Influence of Gut Microbiota on Immune System in infants.

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Declaration

I, *Paidamoyo Kachambwa,* hereby declare that the work on which this dissertation is based on my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Preface

This dissertation is submitted for the degree of Master of Science in Medicine (MSc Med) in Bioinformatics at the Division of Computational Biology in the Department of Integrative Biomedical Sciences (IBMS), The Faculty of Health Sciences, University of Cape Town, South Africa. The study was approved by the Human Research Ethics Committee (HREC) of the University of Cape Town, South Africa (604/2015) and supported by the National Research Fund (Bioinformatics and Functional Genomics Grant). The work reported in this dissertation resulted from data collected and owned by Dr. Heather Jaspan for the Project titled: Early Introduction of Non-Breast milk Foods Activates HIV Target Cells in South African Infants. The data was generated from; collected baby stool samples (microbial compositions) and collected blood samples (gene expression levels).

The work is split into 4 chapters were the first looks to show and discuss literature based on the influence of gut microbiota on the immune system in infants. The second chapter looks at the methods that went into the research and computation that went into looking at the influence of gut microbiota on the immune system in infants. The third chapter is data analysis and results. The last chapter focuses on the conclusion and future work. The study looks at the correlation of microbial profiles and gene expression data for infants in their first 14 weeks. The data collected 0, 6 and 14 weeks was; microbial compositions, gene expression levels and feeding modality. The submitted material is the work of the MSc candidate, unless stated otherwise by acknowledgments.

Contents

Glossary

Commensal:

With respect to the gut, these are microbiota that live in the gut but do not harm the host. These organisms benefit from inhabiting the host and in some instances may provide beneficial functions or beneficial bi-products that come from the organism's functions. This is known as mutualistic behaviour with the human host [1].

Cytokines:

These are small proteins that are secreted and released by the host's immune cells and are involved in cell signalling. They have a specific effect (in order to illicit an immune response) on communications, exchanges and interactions that can occur between cells [2] and can be pro or anti-inflammatory. "Cytokine is a general name; other names include: lymphokine (produced by lymphocytes), monokine (produced by monocytes), chemokine (have chemotactic activities) and interleukin (produced by but act on other leukocytes). They can act on the cells that secrete them (autocrine action), on nearby cells (paracrine action) or on distant cells (endocrine action)" [2].

Gut microbiota homeostasis:

This is where a system (microbiota) maintains a relatively stable state of equilibrium which is balance of its internal intertwined and interdependent factors through constant regulatory mechanisms. With respect to the gut, this is constantly maintaining a balanced environment between the microbes present, products from their various processes, the host's immune system and the epithelial tissue that lines the intestine. The control factors to take into account are the uptake of nutrients by the host and microbes, host's immune responses, cell growth of the different microbial communities, quorum sensing of the microbial communities present and

the regeneration of the epithelial tissue. This homeostatic state is important to maintain as some essential processes such as some metabolic and immune processes can only take place in a homeostatic environment. Dysbiosis is the opposite of homeostasis and is the state when one or more of the control factors are disrupted which can result in a change in the composition microbial groups [3] .

Microbiome:

This is a compilation of different microbial groups, their genomes and functions. This population is present in a particular environmental habitat or area. For example, the various human body parts have their own microbiomes, such as, the mouth cavity microbiome is different to the skin or gut one, but they all make up one microbiome, the human one [4], [5].

Microbiota:

This is a compilation of various organisms which form a population and are living in an environment, in our case, the gut. These organisms range from bacteria to eukaryotes [5].

Operational taxonomic unit (OTU):

In biological classification, these are a group of organisms that have their 16S rRNA gene sequences clustered together as they have closely related sequences [1]. These clusters roughly represent the grouping of taxa at phylogenetic levels. A user can define sequence similarity cut-off to a preferred percentage depending on the quality of their dataset [6]. The general cut-off is 95 % similarity and the sequences are assigned to the same genus and those with 97% similarity to the assigned to the same species [6], [7].

Pathogen:

This is any agent that can cause disease. In the gut, microbes are usually chief culprits and achieve disease state by evading the host's immune system via interactions such as the host-pathogen interactome [8].

Relative abundance:

This is a component of biodiversity and measures of the total number of organisms, sequences or OTUs present in a community or a sample, in relation to all the others that are present in the same community or sample [9].

Abstract

Background and Methods: Microbiota play many significant, direct or indirect, beneficial and detrimental roles in humans. Microbiome development is established at infancy where diet plays a directive role in the proliferation of gut microbes. It has been shown that the presence of a defined set of microbes has been known to increase the overall immunological capacity, which vaccines depend on to be effective. To date, little work has been done on the effect of the microbiota on immune system at infancy, thus an analysis of the microbial ecology present in the infant's gut and its correlation with immune activation is needed. Expression of genes involved in mediating and regulating immunity can be measured as an indicator of immune activity. Vaccines work by stimulating an immune response which can be measured by gene expression levels. This affects the infant's ability to establish a strong immune system, which is also dictated at infancy. 16s rRNA sequence data generated from 134 infant stool samples, at vaccination points 0, 6 and 14 weeks from infants that were either breast or formula fed, was analysed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline to detect different taxonomic groups that make up a particular microbiome. Statistical analysis in R was used to quantify the diversity of the different microbial groups in the gut. Expression levels of immune-related genes were measured from blood samples that were stimulated by a Bacillus Calmette–Guérin (BCG) antigen and correlated with microbiota compositions.

Results and Conclusion: Microbiome data showed initial differentiation between breast and mixed fed infants.15% of 5 of the most abundant bacteria for breast fed infants were Bifidobacteriales, which are known for their probiotic properties. The data did not fully cluster as the oldest samples were taken quite early at 14 weeks. Individual bacteria were correlated with individual gene expression level data. The study shows the relative abundance of particular bacteria, comparing against feeding modality and demonstrated how the microbiota correlates with gene expression levels. At week 14, Bifidobacterium of abundance below 0 (heatmap $\log_{10}$ scale) generally correlated with high CASP3 gene expression levels in breast

fed babies while abundances above 1 correlated with low gene expression levels. Gene expression at abnormal levels usually has undesirable effects which result in dysfunctional immune reactions that lead to conditions ranging from autoimmune diseases to cancer.

Chapter 1: Literature Review - Influence of Gut Microbiota on the Immune System in Infants

1.1 Infant Microbiome

The advancements in ease, accuracy and speed of DNA Sequencing has unlocked a wealth of opportunities. In particular, the ability to identify different species present in samples. These species can not only be identified but quantified. Downstream, we have the ability to note the functional properties of the different species present and compute statistical analysis based on the quantities present. Here we take a closer look at microbial communities in the human host's gut. The gut microbiota [10] is host to the largest number and most diverse community of organisms [11]. The gut microbiota is also quite interesting, with respect to diversity, as compositions differ from host to host regardless of their health status. Much research has gone into looking at possible commonalities, for example, of a healthy gut.

The microbiota is known to have beneficial and detrimental effects [12] on humans where these effects may be directly or indirectly linked to a group or groups of bacterial species. An appreciation of this will afford us great insights into health solutions [13] especially when looking at personalised health solutions (drug wise and nutritional) [12]. Development of the microbiome is dictated at infancy [14] and is important to establish healthy growth moving forward. Various studies have looked to understanding the change in diversity and composition over time in an effort to gain insight on when and how a particular host's microbial profile is set. The microbiome at infancy influences healthy growth, metabolic functionality [14], maturation of the gut, brain and immune development [15]. Recent studies have shed a lot of light on understanding the changes in the microbiome, in particular, when looking at the first

year of life [16], [17]. The microbial composition varies between individuals and the environment that the microbiome interacts with is quite dynamic [18].

There are long term effects associated with the initial colonization of the gut. The gut microbiome is developed via colonisation of the gut by bacteria and this is influenced by a number of factors which include the type of diet (breast fed or mixed fed is breast milk and/or other foods) and the environment (e.g. urban vs. rural settings) the infant is exposed to [19]–[21]. With respect to 'other foods' in mixed fed, at infancy the alternative to breast is formula milk. There are other factors that are suggested to be at play in the development of the microbiome that occurs before, during or just after birth [22]–[25]. It was noted that their roles are probably as significant as those played by factors after birth such as diet. The suggested factors include maternal factors such as the type of diet the mother is on and the different antibiotics in use [26], [27], fetus related factors such as gestational age, and lastly, birth related factors like mode of delivery (vaginal vs. caesarean birth) [21], [28]–[30].

An interesting factor mentioned above is gestational age, which has a strong influence on the maturity of the infant gut microbiome. When comparing the fecal microbiota of a preterm infant vs. a full term one, there is a difference in pathogenic bacteria, which are more abundant in the preterm infants [31]. A suggested explanation may be that, the mother's own microbiome changes in diversity and composition over time. This may be to prepare an appropriate microbiome composition that is fit to be transferred and colonize the infant. Preterm infants, may be born before the maternal microbiome has reached this stage. A limited number of studies have looked at fecal samples as a true representation of the true diversity and composition in the gut microbiome. It has been seen that there is variability in terms of abundance and composition when comparing the small intestine and the colon but fecal samples still are a great source to take a snapshot of the gut [5], [32].

Another interesting factor that has also been found to have a strong influence is the environment [33]. In particular, when looking at the familial environment; the presence of siblings is a huge contributing factor to the development of the microbiota. The familial environment has attributes such as their geographical location and/or their culture. A rural location and culture will vary to a crowded unsanitary setting; it will also differ with a more affluent urban setting. It is also important to note that cleaner is not always best, as has been found in microbial profiles in developing countries. Here the familial environment in developing countries is also attached to a certain diet and culture. The environment may also dictate which disease is prevalent with the household members and of these diseases there is a subset that may be transferred to the infant. This is seen generally as certain diseases are not prevalent everywhere [34], [35].

The health status of an infant affects the development of the microbiota, which in turn influences the development of a robust immune system [36], [37]. Table 1 shows the main pathologies in neonates. Some of the causative agents are household members as discussed above. Treatment of these pathologies also has an effect on the infant's gut microbiota. Table 1 shows bacterial groups that are pathogenic and how they can cause disease at such an early stage in infancy. Furthermore, the treatment duration can be as long as 28 days. Both treatment and the effect of the disease weigh heavily on how the gut is colonized, how it develops.

Table 1: Matamoros *et al.* (2013) describes " the main pathologies and antibiotic treatment in neonates" in the table [3].

Pathologies	Age (days)	Pathogens	Antibiotic treatment	Treatment duration (days)
Peripartum prophylaxis	Prenatal	Group B streptococcus	Penicillin G, or, if penicillin allergy, cefazolin, clindamycin, clindamycin, or vancomycin	Every 4 h until delivery
Early onset perinatal infection	Before day 8	Group B streptococcus *Escherichia coli, Listeria monocytogenes*	Ampicillin and gentamicin*	8–10 days (2 days of aminoglycoside) monocytogenes
Late-onset perinatal infection (commonly bacteremia and meningitis, rarely arthritis or osteomyelitis)	Days 8–80	Group B streptococcus	Ampicillin and gentamicin*	1–14 days (2 days of aminoglycoside); 21–28 days in the case of arthritis or osteomyelitis
Nosocomial neonatal infection (commonly catheter-related septicemia or pneumonia, especially in cases of prematurity, intrauterine growth retardation, or congenital malformation)	Days 5–80	*Staphylococcus aureus, Pseudomonas aeruginosa* (rarely)	Vancomycin, amikacin, and ceftazidime	7–10 days
Pneumonia (or surinfected bronchiolitis	Days 5–80	Group B streptococcus, *Streptococcus pneumoniae*	Ampicillin	10 days
Pyelonephritis	Days 7–80	*Escherichia coli*	Ampicillin and gentamicin	10–15 days
*In the case of severity, the probabilistic antibiotic treatment combines a third-generation cephalosporin (cefotaxime) with ampicillin and gentamicin. The antibiotic treatment is secondarily adapted in response to microbiologic documentation.				

There are 2 categories with respect to how infants are affected by treatments where treatment refers to a response to an ailment. The first is when the treatment is taken by the mother before or after birth. After birth usually affects breast fed infants as breast milk becomes a transport channel for the causative treatment. Studies have shown that, mainly in antibiotic treatments, this results in a lower total number of detected microbes [38]. The second category is when the treatment taken by the infant. Lower proportions of bifidobacteria are associated with antibiotherapy in infants [39]. This therapy affects the development of the microbiota and there was found to be a decreased in phylogenetic diversity in the infants gut [40]. This leads to the unfortunate onset of neonatal sepsis which is related to decreased phylogenetic diversity [41]. Other than treatment for ailments, there are beneficial therapies such as supplement with probiotics and prebiotics for infants. Probiotics are live microbes and when taken in correctly calibrated amounts, they give a health benefit to the host [42]–[44]. Popular probiotic examples include different strains of *Lactobacillus and Bifidobacterium*. They are known to be involved in many different processes in the human host [45] some of which include competitive inhibition with other microorganisms, having an effect on the stability of the mucosal barrier and communication and exchange with dendritic cells that present antigens [46]. Whereas, prebiotics are a chosen fermented component, that effect a particular shift in the microbial composition and/or processes that occur of the gut, thus giving a health benefit to the host [47].

Vast literature shows the benefits of probiotics with necrotizing enterocolitis in preterm neonates as it reduces risk of death [48]–[51]. Studies show very few incidents of secondary effects from use, that being said, there is a long way to go in terms of standardising [5] work done to investigate probiotic use in infants and pregnant women [52], [53]. Overall, they have shown positive effects in infants. Some of these effects are protection against infection [54] and diarrhoea [55]. Studies that look at growth- and age- discriminatory bacterial species can assist in the probiotic design as they are linked to finding a “health-status” that is associated

with a particular microbiome [56]. Extensively tested probiotic supplements have been found to be beneficial in improving specific conditions in infants [57]. Studies have also shown that probiotic use during pregnancy is beneficial to women who may have disrupted vaginal microbiota as it helps maintain the vagina free of pathogenic microorganisms like *Candida albicans* [58]. This is quite important as maternal bacteria are some of the first colonizers of the infant gut. In randomized trials, the supplementation was found to be beneficial and can alter the infant's microbiome during and post pregnancy for vaginally delivered infants [59]–[62].

There is a long lasting effect that the initial microbial colonisation has on an individual. The resulting metabolic and immune programming stems from this colonisation. To support this notion of programming, studies have suggested that the recovery of the microbiome after disruption but not the phenotype points towards programming [14], [63], [64].This programming then has an extensive influence on that individual's risk of getting a particular infection or disease [65]. A study analysing bacterial fatty acid profiles from faecal samples of infants 3 weeks after birth, has shown that these profiles differ significantly between infants developing atopy (hereditary predisposition to developing allergic diseases [66]) and those that are not [67].

Another study correlated *Bifidobacterium* numbers in infancy at the ages of 6 and 12 months to the same infants' obesity and weight at the age of 7 years. It was found that *Bifidobacterium* was in lower numbers for those suffering from weight issues [68]. The studies show the predictive power of studying the microbiome with a large sum of the studies showing the predictive power with respect to enteric diseases [69]. The ability to predict "healthy" microbial compositions which take into account the different factors that influence the composition of the microbiome takes us a step closer to developing strategies that result in personalised

solutions. One of the key components of the strategies would be to look at microbial profiles of individuals first.

As noted before, there are many studies [17], [40], [70]–[74] that focus just on the internal and external maternal factors that contribute to the initial bacterial colonisation of a new born [74]. Bacteria that is later detected in the infant, has also been detected in the fetal membranes [75], [76], amniotic fluid [76]–[78] and umbilical cord blood [22]. These listed factors are internal factors. To support the presence of these using an example of the amniotic fluid, the infant's initial bowel release after birth, shows the presence of ingested amniotic fluid that contains a complex composition of microbes. This is important, as previously, it was assumed to be sterile [25], [74]. Some of the processes for bacterial colonisation are not yet well understood but there is a lot of progress towards better understanding the mechanisms. Figure 1 illustrates factors that are important to take into account during and after birth and the bacteria that the different factors are associated with.

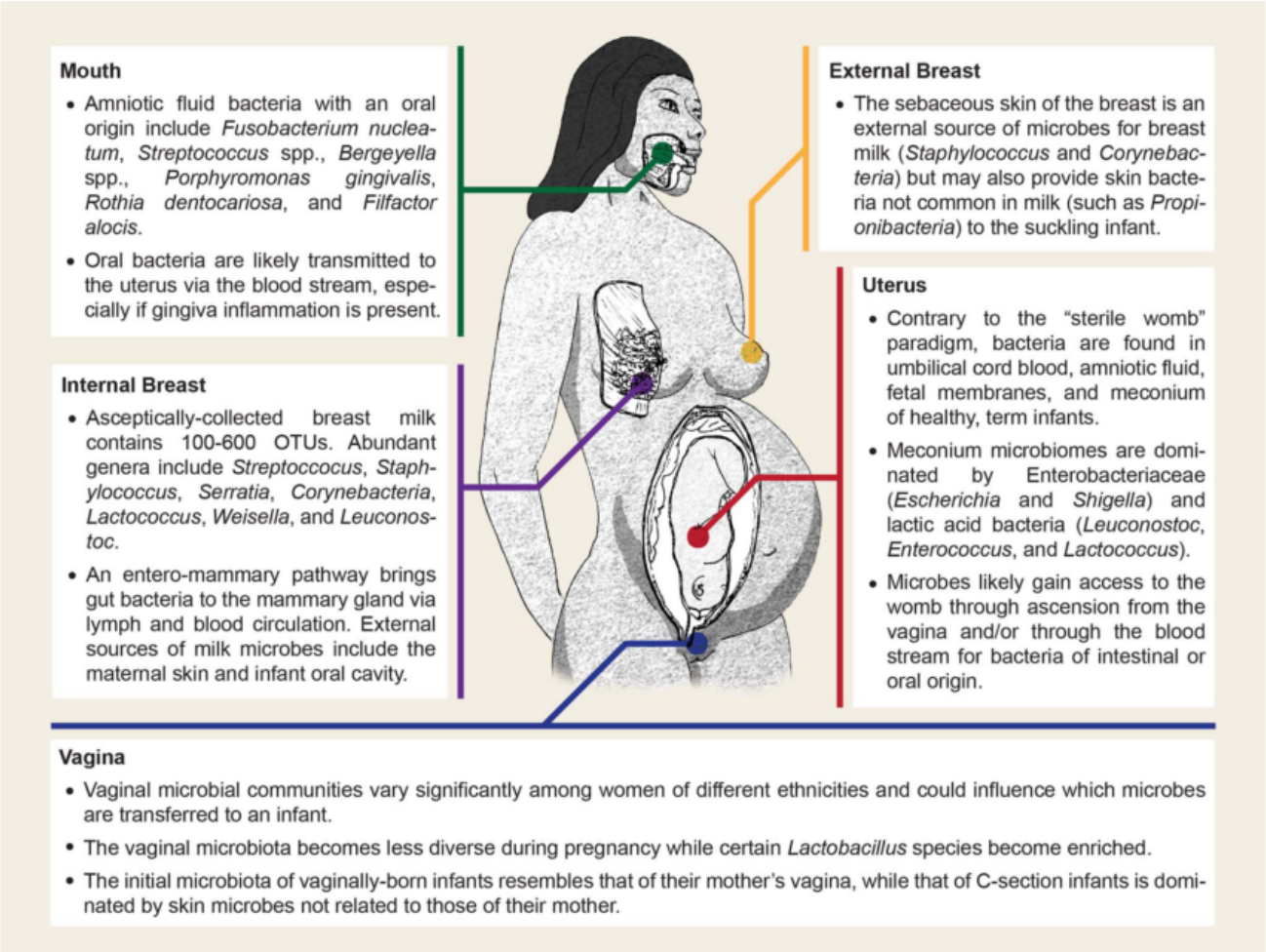

FIGURE 1: Funkhouser *et al.* (2013) shows sources of microbial transmission in humans from mother to child in the figure above [70].

The diagram shows the different sources that the mothers have with respect to transmitting microbes to the infant, the different microbes that are transmitted and the sources that they are transmitted from, such as, the oral cavity [77], [79], the mammary glands which are situated at the breast [80]–[82], the mother's vaginal tract [83]–[85], sebaceous skin that surrounds the breast [81], [86] , and the intrauterine environment [22], [25], [74]–[77], [80], [87], [88]. The illustration was produced by Robert Bruckern [70].

Previous studies have shown that infants born via vaginal delivery have strains that originate from the mother's vagina and gut [89], [90] while those born via caesarean delivery have strains originating from environments that the new born comes into contact with which include the delivering hospital staff, the maternal skin and other neonates[91]. This is also noted in the figure 1. It is interesting to note that these different patterns in microbial compositions are able to persist until the age of 1 [92], [93]. Researchers have hypothesized that this difference may be indicative of the increased risk of having asthma [94] , obesity [95] and lower levels of circulating Th1-associated chemokines [93] for the children whose mode of birth was via caesarean section.

Gut bacterial composition is very dynamic in the first weeks after birth with nutrition playing a major role in its development [92]. Bifidobacteria and *Lactobacilli* are typically dominant in breast fed infants while mixed fed infants have a more diverse composition of bacteria and an increased abundance of *Bacteroides*, Clostridium and Escherichia coli [92], [96]. An increase in bacteriodes is also seen as the diet of an infant moves into solid foods, it is presumed that the presence of bacteriodes may point to their role in nutrient utilization [40]. Previously, the role of microbes in a host was said to be pathogenicity but now there is an appreciation of the beneficial role that particular microbes play [4]. These are starting points where bacterial compositions and diversity are dictated, introduction of solid foods will continue the diversification. [40], [97]. While it is still unclear when the exact age where the adult-like composition is fully established, it starts showing at a young age of 2-3 years where the microbiome increases in richness and diversity [98], [99]. It has recently been shown that some phyla, for example, bacteriodes may take up to 5 years to stabilize but initial directive and colonization is still dictated at infancy [100].

As noted above, the microbiome plays different roles to influence the immune system at infancy. For example, an immature gut microbiome has been seen in children with severe acute malnutrition. It was considered immature when compared to individuals of the same age. This indicated that a delay in the maturation of the gut microbiome had severe physiological implications, which is due to an upset in the interactive role of bacteria with the host immunity and metabolism [101], [102]. It is pertinent to know the effect that different compositions have, their roles in the microbiome, how they affect the other systems they interact with as well as the role that is played in those systems, such as the immune or digestive system in the human host. A notable and important system that is also dictated at infancy is the immune system. In affluent countries, it has been found that, the presence or lack of specific bacteria has been found to link to the increase in the prevalence of allergic diseases [103]. This is because specific species are important to the initially regulating and subsequently developing of the immune system [104]. These specific bacteria are not present due to lifestyle adoptions in these affluent countries, such as access to and continuous use of medication, in particular antibiotics, adopting a particular diet, modernised and cleaner living areas and access to a voluntary C-section.

1.2 Impact of developing gut microbiota on health

Here we take a closer look at the impact the gut microbiota has on the health of an infant and the impact of health on the microbiota. There has not been a consensus about what microbial composition is indicative of a healthy adult but there has been growing directive on what the profile may constitute [105]. Studies have been looking to create models, using data with healthy infants, in order to predict what a healthy microbiome would be composed of. This builds up from the initial establishment that certain bacterial ratios that point towards a healthy system. This has been limited to only predictions within a particular sample space as there

are many factors that affect the composition. Interestingly, they have been able to model and predict, in a given sample space, what a healthy composition will constitute of at early time points of 6 and 18 months in Malawian infants [56], and similar models have been created for the Bangladesh samples [102]. Figure 2 takes a look at some factors that are at play in terms of defining the diversity and abundance in the microbiota. It is interesting to note how the health of an infant is affected by the different shifts in abundances. Moving forward, it may be interesting to look into how normal gut development links to particular age-discriminatory taxa and how this translates to other samples in different geographic areas. Figure 2 also shows how the shifts in abundances differ over time. When comparing the Malawian and Bangladesh samples, there were common age-discriminatory taxa but the bacterial species were ranked differently in terms of prominence [56].

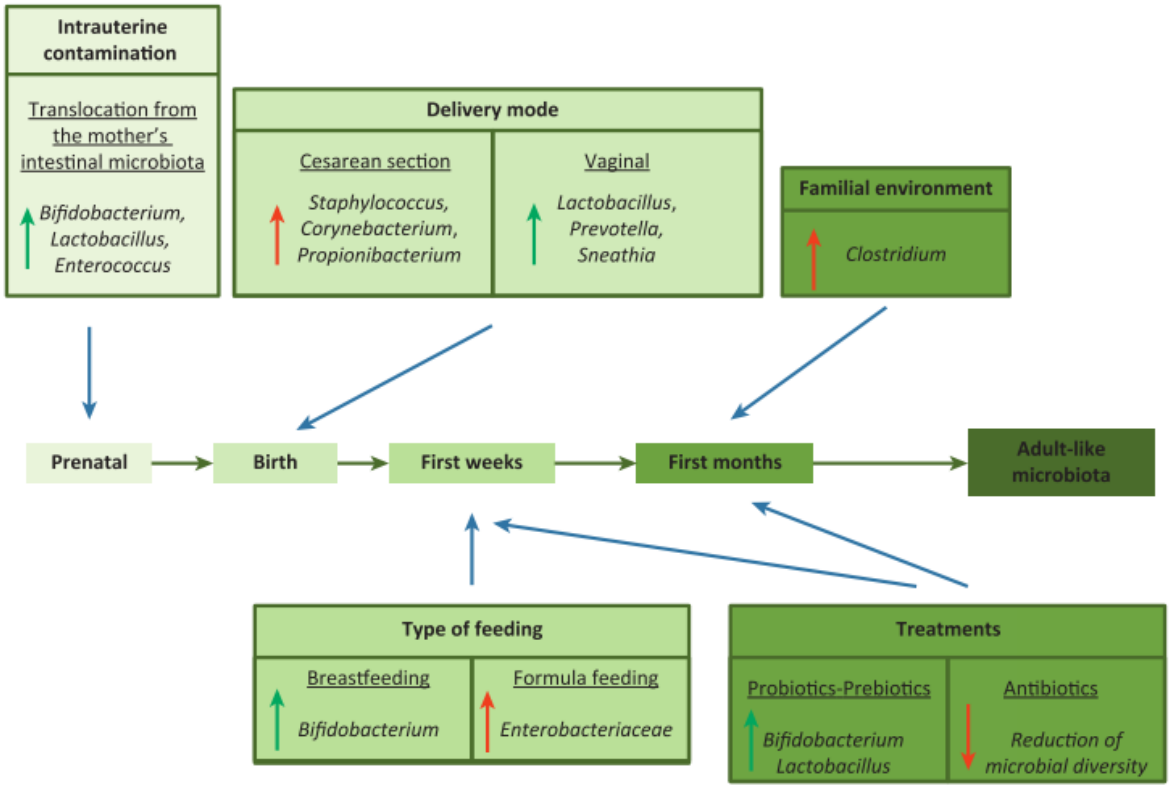

FIGURE 2: Matamoros *et al.* (2013) shows how the infant gut microbiota is affected by external factors and the impact that those factors have on the system. Green upward arrows represent

a beneficial change that is made while the red downward ones represent a change that is considered to have a detrimental effect on the development of a healthy system in infants [3].

When looking at the phyla present in a microbial profile, it is common to over look under represented populations. Of late, there has been more emphasis on looking at their possible vital role in health. Health is achieved through a host of factors; an important one being gut microbiota homeostasis. As mentioned previously, dysbiosis usually occurs when controlled parameters are disrupted. This has a domino effect as it causes a shift in microbial populations, which in turn, is associated with pathological states such as obesity and diarrhoea. The causative agents of the initial disturbance may be immune imbalance, xenobiotics or changes in dietary intake [3]. An example of such phyla, that may be underrepresented, is *Actinobacteria* (*Bifidobacterium*), where *Bifidobacterium* is noted in figure 2 to have an impact on the intestinal microbiota of an infant [106] [107]. One interesting factor that has been studied widely is the Firmicutes/Bacteroidetes ratio. These studies have looked at their variation with respect to diet, in particular, comparing western and rural diets. The rural diet is vegetable-based [97]. Metabolic disorders and obesity were found to be strongly linked to the ratio [108] [109]. Another important one is the ratio between *Prevotella* and *Bacteroides* that also correlates well with microbial profiles but of healthy adults [110], [111]. *Prevotella* was found to be enriched in high fibre and plant based diet and had an association with carbohydrates and simple sugars with children and adults having healthy microbial profiles [112]. In contrast, *Bacteriodes* was associated with a rich, long term animal diet which encompasses saturated fat and several amino acids [97], [111], [113].

It is interesting to note the various roles the microbiome plays with respect to health. Above we have noted external factors that affect the development and overall composition of a microbiome. Taking a closer look at the mode of delivery, it has been shown to have an effect

on preterm infants [26], [114]–[120]. Preterm birth is one of the worldwide [121] leading causes of infant mortality. A strong correlation has been found between preterm deliveries and intrauterine infections, in particular in births that occur at less than 30 weeks [88], [122]. Efforts have been made to identify bacteria that are to blame for unexpected preterm birth. Interestingly enough, largely the bacteria that are found in intrauterine infections, are the same as those that are commonly found in the female vaginal tract [88]. It is important to note that women diagnosed with bacterial vaginosis during pregnancy have a higher risk of preterm birth [123]. Interestingly, the composition of the vaginal microbiota is known to change as pregnancy progresses, it becomes less diverse [85], [124], [125]. These changes are suggested to be adaptive responses as the mother's body prepares a specific inoculum that will be beneficial for the fetus at birth [13]. One way in which this is suggested to take place is the presence of microbes, in the mother, that allow for maximal harvesting of energy. These are then transferred at birth, in particular vaginal birth, and allow for the same energy capacity in the infant [125], [126]. Some of these microbes are known to do so by playing a digestive role in the infant's gut [127].

As noted previously, infants born from a vaginal birth have a different and better adapted microbial composition than those born from a C-section [83]. It is important to note the long term health consequences that are associated with a C-section birth [70]. Of particular concern are immune-mediated diseases that these infants are significantly more likely to develop such as asthma [128], [129] , allergic rhinitis [130], celiac disease [131], [132], inflammatory bowel disease [133] and type 1 diabetes [134]–[136]. This is of great concern as C-section births are on the rise in developed countries [137].

1.3 Infant Immune system

1.3.1 Gut and oral microbiome on immunity

The gut mucosa plays a significant role in immunity as within it, it inhabits a multitude of immune cells. It also maintains a homeostatic environment that allows for efficiency in processes, such as, nutrient uptake. In innate immunity, it is one of the first of defences as it forms a mechanical barrier that works to keep external pathogens out [138]. The following sections will discuss the importance of developing strong innate immunity at infancy. By creating a barrier from the luminal contents, the epithelial wall is one of the key components in the barrier's structure [139]. The oral mucosa is another interesting microbiome that also plays a role in immunity. As noted earlier, the epithelial plays a fundamental role maintaining homeostasis, the oral mucosa has three such epithelial tissues which are regionally variable, for example the palate vs the buccal mucosa [140], [141]. Keratinized areas like the palate form a strong barrier and are much less permeable when compared with counterparts with non-keratinized lining [142]. An interesting protective mechanism that these mucosa use is clearing of topmost cells in order to prevent pathogens from inhabiting the surface [143].

The gut microbiota is suggested to have a link to the oral mucosa as their microbiomes are the most similar of the human host's microbiomes despite them being highly divergent [144]. This is important as the microbiomes at different locations can become predictive of each other [144]. This means, for example, an event/set of events in the gut mucosa may be accompanied by a particular is event/set of events in the oral mucosa. This association is also true for shifts in the microbial communities present in the microbiomes. For example, in individuals with rheumatoid arthritis, the abundance of *lactobacillus salivarius* was found to be strongly correlated with IgG levels (humoral immunity) in both the gut and oral microbiomes. There was

also a positive correlation of the microbe between the two microbiomes [145]. This indicates that the different microbiomes have information on the state of other microbiomes [145]. The oral cavity is connected to the gut via the gastrointestinal tract. This relationship has seen there be manifestations of specific symptoms in the oral microbiome that is indicative of an issue in the gut, for example, in inflammatory bowel disease such as Crohn disease [146]. Growing research suggests that the oral microbiome can be a source of symptomatic information for non-oral ailments [147], [148]. Below is a list of genes whose expression is indicative of a change in the immune system which can be affected by microbial groups in a different locality, such as the gut.

There are 10 genes of interest, due to their significant roles in immunity, which will be discussed. These include cytokines which are involved in immune regulation, chemokine receptors involved in immune activation and genes involved in cell turnover (shedding of top layers on the epithelial wall making way for underlying layers).

IL18 (Interleukin 18):

This gene encodes a proinflammatory cytokine [149] which is powerful with respect to proliferating mechanisms that induce IFN-γ production. It plays a key role, alongside IL12, in enriching NK cell cytotoxicity and the Th1-mediated immune response [150]. The cytokine is produced by macrophages with its precursor being expressed in the endothelial cells and the presence of its precursor is indicative of a healthy host [151].

KRT5 (Keratin 5):

This gene encodes a structural intermediate filament protein that is produced by keratinocytes. The protein is expressed in the epidermis (basal layer) and is associated with the process of cell turnover [152]. In combination with keratin 14, they form strong keratin intermediate filaments which hold up the epidermis [153]. The protein is found in abundance in the epithelial cells but this abundance is dependent on factors such as, the health status in the environment that the proteins are in [154]. This implies it to be a good marker when looking at the health of epithelial cells.

CASP3 (Caspase 3):

The gene encodes the enzyme caspase which is a member of the cysteine-aspartic acid protease family. It exists in an inactive form in all cells and plays a central role in cell apoptosis, in particular in the cascade of cleaving procaspases [155]. Apoptosis usually occurs to maintain a certain number of cells [156] but this may also occur when cells are stressed or damaged due to factors such as disease [155]. In the latter state, Caspase 3 may also be a good marker for health status and immune regulation.

CXCR7 (C-X-C chemokine receptor type 7):

The gene encodes a chemokine receptor. The Chemokine CXCL12 has been shown to bind to the receptor [157]. CXCL12 is a ligand and plays various roles in immune activation ranging from maintaining immune stability, which includes activating proinflammatory signalling [158], [159]. Thus the expression of this receptor should be a good indicator of immune activity.

CCL22 (C-C motif chemokine 22):

The gene encodes a chemokine that is secreted by macrophages and dendritic cells [160] which are key players in the immune system. CCR4 is one of the chemokine receptors that it interacts, this interaction causes an effect on chosen target cells [160]. The secreted proteins, from the interactions, are involved in maintaining the immune system via their role in processes such as immunoregulation, and thus the chemokine has been suggested as a good marker for immune status [161].

IL12A (Interleukin-12 subunit alpha):

This gene encodes a subunit of IL12 which is a cytokine [162]. This cytokine is known to target NK and T cells. In T cells, it is needed in the induction of INF-γ and lastly is needed in the differentiation of th1 and th2 cells [163]. In a review, IL12 was proposed to be a “key modulator of immune function” [164] implying it is a good marker for immune status.

KRT10 (Keratin 10):

The gene encodes a structural protein, like KRT5, and belongs to the cytokeratin family [165]. The cytoskeleton structure of the epithelial cells is made up of these and other products, such as microtubules [165]. Depending on the differentiation stage of the epidermis, keratin chains are formed by different combinations of structural keratin proteins, for example keratin 1 and keratin 10 [166]. This is beneficial in case the cells are at different differentiation stages. The combination of keratin 5 with another keratin protein and/or the combination of keratin 10 with another keratin protein, in order to form structural links, may be able to represent cell turn over as the proteins are part and parcel of cell wall formation.

IL7R (Interleukin-7 receptor):

This gene encodes a cytokine receptor and is bound by the cytokine, IL-7 [167]. This cytokine plays many significant roles in immunity, which also implies it would be a good indicator of immune activity. These roles include the development of B and T cells and maintaining NKT cells [168]. Expression of the receptor has been shown to play an important role in the homeostasis between memory CD4+ T cells and the mature naïve ones [169], [170].

CCL5 (Chemokine (C-C motif) ligand 5):

This gene encodes a chemotactic chemokine which controls how immune cells move [171]. The chemokine is also known as RANTES which stands for "regulated on activation, normal T cell expressed and secreted" [171]. One of the roles that CCL5 plays is in directing leukocytes to inflammatory sites. CCL5 chemokines that are made by immune cells, such as CD8+ T cells, are shown to impede HIV entry to target cells [172]. CCL5 has a myriad of important roles in the immune system and its production during disease state may indicate that it is a good measure for immunity.

CXCR3 (C-X-C motif chemokine receptor 3):

This gene encodes a chemokine receptor, which is a common receptor for a many chemokines, such as CCL4 [173] and is under selective expression by T lymphocytes (activated) [174]. Once CXCR3 is induced during an immune response, it aids in guiding immune cells such as cytotoxic lymphocytes, to the inflammation or infection site (adaptive immunity) [175]. During diseased state, in particular, autoimmune diseases, CXCR3 ligands were found to be highly expressed while expression of the receptor was also found to be high in relation to the T cells present [175]. Increased expression during diseased states may indicate it is a good marker to measure immunity.

1.3.2 Vaccination and the immune system

Vaccination is important at infancy and helps shape the immune system. Immunisation primarily takes place from infancy into early childhood. This is also the same time that the microbiome is being shaped and playing a role in influencing the immune system. In addition, it is hypothesized that the microbiota has played a role in contributing to the diversity of global vaccine efficacy [176], [177].

Studies have found that a compromised microbiome, which may be due to an unsanitary environment, dampened immune responses and efficacy of vaccines when looking at responses such as antibody response [178]–[182]. This could be due to the fact that the microbiome regulates the immune system that the vaccine is targeting to stimulate. Figure 3 summarises the role the microbiome is suggested to play in influencing vaccine efficacy. Vaccination works by stimulating the immune system to respond using dead or weakened antigens. This prepares the host's memory cells for any future possible pathology by an intruder with the same antigen [183].

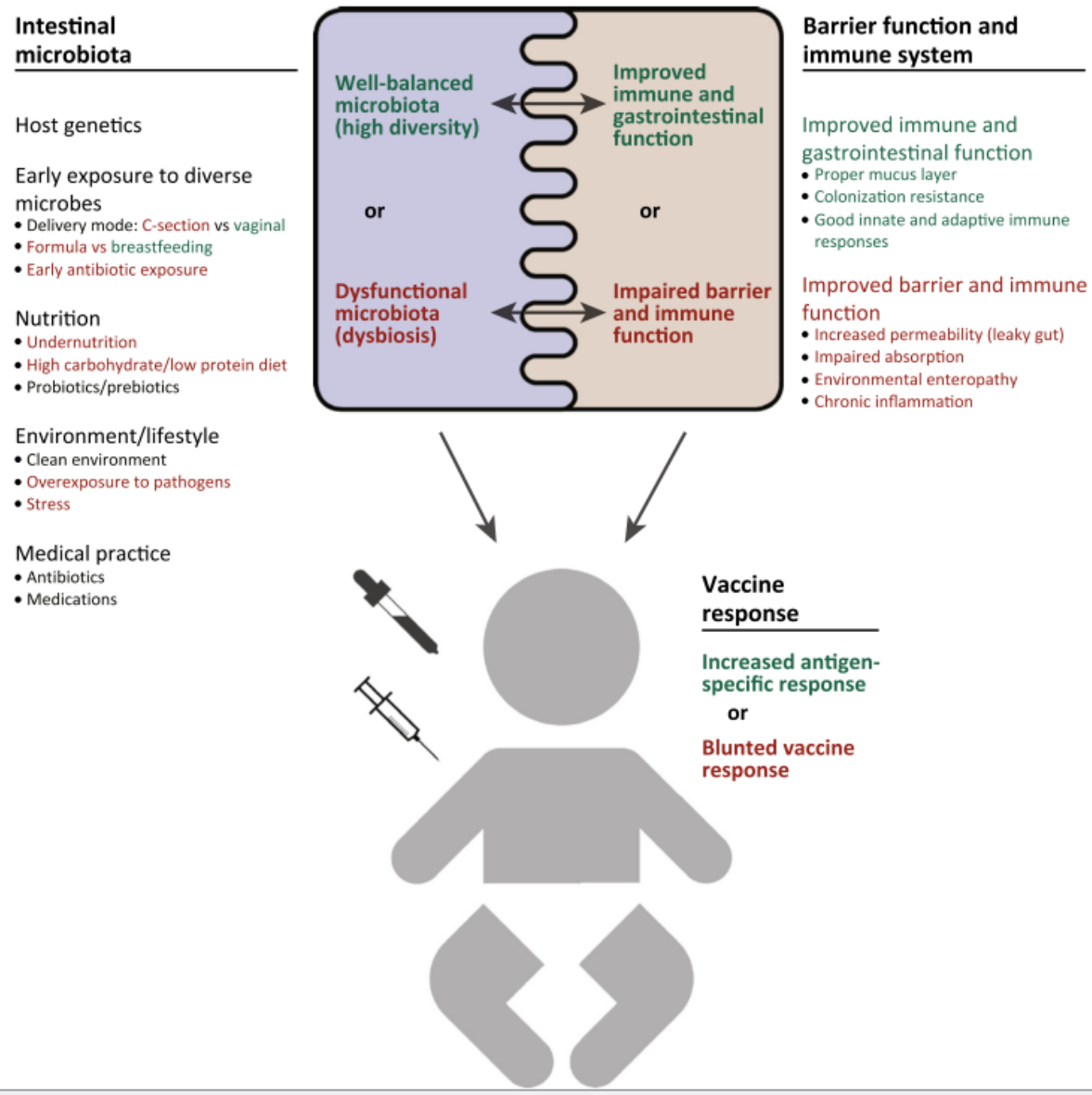

FIGURE 3: Valdez *et al*. (2014) show “a scheme illustrating how the microbiota may influence vaccine responses” [177].

Numerous studies have shown the bidirectional connection between the gut microbiota and the immune system. This connection is also known to play a role in modulating the immune system’s response to vaccines. Vaccines work by eliciting an immune response which is targeted specifically against the antigen that it introduces into the system. The influence of the microbiome on the immune system has an effect on vaccine efficacy, which is dependent on a good immune response [177].

The immune system is divided into: Innate and Adaptive immunity, where the innate comprises of neutrophils, macrophages, dendritic cells, natural killer cells, innate lymphoid cells, defensins and epithelial cells and the adaptive uses B and T cells [184]. One of the important roles of some of these cells is the secretion of cytokines in the immune system, (see figure 4) which can be expressed by most immune cells, but predominantly by helper T cells and Antigen presenting cells, such as macrophages and dendritic cells. An example of the use of cytokines, is when bodies that are foreign to the immune system are present, they are phagocytosized by Macrophages. Thereafter, macrophages use cytokines to stimulate B and T cells responses which are dependent on specific antigens. Other cells are also stimulated to respond but in a manner that is not specific. The engagement of T cells by the macrophages stimulates them to secrete factors that aid in stimulating immune responses towards the specific foreign body present in the system. This cascade effect results in the activation and proliferation of other immune cells.

They are produced as a result of a pathological and/or physiological event occurring in the host. As seen in figure 4, cytokines are pleiotropic, redundant, and can stimulate a cascade effect resulting in the secretion of more cytokines [2]. Cytokine production has been suggested to be good indication of the activity occurring in the immune system [185].

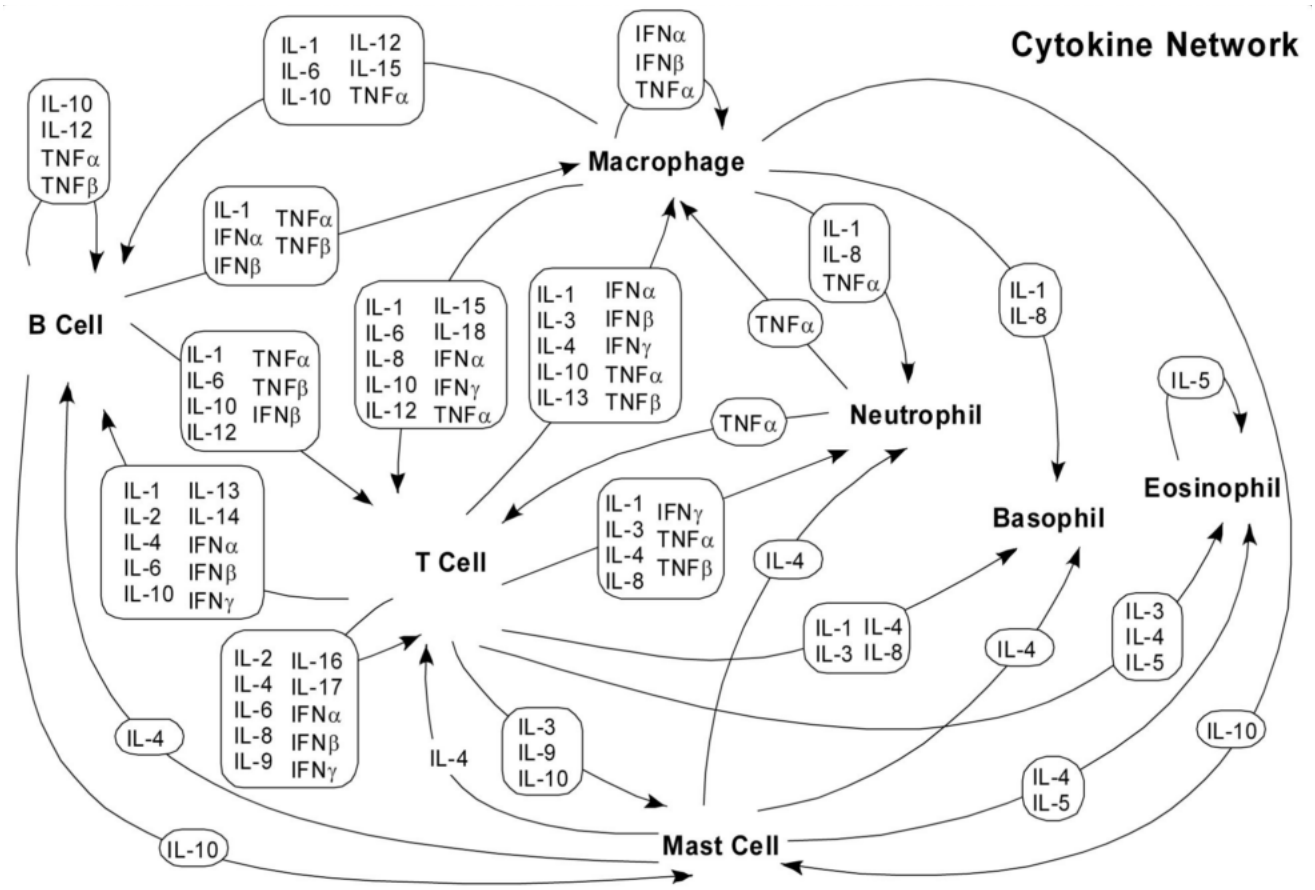

FIGURE 4: Zhang *et al.* (2007) describe the cytokine network in the figure above [2].

There is a myriad of immune cells that work together, and communicate via secreted cytokines, to maintain a homeostatic immune system. There are different types of cells that are involved, such as macrophages, neutrophils and mast cells, and each has a specific role to play, some being more significant than others as roles may overlap [2].

1.3.3 The immune system and its interaction with the microbiome

From the above, we see how complicated the immune system is and the different components that influence and shape it. The microbiome plays an influential role in different ways. It plays a vital role in the maturation of the host's immune responses (adaptive and innate). It is also hosts unique immunoregulatory mechanisms that are present solely to avert unwanted

immune system activation by innocuous antigens. These antigens include those that are expressed by the microbiota [186].

The complexity of the microbiome forms a myriad of interactions. This interaction includes but is not limited to the host system. These interactions span out to affect development of the gut [16], dental health [187], [188], digestive systems [109], [189], resistance to pathogens [190], [191] and the development of immune cells [192]–[194]. It is also important to note that the microbiome possesses a large number of antigens and there is danger of cross-reactivity between the host, commensal bacteria and pathogens, this has a detrimental effect on the host's system [195]. Table 2 shows some of the bacteria involved in immunoregulation and the different roles that they play.

An example of the indirect role the microbiome may play in the immune system is with respect to enteric disease. As noted before, enteric infections are most prevalent during the 2-3 year age-period. Causes for different infections have historically been attributed to the pathogen but studies have shown an interplay between host factors which include; immune response which can be acquired or innate, the commensal gut microbes and age-dependent shift which includes diet or state of the microbiome [196]. An example of age-dependant shift is *Campylobacter* infections which are high in <2 years and again in young adulthood, which are also marked as periods with dramatic shifts in gut microbial population structure [196], [197].

Table 2: Valdez *et al* (2014) describe "the immunoregulatory effects of microbiota in the gut" in the table below [177].

Microbiota species	Immune regulation	Ref
Segmented filamentous bacteria (SFB)	*Alter T cell subsets in the terminal ileum.* SFB induce accumulation of Th17 cells in the terminal ileum, through an unknown mechanism. It is hypothesized that SFB induce the production of SAA in the terminal ileum. This protein could act on lamina propria dendritic cells, which in turn stimulate a Th17 cell inducing environment.	[193]
Bacteroides fragilis (B. fragilis)	*Direct the development of FoxP3+ Tregs in the colon.* Polysaccharide A (PSA) from *B. fragilis* mediates conversion of CD4+ T cells into Foxp3+ Tregs. This effect is mediated through TLR2 signalling in T cells and not in dendritic cells. PSA–TLR2$^{-/-}$ interaction results in increased secretion of IL-10 by Tregs, and markedly reduces the expansion of Th17 cells in the gut.	[192], [198]–[200]
Clostridium cluster IV (C. leptum group) and XIVa (C. coccoides group)	*Promote the expansion of colonic and systemic Tregs.* Clostridia activate IEC to secrete TGFb and other Treg-inducing molecules such as MMP2, MMP9, MMP13 and IDO, thus increasing the number of Tregs in the colon, but not in the small intestine. Tregs are also increased in the spleen, liver and lungs. Clostridia induction of Tregs seems to be independent of PRR because mice deficient for MyD88, Rip2, or Card9 have normal numbers of Tregs in the colon. Clostridia in the gut also seem to affects systemic sites. Low levels of systemic IgE and IL-4, and high levels of IL-10-producing splenocytes, are found in a model of OVA-induced asthma in animals carrying Clostridia.	[201], [202]
Sphingomonas yanoikuyae	*Modulate the phenotype and response of iNKT cells.* iNKT cells from Germ Free mice show significant impairment in antigen-stimulated cytokine responses compared to SPF mice. Oral gavage with S. yanoikuyae could restore the hyporesponsiveness of iNKT from GF mice. This response is independent of TLR and IL-12 stimulation because mice deficient in these receptors have similar phenotypic changes.	[203]
Abbreviations: *Card9, caspase recruitment domain family, member 9, a key inducer of Dectic-1 signaling; IDO, indoleamine 2,3-dioxygenase; IEC, intestinal epithelial cells; iNTK cells, invariant natural killer T cells; MMP, matrix metalloproteinase; Myd88, myeloid differentiation protein 88 a signaling adaptor protein for Toll-like receptors; Rip2, receptor-interacting protein 2 (an adaptor protein for NOD receptors); SAA, serum amyloid A; TGFb, transforming growth factor* [177].		

As an example we take a closer look at host locale for the disease. The epithelium of the intestine is a heterogeneous mixture of cells that are set up to respond to the microbiota which is in close proximity. The mixture is also in close proximity to the luminal contents where luminal refers to the inner open space or cavity of a tubular organ like the intestines [198]. These tight junctions noted above maintain a barrier [199] from enteric pathogen or toxin. The cause of barrier defects can be attributed to factors that include the disruption of a healthy microbiome [200] as the microbiome is shown to contribute to the processes of maintaining function of the barrier [201]–[203]. When the barrier is disrupted and penetrated through by a pathogen or toxin, this may result in the contents of the luminal leaking through to the lamina propria. The lamina propria is where immune cells reside and the leakage can result in detrimental physiological and inflammatory responses. These responses lead to diseases ranging from disrupted absorptive function to diarrhoea [200], [204]. This notes one of the many indirect roles the microbiome plays in affecting the immune system.

The microbiome is also known to have a more direct effect on the immune system. During a malnutrition state in childhood, there are also changes in the structure of the intestines which includes the diversity and richness of the microbiome. These alterations result in detrimental effects, which result in a high negative impact, altered levels of intraepithelial lymphocytes, blunted villi and crypt hypertrophy (which is an enlargement of an organ or a tissue due to a subsequent increase in the size of its cells [205]). These effects can be seen in cases of early childhood malnutrition and enteric disease and have been linked to developmental issues such as impaired cognitive development [206], [207]. The profound cascade effect on cognitive function affects the individual's productivity later and the impact in early life increases the risk of chronic diseases in the future [208], [209]. The alterations in the intestinal structure lead to dysfunctional nutrient absorption which can be seen in figure 5 [196] and impaired immune responses. Ultimately these lead to a decline in growth during the early childhood phase [210], [211]. It is notable that in addition to the important roles of energy and nutrient extraction [212]–

[215], the microbiome also modulates the host's immune system, protecting it against pathogens [216], [217].

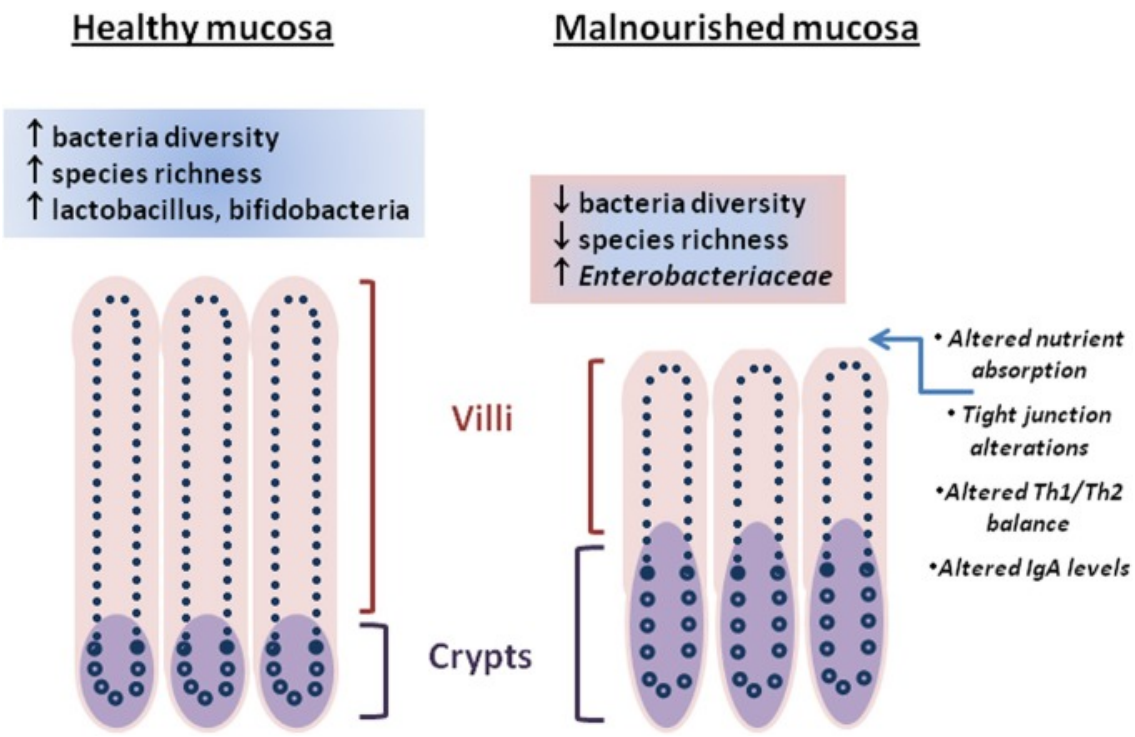

FIGURE 5: Kolling *et al* (2012) show that the molecular, immune and architectural changes in the gut mucosa are indicative of the health state in the gut mucosa. Examples of these changes include, changes in nutrient absorption (molecular), changes in the depth of crypts or height of villi (architectural) and changes in levels of IgA (immunity) [196].

As mentioned earlier in this section, colonization and stabilization of the gut microbiome is intertwined with development of the immune system [218]. It has been proposed that the modulation of the mucosal immune response in infants is carried out by microbiome primarily in the gut, in terms of the immune systems response to allergens in the environment (one of the most common allergens, for example is pollen from grass [219]) [220]. An association has also been made between the differences in the Bifidobacterial populations and the allergic

status and atopic diseases in children [73], [104], [221], [222]. Interestingly, studies have shown that low microbial diversity later on in infancy (5 and 6 months) was not associated with atopic diseases when compared in contrast to earlier on (1 week and 1 month) when it was [103]. These results suggest early infancy to be a critical point for health with respect to the microbiome

Work has been reviewed to take a further look at the intestinal mucosa and the role of the gut microbiota [223]–[225]. It was noted that secondary lymphoid tissues in the gut and intestinal mucosa development was dependant on the pattern-recognition receptors on intestinal epithelial cells' ability to recognise particular microbial components [186]. It is suggested that some of health issues encountered by infants delivered by C-section may be due to the inability of the microbes acquired to interact with the above mentioned receptors [70]. This goes on to hinder immune development in the infant which leads to increased risk of immune-mediated disorders that have been mentioned previously. It is also suggested that this is due to the transfer of fecal and vaginal microbes that has occurred over thousands of years. This may have created specific interactions between the infant's gut and the particular microbes that initially colonise the gut at birth. These interactions then become quite important for gut development. Perhaps with time, interactions between microbes acquired via a C-section birth and the infant's gut will begin to form.

Breast milk bacteria are also suggested to play a role in the maturation of an infant's immune system. In particular, in acquired and natural immune responses, where some bacterial strains are able to modulate these responses [226]–[229]. The functions, with respect to modulation, are variably flexible which hinges on the current state and environment in the gut. In the case where there is lack of an inflammatory stimulus for cells developed in the existence of lipopolysaccharides, the macrophage production of Th1 cytokines is enhanced in the

presence of *Lactobacillus fermentum* CECT 5716 and *Lactobacillus salivarius* CECT 5713. These Th1 cytokines include IL12, IL2 and TNF-α (inflammatory mediator) [226]. A study has confirmed the broad array of effects that these two strains have on the immune system. Some of these include the strong induction of a wide array of anti- and pro- inflammatory chemokines and cytokines, potent activation of NK cells and activators (moderate) of regulatory, CD8+ and CD4+ T cells [230], [231]. A dominant bacterial group in milk, *viridians streptococci*, is seen as a key component in a healthy infant's gut when compared to the gut of infants suffering from atopy [232]. Generally, the bacterial components of human milk have the potential to be involved at a metabolic level in infants which will aid in creating and shaping a healthy microbiome in the infant's gut [233], [234]. Some metabolically active strains from human milk like lactobacilli are known to be quite active in the infant gut. They are known to increase the chief source of energy that is used by colonocytes and compounds that are pertinent in intestinal function maintenance. In the case of colonocytes, they amplify the creation of functional metabolites, as an example butyrate (energy source) [235], [236]. It is also interesting to note that mammals are reliant on microbes to break down undigestible components and butyrate is a product from this process [237]–[241]. Identification of metabolites that are derived from commensals by innate immune cells is quite important in, for example, responding to not only intestinal injury but a host of ailments ranging from allergies to arthritis [242] . Gut bacteria are also noted to be involved in active process of inducing dendritic cell (antigen presenting cells) maturation [243], an important stimulus for developing lymphoid tissue that is associated with the gut and promoting antiallergenic processes [244]. The gut microbiota also plays an indirect role in the immune system. Signals derived from it are responsible for the development of most IFNγ-producing (Th1) and IL-17 (Th17) T cells that are found in the gut [193], [245], [246]. Consequently, changes in the gut microbiota result in alterations in the immune system [247]. For example, a decrease in gut commensals due to the use of antibiotics results impaired B and T cell responses against infection like influenza [248].

Figures 6 and 7 show various ways in which the microbiota influences the immune system to promote and aid in providing immunity. Failure to regulate the response noted below, via disruption of the microbial environment, may result in pathological issues such as allergies or inflammatory bowel diseases [249]–[252].

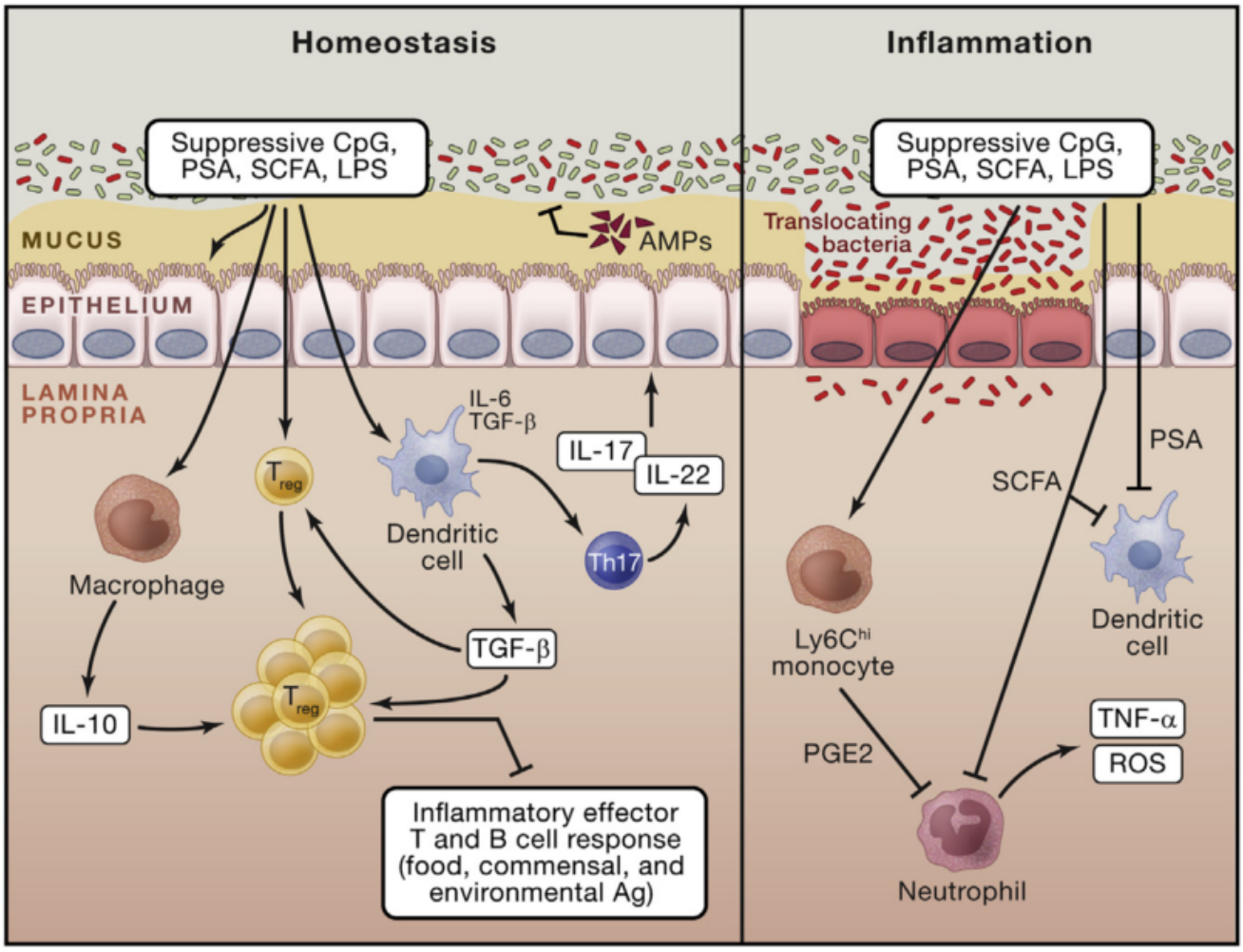

FIGURE 6: Belkaid and Hand (2014) show “the promotion of immune regulation by the Microbiota during Steady State and inflammation” in the figure above [186].

Left: Homeostasis, commensals are shown to promote immune activation by inducing regulatory T cells in a localised manner via the release of metabolites and/or microbial

products that are sensed by T or dendritic cells. Furthermore, commensals are shown to promote Th17 cells induction. The Th17 cells are involved in the regulation and maintenance of a homeostatic environment in the epithelial cells. **Right:** Inflammation, The microbiota uses similar mechanisms in its role in inflammation where metabolites from commensal metabolites affect inflammatory cells either in a systematic or localised manner. As an example, short chain fatty acids (SCFAs) which are produced by microbes in the gut can inhibit the activation of neutrophils. Inflammatory monocytes that enter a tissue can respond to ligands that are derived from microbes, in doing so, the monocytes produce mediators. The mediators produced, such as Prostaglandin E2, are known to limit the activation of neutrophils [186].

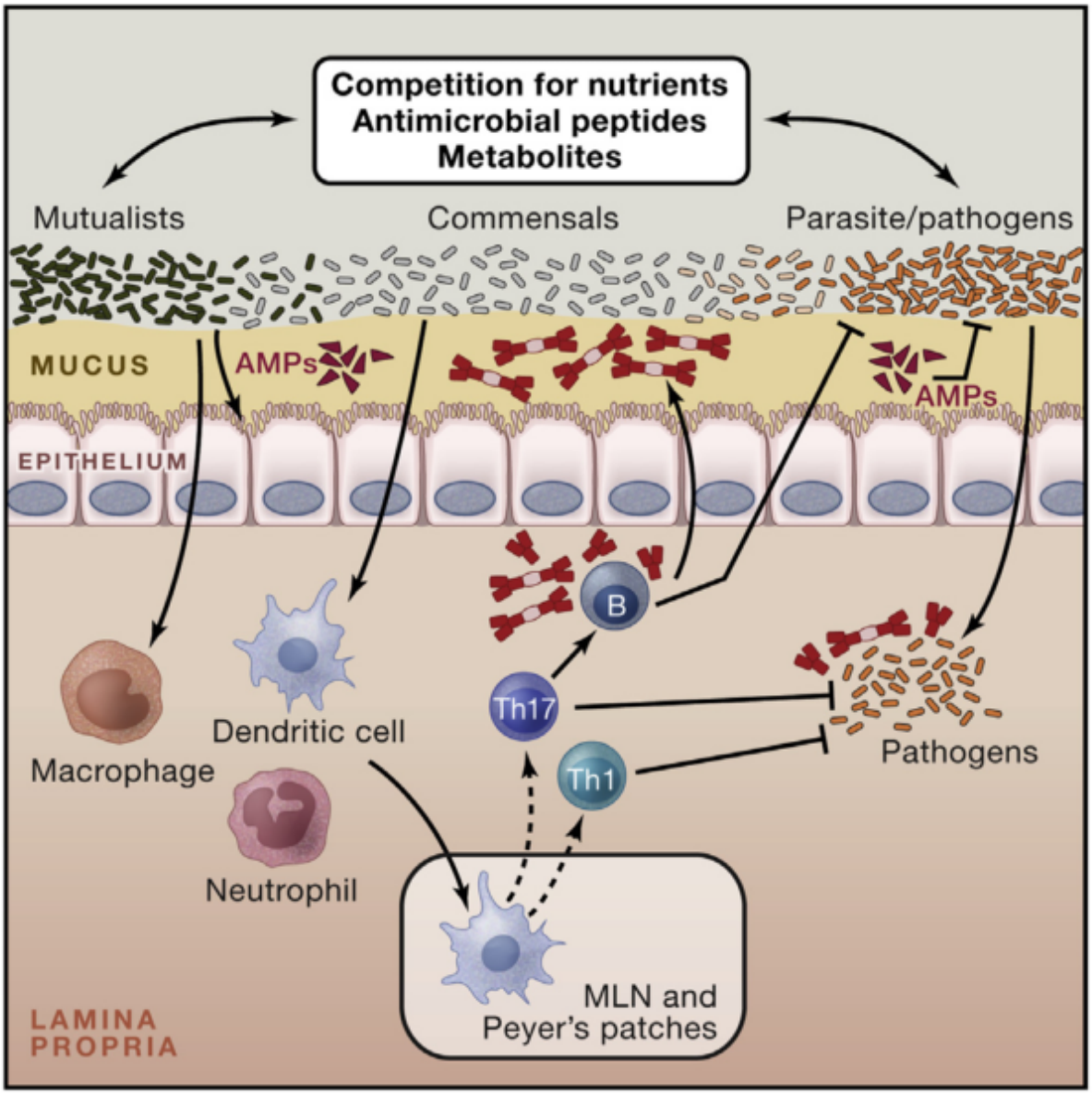

FIGURE 7: Belkaid and Hand (2014) show "the promotion of protective immunity by the microbiota" in the figure above [186].

The microbiota and its host have a symbiotic relationship whose balance is hinged on many factors, some of which include the presence of mutualistic, commensal and parasitic microbes. The status of a microbe with respect to being mutualistic, parasitic or a commensal is dependent on a host of factors as well, such as, the disease state of the host. Disease has been shown to influence microbe's capacity to shift from a commensal to a parasitic state (dependent on co-infection, the state of activation by the host or localisation) and in other

instances the commensals are known to act against potentially pathogenic microbes. In the instances where they act against the potentially pathogenic microbes, they employ various mechanisms, such as, competing for nutrients or releasing metabolites or molecules that are antimicrobial in order to have a negative impact on the survival of the pathogen. Another mechanism, that is indirect, is when commensals promote the release of antimicrobial metabolites by other cells, such as, epithelial cells and reinforcing the tightening of the epithelial junctions. Commensals are also known to modulate the function of immune cells systematically (cascade effect of immune activation) or locally. In an uncontrolled state, these functions of the microbiota can result in autoimmune diseases [186].

1.4 Diet and the microbiota

As mentioned previously, diet assumes a significant role in the development of the immune system. At infancy, there are limited types of feeding possible and can be split into these categories breast fed and mixed fed. Mixed fed refers to the infant consuming anything else in addition to or replacement of breast milk. Long term intake of a particular diet results in changes in the structure of the microbiome [253]. Studies have been done to look at the short term effect and it has been found that there is also a change in structure but the microbiome's composition reverts back after the short term period is over [254]. Predicting the time and reproducibility of the change in the microbiota is still unclear. That being said, work that has been carried out in inbred mice has shown changes in the microbiome (due to a shift in macronutrients in the diet) manifesting in the space of one day [255], [256].

By contrast, work done in human cohorts has shown this time frame change being seen only after weeks[257] and/or even months [109]. In the human cohort studies, there was failure to establish significant effects of specific diets [111]. The studies have managed to show the

changes in limited bacterial taxa as they respond to the change in diet [258], [259]. In addition to the use of probiotics, work has been done to see the effect of diet as an intervention method in altering the gut microbiota. The results showed significant difference in β diversity but no significant difference in α diversity as shown in figure 8. As noted above, after the diet shift period, the subjects' microbial structure reverted back to the original state [253].

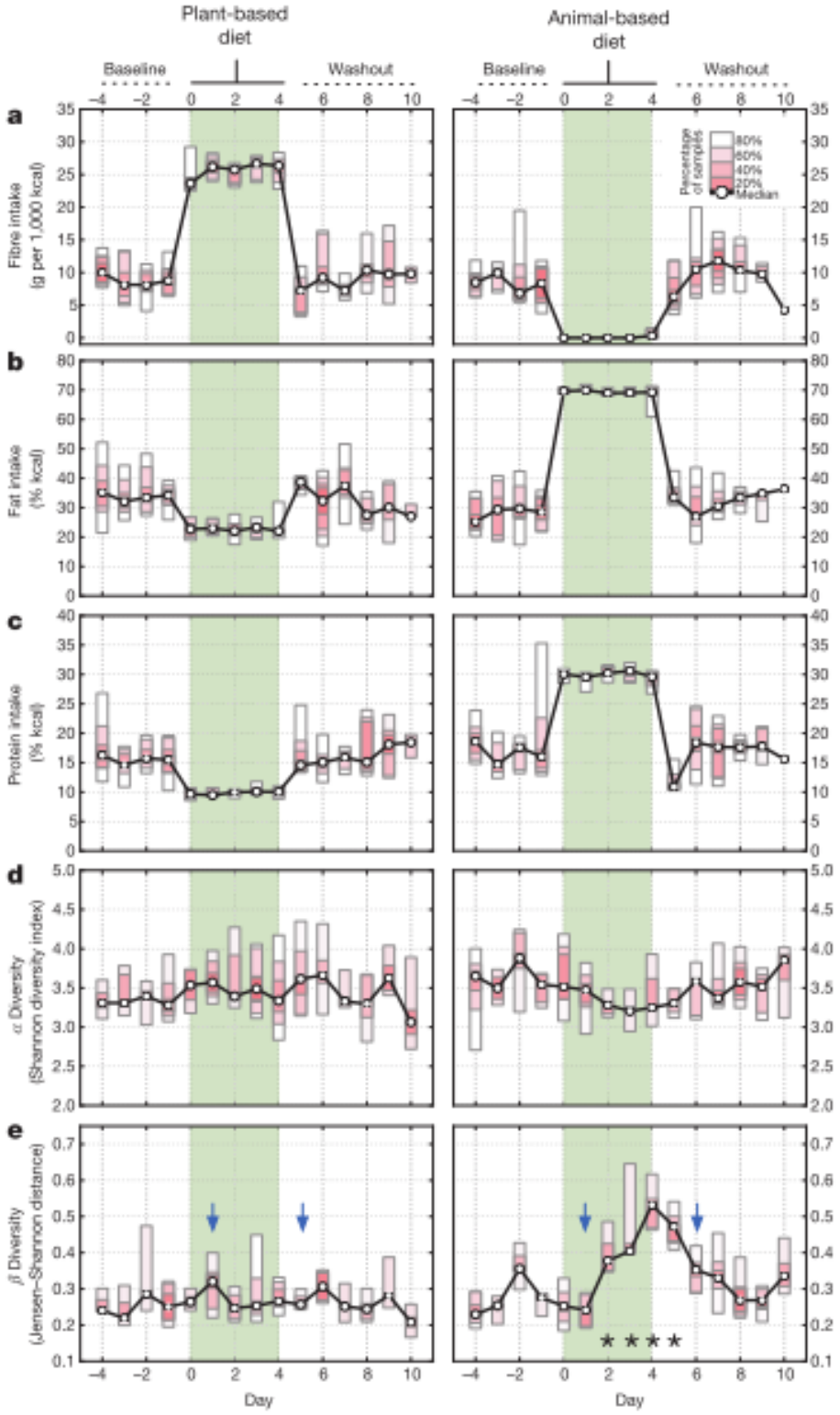

FIGURE 8: David *et al*. 2014 shows "how short-term diet alters the gut microbiota" [253].

a–e shows ten individuals that were investigated through the course of each diet where PB is plant based and AB is the animal based diet in the description that follows:

a) PB diet, fibre intake increased from the median baseline amount whereas the AB diet was negligible. **b)** AB diet, doubling in daily fat intake but a drop in the PB diet. **c)** AB diet, rise in protein intake and a decrease in the PB diet **d)** No significant change in alpha diversity for both diets. **e)** AN diet, decrease in the similarity between gut microbiota of individuals when compared to the baseline communities. The differences in community could be tracked by tracing dye and were seen in 1 day after the dye indicated that the AB diet had reached the gut. The blue arrows present show the days when the first and last meals of either diet were taken [253].

It is important to note that the genus *Prevotella* is one of the top sources of inter-individual gut microbiota variation [110]. This genus is also hypothesized to show sensitivity from the long term intake of fibre [111], [112]. It was observed that there was significant positive correlation, over a year, between the subjects' fibre intake and the baseline levels of gut *Prevotella.* In this study, diet was noted to alter the expression of genes and the activity of the microbes. Secondly, food borne microbes that are specific to a diet are detectable in the gut. Lastly, particular diets e.g. have different bi-products an animal diet produces bile acids [253]. These bi-products may have a downstream effect and this study shows the presence of a secondary bile acid (DCA) that is described to advance the damage of DNA and hepatic carcinomas [260].

Studies have been done to look at the diet of infants who are either exclusively breast fed or are mixed fed. In some cases, formula fed infants are considered in the mixed fed category. It has been found that this feeding type impairs the maturation of the infant's immune system [261] which also alters the metabolism later on in the course of life [262]. Generally, formula feeding is accompanied by an increase in bacterial diversity [38] but with a decrease in the prevalence of beneficial bacteria like Bifidobacteria, and increased prevalence in potentially

problematic ones like *E. coli*, *Bacteroides fragilis* [263], [264] and *C. difficile* [263]. The use of certain probiotics with formula milk has been seen to give a similar effect and benefit to the development of the gut microbiota as that of breast fed infants [265]. Table 3 lists some measures to aid in restoring or developing a mature gut microbiome in infants, this spans not only feeding type but other major influencers like mode of delivery and the presence of antibiotics [266]–[268].

Table 3: Mueller *et al.* (2015) show the "perturbations to the assembly of the neonatal microbiome, prevention strategies, and approaches for microbiome restoration" in the table below [13].

Perturbation to microbiome assembly	Prevention strategies	Restoration approaches
C-section delivery	- Support efforts to increase use of midwives - Champion evidence-based labor management -Optimize managing labor (reduce pain, increase maternal comfort - Educate women about the potential consequences of C-section delivery - Change policies around physician incentives and malpractice insurance	- Inoculation of neonate with maternal vaginal flora immediately following C-section delivery - Breastfeeding - Pre- and probiotic supplementation of neonate
Gestational, perinatal, or postnatal antibiotics	-Implement robust antimicrobial stewardship programs - Develop safe strategies that limit use of antibiotics in women in labor (e.g., rapid PCR testing for group B Streptococcus at the time of of admission to the delivery unit) - During C-section delivery, give antibiotics after cord clamping to eliminate fetal exposure to antibiotics - Use more prudency in antibiotic administration during pregnancy	- Breastfeeding - Pre- and probiotic supplementation of mother during pregnancy and the neonate after birth
Formula feeding	- Adopt WHO/UNICEF Baby Friendly Hospital Initiative - Develop other policies that incentivize breastfeeding - Do not offer formula to newborns without request or medical indication - Promote use of donor breast milk rather than formula when maternal milk is not an option	- Reintroduce breastfeeding - Pre- and probiotic supplementation

Exclusively breastfeeding is recommended and has many advantages. Breast milk contains a host of beneficial constituents which antimicrobial peptides, immunocompetent cells, polyamines, fatty acids, human milk olisaccharides(HMOs) [269] and lysozyme [186], [270]. The bacteria present in milk were once thought to be contaminants, but now are known to be inhabitants [271]. For example, with one of the beneficial properties; antimicrobial peptides [272] are responsible for inactivating pathogens either additively, individually or synergistically [273]. HMO's, the third largest component in milk [233] are known to have an important function in driving microbial diversity in the gut of the infant [274]. HMOs secreted early in the lactation cycle have been demonstrated to be used by particular phylotypes of Lactobacilli and Bifidobacterial[275]. Interestingly, there has been recent work that revealed two Bifidobacterial strains have had many alterations in order to utilize milk within the infant microbiome [276], [277]. It is hypothesized that the structure and functioning of the microbiome has been adapted to suit the human host [11], [224]. Exposure to bacteria in milk has also been known to improve the intestinal barrier functionality as it decreases the permeability of the intestine [278] while increasing the production of mucine [272]. It is suggested that an infant's exposure to the many different bacterial phylotypes may assist in aiding against diarrheal and respiratory diseases, lessen the probability of getting other diseases like obesity [279] or diabetes [81], [280] and reduce the incidence of infection in an infant [281]. Breast feeding provides a secondary route for transmission of maternal microbes [282] and stimulates maturation of infants gut [17], [283]. This was also noted in primates such as rhesus monkeys [284]. The microbes that are transmitted are implicated in playing a role in the immune system development [226] and resistance against possible infection [250], [281]. Another important role that they play is protection against possible ailments such as asthma [285], [286] and allergies later on in childhood [282]. Literature also shows that breast milk has a selecting effect for particular microbes that inhabit the infant gut and seeds for them too [287], [288]. Studies have also shown that the milk microbial composition changes over time. It is suggested that this is to

prime the infant to move towards a mixed fed diet that needs digestion of solid foods, which is assisted by a particular microbial composition. The change over time can be seen from initial (immediately after birth) inhabitation of beneficial lactic acid bacteria (*Staphylococcus*, *Streptococcus*, and *Lactococcus*) and later, after 6 months, an increase in oral cavity inhabitants (*Veillonella*, *Leptotrichia*, and *Prevotella*) maybe, as noted above, to prime the infant for the uptake of solid food [82]. Furthermore, the sebaceous skin tissue that is found around the nipple and breast milk do not share many taxa [81], [86]. Thus the presence of some microbes like *Streptococcus*, a dominant phylotype in infant saliva [289], may be as a result of the suckling action on the breast (flow from the oral cavity and back to the milk ducts [290]) [291].

An 'obesogenic' environment, which is largely as a result of modern living and diet, leads to excessive body fat accumulation which results in various diseases. One of the most prominent and problematic one being obesity [292]. Obesity is associated with a decrease in the phylum Bacteroidetes but an increase in the phylum Firmicutes, this was observed in some studies [109], [293] but not in all of them [294]. The inverse, with respect to increased and decreased phyla, has also been reported [295]. Obese individuals were also reported to have an increase in Actinobacteria [296]. In the mouse model, for obese mice, the Firmicutes to Bacteroides ratio was also observed as obesity related alteration. This ratio was reported as increased in the obese mice [297]–[299]. The obese phenotype can be transmitted via transplantation of the gut microbiota. This has been done in mice, which indicates that the microbial populations in the gut play an active role in obesity pathogenesis [189], [300]. Taking a step further to catalogue bacterial genes that are found in the human gut [301] will aid in addressing the hypothesis that there is variation of the gut microbiome at a species and gene level. These subsets can therefore define different subsets of persons, including adults that are at risk of developing disease such as those that are obesity related [292], [302]–[304].

1.5 Conclusion

The previous section touched on the interaction between the microbiome and the immune system and the current section speaks to the effect of diet on the microbiome. Below is a summary figure 9 that shows some of the key interdependent factors that tie in the two sections. Work has been done to show how this dependency is affected in disease states such as colitis [109], [305]–[308] . In some cases diet affects both the microbiome and immunity directly, such is the case with respect to a deficiency in vitamin A [308].

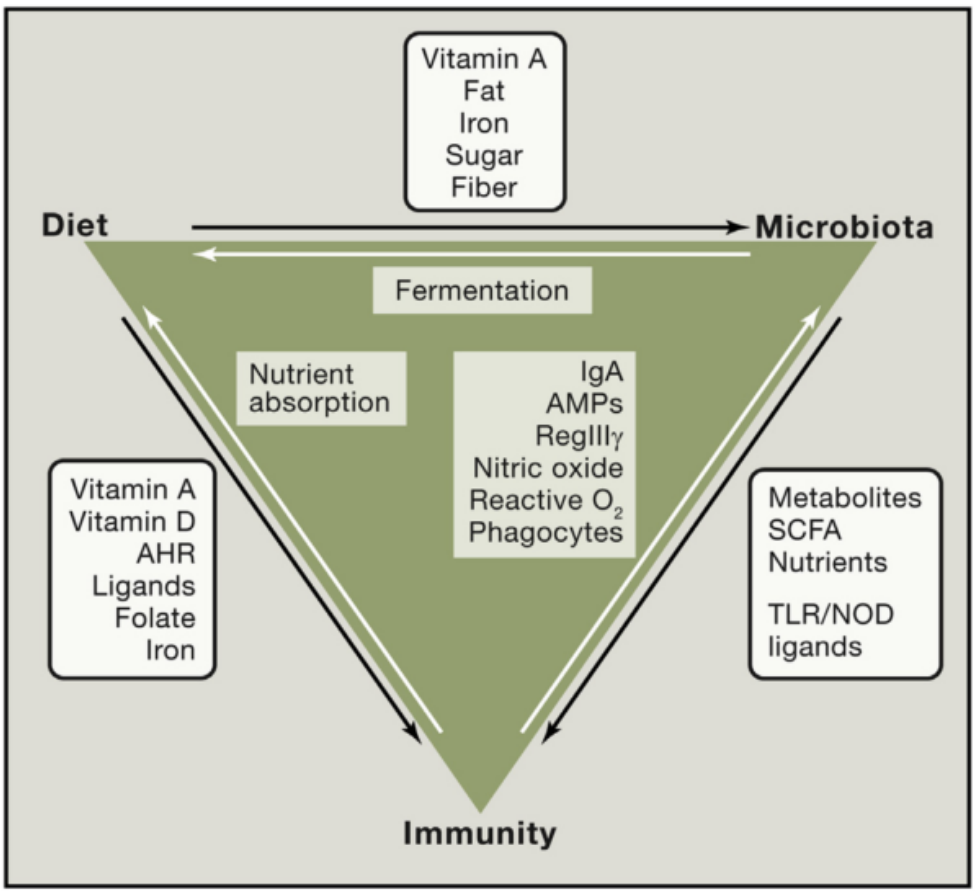

Figure 9: Belkaid and Hand (2014) show the interdependence of diet, immune, and microbiota in the figure above [186].

Evidence of bidirectional interaction between the three key factors now exist and is illustrated in the diagram, where the factors are, diet, the immune system and gut microbiota. For example, the gut microbiota, through commensal microbes, influences the functioning and development of the immune system. In turn, the immune system has a profound effect on nutrient uptake in the gut. It should be noted that the gut also plays a significant role in affecting the nutrient uptake process which is dependent on microbial compositions and their activities in the gut. The third factor, diet, also plays a significant role which is dependent on the nutrients taken in by the host and is in turn affected by microbial compositions in the gut and the functioning of the immune system. [186].

1.6 Study aims and objectives

There is need for more work to be done to not only look at the microbiome in infancy but the different areas that it effects such as the immune system This is pertinent at infancy as this is where it is dictated and has some detrimental long term effects on the individual. The aim of the project was to investigate the influence of gut microbiota on the immune system in infants. This was done using fold change in gene expression levels as indicators to determine whether there is an influence on the immune system. The data shows the microbes present in the collected stool samples and the gene expression level responses from blood samples of infants.

The main objectives of this study were to use infant stool samples to determine:

1. The influence of diet on the microbiome by comparing samples based on feeding type.

2. The subsequent impact of the microbiome on the immune response by looking at the effect of feeding type on gene expression levels. Gene expression levels are indicative of the production of cytokines that are produced to respond to events in the immune system.

3. The effect of the presence of different microbes on gene expression levels.

Chapter 2: Methods

2.1 Introduction

As described in chapter 1, the microbiota plays many significant roles; one of which is an immunoregulatory one. Studies have migrated from showing what impacts the microbiota to what the microbiota, in turn, impacts. It is important to note that the microbiota is dictated at infancy and that some of the key processes that it in turn affects are also determined at this point. Limited work has been done to look at that pipeline as a whole. Numerous studies have looked at what goes into influencing how the microbiota is shaped while others looked at the different roles the microbiota plays. It is of particular importance to note how the microbiome is dictated and is influenced; this will be of great importance in future to aid in personalising efforts to counter, for example, immune related ailments.

In this work we look at the microbiome of infants at 0, 6 and 14 weeks, having been given information on the feeding modality. We then also look at the gene expression levels when a vaccine (BCG) antigen is introduced to the infant's system. The time points are quite early but studies have shown that the diversity and abundances of an infant's microbiome already begin to take shape at 1 week.

2.2 Sample collection and processing

Information and data from the primary study were kindly supplied by Dr. Heather Jaspan who is a Principal Investigator from the Division of Immunology, Health Sciences faculty at the University of Cape Town. The primary study is titled: Early Introduction of Non-Breast milk Foods Activates HIV Target Cells in South African Infants (HREC REF (Ethics code):

571/2010). The sample collection and data generation were carried out by Dr Jaspan's laboratory, while the data analysis was performed as part of this thesis.

2.2.1 Background

A total of 156 infants were recruited to the study. One mother that was initially enrolled in the study tested positive for HIV, and therefore was excluded from the study. 101 (65% returned for follow-up at 6 weeks and 85 (55%) completed the study until 14 weeks. Although most mothers that did not return for follow-up visits could not be reached (60%), the most common reason provided for not returning for later visits was that the mother and/or infant had relocated to outside of the community (21%). Due to the non-visits (for the study) for various reasons, such as relocating, there are some gaps in the data.

2.2.2 Cohort Recruitment

A total of 156 infants were recruited at within 12 hours of vaginal delivery from the maternity ward at the Site B Clinic in Khayelitsha, Western Cape, South Africa, into a prospective cohort. At the time of enrolment, the mothers of all enrolled infants indicated that they planned to exclusively breastfeed the infant.

2.2.3 Feeding

Exclusively breastfed (EBF) infants were considered as those reported having received only breast milk and prescribed medicine at that and any previous time point. By 6 weeks of age, 44 (43.5%) of the infants in the cohort were no longer EBF. This dropped further to 20% of

infants by 14 weeks of age. Of the foods that were introduced by 6 weeks, 73% were solids, predominantly in the form of porridge or cereals.

2.2.4 Sample Collection

If an infant had stool in their diaper at their study visit, a stool sample was collected, with caution to avoid stool that was in direct contact with the diaper. If stool was not present at the study visit, mothers were provided with a sterile sample cup, and instructed to collect stool and return the sample cup to the clinic the morning after collection. After collection, stool was stored at 4C for no more than 6 hours before transport to the laboratory, where the stool was immediately placed at -20C. Stools were thawed, and then treated with a cocktail of mutanolysin (25kU/ml, Sigma Aldrich), lysozyme (450kU/ml,Sigma Aldrich), and lysostaphin (4kU,Sigma Aldrich). Stool was then mechanical disrupted with a bead-beater (Yuan 2012). DNA was extracted, using the MioBio Powersoil DNA kit. The quantity of extracted DNA was then assessed by Qubit dsDNA high sensitivity reagent (Invitrogen). Isolated DNA, suspended in water, was frozen at -80C until sequencing.

Blood samples were also collected. Oral cytobrush samples (OralCDx Brush; OralCDx Diagnostics, New York, USA) and saliva samples (Salivette; Sarstedt, Germany) were collected at each study visit. The data supplied from the blood sample collection only contains information on cytokine response for the infants at the 3 time points. Peripheral blood mononuclear cells (PBMCs) were isolated from each sample within 8 hours of sample collection, using Ficoll (Sigma) density gradient separation. PBMCs were slowly cooled to -80C in Dimethyl sulfoxide (DMSO) (Sigma) + 10% Fetal bovine serum (FBS) (vendor), and transferred to liquid nitrogen for storage. PBMCs were stored in liquid nitrogen for no more than two years before analysis.

2.2.5 Oral Cytobrush RNA Processing

Samples of the oral epithelium were collected to evaluate if immune activation was evident at the site of HIV exposure in breastfed infants (HIV status was examined as part of a separate study). Samples were collected with the Oral CDx brush, which was immediately placed into RLT lysis buffer (Qiagen, Netherlands) and stored at 4C until transport to the Jaspan laboratory. Samples were then placed at -80C and shipped on dry ice to the Sodora laboratory in Seattle, Washington, USA for processing. RNA was isolated from each sample, using an RNeasy kit (Qiagen, Netherlands). RNA was eluted in water, and then quantified on a Nanodrop (NanoDrop, Delaware, USA). All oral sample mRNA processing and analysis was performed in the Sodora Lab in Seattle, WA.

2.2.6 Oral Cytobrush qPCR Sample Processing and Analysis

Quantitative PCR (qPCR) was employed to measure expression differences by feeding pattern and age. 10 genes were selected for qPCR which are IL18, KRT5, CASP3, CXCR7, CCL22, IL12A, KRT10, IL7R, CCL5 and CXCR3. RNA was converted to cDNA with SuperScript III (Invitrogen, California, USA). Taqman Universal Master Mix (Applied Biosystems, California, USA) and Single Tube Taqman Assays (Applied Biosystems, California, USA) were used to set up qPCR amplification reactions, which were run on a 7500 Fast Real Time PCR System (Applied Biosystems, California, USA) for 40 cycles. Thresholds were set for each gene in the linear range of the curve. Fold changes were then calculated, using the Comparative Ct method, using RPLP0 or MAPK3 as endogenous control gene and the median exclusively breastfed value for each gene as the reference sample. The fold change was calculated relative to the mean of the exclusively breast fed samples at each time

point All oral sample mRNA processing and analysis was performed in the Sodora Lab in Seattle, WA.

2.3 Method to study Microbiomes (16s rRNA Sequencing)

There are 2 main methods that are used when analysing microbiome genomic data, these are shotgun metagenomics and 16s rRNA. Studies have compared these methods against each other with respect to their strengths and weaknesses in classifying data [309]. Metagenomics takes a step further to look at the functionality of the identified microbes [310] and has been added to the Future work section. For purposes of this study, 16s rRNA sequencing was used as the scope was to identify the different microbes present and is less expensive compared to metagenomics [309].

16S rRNA is a constituent, in addition to ribonucleoproteins, of the 30S small subunit of a prokaryotic ribosome and is a housekeeping gene [311].The 16S rRNA gene is targeted as it is a ubiquitous gene in bacteria and the gene itself is large enough to use for informatics purposes [311]. Sequencing of this gene aids in obtaining information on microbes without culturing them [312], this is particularly important for bacterial species that cannot and have not yet been cultured. It is also more sensitive to detection than culturing methods [313] as the 16S rRNA gene is highly conserved in bacteria as it is essential in the translation process [314]. There is a benefit to the genes being highly conserved, this allows for the construction of universal primers [315]. The gene also provides enough phylogenetic information that allows identification of a bacteria [316]. One is able to infer phylogenetic relationships because the gene is a molecular clock (evolves at relative constant slow rate) [317]. Over time, as the gene has become the standard, the phylogenetic information on the gene has accumulated

and has been stored in databases that one can check against, which makes it easier to identify novel bacteria [318].

Within the 16S rRNA gene, there are variable and conserved regions. The variable regions have different bases present for different species, which facilitates development of methods to amplify the region and distinguish species. The conserved region, as mentioned before, allows for universal primers to be designed that target all bacteria. Another important factor that has developed over time, as to why the gene is used, is that it well studied and characterised (mainly due to ease of sequencing [311] and a simplified amplification technique [319]) which has resulted in information being readily available through databases such as GreenGenes.

With respect to the variable region, the V4 (variable region 4) hypervariable region was used in this study. This species-specific region allows for identification of different bacteria [320], [321]. It is also one of the most consistent and dependable sections that are representative of the complete region of 16S rRNA sequences that can be used in the phylogenetic analysis of most bacterial phyla [322]. There is no apparent consensus (this is also apparent in literature [323]–[328]) as to which hypervariable region is most optimal for use, the top contenders are the V3 and V4 regions [322], [329], [330]. Efforts have been made to investigate which hypervariable regions work best for different organisms and groups of organisms that are commonly investigated. The V4 region is of particular interest and is popular due to its short length. The length supports quicker less expensive runs that come with simpler working protocols but advances in technology and primer design are working towards better implementation of longer variable region like V3-V4 [331].

The first step in analysing microbial communities begins with extraction of DNA from a primary source, which in this case were infant stool samples. Once the samples are extracted, they are amplified via PCR at the V4 region of the 16S rRNA gene and a fastq file with sequenced

data is produced [332]. The forward primers comprise of MiSeq sequencing adapters (12 nucleotide Golay barcode, error correcting barcode) which are followed by bases that match the 16S rRNA gene [333]. The reverse primer on the other hand is not barcoded [334]. To maximise the accuracy of assignment and retention of sequences, it is advised to use Golay barcodes as the QIIME (Quantitative Insights Into Microbial Ecology) default settings are set to detect these error barcodes [335]. 16S rRNA gene sequences were generated on the Illumina MiSeq platform [335].

2.4 Data processing workflow

The QIIME (version 1.9.1 was used) pipeline takes generated sequence files (fastq files) as input as well as a generated mapping file which has data from of the different samples. The pipeline was used for quality filtering of DNA sequences, demultiplexing, taxonomic assignment (includes generating OTU tables), and calculating α and β diversity [336], [337]. A description of the pipeline is illustrated in figure 10 and includes the downstream analysis [338]. The V4 region of the 16S rRNA gene was amplified using universal 515F/806R primers, and quality checked with Bioanalyzer (Agilent). The samples were sequenced from both ends with the Illumina MiSeq platform to generate sequence data. The 16S rRNA diversity analysis starts off with quality control and processing in QIIME and afterwards analysis in R using the phyloseq package.

FIGURE 10: Flowchart of data processing up to the analysis stage.

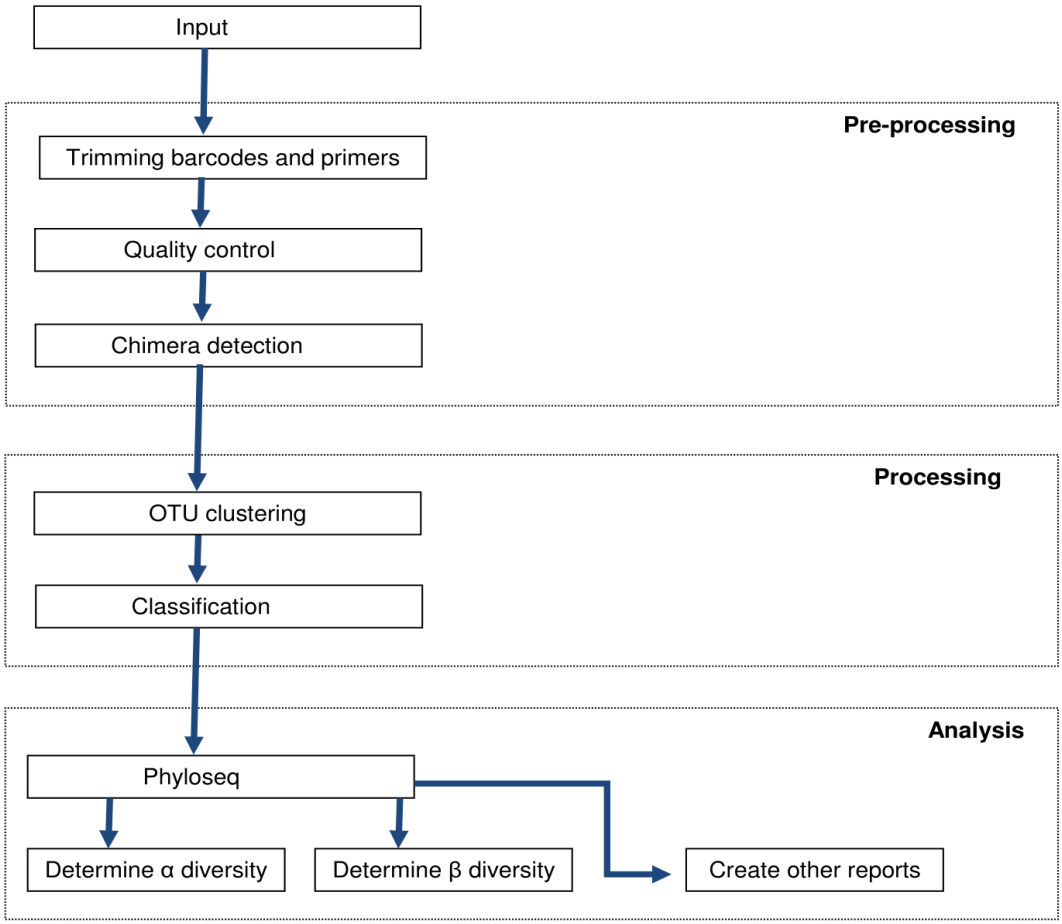

FIGURE 10: Flowchart of data processing up to the analysis stage.

2.4.1 The QIIME pipeline

QIIME is used for raw data preparation and analysis from high-throughput sequencing of the 16S rRNA gene [339]. It is an open-source bioinformatics software package that is useful in aiding to prepare and visualise diversity in large datasets [340]. Figure 10 shows the flowchart, the QIIME pipeline uses from the pre-processing to processing stages. The pipeline can also be used for interactive visualizations and statistical analyses [335]. In our case, phyloseq was used for analysis as it allows for further expansion on analysis of the microbial samples. Phyloseq allows for this expansion as it is a package in R and is thus able to integrate different

types of data from a range of sources as it is able to leverage and make use of other useful packages in R [335]. QIIME has third party package dependencies. These third party packages dependencies are used because they are benchmarks in terms of performing their particular task. Although having a large number of dependencies creates complexity in a pipeline, the upside is the incorporation of familiar benchmark software which in many cases has become the standard go to for use. An example, of a third party package dependency, is the UCLUST program that is used for clustering sequences into OTUs [341], [342]. Using already established package dependencies also avoids attempting to recreate packages as a new created package (which is not as tried and tested) is likely not able to preserve the integrity of the functionality needed.

The key tools [335] that were used in the default QIIME pipeline were: UCLUST which is described above as being used to pick OTUs (this is under processing in figure 10); USEARCH [341] which is also used for picking OTUs and chimera checking (pre-processing in figure 10); RDP Classifier [330] which is used for assignment of taxonomy; GreenGenes Database [343] which is used as a reference database for reference-based picking of OTUs and assignment of taxonomy; PyNAST [344] which is used for multiple alignment of sequences, and lastly UniFrac [345] which is used as a phylogenetic metric that can be used for beta diversity analysis.

The QIIME pipeline steps shown in the flowchart in figure 10 are described further below:

Pre-processing (Trimming barcodes and primers, quality control and chimera detection):

As multiple sequences are combined in a single run, they need to be linked back to the individual samples. This is done via use of the DNA barcodes in the mapping file which are

unique to each sample so as to assign the corresponding sequences back to the samples, this is known as demultiplexing. Error correcting codes are also incorporated if they are available. The demultiplexing process also involves the removal (trimming) of barcodes and primers thus leaving just the matching 16S rRNA gene [335].

Quality control follows after trimming the barcodes and primer sequences. This helps to improve accuracy by improving diversity estimates [346]. This is done via the use of a quality score that Illumina creates for each nucleotide which is called the Phred score [347]. This score is related to the probability that each of the read nucleotides was read incorrectly [335]. Any sequences that do not meet a set desired parameter are removed. The parameter details include factors such as the maximum number of ambiguous bases which is typically represented as n [346]. Default parameter values are also available in the QIIME pipeline and were used, as this is recommended practice [335]. Chimeras are technical and usually rare artefacts [348], [349], of amplified sequences that are incorrectly produced from multiple parent sequences. The recommended method to identify chimeras is UCHIME [350] which is integrated into the USEARCH package. Quality control also includes the removal of OTUs that are represented by single sequences, these are deemed to be erroneous as they are less reliable and may result from sequencing errors [340]. Additional information on the number of OTUs, samples and reads can be found in the supplementary figure 1 and 2.

Processing (OTU clustering and classification):

Sequences are clustered together if they are more similar than a set threshold percentage [351]. Conventionally 97% [352] is used, and was used here for bacterial species but this varies from taxa to taxa [353]. There are 3 approaches (*de novo*, closed and open reference) in QIIME for OTU picking. The open reference approach was used for OTU picking, it tries to match sequences against a reference database, if there is no match, the sequence is added

to the database as a new reference sequence [335], [354], [355], this is the recommended approach in QIIME. After clustering of sequences into OTUs, a representative one was picked where the default in QIIME is to pick the most abundant.

After OTU clustering, the last step is classification where taxonomy is assigned and sequences are aligned. With assignment to taxonomy, the OTUs are linked to an organism. The recommended method, RDP classifier [330] and the GreenGenes dataset [335], [343]. This assists in inferring the different roles of members in the microbial community. Sequence alignment is important in order to infer the resulting phylogenetic tree. PyNAST (recommended and default) was used for sequence alignment [344]. The method uses a template sequence to align the sequences, GreenGenes core set was used and is recommended as a source for template sequences [343]. Once sequences are aligned, a phylogenetic tree is constructed using the FastTree method [356]. The final step is creation of an OTU table using the Genomics Standards Consortium *candidate standard* Biological Observation Matrix (BIOM) format [357]. This was used as input to create a phyloseq object for downstream analysis in R. Support for QIIME in R is predominantly achieved by the use of the phyloseq package [358].

2.4.2 Downstream analysis

Data analysis was done in R (version: 3.2.2) using the interface of R Studio (version: 0.99.473). The central package used was phyloseq. Phyloseq is an open source software package whose purpose is the object-oriented representation and analysis of microbiome sample data [358]–[360] and analysis of phylogenetic sequencing data in R [360]. This package takes in data from the QIIME pipeline. Other packages used include vegan [361], ggplot2 [362], MetagenomeSeq [363] and gridExtra [364].

Chapter 3: Results and Discussion

As mentioned previously, there were 2 focus areas for this project, oral mucosal gene expression levels as indicators of immune activity, and the microbiome in infants with different feeding practices. The results of these analyses are expanded on below.

3.1 Oral mucosal gene expression

Firstly, we investigated oral mucosal gene expression over time (see figure 11), separated by feeding modality. The samples used were those that had qPCR data for week 6 and week 14, where day 0 gene expression levels were always 0 as they were not measured due to the babies not yet being fed. Initially, we investigated whether there is a change in expression over time, that is, a fold change at the age of 6 weeks compared to fold change at the age of 14 weeks within the 2 groups, breast and mixed fed. Of particular interest are the mixed fed infants that have spikes in their response data (highlighted in table 4).

Secondly, we investigated if there was a difference in mucosal gene expression between the breast fed (BF) and mixed fed (MF) infants. As mentioned earlier, diet plays a role in shaping the microbiome and the microbiome structure, in turn, has an influence on the immune system. An influence of the immune system can be seen in a change in the mucosal immunity.

Figure 11 shows fold change of gene expression levels as they change over time. The initial samples show infants who were exclusively breast fed (baby number 32 and 54) then infants that moved from breast feeding at week 6 to mixed feeding by week 14 (baby number 52 and 76). The rest were mixed fed by week 6. There is a clear distinction between the exclusively

breast fed infants and their mixed fed counterparts. It is interesting to note that there are several spikes in fold change of gene expression level data which were only found in the mixed fed data, see red highlighted cells in table 4. As an example, baby number 76 at age 14 weeks for KRT5 had fold change of 2207.753. Most of the spikes (high expression fold change above 6) are found in week 14 with KRT5 having the largest increase. These spikes are present only in the mixed fed infants and as discussed earlier, this feeding modality has an effect on the development of immunity, where expression patterns of particular genes are indicative of a healthy immune system. Over expression of genes such as KRT5 has been associated with dysbiosis which may result in disease, for example cancer [365]. Functional studies that look not only at gene expression but incorporate metabolic production and protein expression are able to paint a clearer picture with respect to deducing whether spikes in expression data are point towards an anomaly [366]. The gene expression fold change for CASP3, KRT10 and CXCR3 showed no increase. Only samples that had data at both week 6 and 14 were illustrated in table 4. The increases may be due to the makeup of their mixed fed diet. As mentioned earlier in chapter 1, diet influences the composition of the microbiome and in turn the different microbial structures have varied influence on the immune system. This is not the case with the breast fed infants as breast milk constitutes a similar makeup.

For the chosen 10 gene expression levels verified by qPCR, there was a difference in expression for some genes between the breast and mixed fed infants. This included the chemokines CCL5 (Mean log10 fold change at 14 weeks in EBF=-0.419 versus in MF=0.371, p=0.004), and CCL22 (mean log10 fold change at 14 weeks in EBF=-0.144 versus 0.251; p=0.023). The chemokine receptor CXCR7 was also upregulated in mixed fed infants at 14 weeks (mean log10 fold change -0.345 in EBF versus 0.020 in MF, p=0.0001).

There were no significant differences gene expression fold change levels of the innate cytokines IL12A and IL18 between the exclusively breast fed and mixed fed infants at either time point. Both KRT5 and KRT10 were increased in mixed fed infants at 14 weeks (mean

log10 fold change in KRT5 -1.790 in exclusively breast fed infants versus -0.139 in MF, $p<0.0001$ and mean log10 fold change in KRT10 -0.361 in exclusively breast fed infants versus 0.063 in MF, $p=0.027$). This can also be seen in the highlighted samples in the table. The differences in CXCR7 and KRT5 remained significant after adjustment for multiple comparisons (adj. $p=0.002$ for both).

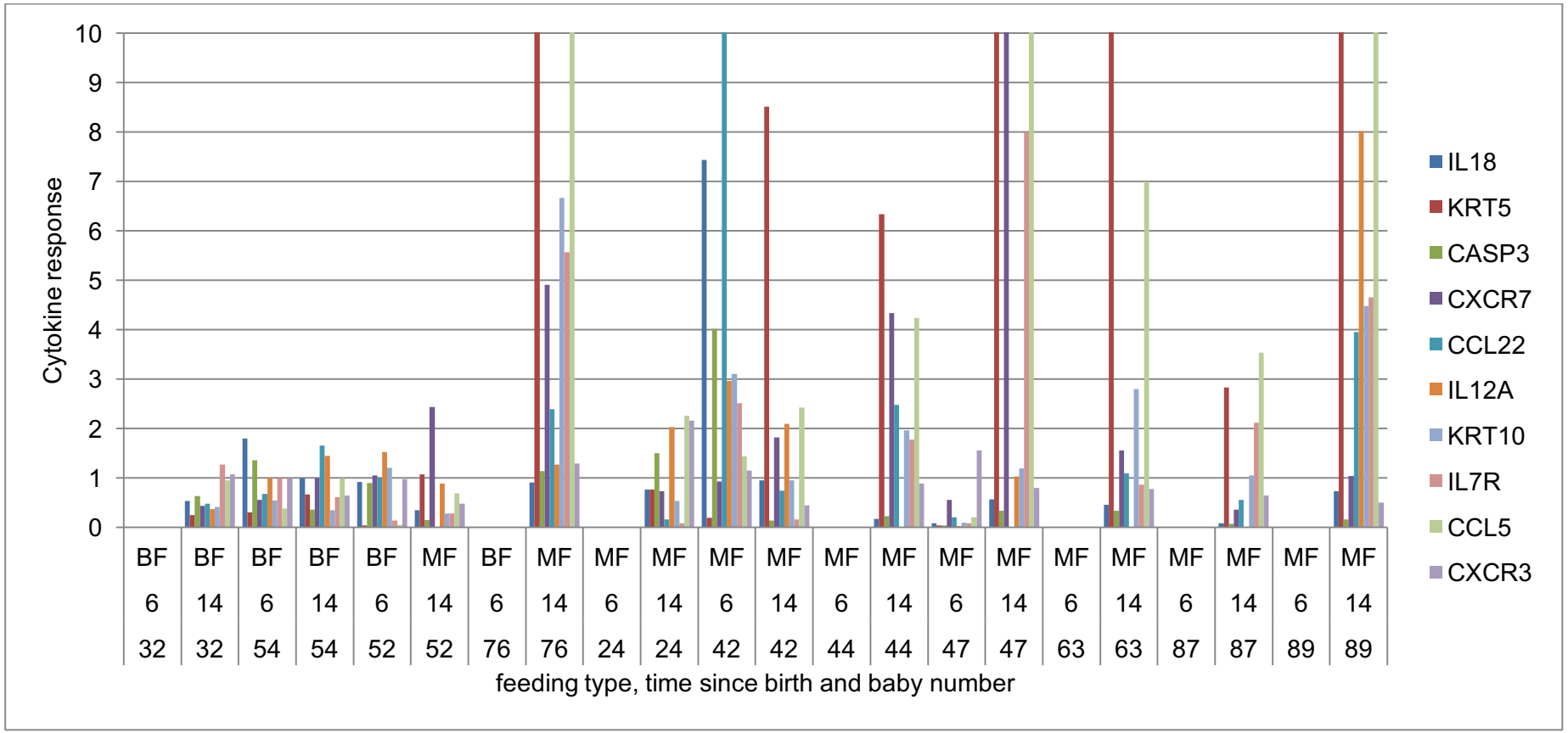

FIGURE 11: Shows fold change of gene expression levels as they changes over time (time points from an age of 6 weeks to 14 weeks) for exclusively breast fed (BF), BF to mixed fed (MF) and strictly mixed fed infants. The number under the time points represents the sample (baby) number. The index shows the different genes that were expressed.

Table 4: Shows fold change of gene expression for IL18, KRT5, CASP3, CXCR7, CCL22, IL12A, KRT10, IL7R, CCL5 and CXCR3 over time, levels that had spiked values above 10 in figure 11 above are highlighted in red and high values (between 6-10) are highlighted in blue. Breast fed (BF) feeding modality is highlighted in green while mixed fed (MF) is in yellow.

Baby number	Time since birth	Feeding	IL18	KRT5	CASP3	CXCR7	CCL22	IL12A	KRT10	IL7R	CCL5	CXCR3
32	6	BF	0	0	0	0	0	0	0	0	0	0
32	14	BF	0.535243	0.244315	0.631289	0.43296	0.479267	0.365039	0.412436	1.270176	0.955193	1.074565
54	6	BF	1.793904	0.305302	1.360412	0.554128	0.671199	1	0.541666	1	0.378135	1
54	14	BF	1	0.667715	0.355501	1	1.658175	1.448896	0.343719	0.613097	1	0.640534
52	6	BF	0.918552	0.035215	0.898861	1.052647	1	1.527197	1.199659	0.135274	0.05063	0.971952
52	14	MF	0.348467	1.076195	0.150117	2.431874	0	0.888227	0.283112	0.277202	0.683524	0.479039
76	6	BF	0	0	0	0	0	0	0	0	0	0
76	14	MF	0.903009	2207.753	1.139901	4.903127	2.393609	1.264958	6.6683	5.55923	20.0216	1.287211
24	6	MF	0	0	0	0	0	0	0	0	0	0
24	14	MF	0.765412	0.760553	1.504608	0.727808	0.163078	2.025093	0.530647	0.081323	2.256358	2.157476
42	6	MF	7.43502	0.187839	4.011789	0.930873	17.11978	2.963942	3.101734	2.513723	1.429417	1.148336
42	14	MF	0.945736	8.509831	0.134669	1.814458	0.74395	2.092963	0.954087	0.154922	2.428033	0.449357
44	6	MF	0	0	0	0	0	0	0	0	0	0
44	14	MF	0.167671	6.329905	0.22834	4.333907	2.482542	0	1.961526	1.770067	4.234802	0.880492
47	6	MF	0.084431	0.044554	0.043686	0.550789	0.208046	0.029296	0.097442	0.085671	0.204478	1.559492
47	14	MF	0.568324	27.37178	0.336254	11.86079	0	1.032985	1.193102	7.992558	21.51336	0.802052
63	6	MF	0	0	0	0	0	0	0	0	0	0
63	14	MF	0.45384	36.29756	0.338913	1.550389	1.092119	0	2.800046	0.860292	6.989495	0.778354
87	6	MF	0	0	0	0	0	0	0	0	0	0
87	14	MF	0.086298	2.826227	0.071964	0.360869	0.555103	0	1.047017	2.116286	3.536628	0.648651
89	6	MF	0	0	0	0	0	0	0	0	0	0
89	14	MF	0.736367	84.28915	0.156161	1.03954	3.947392	8.016209	4.473591	4.654823	19.51849	0.501571

3.2 Microbial communities (Microbiota)

3.2.1 Alpha diversity

As mentioned in chapter 1, feeding plays an important role in shaping the microbiome. Diversity measures (figure 12 and 13) are a good way of observing the distribution of microbial species and how factors like diet play a role in shaping composition.

Alpha diversity looks at the richness in species, that is, the mean number of species in a habitat or locality [367], in this case, the microbiota of an infant's gut, by analysing their stool samples. The plot_richness function from phyloseq was used in R to determine the alpha diversity in our samples. This function "creates plots of richness estimates of each sample in a phyloseq data object, allowing for horizontal grouping and colour shading according to additional sample variables" [358]. Each point on the graph in figure 12 represents a stool sample at a specified coloured time point and is denoted either as mixed or exclusively breast fed. The corresponding alpha diversity measure is on the y-axis. Alpha diversity is one of the initial enquiries made with respect to phylogenetic sequence data so as to note the richness estimates of each sample [360]. Studies have shown that diversity increases over time [20]. For example, a particular study that took infant's fecal samples (in order to investigate their gut microbiome) at intervals (from birth until 2 years of age) showed a steady increase from 4 months [20], [93]. Before 4 months, there were increases and decreases in alpha diversity at different time points with no noticeable pattern. Other studies have also shown that alpha diversity values can vary [361]. The Shannon index was used because it is widely used in studies, like ours, as it best shows richness and rare and abundant species [368]. A decrease in alpha diversity, amongst other factors like antibiotic treatment [369], [370] has been correlated to disease phenotype [371]–[373]. This may account for alpha diversity not separating out over time, from week 0 to week 14 There was a significant difference between

the breast and mixed fed infants, Wilcox p-value = 0.01 ($p<0.05$). It has been shown that alpha diversity significantly increases with time, and this is evident in other data from time points that succeed 3 months [93]. Our study unfortunately only shows a time point (14 weeks = 3.5 months) ending just after 3 months with no succeeding time points. The points that showed very low measures of alpha diversity may be affected by factors that affect initial colonisation such as antibiotic consumption by mothers, which have been known to decrease alpha diversity [369], [370], [374].

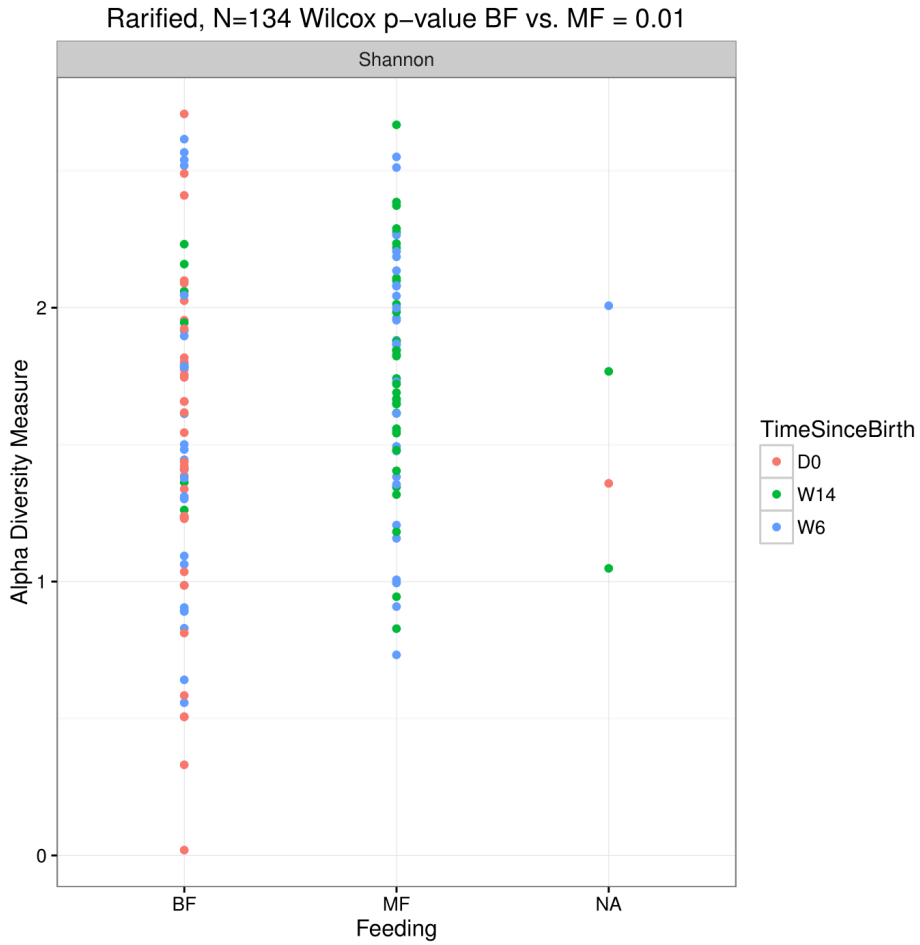

FIGURE 12: Shows alpha (α) diversity (Shannon index) from rarefied data over the 3 time points (age of infant). The 3 points are D0=Day 0 in red, W14=Week 14 in green and W6=Week 6 in blue. This was compared with BF= Breast fed, MF= Mixed and NA= Missing feeding modality data.

3.2.2 Beta diversity

Another measure to analyse in microbiomes is beta diversity (figure 13), which goes a step further from measuring the number of species (figure 12) within a system to comparing this measurement between samples. This basically looks at the number of different microbial species between the samples in order to observe if there is a difference between the breast and mixed fed infants. This helps to investigate the state of the microbiome, as literature has shown that diet affects the number of different microbial species, and this in turn has an effect on the immune system.

Beta diversity is a biodiversity measure that is between groups of samples (inter-sample) [85], [367], [375]. The plot_ordination function in phyloseq, which is the main function for plotting the results of an ordination, was used to create an MDS plot in R to investigate beta diversity. Additional factors can be set in the package to effortlessly show a desired sample variable or taxonomic rank in different size, shape, or colour to enhance visual aesthetics [358]. Figure 13 shows diversity between individuals by calculating pairwise ecological distances [358], [376], [377][361] to create the MDS [358], [378]. Each point on the graph in figure 13 represents a stool sample at a specified coloured time point (age). There were no significant differences in the breast fed and mixed fed samples. This is evident as no clusters are formed by the samples in figure 13, either by the age of the infant or by the type of feeding. Studies have shown newborns to exhibit the highest beta diversity, and differences in the types of species present, between sampled individuals [20], [117], [119] when compared to later ages. The variation has also been shown to start to show a decrease at 4 months which is ~16 weeks. The samples used ended at 14 weeks, which may be too early for samples to start separating out to form clusters, as beta diversity is known to change over time [367]. It is shown that over time beta-diversity decreases as it moves towards forming a more stable community [20] such changes are also known to be affected by a change in diet [253]. It would

be beneficial to study the microbiota in decreased time gaps such as those used in this study, but for a longer time frame [97].

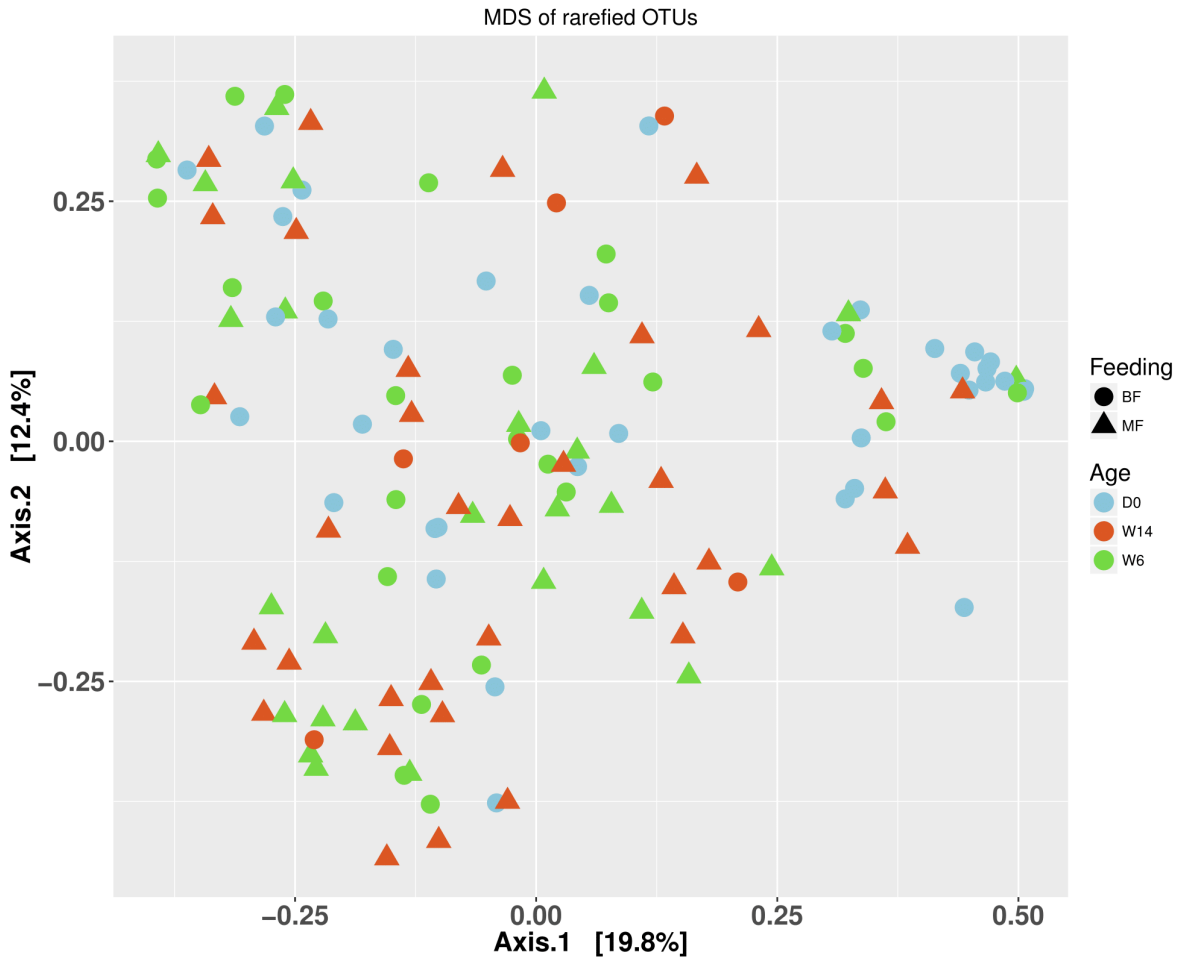

FIGURE 13: Shows beta (β) diversity through multidimensional scaling (plotted using Bray-Curtis distance between samples). The percentages on the 2 axes are similarity values. The 3 points are D0=Day 0 in blue, W14=Week 14 in red and W6=Week 6 in green. The percentage of variation shown by the plotted principal coordinates can be seen on the axes [85] for the rarefied samples.

3.2.3 Relative bacterial abundance

As mentioned in chapter 1, there are individual species whose presence and/or abundance, whether alone or in a group, have an effect on the microbiome and subsequently the immune system. These can be viewed in a profile (figure 14). Previous studies have shown microbial profiles that point towards a healthy system which in turn is able to elicit an immune response. We investigated the relative abundances of the families present in the infant samples to determine whether this was affected by diet. The relative abundance was based on all the identified genera. It describes the number of microbes of a particular kind as a percentage of the total number of organisms of a community, which in this case is the gut [9], [374]. The plot_bar function in phyloseq was used to create a custom function to create the bar plots in R. It takes the phyloseq dataset as input and a compilation of random expressions that can combine and group the data on the basis of taxonomic rank and different sample variables [358]. The microbes described in figure 14 fall within the 4 major phyla that are associated with the infant gut microbiota, *Actinobacteria*, *Bacteroidetes Firmicutes* and *Proteobacteria* [119], [374]. Other bacteria, also represented figure 14, such as Bifidobacterium, Lactobacillus, Lactococcus, and Streptococcus are known to have high relative abundance in the infant gut. Their genomes are known to be well represented in some biosynthetic pathways such as that for cobalamin [97]. This prevalence can also be clearly seen in the phylogenetic tree in figure 15. The relative abundance of lactic acid bacteria that is present in milk, for example, Lactobacillaceae (represented in the figure 14) has been found to be important in innate immunity [284]. Although diet is known to affect the variance of relative abundances, it may be that diet may not have an immediate effect on changing the relative abundance in an infant. Although there is no significant difference between the mixed fed and breast fed relative abundances, studies show that continued breast feeding at 9 months is associated positively with high relative abundances of dominant species such as *Bifidobacterium* and *Lactobacillus* [269], [274] which are also shown in figure 14 to be present.

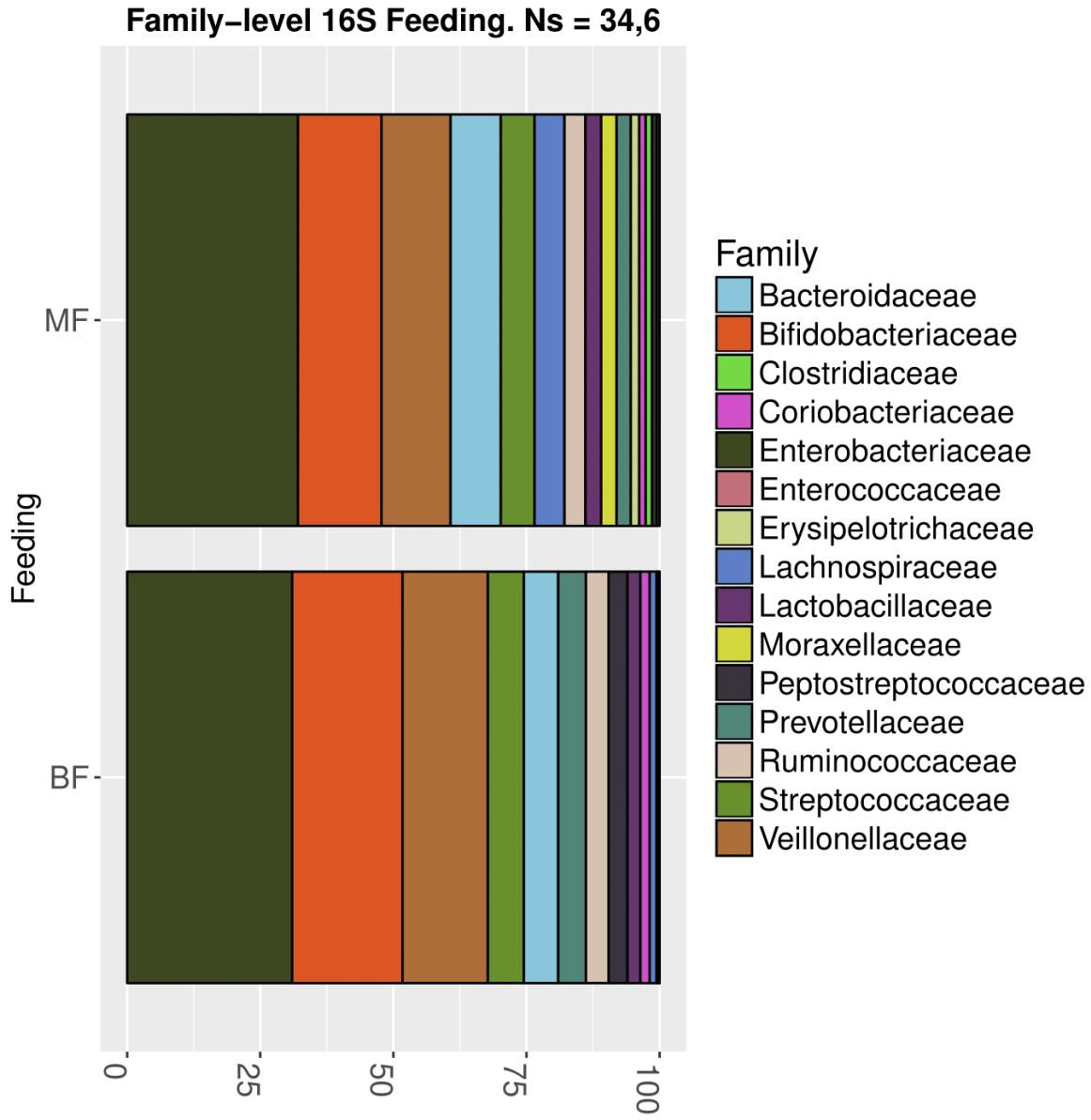

FIGURE 14: Plot showing the relative abundance of organisms at the family level across the samples and are separated by feeding at age, week 14 where BF= Breast fed and MF= Mixed.

3.2.4 Relatedness of bacteria in the microbiome

Another important aspect in microbial studies is the phylogenetic relatedness of organisms in a sample. This builds on from figure 14 which looks at the relative abundances and takes a step further to look at how these organisms are related (see figure 15).

The phylogenetic tree was produced in order to establish whether the microbiome communities that were derived from the stool samples were significantly different [85]. The plot_tree function in phyloseq was used to create figure 15 in R. It allows for easy and simple graphical representation and/or investigation of a phylogenetic tree, with sample data overlaid. Some instances have shown the rendered tree to be a powerful representation of the possible underlying evolutionary structure represented by the sample data [358].The phylogenetic tree provides an estimate of the degree of divergence between the different representative sequences found in the samples [345], [379], [380]. It is also used to capture the evolutionary relationship between OTUs [6]. Sequences (OTUs) that are highly similar with each other are situated close to each other and linked by connections in branch points which shows that the OTUs are phylogenetically associated [120]. It is also interesting to note that even phylogenetically disparate microbes have been found to perform similar functions, this is due to the fact that the gut has a great diversity of well adapted bacteria [12]. Even though it may be important to note the phylogenetically related bacteria, the disparate ones also play a significant role. For example, a diverse range of bacteria from sulfate-reducing bacteria to methanogenic Archaea have been known to consume H^2 produced by other fermenting microbes [381].

From the phylogenetic tree in figure 15, Firmicutes which contain, the second largest group of sulfate-reducing bacteria [382] are distantly related to Proteobacteria (predominantly of the genus *Desulfovibrio,* which are also involved in sulfate reduction [383]) but carry out the same

role. This kind of data can assist with looking at compositional patterns of infants that may be suffering from a particular ailment and this helps visualise the natural clusters that are formed in the gut microbiota [6], [384]. As seen in figure 15, Proteobacteria cluster together. It is expected that bacteria that cluster together usually have overlapping roles, functions and pools from which they obtain nutrients, so these would naturally compete for resources. This is not the case, as seen the different Proteobacteria lineages are just as abundant as each other which suggests they are not competing for resources. Studies have shown that similar species are more likely to co-appear in samples [385] and in some samples they appear with significantly positively correlated abundances [12], [386].

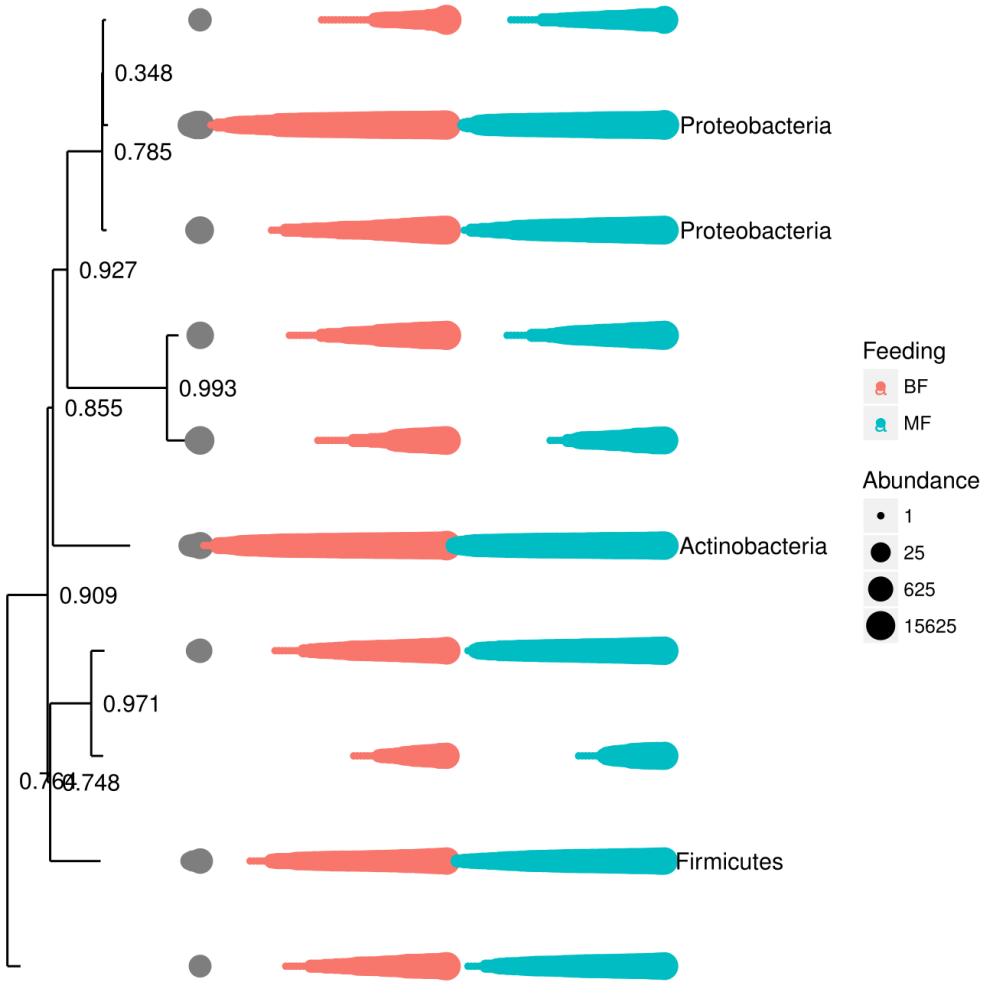

FIGURE 15: Phylogenetic tree at Phyla level which showing the relative abundances. The labelled species are those found to be most abundant.

3.2.5 Specific organism abundance

Violin biplots, like heatmaps, aid in getting quick insight into the nature of data and directive for future exploration [387]. One of their main advantages is their shape which shows how the abundance is distributed [388] for a particular organism. A violin plot takes its initial form from a box plot but is able to show dense areas in abundance data through curves and bumps [388]. The plots were used to investigate possible areas of future exploration by looking at

abundant organisms (figure 16) and picking a popular group in literature such as Bifidobacteriales. From here we take a further look at the genera present and their role.

It is important to look at microbes that may be underrepresented as they may have key roles in the microbiome. We used violin biplots to visualise summary data as in box plots, this aids in gaining insight into the distribution of abundances which is beneficial for directive with respect to future exploration. To create the plots, a custom function was created in R which utilised the phyloseq function psmelt [359]. Figure 17 shows the relative abundances of the most prevalent taxa. The following figures (figures 17, 18 and 19) show biplots of the Bifidobacteriales taxonomic order over time. It is interesting to note the changes in relative abundances for both Bifidobacterium and Gardnerella. At day 0 Gardnerella abundances show high variability, with most samples having the median value of ~0.001 (log_{10} scale) with a range from ~0.00025 (lower whisker) to ~0.085 (upper whisker). By week 14, there is decreased variability amongst samples with most samples having the value of ~0.00015 with a range from ~0.0001 (lower whisker) to ~0.00025 (upper whisker). This can also be seen in Bifidobacterium except, it shows greater variability (from ~0.00015 (lower whisker) to ~0.145 (upper whisker) at Day 0) and higher abundances. This is consistent with literature that shows the gut microbiota abundance variability stabilising over time, Studies have shown that some overlooked underrepresented microbes have the ability to cause pathogenicity [112], [389]. In our case, Bacterial vaginosis which has detrimental effects in infants born via the vaginal method are associated with anaerobic bacteria such as Gardnerella [124], [184], [390]. Efforts need to be put into understanding the effect of underrepresentation of organisms such as Bifidobacterium [3] when compared to pathogenic counterparts. Generally, it would be useful to move to understand the different impact each microbe has on the infant gut, especially those that have dynamic relative abundances.

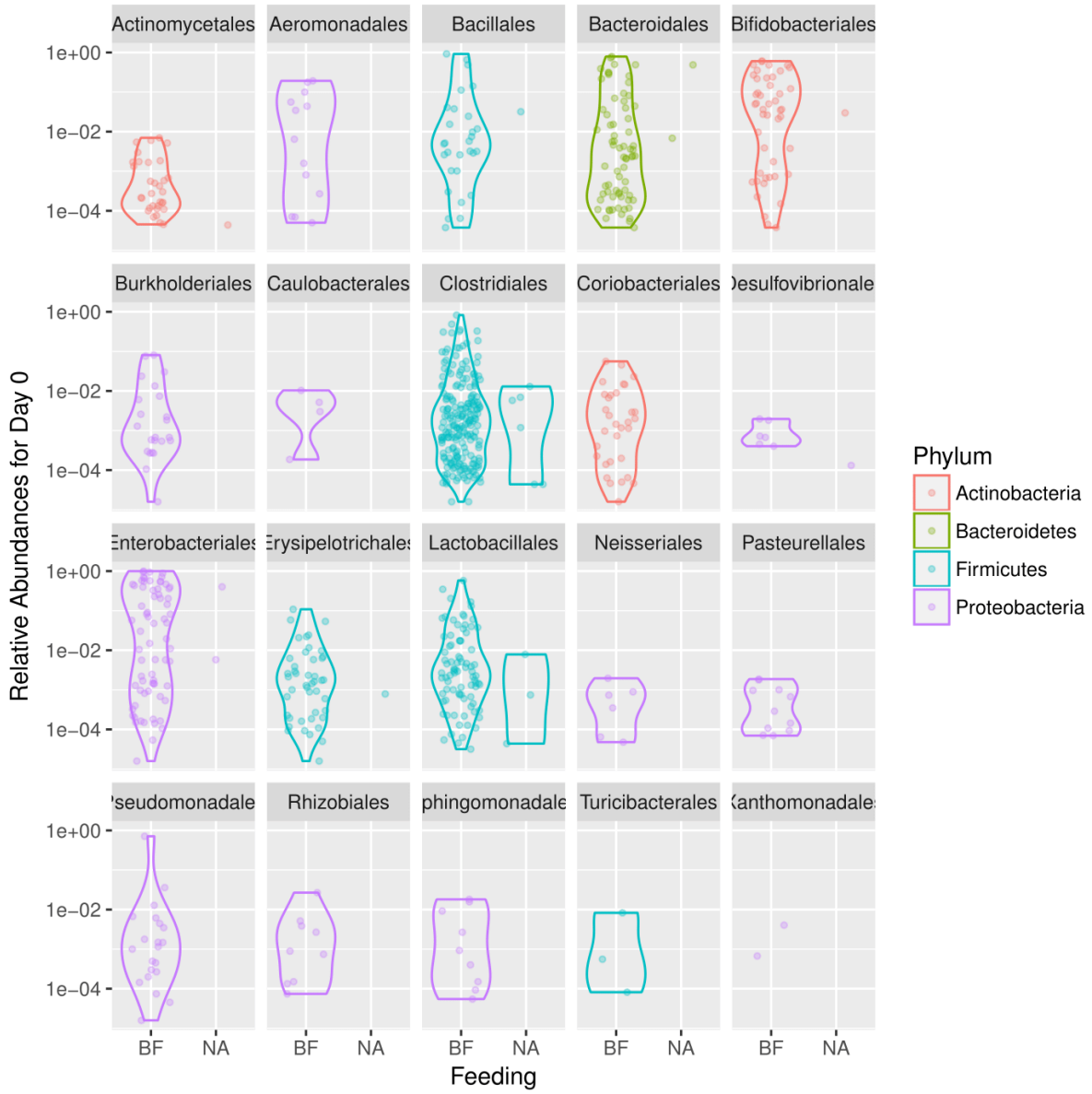

FIGURE 16: Violin biplots showing the relative abundance of the most prevalent phyla at day 0. Where BF= Breast fed and NA= Missing feeding modality data.

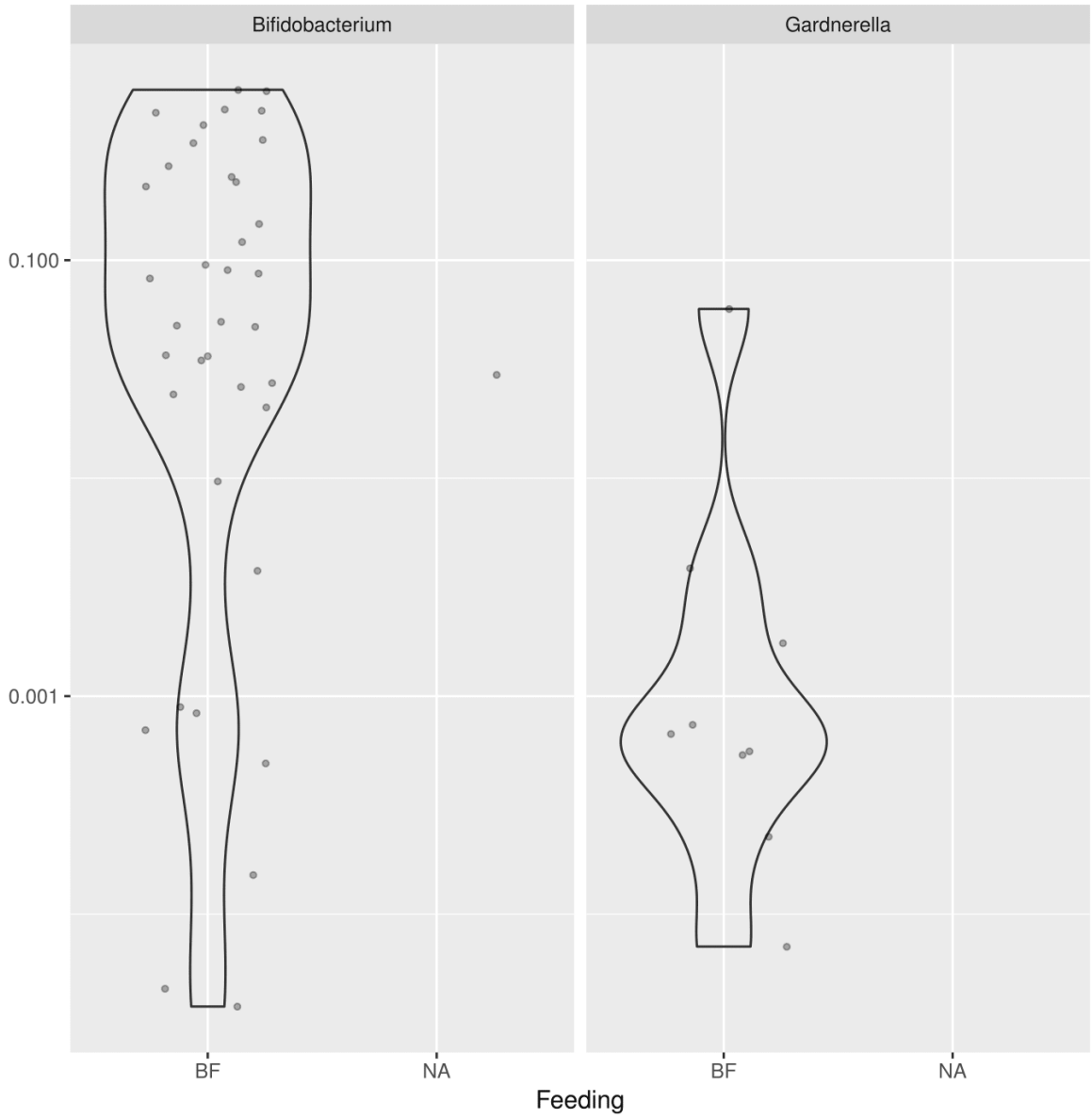

FIGURE 17: Violin biplot of the relative abundances of Bifidobacteriales taxonomic Order, grouped by feeding modality and genera at day 0. Where BF= Breast fed and NA= Missing feeding modality data.

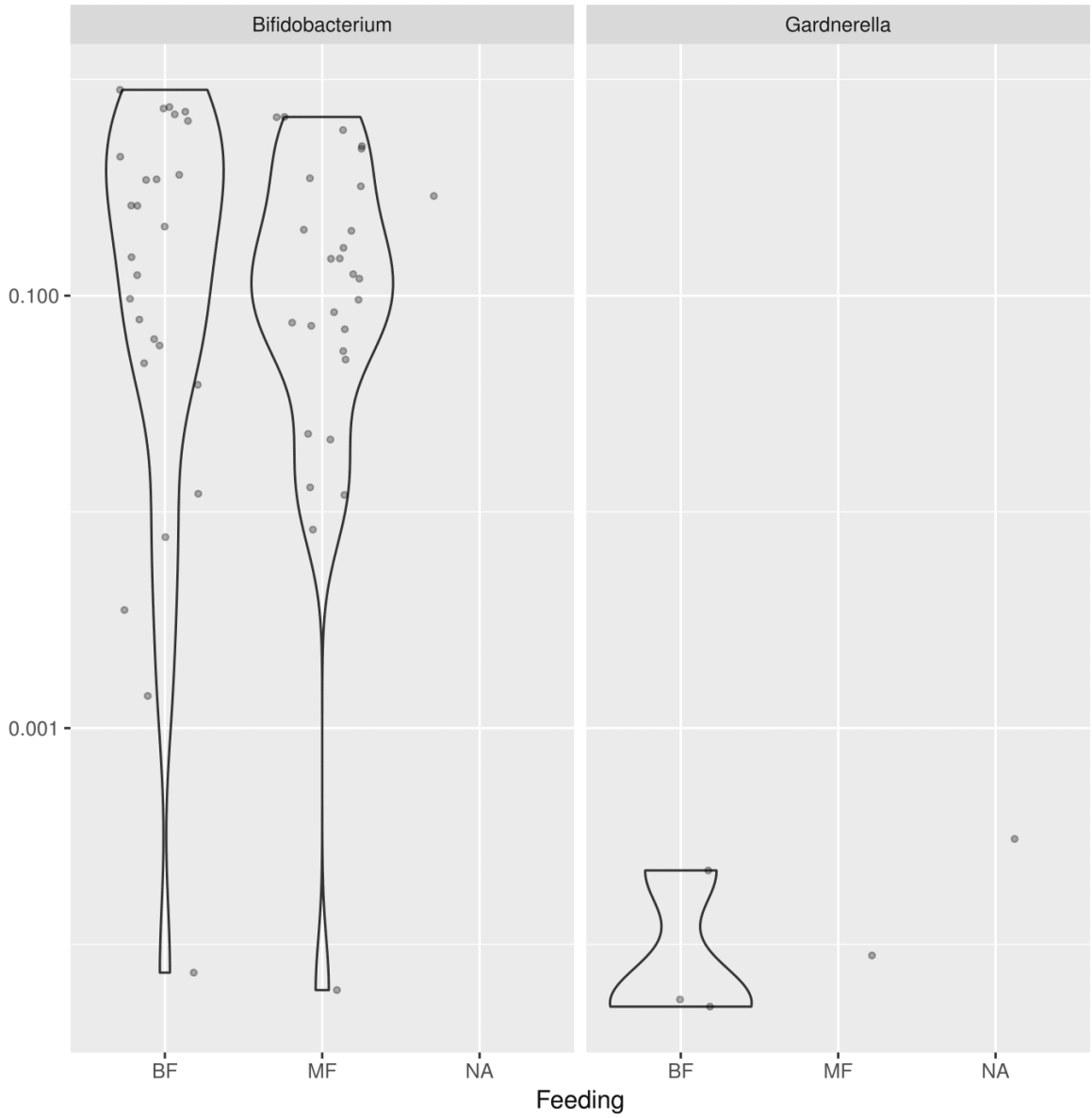

FIGURE 18: Violin biplot of the relative abundances of Bifidobacteriales taxonomic Order, grouped by feeding modality and genera at week 6. Where BF= Breast fed, MF= Mixed and NA= Missing feeding modality data

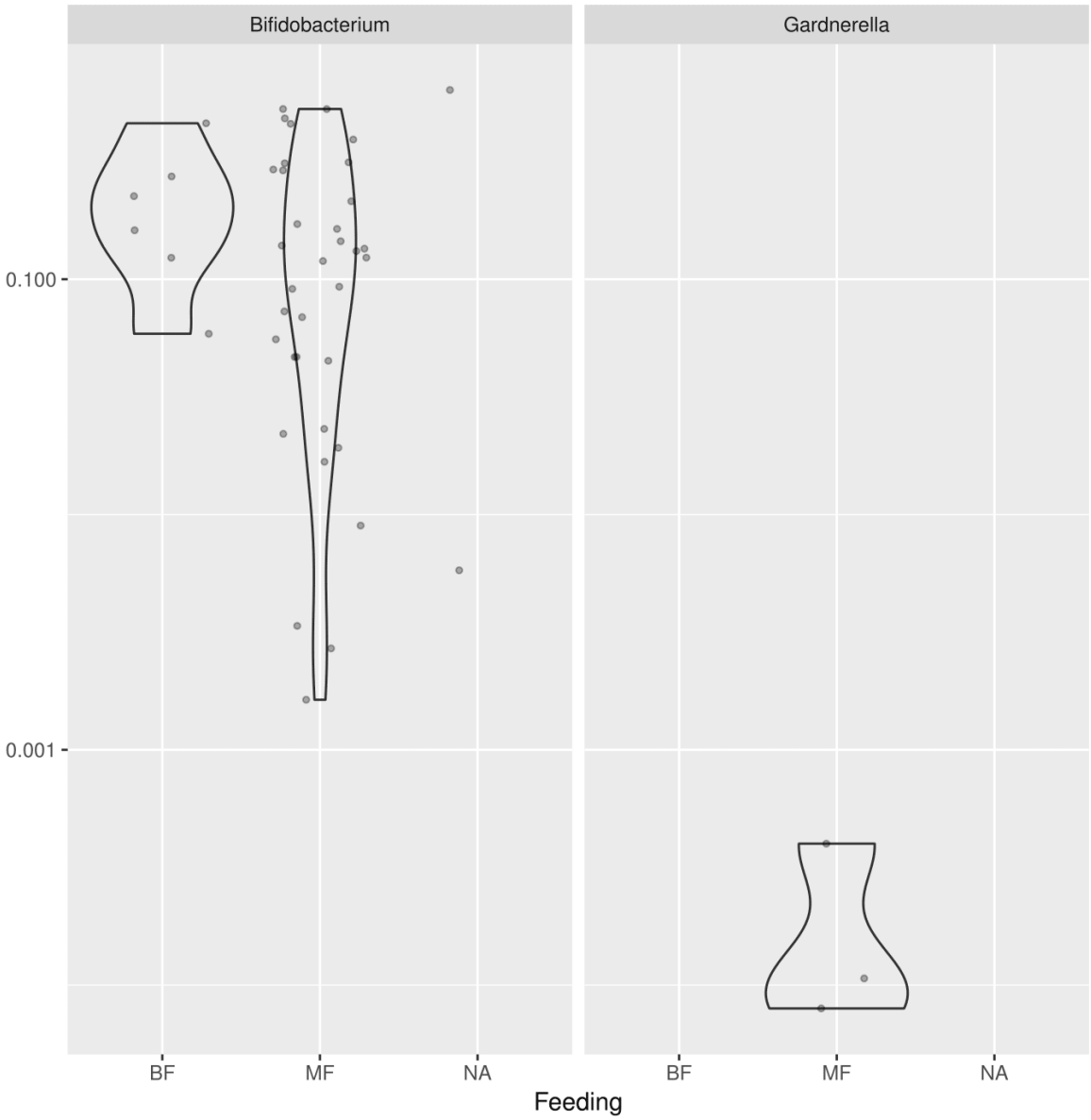

FIGURE 19: Violin biplot of the relative abundances of Bifidobacteriales taxonomic Order, grouped by feeding modality and genera at week 14. Where BF= Breast fed, MF= Mixed and NA= Missing feeding modality data

3.3 Microbial communities and mucosal immune gene expression

Building on from beta diversity, heatmaps are a good way to visualise clustering. They are beneficial as they also display the different organisms present in the samples and shows their

relative abundance against factors like feeding modality and particular expression levels for a gene.

The function used reorders the heatmap based on radial coordinate angle in the first two axes of an ordination rather than clustering or being placed arbitrarily [358]. Traditionally, hierarchical clustering has been used as a means of organisation but this has been found to have the potential to be misleading and lead to misrepresentation of the data in question [391]. A customised function, which utilised the NMF (nonnegative matrix factorization) package [392] and a phyloseq object for taxa annotation for plotting the OTUs was used to create the heatmaps in R. The abundances present are consistent with the current understanding of the microbial composition infant's gut [49], [57], [97], [223]. The lack of clustering of the samples in the heatmaps is also consistent with other studies [372], [373], [393], [394] as well as with the MDS in figure 13 where the samples are measured at stages (ages) that are too early to cluster in terms of feeding modality with respect to abundances of different microbes.

Following from looking at fold change of mucosal immune gene expression levels over time in figure 11, the heatmap in figure 21 looks to investigate whether organisms present cluster according to the expression changes of genes. KRT5 and CASP3 were picked from the 10 chosen genes expression as KRT5 had the most noticeable increases in data while CASP3 had constant values. As discussed in chapter 1, the microbiome influences the immune system and the expressed genes are an indication of the state of the immune system. The heatmaps aid in stacking fold change in gene expression levels against the relative abundances of microbes that were present in the samples. At week 14, Bifidobacterium of abundance below 0 (heatmap $\log_{10}$ scale) generally correlates with high CASP3 gene expression levels in breast fed babies while abundances above 1 correlated with low gene expression levels. KRT5 follows a similar trend as their gene expression levels overlay each other in figure 21.These changes are not significant but are in line with the results in beta diversity where there is also no significance in the diversity between samples. The 14 week

age may be too early for noting the dynamic changes occurring in the microbiome. This dynamic nature of the abundances of different microbes may be the reason why a significant correlation with fold change in gene expression is not seen.

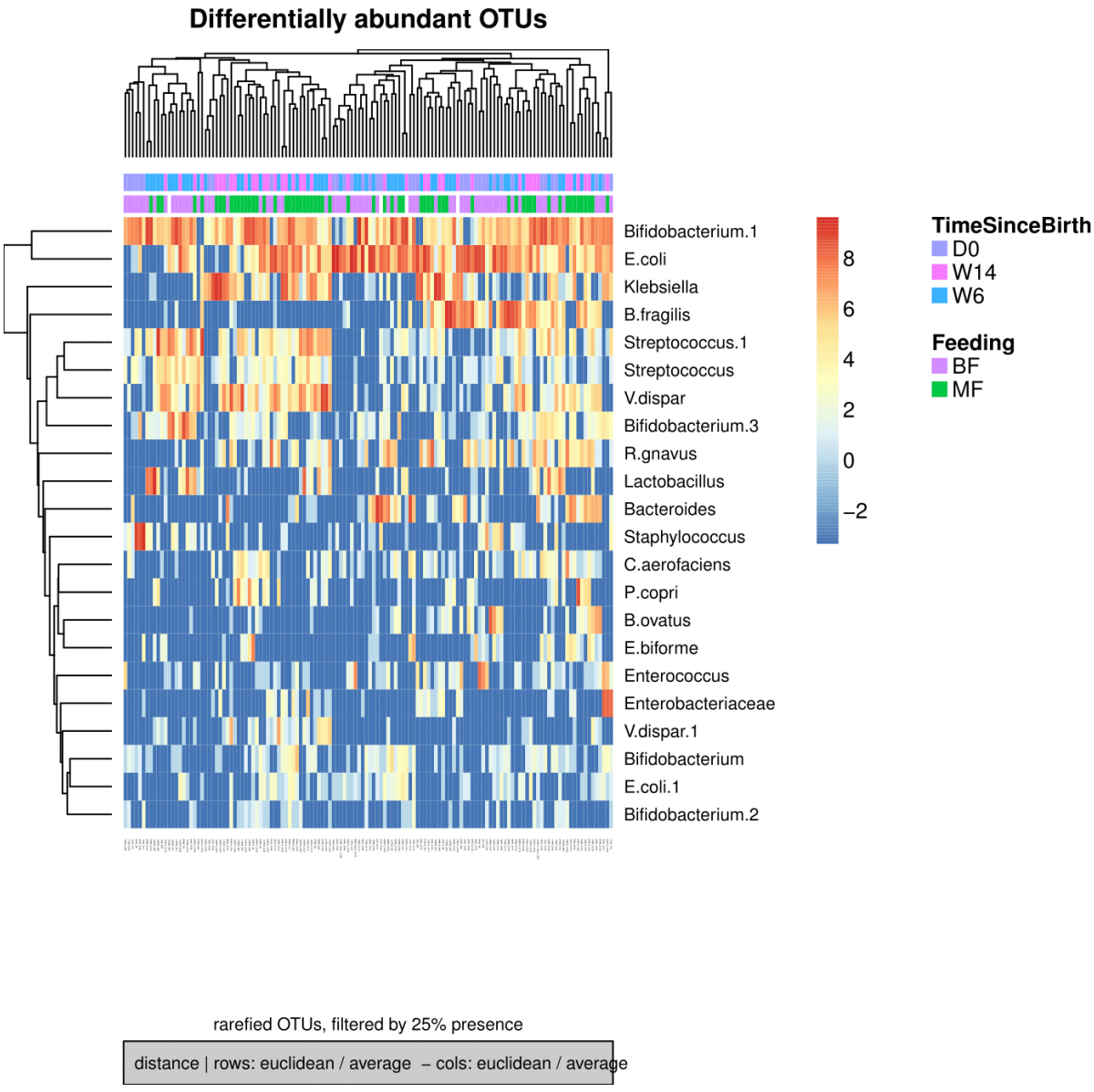

FIGURE 20: Heatmap with dendrograms of rarefied OTUs taxonomic classes (rows) filtered at 25% presence (transformed (log) abundances values) to show the effect of feeding (first row). Time since birth is shown as D0 = day 0, W6 = week 6 and W14 = week 14 (first row). BF, breast fed samples, are represented in purple while MF, mixed fed were represented in green (second row).

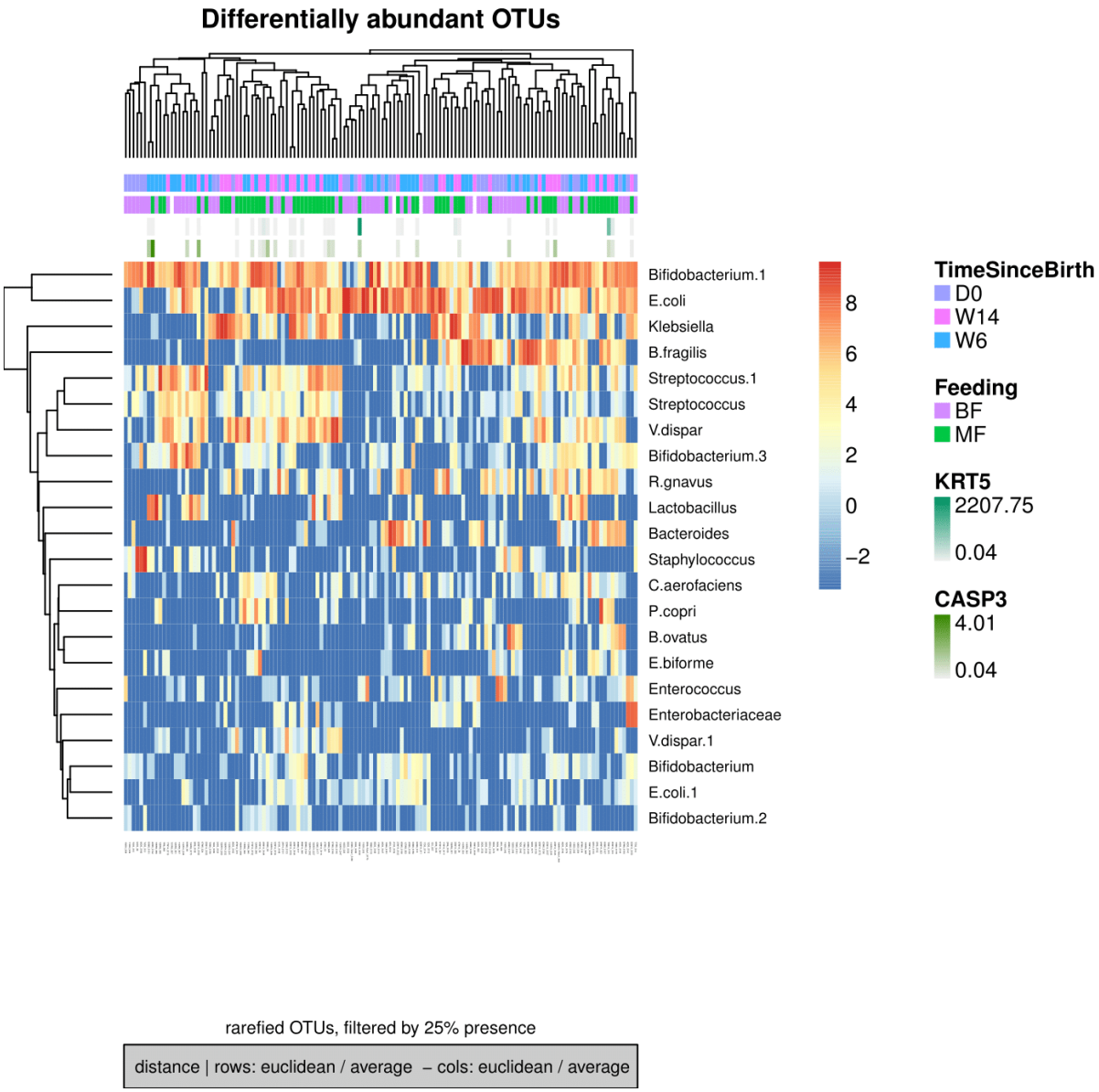

FIGURE 21: Heat map with dendrograms of rarefied OTUs taxonomic classes (rows) filtered at 25% presence (transformed (log) abundances values). Time since birth is shown as D0 = day 0, W6 = week 6 and W14 = week 14 (first row). The diagram shows the effect of feeding modality (second row) for two fold change of mucosal immune gene expression levels, KRT5 and CASP3 (third and fourth rows). BF, breast fed samples, were represented in purple while MF, mixed fed were represented in green. The green represents the fold change of mucosal immune gene expression levels.

Chapter 4: Conclusion and future work

4.1 General Conclusions

From the results section, we have seen a significant difference in the fold change in gene expression levels, in all the genes except IL12A and IL18, between infants that are exclusively breast and those that are mixed fed. Earlier in chapter 1, there was a note and big emphasis on the influence of diet on the microbiome. Studies have shown that different diets result in varied diversity and composition of an individual's microbiome. The altered microbiome then has an effect of different systems. In this study, we looked first at the immune system. Cytokines, chemokine receptors and structural proteins play key roles in the immune system; a shift in their expression levels can be an indication that there has been some influence that has caused a change in response. The objectives were to see the influence of diet on the microbiome by comparing samples based on feeding type (observed in significant alpha diversity measures between the feeding modalities), the subsequent impact of the microbiome on the immune response by looking at the effect of feeding type on gene expression levels (observed in significant fold change in gene expression between the feeding modalities) and the effect of the presence of different microbes on gene expression levels (observed as trends in the data). The lack of significance (in correlating individual microbes to fold change in gene expression) may be due to the early time point age of 14 weeks where bacterial abundances are still dynamic and do not correlate well. This is also supported by the lack of significance in beta diversity. More genes and later times points are essential for future work. Significant difference, Wilcoxon p-value<0.05 at 0.01, in alpha diversity (figure 12) may account for the significant differences in gene expression levels (described in chapter 3) between breast and mixed fed infants. This supports the notion that diet influences the development of the microbiome (significant differences in the alpha diversity of microbes based on feeding

modality) which in turn influences the immune system (significant difference in fold change in gene expression levels between breast and mixed fed infants). Alpha diversity looks at the mean species diversity but there was no significance difference in the beta diversity between feeding types although the different populations may have started to cluster. This may be due to the fact that it is quite early in the infant's life and this is the period when the gut is very dynamic the (figure 13).

The phylogenetic tree in figure 15 is in line with initial bacteria composition that has previously been found to be prevalent during the initial colonisation in infants. The heatmaps then go further to initially look at the relationship between feeding modality and the microbiome over time (figure 20) and examples (figure 21), of the relationship between gene expression levels and the microbiome. Other studies have looked at underrepresented bacteria like that in figure 16, which shows the distribution of abundance for one of the most prevalent phyla. The biplots that follow take a look at the taxa Bifidobacteriales. Biplots separate out the phyla to take a closer look at the different taxa at play. It is interesting to note the dynamic nature of both taxa, especially Gardnerella. Looking into the future, with respect to personalised medicine, it may be beneficial to understand their role this may be a more precise and focused target than looking at a broad phyla.

Significant alpha diversity measures between breast and mixed fed infants show that feeding modality plays a role in shaping the microbiome, which, in turn, influences its immunoregulatory role in the immune system which can be seen through the gene expression levels (significant difference in responses between breast and mixed fed infants).

4.2 Future Work

The human body is like a machine made of many components, some more vital than others but each playing a specialized role. Some of these vital components like the gut microbiome do not work in isolation. It is also important to note that one cannot attribute the development of the microbiota to one factor and this creates a need to study how these components work in a system to develop a particular microbial profile. Understanding how each particular profile can be developed will contribute immensely to the development of personalised therapy and medicines[3], [5]. When one understands how a “healthy” microbiota status can be reached, as mentioned in chapter 1, this differs by factors such as locality; one can develop therapies and medicines that aid in ailments related to a microbiota in dysbiosis.

As important as it is to focus on the gut microbiome with respect to its influence on the immune system, it may be beneficial to later on then branch out to look at some of the key factors that play a role in influencing the microbiome. In particular how different components relate to each other and subsequently how they influence the immune system at infancy. The microbiome and the immune system play significant roles at infancy, future work would be beneficial to build on the following:

4.2.1 Proteomics analysis

One of the major advantages of proteomics, which is the study of proteomes and their functions, is that it gives a high resolution representation of microbial populations so as to look at their function and structure [395]. In addition to other systems-level data, proteomics provides beneficial information on identifiable proteins such as enzymes, and thus enzyme

activity. Enzymes can also be used to construct metabolic pathways which can reveal metabolic functions and carbohydrate transport. It has been shown that activity associated with enzymes may have a role in the symbiotic relationship between the microbial community of termite's (*Nasutitermes*) hindgut and the termite host. [396]. The microbial community is thought to provide the means to program the metabolic system and provide knowledge to the naive immune system [16], [397]. Although metagenomics is an important step in understanding microbiomes as it provides candidate species that are at play, it does not provide evidence of their actual involvement in any role or at what level they are involved in. This is where functional approaches come in; their aim is to identify active molecules and species present in order to decipher the ecological interactions present in the gut microbiome. Furthermore, this gives beneficial insight when looking at disease mechanisms (usually infection is initiated and propagated by protein molecules) associated with the microbiome and finding strategies to evade infection and maintain a healthy microbiome [398].

In time it would be beneficial to incorporate the rest of the "-omics". These include genomics (listed below as host genetics), metabolomics, metagenomics and transcriptomics. These will definitely open a new and more informed perspective to understanding the infant gut and immune system [40].

4.2.2 Viral, Fungal and Bacterial microbiome Interactions

Recent findings from microbiome studies shows interactions between bacteria and viruses and how these interactions can influence disease and health in a host [399], [400]. These interactions can also affect bacterial compositions in the microbiome. Fungi have been found to interact with the gut and are involved in regulating immunity. An example is the prevention of inflammation when there is an acute mucosal injury, when commensal fungi interacts with C-type lectin receptor Dectin 1 [401]. In the intestines, antiviral immunity is dependent on

bacterial signalling that is carried out by Gram-negative bacteria [402]. On the other hand, enteric viral infections have been found to protect the host against intestinal damage and furthermore, pathogenic bacteria [403]. Enteric viruses can have their replication enhanced by the gut bacteria. Some of these viruses include rotavirus and poliovirus [403]–[408] where chronic rotavirus infection is associated with immunodeficiency [409]. It would also be of great importance to have a deeper understanding of bacterial interactions and their contributions in the gut [410], [411].

4.2.3 Biographical information.

It has been shown that first born children have less bacterial diversity when compared to their successive siblings. This is attributed to the bacterial transfer between siblings and the household hygienic practices [374]. Furthermore, use of antibiotics by the mother while she is pregnant has an effect of the infant's gut microbiome and future health [412]–[414]. Antibiotic use during breast feeding has the same undesirable effects in infants [21]. This is not the same case in adults where the microbiome may initially be disrupted due to antibiotic use [12] and later, due to resilience, recover to its normal state. Unfortunately infants at this point have not developed such a resilience and may fail to fully recover [415]. The microbiome is a complex system and disruptions cause shifts in microbial compositions. In infants, the disruption in ecology may cause a shift that facilitates colonization of the gut by enteric pathogens [393]. Enteric infections are most prevalent during the period of 2-3 years [196]. An increase in biographical information will paint a better picture of what affects colonisation of the infant gut microbiota as interplay of these factors, and others influence microbial compositions in the gut.

4.2.4 Host Genetics

Following on from biographical information, in particular familial environment, family members have been found to have similar microbiomes compared to those of unrelated individuals [97], [296], [416], [417]. Of course this can be attributed to the fact that they share a familiar environment and have similar dietary preferences, which are strong influencers that shape the microbiome [111], [253], [418]. Even so, a large degree of genetic identity is shared between related individuals. Discrepancies in the effect of familial environment on the microbiome can be attributed to factors such as exposure time, for example, in the case were pets are involved [34], [419], [420] where there may be a pet in the house but it is kept outside or away from the infant reducing exposure time. This then raises the possibility that familial microbial similarities are underlined by shared genetic similarity. Studies have shown how these are related [381], [421], others take a generalised approach and look at the link between abundances of gut microbiota and genetic loci [422], [423] while yet other studies have looked at gene-microbiota interactions [424]–[428]. It would be important to note the role that host genetics play with respect to the development and maturation of the microbiome as studies have shown that abundances for particular groups of the microbiome are also influenced, in part, by the host's genetics [186], [421], [429].

4.2.5 Additional Samples

One can look to incorporate additional samples from other locations as their microbial compositions may play a significant role in initial colonisation and shape the infant's gut microbiome. Studies have been looking extensively at the following microbial communities: maternal faeces, meconium (earliest stool sample which contains materials ingested during the infant's time in the uterus) [26], [74], [83], placenta, amniotic fluid [23], colostrum [430]–

[436], milk samples [282] and vaginal samples (some of these are also dependent on mode of delivery.) [78], [394]. Recent studies have suggested that live bacteria in breast milk may have originated from the maternal gut. The endogenous route suggested has been recently confirmed by independent research groups [282], [437], [438]. This is then important in identifying and understanding the different groups that are at play in influencing the infant microbiota and its immune system.

The incorporation of different factors may be a big step but initial smaller steps are already at underway with research going into understanding how these different components affect the microbiome. The next step then is to see, how collectively these factors influence the microbiome and subsequently the immune system. It would also be interesting to include research of where the factors mentioned above also play a direct role in influencing the immune system.

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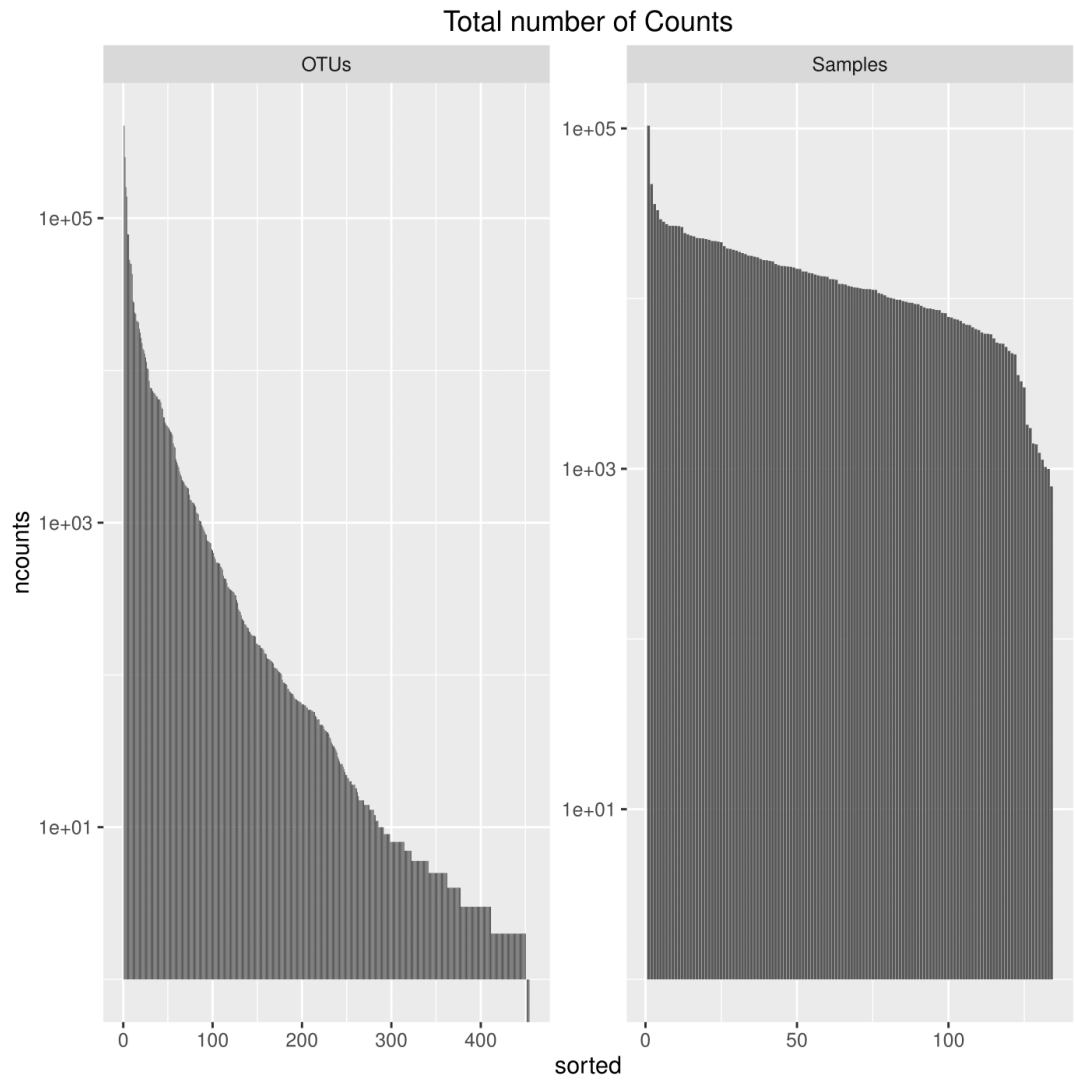
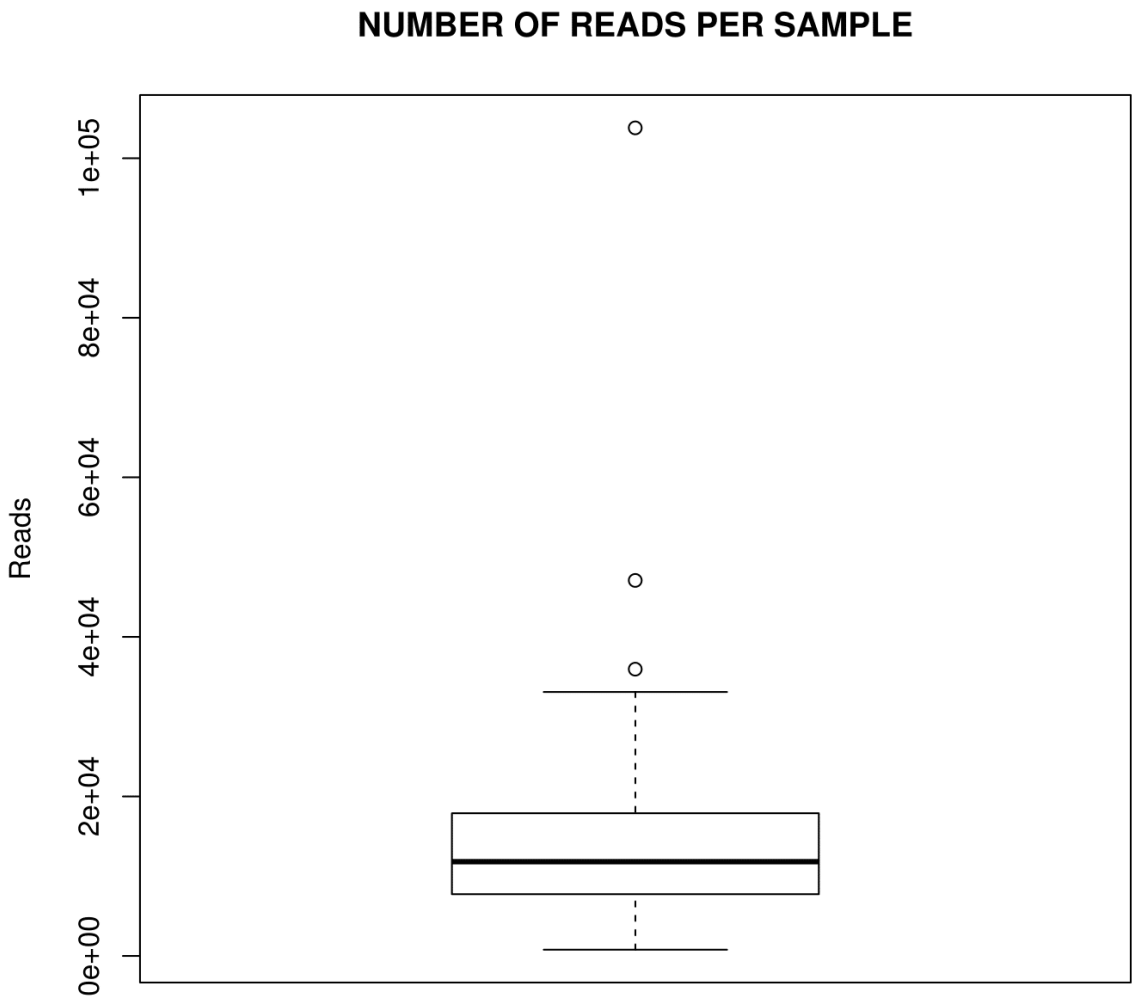
Supplementary data



SUPPLEMENTARY FIGURE 1: Two graphs showing the total number of counts for the OTUs and samples



SUPPLEMENTARY FIGURE 1: Boxplot showing the number of reads per sample.