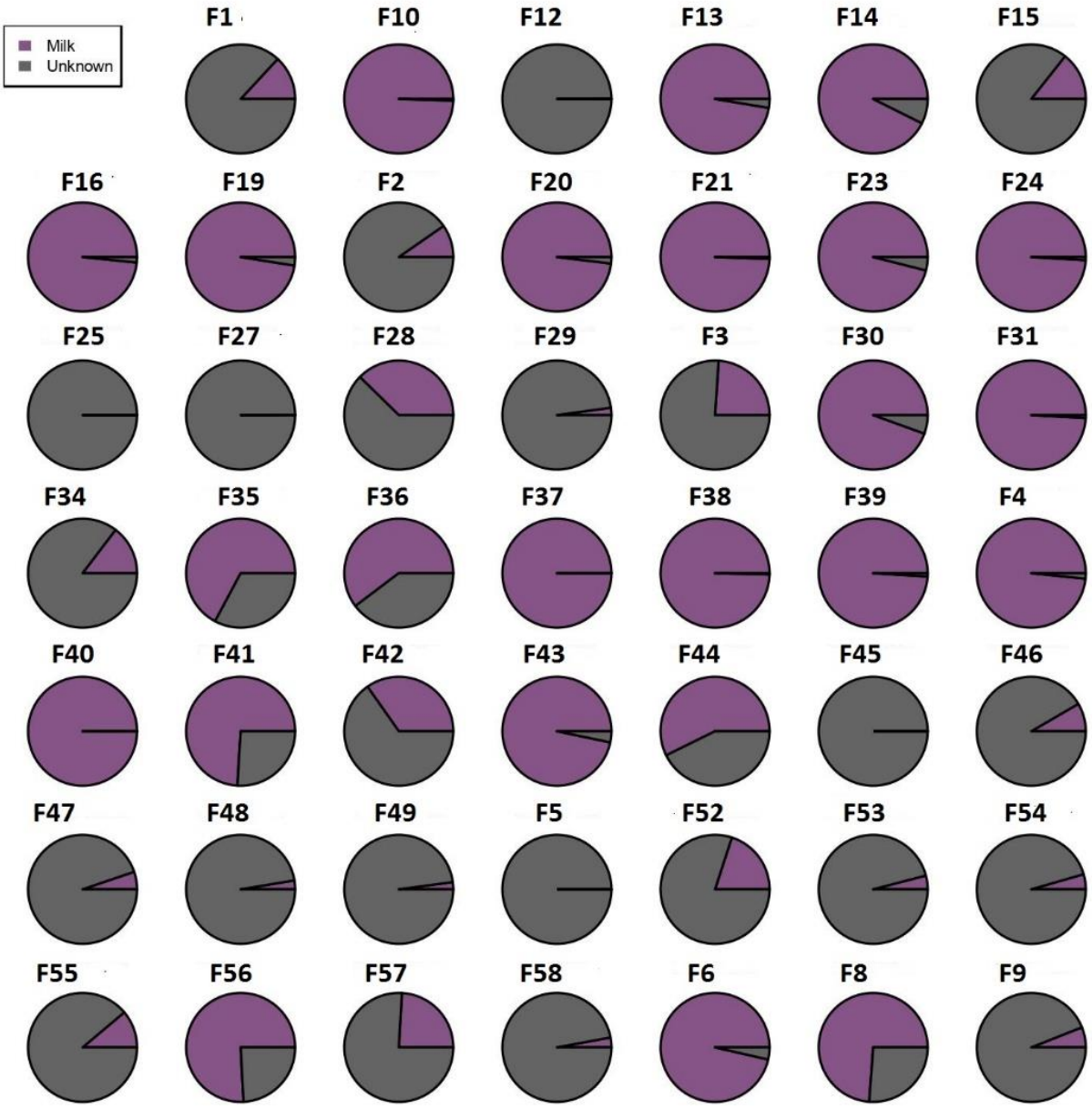


Figure 5.14: Analysis of the origin of bacteria found in each baby's faecal sample (F) using Microbial Source Tracker Analysis. The purple colour denotes human milk-origin bacteria, while the grey colour denotes bacteria with unknown origin. Each of the pies represents each baby's faeces denoted by F followed by the participant number.

5.4.5 Bacterial abundance in faeces of formula-fed babies

A total of 537, 366 read pairs were obtained from all 8 formula-fed baby faeces with a mean length of 416bp (±6) and a mean quality score value of 31.95. The reads were classified into 626 Operational Taxonomic Units (OTUs). At the phylum level, Actinobacteria, Firmicutes, and Proteobacteria accounted for 99% of the bacterial taxa in the faeces. Actinobacteria was the most abundant phylum accounting for 37.2% next to Firmicutes (36.5%) and Proteobacteria (25.3%). (Figure 5.15).

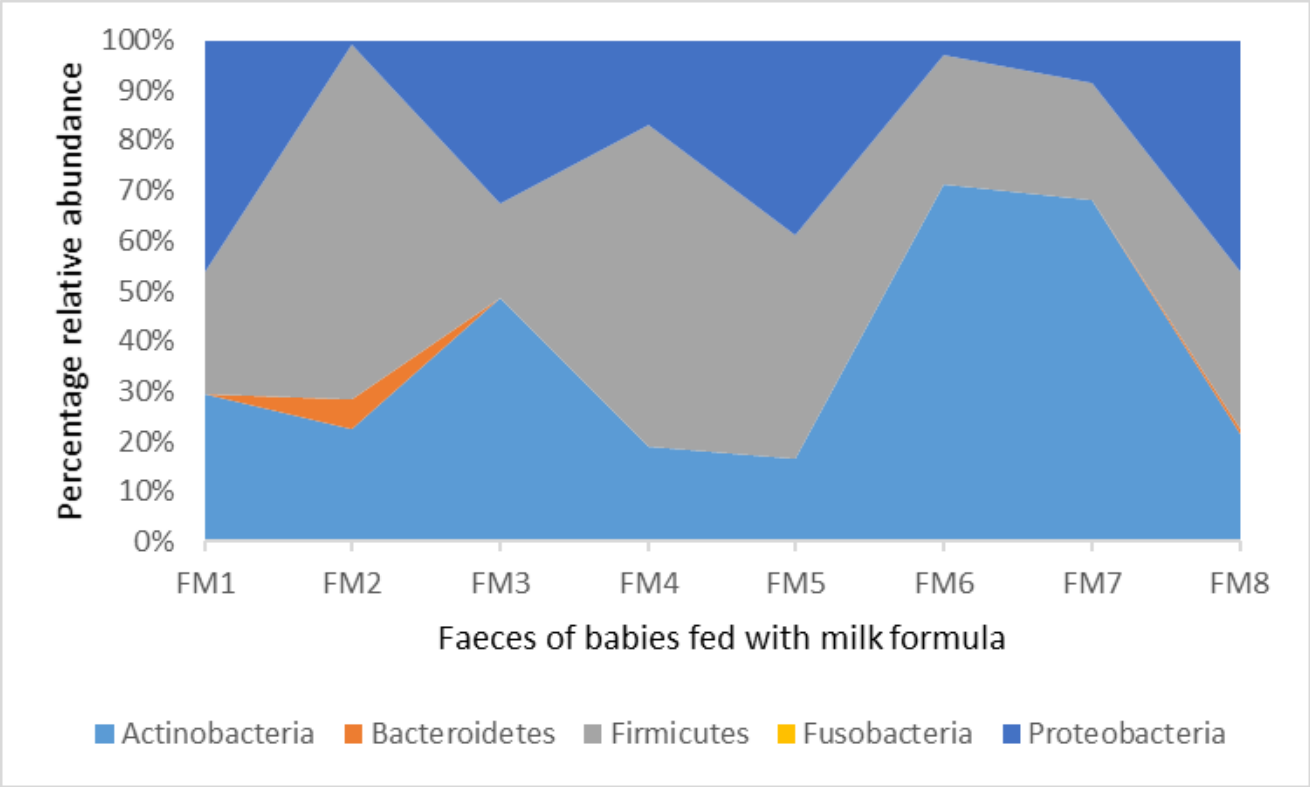


Figure 5.15:The relative abundance of the bacterial phyla detected in the faeces of 8 formula-fed babies. FM1 to FM8, represent each participant i.e., faeces of babies fed formula milk

The relative abundance of bacteria was also analysed at the family level. Families that were predominant in the faeces of formula-fed babies, occurring in at least 5 of the 8 babies at a relative abundance of at least 1%, are *Bifidobacteriaceae* (26.5%) represented by genus *Bifidobacteria*, *Enterobacteriaceae* (24.6%), *Lactobacillaceae* (11.6%) represented by genus *Lactobacillus*, *Coriobacteriacaea* (9.1%) represented by *Collinsella*, *Lachnospiraceae* (9%) represented by *Blautia*, *Ruminococcaceae* (5.4%) largely represented by *Faecalibacterium* and *Streptococcaceae* (4.4%) represented by *Streptococcus*.

5.4.6 Comparing the bacterial taxa of babies fed exclusively with breast milk and those exclusively fed with milk formula

In terms of gut microbiota diversity, the gut microbiota of babies fed with formula and breast milk were majorly composed of the phyla Actinobacteria, Firmicutes and Proteobacteria. However, looking closely at the bacterial taxa at the genus level, the most abundant bacteria in the gut of both babies which belongs to the genus *Bifidobacterium* ($P=0.015$, $q=0.072$) seems to be slightly abundant (5.57 OTUs) in babies who are fed with breast milk when compared to babies who were exclusively fed with formula while the genus *Lactobacillus* ($P<0.001$, $q<0.001$) appears to be more abundant in the faecal samples of babies fed with formula (18 OTUs) when compared to babies fed with breast milk (Figure 5.16) (Appendix 5 Table 2).

The gut of breastfed babies was also mainly dominated by *Bifidobacteriaceae* (55.3%), *Enterobacteriaceae* (23.7%) and *Streptococcaceae* (2%), while the gut of babies who were fed with milk formula was dominated by *Bifidobacteriaceae* (26.5%), *Enterobacteriaceae* (24.6), *Lactobacillaceae* (11.6%), *Coriobacteriaceae* (9.1%), and *Ruminococcaceae* (5.4%).

Furthermore, the gut of formula-fed babies appears to be predominated by *Faecalibacterium*, a member of *Ruminococcaceae* occurring in 5 of the 8 babies which were not detected in the faeces of breastfed babies.

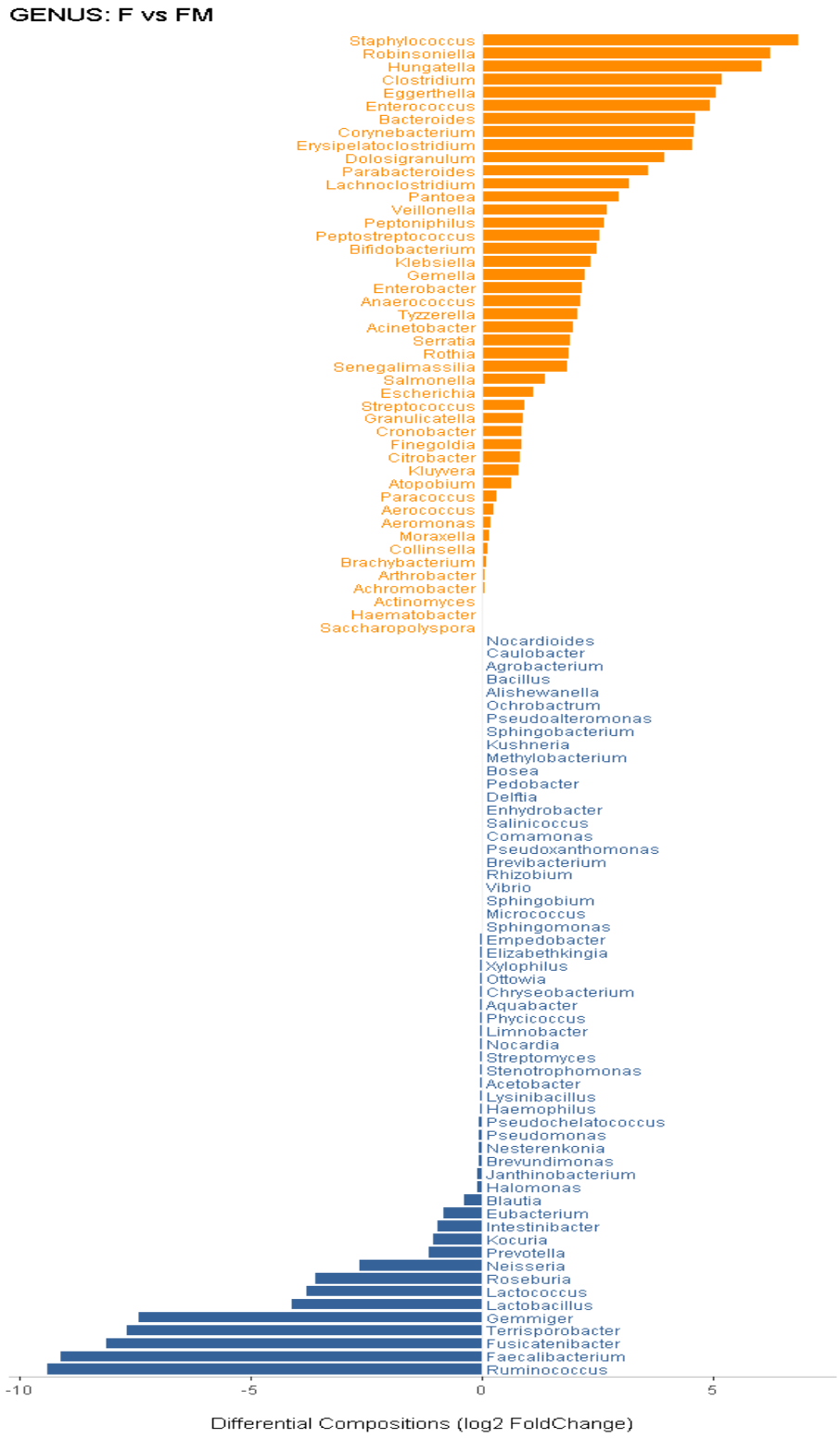


Figure 5.16: Differential Composition analysis (log2 Fold Change) between faeces of breastfed babies and faeces of babies fed with milk formula. Genus level composition are ordered according to up and down-regulated composition differences. Orange bars represent the faeces of breastfed babies(F) while blue bars represent the faeces of formula-fed (FM) babies. Positive values indicate upregulated OTUs in the time point and negative values indicate

downregulated OTUs in the time point compared to the baseline sample. Differential Compositions at the genus level are measured in Log2 Fold Change between the faeces of breastfed babies and faeces of formula-fed babies. *Staphylococcus* showed Log2 fold change of (6.87) =117 OTU's more in faeces of breastfed babies (F) compared to faeces of babies fed formula milk (FM), while *Ruminococcus* showed a Log2 fold change (9.41) =680 OTU's in the faeces of babies fed formula milk compared to faeces of babies fed with breast milk. FDR corrected p-value (adjusted p-value) of P<0.1 was considered significant. log2foldchange of 0.5 was set as a threshold. (Figure generated by Eurofins, Germany).

Furthermore, the alpha diversity of gut microbiota in faeces of breastfed babies and formula-fed babies was estimated and Kruskal-Wallis test was used to find significant differences between both groups (Figure 5.17). To estimate the bacterial diversity and dominance, the Shannon (p<0.001) and Simpson (p<0.001) indexes showed higher bacterial diversity in faecal samples of babies fed with formula (FM) compared to breastfed babies (F).

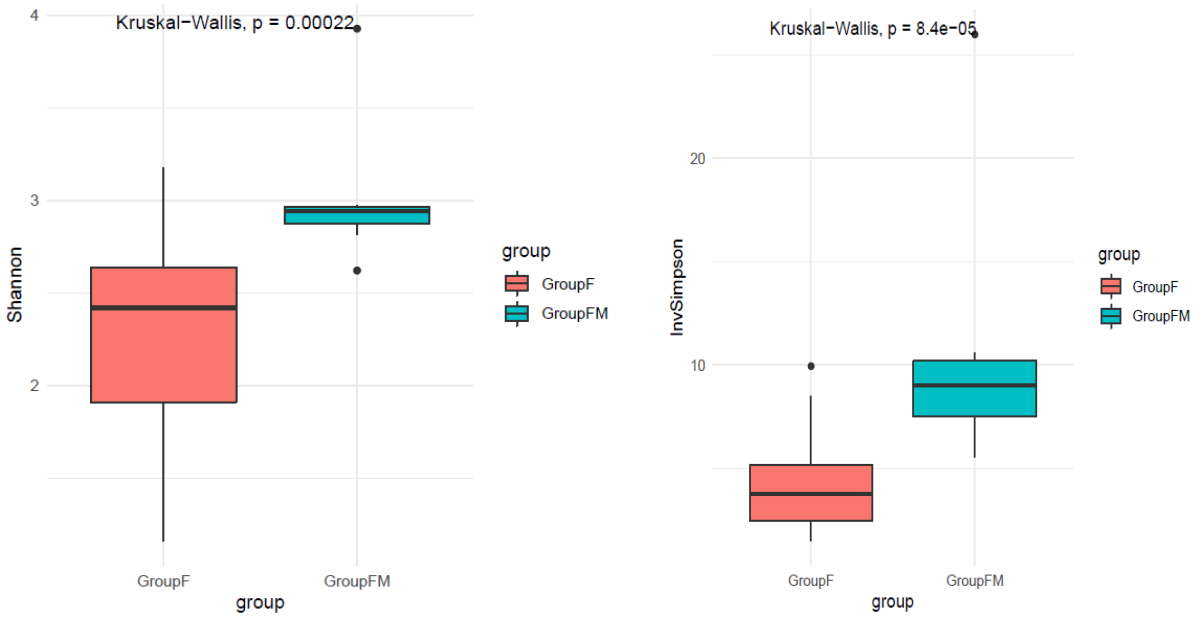


Figure 5.17: Alpha-diversity estimate of the microbiome in faeces of breastfed babies and formula-fed babies. Alpha diversity is based on Shannon Index(P<0.05) and Inverse Simpson index (P<0.05). The significant difference was estimated using Kruskal-Wallis test with P<0.05 considered statistically significant

5.4.7 Association between the microbiome of mother's milk and the maternal characteristics

The association between the maternal and infant characteristics and alpha diversity of human milk microbiota was determined by using Chao1 alpha diversity index for estimating microbial richness and Shannon indexes for microbial diversity (Appendix 6). The maternal milk microbiota richness and diversity was not statistically associated with maternal characteristics such as the level of education, parity, working condition (working outside of the home vs working within the home), maternal age range, and diet richness (fibre, red meat, chicken, yoghurt and fat) (Appendix 8).

Next, a Nonparametric test (Mann-Whitney U test) was used to find any association between the top ten most predominant bacterial genera in human milk and maternal characteristics, and Benjamini-Hochberg method was used to estimate the false discovery rate to generate a corrected p-value (q-value), with $q < 0.05$ considered statistically significant. It was found that parity was significantly associated with the level of *Brevundimonas* in milk being the only maternal characteristic with a statistical significance difference ($p=0.004$, $q=0.04$). Mothers with more than one child (multiparous) have a higher level of *Brevundimonas* in their milk than mothers with a single child (uniparous) (Figure 5.19).

Although with no statistically significant difference (after Benjamini Hochberg correction), the abundance of bifidobacteria in milk was associated with the mode of delivery($P =0.048$; $q=0.48$). Similarly, the high consumption of fat, chicken and red meat was associated with the level of *Rothia* ($P=0.008$; $q=0.08$), bifidobacteria ($P=0.021$; $q=0.2$), and *Actinomyces* ($P =0.012$; $q=0.12$) respectively without any statistically significant difference, while the abundance of Acinetobacter ($P=0.026$, $q=0.08$) and bifidobacteria ($P= 0.021$, $q=0.08$) in milk, associated with parity, was also not statistically significant (Appendix 8).

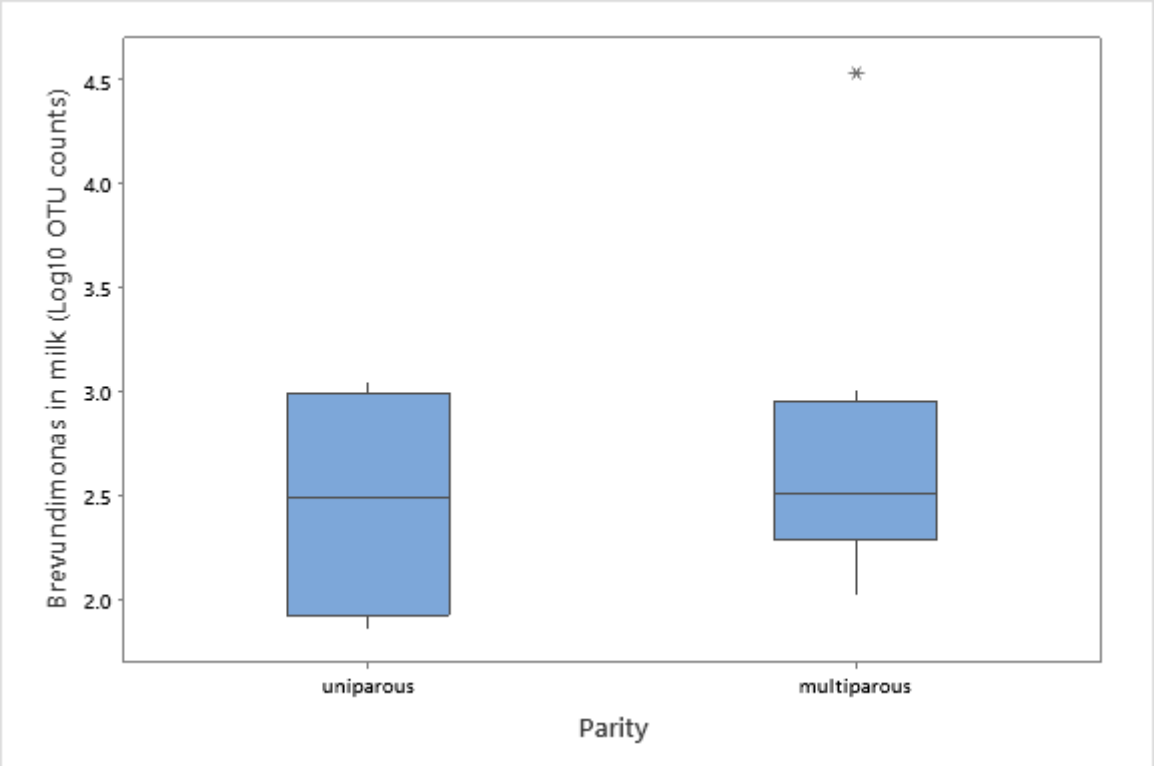


Figure 5.18: Association between parity and *Brevundimonas* in milk. On the y-axis is the number of reads or OTU counts belonging to *Brevundimonas* in the milk of mothers formatted as a log scale while the x-axis shows parity. Mothers who reported having more than a child (multiparous) had a higher level of *Brevundimonas* in their milk compared to mothers who reported having a child (uniparous). According to Mann-Whitney U test, there is a statistically significant difference based on P-value= 0.004, and q-value 0.04. $q<0.05$ was considered to be statistically significant

5.4.8 Association between the microbiota of breastfed baby's gut and the maternal or infant characteristics

Using the Chao 1 and Shannon alpha diversity indexes, it was discovered that the bacterial diversity and richness of the baby's faecal microbiota were not associated with the baby's delivery mode, gender, or age. When the top ten most predominant bacterial genera in baby faeces were examined, the only bacteria with a significant association was *Klebsiella*, which was associated

with the mode of delivery. The faeces of breastfed babies born by CS have a higher level of *Klebsiella* compared to babies born naturally (P= 0.001, q=0.01) as shown in figure 5.20.

With no statistically significant difference (P=0.043, q=0.43), breastfed babies whose mothers reported working within the home were also found to have a higher level of bifidobacteria in their faeces compared to those whose mothers worked outside of the home (Appendix 9).

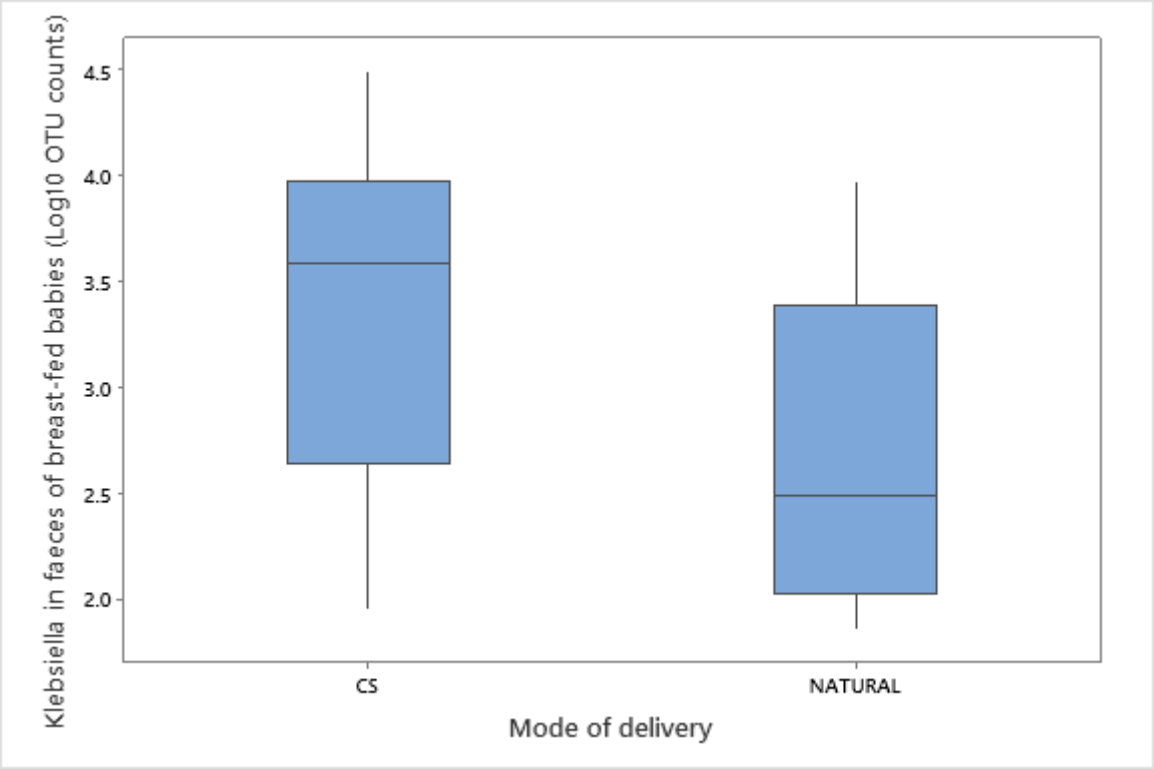


Figure 5.19: Association between babies' mode of delivery and *Klebsiella* in faeces of breastfed babies. On the y-axis is the number of reads or OTU counts belonging to *Klebsiella* in faeces of breastfed babies formatted as a log scale while the x-axis shows the mode of delivery; CS-Caesarean section. According to the Mann-Whitney U test, there is a statistically significant difference based on a q-value of 0.01. Breastfed babies born via CS had a higher level of *Klebsiella* in their gut compared to babies born naturally. ($q<0.05$)

5.4.9 Summary of findings

1. Human milk of Nigerian Mothers harbours bacteria and the presence of viable bacteria such as staphylococci, streptococci, lactobacilli and *Rothia* were confirmed in breast milk
2. Species of enterococci, lactobacilli, bifidobacteria and streptococci were also confirmed in faeces by culture.
3. The human milk microbiota of Nigerian mothers differs significantly from the gut microbiota of breastfed babies with the milk being highly diverse compared to the gut microbiota as revealed by 16S rDNA amplicon sequencing.
4. Bacterial taxa such as *Streptococcus* (80% of mother-baby pairs), *Rothia* (60% of mother-baby pairs) *and Bifidobacterium* (48% of mother-baby pairs) may be transferred through breastfeeding to the gut of babies.
5. The gut microbiota of breastfed babies in Nigeria differs significantly from the gut microbiota of babies who only drank milk formula, with the genus *Faecalibacterium* related to those on milk formula but not observed in solely breastfed babies.
6. 16S rDNA amplicon sequencing was confirmed to provide a more comprehensive picture of the human milk and gut microbiota than the traditional culture approach or genus-targeted qPCR.

Chapter 6 : Discussion

Human milk is known to provide essential nutrients to babies during their first few months of life. It is recommended by WHO to exclusively breastfeed babies for up to 6 months and up to two years after weaning (World Health Organization, 2021). Human milk does not only contain the nutrients required for a baby's growth such as vitamins and minerals but also contains immune cells, cytokines, immunological factors such as secretory immunoglobulin A and microorganisms. Human milk contains diverse bacteria and the presence of fungi, viruses, protozoa, and archaea have been reported to collectively form the human milk microbiome (Consales et al., 2022). This study investigated the microbiome in Nigerian mothers' milk and the gut microbiome of babies who were breastfed or fed milk formula by different methods including the traditional approach of culture and molecular approach by NGS 16S rDNA sequencing analysis with a focus on bacterial diversity.

6.1 Bacteria present in human milk in the current study proved a high level of diversity

At phylum level, Firmicutes (61.4%), Actinobacteria (26.4%) and Proteobacteria (10.5%) predominate the human milk in this study. The presence of 4 families including *Streptococcaceae* (19.8%), *Staphylococcaceae* (11.4%), *Micrococcaceae* (11.1%) and *Corynebacteriacaea* (8.3%) represented by genera *Streptococcus*, *Staphylococcus, Rothia* and *Corynebacterium* were revealed to be abundant in the breast milk of mothers analysed by Illumina MiSeq sequencing based on 16S rDNA amplification. Bacterial genera that were common in breast milk occurring in at least 15 of 48 mothers are *Staphylococcus, Streptococcus, Rothia, Corynebacterium Pseudomonas, Acinetobacter, Bifidobacterium Actinomyces, Brevundimonas* and *Kocuria*. *Streptococcus* and *Staphylococcus* also predominate the human milk samples when tested by traditional culturing. In contrast to this study, a study carried out in Ghana which is also in the Western part of Africa just like Nigeria reported a high abundance of *Klebsiella*, *Lactobacillus* and *Enterococcus* in the breast

milk of Ghanaian mothers. However, this difference is not surprising as variations in human milk have been reported even among mothers that live within the same vicinity (Lackey et al., 2019).

Streptococcus for example has been reported to be one of the dominant genera in human milk in several studies including the study of Martin et al. (2016) who ascribed the human milk streptococci to mainly belong to the viridans group. Also, in the study carried out by Hunt et al. (2011), streptococci were among the most abundant genera in human milk. Its role has not been clearly defined, but its presence has been linked to breast milk and gut of healthy mothers and babies respectively (Kirjavainen et al., 2001; Martín et al., 2016). Similarly, *Staphylococcus* was also prevalent among mothers in this study. The genus *Staphylococcus* may be considered to be one of the core microbiome of human milk because it has been reported in studies of human milk microbiome across many geographic locations such as the USA, Europe, Mexico, and Peru irrespective of the methods used (Lackey et al., 2019). Interestingly in a study carried out by Stinson et al. (2021), comparing the presence of viable and not viable DNA cells in human milk among Western Australian women, *Staphylococcus* and *Streptococcus* were reported to be dominant genera of non-viable cells. The existence of non-viable cells in human milk is assumed to have a distinct biological function, but little is understood about their roles (Stinson et al., 2021).

Rothia and *Corynebacterium* also predominate in the milk of mothers in this study, occurring in 41 and 35 of the 48 participants with at least 1% abundance, respectively. This is consistent with the study carried out by Tuominen et al. (2018) who concluded that these two genera are among the core genera of milk of Finnish women and the oral cavity of their babies. *Rothia* was detected in 50% of mothers in Norway at 3 months postpartum (Simpson et al., 2018). Similarly, Biagi et al. (2018) reported the presence of *Rothia* to be most abundant in the breast milk of Italian women, particularly after the baby has latched, and was present in 56% of the recruited infants, at a relative abundance > 0.1%. *Rothia*, being a genus associated with the oral environment, a transfer of this

bacteria to breast milk may have occurred which supports the oro-mammary pathway theory (McGuire & McGuire, 2017). There is not much information regarding the biological function of *Rothia* in human milk, but its abundance has been associated with breast milk of healthy individuals and is significantly correlated with its abundance in infants' guts, suggesting that it may influence immunological homeostasis in infants (Fehr et al., 2020).

In contrast to many studies (Dutta et al., 2021; Corona-Cervantes et al., 2020; Murphy et al., 2017; Jost et al., 2014; Ward et al., 2013). *Pseudomonas* was not among the most abundant genera and only constitute about 1% of the relative abundance of human milk microbiome in the study of the Nigerian population. The biological function of *Pseudomonas* in human milk and the baby's gut has not been described, but its low abundance may be attributed to the study location difference. There have been very few studies undertaken in Africa, particularly in the Western part of the continent, where this study was conducted. One of these is a large study conducted by Lackey et al. (2019) that analysed the milk microbiome of women in Gambia and Ghana, both of which are located in Western Africa, but where *Pseudomonas* was not found in high abundance.

Notably is the high presence of an isolate identified as *Wickerharmomyces anomalus* in the breast milk of some mothers accounting for about 19% of the total bacterial species identified by culture from the breast milk of mothers. A similar organism could not be detected by 16S sequencing, although this could be because *W.anomalus* is a yeast and this study's PCR-NGS approach was designed to target just the bacterial V3-V4 region. The function of this fungus especially in relation to breast milk has not been discussed in the literature. An Asian study has reported its cohabitation with the Anopheles mosquito (Ricci et al., 2011). It is possible that its abundance in the breast milk of Nigerian mothers is attributable to the high prevalence of Malaria, which is transmitted by Anopheles mosquitoes (Awosolu et al., 2021; Okorie et al., 2011).

It should also be noted that breast swabs were not taken from mothers in Nigeria in order to assess the bacteria that may have migrated from breast skin to milk and could be passed on to babies during breastfeeding revealing the true composition of the breast milk that infants consume (Corona-Cervantes et al., 2020). Additionally, mothers in Nigeria don't typically clean their breasts before breastfeeding their babies, and the pilot study discovered that it is difficult to eliminate skin bacteria even after cleaning. Skin swab samples showed a significant decrease in bacterial counts, but some bacteria, including *Staphylococcus* and *Micrococcus* species, could still be found after cleaning the breast skin in the pilot study.

6.2 Bifidobacteria was the most abundant genera in the faeces of breastfed babies

In general, the faecal microbiota of breastfed babies is less diverse than that of human milk in this study, which is consistent with many other investigations (Murphy et al., 2017; Corona-Cervantes et al., 2020; Pannaraj et al., 2017).

In the present study, Illumina MiSeq revealed that the microbiota in faeces of breastfed babies was dominated by the phyla Actinobacteria followed by Proteobacteria, then Firmicutes represented by families *Bifidobacteriacaea*, *Enterobacteriaceae* and *Streptococcacaea* respectively and the genera bifidobacteria, *Escherichia*/*Shigella*, and *Streptococcus*. This is in contrast with studies that reported the dominance of Proteobacteria (Corona-Cervantes et al., 2020) in Mexico, and in China among babies less than 3 months old (Kuang et al., 2016; Niu et al., 2020), but consistent with studies carried out in the Western part of the world such as in the USA and Europe (Kuang et al., 2016).

At the genus level, bifidobacteria is the most abundant bacteria in the faecal samples of breastfed babies in this study. Its presence was detected in 35 participants with at least 20% relative abundance. This is consistent with a recent study carried out on the gut microbiome of exclusively

breastfed Nigerian children within the first year of life (Oyedemi et al., 2022), and agrees with several studies across different geographic locations (Ma et al., 2020). Bifidobacteria can ferment the oligosaccharide in milk (Niu et al., 2020) and considering that exclusively breastfed babies were included in this study, it is not surprising that this genus was among the genera that predominate their gut. *Bifidobacterium* could offer a number of benefits, including assisting in the production of nutrients such as vitamins, helping to modulate the immune system, and being linked to a lower incidence of allergies and asthma (Ma et al., 2020). The reduction of bifidobacteria has been reported to be typical of the gut of older babies who eat solid foods (Niu et al., 2020). A large international study investigating the milk and faeces of mothers and babies less than 6 months of age also reported a high relative abundance of *Bifidobacterium* in the faeces of babies in Gambia and Ghana (Lackey et al., 2019).

Faeces of exclusively breastfed babies were also dominated by streptococci in this study. Lackey et al. (2019) reported streptococci to be one of the core genera in faeces by comparing the microbiota of breastfed babies from 11 different geographic locations, although the said study combined both exclusively breastfed infants and those not exclusively breastfed across the 11 sites (Lackey et al., 2019). Nonetheless, streptococci were common to all, irrespective of the study site.

The abundance of *Enterobacteriaceae* represented by *Escherichia coli* is typical of the gut microbiota and similar to this study, it has been documented in research undertaken in Japan (Nagpal et al., 2017), the United States (Chu et al., 2017), and Europe (Bäckhed et al., 2015).

In the current study, enterococci and lactobacilli account for 0.5% and 0.2% of the overall abundance of bacteria in the faeces of breastfed babies respectively by Miseq and do not form part of the predominant genera in faecal samples of breastfed babies. *Enterococcus* and Lactobacillus are attractive due to their probiotic potential (Maldonado et al., 2012; Rahmani et al., 2020). Lactobacilli have been reported to reduce the incidence of gastrointestinal infection while

enterococci have been reported to have an antibacterial inhibitory effect on pathogens such as *Listeria monocytogenes* in a study on breastfed infants (Rahmani et al., 2020). Moreover, culture on selective media revealed the presence of enterococci and lactobacilli confirming the viability of these bacteria and implying that they may be used as a possible probiotic.

6.3 Streptococci, *Rothia* and Bifidobacteria were commonly shared between human milk and faeces of breastfed babies

Breastmilk has been considered a source of bacteria for babies that are breastfed (Fehr et al., 2020). Mothers have in fact been reported to share about 28% of their breast milk bacteria with their breastfed babies (Corona-Cervantes et al., 2020; Fehr et al., 2020; Ferretti et al., 2018; Pannaraj et al., 2017; Rahmani et al., 2020). In the present study, the bacteria shared between human milk and baby faeces were similar mostly with respect to *Streptococcus* (80% of pairs), *Rothia* (60% of pairs) *and Bifidobacterium* (48% of pairs) which occurred in at least 48% of mother-baby pairs. The same bifidobacterial strain was reported by Martín et al. (2012) in Spain to be shared between mothers and their babies. Additionally, a comparative study of genotyping and antimicrobial activity of human milk bifidobacteria in Iran also reported sharing of similar strains of bifidobacteria between 29% of mothers and their respective babies (Eshaghi et al., 2017).

Members of the bifidobacteria and lactobacilli, which are known to have probiotic qualities, are of particular interest (Farahmand et al., 2021; Raeisi et al., 2013). In the current study, their presence in breast milk was shown to be low compared to faeces by NGS sequencing. The pilot study and the culture approach both revealed similar findings, which have also been supported by other studies (Chen et al., 2018; Murphy et al., 2017; Yan et al., 2021). However, while these bacterial taxa may be present in low concentrations in breast milk, earlier research has shown that breast milk serves as an important source of these bacteria to the guts of breastfed babies (Yan et al., 2021).

Like the present study, *Streptococcus* and *Rothia* have been reported to be shared between mothers and babies in other studies. According to a Canadian study, the presence of *Streptococcus* and *Rothia* among mothers and babies were connected with breastfed babies who were fed entirely from the breast as opposed to pumping the milk (Fehr et al., 2020). Furthermore, *Rothia* was found to be prevalent in babies who had been breastfed at some point, whether exclusively or partially, and to be absolutely absent in babies who had never been breastfed (Fehr et al., 2020). As a result, it is hypothesized that breast milk may transmit species of *Streptococcus* and *Rothia* to babies' guts vertically via breastfeeding.

Interestingly, the genera detected in both mother's milk and baby faeces in the current study, account for over 60% of the relative abundance of bacteria discovered in baby faeces. This supports the vertical transmission concept by demonstrating that human milk seeds the newborn's gut with bacteria. Although the bacteria in this study were not identified by strain, identical bacterial species shared by both groups could indicate probable transfer from breast milk to the gut.

Meanwhile, microbial source tracker analysis revealed that 51% of the bacteria present in baby faeces in this study may have come from breast milk, with 5 participants tracing its probable source to be an unknown source. It has been claimed that, in addition to breast milk, newborns can receive bacteria from various external sources such as the environment, the birth canal, or an entero-mammary pathway from the mother's gut (Corona-Cervantes et al., 2020). In the current study, a high relative abundance of the orders Enterobacteriales, Clostridales, and Coriobacteriales, maybe from an unknown source as revealed by source tracker (Fig 5.13) and supported by studies carried out by Corona-Cervantes et al. (2020) and Parnell et al. (2017).

Many studies have claimed that *Staphylococcus* was shared between mother's milk and baby's faeces due to their dominance in human milk, which could have resulted from retrograde transfer

from the mother's nipple or gut via the enteromammary pathway (Corona-Cervantes et al., 2020; Murphy et al., 2017), but this study did not find this. Although *Staphylococcus* was found in the milk of 47 of the 48 mothers in this study, it was not prevalent in the faeces of the babies and was found in only 15 mother-baby pairs. Location differences may have contributed to this.

Members of Lactobacillales are known for their ability to ferment sugars to lactic acid, and they are believed to have probiotic potential (Bahreini-Esfahani & Moravejolahkami, 2020). It's worth noting that 40 of the 46 mother-baby pairs in the current study shared unclassified Lactobacillales genera that accounted for a high proportion of the bacteria in human milk (25.5%) and 5.3 per cent of the total bacteria in baby faeces. The presence of this taxa was not discovered in the milk and faeces of the remaining 6 mother-baby pairs. Unfortunately, due to the short-read sequencing platform used in this study to target 16S variable regions, further classification of Lactobacillales into genera was not possible.

6.4 The gut microbiota of breastfed babies in Nigeria differs from that of babies fed with milk formula

A total of at least 25 exclusively formula-fed babies were supposed to be recruited to participate in the study in order to compare the gut microbiota of exclusively breastfed babies with that of purely formula-fed babies, however, due to the widespread breastfeeding culture in Nigeria (Berde & Yalcin, 2016) and probably due to the impact of the high cost of formula, babies fed entirely with formula were scarce, and only 8 babies were recruited. It is also to be noted that investigations of gut microbiota in infants exclusively fed with breast milk or exclusively fed with formula are rare or limited (Ma et al., 2020).

Even though the recruited number of formula-fed babies in this study is limited, substantial findings were discovered. When comparing babies who were exclusively given formula to babies who were exclusively fed breast milk, it was shown that formula-fed babies had higher bacterial

diversity. This is also supported by other research (Ma et al., 2020; Roger et al., 2010). Although a highly diverse gut microbiota is reported to be related to a healthier adult gut microbiome (Pickard et al., 2017), this does not appear to be the case for newborns, who have a different ecology and body function when compared to adults. Furthermore, breast milk has also only been known for its beneficial effects on babies (World Health Organization, 2021).

Bifidobacteria has been reported to be linked with healthy infants and it is also connected with the reduction in the incidence of some diseases such as gastrointestinal illnesses and allergies in infants (Ma et al., 2020). In the current study, it was observed that the gut of babies exclusively fed with formula harboured *Bifidobacterium* just like breastfed babies, although the abundance was slightly higher in breastfed babies compared to formula-fed babies (5.57 OTU's) (Figure 5.16). Other researchers have also confirmed this (Rautava, 2016; Zhu et al., 2021). The reason behind the high abundance of bifidobacteria in the gut of breastfed babes has been reported to be due to the use of human milk oligosaccharide present in human milk by some species of bifidobacteria serving as substrates to them. Examples of such species of bifidobacteria are *Bifidobacterium longum* subspecies infantis, *Bifidobacterium breve* and *Bifidobacterium bifidum* (Laursen, 2021).

Additionally, it was observed that the abundance of *Lactobacillus* in the current study is higher in babies exclusively fed with formula when compared to babies fed with breast milk. Other research has found that babies who are breastfed exclusively had higher levels of *Lactobacillus* species compared to formula-fed babies (Bäckhed et al., 2015; Vacca et al., 2022). Even though the number of formula-fed babies in this study was small, *Lactobacillus* was shown to be more abundant in these babies (18 OTUs) (Figure 5.16). The difference could be related to the study site in comparison to earlier studies because studies comparing the microbiota of exclusively breastfed babies to that of solely formula-fed babies have not been carried out in Nigeria, there is no data to compare the current data with. It could also be because many infant formula formulations contain

fructo-oligosaccharides or galacto-oligosaccharides, which can boost the growth of *Lactobacillus* strains (Laursen, 2021).

A relevant finding in this study is the presence of *Faecalibacterium,* a member of the *Ruminococcaceae* family which was present in the faeces of 5 of the 8 exclusively formula-fed babies and was absent in the faeces of babies exclusively fed with breast milk. The presence of this bacteria has been reported to be associated with older children from about one year of age (Vacca et al., 2022) which explains its absence in the gut of breastfed babies and further confirms that the gut of babies fed with formula has a degree of resemblance with that of adult (Laursen, 2021). The sole known species of *Faecalibacterium, Faecaliacterium prausnitzii,* has been linked to healthy individuals (He et al., 2021). The butyrate-producing bacteria have anti-inflammatory capabilities, and their low abundance has been linked to disorders including inflammatory bowel disease (He et al., 2021).

Other beneficial bacteria, such as clostridia, were found in higher abundance in babies fed exclusively with breast milk in the current study. Certain strains of clostridia are important in maintaining mucosal barrier homeostasis during infancy, which is crucial in the development of the intestine (Ma et al., 2020). This conclusion, however, could be due to the small sample size of exclusively formula-fed babies, therefore it should be interpreted with caution.

6.5 Some maternal factors may have an impact on milk and/or faecal microbiota

The relationship between maternal and infant factors collected by questionnaire and the microbiota of the milk and faeces was also looked at. The current study found that there may be an association between a woman's parity (the number of births she has had) and the abundance of *Brevundimonas*, in her milk. Mothers who have more than one child appear to have a higher abundance of this bacteria than mothers who only have one child. There hasn't been much research exploring the

relationship between parity and its impact on milk microbiota, however, Moossavi et al. (2019) found a higher richness of Actinobacteria in mothers with many children. Further research is needed to confirm the findings of this result.

The relationship between diet and milk microbiota was also investigated. The results indicate that there could be an association between the consumption of a diet rich in fats, red meat and chicken and the low abundance of *Rothia*, *Actinomyces* and bifidobacteria in the breast milk of Nigerian mothers respectively. However, due to the issue of questionnaire design, this needs to be further investigated. Moreover, studies have reported an association between diet and breast milk bacteria in other parts of the world. Cortes-Macias et al. (2021) for example reported the association of *Gemella* with the consumption of high protein by Spanish mothers. The consumption of high dietary fibre, plant protein and carbohydrates were also reported to be linked to a higher incidence of *Staphylococcus* and *Bifidobacterium* in human milk (Cortes-Macías et al., 2021). Furthermore, the mother's diet or nutrient intake (for example, consumption of polyunsaturated fatty acids) was reported to be connected to the presence of bifidobacteria in milk (Padilha et al., 2019).

Another commonly researched perinatal variable is the delivery style due to its possible impact on the milk microbiota as well as the composition of the baby's gut microbiota (Corona-Cervantes et al., 2020). Associations were found between the delivery style (Natural vs C-section) and gut microbiota of breastfed babies in the current study. Infants born through the vagina showed a lower abundance of *Klebsiella* than those born via CS. An increase in *Klebsiella* and a decrease in bifidobacteria have been associated with allergy development in early childhood (Low et al., 2017; Reyman et al., 2019). This could explain why there is a higher frequency of allergies and respiratory illnesses in CS-delivered infants (Reyman et al., 2019). In contrast, a Mexico study (Corona-Cervantes et al., 2020) found that gut microbial profiles differ significantly between babies born vaginally and those delivered via C-section, with *Staphylococcus* being the most

abundant in CS-delivered infants and *Pseudomonadaceae* being the most abundant in vaginally delivered infants. More extensive research may be needed in Nigeria to further assess these associations.

Lastly, although this study discovered that there may be an association between religion and milk microbiota among Nigerian mothers. As there is not enough evidence to support this, more research is needed which may involve examining the lifestyles of Christian and Muslim mothers in Nigeria in greater detail to identify potential contributing factors.

No associations were found between all other maternal and infant factors and the microbiota of mothers or babies.

6.6 Evaluation of the analysis and the identification of the microbiota of human milk and faeces

6.6.1 The bacteria found in milk and faeces samples were better represented by 16S rDNA NGS-based approach

This study revealed that the breast milk of mothers from Nigeria contained viable bacteria which is consistent with previous studies (Ding et al., 2019; Jost et al., 2014; Martín et al., 2012), and these bacteria may influence breastfed babies' early gut colonisation (Jost et al., 2013). Using the culture method, the presence of lactobacilli, bifidobacteria, and enterococci was investigated in breast milk and faeces using selective media, with the addition of blood agar to check for other bacteria that may be present in breast milk. By culture, the most common bacterial genera in breast milk are *Staphylococcus*, *Streptococcus*, and *Rothia*. In addition to the presence of these genera, 16S rDNA sequencing also revealed that *Corynebacterium* predominated the human milk microbiome. While culture and MALDI-TOF identification of isolates took into consideration the identification of the major viable bacterial populaces in breast milk, 16S rDNA uncovered that bacterial variety in breast milk is higher and more diverse than discovered by culture methods.

Corynebacterium is one of the most prevalent bacteria in breast milk identified by 16SrDNA sequencing (after *Streptococcus, Staphylococcus* and *Rothia*) in this study. Its detection is consistent with earlier culture-independent investigations (Ding et al., 2019; Huang et al., 2019; Tuominen et al., 2018). However, even though blood agar is expected to facilitate the growth of such non-fastidious organisms, no equivalent strains were found using culture method in the current study. This indicates that the DNA found could be from dead, or partially lysed cells (Stinson et al., 2021). It is also possible that this isolate may have been missed during the sub-culturing stage since a selective media for its isolation was not employed. Besides, the presence of anaerobes particularly strict anaerobes such as bifidobacteria could not be isolated by culture from milk, but their presence could be detected in human milk by 16SrDNA sequencing. Additionally, bifidobacteria could only be isolated from the faeces of one breastfed baby, whereas sequencing approach revealed it to be the most predominant bacterial genera in the faeces of breastfed babies. This could be owing to the bacteria's fastidious nature, which requires a strictly anaerobic environment with a neutral pH (Butta et al., 2017). Additionally, this could also be a result of the selective media used for the isolation of bifidobacteria in the current study. Perhaps the use of commercially available media called Transoligosaccharide propionate agar supplemented by antibiotic mupirocin (TOS-MUP), may have been better in isolating bifidobacteria as it was reported to provide good recovery of these species in a study carried out by Ghoddusi and Hassan (2011). This will therefore be put into consideration in future studies.

In general, 16S sequencing confirmed the microbiota result identified by the traditional culture method, confirming the presence of *Rothia, Staphylococcus, and Streptococcus* in breast milk as well as *Lactobacillus* and *Enterococcus* in faeces. In addition to these bacterial genera, 16S sequencing allowed the detection of a variety of other bacterial species in both milk and faeces, including *Corynebacterium, Acinetobacter, Enterobacter, Actinomyces, Bacteroides,* and

members of the clostridia family, such as *Blautia* (Corona-Cervantes et al., 2020). In summary, this study has confirmed that the use of the traditional culture approach in the investigation of breast milk or faecal microbiota can only provide limited information on the presence and number of microorganisms present due to unculturable organisms as well as microorganisms needing special growth and nutrient requirements. However, culture can be used to analyse live bacterial cells that can be identified with strong certainty up to the species level. 16S sequencing has its limitations as it only reports the existence of DNA, and not whether it came from dead or living cells.

6.6.2 Evaluation of the analysis and the identification of the microbiota of human milk and faeces by culture-based method

In the past decades, scientists have relied on traditional culture methods alongside phenotypic (macroscopic, microscopic, Gram's reaction and biochemical characteristics) approaches to identify bacteria in human samples (Gavin & Ostovar, 1977). Through culture-dependent studies (Ding et al., 2019; Gavin & Ostovar, 1977; Heikkilä & Saris, 2003; Martín et al., 2003; Soto et al., 2014), it has been confirmed that bacteria such as *Staphylococcus*, *Streptococcus*, *Lactobacillus*, *Pseudomonas*, *Bifidobacteria* and *Enterococcus* can be cultured from breast milk, although Staphylococcus and Streptococcus appear to be the most commonly isolated. Having said that, the microbial diversity in breast milk for example, by culture method was restricted to a few bacterial types due to the limitations of the culture method. For instance, Heikkilä & Saris, (2003), and Gavin & Ostovar, (1977) in their culture-dependent studies on human milk bacteria, employing the use of microscopic, genotypic, and biochemical identification, identified only *Staphylococcus* and *Streptococcus* to be predominant in human milk.

Culture-dependent methods have several limitations because they rely on the ability of bacteria to grow on nutrient media, whereas some bacteria are difficult to cultivate in artificial media due to their special nutrient and incubation condition requirements (Ruiz et al., 2019).

Notwithstanding, all the genera associated with the culture-dependent approach mentioned above are consistent with the result of culture-independent studies (Chen et al., 2018; Ding et al., 2019; Jost et al., 2013; Khodayar-Pardo et al., 2014; Li et al., 2017; Martín, et al., 2007; Martín et al., 2012; Simpson et al., 2018; Soto et al., 2014; Urbaniak et al., 2016; Williams et al., 2017), although more diverse bacterial genera have been identified using culture-independent approaches.

Moving forward from the traditional method of microbial identification based on Gram staining, and physical and biochemical characteristics, a more recent approach which has been combined with culture to identify microbial isolates is Matrix-Assisted Laser Desorption Ionisation-Time-of-Flight Mass Spectrometry (MALDI-TOF MS).

MALDI-TOF MS can identify bacterial isolates based on their abundant ribosomal proteins and match this with its database which contains peptide mass fingerprints of different bacterial species (Croxatto et al., 2012; Singhal et al., 2015). MALDI-TOF MS has been combined with culture to identify bacterial isolates from human milk and faeces (Damaceno et al., 2017; Soto et al., 2014). This approach eliminates longer identification time and the need for multiple traditional biochemical tests (Croxatto et al., 2012).

Only a few studies on human milk or faecal microbiota have employed the use of MALDI-TOF MS (Albesharat et al., 2011; Damaceno et al., 2017; Soto et al., 2014), due to the fact that MALDI-TOF cannot be used directly on these samples for the parallel analysis of all bacterial genera present but relies on the extraction of bacterial proteins from isolated bacteria. i.e., identification

of microbiota from a mixed sample (like breast milk or faeces) is not achievable without prior selection/purification of colonies (Damaceno et al., 2017).

Another limitation of MALDI-TOF is its inability to identify bacteria which are not present in its database (Croxatto et al., 2012). In addition to this, closely related species such as *Escherichia coli* and *Shigella, Streptococcus pneumoniae* and *Streptococcus mitis* sometimes cannot be differentiated using MALDI-TOF and may require additional tests to confirm the type of bacteria (Fisher, 2017; Wieser et al., 2012). Other limitation factors are the size of isolates (i.e., isolates must be big enough for successful extraction) and a requirement for a well purified and fresh isolate (i.e., an isolate which has not been previously preserved in cold temperature) to produce quality results (Rychert, 2019). Nonetheless, MALDI-TOF is a robust method of identification of bacterial isolates in breast milk and faeces. It has also been widely used in veterinary microbiology to ensure milk quality and safety (Adkins & Middleton, 2017), and its turn-around time is short (Wieser et al., 2012).

Due to all the issues with culture-based identification methods as confirmed from reviewed studies (Gavin & Ostovar, 1977; Heikkilä & Saris, 2003; Martín et al., 2003; Martín et al., 2012), culture has been revealed to be less effective in investigating the microbial components of human milk and faeces in their whole and may result to an under-representation of the sort of bacteria present. Furthermore, because human milk and faeces are varied, the use of culture will involve several development steps, making the method complicated, time-consuming, and labour-intensive. Culture-based approaches can however provide information on the type, viability, and number of bacteria in human milk. Furthermore, culture is vital in the maintenance of isolated strains, which will benefit future studies such as the investigation of probiotic potentials of isolates and studies involving antimicrobial substances (Cárdenas et al., 2015; Martín et al., 2012).

Moreover, a new strategy for culture-based identification has been identified by a recent study. This method entails growing bacteria in a variety of environments that encourages the development of more fastidious bacteria and then detecting these bacteria using efficient identification techniques like MALDI-TOF MS and 16S rDNA gene sequencing (Cassir et al., 2015). This seems to be an effective strategy that can be used while researching human milk.

6.6.3 Evaluating the analysis and identification of microbiota in human milk and faeces by nucleic-acid-based approaches (culture-independent).

Nucleic acid-based approaches such as quantitative Polymerase Chain Reaction (qPCR) and Next Generation Sequencing analysis have allowed the detection and estimation of bacteria that are difficult to grow, hence allowing more rigorous analysis of the human milk and faeces microbiome (Aakko et al., 2017; Boix-Amorós et al., 2016; Chen et al., 2018; Collado et al., 2009; Ding et al., 2019; Hunt et al., 2011; Khodayar-Pardo et al., 2014; Li et al., 2017; Murphy et al., 2017).

qPCR is a highly specific and sensitive approach that detects and amplifies target genes which are usually 16S rDNA or genus/species-specific primers. It is similar to conventional PCR except that absolute quantification is achieved by the detection of exponentially synthesized amplicons of target genes using fluorescent probes (Kralik & Ricchi, 2017). qPCR can estimate the genome copy of selected bacteria in human milk or faeces. The reaction can be designed to target the genus or species, by using genus or species-specific primers (Collado et al., 2009; Martín et al., 2012; Soto et al., 2014). Several studies have investigated the bacteria present in human milk by qPCR (Collado et al., 2009; Martín et al., 2012; Soto et al., 2014) and it has been reported to be highly sensitive in the specific detection of bacterial species in a complex eco-system such as breast milk and faeces (Collado et al., 2009; Guo et al., 2008), except that it is more useful when dealing with a low number of target regions. Due to this, conventional PCR and qPCR are less likely to provide a comprehensive picture of all the bacterial species present in human milk or faeces.

Next-Generation Sequencing (NGS) on the other hand has continued to gain attention in recent years due to its rapidity and sensitivity (Deurenberg et al., 2017). It appears to be more reliable and less biased as it does not rely on genus or species-specific primers, although it is an expensive approach of studying the human microbiome. NGS can either be used to sequence a whole genome or a specific area depending on interest (Behjati & Tarpey, 2013). This involves the extraction of DNA from the sample of interest, followed by the PCR amplification of the region of interest. Next-Generation sequencing relies on the analysis of the 16S ribosomal DNA sequence of bacteria. The bacterial 16SrDNA gene has highly conserved regions which are interleaved with the hypervariable regions (V1-V9) (Kim et al., 2011). The conserved region serves as a basis for designing the primers to be used in PCR, while the sequencing of the variable regions is used for identifying the bacterial species (Fuks et al., 2018). The hypervariable regions which have been commonly employed in the study of the human milk microbiome include the regions V1 to V5. The V3-V4 primers have been reported to have led to the detection of an increased abundance of Actinobacteria while the V4-V5 primers have been linked to a higher abundance of Firmicutes (Biol-Aquino et al., 2019).

NGS has allowed researchers to study human milk in its entirety (Chen et al., 2018; Li et al., 2017; Williams et al., 2017). Unlike qPCR, the sequence information does not need to be known, creating a genetic fingerprint of all bacterial species present in human milk or faeces (Gullapalli et al., 2012). With this method, the core genera predominantly present in human milk were reported to be '9' (i.e. *Streptococcus, Staphylococcus, Serretia, Corynebacterium, Pseudomonas, Ralstonia, Propionibacterium, Sphingomas, Bradyrhizobium*) by Hunt et al. (2011), '12' by Jost et al. (2013) (i.e. *Staphylococci, Streptococci, Pseudomonas, Corynebacteria, Propionibacteria, Rothia, Flavobacteria, Brevundimonas, Burkholderia Bifidobacteria, Ralstonia* and *Blautia*, and '12' by Li et al. (2017) (i.e *Streptococcus, Pseudomonas, Staphylococcus, Lactobacillus,*

Propionibacterium, Herbaspirillum, Rothia, Stenotrophomonas, Acinetobacter, Bacteroides, Halomonas, Veillonella, Sphingomonas, Delftia, Corynebacterium), which implies that the presence of some bacterial species may have been underestimated using traditional culturing or qPCR. Among the predominant bacterial genera reported by these 3 studies (i.e. (Hunt et al., 2011; Jost et al., 2013; Li et al., 2017) above, 5 bacterial genera were common to them, including *Staphylococcus, Streptococcus, Pseudomonas, Propionibacterium,* and *Corynebacterium*. This further suggests that additional genera may also be classified as part of the predominant genera in human milk, but their presence may be determined by the study participant's location and/or the analytical method used.

However, nucleic acid-based methods have their limitations when being used to analyse complex samples such as human milk or faeces. Factors that must therefore be put into consideration when using a nucleic acid-based approach include the type of DNA extraction technique used (McGuire & McGuire, 2015), the over or underestimation of bacterial count due to the number of bacterial 16SrDNA gene copies, the difference in cell structures of bacteria which can influence the efficiency of extracted DNA (e.g. the cell wall of gram-positive bacteria is more difficult to lyse than gram-negative bacteria) (Wesolowska-Andersen et al., 2014), the issues of cross contaminants (Eisenhofer et al., 2019), and inability to confirm the viability of detected bacteria. Other factors to be considered when processing human milk samples for analysis are the DNA extraction procedure and the type of milk to be employed for DNA extraction (i.e., defatted milk or whole milk) (Gomez-Gallego et al., 2016). A basic DNA extraction procedure can be divided into 3 steps: the first is the disruption of cells (i.e., cell lysis) to release its DNA content, followed by precipitation to separate this DNA from cellular debris before finally eluting the DNA having carried out washing steps. There are several commonly used techniques to disrupt the bacterial cells to release the nucleic acid content; they are the mechanical methods (using a homogenizer or

bead beater), chemical methods, enzymatic methods, or a combination of the three (Yuan et al., 2012). DNA purification is crucial because ineffective DNA extraction caused by inefficient lysis of bacterial cell walls, especially gram-positive bacteria, can lead to the misrepresentation of the relative abundance of species present in milk samples (Jervis-Bardy et al., 2015), hence significantly influencing the outcome of milk microbiota. For instance, Douglas et al. (2020) reported a notable difference in the DNA yield, DNA purity and microbial composition when comparing the effect of different DNA extraction methods on the microbial composition in mock breast milk samples (i.e., inoculated with bacteria) as well as individual breast milk samples. It was also observed in a comparative study that the combination of bead beating and a cocktail of enzymes (lysozyme, mutanolysin and lysostaphin) produced the best representation of the microbial diversity compared to the lysis by enzymes or chemicals (Yuan et al., 2012). The variations in the microbial composition in saliva, stool and vaginal samples that resulted from different extraction methods have also been reported (Fouhy et al., 2016).

6.6.4 Reporting details of methods and analysis in the study of human milk microbiota

It was noted from reviewed articles that studies involving the human milk microbiome have sometimes failed to report relevant information regarding sample collection and processing. A need for reporting is particularly important when the required information or data is likely to have an impact when interpreting results.

Irregularities in the mode of reports across human milk studies are often observed and sometimes, basic information relating to the analytical methods used is missing. For example, it is important to report the process of collecting the breast milk in detail, i.e., whether the breast was cleaned before collection or not, the extent to which the cleaning was carried out, as well as what was used in the cleaning (water, alcohol, chlorhexidine etc.). Some of the data reported by studies are insufficient or not clearly defined, for example, phrases like ‘‘aseptic technique’’ (Hunt et al.,

2011; Martín et al., 2003; Martín, et al., 2007) or ‘‘use of sterile kit’’ (Williams et al., 2017) when describing the breast milk collection method should be explained further. It is therefore recommended that, when a cleaning step is required (depending on the aim of the research), it should be clearly defined due to the impact the skin bacteria may have on the human milk sample (Gomez-Gallego et al., 2016).

Other points which have commonly been omitted in some published articles include the human milk expression method, sampling time (i.e., how many days postpartum the sample was obtained), information about the actions that occurred between sample collection and its processing (for example, the duration before milk samples were processed and the storage conditions during this period). Vague phrases such as ‘‘the sample was frozen’’ (Soto et al., 2014) without reporting the temperature conditions can also be clarified further due to the impact that temperature conditions can have on the human milk microbiome (Wesolowska-Andersen et al., 2014). For example, research has shown that whenever it is impossible to process human milk samples when freshly obtained, freezing at -80°C is the next best option to preserve the integrity of its microbiome (Lyons et al., 2021). Figure 6.1 below is a summary of the basic things to consider when reporting details of methods used in breast milk microbiome research.

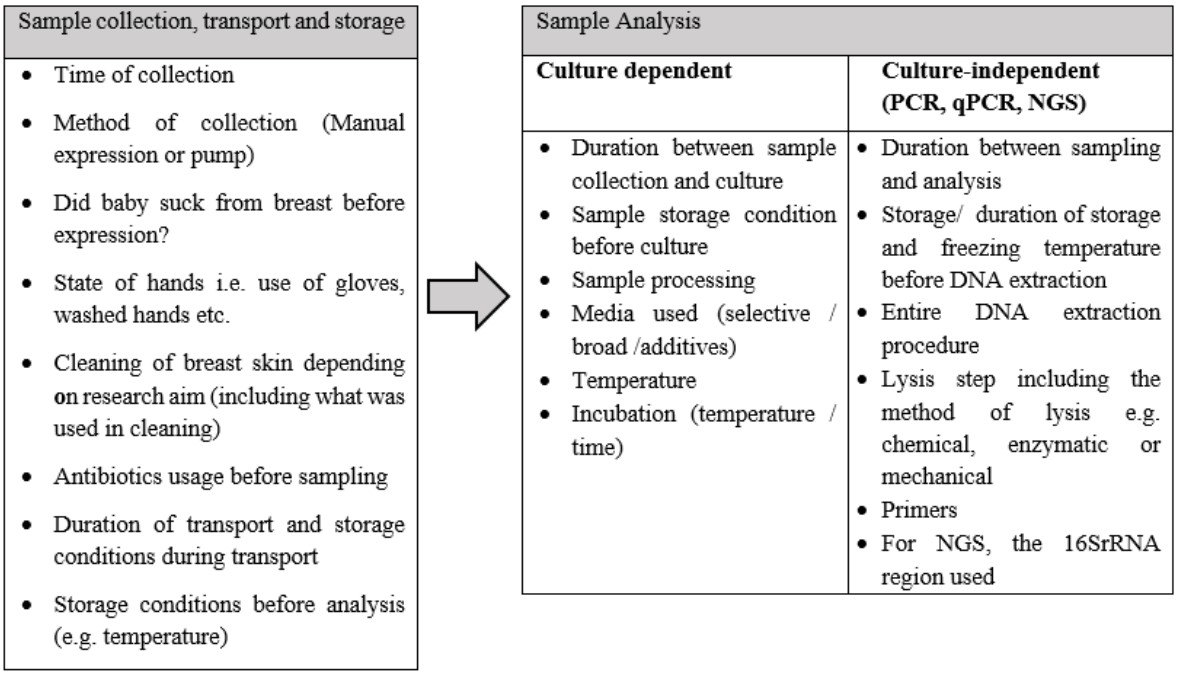


Figure 6.1: Recommendations for reporting data from human milk analysis. When reporting data involving analysis of human milk microbiome, details of sample collection and sample analysis which may influence the result should be considered. The method of sample collection including the time, the expression method, cleaning, and antibiotics may all influence the type of bacteria identified. Details of the method used in analysis i.e., whether a culture-dependent method or culture-independent method was employed, including the type of media used in culture-based studies, sample storage time and storage temperature should be included due to the influence they may have on the microbiome.

Lastly, the major pitfall in the study of the human milk microbiome is the unavailability of a standardized protocol for analyzing human milk samples: particularly the sampling protocol, the DNA extraction procedure and identification methods. Different approaches used in the lysis step of human milk microbiome extraction resulted in different microbial diversity and bacterial community structures. A slight alteration in protocols such as DNA extraction, downstream applications and bioinformatics analysis can also lead to misrepresentation of the human milk microbiome (Natureportfolio, 2022). Until a standard protocol is developed, there will be bias in the results of the milk microbiome, and there will be no reliable data to compare the results of different studies around the world, hence hindering the progress of human milk microbiome research.

Chapter 7

7.1 Conclusions

This study investigated the microbiota of human milk and gut of babies by employing the traditional culture-based method and 16S rDNA NGS-based approach. This study forms the first type of study on human milk and faeces of breastfed and formula-fed babies to be conducted in West Africa. This research confirmed that human milk has a highly customised microbiota with a lot of inter-individual variabilities and the microbiota of milk is more diverse than the gut microbiota of breastfed babies. It was revealed that the milk microbiome of mothers from Nigeria is characterised by the high dominance of Firmicutes such as Lactobacillales and Bacillales next to Actinobacteria largely represented by Micrococcales and Corynebacteriales and then Proteobacteria as seen in the presence of Caulobacterales and Pseudomonadales. In the faeces of breastfed babies, there was high dominance of members of Actinobacteria, Proteobacteria, and Firmicutes represented by bifidobacteria, *Escherichia*/*Shigella* as well as streptococci.

This research also provides evidence that mothers may transfer bacteria vertically through breastfeeding to their breastfed babies and about 51% of bacteria in the baby's gut may have originated from human milk. The bacteria that may be transferred from mothers to babies include *Streptococcus* (present in 80% of mother-baby pairs), *Rothia* (present in 60% of mother-baby pairs) *and Bifidobacterium* (present in 48% of mother-baby pairs).

Culture technique also revealed the presence of common viable bacteria including species of staphylococci, streptococci, lactobacilli and *Rothia* in breast milk as well as species of enterococci, lactobacilli, bifidobacteria and streptococci in faeces. Culture detection of strict anaerobes such as bifidobacteria was restricted in both breast milk and faeces.

When comparing the gut microbiota of breastfed babies with that of babies fed with formula, breastfed babies had lower microbial diversity than formula-fed babies. The gut of breastfed babies

was mainly dominated by *Bifidobacteriaceae* (55.3%), *Enterobacteriaceae* (23.7%) and *Streptococcaceae* (2%), while the gut of babies who were fed with milk formula were dominated by *Bifidobacteriaceae* (26.5%), *Enterobacteriaceae* (24.6), *Lactobacillaceae* (11.6%), *Coriobacteriaceae* (9.1%), and *Ruminococcaceae* (5.4%). The presence of *Faecalibacterium* was only detected in babies on milk formula but not observed in solely breastfed babies. This study, therefore, provided preliminary information that will aid future research on the gut microbiota of babies in relation to the mode of feeding in Nigeria.

7.2 Strengths and limitations of the study

This study is the first of its kind in Nigeria, involving the characterization of microbiota in human milk and infant faeces among Nigerian mothers and babies, thereby providing insight into the composition of their milk and gut. Although several studies have been carried out in various parts of the world on human milk and gut microbiota, however, when studying the microbial communities of the milk and gut, access to reliable data from diverse geographic locations is required to fully understand the ecosystem of these environments, which has been shown to differ between different geographic locations. This study is significant because it adds to previous research on human milk and gut microbiota and provides new data for future studies to compare. There is also no information in the Nigerian community about the gut microbiota of babies who are exclusively fed formula, which was also investigated in the current study.

This study overcame the difficulty of collecting human samples in Nigeria, sampling more than 50 mothers and their babies, as well as 8 formula-fed babies, and contributing significantly to the body of knowledge on the microbiota of milk and gut of babies around the world, particularly the gut microbiota of formula-fed babies, for which there are relatively few published studies.

Furthermore, novel techniques and bioinformatic analysis were used to investigate the samples during the study, resulting in a wealth of comprehensive and reliable data on the milk microbiome

and its relationship to the gut microbiome of Nigerian babies, which can be used as a foundation for future research. The findings of this study further supported the notion that breast milk is the healthiest food for infants by confirming the presence of beneficial bacteria in the milk of healthy mothers and their potential for transfer to the baby's gut.

On the contrary, due to Nigeria's widespread breastfeeding culture, it was difficult to recruit an adequate number of exclusively formula-fed participants, resulting in a lower number of samples when compared to breastfed babies. Although meaningful results were obtained with a small number of participants, statistical interpretation may be biased.

Furthermore, this study did not also obtain samples of milk and faeces over time, hence the microbial presence was not looked at over time to confirm any changes or stability. It was not also possible to carry out investigations such as the identification of isolates on the field due to the lack of equipment in Nigeria, which led to the isolates being frozen before subsequent sub-culturing and identification in the UK. It is assumed that this may have led to the loss of some bacterial isolates. It was also observed that although a selective media was used in the isolation of *Lactobacillus* in the current study, the growth of yeast was also observed. The reason behind this is unknown, as it is unlikely to be due to the incubating temperature of 37°C used to incubate rogosa plates in the current study, as opposed to the 35°C suggested by the manufacturer.

Besides, because this study did not look at bacteria at the strain level, it cannot be confirmed that the bacterial species found in both mothers and babies are of the same strain, and thus vertical transmission from a mother to her baby cannot be confirmed. This will be one of the areas to investigate in the future.

Limited socioeconomic data were acquired as a result of the questionnaire's design, indicating that there was not enough data to draw a conclusion and that results should be carefully interpreted. Additionally, the dietary data collected from mothers in Nigeria was insufficient to draw any conclusions. The frequency of each dietary consumption could not be determined because the

questionnaire used in this study to gather information on nutrients and food intake was not a food frequency questionnaire.

7.3 Recommendations for future studies

i. More research into human milk and gut microbiota in Nigeria and other parts of West Africa is recommended, to allow for adequate comparison.

ii. The 16SrDNA method used in this study provided comprehensive information on the microbiome of mothers' milk and faeces of babies in Nigeria, however, this study recommends the use of strain level identification methods such as whole genome sequencing to look at the bacteria common to both milk and faeces to determine if they are of similar strains.

iii. This study focused more on the type of bacteria present in mothers' milk and baby faeces. Analysis of the functional properties of these bacteria is recommended to determine their importance and to see if any variations can be linked to socioeconomic or environmental factors in Nigeria.

iv. In addition, further research should be done to determine the origin of these bacteria, as well as when the colonisation of the mammary gland with these bacteria starts, to determine whether this colonisation can be modulated in any way.

v. More research on the gut microbiome of formula-fed babies is also recommended to determine if formula milk can be improved in any way.

vi. This study suggests that, for adequate comparison between studies, a standard approach to sample collection and processing be developed in the study of the human milk microbiome.

vii. A need for adequate reporting in published articles especially when the required information is likely to have an impact when interpreting results is recommended.

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.

Acharya, K. R., Dhand, N. K., Whittington, R. J., & Plain, K. M. (2017). PCR inhibition of a quantitative PCR for detection of Mycobacterium avium subspecies Paratuberculosis DNA in feces: diagnostic implications and potential solutions. *Frontiers in Microbiology, 8*, 115.

Adkins, P. R., & Middleton, J. R. (2017). Laboratory handbook on bovine mastitis. *National Mastitis Council,* Incorporated.

Akinyandenu, O., & Akinyandenu, A. (2014). Irrational use and non-prescription sale of antibiotics in Nigeria, a need for change. *Journal of Scientific and Innovative Research, 3*(2), 251-257.

Albesharat, R., Ehrmann, M. A., Korakli, M., Yazaji, S., & Vogel, R. F. (2011). Phenotypic and genotypic analyses of lactic acid bacteria in local fermented food, breast milk and faeces of mothers and their babies. *Systematic and Applied Microbiology*, 34(2), 148-155.

Ali, E., & Nizar, N. N. A. (Eds.). (2018). Preparation and processing of religious and cultural foods. Woodhead Publishing (pp.73). Retrieved from https://books.google.co.uk/books?hl=en&lr=&id=gtJKDwAAQBAJ&oi=fnd&pg=PP1&ots=syMOovtFr6&sig=PEboK_EM3sYICYkYqNNdPzmEC20&redir_esc=y#v=onepage&q&f=false

Araújo, E. D., Gonçalves, A. K., Cornetta, M. d. C., Cunha, H., Cardoso, M. L., Morais, S. S., & Giraldo, P. C. (2005). Evaluation of the secretory immunoglobulin A levels in the colostrum

and milk of mothers of term and pre-trerm newborns. *Brazilian Journal of Infectious Diseases, 9*, 357-362.

Arroyo, R., Martín, V., Maldonado, A., Jiménez, E., Fernández, L., & Rodríguez, J. M. (2010). Treatment of infectious mastitis during lactation: antibiotics versus oral administration of Lactobacilli isolated from breast milk. *Clinical Infectious Diseases, 50*(12), 1551-1558.

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., Fernandes, G. R., Tap, J., Bruls, T., & Batto, J. (2011). Enterotypes of the human gut microbiome. *Nature, 473*(7346), 174-180.

Awosolu, O. B., Yahaya, Z. S., Haziqah, M. T. F., Simon-Oke, I. A., & Fakunle, C. (2021). A cross-sectional study of the prevalence, density, and risk factors associated with malaria transmission in urban communities of Ibadan, Southwestern Nigeria. *Heliyon, 7*(1), e05975.

Ayeni, F. A., Biagi, E., Rampelli, S., Fiori, J., Soverini, M., Audu, H. J., Cristino, S., Caporali, L., Schnorr, S. L., Carelli, V., Brigidi, P., Candela, M., & Turroni, S. (2018). Infant and Adult Gut Microbiome and Metabolome in Rural Bassa and Urban Settlers from Nigeria. *Cell reports*, 23(10), 3056–3067.

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., Li, Y., Xia, Y., Xie, H., & Zhong, H. (2015). Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host & Microbe, 17*(5), 690-703.

Bahreini-Esfahani, N., & Moravejolahkami, A. R. (2020). Can synbiotic dietary pattern predict lactobacillales strains in breast milk? *Breastfeeding Medicine, 15*(6), 387-393.

Ballard, O., & Morrow, A. L. (2013). Human milk composition: Nutrients and Bioactive factors. *Paediatric Clinics of North America*, 60(1), 49-74.

Bassey, S. E., & Izah, S. C. (2017). Some determinant factors of malaria prevalence in Nigeria. *Journal of Mosquito Research, 7* (7), 48-58.

Baumann-Dudenhoeffer, A. M., D'Souza, A. W., Tarr, P. I., Warner, B. B., & Dantas, G. (2018). Infant diet and maternal gestational weight gain predict early metabolic maturation of gut microbiomes. *Nature Medicine, 24*(12), 1822-1829.

Beasley, S. S., & Saris, P. E. (2004). Nisin-producing Lactococcus lactis strains isolated from human milk. *Applied and environmental microbiology*, 70(8), 5051-5053.

Behjati, S., & Tarpey, P. S. (2013). What is next generation sequencing? *Archives of Disease in Childhood-Education and Practice, 98*(6), 236-238.

Behnsen, J., Deriu, E., Sassone-Corsi, M., & Raffatellu, M. (2013). Probiotics: properties, examples, and specific applications. *Cold Spring Harbor perspectives in medicine*, 3(3), a010074.

Berde, A. S., & Yalcin, S. S. (2016). Determinants of early initiation of breastfeeding in Nigeria: a population-based study using the 2013 demograhic and health survey data. *BMC Pregnancy and Childbirth, 16*(1), 1-9.

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M. C., Charles, T., Chen, X., Cocolin, L., Eversole, K., & Corral, G. H. (2020). Microbiome definition re-visited: old concepts and new challenges. *Microbiome, 8*(1), 1-22.

Biagi, E., Aceti, A., Quercia, S., Beghetti, I., Rampelli, S., Turroni, S., Soverini, M., Zambrini, A. V., Faldella, G., & Candela, M. (2018). Microbial community dynamics in mother's milk and infant's mouth and gut in moderately preterm infants. *Frontiers in Microbiology,* , 2512.

Biol-Aquino, M. A., Perdiz, C. J., Borlagdan, M., Alcantara, J. D., & Mallillin, A. (2019). Differences in the bacterial profiles of infant gut by birth process, milk diet, and choice of 16S rRNA gene target region. *Human Microbiome Journal, 13*, 100062.

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*, 492.

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), 1-13.*

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.

Boyanta, K. (2018). *How does milk production work?* https://kellymom.com/hot-topics/milkproduction/

Brandtzaeg, P. (2013). Secretory IgA: designed for anti-microbial defense. *Frontiers in immunology*, 4, 222.

Butta, H., Sardana, R., Vaishya, R., Singh, K. N., & Mendiratta, L. (2017). Bifidobacterium: an emerging clinically significant metronidazole-resistant anaerobe of mixed pyogenic infections. *Cureus, 9*(4)

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.

Cabrera-Rubio, R., Mira-Pascual, L., Mira, A., & Collado, M. C. (2016). Impact of mode of delivery on the milk microbiota composition of healthy women. *Journal of Developmental Origins of Health and Disease, 7*(1), 54-60.

Carbajal, R., Veerapen, S., Couderc, S., Jugie, M., & Ville, Y. (2003). Analgesic effect of breast feeding in term neonates: randomised controlled trial. *British medical journal, 326*(7379), 13.

Cárdenas, N., Laiño, J. E., Delgado, S., Jiménez, E., Juárez del Valle, M., Savoy de Giori, G., Sesma, F., Mayo, B., Fernández, L., & LeBlanc, J. G. (2015). Relationships between the genome and some phenotypical properties of Lactobacillus fermentum CECT 5716, a probiotic strain isolated from human milk. *Applied Microbiology and Biotechnology, 99*(10), 4343-4353.

Cassir, N., Benamar, S., Khalil, J. B., Croce, O., Saint-Faust, M., Jacquot, A., Million, M., Azza, S., Armstrong, N., & Henry, M. (2015). Clostridium butyricum strains and dysbiosis linked to necrotizing enterocolitis in preterm neonates. *Clinical Infectious Diseases, 61*(7), 1107-1115.

Cechova, M., Beinhauerova, M., Babak, V., Slana, I., & Kralik, P. (2021). A novel approach to the viability determination of mycobacterium avium subsp. paratuberculosis using platinum compounds in combination with quantitative PCR. *Frontiers in Microbiology*, 12.

Chehab, R., Fehr, K., Moossavi, S., Moraes, T., Mandhane, P., de Souza, R., Turvey, S., Subbarao, P., Khafipour, E., & Azad, M. (2021). Maternal Prenatal Supplement Intake, but Not Dietary Patterns, Is Associated With Human Milk Microbiota Composition in the CHILD Cohort Study. *Current Developments in Nutrition, 5*(Supplement_2), 1154.

Chen, P., Lin, Y., & Huang, M. (2018). Profiles of commensal and opportunistic bacteria in human milk from healthy donors in Taiwan. *Journal of Food and Drug Analysis, 26*(4), 1235-1244.

Chu, D. M., Ma, J., Prince, A. L., Antony, K. M., Seferovic, M. D., & Aagaard, K. M. (2017). Maturation of the infant microbiome community structure and function across multiple body sites and in relation to mode of delivery. *Nature Medicine, 23*(3), 314-326.

Chua, S., Arulkumaran, S., Lim, I., Selamat, N., & Ratnam, S. S. (1994). Influence of breastfeeding and nipple stimulation on postpartum uterine activity . *BJOG: An International Journal of Obstetrics and Gynaecology, 101(9)*, 804-805.

Codony, F., Dinh-Thanh, M., & Agustí, G. (2019). Key factors for removing bias in viability PCR-based methods: A Review. *Current Microbiology*, 77(4), 682–687.

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.

Consales, A., Cerasani, J., Sorrentino, G., Morniroli, D., Colombo, L., Mosca, F., & Giannì, M. L. (2022). The hidden universe of human milk microbiome: origin, composition, determinants, role, and future perspectives. *European Journal of Pediatrics, ,* 1-10.

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.

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.

Croxatto, A., Prod'hom, G., & Greub, G. (2012). Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. *FEMS Microbiology Reviews, 36*(2), 380-407.

Damaceno, Q. S., Souza, J. P., Nicoli, J. R., Paula, R. L., Assis, G. B., Figueiredo, H. C., Azevedo, V., & Martins, F. S. (2017). Evaluation of potential probiotics isolated from human milk and colostrum. *Probiotics and Antimicrobial Proteins, 9*(4), 371-379.

Davani-Davari, D., Negahdaripour, M., Karimzadeh, I., Seifan, M., Mohkam, M., Masoumi, S. J., ... & Ghasemi, Y. (2019). Prebiotics: definition, types, sources, mechanisms, and clinical applications. *Foods*, 8(3), 92.

Derrien, M., Alvarez, A., & de Vos, W. M. (2019). The gut microbiota in the first decade of life. *Trends in Microbiology, 27*(12), 997-1010.

Deurenberg, R. H., Bathoorn, E., Chlebowicz, M. A., Couto, N., Ferdous, M., García-Cobos, S., Kooistra-Smid, A. M., Raangs, E. C., Rosema, S., & Veloo, A. C. (2017). Application of next generation sequencing in clinical microbiology and infection prevention. *Journal of Biotechnology, 243*, 16-24.

Díaz-Ropero, M. P., Martin, R., Sierra, S., Lara-Villoslada, F., Rodríguez, J. M., Xaus, J., & Olivares, M. (2007). Two Lactobacillus strains, isolated from breast milk, differently modulate the immune response. *Journal of Applied Microbiology, 102*(2), 337-343.

Ding, M., Qi, C., Yang, Z., Jiang, S., Bi, Y., Lai, J., & Sun, J. (2019). Geographical location specific composition of cultured microbiota and Lactobacillus occurrence in human breast milk in China. *Food & Function, 10*(2), 554-564.

Dizdar, E. A., Sari, F. N., Degirmencioglu, H., Canpolat, F. E., Oguz, S. S., Uras, N., & Dilmen, U. (2014). Effect of mode of delivery on macronutrient content of breast milk. *The Journal of Maternal-Fetal & Neonatal Medicine, 27*(11), 1099-1102.

Douglas, C. A., Ivey, K. L., Papanicolas, L. E., Best, K. P., Muhlhausler, B. S., & Rogers, G. B. (2020). DNA extraction approaches substantially influence the assessment of the human breast milk microbiome. *Scientific Reports, 10*(1), 1-10.

Drago, L., Toscano, M., De Grandi, R., Grossi, E., Padovani, E. M., & Peroni, D. G. (2017). Microbiota network and mathematic microbe mutualism in colostrum and mature milk collected in two different geographic areas: Italy versus Burundi. *The ISME Journal, 11*(4), 875-884.

Eidelman, A. I., & Szilagyi, G. (1979). Patterns of bacterial colonization of human milk. *Obstetrics and Gynecology, 53*(5), 550-552.

Eisenhofer, R., Minich, J. J., Marotz, C., Cooper, A., Knights, R., & Weyrich, L. S. (2019). Contamination in low microbial biomass microbiome studies: issues and recommendations. *Trends in Microbiology 27(2),* `105-117.

Emerson, J. B., Adams, R. I., Román, C. M. B., Brooks, B., Coil, D. A., Dahlhausen, K., Ganz, H. H., Hartmann, E. M., Hsu, T., & Justice, N. B. (2017). Schrödinger's microbes: tools for distinguishing the living from the dead in microbial ecosystems. *Microbiome, 5*(1), 1-23.

Eshaghi, M., Bibalan, M. H., Rohani, M., Esghaei, M., Douraghi, M., Talebi, M., & Pourshafie, M. R. (2017). Bifidobacterium obtained from mother's milk and their infant stool; A comparative genotyping and antibacterial analysis. *Microbial Pathogenesis, 111,* 94-98.

Ezechi, E. H., Nwabuko, C. G., Enyinnaya, O. C., & Babington, C. J. (2017). Municipal solid waste management in Aba, Nigeria: Challenges and prospects. *Environmental Engineering Research, 22*(3), 231-236.

Fallani, M., Young, D., Scott, J., Norin, E., Amarri, S., Adam, R., Aguilera, M., Khanna, S., Gil, A., & Edwards, C. A. (2010). Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. *Journal of Pediatric Gastroenterology and Nutrition, 51*(1), 77-84.

Falola, T. (2001). Culture and customs of Nigeria. *Greenwood Publishing Group*.

Farahmand, N., Ouoba, L. I., Naghizadeh Raeisi, S., Sutherland, J., & Ghoddusi, H. B. (2021). Probiotic lactobacilli in fermented dairy products: Selective detection, enumeration and identification scheme. *Microorganisms, 9*(8), 1600.

Favier, C. F., Vaughan, E. E., De Vos, W. M., & Akkermans, A. D. (2002). Molecular monitoring of succession of bacterial communities in human neonates. *Applied and Environmental Microbiology, 68*(1), 219-226.

Fehr, K., Moossavi, S., Sbihi, H., Boutin, R. C., Bode, L., Robertson, B., Yonemitsu, C., Field, C. J., Becker, A. B., & Mandhane, P. J. (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.

Fernández, L., Delgado, S., Herrero, H., Maldonado, A., & Rodriguez, J. M. (2008). The bacteriocin nisin, an effective agent for the treatment of staphylococcal mastitis during lactation. *Journal of Human Lactation, 24*(3), 311-316.

Fernández, L., Langa, S., Martín, V., Maldonado, A., Jiménez, E., Martín, R., & Rodríguez, J. M. (2013). The human milk microbiota: origin and potential roles in health and disease. *Pharmacological Research, 69*(1), 1-10.

Ferretti, P., Pasolli, E., Tett, A., Asnicar, F., Gorfer, V., Fedi, S., Armanini, F., Truong, D. T., Manara, S., & Zolfo, M. (2018). Mother-to-infant microbial transmission from different body sites shapes the developing infant gut microbiome. *Cell Host & Microbe, 24*(1), 133-145. e5.

Fisher, M. A. (2017). Differentiation of Closely Related Organisms using MALDI-TOF MS. *MALDI-TOF and Tandem MS for Clinical Microbiology,* , 147-165.

Fitzstevens, J. L., Smith, K. C., Hagadorn, J. I., Caimano, M. J., Matson, A. P., & Brownell, E. A. (2017). *Nutrition in Clinical Practice,* 32(3), 354-364.

Fouhy, F., Clooney, A. G., Stanton, C., Claesson, M. J., & Cotter, P. D. (2016). 16S rRNA gene sequencing of mock microbial populations- impact of DNA extraction method, primer choice and sequencing platform. *BMC microbiology, 16(1), 1-13.*

Fuks, G., Elgart, M., Amir, A., Zeisel, A., Turnbaugh, P. J., Soen, Y., & Shental, N. (2018). Combining 16S rRNA gene variable regions enables high-resolution microbial community profiling. *Microbiome, 6*(1), 1-13.

Gaitskell, K., Green, J., Pirie, K., Reeves, G., & Beral, V. (2015). Parity, breastfeeding and ovarian cancer in the Million Women Study. *Cancer Research,* 75(15_Supplement), 873-873.

Gao, X., McMahon, R. J., Woo, J. G., Davidson, B. S., Morrow, A. L., & Zhang, Q. (2012). Temporal changes in milk proteomes reveal developing milk functions. *Journal of Proteome Research, 11*(7), 3897-3907.

Gavin, A., & Ostovar, K. (1977). Microbiological characterization of human milk. *Journal of Food Protection.40(9),* 615-616.

Gay, M., Koleva, P. T., Slupsky, C. M., Toit, E. D., Eggesbo, M., Johnson, C. C., Wegienka, G., Shimojo, N., Campbell, D. E., Prescott, S. L., Munblit, D., Geddes, D. T., Kozyrskyj, A. L., & InVIVO LactoActive Study Investigators (2018). Worldwide Variation in Human Milk Metabolome: Indicators of Breast Physiology and Maternal Lifestyle?. *Nutrients*, 10(9), 1151.

Ghoddusi, H. B., & Hassan, K. (2011). Selective enumeration of bifidobacteria: a comparative study. *Milchwissenschaft-Milk Science International*, 66(2), 149.

Godhia, M. L., & Patel, N. (2013). Colostrum–its Composition, Benefits as a Nutraceutical–A Review. *Current Research in Nutrition and Food Science Journal, 1*(1), 37-47.

Gomez-Gallego, C., Garcia-Mantrana, I., Salminen, S., & Collado, M. C. (2016). The human milk microbiome and factors influencing its composition and activity. Paper presented at the *Seminars in Fetal and Neonatal Medicine, , 21*(6) 400-405.

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), e80299.

Goodman, C., Brieger, W., Unwin, A., Mills, A., Meek, S., & Greer, G. (2007). Medicine sellers and malaria treatment in sub-Saharan Africa: what do they do and how can their practice be improved? *The American Journal of Tropical Medicine and Hygiene, 77*(6 Suppl), 203.

Gray, L., Miller, L. W., Philipp, B. L., & Blass, E. M. (2002). Breastfeeding is analgesic in healthy newborns. *Pediatrics, 109*(4), 590-593.

Guaraldi, F., & Salvatori, G. (2012). Effect of breast and formula feeding on gut microbiota shaping in newborns. *Frontiers in Cellular and Infection Microbiology, 2*, 94.

Gullapalli, R. R., Desai, K. V., Santana-Santos, L., Kant, J. A., & Becich, M. J. (2012). Next generation sequencing in clinical medicine: Challenges and lessons for pathology and biomedical informatics. *Journal of Pathology Informatics, 3*(1), 40.

Guo, X., Xia, X., Tang, R., Zhou, J., Zhao, H., & Wang, K. (2008). Development of a real-time PCR method for Firmicutes and Bacteroidetes in faeces and its application to quantify intestinal population of obese and lean pigs. *Letters in Applied Microbiology, 47*(5), 367-373.

Harrison, D., Reszel, J., Bueno, M., Sampson, M., Shah, V. S., Taddio, A., Larocque, C., & Turner, L. (2016). Breastfeeding for procedural pain in infants beyond the neonatal period. *Cochrane Database of Systematic Reviews,* (10)

He, X., Zhao, S., & Li, Y. (2021). Faecalibacterium prausnitzii: a next-generation probiotic in gut disease improvement. *Canadian Journal of Infectious Diseases and Medical Microbiology, 2021*

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

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*, 4.

Hoppener-Ogawa, S., Leveau, J. H., Smant, W., van Veen, J. A., & de Boer, W. (2007). Specific detection and real-time PCR quantification of potentially mycophagous bacteria belonging to

the genus Collimonas in different soil ecosystems. *Applied and Environmental Microbiology, 73*(13), 4191-4197.

Huang, M., Cheng, C., Tseng, S., Lin, Y., Lo, H., & Chen, P. (2019). Most commensally bacterial strains in human milk of healthy mothers display multiple antibiotic resistance. *Microbiologyopen, 8*(1), e00618.

Hunt, K. M., Foster, J. A., Forney, L. J., Schütte, U. M., 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.

Illumina.com (2013). 16s metagenomic sequencing library preparation. Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System. Illumina: San Diego, CA, USA.

india

Jahani, S., Shakiba, A., & Jahani, L. (2015). The Antimicrobial effect of lactoferrin on Gram-negative and Gram-positive bacteria. *International Journal of Infection,* 2(3).

Jarlenski, M. P., Bennett, W. L., Bleich, S. N., Barry, C. L., & Stuart, E. A. (2014). Effects of breastfeeding on postpartum weight loss among US women. *Preventive medicine*, 69, 146-150.

Jervis-Bardy, J., Leong, L. E. X., Marri, S., Smith, R. J., Choo, J. M., Smith-Vaughan, H. C., Nosworthy, E., Morris, P. S., Leary, S., Rogers, G. B., & Marsh, R. L. (2015). Deriving accurate microbiota profiles from human samples with low bacterial content through post-sequencing processing of Illumina MiSeq data. *Microbiome, 3*(1), 19. 10.1186/s40168-015-0083-8

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.

Jiménez, E., Delgado, S., Maldonado, A., Arroyo, R., Albújar, M., García, N., Jariod, M., Fernández, L., Gómez, A., & Rodríguez, J. M. (2008). Staphylococcus epidermidis: a differential trait of the fecal microbiota of breastfed infants. *BMC Microbiology, 8*(1), 1-11.

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,* 4650- 4655.

Jiménez, E., Langa, S., Martín, V., Arroyo, R., Martín, R., Fernández, L., & Rodríguez, J. M. (2010). Complete genome sequence of Lactobacillus fermentum CECT 5716, a probiotic strain isolated from human milk. *Journal of bacteriology,* 192(18), 4800-4800.

Jost, T., Lacroix, C., Braegger, C. P., Rochat, F., & Chassard, C. (2014). Vertical mother–neonate transfer of maternal gut bacteria via breastfeeding. *Environmental Microbiology, 16*(9), 2891-2904.

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.

Kashtanova, D. A., Popenko, A. S., Tkacheva, O. N., Tyakht, A. B., Alexeev, D. G., & Boytsov, S. A. (2016). Association between the gut microbiota and diet: fetal life, early childhood, and further life. *Nutrition, 32*(6), 620-627.

Kennedy, K. I., Labbok, M. H., & Van Look, P. (1996). Lactational amenorrhea method for family planning. *International Journal of Gynecology & Obstetrics, 54*(1), 55-57.

Kesen, M. A., & Aiyegoro, O. A. (2018). Beneficial characteristics and evaluation criteria of probiotics. *International Journal of Food Biosci*ence, 1, 25-33.

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), 599-605.

Kim, M., Morrison, M., & Yu, Z. (2011). Evaluation of different partial 16S rRNA gene sequence regions for phylogenetic analysis of microbiomes. *Journal of Microbiological Methods, 84*(1), 81-87.

Kirjavainen, P. V., Apostolou, E., Arvola, T., Salminen, S. J., Gibson, G. R., & Isolauri, E. (2001). Characterizing the composition of intestinal microflora as a prospective treatment target in infant allergic disease. *FEMS Immunology & Medical Microbiology, 32*(1), 1-7.

Kirk-Greene, A. H. (2022). *Nigeria.* Available online: https://www.britannica.com/place/Nigeria.%20https://www.britannica.com/place/Nigeria

Kordy, K., Gaufin, T., Mwangi, M., Li, F., Cerini, C., Lee, D. J., Adisetiyo, H., Woodward, C., Pannaraj, P. S., & Tobin, N. H. (2020). Contributions to human breast milk microbiome and enteromammary transfer of Bifidobacterium breve. *PLoS One, 15*(1), e0219633.

Kralik, P., & Ricchi, M. (2017). A basic guide to real time PCR in microbial diagnostics: definitions, parameters, and everything. *Frontiers in Microbiology, 8,* 108.

Kuang, J., Yan, X., Genders, A. J., Granata, C., & Bishop, D. J. (2018). An overview of technical considerations when using quantitative real-time PCR analysis of gene expression in human exercise research. *PLoS one*, 13(5), e0196438.

Kuang, Y., Li, S., Guo, Y., Lu, J., He, J., Luo, B., Jiang, F., Shen, H., Papasian, C. J., Pang, H., Xia, H., Deng, H., & Qiu, X. (2016). Composition of gut microbiota in infants in China and global comparison. *Scientific reports, 6(1), 1-10.*

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. (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*, 45.

Laursen, M. F. (2021). Gut microbiota development: influence of diet from infancy to toddlerhood. *Annals of Nutrition and Metabolism, 77*(3), 21-34.

Lawrence, A. M., Oluwatoyin, A. O., & Charles, A. F. (2018). The influence of socio-economic characteristics on satisfaction in selected universities students' hostels in Southwestern, Nigeria. *American Journal of Computer Architecture, 5*(1), 1-19.

Le Doare, K., & Kampmann, B. (2014). Breast milk and Group B streptococcal infection: vector of transmission or vehicle for protection? *Vaccine*, *32*(26), 3128-3132.

Lee, E., Kim, B., Kang, M., Choi, K. Y., Cho, H., Kim, Y., Yang, S. I., Jung, Y., Kim, H. Y., & Seo, J. (2016). Dynamics of gut microbiota according to the delivery mode in healthy Korean infants. *Allergy, Asthma & Immunology Research, 8*(5), 471-477.

Li, S. W., Watanabe, K., Hsu, C. C., Chao, S. H., Yang, Z. H., Lin, Y. J., Chen, C. C., Cao, Y. M., Huang, H. C., & Chang, C. H. (2017). Bacterial Composition and Diversity in Breast Milk

Samples from Mothers Living in Taiwan and Mainland China. *Frontiers in Microbiology, 8: 965.*

Lönnerdal, B. (2013). Key Components for the Biological Activity of Human Milk. *Protecting Infants through Human Milk: Advancing the Scientific Evidence, 554,* 11.

Louis, P., Flint, H. J., & Michel, C. (2016). How to manipulate the microbiota: prebiotics. *Microbiota of the human body*, 119-142.

Low, J., Soh, S., Lee, Y. K., Kwek, K., Holbrook, J. D., Van der Beek, E M, Shek, L. P., Goh, A., Teoh, O. H., & Godfrey, K. M. (2017). Ratio of Klebsiella/Bifidobacterium in early life correlates with later development of paediatric allergy. *Beneficial Microbes, 8*(5), 681.

Lu, Z. (2020). Microbiota research: from history to advances. Paper presented at the *E3S Web of Conferences, , 145* 01014.

Łubiech, K., & Twarużek, M. (2020). Lactobacillus bacteria in breast milk. *Nutrients*, 12(12), 3783.

Lyons, K. E., Fouhy, F., O'Shea, C., Ryan, C. A., Dempsey, E. M., Ross, R. P., & Stanton, C. (2021). Effect of storage, temperature, and extraction kit on the phylogenetic composition detected in the human milk microbiota. *MicrobiologyOpen, 10*(1), e1127.

Lyons, K. E., Ryan, C. A., Dempsey, E. M., Ross, R. P., & Stanton, C. (2020). Breast milk, a source of beneficial microbes and associated benefits for infant health. *Nutrients*, 12(4), 1039.

Ma, J., Li, Z., Zhang, W., Zhang, C., Zhang, Y., Mei, H., Zhuo, N., Wang, H., Wang, L., & Wu, D. (2020). Comparison of gut microbiota in exclusively breastfed and formula-fed babies: a study of 91 term infants. *Scientific Reports, 10*(1), 15792.

Makino, H., Kushiro, A., Ishikawa, E., Muylaert, D., Kubota, H., Sakai, T., Oishi, K., Martin, R., Ben Amor, K., & Oozeer, R. (2011). Transmission of intestinal Bifidobacterium longum subsp. longum strains from mother to infant, determined by multilocus sequencing typing and amplified fragment length polymorphism. *Applied and Environmental Microbiology, 77*(19), 6788-6793.

Maldonado, J., Cañabate, F., Sempere, L., Vela, F., Sánchez, A. R., Narbona, E., López-Huertas, E., Geerlings, A., Valero, A. D., & Olivares, M. (2012). Human milk probiotic Lactobacillus fermentum CECT5716 reduces the incidence of gastrointestinal and upper respiratory tract infections in infants. *Journal of Pediatric Gastroenterology and Nutrition, 54*(1), 55-61.

Martín, R., Heilig, H. G., Zoetendal, E. G., Jiménez, E., Fernández, L., Smidt, H., & Rodríguez, J. M. (2007). Cultivation-independent assessment of the bacterial diversity of breast milk among healthy women. *Research in microbiology*, 158(1), 31-37.

Martín, R., Jiménez, E., Heilig, H., Fernández, L., Marín, M. L., Zoetendal, E. G., & Rodríguez, J. M. (2009). Isolation of Bifidobacteria from breast milk and assessment of the bifidobacterial population by PCR-denaturing gradient gel electrophoresis and quantitative real-time PCR. *Applied and Environmental Microbiology*, 75(4), 965-969.

Martín, R., Jiménez, E., Olivares, M., Marín, M. L., Fernández, L., Xaus, J., & Rodríguez, J. M. (2006). Lactobacillus salivarius CECT 5713, a potential probiotic strain isolated from infant feces and breast milk of a mother–child pair. *International Journal of Food Microbiology, 112*(1), 35-43.

Martín, R., Langa, S., Reviriego, C., Jimínez, E., Marín, M. L., Xaus, J., Fernández, L., & Rodríguez, J. M. (2003). Human milk is a source of lactic acid bacteria for the infant gut. *The Journal of Pediatrics, 143*(6), 754-758.

Martín, R., Olivares, M., Marín, M. L., Fernández, L., Xaus, J., & Rodríguez, J. M. (2005). Probiotic potential of 3 lactobacilli strains isolated from breast milk. *Journal of Human Lactation*, *21*(1), 8-17.

Martín, V., Maldonado-Barragán, A., Moles, L., Rodriguez-Baños, M., Campo, R. D., Fernández, L., ... & Jiménez, E. (2012). Sharing of bacterial strains between breast milk and infant feces. *Journal of Human Lactation*, 28(1), 36-44.

Martín, V., Mediano, P., del Campo, R., Rodríguez, J. M., & Marín, M. (2016). Streptococcal Diversity of Human Milk and Comparison of Different Methods for the Taxonomic Identification of Streptococci. *Journal of Human Lactation*, *32*(4), NP84-NP94. 10.1177/0890334415597901

Martineau, F., Picard, F. J., Ke, D., Paradis, S., Roy, P. H., Ouellette, M., & Bergeron, M. G. (2001). Development of a PCR assay for identification of staphylococci at genus and species levels. *Journal of Clinical Microbiology*, *39*(7), 2541-2547.

McGuire, M. K., & McGuire, M. A. (2015). Human milk: mother nature's prototypical probiotic food? *Advances in Nutrition*, *6*(1), 112-123.

McGuire, M. K., & McGuire, M. A. (2017). Got bacteria? The astounding, yet not-so-surprising, microbiome of human milk. *Current Opinion in Biotechnology*, *44*, 63-68.

Mehanna, N. S., Tawfik, N. F., Salem, M. M., Effat, B. A., & Gad El-Rab, D. A. (2013). Assessment of potential probiotic bacteria isolated from breast milk. *Middle-East Journal of Scientific Research*, *14*(3), 354-360.

Moland, N. A. (2015). Nigeria: An overview. *Education in West Africa*, *29*, 363.

Moossavi, S., & Azad, M. B. (2020). Origins of human milk microbiota: new evidence and arising questions. *Gut Microbes, 12*(1), 1667722.

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.

Morelli, L., & Capurso, L. (2012). FAO/WHO guidelines on probiotics: 10 years later. *Journal of clinical gastroenterology*, 46, S1-S2.

Moruff, M. (2012). Cultural understanding of space and waste disposal habit among the urban populace in Ibadan Metropolis, South Western Nigeria. *Journal of Sustainable Development in Africa, 14*, 82-95.

Muhammad, S., & Amusa, N. A. (2005). The important food crops and medicinal plants of north-western Nigeria. *Research Journal of Agriculture and Biological Sciences, 1*(3), 254-260.

Munoa, F. J., & Pares, R. (1988). Selective medium for isolation and enumeration of *Bifidobacterium* spp. *Applied and Environmental Microbiology, 54*(7), 1715-1718.

Murphy, K., Curley, D., O'Callaghan, T. F., O'Shea, C., 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*(1), 1-10.

Nagpal, R., Tsuji, H., Takahashi, T., Nomoto, K., Kawashima, K., Nagata, S., & Yamashiro, Y. (2017). Ontogenesis of the gut microbiota composition in healthy, full-term, vaginally born

and breastfed infants over the first 3 years of life: a quantitative bird's-eye view. *Frontiers in Microbiology*, *8*, 1388.

Natureportfolio. (2022). *Setting standards for reproducibility in gut microbiome research.* https://www.nature.com/articles/d42473-018-00136-7

Neville, M. C., Morton, J., & Umemura, S. (2001). Lactogenesis: the transition from pregnancy to lactation. *Pediatric Clinics of North America, 48*(1), 35-52.

Niu, J., Xu, L., Qian, Y., Sun, Z., Yu, D., Huang, J. D., Zhou, X., Wang, Y. X., Zhang, T., Ren, R., Li, Z. R., Yu, J., & Gao, X. (2020). Evolution of the Gut Microbiome in Early Childhood: A Cross-Sectional Study of Chinese Children. *Frontiers in Microbiology*, 11, 439.

Oguntona, C. R., Odunmbaku, J. A., & Ottun, B. O. (1999). Proximate composition of ten standardized Nigerian dishes. *Nutrition & Food Science.*

Oikarinen, S., Tauriainen, S., Viskari, H., Simell, O., Knip, M., Virtanen, S., & Hyöty, H. (2009). PCR inhibition in stool samples in relation to age of infants. *Journal of Clinical Virology*, *44*(3), 211-214.

Oikonomou, G., Addis, M. F., Chassard, C., Nader-Macias, M. E. F., Grant, I., Delbès, C., ... & Even, S. (2020). Milk microbiota: what are we exactly talking about? *Frontiers in microbiology*, 11, 60.

Ojo-Okunola, A., Claassen-Weitz, S., Mwaikono, K. S., Gardner-Lubbe, S., Stein, D. J., Zar, H. J., Nicol, M. P., & du Toit, E. (2019). Influence of socio-economic and psychosocial profiles on the human breast milk bacteriome of South African Women. *Nutrients, 11*(6), 1390.

Ojo-Okunola, A., Nicol, M., & Du Toit, E. (2018). Human breast milk bacteriome in health and disease. *Nutrients, 10*(11), 1643.

Okorie, P. N., McKenzie, F. E., Ademowo, O. G., Bockarie, M., & Kelly-Hope, L. (2011). Nigeria Anopheles vector database: an overview of 100 years' research. *Plos One, 6*(12), e28347.

Olasantan, F. O. (2001). Tropical vegetables and spices: potential value and contributions to sustainable agriculture in Nigeria. *Outlook on Agriculture, 30*(1), 55-67.

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.

Olivares, M., Díaz-Ropero, M. P., Martín, R., Rodríguez, J. M., & Xaus, J. (2006). Antimicrobial potential of four Lactobacillus strains isolated from breast milk. *Journal of Applied Microbiology, 101*(1), 72-79.

Oxoid.com (2022). Dehydrated Culture media. Product details, Oxoid. Available at: http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0627&org=82&c=UK &lang=EN. (Accessed: October 23, 2022).

Oyedemi, O. T., Shaw, S., Martin, J. C., Ayeni, F. A., & Scott, K. P. (2022). Changes in the gut microbiota of Nigerian infants within the first year of life. *PloS one*, 17(3), e0265123.

Padilha, M., Danneskiold-Samsøe, N. B., Brejnrod, A., Hoffmann, C., Cabral, V. P., Iaucci, J. d. M., Sales, C. H., Fisberg, R. M., Cortez, R. V., & Brix, S. (2019). The human milk microbiota is modulated by maternal diet. *Microorganisms, 7*(11), 502.

Pannaraj, P. S., Li, F., Cerini, C., Bender, J. M., Yang, S., Rollie, A., Adisetiyo, H., Zabih, S., Lincez, P. J., & Bittinger, K. (2017). Association between breast milk bacterial communities and establishment and development of the infant gut microbiome. *JAMA Pediatrics, 171*(7), 647-654.

Parnell, L. A., Briggs, C. M., Cao, B., Delannoy-Bruno, O., Schrieffer, A. E., & Mysorekar, I. U. (2017). Microbial communities in placentas from term normal pregnancy exhibit spatially variable profiles. *Scientific Reports, 7*(1), 1-11.

Penders, J., Thijs, C., Vink, C., Stelma, F. F., Snijders, B., Kummeling, I., van den Brandt, Piet A, & Stobberingh, E. E. (2006). Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics, 118*(2), 511-521.

Pereyra-Elías, R., Quigley, M. A., & Carson, C. (2022). To what extent does confounding explain the association between breastfeeding duration and cognitive development up to age 14? Findings from the UK Millennium Cohort Study. *PloS one*, 17(5), e0267326.

Perez, P. F., Dore, J., Leclerc, M., Levenez, F., Benyacoub, J., Serrant, P., Segura-Roggero, I., Schiffrin, E. J., & Donnet-Hughes, A. (2007). Bacterial imprinting of the neonatal immune system: lessons from maternal cells? *Pediatrics, 119*(3), e724-e732.

Pickard, J. M., Zeng, M. Y., Caruso, R., & Núñez, G. (2017). Gut microbiota: role in pathogen colonization, immune responses, and inflammatory disease. *Immunological reviews*, 279(1), 70-89.

Primo, E. D., Otero, L. H., Ruiz, F., Klinke, S., & Giordano, W. (2018). The disruptive effect of lysozyme on the bacterial cell wall explored by an in-silico structural outlook. *Biochemistry and Molecular Biology Education*, 46(1), 83-90.

Promega. (2022). *Choosing the Right Method for Nucleic Acid Quantitation.* https://www.promega.co.uk/resources/pubhub/choosing-the-right-method-for-nucleic-acid-quantitation/

Quesada, J. A., Méndez, I., & Martín-Gil, R. (2020). The economic benefits of increasing breastfeeding rates in Spain. *International breastfeeding journal*, 15(1), 1-7.

Quinn, E. A., Largado, F. E., Power, M., & Kuzawa, C. W. (2012). Predictors of breast milk macronutrient composition in Filipino mothers. *American Journal of Human Biology, 24*(4), 533-540.

Raeisi, S. N., Ouoba, L. I. I., Farahmand, N., Sutherland, J., & Ghoddusi, H. B. (2013). Variation, viability and validity of bifidobacteria in fermented milk products. *Food Control, 34*(2), 691-697.

Rahmani, M., Saffari, F., Aboubakri, O., & Mansouri, S. (2020). Enterococci from breastfed infants exert higher antibacterial effects than those from adults: A comparative study. *Human Microbiome Journal, 17*, 100072. 10.1016/j.humic.2020.100072

Ramsay, D. T., Kent, J. C., Owens, R. A., & Hartmann, P. E. (2004). Ultrasound imaging of milk ejection in the breast of lactating women. *Pediatrics, 113*(2), 361-367.

Rautava, S. (2016). Early microbial contact, the breast milk microbiome and child health. *Journal of Developmental Origins of Health and Disease, 7*(1), 5-14.

Reyman, M., van Houten, M. A., van Baarle, D., Bosch, A. A., Man, W. H., Chu, M. L. J., Arp, K., Watson, R. L., Sanders, E. A., & Fuentes, S. (2019). Impact of delivery mode-associated gut microbiota dynamics on health in the first year of life. *Nature Communications, 10*(1), 1-12.

Ricci, I., Damiani, C., Scuppa, P., Mosca, M., Crotti, E., Rossi, P., Rizzi, A., Capone, A., Gonella, E., & Ballarini, P. (2011). The yeast Wickerhamomyces anomalus (Pichia anomala) inhabits

the midgut and reproductive system of the Asian malaria vector Anopheles stephensi. *Environmental Microbiology, 13*(4), 911-921.

Rinttilä, T., Kassinen, A., Malinen, E., Krogius, L., & Palva, A. (2004). Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *Journal of Applied Microbiology, 97*(6), 1166-1177.

Rodríguez, J. M. (2014). The origin of human milk bacteria: is there a bacterial entero-mammary pathway during late pregnancy and lactation? *Advances in Nutrition, 5*(6), 779-784.

Rodríguez, J. M., Murphy, K., Stanton, C., Ross, R. P., Kober, O. I., Juge, N., Avershina, E., Rudi, K., Narbad, A., & Jenmalm, M. C. (2015). The composition of the gut microbiota throughout life, with an emphasis on early life. *Microbial Ecology in Health and Disease, 26*(1), 26050.

Rodríguez-Sojo, M. J., Ruiz-Malagón, A. J., Rodríguez-Cabezas, M. E., Gálvez, J., & Rodríguez-Nogales, A. (2021). Limosilactobacillus fermentum CECT5716: Mechanisms and Therapeutic Insights. *Nutrients*, 13(3), 1016.

Roger, L. C., Costabile, A., Holland, D. T., Hoyles, L., & McCartney, A. L. (2010). Examination of faecal Bifidobacterium populations in breast-and formula-fed infants during the first 18 months of life. *Microbiology, 156*(11), 3329-3341.

Roscoe, J. T. (1975). *Fundamental research statistics for the behavioral sciences [by] John T. Roscoe*

Ruiz, L., Bacigalupe, R., García-Carral, C., Boix-Amoros, A., Argüello, H., Silva, C. B., de los Angeles Checa, Maria, 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), 1-13.

Ruiz, L., Espinosa-Martos, I., García-Carral, C., Manzano, S., McGuire, M. K., Meehan, C. L., McGuire, M. A., Williams, J. E., Foster, J., & Sellen, D. W. (2017). What's normal? Immune profiling of human milk from healthy women living in different geographical and socioeconomic settings. *Frontiers in Immunology, 8*, 696.

Ruiz, L., García-Carral, C., & Rodriguez, J. M. (2019b). Unfolding the human milk microbiome landscape in the omics era. *Frontiers in Microbiology, 10*, 1378.

Rychert, J. (2019). Benefits and limitations of MALDI-TOF mass spectrometry for the identification of microorganisms. *Journal of Infectiology and Epidemiology, 2*(4)

Saarela, T., Kokkonen, J., & Koivisto, M. (2005). Macronutrient and energy contents of human milk fractions during the first six months of lactation. *Acta Paediatrica, 94*(9), 1176-1181.

Sekaran, U., & Bougie, R. (2016). *Research methods for business: A skill building approach*. john wiley & sons.

Simpson, M. R., Avershina, E., Storrø, O., Johnsen, R., Rudi, K., & Øien, T. (2018). 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.

Sindi, A. S., Geddes, D. T., Wlodek, M. E., Muhlhausler, B. S., Payne, M. S., & Stinson, L. F. (2021). Can we modulate the breastfed infant gut microbiota through maternal diet? *FEMS Microbiology Reviews*, 45(5), fuab011.

Singhal, N., Kumar, M., Kanaujia, P. K., & Virdi, J. S. (2015). MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. *Frontiers in Microbiology, 6*, 791.

Smith, K. G., Kamdar, A. A., & Stark, J. M. (2019). 8 - Lung Defenses: Intrinsic, Innate, and Adaptive. In R. W. Wilmott, R. Deterding, A. Li, F. Ratjen, P. Sly, H. J. Zar & A. Bush (Eds.), *Kendig's Disorders of the Respiratory Tract in Children (Ninth Edition)* (pp. 120-133.e2). Elsevier.

Solís, G., de Los Reyes-Gavilan, C G, Fernández, N., Margolles, A., & Gueimonde, M. (2010). Establishment and development of lactic acid bacteria and bifidobacteria microbiota in breast milk and the infant gut. *Anaerobe, 16*(3), 307-310.

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.

Sridhar, A., & Salcedo, J. (2017). Optimizing maternal and neonatal outcomes with postpartum contraception: impact on breastfeeding and birth spacing. *Maternal Health, Neonatology and Perinatology,* 3(1), 1-10.

Stinson, L. F., Trevenen, M. L., & Geddes, D. T. (2021). The Viable Microbiome of Human Milk Differs from the Metataxonomic Profile. *Nutrients, 13*(12), 4445. 10.3390/nu13124445

Tanaka, M., & Nakayama, J. (2017). Development of the gut microbiota in infancy and its impact on health in later life. *Allergology International*, 66(4), 515-522.

Timmerman, H. M., Rutten, N. B., Boekhorst, J., Saulnier, D. M., Kortman, G. A., Contractor, N., Kullen, M., Floris, E., Harmsen, H. J., & Vlieger, A. M. (2017). Intestinal colonisation

patterns in breastfed and formula-fed infants during the first 12 weeks of life reveal sequential microbiota signatures. *Scientific Reports, 7*(1), 1-10.

Toscano, M., De Grandi, R., Peroni, D. G., Grossi, E., Facchin, V., Comberiati, P., & Drago, L. (2017). Impact of delivery mode on the colostrum microbiota composition. *BMC Microbiology, 17*(1), 1-8.

Tuominen, H., Rautava, S., Collado, M. C., Syrjänen, S., & Rautava, J. (2018). HPV infection and bacterial microbiota in breast milk and infant oral mucosa. *PLoS One, 13*(11), e0207016.

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), 1-9.

Vacca, M., Raspini, B., Calabrese, F. M., Porri, D., De Giuseppe, R., Chieppa, M., Liso, M., Cerbo, R. M., Civardi, E., & Garofoli, F. (2022). The establishment of the gut microbiota in 1-year-aged infants: from birth to family food. *European Journal of Nutrition,* , 1-14.

Wagner, C. L. (2015). Human Milk and Lactation: Background, Pathophysiology. http://emedicine.medscape.com/article/1835675overview.

Wallenborn, J. T., Gunier, R. B., Pappas, D. J., Chevrier, J., & Eskenazi, B. (2022). Breastmilk, stool, and meconium: bacterial communities in South Africa. *Microbial ecology,* 83(1), 246-251.

Wang, B., Yao, M., Lv, L., Ling, Z., & Li, L. (2017). The human microbiota in health and disease. *Engineering, 3*(1), 71-82.

Ward, T. L., Hosid, S., Ioshikhes, I., & Altosaar, I. (2013). Human milk metagenome: a functional capacity analysis. *BMC Microbiology, 13*(1), 1-12.

Weimer, J. P. (2001). The economic benefits of breastfeeding: A review and analysis.

Wesolowska-Andersen, A., Bahl, M. I., Carvalho, V., Kristiansen, K., Sicheritz-Pontén, T., Gupta, R., & Licht, T. R. (2014). Choice of bacterial DNA extraction method from fecal material influences community structure as evaluated by metagenomic analysis. *Microbiome, 2*(1), 1-11.

West, P. A., Hewitt, J. H., & Murphy, O. M. (1979). The influence of methods of collection and storage on the bacteriology of human milk. *Journal of Applied Bacteriology, 46*(2), 269-277.

Wieser, A., Schneider, L., Jung, J., & Schubert, S. (2012). MALDI-TOF MS in microbiological diagnostics—identification of microorganisms and beyond (mini review). *Applied Microbiology and Biotechnology, 93*(3), 965-974.

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. (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.

World Health Organization. (2021). Indicators for assessing infant and young child feeding practices: definitions and measurement methods.

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,* , 1323.

Yao, Y., Cai, X., Ye, Y., Wang, F., Chen, F., & Zheng, C. (2021). The role of microbiota in infant health: from early life to adulthood. *Frontiers in Immunology*, 4114.

Yuan, L., Zhang, X., Luo, B., Li, X., Tian, F., Yan, W., & Ni, Y. (2022). Ethnic Specificity of Species and Strain Composition of Lactobacillus Populations from Mother-infant Pairs, Uncovered by Multilocus Sequence Typing. *Frontiers in microbiology*, 9.

Yuan, S., Cohen, D. B., Ravel, J., Abdo, Z., & Forney, L. J. (2012). Evaluation of methods for the extraction and purification of DNA from the human microbiome. *PloS One, 7*(3), e33865.

Zheng, J., Wittouck, S., Salvetti, E., Franz, C. M., Harris, H. M., Mattarelli, P., ... & Lebeer, S. (2020). A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. *International journal of systematic and evolutionary microbiology*, 70(4), 2782-2858.

Zhu, B., Zheng, S., Lin, K., Xu, X., Lv, L., Zhao, Z., & Shao, J. (2021). Effects of infant formula supplemented with prebiotics and OPO on infancy fecal microbiota: a pilot randomized clinical trial. *Frontiers in Cellular and Infection Microbiology, 11*, 194.

Appendices
APPENDIX 1

Questionnaire used for the study

THANK YOU VERY MUCH FOR AGREEING TO TAKE PART IN THE STUDY

In order to be able to analyse the results of our study we need to ask you some questions. Some of these questions are designed to get an idea of you and your baby's general health; some are about your lifestyle, work and also your family.

Please tick the appropriate box in answer to each question. ALL INFORMATION COLLECTED WILL BE TREATED CONFIDENTIAL

SECTION A: Questions about the mother

1. At which age group are you?

   15-20 years ☐ 21-25years ☐ 26-30 years ☐ 35-40 years ☐ 41-45 years ☐

2. How many children do you have?

   Please state:

3. Is this your

   First, ☐second, ☐third,☐ fourth ☐ other ☐ child?

4. Has the baby or yourself been unwell during the past two weeks?

   Yes ☐No ☐

   If yes, please explain:

5. Has any of you been on any kind of medication/antibiotics since the last 2 weeks?

   Yes ☐No ☐

   If yes, please explain:

6. What is your food mainly composed of? Please tick all that applies

   Carbohydrate☐, Protein☐, Fats ☐Fibre☐ Diary products ☐

7. Is your diet rich in:

   Please tick all that applies; Fruit☐, Yoghurt, ☐ Red meat, ☐ Chicken☐

   Fish, ☐ Vegetables, ☐ Probiotics☐

8. Do you smoke?

   Yes ☐No ☐

9. Do you consume alcohol?

   Yes ☐No ☐

10. Do you work outside of home?

    Yes ☐No ☐

11. How would you describe your economic status?

    High ☐ Medium ☐ Low ☐

12. At what kind of area do you live?

    Urban☐ Rural☐Other ☐ please state:

13. How long have you lived there?

    Please state:

14. What part of Nigeria are you from?

    North Central ☐ North East ☐ North West ☐ South East ☐ South, South

    ☐ South, West ☐ other ☐ please state:

15. Have you ever lived outside of Nigeria?

Yes ☐ No ☐

If yes when did you move to Nigeria?

Please state:

16. What is your religious belief?

Please state:

17. If breastfed, are you the only person who feeds the baby?

Yes ☐ No ☐

18. How long have you been breastfeeding your baby?

Please state:

19. Level of Education?

No education ☐ Primary ☐ Secondary ☐ Tertiary ☐

20. Partner's level of education?

No education ☐ Primary ☐ Secondary ☐ Tertiary ☐

21. Do you have access to regular internet

Yes ☐ No ☐ other ☐

22. Ethnic Background

North central ☐ North west ☐ North east ☐ South east ☐ South west ☐

South south

23. What sort of work do you do?

SECTION B: Questions about your baby

24. How old is the baby

0-6months ☐ 7-12months ☐ >12months ☐

25. What is the gender of baby?

Male ☐ Female ☐

26. How was the baby born?

Natural ☐ Assisted ☐

27. How is she/he fed?

Breast milk ☐ Formula ☐ Both ☐ other ☐ please state:

28. If formula, does it contain:

Prebiotics ☐ Probiotics ☐ Not known ☐

Please give the name and brand of the formula below

Name----------, Brand…….

29. How long do you store the formula? …….. In what condition? ……..

30. How do you prepare the formula?

……..

……..

THANK YOU FOR PARTICIPATING

APPENDIX 2A

Material Transfer Agreement to transport human samples

Material Transfer Agreement for the Supply of Human Tissue Materials FOR USE where the material is human organs, tissue or cells (other than human gametes or embryos) but NOT where the intended use is transplantation or human application

This Agreement is made by and between:

a) <***Adeoyo Maternity Hospital, Yemetu, Oyo State Nigeria***> ("the Provider Institution") and

b) **Middlesex University Higher Education Corporation**, whose registered offices are at The Burroughs, London, NW4 4BT ("the Recipient Institution").

This Agreement records the terms under which the Provider Institution will make available to the Recipient Institution the Material identified in Appendix 2 (the "Material"). The term "Material" means material, other than human gametes or embryos, which consists of or includes human cells and which is considered "Relevant Material" for the purposes of the Human Tissue Act 2004[1] together with related data. The Recipient Institution will hold the Material on the terms of this Agreement and solely for the purpose of **"Investigating the bacterial diversity present in the samples''** ("the Study") and as described in Appendix 1, within the research group of **"Adebusayo Nafisah Hassan"**. The Recipient Institution hereby agrees to comply and procure that the Recipient Scientist and all personnel who work with the Material comply with the following terms and conditions:

1. The Recipient Institution will not use the Material for administration to human subjects or human application as that term is defined in the Human Tissue (Quality and Safety for Human Application) Regulations 2007 (or equivalent as each may be replaced or amended from time to time), or for clinical or diagnostic purposes.[2]

[1] The Human Tissue Act 2004 applies to the "authorised activities" principally the removal, storage and use of "Relevant Materials" (as defined under the Act, including human cells, tissue and organs, but not cell lines) which come from a living or deceased person for "Scheduled Purposes" (these are set out in Schedule 1 of the Act, including, but not limited to, "research in connection with disorders, or the function of the human body", "education or training relating to human health", and "transplantation").

[2] The Human Tissue (Quality and Safety for Human Application) Regulations 2007 apply to the procurement, testing, processing, storage, distribution, and import or export of tissues and cells (including cell lines). "Cells" mean

human cells (whether individually or in an unbound collection) including cell lines, but not including gametes, embryos outside the body, blood or blood components. “Tissue” for the Regulations, means all constituent parts of the human

2. The Recipient Institution may use the Material for the purposes of the Study and as described in Appendix 1, from the date of receipt of the Material. The Recipient Institution will comply fully with all applicable environmental, health and safety laws, the Human Tissue Act 2004 and other Applicable Laws[1] with respect to its use (including, but not limited to, disposal or return).

3. The Recipient Institution shall use a courier with suitable skill and experience to safely transport the Material in accordance with all Applicable Laws. The Recipient Institution will bear the cost of carriage and any necessary insurance. The Provider Institution makes no charge for the Material. Risk in and responsibility for the Material shall pass to the Recipient Institution once it is loaded onto transport as organised by the Recipient Institution. If so requested by the Provider Institution the Recipient Institution shall provide it with written confirmation of the safe receipt of the Materials promptly after their delivery to the Recipient Institution's laboratory.

4. The Recipient Institution understands that the Material may have hazardous properties, contain infectious agents or pose other health and safety risks. Subject to clause 9, the Provider Institution makes no representations and gives no warranties either express or implied in relation to it: for example (without limitation), no warranties are given about quality or fitness for a particular purpose, or freedom from infection. The Provider Institution will not be liable for any use made of the Material by the Recipient Institution. The Recipient Institution will use the Material in accordance with good laboratory practice standards, all due skill and care and with dignity, sensitivity and respect. The Recipient Institution will comply with all Applicable Laws, approvals, rules, codes of practice and regulations governing the transportation, storage, use and disposal of the Material. The Recipient Institution warrants that it will only use, or permit the use of the Material in work that has ethical approval, as stated in Appendix 1.

5. Except to the extent prohibited by Law and subject to clause 9, the Recipient Institution assumes all liability for damages which may arise from its receipt, use, storage or disposal of the Material. The Provider Institution will not be liable to the Recipient Institution for any loss, claim or demand made by the Recipient Institution, or made against the Recipient Institution by any other party, due to or arising from its use, storage or disposal of the Material by the Recipient Institution, except to the extent the law otherwise requires.

body formed by cells, but not including gametes and embryos outside the body (which are regulated by the Human Fertilisation and Embryology Authority pursuant to the Human Fertilisation and Embryology Act 1990), or organs.

[1] Applicable Laws means all laws, rules, regulations, codes of practice, research governance or ethical guidelines, or other requirements of any Regulatory Authority, that may apply to the use of the Material by the Recipient Institution from time to time, including (but not limited) the Human Tissue Act 2004 or the Human Tissue (Scotland)_Act 2006, the Human Tissue (Quality and Safety for Human Application) Regulations 2007, the Human Fertilisation and Embryology Act 1990 (as amended), the EU Tissues and Cells Directive (2004/23/EC) and Commission Directives 2006/17/EC and 2006/86/EC. The Human Tissue Authority Directions and Codes of Practice, and the Medicines for Human Use (Clinical Trials) Regulations 2004, as updated and amended from time to time and, where relevant, the national implementations of the same.

6. The liability of either party for any breach of this Agreement, or arising in any other way out of the subject matter of this Agreement, will not extend to loss of business or profit, or to any indirect or consequential damages or losses.

7. The Recipient Institution agrees to obtain the written consent of the Provider Institution if there is any material change to the proposed use of the Material in the Study as described in Appendix 1.

8. The Recipient Scientist will acknowledge the source of the Material in any publication reporting on its use. If the Recipient Scientist wishes to include in a publication any information which has been provided by the Provider Institution with the Material and which was clearly marked as "confidential" and "proprietary" at the point of disclosure ("Confidential Information"), the Recipient Scientist must obtain written permission from the Provider Institution, providing a copy of the text to allow a reasonable period for review before publication takes place, such permission not to be unreasonably withheld or delayed. If so requested by the Provider Institution, the Recipient Institution shall provide the Provider Institution with a confidential copy of the findings of the Study.

9. The Provider Institution warrants that where required by Applicable Laws the Material has been obtained from humans with the appropriate consent as required by the Human Tissue Act 2004 and with ethical approval and the Provider Institution shall be liable for any claims arising due to the breach of this warranty. The Provider Institution hereby grants to the Recipient Institution a non-exclusive research licence to use the Material for the Study only. The Provider Institution further warrants that it has not provided any information (and does not intend to provide any information) which has led or may lead to the Recipient Institution being able to identify the person from whom the relevant material came.

10. The Recipient Institution undertakes to store the Material in accordance with all Applicable Laws and not to attempt to identify or contact the donor of the Material or to compromise or otherwise infringe the confidentiality of information on the donors and their right to privacy.

11. Nothing included in this Agreement shall prevent the Provider Institution from being able to distribute the Material to other entities as described in Appendix 1. If, as per the details included in Appendix 1, the Material is to be transferred to another institution for the purposes of the Study, the responsibility for compliance with the terms of this Agreement rests with the Recipient Institution.

12. The Provider Institution has the right to terminate this agreement forthwith at any time by means of written notice to Recipient Institution if the ethical approval is withdrawn or if the Recipient Institution is in breach of this Agreement. In the case of any termination, the Recipient Institution shall immediately discontinue all use of the Material and, at the Provider Institution's discretion, promptly return (at the Provider Institution's own cost) or destroy (at the Recipient Institution's own cost) all unused Material and provide written confirmation that this has been completed. If requested,

the Recipient Institution must certify that it has complied in full with any such requirement of the Provider Institution. Should an individual donor or their next of kin rescind their consent, the Provider Institution will require and the Recipient Institution agrees to discontinue using the appropriately identified sample and return or destroy it in accordance with the Provider Institution's instructions.

13. This Agreement shall be governed by English Law, and the English Courts shall have exclusive jurisdiction to deal with any dispute which may arise out of or in connection with this Letter Agreement.

Accepted and Agreed on behalf of	Accepted and Agreed on behalf of
Middlesex University	Adeoyo Maternity Hospital
Name:	Name:
Position:	Position:
Signature	Signature:
Date:	Date:

Study description and details of Materials

TO BE COMPLETED BY THE RECIPIENT INSTITUTION'S SCIENTIST:

1. **STUDY DESCRIPTION: Investigating bacterial diversity in breast milk with relationship with gut microbiota of fed babies in Nigeria.**

Breast milk of healthy mothers and faeces of fed babies will be obtained and cultured in the laboratory. Breast milk and faecal samples will also be transported to the United Kingdom through DHL/FEDEX, for the purpose of DNA extraction and 16S sequencing.

2. **DETAILS OF MATERIALS REQUESTED (type of material, quantity, numbers of material):**
Breast milk : 5ml obtained from 50 participant
Faeces: About 0.6g of faeces from 50breast fed babies and10-25 bottle fed babies

3. **DETAILS OF COURIER TO BE USED AND COURIER ACCOUNT CODE:**
DHL / FEDEX Nigeria

4. **LOCATION OF LABORATORY WHERE MATERIALS ARE TO BE HELD/USED:**
Microbiology laboratory, Middlesex University, London

5. **HTA LICENCE / ETHICS**

APPROVAL: Complete one of the

following:

□ This Study has been given a favourable opinion by an ethics committee which, within the UK, is recognised under the Human Tissue Act 2004. Please provide the reference of the opinion and name of the committee: **Middlesex University Natural Sciences Ethics Sub-Committee , Project no: 2366; Ministry of Health, Oyo State Nigeria, ref: AD 13/ 475/ 789**

Or:

□ The Materials are to be stored in premises licensed by the Human Tissue Authority, until favourable ethical approval has been obtained for the proposed Study at which point the Recipient Scientist shall notify the Provider Institution. Please provide the licence number:

Or:

□ Where the Materials are supplied by the Provider Institution from a research tissue bank

which may be a diagnostic archive and which has been granted REC approval for specific research projects, this REC approval may cover the research Study with the materials at the Recipient Institution. If this is the case, the Designated Individual (or their duly authorised delegate) of the Provider Institution confirms that its REC approval for the tissue bank will cover the proposed Study by signing here:

.....

Delivery and Storage of Materials

TO BE COMPLETED BY THE PROVIDER INSTITUTION:

1. QUANTITY OF MATERIALS TO BE DELIVERED:

6 mL of breast milk from 50 participants and 0.6g of stool from 50 to 70 babies

2. COST OF SAMPLE PREPARATION:

No cost involved. Any cost will be taken care of by the researcher

3. CONDITIONS OF STORAGE

[-80 ^{0}C]

4. RETURN/DESTRUCTION OF SURPLUS MATERIALS ON COMPLETION OF STUDY

If there are any Materials left over from the Study, the Recipient Institution needs to provide confirmation to the Provider Institution that any remaining Material will be destroyed (and if destroyed the Recipient Institution needs to provide confirmation to the Provider Institution that this has been completed).

APPENDIX 2B

Ethical clearance certificate

TELEGRAMS..... TELEPHONE.....

MINISTRY OF HEALTH

DEPARTMENT OF PLANNING, RESEARCH & STATISTICS DIVISION

PRIVATE MAIL BAG NO. 5027, OYO STATE OF NIGERIA

Your Ref. No. .....
All communications should be addressed to
the Honorable Commissioner quoting
Our Ref. No. AD 13/479/ 785

15th June, 2018

The Principal Investigator,
School of Health and Social Sciences,
Natural Sciences Ethics Sub-Committee
Middlesex University,
London.

Attention: Hassan Adebusayo

ETHICAL APPROVAL FOR THE IMPLEMENTATION
OF YOUR RESEARCH PROPOSAL IN OYO STATE

This is to acknowledge that your Research Proposal titled: "Investigation of Bacterial Diversity in Human and Reconstituted Formula Milk with Relationship to Gut Micribiota of Fed Babies in Nigeria" has been reviewed by the Oyo State Ethical Review Committee.

2. The committee has noted your compliance. In the light of this, I am pleased to convey to you the full approval by the committee for the implementation of the Research Proposal in Oyo State, Nigeria.

3. Please note that the National Code for Health Research Ethics requires you to comply with all institutional guidelines, rules and regulations, in line with this, the Committee will monitor closely and follow up the implementation of the research study. However, the Ministry of Health would like to have a copy of the results and conclusions of findings as this will help in policy making in the health sector.

4. Wishing you all the best.

Dr. Abbas Gbolahan
Director, Planning, Research & Statistics
Secretary, Oyo State, Research Ethical Review Committee

APPENDIX 3A

Standard curve for the absolute quantification of genera streptococci, lactobacilli, bifidobacteria, and enterococci in milk as generated from qPCR run

Standard Curves

Gene Name	Slope	Efficiency	Error	R^2	Y-Intercept
None	-3.2930	2.01	0.54	0.99	12.61

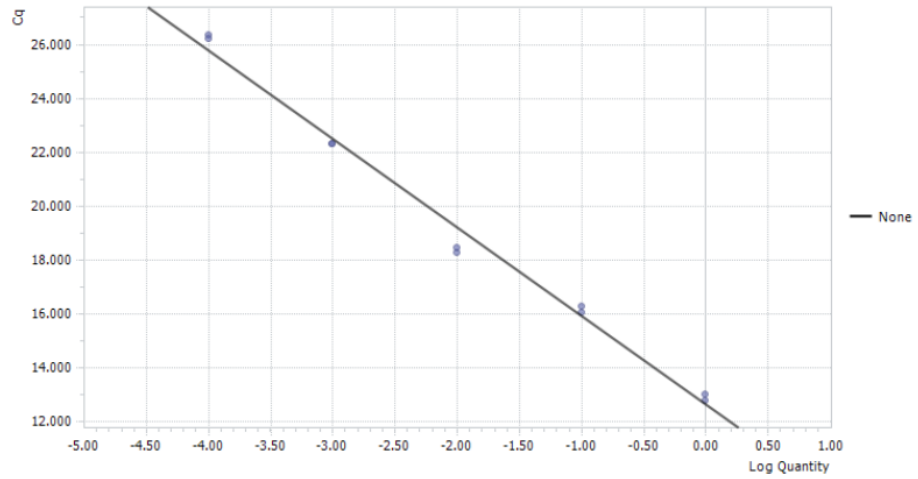


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Figure 1: Standard curve for the absolute quantification of streptococci in milk as genrated from qPCR run

Standard Curves

Gene Name	Slope	Efficiency	Error	R^2	Y-Intercept
None	-2.4195	2.59	0.44	1.00	8.09

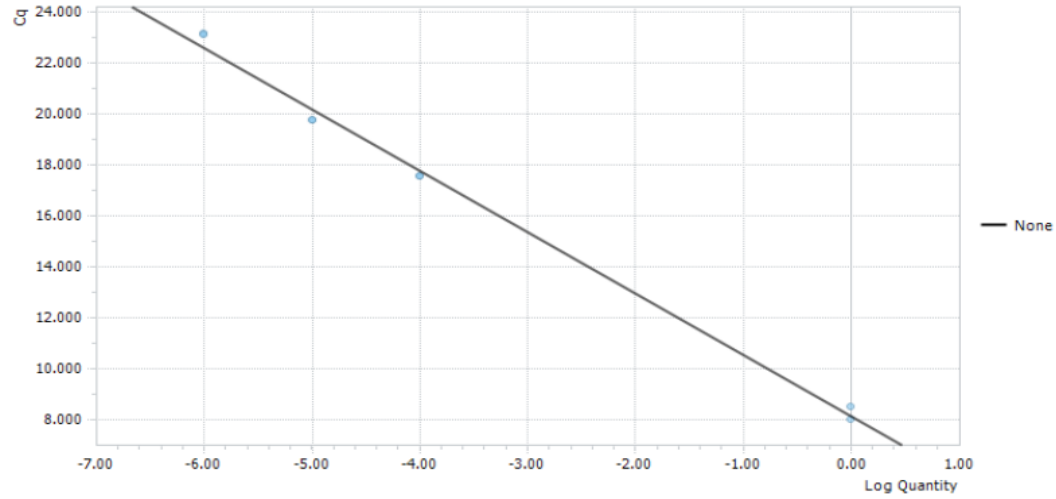


Figure 2: Standard curve for the absolute quantification of lactobacilli in milk as genrated from qPCR run

Standard Curves

Gene Name	Slope	Efficiency	Error	R^2	Y-Intercept
None	-3.7057	1.86	0.67	0.99	7.69

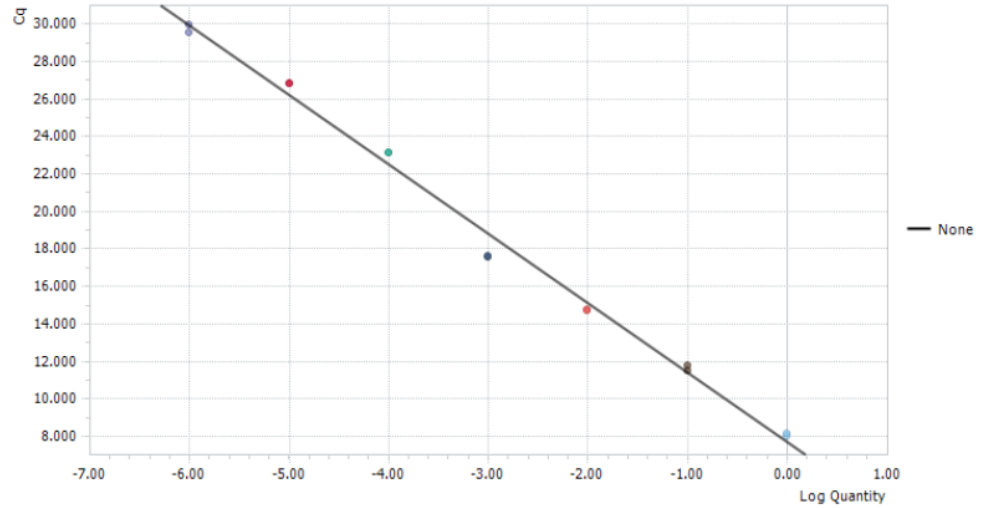


Figure 3: Standard curve for the absolute quantification of bifidobacteria in milk as genrated from qPCR run

Standard Curves

Gene Name	Slope	Efficiency	Error	R^2	Y-Intercept
None	-3.5965	1.90	0.38	1.00	8.41

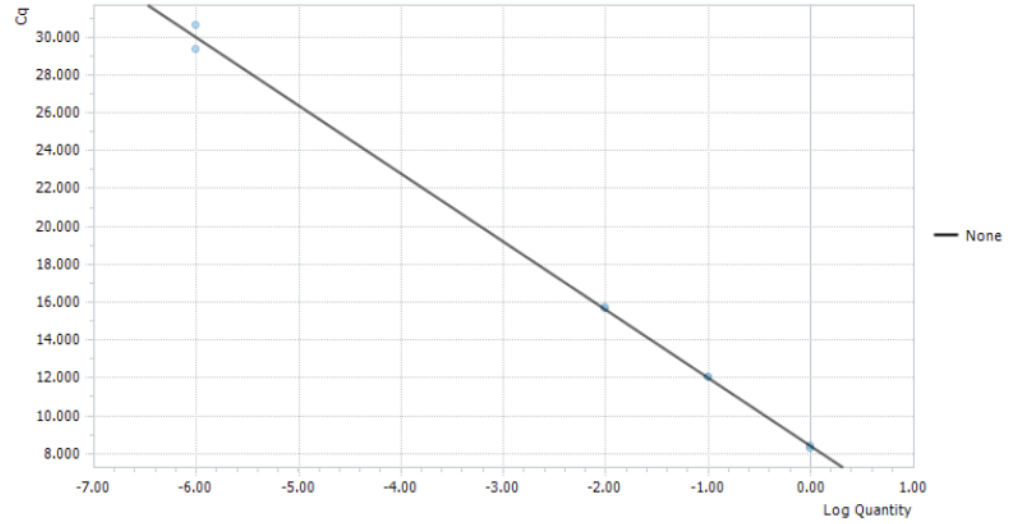


Figure 4: Standard curve for the absolute quantification of enterococci in milk as genrated from qPCR run

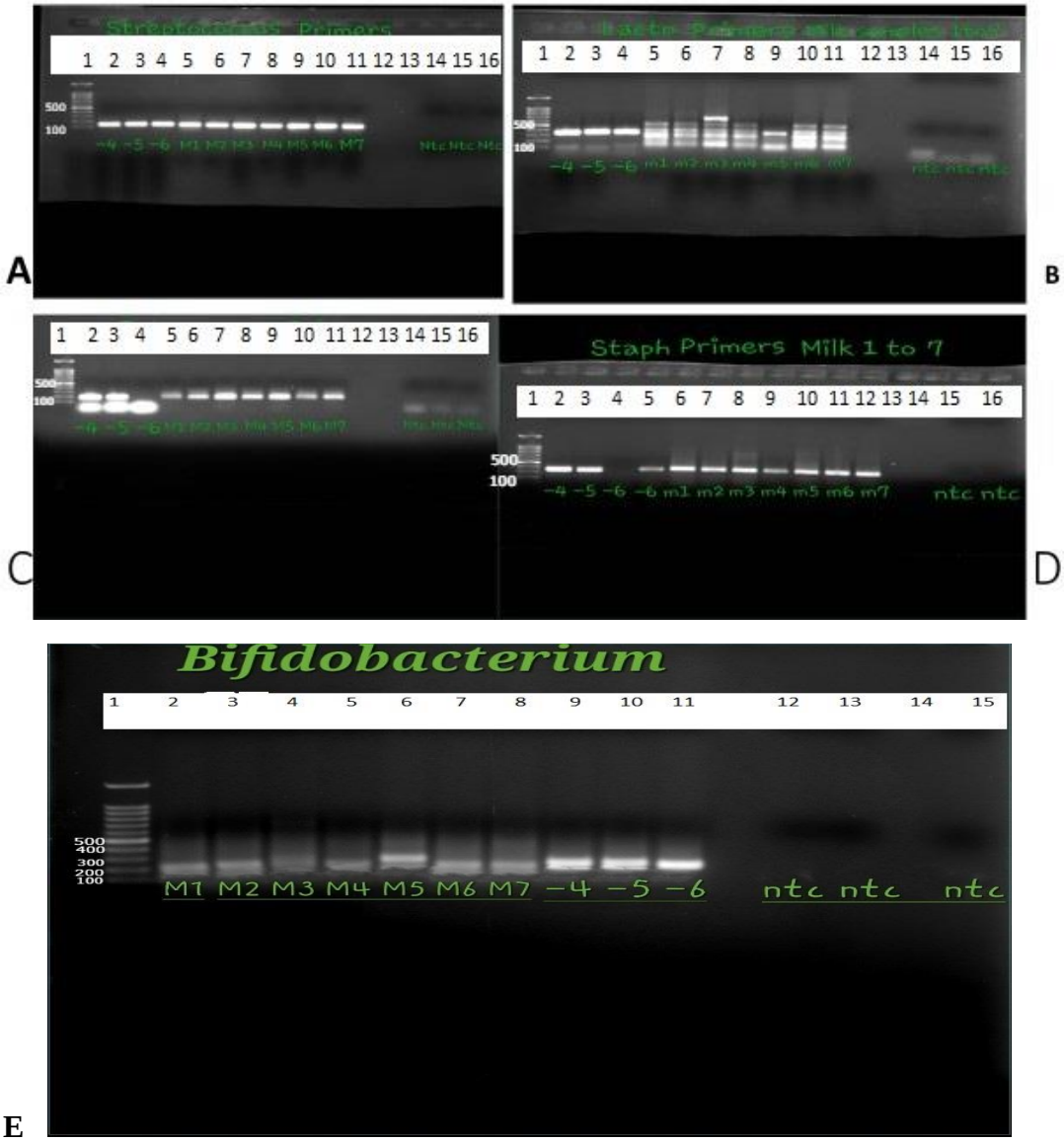


Figure 5: Gel image showing PCR products from milk samples of participants 1 to 7 using primers specific for genera (A)streptococci: lane 2 to 4 are positive controls while lanes 5 to 11 represents participant 1 to 7 respectively, lane 14 to 16 are non-template control. Expected product size is 197bp (B) Lactobacilli: lane 2 to 4 are positive control while lane 5 to 11 represents participant 1 to 7 respectively, expected product size: 341bp. Non-specific bands here is possibly due to high concentration of primer compared to product (C) Enterococci: lane 2 to 4 are positive controls. The three brighter bands below the positive control are primer dimer. Lane 5 to 11 represents participants 1 to 7 respectively. Expected product size: 144bp (D) Staphylococci as explained in figure 4.4 of the main document. (E) Bifidobacteria, lanes 2 to 8 represents participant 1 to 7 respectively while lane 9-11 are positive controls. Expected product size: 243bp. Ntc (A-E) indicates a non-template control with no bands.

APPENDIX 3B

Standard curve for the absolute quantification of genera streptococci, lactobacilli, bifidobacteria, staphylococci and enterococci in faeces as generated from qPCR run

Standard Curves

Gene Name	Slope	Efficiency	Error	R^2	Y-Intercept
None	-2.9284	2.20	0.14	1.00	11.87

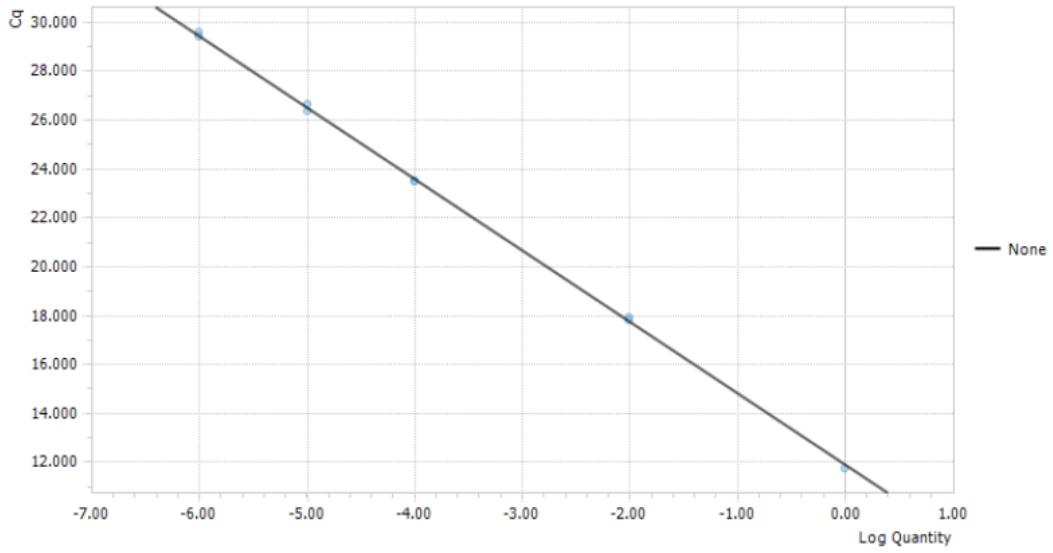


Figure 6: Standard curve for the absolute quantification of streptococci in faeces as genrated from qPCR run

Standard Curves

Gene Name	Slope	Efficiency	Error	R^2	Y-Intercept
None	-3.3195	2.00	0.15	1.00	7.70

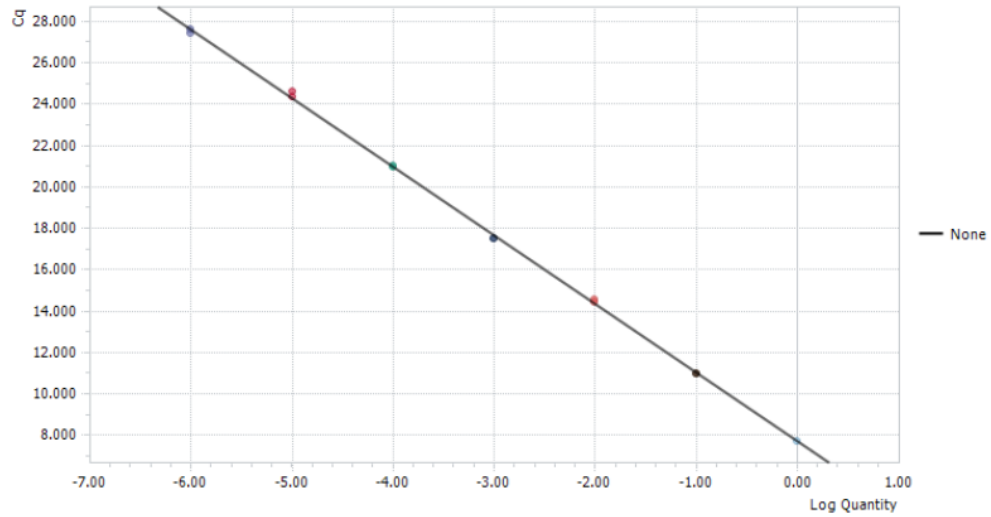


Figure 7: Standard curve for the absolute quantification of lactobacilli in faeces as genrated from qPCR run

Standard Curves

Gene Name	Slope	Efficiency	Error	R^2	Y-Intercept
None	-3.5906	1.90	0.22	1.00	7.58

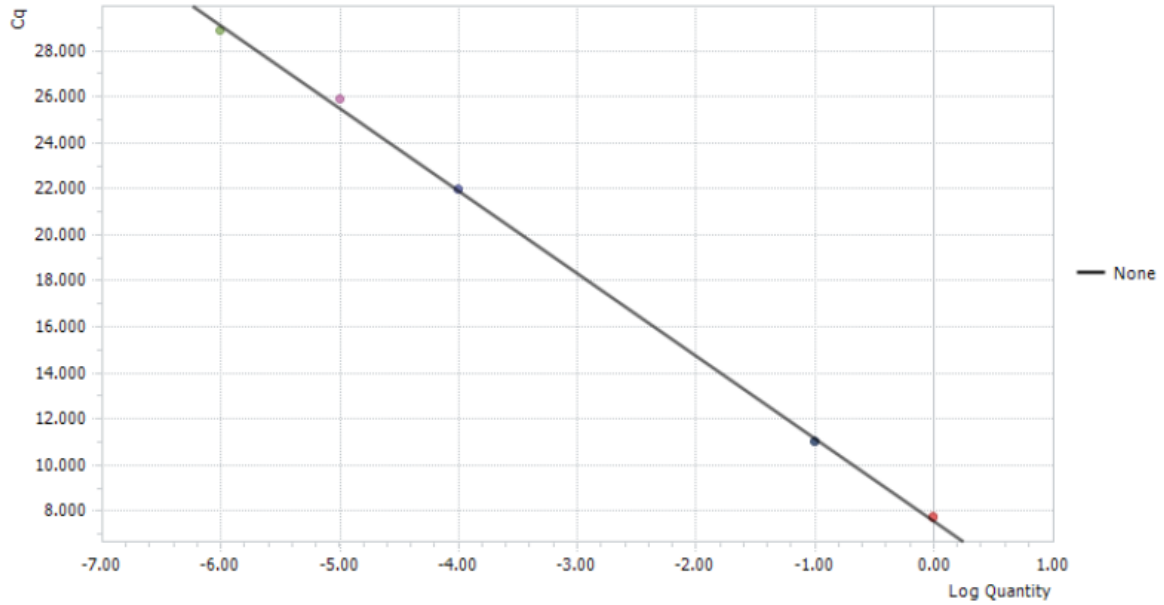


Figure 8: Standard curve for the absolute quantification of bifidobacteria in faeces as genrated from qPCR run

Standard Curves

Gene Name	Slope	Efficiency	Error	R^2	Y-Intercept
None	-2.9239	2.20	0.44	0.99	11.15

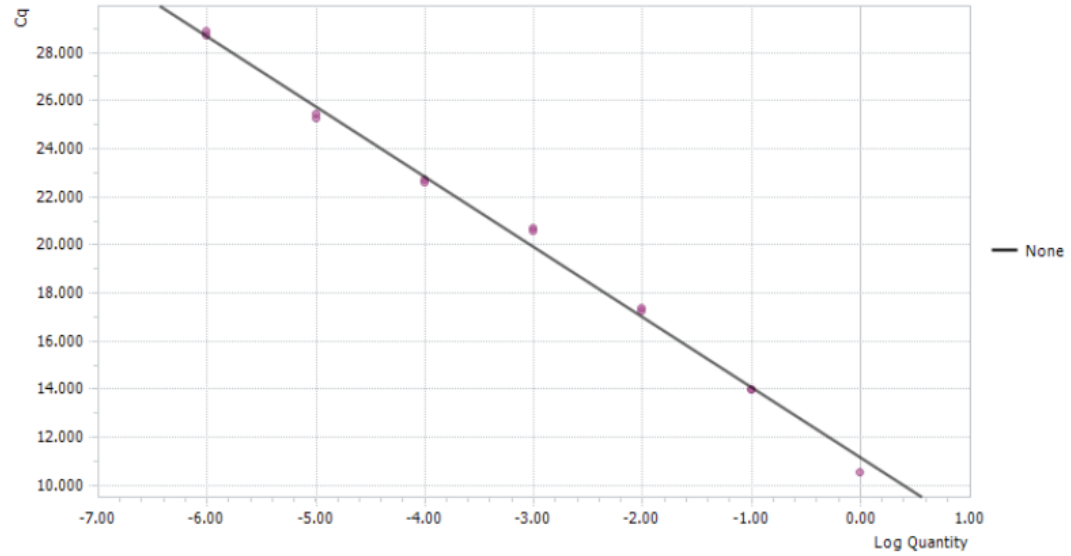


Figure 9: Standard curve for the absolute quantification of staphylococci in faeces as genrated from qPCR run

Standard Curves

Gene Name	Slope	Efficiency	Error	R^2	Y-Intercept
None	-3.1737	2.07	0.22	1.00	8.82

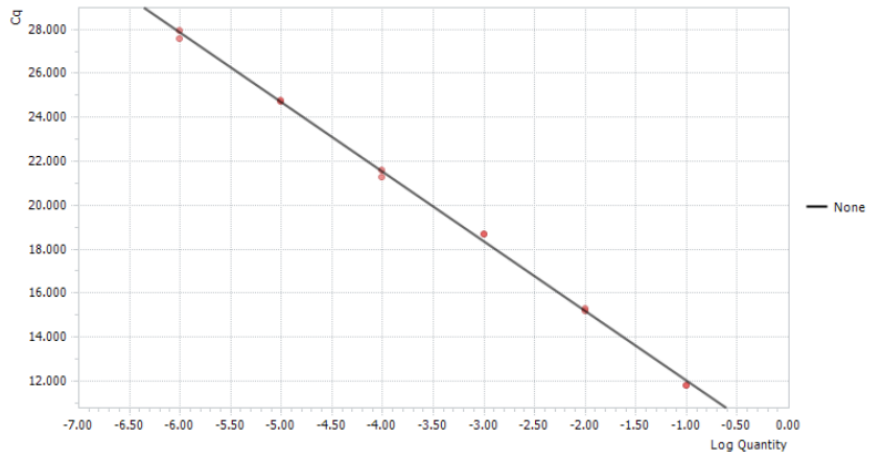


Figure 10: Standard curve for the absolute quantification of enterococci in faeces as genrated from qPCR run

Fig 11: Gel image showing PCR products from faeces samples of participants 1 to 7 using primers specific for genera (A) streptococci (197bp) (lane 2-8 represent participants 1 to 7 respectively) and staphylococci (370bp) (lane 9-13 represent participant 1 to 5 respectively). (B) Staphylococci (370bp) (lane 2 and 3 representing participants 6 and 7 respectively) and enterococci (144bp) (lane 7, 8 and 10-14 representing participants 1 to 7 respectively), lane 4, 5, and 6 represents positive control for streptococci, staphylococci and enterococci respectively. (C) lactobacilli (341bp) (lane 1-8 representing participants 1-7 respectively) and bifidobacteria (243bp) (lane 10-12 representing participants 1 to 3 respectively). (D) bifidobacteria (243bp) (lane 2-5 representing participants 4 to 7 respectively), lane 6-7 represents positive control for bifidobacteria and lane 9-10 represents positive control for lactobacilli. Ntc (A -D) represents non template control with no band.

APPENDIX 4

Table 1: OTU counts of bacterial phyla in human milk and baby faeces

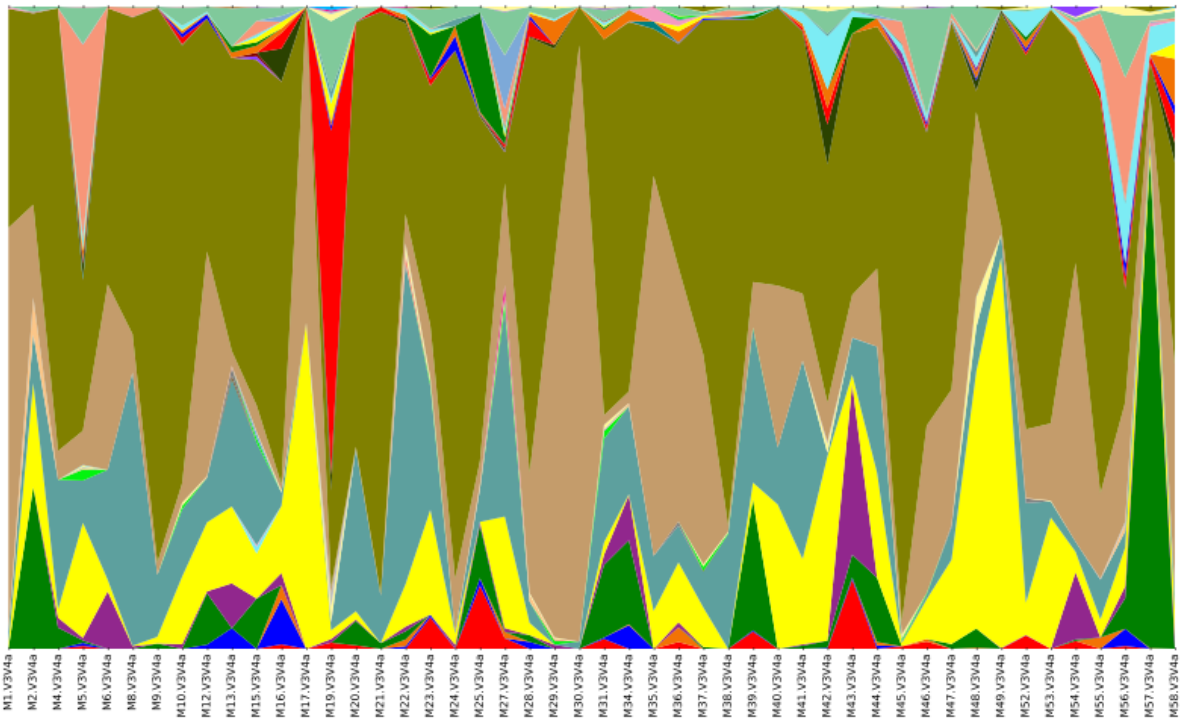
	N	Median(OTU counts)		P-value
		Human milk	Baby faeces	
Actinobacteria	48	8062.5	28522.5	<0.001
Proteobacteria	48	1210.5	3902	0.003
Firmicutes	48	18211	3662	<0.001
Bacteroidetes	48	39.5	0.0	0.638

Comparison between the abundance of bacterial phyla in human milk and baby faeces was calculated using nonparametric t-test, Man-Whitney U test, $p<0.05$. Data were calculated using Minitab v20.2.0.0

Table 1B : Abundance of bacterial phyla in human milk and baby faeces in percentage

Index	Human milk	Baby faeces	*p*-value
	n=48	n=48	
Actinobacteria	26.4%	62.6%	< 0.001
Firmicutes	61.4%	11.6%	< 0.001
Proteobacteria	10.5%	24.3%	0.003
Bacteroidetes	0.7%	1.4%	0.638
Others	1%	0.1%	

n total number of participants, values are percentage total abundance of bacterial phyla, *p*-value were calculated by Mann Whitney U-test. and $p < 0.05$ are considered statistically significant.


M1.V3V4a
M2.V3V4a
M4.V3V4a
M5.V3V4a
M6.V3V4a
M8.V3V4a
M9.V3V4a
M10.V3V4a
M12.V3V4a
M13.V3V4a
M15.V3V4a
M16.V3V4a
M17.V3V4a
M19.V3V4a
M20.V3V4a
M21.V3V4a
M22.V3V4a
M23.V3V4a
M24.V3V4a
M25.V3V4a
M27.V3V4a
M28.V3V4a
M29.V3V4a
M30.V3V4a
M31.V3V4a
M34.V3V4a
M35.V3V4a
M36.V3V4a
M37.V3V4a
M38.V3V4a
M39.V3V4a
M40.V3V4a
M41.V3V4a
M42.V3V4a
M43.V3V4a
M44.V3V4a
M45.V3V4a
M46.V3V4a
M47.V3V4a
M48.V3V4a
M49.V3V4a
M52.V3V4a
M53.V3V4a
M54.V3V4a
M55.V3V4a
M56.V3V4a
M57.V3V4a
M58.V3V4a

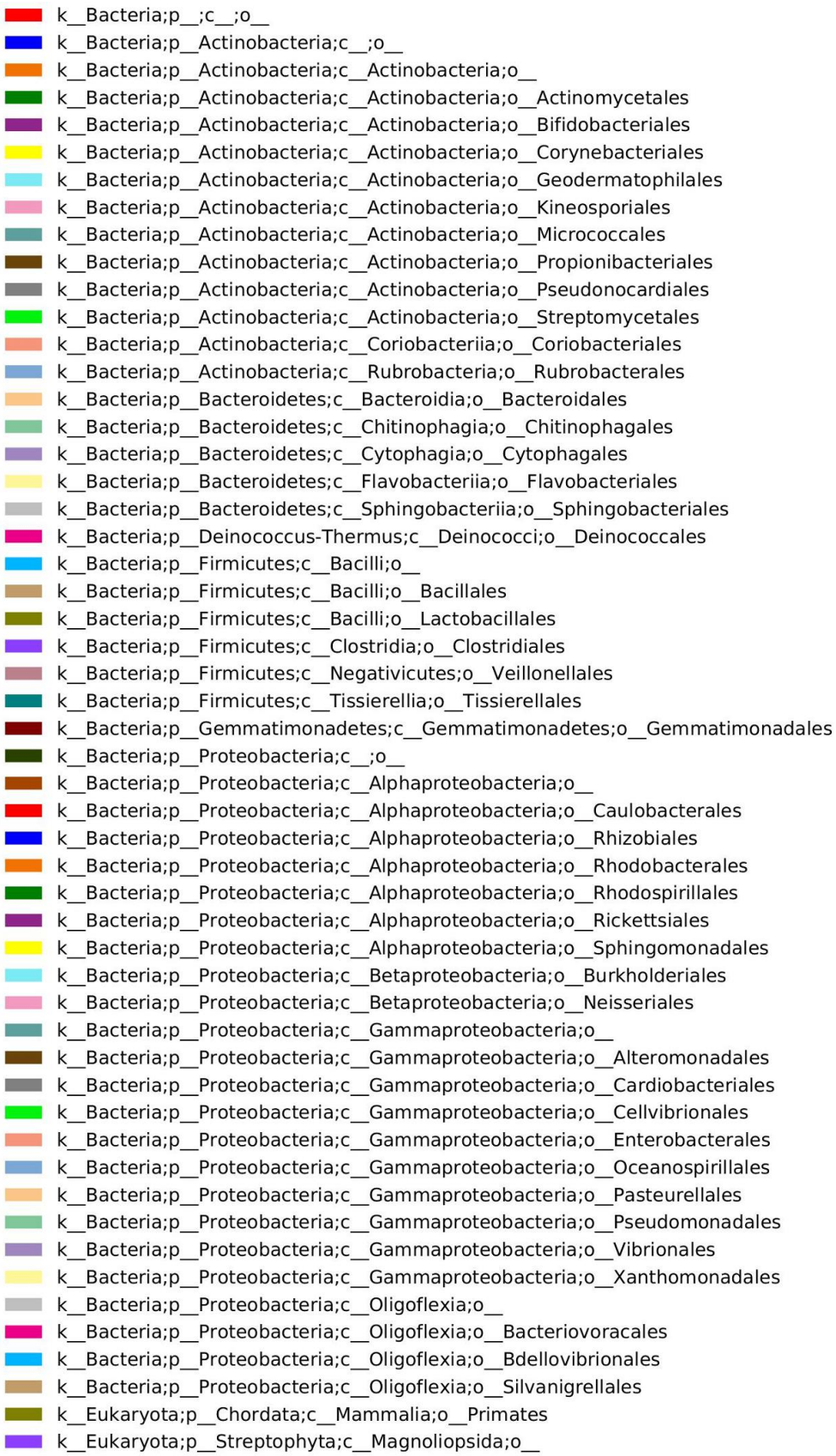


Figure 1: Relative abundance of bacterial taxa present in human milk samples at the order level. Lactobacillales was detected in 45 of the 48 participants

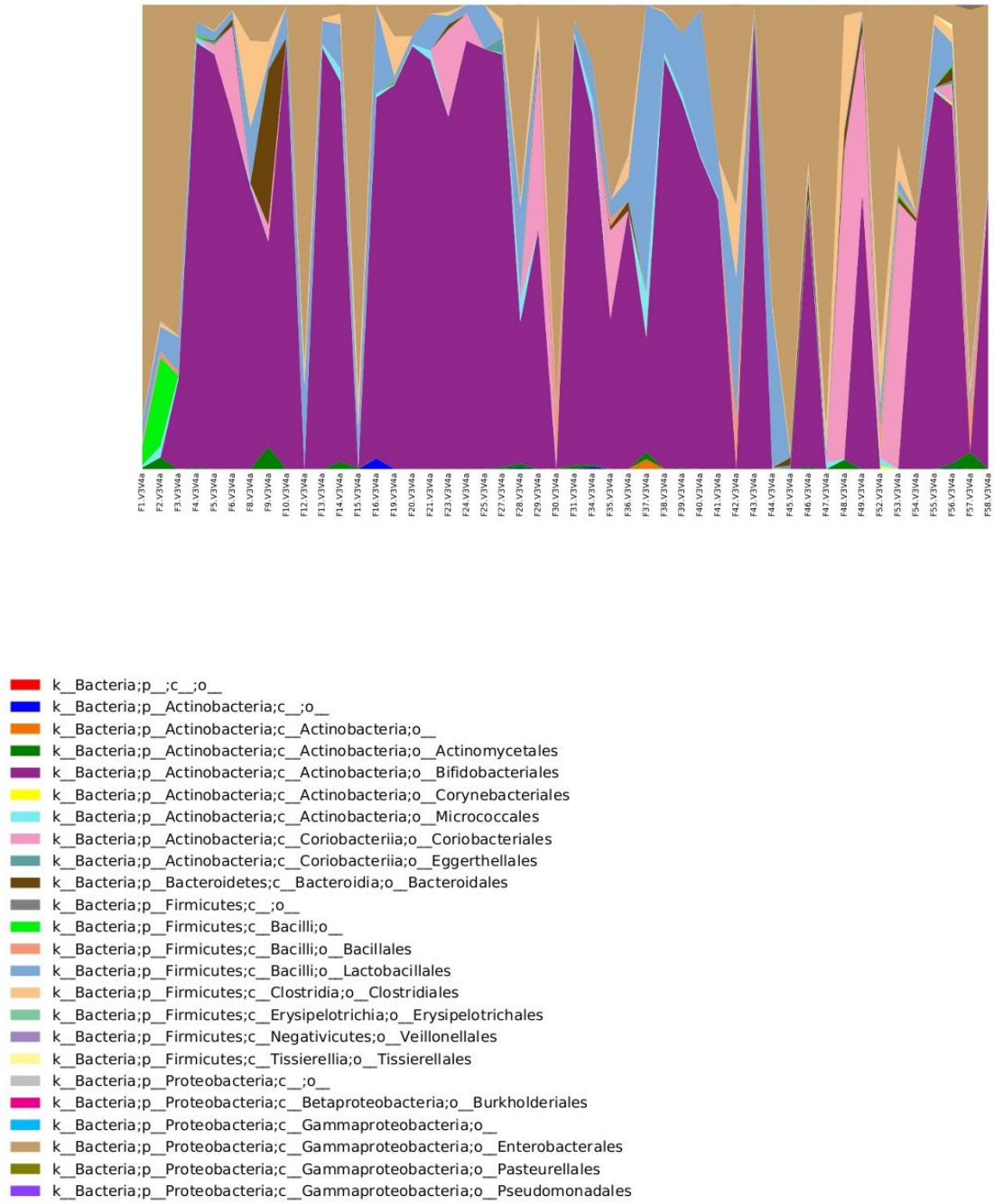


Figure 2: Relative abundance of bacterial taxa present in the faeces of breastfed babies at the order level. Lactobacillales was detected in 25 of the 48 participants

APPENDIX 5

Table 1: Significantly differential OTU comparison between faeces of breastfed babies (F) and milk (M)

id	mean_counts	log2_foldChange	p_value	adj_p_value
Acetobacter	635	-9.218651891	-	-
Achromobacter	12	-3.365033242	9.09E-20	1.50E-19
Acinetobacter	465	-6.183795733	5.74E-49	4.59E-48
Actinomyces	1281	-1.830340264	0.001474399	0.001608435
Aerococcus	36	-4.838663831	-	-
Aeromonas	9	-2.819568132	1.26E-15	1.89E-15
Agrobacterium	7	-2.584984501	-	-
Alishewanella	11	-3.362286443	1.94E-17	3.10E-17
Anaerococcus	67	-2.677004709	-	-
Aquabacter	17	-4.005724166	-	-
Arthrobacter	7	-2.64151718	6.68E-12	8.67E-12
Atopobium	115	0.046309088	0.934264153	0.934264153
Bacillus	2536	-11.12326885	-	-
Bacteroides	1892	10.02563888	-	-
Bifidobacterium	68937	7.001532485	8.64E-36	2.44E-35
Blautia	2772	9.715665632	-	-
Bosea	11	-3.446070101	3.02E-17	4.68E-17
Brachybacterium	16	-3.773937824	2.33E-21	4.48E-21
Brevibacterium	15	-3.851769995	-	-
Brevundimonas	180	-7.508835539	-	-
Caulobacter	5	-2.188066793	-	-
Chryseobacterium	41	-5.35749369	2.65E-36	7.96E-36
Citrobacter	302	7.475762781	-	-
Clostridium	3010	8.42464854	1.09E-41	4.36E-41
Collinsella	2435	8.446584994	-	-
Comamonas	20	-4.320610155	9.63E-28	2.20E-27
Corynebacterium	2945	-5.347109321	2.24E-34	5.66E-34
Cronobacter	165	7.453427804	-	-
Delftia	10	-3.304663387	3.39E-14	4.92E-14
Dolosigranulum	24	1.064470026	-	-
Eggerthella	83	6.476206234	-	-
Elizabethkingia	37	-5.206148612	1.25E-35	3.33E-35
Empedobacter	22	-4.46296444	-	-
Enhydrobacter	120	-6.875048854	1.24E-48	8.48E-48
Enterobacter	1632	5.524168245	-	-
Enterococcus	1989	7.447493234	-	-
Erysipelatoclostridium	24	5.604834165	-	-
Escherichia	17881	9.644602815	2.10E-95	5.04E-94

Eubacterium	39	5.882706699	-	-
Faecalibacterium	95	1.049663293	0.006951355	0.007414779
Finegoldia	38	0.889213072	-	-
Fusicatenibacter	48	1.086281178	-	-
Gemella	702	-4.637136524	3.17E-20	5.43E-20
Gemmiger	31	0.79715785	-	-
Granulicatella	196	-2.382187922	1.51E-05	1.72E-05
Haematobacter	93	-6.434679369	1.44E-39	4.94E-39
Haemophilus	62	2.539534276	1.70E-06	1.99E-06
Halomonas	103	-6.685634368	-	-
Hungatella	69	7.136039268	-	-
Intestinibacter	98	6.8989513	-	-
Janthinobacterium	181	-7.511755849	4.62E-48	2.77E-47
Klebsiella	5130	5.719245795	3.70E-21	6.83E-21
Kluyvera	110	6.677770162	-	-
Kocuria	269	-7.996376686	-	-
Kushneria	7	-2.700438598	-	-
Lachnoclostridium	23	5.3553365	-	-
Lactobacillus	2781	3.279275445	1.14E-09	1.40E-09
Lactococcus	23	2.725144074	-	-
Limnobacter	22	-4.433244307	-	-
Lysinibacillus	49	-5.605608084	-	-
Methylobacterium	14	-3.797214287	9.35E-24	1.87E-23
Micrococcus	33	-5.038015088	5.16E-37	1.65E-36
Moraxella	23	-4.295178008	1.96E-20	3.49E-20
Neisseria	75	-5.640154586	-	-
Nesterenkonia	59	-5.895054892	-	-
Nocardia	23	-4.489916352	-	-
Nocardioides	6	-2.491005353	2.63E-12	3.50E-12
Ochrobactrum	9	-3.120717932	8.50E-14	1.20E-13
Ottowia	18	-4.159726862	-	-
Pantoea	46	-1.248364325	-	-
Parabacteroides	43	6.059309824	-	-
Paracoccus	113	-6.436543057	2.29E-53	2.75E-52
Pedobacter	10	-3.216158591	1.18E-13	1.61E-13
Peptoniphilus	26	1.855153261	-	-
Peptostreptococcus	61	5.840219853	-	-
Phycicoccus	38	-5.257577527	1.33E-33	3.20E-33
Prevotella	303	1.267271444	-	-
Pseudoalteromonas	16	-3.910944372	3.71E-27	8.09E-27
Pseudochelatococcus	43	-5.404262559	-	-
Pseudomonas	699	-9.483961434	3.58E-137	1.72E-135
Pseudoxanthomonas	13	-3.668546426	-	-
Rhizobium	20	-4.313285211	5.68E-25	1.19E-24

Robinsoniella	79	7.321359526	-	-
Roseburia	36	4.495221385	-	-
Rothia	4729	-2.303980427	3.16E-07	3.79E-07
Ruminococcus	118	0.954535637	-	-
Saccharopolyspora	4	-1.862530335	-	-
Salinicoccus	11	-3.402821558	-	-
Salmonella	191	-5.281706519	-	-
Senegalimassilia	85	6.967236891	-	-
Serratia	8	-0.265649493	-	-
Sphingobacterium	8	-2.917838943	-	-
Sphingobium	16	-3.957611725	-	-
Sphingomonas	47	-5.56647527	3.14E-50	3.01E-49
Staphylococcus	14841	-3.102515341	6.05E-11	7.65E-11
Stenotrophomonas	66	-6.070444093	1.09E-47	5.83E-47
Streptococcus	45220	-1.252311567	6.80E-05	7.59E-05
Streptomyces	59	-5.908644408	5.49E-44	2.64E-43
Terrisporobacter	36	0.859634268	0.030809036	0.031464547
Tyzzerella	68	6.934507728	1.29E-41	4.76E-41
Veillonella	68	1.141870973	0.013081961	0.013650741
Vibrio	49	-5.638179561	1.97E-57	3.15E-56
Xylophilus	45	-5.507430001	8.55E-42	3.73E-41

ID, indicates the bacterial genera detected in milk(M) or faeces of breastfed babies(F). Mean_counts, are the mean of the OTU counts detected in the milk or faeces of breastfed babies. Composition differences are given in log2 fold change between faeces and milk comparison. Positive fold change indicates the genus is more expressed in faeces of breastfed babies compared to milk, while the negative fold change indicates the genus is more expressed in milk compared to faeces. FDR corrected p-value (adjusted p-value) of $P<0.1$ was considered significant.

Table 2: Significantly differential OTU comparison between faeces of breastfed babies and faeces of babies fed with milk formula

id	mean_counts	log2_foldChange	p_value	adj_p_value
Acetobacter	635	-0.051902702	-	-
Achromobacter	12	0.073149949	0.924974306	0.98307515
Acinetobacter	465	1.985615918	0.02007634	0.074829996
Actinomyces	1281	0.060225348	0.954227844	0.98307515
Aerococcus	36	0.262305381	-	-
Aeromonas	9	0.198768809	0.792044098	-
Agrobacterium	7	-0.011122733	-	-
Alishewanella	11	-0.015433097	0.984865411	-
Anaerococcus	67	2.138304094	-	-
Aquabacter	17	-0.043082459	-	-

Arthrobacter	7	0.078379672	0.921786863	-
Atopobium	115	0.661583159	0.519152445	0.98307515
Bacillus	2536	-0.01239675	-	-
Bacteroides	1892	4.620389563	-	-
Bifidobacterium	68937	2.483425619	0.015231376	0.072296884
Blautia	2772	-0.398892498	-	-
Bosea	11	-0.017974946	0.982787657	0.98307515
Brachybacterium	16	0.116150898	0.887729745	0.98307515
Brevibacterium	15	-0.027265659	-	-
Brevundimonas	180	-0.081405464	-	-
Caulobacter	5	-0.00648821	-	-
Chryseobacterium	41	-0.042496605	0.960673641	0.98307515
Citrobacter	302	0.827999125	-	-
Clostridium	3010	5.220841351	3.61E-06	2.96E-05
Collinsella	2435	0.130288333	-	-
Comamonas	20	-0.025237912	0.975288167	0.98307515
Corynebacterium	2945	4.602374175	6.82E-08	6.99E-07
Cronobacter	165	0.887721425	-	-
Delftia	10	-0.020299383	0.981533259	-
Dolosigranulum	24	3.968492131	-	-
Eggerthella	83	5.068938991	-	-
Elizabethkingia	37	-0.038987389	0.963389922	0.98307515
Empedobacter	22	-0.037996562	-	-
Enhydrobacter	120	-0.020784326	0.982185164	0.98307515
Enterobacter	1632	2.189631574	-	-
Enterococcus	1989	4.937001558	-	-
Erysipelatoclostridium	24	4.56023557	-	-
Escherichia	17881	1.124986174	0.18848427	0.515190338
Eubacterium	39	-0.851769068	-	-
Faecalibacterium	95	-9.147170978	4.05E-66	1.66E-64
Finegoldia	38	0.885749747	-	-
Fusicatenibacter	48	-8.134154278	-	-
Gemella	702	2.251327971	0.016909385	0.072296884
Gemmiger	31	-7.429524612	-	-
Granulicatella	196	0.894953564	0.375741578	0.962837794
Haematobacter	93	0.035128201	0.97093448	0.98307515
Haemophilus	62	-0.067156302	0.944724359	0.98307515
Halomonas	103	-0.101851675	-	-
Hungatella	69	6.073548996	-	-
Intestinibacter	98	-0.969305852	-	-
Janthinobacterium	181	-0.10066736	0.919989884	0.98307515
Klebsiella	5130	2.36413225	0.031639355	0.099785659
Kluyvera	110	0.796305285	-	-
Kocuria	269	-1.069206299	-	-

Kushneria	7	-0.01696235	-	-
Lachnoclostridium	23	3.186683415	-	-
Lactobacillus	2781	-4.127151798	2.78E-05	0.000189787
Lactococcus	23	-3.817890865	-	-
Limnobacter	22	-0.043616626	-	-
Lysinibacillus	49	-0.06490032	-	-
Methylobacterium	14	-0.017298693	0.982448498	0.98307515
Micrococcus	33	-0.032673819	0.968048163	0.98307515
Moraxella	23	0.155979409	0.866540107	0.98307515
Neisseria	75	-2.655020831	-	-
Nesterenkonia	59	-0.072280498	-	-
Nocardia	23	-0.044640812	-	-
Nocardioides	6	-0.002991369	0.996812149	-
Ochrobactrum	9	-0.015779347	0.98516586	-
Ottowia	18	-0.04059024	-	-
Pantoea	46	2.974883941	-	-
Parabacteroides	43	3.627385831	-	-
Paracoccus	113	0.341552959	0.692046173	0.98307515
Pedobacter	10	-0.018919363	0.982712446	-
Peptoniphilus	26	2.655519328	-	-
Peptostreptococcus	61	2.548502465	-	-
Phycicoccus	38	-0.04350414	0.960423761	0.98307515
Prevotella	303	-1.164677877	-	-
Pseudoalteromonas	16	-0.01617047	0.98307515	0.98307515
Pseudochelatococcus	43	-0.071410971	-	-
Pseudomonas	699	-0.072087503	0.927064377	0.98307515
Pseudoxanthomonas	13	-0.02648179	-	-
Rhizobium	20	-0.029043008	0.972728517	0.98307515
Robinsoniella	79	6.255476324	-	-
Roseburia	36	-3.623821463	-	-
Rothia	4729	1.890029699	0.023036338	0.078707489
Ruminococcus	118	-9.411248129	-	-
Saccharopolyspora	4	0.000803043	-	-
Salinicoccus	11	-0.023052524	-	-
Salmonella	191	1.390727363	-	-
Senegalimassilia	85	1.848540061	-	-
Serratia	8	1.93698382	-	-
Sphingobacterium	8	-0.016285225	-	-
Sphingobium	16	-0.031644038	-	-
Sphingomonas	47	-0.033157196	0.966090096	0.98307515
Staphylococcus	14841	6.874474775	6.65E-15	9.09E-14
Stenotrophomonas	66	-0.04878075	0.954227853	0.98307515
Streptococcus	45220	0.923734569	0.114753822	0.336064765
Streptomyces	59	-0.048575884	0.954952119	0.98307515

Terrisporobacter	36	-7.69967196	9.24E-42	1.89E-40
Tyzzerella	68	2.09788673	0.017633386	0.072296884
Veillonella	68	2.736760166	0.001522728	0.008918833
Vibrio	49	-0.02962867	0.968413619	0.98307515
Xylophilus	45	-0.039764208	0.961863416	0.98307515

ID, indicates the bacterial genera detected in faeces of breastfed babies (F) or in the faeces of babies fed milk formula (FM). Mean_counts, are the mean of the OTU counts detected in the faeces of breastfed babies and faeces of babies fed formula milk. Composition differences are given in log2 fold change between F and FM comparison. Positive fold change indicates the genus is more expressed in F compared to FM, while the negative fold change indicates the genus is more expressed in FM compared to F. FDR corrected p-value (adjusted p-value) of $P<0.1$ was considered significant.

APPENDIX 6

Table 1: Comparison if alpha diversity indexes between mother's milk and faeces of babies

Index	**Mother's milk**	**Baby faeces**	***p*-value**
	n=48	**n=48**	
Observed	116.08 ± 22.01	87.13 ± 15.09	< 0.001
Chao1	117.06 ± 22.24	87.60 ± 15.21	< 0.001
Shannon	4.26 ± 0.48	3.26 ± 0.71	< 0.001
Simpson	0.86 ± 0.06	0.71 ± 0.14	< 0.001

Values are expressed as mean ± SD, p-values were calculated by Mann-Whitney U test.

Table 2: Comparison if alpha diversity indexes between religion (Christians and Muslims) in mothers' milk

Group	**Index**	**Christians**	**Muslims**	**p-value**
		N=17	N=31	
Mother's milk N=48	Chao1	123.26 (± 20.39)	113.65 (± 22.78)	0.030
	Shannon	4.39 (± 0.28)	4.18 (± 0.55)	0.079

Values are expressed as mean ± SD, p-values were calculated by Mann-Whitney U test.

Table 3: Comparison of alpha diversity indexes between working conditions (outside or inside the home) in mother's milk.

Group	**Index**	**Outside**	**Inside**	**p-value**
		N=31	N=17	
Mother's milk N=48	Chao1	114.34 (± 19.69)	122.01 (± 26.18)	0.407
	Shannon	4.24 (± 0.48)	4.28 (± 0.50)	0.712

Values are expressed as mean ± SD, p-values were calculated by Mann Whitney-U test.

Table 4: Comparison of alpha diversity indexes between parity (uniparous and multiparous) in mother's milk.

Group	**Index**	**Uniparous**	**Multiparous**	**p-value**
		N=27	N=21	
Mother's milk N=48	Chao1	117.87 (± 22.94)	117.70 (± 21.99)	0.596
	Shannon	4.35 (± 0.48)	4.14 (± 0.46)	0.149

Values are expressed as mean ± SD, p-values were calculated by Mann-Whitney U test.

Table 5: Comparison of alpha diversity indexes between stages of education (secondary and tertiary) in mother's milk.

Group	**Index**	**Secondary**	**Tertiary**	**p-value**
		N=13	N=35	
Mother's milk N=48	Chao1	103.09 (± 37.62)	118.98 (± 22.55)	0.880
	Shannon	3.82 (± 1.21)	4.30 (± 0.51)	0.634

Values are expressed as mean ± SD, p-values were calculated by Mann-Whitney U test.

Table 6: Comparison of alpha diversity indexes between maternal age range

Group	Index	15-25	26-35	36-40	p-value
		N=12	N=32	N=4	
Mother's milk N=48	Chao1	122.69 (± 27.01)	113.31 (± 18.08)	130.09 (± 34.53)	0.988
	Shannon	4.48 (± 0.54)	4.19 (± 0.43)	4.14 (± 0.61)	0.426

Values are expressed as mean ± SD, p-values were calculated by Kruskal-Wallis test.

Table 7: Comparison of alpha diversity indexes between maternal diet

Group	Index	Fibre	Fat	Redmeat	Yoghurt	Chicken	p-value
		N=16	N=28	N=32	N=7	N=24	
Mother's milk N=48	Chao1	109.35 (± 17.24)	114.18 (± 23.09)	119.08 (± 25.11)	137.60 (± 31.82)	119.73 (± 26.37)	0.099
	Shannon	4.22 (± 0.39)	4.29 (± 0.45)	4.33 (± 0.45)	4.35 (± 0.47)	4.35 (± 0.49)	0.510

Values are expressed as mean ± SD, p-values were calculated by Kruskal-Wallis test.

Table 8: Comparison of alpha diversity indexes between age range (<3 Months and >3 Months) in baby faeces

Group	Index	<3 Months	>3 Months	p-value
		N=33	N=15	
Baby faeces N=48	Chao1	90.96 (± 16.13)	80.21 (± 9.83)	0.743
	Shannon	3.40 (± 0.66)	2.97 (± 0.72)	0.930

Values are expressed as mean ± SD, p-values were calculated by Mann-Whitney U test.

Table 9: Comparison of alpha diversity indexes between gender (male and female) in baby faeces

Group	Index	Male	Female	p-value
		N=27	N=21	
Baby faeces N=48	Chao1	88.01 (± 16.31)	87.08 (± 14.04)	0.959
	Shannon	3.31 (± 0.71)	3.19 (± 0.72)	0.811

Values are expressed as mean ± SD, p-values were calculated by Mann-Whitney U test.

Table 10: Comparison of alpha diversity indexes between mode of delivery (natural and C-section) in baby faeces

Group	**Index**	**Natural**	**C-section**	**p-value**
		N=37	N=11	
Baby faeces				
N=48	Chao1	89.32 (± 14.86)	81.84 (± 15.66)	0.189
	Shannon	3.29 (± 0.69)	3.14 (± 0.79)	0.548

Values are expressed as mean ± SD, p-values were calculated by Mann-Whitney U test.

APPENDIX 7

Table 11: OTU counts of bacterial phyla in human milk and baby faeces

Abundance of bacterial phyla in human milk

Variable	N	Mean	SE Mean	StDev	CoefVar	Minimum	Median	Maximum	Range
k__Bacteria;p__Actinobacteria	48	8423	826	5725	67.96	195	8063	22001	21806
k__Bacteria;p__Firmicutes	48	19560	1203	8333	42.60	2542	18211	37839	35297
k__Bacteria;p__Proteobacteria	48	3336	1101	7630	228.73	0	1211	50013	50013
k__Bacteria;p__Bacteroidetes	48	225.6	77.9	539.9	239.33	0.0	39.5	3043.0	3043.0

Abundance of bacterial phyla in baby faeces

Variable	N	Mean	SE Mean	StDev	CoefVar	Minimum	Median	Maximum	Range
k__Bacteria;p__Bacteroidetes_1	48	656	383	2656	404.99	0	0	18233	18233
k__Bacteria;p__Firmicutes_1	48	5561	850	5892	105.95	275	3662	29040	28765
k__Bacteria;p__Proteobacteria_1	48	11628	2220	15383	132.29	0	3902	54364	54364
k__Bacteria;p__Actinobacteria_1	48	29920	3244	22478	75.13	0	28523	67986	67986

N, number of samples; SD, standard deviation; CoefVar, coefficient of variation; Range, denotes the difference between maximum values and minimum values in the OTU counts of each phylum.

APPENDIX 8

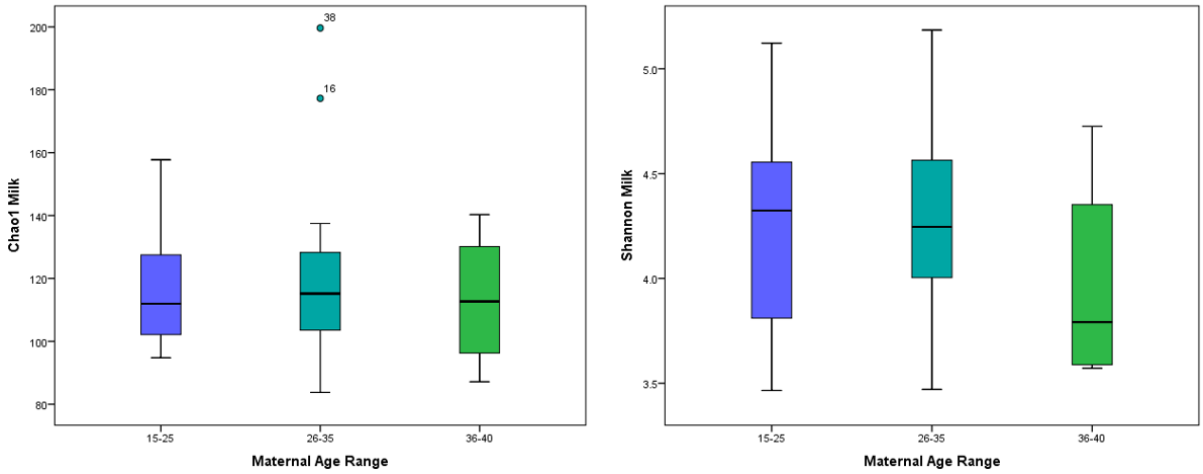


Figure 1: Alpha diversity in maternal milk samples from mothers between the ages of 15-25 (n=12), 26-35 (n =32) or 36-40 (n=4). (A)Chao 1($p = 0.988$), and (B) Shannon ($p= 0.426$), indexes. The diversity indexes were calculated using Kruskal-Wallis test where $p < 0.05$ was considered significant (Table S6). Labels below the graphics indicates the age range.

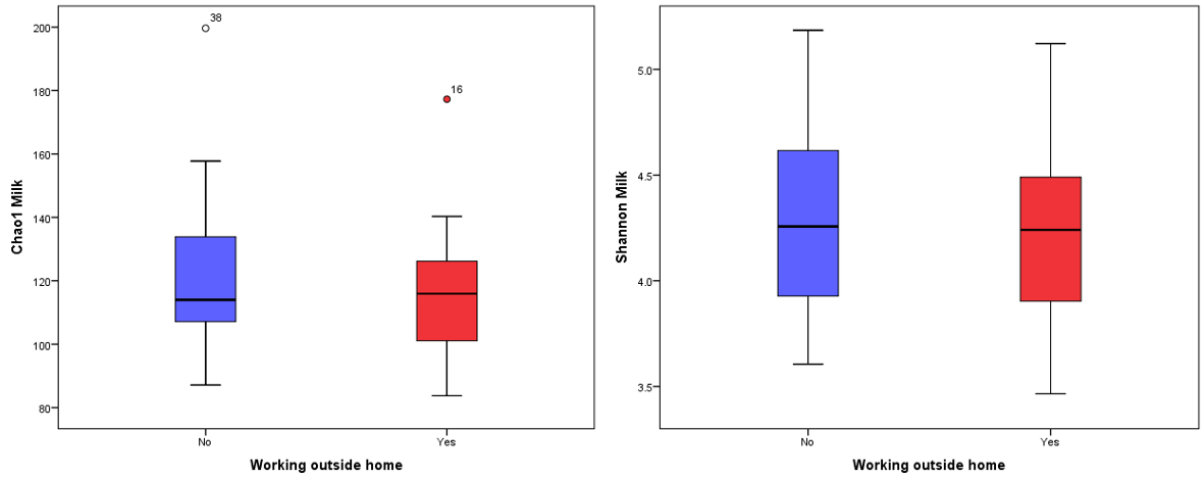


Figure 2: Alpha diversity in maternal milk samples from mothers working outside of home (n=31) and inside (n=17). (A) Chao 1($p = 0.407$), and (B) Shannon ($p= 0.712$), indexes. The diversity indexes were calculated using Man-Whitney test where $p < 0.05$ was considered significant (Table S6). Labels below the graphics indicates 'Yes' for those working outside and 'No' for those working within the home.

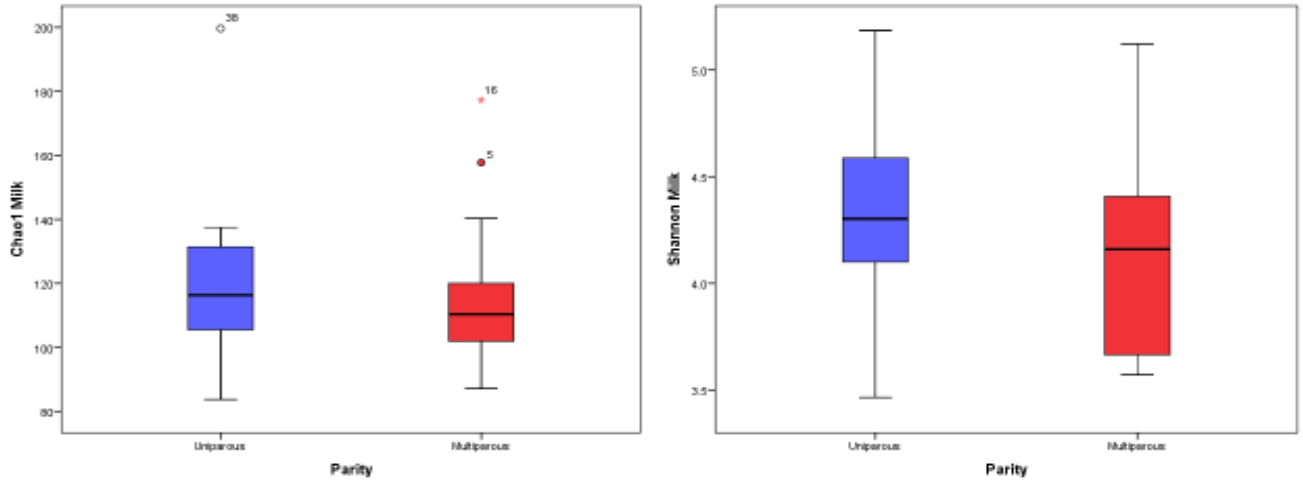


Figure 3: Alpha diversity in maternal milk samples from mothers with a single child (Uniparous) (n=27) and more than one child (Multiparous) (n=21). (A)Chao 1($p = 0.596$), and (B) Shannon ($p= 0.149$), indexes. The diversity indexes were calculated using Man-Whitney test where $p < 0.05$ was considered significant (Table S6). Labels below the graphics indicates 'Uniparous' and 'Multiparous'.

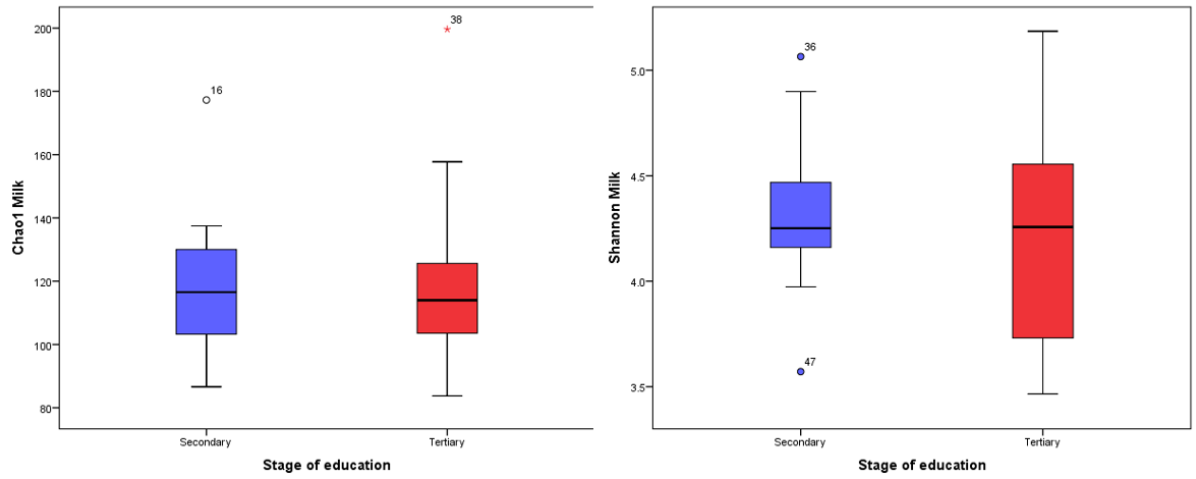


Figure 4: Alpha diversity in maternal milk samples from mothers with secondary as highest level of education (n=13) or tertiary (n=35). (A)Chao 1($p = 0.880$), and (B) Shannon ($p= 0.634$), indexes. The diversity indexes were calculated using Man-Whitney test where $p < 0.05$ was considered significant (Table S6). Labels below the graphics indicates 'Secondary' and 'Tertiary'.

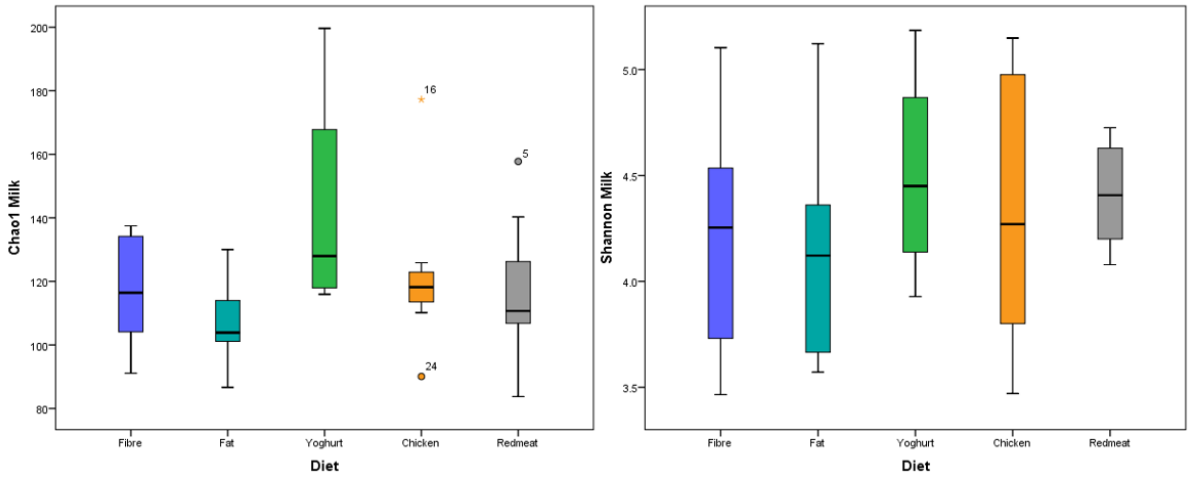


Figure 5: Alpha diversity in maternal milk samples from mothers who stated their diet were rich in Fibre (n=16), Fat (n =28), Yogurt (n=7), Chicken (24), and Redmeat (32).(A)Chao 1($p = 0.099$), and (B) Shannon ($p= 0.510$), indexes. The diversity indexes were calculated using Kruskal-Wallis test where $p < 0.05$ was considered significant (Table S6). Labels below the graphics indicates the diet richness.

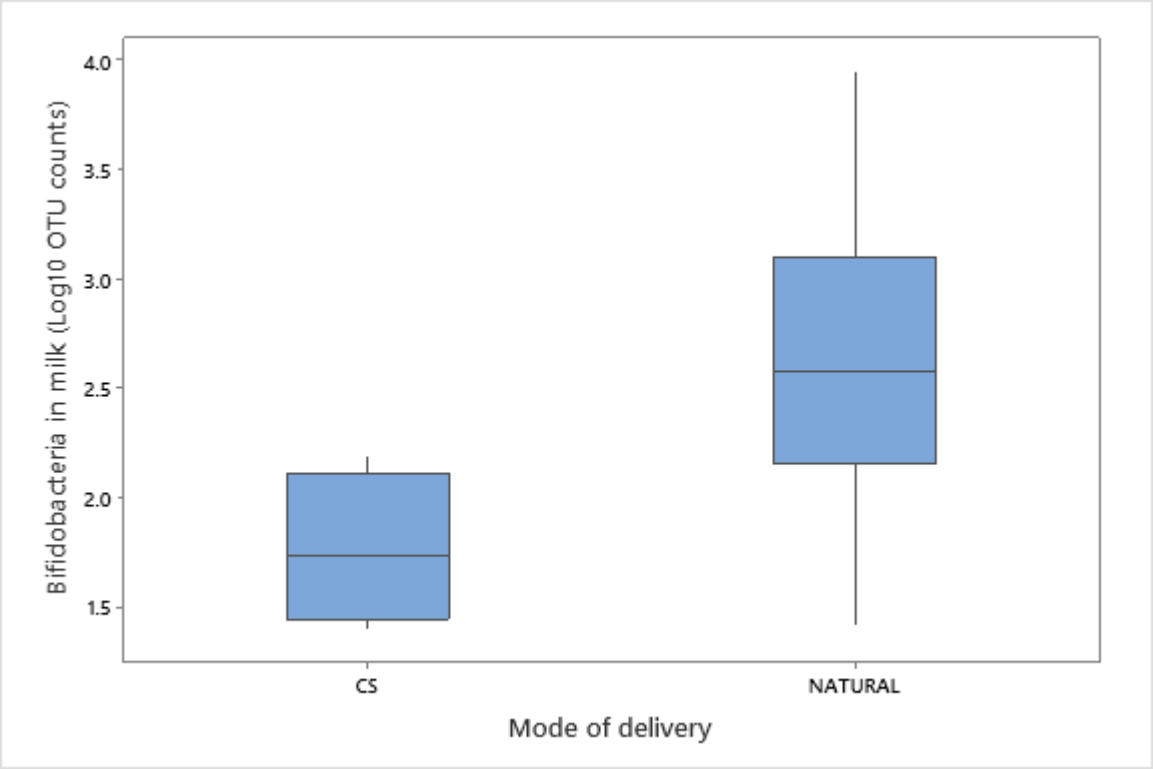


Figure 6: Association between mother's mode of delivery and bifidobacteria in milk. On y-axis is the number of reads or OTU counts belonging to bifidobacteria in milk of mothers formatted as a log scale while the x-axis shows the mode of delivery; CS-Caesarean section. Mothers who delivered naturally had higher level of bifidobacteria in their milk compared to mothers who delivered through CS. However, according to Mann-Whitney U test and BH corrected p value, there is no statistically significant difference ($P = 0.048$; $q=0.48$). $q<0.05$ was considered to be statistically significant

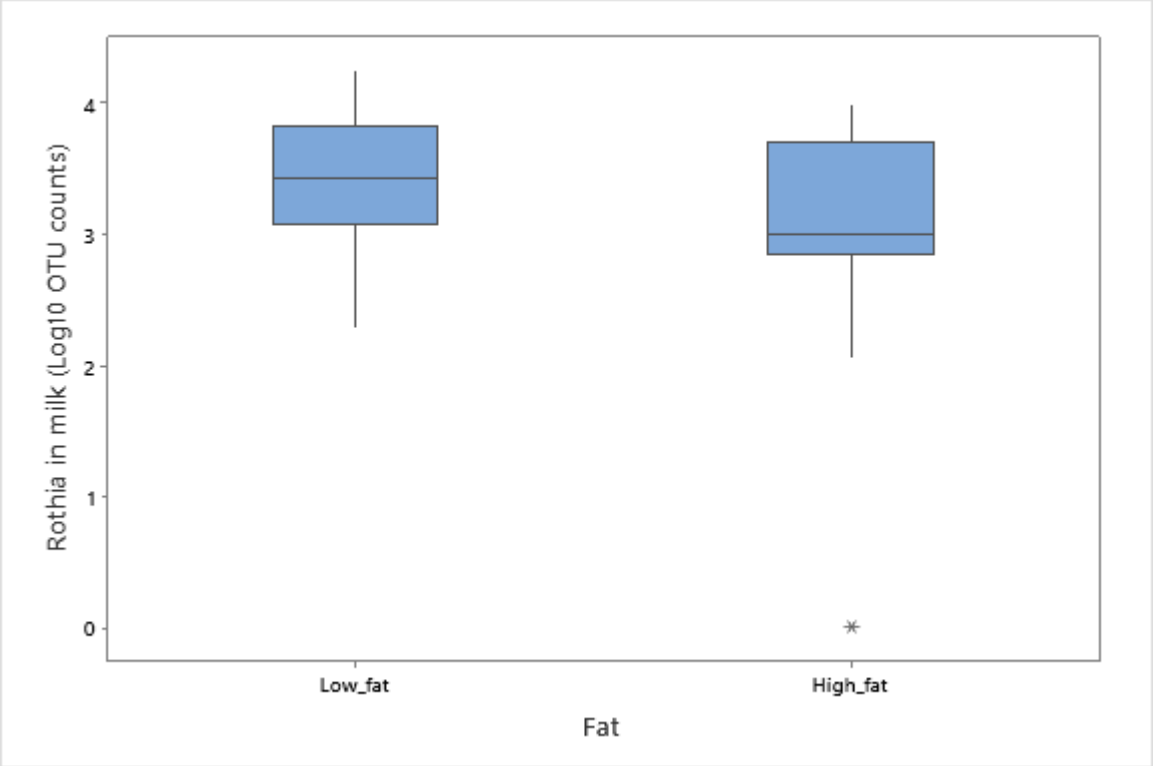


Figure 7: Association between mother's diet (fat) and *Rothia* in milk. On y-axis is the number of reads or OTU counts belonging to *Rothia* in milk of mothers formatted as a log scale while the x-axis shows the diet. Mothers who reported diet high in fat had lower level of *Rothia* in their milk compared to mothers who did not report diet high in fat. However, according to Mann-Whitney U test and BH corrected p value, there is no statistically significant difference (P-value =0.008; q=0.08). $q<0.05$ was considered to be statistically significant

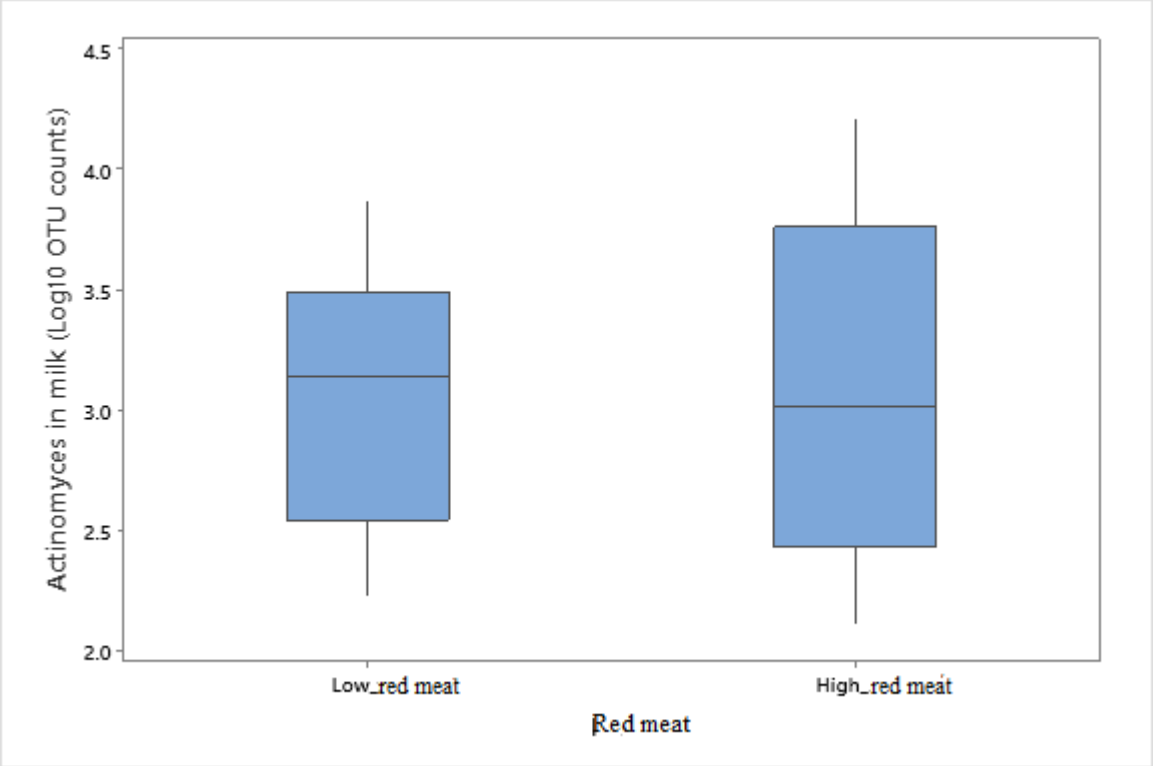


Figure 8: Association between mother's diet (red meat) and *Actinomyces* in milk. On y-axis is the number of reads or OTU counts belonging to *Actinomyces* in milk of mothers formatted as a log scale while the x-axis shows the diet. Mothers who reported diet rich in red meat had lower level of *Actinomyces* in their milk compared to mothers who did not report diet rich in red meat. However, according to Mann-Whitney U test and BH corrected p value, there is no statistically significant difference (P-value =0.012; q=0.12). $q<0.05$ was statistically significant

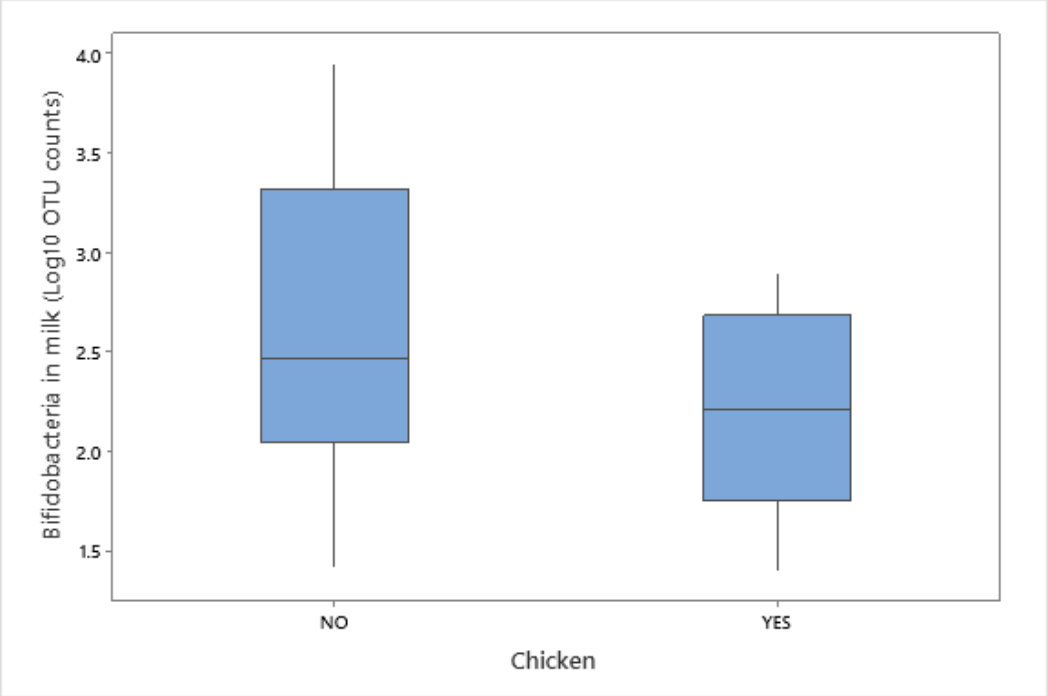


Figure 9: Association between mother's diet (chicken) and *bifidobacteria* in milk. On y-axis is the number of reads or OTU counts belonging to bifidobacteria in milk of mothers formatted as a log scale while the x-axis shows the diet. Mothers who reported diet rich in chicken had lower level of bifidobacteria in their milk compared to mothers who did not report diet rich in chicken. However, according to Mann-Whitney U test and BH corrected p value, there is no statistically significant difference (P-value =0.021; q=0.2). $q<0.05$ was statistically significant

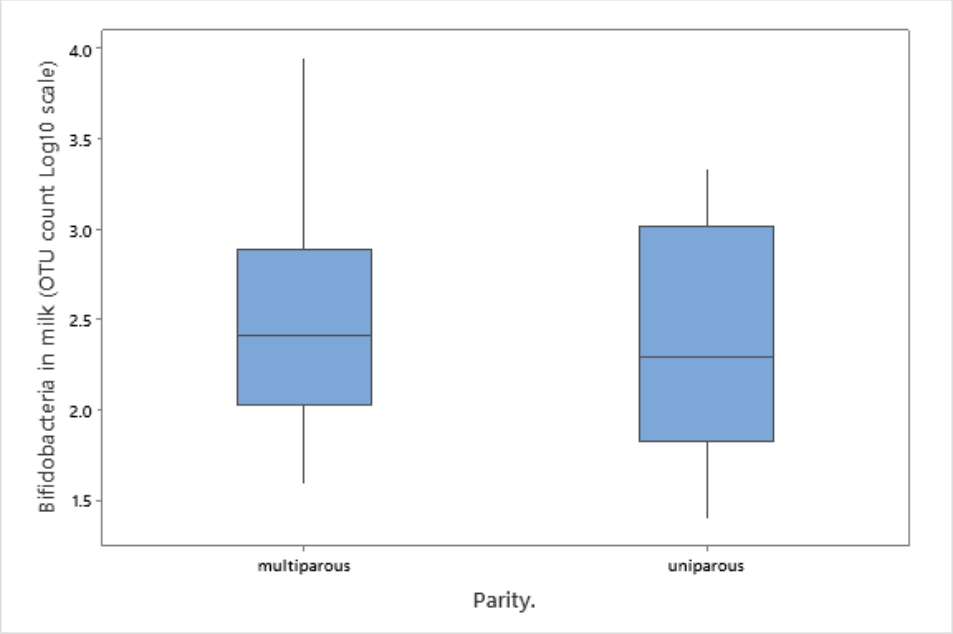


Figure 10: Association between parity and bifidobacteria in milk. On y-axis is the number of reads or OTU counts belonging to bifidobacteria in milk of mothers formatted as a log scale while the x-axis shows parity. Mothers who reported having more than a child (multiparous) had higher level of bifidobacteria in their milk compared to mothers who reported having a child (uniparous). However, according to Mann-Whitney U test and BH corrected p value, there is no statistically significant difference (P= 0.021, q=0.08). q<0.05 was statistically significant.

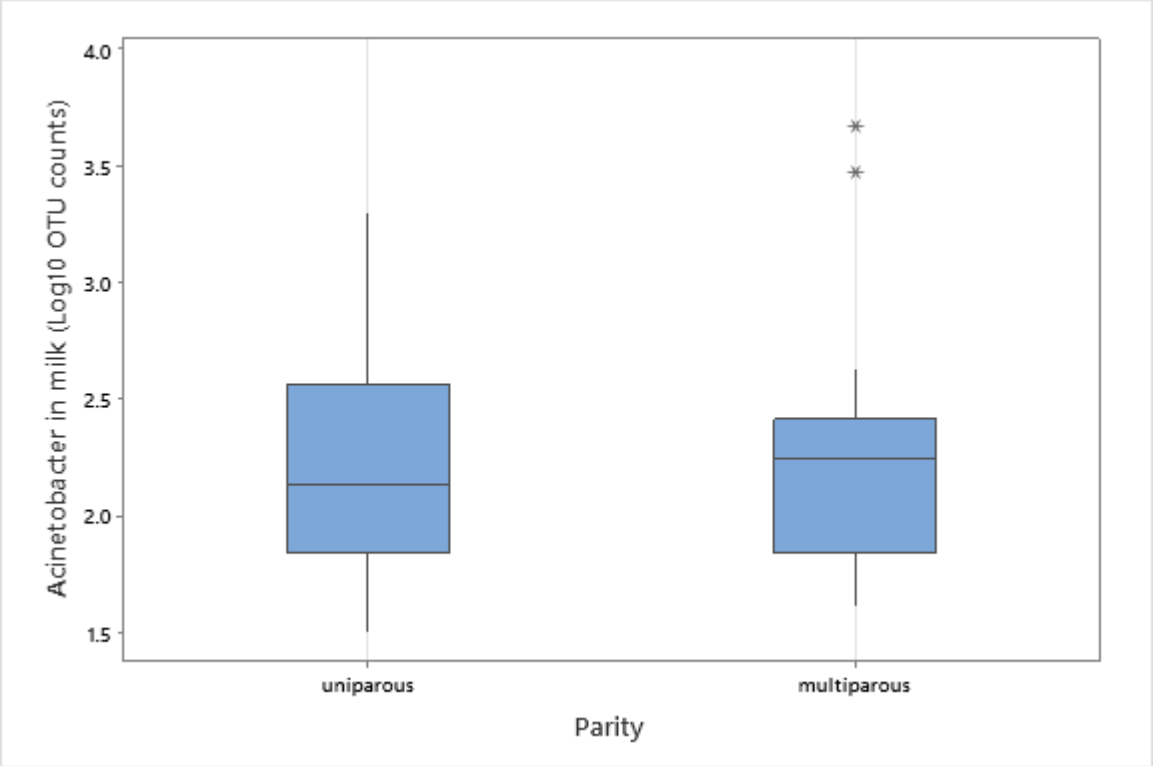


Figure 11: Association between parity and *Acinetobacter* in milk. On y-axis is the number of reads or OTU counts belonging to *Acinetobacter* in milk of mothers formatted as a log scale while the x-axis shows parity. Mothers who reported having more than one child (multiparous) had higher level of *Acinetobacter* in their milk compared to mothers who reported having a child. However, according to Mann-Whitney U test and BH corrected p value, there is no statistically significant difference ($p=0.026$, $q=0.08$). $q<0.05$ was statistically significant.

APPENDIX 9

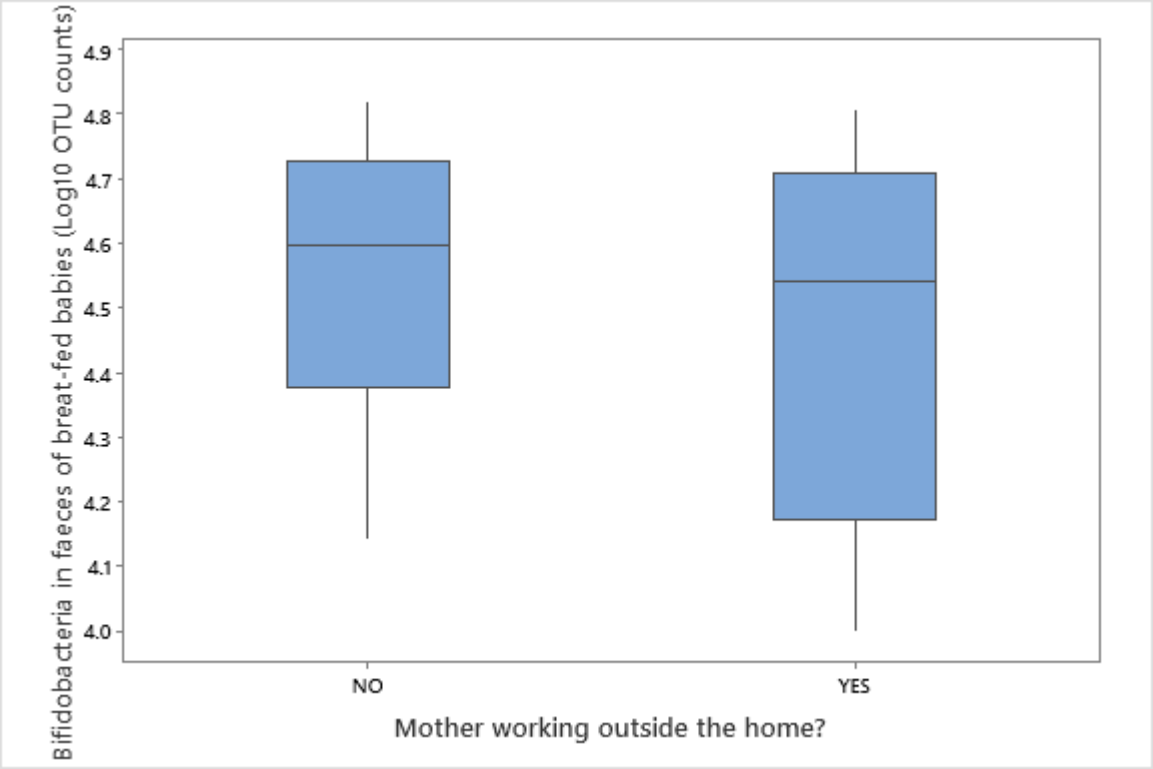


Figure 1: Association between mother's working mode (working outside of home (YES) or working inside of home (NO)) and abundance of *bifidobacteria* in baby faeces. On y-axis is the number of reads or OTU counts belonging to bifidobacteria in faeces of breastfed babies while the x-axis shows the working mode of mothers. Breastfed babies whose mothers work within their homes (NO) had higher level of bifidobacteria in their gut compared to babies whose mothers work outside the home (YES). However, according to Mann-Whitney U test and BH corrected p value, there is no statistically significant difference (P-value =0.043; q=0.43). q<0.05 was statistically significant.

APPENDIX 10

Version: 1

Date: 12.8.14

MIDDLESEX UNIVERSITY

SCHOOL OF HEALTH AND SOCIAL SCIENCES

NATURAL SCIENCES ETHICS SUB-COMMITTEE (NSESC)

PARTICIPANT INFORMATION SHEET FOR BREASTFED BABIES AND MOTHERS

1. Study title

Investigating the bacterial diversity in breast milk and its relationship with gut microbiota of breast fed babies in Nigeria

2. Invitation paragraph

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this

1. What is the purpose of the study?

Human breast milk has traditionally been considered sterile. However recent studies suggest that human milk carries bacteria that help babies to build up a beneficial population of bacteria in their gut which protects them from disease causing bacteria. Moreover exposure to this group of microorganisms boosts maturation of immune system and reduces the likelihood of disorders such as allergy, diabetes, and obesity in adulthood. The aim of this project is:

1. To investigate the presence and diversity of bacteria in human milk of Nigerian mothers.
2. To investigate the relationship between breast milk microbiota and gut microbiota of breast fed babies in Nigeria
3. To investigate the relationship between the gut microbiota of breast fed babies and babies who are fed with formula (bottle-fed).

4. Why have I been invited?

You have been chosen because you are

- Healthy
- Breast feeding
- Not receiving antimicrobials since two weeks before commencement of the study
- Not drinking alcohol

And your baby is:

- Healthy
- Breastfed
- Not on any medication

5. Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive (include this section only if applicable.

6. What will happen to me if I take part?

Nothing will happen to you, we will provide you with clean containers, you will give us a sample of your breast milk in three occasions and also a sample of your baby's faeces taken from their nappies in three different occasions and we will process the samples.

7. What do I have to do?

As above

10. What are the possible disadvantages and risks of taking part?

There are no risks at all.

11. What are the possible benefits of taking part?

Results will create a better understanding of the presence of bacteria in human milk and faeces of babies in Nigeria

12. Will my taking part in this study be kept confidential?

No information concerning participants will be disclosed

13. What will happen to the results of the research study?

Results of the research will be published as a scientific paper.

14. Who has reviewed the study?

The Middlesex University, School of Science and Technology, Natural Sciences Ethics sub-Committee has reviewed the project.

15. Contact for further information

Adebusayo Hassan

Middlesex University, London

Osun State University, Osogbo

Busayohassan18@gmail.com

M: +2348162504908

M: +447857413503

Thank you for taking part in this study.

Adebusayo Hassan

Version: 1

Date: 12.8.14

MIDDLESEX UNIVERSITY

SCHOOL OF HEALTH AND SOCIAL SCIENCES

NATURAL SCIENCES ETHICS SUB-COMMITTEE (NSESC)

PARTICIPANT INFORMATION SHEET FOR BOTTLE-FED BABIES

1. Study title

Investigating the bacterial diversity in breast milk and its relationship with gut microbiota of breast fed babies in Nigeria

2. Invitation paragraph

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this

2. What is the purpose of the study?

Human breast milk has traditionally been considered sterile. However recent studies suggest that human milk carries bacteria that help babies to build up a beneficial population of bacteria in their gut which protects them from disease causing bacteria. Moreover exposure to this group of microorganisms boosts maturation of immune system and reduces the likelihood of disorders such as allergy, diabetes, and obesity in adulthood. The aim of this project is:

1. To investigate the presence and diversity of bacteria in human milk of Nigerian mothers.

2. To investigate the relationship between breast milk microbiota and gut microbiota of breast fed babies in Nigeria

3. To investigate the relationship between the gut microbiota of breast fed babies and babies who are fed with formula (bottle-fed).

4. Why have I been invited?

You have been selected because your baby is:

- Healthy
- Bottle-fed
- Not on any medication

5. Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive (include this section only if applicable.

6. What will happen to me if I take part?

Nothing will happen to you, we will provide you with clean containers, you will give us your babies faeces taken from their nappies in three different occasions and we will process the samples.

7. What do I have to do?

As above

10. What are the possible disadvantages and risks of taking part?

There is no risk at all.

11. What are the possible benefits of taking part?

Results will add to the knowledge that is currently available.

12. Will my taking part in this study be kept confidential?

No information concerning participants will be disclosed

13. What will happen to the results of the research study?

Results of the research will be published as a scientific paper.

14. Who has reviewed the study?

The Middlesex University, School of Science and Technology, Natural Sciences Ethics sub-Committee has reviewed the project.

15. Contact for further information

Adebusayo Hassan
Middlesex University, London
Osun State University, Osogbo
Busayohassan18@gmail.com
M: +2348162504908
M: +447857413503

Thank you for taking part in this study.

Adebusayo Hassan