CHANGES IN THE GUT MICROBIOTA OF SOME NIGERIAN INFANTS WITHIN THE FIRST YEAR OF LIFE

BY

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ABSTRACT

The gut microbiota plays an important role in health and its negative alteration can lead to different pathologies. Different factors which include birthing methods, diet and antibiotic treatment contribute to infant gut colonisation. Many studies in Western countries have reported the effect of these factors on the gut microbiota of infants but there is dearth of information in Nigeria. This study was designed to investigate the changes in the gut microbiota of some Nigerian infants within the first year of life.

This was a longitudinal study of convenience sampled participants. Faecal samples were obtained monthly from 28 infants (gestational age (37.6±2.8 weeks) and birth weight (2.9±0.6 kg) within the first year (8.8±1.3 months) of life at Federal Teaching Hospital, Ido-Ekiti with ethical approval (ERC/2016/09/29/44B) and approved parental consent. The DNA from all samples was extracted with a commercial kit, followed by PCR amplification of the V1-V2 region of the 16S rRNA gene, library preparation and sequencing on Illumina MiSeq. The raw sequences generated underwent downstream bioinformatics analysis with DADA2 pipeline (quality score ≤ 2) to assign taxonomy and to compare the gut microbiota in different groups at different time points (0-4 n=28, 5-8 n=23 and 9-12 months n=13), caesarean section birth (CSB) (n=13) and vaginal birth (VB) (n=15), exclusively breastfed (n=15) and mixed fed (MF) (n=8) and then preweaning and weaning (n=23) groups. The diversity in all the groups was determined by Quantitative Insight into Microbial Ecology (QIIME). Selected antibiotic resistance genes (*aac* (6'), *mef* A/E, *erm*A, *erm*B, *bla*Z) and tetracycline's ribosomal protection protein (*tet*) gene were detected in samples by PCR. Short chain fatty acids (SCFAs) present in each faecal sample were identified by gas chromatography.

Alpha diversity significantly increased with infants' age. Beta diversity showed tight clusters from birth to 4 months, revealing taxonomic similarities and dispersion at 5-8 and 9-12 months, thereby confirming the increased diversity with age. There was no statistically significant difference in the gut microbiota between the birthing methods. However, *Klebsiella* (33.8%) and *Staphylococcus* (13.5%) were most abundant in CSB,

while *Streptococcus* (29.9%) and *Enterococcus* (20.2%) were most abundant in VB. Exclusively breastfed infants had Significant Differential Abundance (SDA) of*Collinsella*, *Bacteroides*, *Sutterella* and *Actinomyces* while *Bifidobacterium* was differentially abundant in MF. Firmicutes were predominant in preweaning and weaning period. However, there was a shift from Proteobacteria to Actinobacteria as the next SDA phylum in preweaning and weaning groups respectively. The effect of antibiotics was marked with decrease in number of observed taxa at point of administration or the next time point while *tet* was the most prevalent (27.0%) resistance gene. Butyrate only appeared, while other SCFAs (acetate, lactate and propionate) increased during weaning indicative of complex carbohydrate metabolic functions.

Observed gut microbiota taxonomic differences between preweaning and weaning in some Nigerian infantsas well as butyrate production were influenced by diet. Introduction of solid foods encouraged the colonisation and adaptation of specific marker organisms associated with carbohydrate metabolism helpful for a healthy life.

Keywords: Nigerian infants, Gut microbiota, Feeding regime, Short chain fatty acids
Word Count: 488

DEDICATION

This research is dedicated to the Invisible, Immortal, and Immutable God. The omnipresent, omniscient, all sufficient God who made this research a reality.

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CERTIFICATION

I certify that this work was carried out by Mrs O.T. Oyedemi in the Department of Pharmaceutical Microbiology, University of Ibadan

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TABLE OF CONTENTS

TITLE PAGE		i
ABSTRACT		ii
DEDICATION		iv
ACKNOWLEDGEMENTS		v
CERTIFICATION		viii
TABLE OF CONTENTS		ix
LIST OF TABLES		xiv
LIST OF FIGURES		xv
LIST OF MAIN ABBREVIATIONS		xviii
CHAPTER ONE		
	INTRODUCTION	1
1.1.	The human gut microbiota	1
1.2.	Growth and development stages in children	2
1.3.	Children vulnerability to diseases	3
1.4.	The gut microbiota of children	3
1.4.1.	Evolution of the gut microbiota from birth till one year	4
1.4.2.	Disease conditions predictable by the gut microbiota	5
1.4.3.	Healthy state predictable by the gut microbiota	5
1.4.4.	Impact of the gut microbiota on health	6
1.5.	Scope of the problem	7
1.6.	Rationale for the study	8
1.7.	Study hypotheses	9
1.8.	Research questions	9
1.9.	Aim and objectives	10
CHAPTER TWO		
	LITERATURE REVIEW	11

2.1. The human gastrointestinal tract 11
2.2. The microbiome 12
2.2.1. The human microbiome 12
2.2.2. The gut microbiome 14
2.3. Gut microbiome of infant 14
2.4. Phyla composition of infant growth 15
2.4.1. Actinobacteria 16
2.4.2. Firmicutes 16
2.4.3. Bacteroidetes 17
2.4.4. Proteobacteria 19
2.5. Factors influencing the gut microbial composition and diversity of children 20
2.5.1. Mother's microbiota 21
2.5.2. Gestational age 21
2.5.3. Host genetics 22
2.5.4. Delivery mode 23
2.5.5. Feeding method 23
2.5.6. Antibiotic usage 24
2.5.7. Geographical location 25
2.6. Functions of the human gut microbiota 26
2.7. Birthing methods 27
2.7.1. Caesarean section birth in Nigeria 27
2.7.2. Caesarean section birth in other countries 28
2.7.3. Vaginal birth in Nigeria 31
2.7.4. Vaginal birth in other countries 31
2.8. Feeding methods among infants in Nigeria 32
2.8.1. Exclusive breastfeeding 33
2.8.2. Formula feeding 34

2.8.3. Mixed feeding 34
2.9. Antimicrobial resistance 35
2.9.1. Antimicrobial resistance in infants 36
2.9.2. Antimicrobial resistant genes 36
2.9.2.1. Tetracycline resistance (*tet*) gene 36
2.9.2.2. Aminoglycoside modifying enzyme (*aac*(6')) gene 38
2.9.2.3. β-Lactamase (*bla*Z) gene 40
2.9.2.4. Macrolide resistance gene 41
2.9.3. Health implication of AMR genes in children 41
2.10. Short-chain fatty acids (SCFAs) 42
2.10.1. Acetate 43
2.10.2. Propionate 43
2.10.3. Butyrate 44
2.10.4. Importance of Short Chain Fatty Acids (SCFAs) in the gut 44
2.11. Studies on infants gut microbiota 45
2.11.1. Studies on infants gut microbiota in the World 45
2.11.2. Studies on infant gut microbiota in Africa 46
2.11.3. Studies on infant gut microbiota in Nigeria 47
CHAPTER THREE
MATERIALS AND METHODS 49
3.1. MATERIALS 49
3.1.1. Supplies and reagents 49
3.1.2. DNA positive control 49
3.2. METHODS 49
3.2.1. Definition of terms as used in this study 49
3.2.2. Ethical consideration 50

3.2.3. Subject enrolment 51

3.2.4. Sample collection and storage 51

3.2.5. Total DNA extraction from faecal samples 54

3.2.6. Polymerase chain reaction amplification of V1-V2 region of 16S rRNA gene 55

3.2.7 Library preparation for Illumina sequencing 55

3.2.8. Bioinformatics analysis of the sequence reads 56

3.2.9. Denaturing Gradient Gel Electrophoresis (DGGE) analysis 58

3.2.9.1. Amplification of the V3 region of the 16S RNA gene for DGGE 58

3.2.9.2. Preparation of the denaturing solution 58

3.2.9.3. Preparation of the cassettes and gel loading 59

3.2.9.4. Gel removal and visualization 60

3.2.10. Verification of manufacturer's claim on NAN 1 formula milk 60

3.2.11. Short-Chain Fatty Acids analyses 61

3.2.11.1. Sample preparation for short-chain fatty acids 61

3.2.11.2. Preparation of internal and external standards 61

3.2.11.3. Extraction of short-chain fatty acids from the faecal samples 61

3.2.12. Selected antimicrobial resistance gene (AMRG) screening 64

CHAPTER FOUR

RESULTS 66

4.1. Metadata of the infants 66

4.2. The gut microbiota composition of the infants 71

4.3. Bacterial diversity of the samples 110

4.4. Beta diversity metrics of the samples 114

4.5. The infants gut microbial composition based on birthing and feeding method 119

4.6. Notable changes between preweaning and weaning groups based on fingerprinting technique 136

4.7. Characterisation of *Bifidobacterium* in formula consumed by the infant 141

4.8. Prevalence of antibiotic resistance genes in samples 143

4.9. Presence of SCFA in samples in samples 145

CHAPTER FIVE

DISCUSSION 148

5.1. The characteristics of the study infants 148

5.2 The nature of the gut microbiota composition of the infants within the first year of life 149

5.3. Effect of birth mode on the gut microbiota composition of the infants 153

5.4. The impact of feeding on the gut microbiota composition of the infant 154

5.5. Impact of short chain fatty acids in the gut of the study infants 159

5.6. Effect of antibiotic usage on the gut microbiota of the infants 162

5.7. Conclusion 165

5.8. Contributions to knowledge 166

5.9. Recommendations 167

5.10. Future directions 168

REFERENCES 169

APPENDIX I 194

APPENDIX II 195

APPENDIX III 196

APPENDIX IV 209

APPENDIX V 211

APPENDIX VI 212

APPENDIX VII 219

APPENDIX VIII 226

APPENDIX IX 230

258

APPENDIX X

LIST OF TABLES

Table 3.1. Number of faecal samples collected from each baby 53

Table 3.2. Constituent of short chain fatty acids standard solution 63

Table 3.3. The AMR investigated and the primers used 65

Table 4.1. Metadata of babies at birth 68

Table 4.2. Sequence variants with significant differential abundance between exclusively breastfed and mixed fed samples 125

Table 4.3. Sequence variants with significant differential abundance between exclusive breastfed and local food/solid fed samples 127

Table 4.4. Sequence variants with significant differential abundance between mixed fed and local food/solid food samples 130

Table 4.5 Sequence variants with significant differential abundance between preweaning and weaning samples 133

LIST OF FIGURES

Figure 2.1.	The human microbiome	13
Figure 2.2.	Global and regional rate of caesarean section between 1990 – 2014	30
Figure 4.1.	Mean proportion of major phyla in the gut microbiota of the infants from birth till 10 months	72
Figure 4.2.	The phylum level gut composition of infants	73
Figure 4.3.	Mean proportion of major genera in the gut of the infants from birth till 10 months	75
Figure 4.4(1).	The genus level gut composition of Baby 1	77
Figure 4.4(2).	The genus level gut composition of Baby 2	78
Figure 4.4(3).	The genus level gut composition of Baby 3	79
Figure 4.4(4).	The genus level gut composition of Baby 4	80
Figure 4.4(5).	The genus level gut composition of Baby 5	81
Figure 4.4(6).	The genus level gut composition of Baby 6	82
Figure 4.4(7).	The genus level gut composition of Baby 7	83
Figure 4.4(8).	The genus level gut composition of Baby 8	85
Figure 4.4(9).	The genus level gut composition of Baby 9	86
Figure 4.4(10).	The genus level gut composition of Baby 10	90
Figure 4.4(11).	The genus level gut composition of Baby 11	91
Figure 4.4(12).	The genus level gut composition of Baby 12	92
Figure 4.4(13).	The genus level gut composition of Baby 13	93
Figure 4.4(14).	The genus level gut composition of Baby 14	95
Figure 4.4(15).	The genus level gut composition of Baby 15	96
Figure 4.4(16).	The genus level gut composition of Baby 16	97
Figure 4.4(17).	The genus level gut composition of Baby 17	98
Figure 4.4(18).	The genus level gut composition of Baby 18	99
Figure 4.4(19).	The genus level gut composition of Baby 20	100

Figure 4.4(20). The genus level gut composition of Baby 21 101
Figure 4.4(21). The genus level gut composition of Baby 22 102
Figure 4.4(22). The genus level gut composition of Baby 23 103
Figure 4.4(23). The genus level gut composition of Baby 24 104
Figure 4.4(24). The genus level gut composition of Baby 25 105
Figure 4.4(25). The genus level gut composition of Baby 26 106
Figure 4.4(26). The genus level gut composition of Baby 27 107
Figure 4.4(27). The genus level gut composition of Baby 28 108
Figure 4.4(28). The genus level gut composition of Baby 30 109
Figure 4.5. Alpha diversity metrics of samples grouped by time point 111
Figure 4.6. Rarefaction plots of every sample for the alpha diversity metrics 112
Figure 4.7. Observed numbers of taxa from each infant across time points, dependent on the antibiotics used 113
Figure 4.8. PCoA plots and clustered trees based upon Bray Curtis Diversity metrics 115
Figure 4.9. The phylum level gut composition of infants grouped by simplified feeding regime 123
Figure 4.10. Alpha diversity metrics with samples grouped by simplified feeding regime 124
Figure 4.11. Significant biomarkers identified by LEfSe between exclusive breastfed and mixed fed samples 126
Figure 4.12. Significant biomarkers identified by LEfSe between exclusively breastfed and local food/solid food samples 129
Figure 4.13. Significant biomarkers identified by LEfSe between mixed fed and local food/solid food samples 132
Figure 4.14. Significant biomarkers identified by LEfSe between preweaning and weaning samples 135
Figure 4.15. Preweaning and weaning fingerprinting profile of mixed fed baby 137
Figure 4.16. Preweaning and weaning fingerprinting profile of exclusively 138

breastfed baby

Figure 4.17. Preweaning and weaning fingerprinting profile of twin 1 baby 139

Figure 4.18. Preweaning and weaning fingerprinting profile of twin 2 baby 140

Figure 4.19. The presence of *Bifidobacterium* in NAN 1 formula milk 142

Figure 4.20. Prevalence of antibiotic resistance genes in the study population 144

Figure 4.21. Trends of SCFAs in babies across time points 146

Figure 4.22. Average SCFAs production between preweaning and weaning samples for all the babies 147

LIST OF MAIN ABBREVIATIONS

GIT	-	Gastrointestinal Tract
WHO	-	World Health Organization
FOS	-	Fructo-Oligosaccharides
HMO	-	Human Milk Oligosaccharides
GM	-	Gut Microbiota
SCFA	-	Short Chain Fatty Acids
NDC	-	Non-Digestible Carbohydrates
IgA	-	Immunoglobulin A
IELs	-	Intraepithelial Lymphocytes
V1	-	Variable region 1
V2	-	Variable region 2
DNA	-	Deoxyribonucleic Acid
RNA	-	Ribonucleic acid
HMP	-	Human Microbiome Project
CSB	-	Caesarean Section Birth
VB	-	Vaginal Birth
EBF	-	Exclusive Breastfeeding
MF	-	Mixed Feeding
NGS	-	Next Generation Sequencing
LGT	-	Lateral Gene Transfer

PUL	-	Polysaccharide Utilization Loci
UNICEF	-	United Nations International Children’s Emergency Fund
SI	-	Small Intestine
LI	-	Large Intestine
AME	-	Aminoglycoside Modifying Enzymes

CHAPTER ONE

INTRODUCTION

1.1. The human gut microbiota

The human body is colonised by a great number of microorganisms referred to as the human microbiota, while the total number of the genomes of these microorganisms in the body is known as human microbiome (Human Microbiome Project Consortium, 2012). The human gastrointestinal tract (GIT), especially the large intestine houses the greatest numbers of microbes in the body in relation to other human body niches. The microbiota include bacteria, viruses, eukarya (protozoa, yeast and fungi) and archaea (Kelley, 2008). Many works done on the human gut microbiota focused principally on bacteria. The most encountered microbial groups in the human gut are the Actinobacteria, Proteobacteria, Bactereoidetes and Firmicutes. These have been identified by culture- and non-culture-dependent techniques in the faecal samples of humans and they sum up to 90% of the entire gut microbiome (Jandhyala *et al*., 2015).

Generally, infants are believed to have a sterile gut or to be born with intestines that contain very low levels of microbes. However, the infant GIT is rapidly colonised by microbes from the mother’s skin or vaginal following delivery (Perez-Muñoz *et al*., 2017). Over the years, the emphasis was on the pathogenic strains that cause infectious diseases, with little considerations given to the beneficial bacteria occupying different human body sites. Previously, most of the studies on the beneficial microorganisms were culture-based while on the contrary, many of the human beneficial microbes remain uncultured. Recently, culture-independent techniques became widely used and the importance of this myriad of beneficial symbionts are being studied.

The gut microbiota contributes to the immune system, preventing colonisation by pathogenic microorganisms and improving the health and developmentof infants. The multifunctional capacity of the gut microbiota has earned them the tag “super organ”

(Rodríguez *et al*., 2015). Perturbation of the gut microbes has been linked with different pathologies such as immune diseases, for example atopy, allergy, asthma and sclerosis (Penders *et al*., 2006), autoimmune diseases, metabolic diseases such as diabetes and obesity (Scott *et al*., 2015; Blandino *et al*., 2016), and gastrointestinal diseases such as diarrhoea, inflammatory bowel diseases, and necrotizing enterocolitis. Before the gut microbiota could perform the beneficial functions, there is need for proper development from birth and it's abilityto cohabit with the host. The first residents of the gut after birth are the facultative anaerobes, the Proteobacteria (Enterobacteriaceae) and some Firmicutes (*Staphylococcus, Enterococcus*). After the exhaustion of the host's environment oxygen, the environment favours the colonisation of strict anaerobes such as the Actinobacteria (*Bifidobacterium*), Bacteroidetes (*Bacteroides*) and the Firmicutes (*Clostridium*) (Timmerman *et al*., 2017). Various factors contribute to the colonisation and development of gut microbiota in infants. These factors include maternal microbiota, gestational age, genetics, delivery method (vaginal/natural birth, caesarean section), diet (breastfed, formula-fed, mixed-fed) and antibiotic usage (Hill *et al*., 2017). These factors are thought to determine microbial colonisation and adaptation to the host before the microbiota reaches maturity. The infants gut microbiota undergoes maturity from birth till 2 to 3 years when it begins to resemble the adult gut microbiota both in composition and function (Aloisio *et al*., 2016).

1.2. Growth and development stages in children

Generally, growth in children is divided into four different stages: infancy, toddler, preschooler and young adolescent (Nelson *et al*., 2016). Growth in children results from the interaction between genetics, nutrition and health. The World Health Organization (WHO) has different indicators of measuring the growth and development of a child. These include, but are not limited to, "length/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age" (De Onis, 2008). These indicators describe the physical development of a child.The WHO has recommended that the growth of humans must be observed and checked against international standards. This is because

parameters to measure length and head circumference, body weight, height and motor development among others have been standardized by WHO (Villar *et al*., 2018)

1.3. Children vulnerability to diseases

Children are vulnerable to infection and diseases. This is as a result of the immaturity of their immune system and the developing digestive, central nervous and reproductive systems as well as internal organs (Simon *et al*., 2015). Children are in a dynamic, active growth stage; hence, exposure to adverse environmental conditions could cause permanent damage to the developing organs. The early years of children are spent in proximity to the ground; thus, if proper hygiene is not ensured, they become at a great risk of some deadly infectious diseases (Ferguson *et al*., 2013). During these early years, the oral stage is very active and anything gotten hold of goes to the mouth. Gastrointestinal infections, most especially diarrhoea, happen to be the most occurring infection in children at this stage. Diarrhoea has been at the top of the list of killer infectious diseases in children most especially in developing countries (Nweze, 2010). Additionally, infants are vulnerable to upper or lower respiratory tract infections, especially pneumonia (Tromp *et al*., 2017). However, vaccine administration has aided the reduction of the mortality rate of pneumonia infection. Children in their early years of life are also vulnerable to sepsis, malaria, and neglected tropical diseases (Nelson, 2010).

1.4. The gut microbiota of children

The gut microbiota is termed a “super organ” (Rodríguez *et al*., 2015) because of its metabolic, physiology and protective functions. The microbiota plays essential functions in building the immune system, prevent the proliferation of pathogenic organisms, and improve the health and growth of infants. The development of gut microbiota in infants plays an important function in the progression of the host immune system, thus promoting a healthy living state in early or later life (Kelly, 2008). A healthy infant gut is dominated by the genus *Bifidobacterium* in the pre-weaning stage (Azad *et al*., 2013). Members of

this bacterial group possess some gene clusters involved in the metabolism of human milk oligosaccharides (HMOs), a good prebiotics for the developing infants' microbiota (Matamoros *et al*., 2013). The composition and diversity of the microbiota as well as their metabolic activities have a great impact on the gut immune regulations and functions. The gut immune system is said to be immature when it is default of microbial stimulation (Kim *et al*., 2017).

The infant gut microbiota undergoes series of processes before it becomes adult-like, when it is said to have attained maturity. This process starts from birth and continues till it reaches maturity. However, mutual cohabitation between the host and the microbes to establish a host-microbe relationship is essential. The classical composition of early colonisers is the facultative anaerobes Proteobacteria (*E.coli*) and some of the Firmicutes (*Staphylococcus*). When these microorganisms deplete the oxygen, they create an environment suitable for obligate anaerobes such as Actinobacteria (*Bifidobacterium*), Bacteroidetes (*Bacteroides*) and Firmicutes (*Clostridium*) to be established (Jandhyala *et al*., 2015).

After the first year of life, the microbial composition tends towards that of an adult and by the end of 3 years, it is similar to that of an adult. However,it has been stated that it takes 5 years for the gut microbiota of children to mimic that of an adult (Avershina *et al*., 2016). This period is referred to as the window period. The period appropriate for modulation of the gut microbiota to enhance the development of vital organs as well as to prevent diseases both at present and later life.

1.4.1. Evolution of the gut microbiota from birth till one year

After birth, relative to the mode of delivery, the baby is colonised by the mother's microbiota. The guts of children delivered through natural birth leads to colonisation by the vagina microbiota such as *Lactobacillus*, *Atopobium*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Megasphaera*, while babies delivered by caesarean section acquire the

mother's skin flora, such as *Staphylococcus, Propionibacterium* and *Corynebacterium* (Azad *et al*., 2014). As soon as the babies start feeding on milk, there is proliferation of other microbes such as *Bifidobacteriium*, *Lactobacillus*, *Bacteroides* e.t.c (Milani *et al*., 2017). However, when weaning starts, there is usually a shift in the microbial composition with more diversity and richness of the gut microbiota (Milani *et al*., 2017). The microbial population becomes highly diverse as the timepoint progresses to post-weaning and at the end of 2 years, the microbiota converges toward the adult microbiota (Rodrıguez *et al*., 2015).

1.4.2. Disease conditions predictable by the gut microbiota

Alteration in the microbial composition of infant gut has led to various diseased conditions, such as inflammatory bowel disease (IBD), multiple sclerosis, asthma and allergic diseases, rheumatoid arthritis, diabetes and obesity (Thursby and Juge, 2017a). Studies on IBD, especially ulcerative colitis (UC), revealed a reduction in microbial diversity of the gut compared with the healthy controls (Gong *et al*., 2016). Healthy gut biomarkers, belonging to Bacteroidetes and Firmicutes were reduced in UC patients while Proteobacteria increased. Likewise, alteration of the gut microbiota in germ free mice contributed to the onset of IBD (Khan *et al*., 2019). The abundance of *E. coli* or *Enterococcus faecalis* in the experimental mice also contributed to the development of colitis. This condition was corrected with the increased number of *Bacteroides vulgates* (Jandhyala *et al*., 2015). The gut microbiota of babies with UC disease have been reported to be dominated with Clostridiaand Enterococciand with low abundance of Lactobacilli, Bifidobacteria and *Bacteroides* in contrast to healthy babies (Kim *et al*., 2017).

1.4.3. Healthy state predictable by the gut microbiota

Recently, with the help of high thorough-put techniques and sequencing platforms, the health status of humans have been predicted through the microbial composition and diversity of the gastrointestinal tracts. This has led to some modulation of the gut at

infancy with prebiotics and or probiotics. Prebiotics are fermentable dietary fibres that promote the growth and activity of beneficial microorganisms, thus, conferring positive health outcomes. Examples of prebiotics are fructooligosaccharides (FOS) and galactooligosaccharides (GOS). These oligosaccharides are used in formulating infants' formula. Other examples are human milk oligosaccharides (HMO), inulin, lactulose and resistant starch. "Probiotics are live microorganisms that confer health benefit when consumed in an adequate quantity" (FAO/WHO, 2001). With respect to microbial composition and diversity, healthy or normal gut in adult humans contain an abundance of Bacteroidetes and Firmicutes (Jandhyala *et al*., 2015). They perform various functions in the human body. *Bacteroides* and *Faecalibacterium prausnitzii* are reported to have anti-inflammatory effects on the host (Scott *et al*., 2015)

1.4.4. Impact of the gut microbiota on health

The overall impact of gut microbiota on health is believed to be hinged on the production of ShortChain Fatty Acids (SCFAs), as well as host-microbial association with attending immune stimulations and response (Sun *et al*., 2017). Short chain fatty acids are secondary metabolites produced from the biological breakdown of Non-Digestible Carbohydrates (NDC) by the gut residents. These carbohydrates escape degradation by the digestive enzymes and are established in the colon where the gut microbiota uses them as a substrate to produce propionate, formate, acetate, lactate and butyrate (Morrison and Preston, 2016).

The SCFAs are readily ingested by the mucosal epithelial cells in the gastrointestinal tract and are involved in cellular regulations such as gene expression, proliferation, chemotaxis, apoptosis and differentiation of the immune cells (Corrêa-Oliveira *et al*., 2016). Production of acetate is common among bacteria groups such as *Bifidobacterium, Lactobacillus*, while propionate, butyrate and lactate production is through a distinct and highly specificpathway (Murugesan *et al*., 2017). In the gastrointestinal tracts, propionate is majorly produced by members of the phylum Bacteroidetes. Butyrate production is associated with the phylum Firmicutes, especially strains belonging to *Faecalibacterium*

prausnitzii, Eubacterium rectal, E. hallii and *Ruminoccocus bromii* (Flint *et al*., 2015). Butyrate serves as energy source to the colon and also impact human metabolic health by acting as anti-inflammatory and anticancer agents. Acetate and lactate provide unfavourable environment for the proliferation of pathogens. The role of SCFAs on human health is extensive.

1.5. Scope of the problem

Microbiome biology is an emerging field which has expanded as a result of next generation sequencing platforms and bioinformatics tools that are now available for characterisation and analysis of microorganisms in an environment. In developed countries, these platforms have been used to characterise non-cultivable microbes living in different niches of the human body as well as other environmental niches. Many studies have been conducted on the gut microbiota of infants in other countries, particularly those in Europe (United Kingdom and Italy) and North America (United States of America and Canada) (Fallani *et al*., 2010; Wopereis *et al.*, 2014; Hansen *et al.*, 2015; Hill *et al.*, 2017).

Reports from these studies are insightful and have informed the role of microbiome in child's growth and development. However, there are insufficient data from Nigeria on the subject. In addition, most of the studies carried out in western nations have been single-time-point studies rather than longitudinal ones. A single-time-point study may not reveal the changes that take place in the development of infant gut microbiota overtime.

Diet is one of the factors reported to have great influence on the gut microbiota (Agu *et al*., 2019). During weaning, Nigerian infants consume basic diet made from millet, maize and sorghum which is highly rich in fibre. Some of these fibres do escape the digestive enzymes in the gut and find their way into the colon where the gut microbiota use them as a substrate for fermentation process to produce metabolites such as short chain fatty acids. These SCFAs play a key role in gut homeostasis. The impact of these local high fibre foods on Nigerian infant's health through the SCFAs is highly important.

1.6. Rationale for the study

The development of the infant gut microbiota, and the changes that occur in the human microbiota from child birth to two or three years thereafter when the microbiota resembles that of an adult is very crucial. Most studies report the microbiota at birth, especially the microbiota of first-pass faeces, meconium within 24 hours of birth (Hansen *et al*., 2015), 3 days (Biasucci *et al*., 2010), 1 month (Penders *et al*., 2006) and at 6 weeks (Fallani *et al*., 2010; Walker *et al*., 2015). However, there are some internal and external factors that come to play during the development of the infant's microbiota; these could cause a switch in the gut composition. Many studies in the western countries have reported effects of various factors on the composition and diversity of the infant gut microbiota but no study has been done in Nigeria where prenatal and postnatal care is quite different and antibiotics use in children is not regulated. Factors such as delivery mode, diet, and antibiotics use can alter the composition of infants gut (Rutayisire *et al*., 2016). The switch that occurs between the preweaning and weaning period is also dynamic and drastic; however, very few works have been done to understand the changes that occur between these periods (Fallani *et al*., 2011). A longitudinal study is therefore needful to observe any changes that occur during these stages.

Moreover, limited data on the gut microbiota composition and diversity of Nigerian infants within the first year of life leave questions on what constituted the gut of these infants during their first years and early developmental stages. Other point to consider is the impact of introducing the basic diet (maize, millet and sorghum) on the gut microbiota composition and diversity. This is very important because geographical location is thought to have an influence on the gut microbiota due to differences in the diet and lifestyle. For instance, American and European diet and lifestyle are at variance with the African diet and lifestyle. While the diet in the western world is rich in proteins and fats, African diet is rich in carbohydrates (fibre) and low-fat diet (De Filippo *et al*., 2010; Ayeni *et al*., 2018).

1.7. Study hypotheses

- Vaginally delivered infants will have more beneficial microbes.
- There will be changes in the gut microbiota of Nigerian infants within the first year of life with associated microbial signatures.
- Nigerian basic diet during preweaning and weaning will influence the infant gut microbiota composition.
- There will be a correlation between the gut microbiota composition and short chain fatty acids production in Nigerian infants gut within the first year of life.
- Antibiotic use within the first year of life will influence the gut microbiota composition and prevalence of antibiotic resistance genes in the gut of Nigerian infants.

1.8. Research questions

This research intends to answer the following questions:

1. Does delivery mode have any influence on the gut microbiota of Nigerian infants?
2. What are the changes in the gut microbiota that could occur overtime?
3. Could there be any distinction between the gut microbial contents of Nigerian infants at preweaning and weaning periods?
4. What type of short chain fatty acids would be present in the gut of the infants within the first year of life?
5. What is the impact of antibiotic use on prevalence of antibiotic resistance genes in infants gut and their microbial gut composition?

1.9. Aim and objectives

The general aim of this study is to evaluate the changes in the gut microbiota of some Nigerian infants within the first year of life.

The specific objectives of the study are:

1. To evaluate the influence of mode of delivery on infants gut microbiota composition

2. To analyse the changes in the gut microbiota composition of selected Nigerian infants within the first year of life.

3. To compare variations in the gut microbiota of Nigerian infants during preweaning and weaning periods.

4. To evaluate the presence of short-chain fatty acids in the infant faeces within the first year of life.

5. To determine the occurrence of antibiotic resistance genes in the infants faeces and the influence of antibiotic use on the infants gut microbiota composition.

CHAPTER TWO

LITERATURE REVIEW

2.1. The human gastrointestinal tract (GIT)

The human gastrointestinal tract (GIT) is a complex structure that comprises the stomach and the intestines. The tract is like a canal that runs from the mouth to the anus and it is divided into upper and lower gastrointestinal tracts. It is further divided into the foregut, midgut and hindgut. The upper GIT consists of mouth, pharynx, oesophagus and the stomach while the lower GIT comprises of the Small Intestine (SI), Large Intestine (LI) and the anus. The upper GIT component forms part of the foregut with the exception of the forepart of the duodenum. The major function of the upper GIT is the ingestion and digestion of food particles, it also performs a protective function. The lower GIT comprising of small intestine is divided into 3 compartments namely: duodenum, jejunum and ileum. Completion of digestion process takes place in the small intestine. The large intestine is also divided into 3 parts namely: the caecum, colon and the rectum. The LI partakes in absorption, assimilation and excretion of food and other metabolic substances (Kumar, 2017).

The gut is an enriched community for microbes as theyenhance the function of the gut. Also, the gut originating from the mouth is an entrance for food coming from the environment. Therefore, the gut is not only colonised by the resident organisms but with myriads of microbes coming from the environment. The total number of microbes residing or situated in the gut has been earmarked to be more than 10^{14} (Bäckhed *et al*., 2015) and that exceeds the number of human genome. It is generally believed that bacterial genome in the human body outnumbers that of human genome 10 times, in ratio 10:1 (Bäckhed *et al*., 2015; Hansen *et al*., 2015; Hill *et al*., 2017; Thursby and Juge, 2017a).

The large genomic content of the gut microbiota and its vast metabolic activities enabled their cohabitation with the host. The gut microbiota thus performs beneficial roles such as

progression of the host immune system, restoration of host gut homeostasis, anti-inflammatory and protective function (Pickard *et al*., 2017).

2.2. The Microbiome

The entire number of the genes of microorganisms residing in the body is called human microbiome. Microbiome can thus be applicable to different communities such as the human, animal, aquatic, soil and plant bodies (Lederberg and McCray 2001).

2.2.1. The Human Microbiome

The idea of the human microbiome was first proposed by Joshua Lederberg. He defined microbiome as the 'ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space" This was initially referred to as "the normal flora" now refers to as Human Microbiome (Figure 2.1). Culture-based method has been the usual method of cultivation since 19th century. However, it is not a sufficient tool for gross populated microbial communities as a result of some non-cultivable species that may be present in such environment. The DNA-based analyses, most especially the next-generation sequencing has broadened our understanding of the type of grossed populated communities by generating enormous new data set that gave insight to the composition, diversity and functionality of a greater number of microbial communities (Huttenhower *et al*., 2012).

This method was employed in 2007 for Human Microbiome Project (HMP) launched by the US National Institute of Health to study microorganisms occupying different body niches. Likewise, to demonstrate the association between microbiome and diseased or healthy state. It is a known fact that the number of bacteria in the human body outnumbered that of human cells in multiples of 10 and the greatest number of these

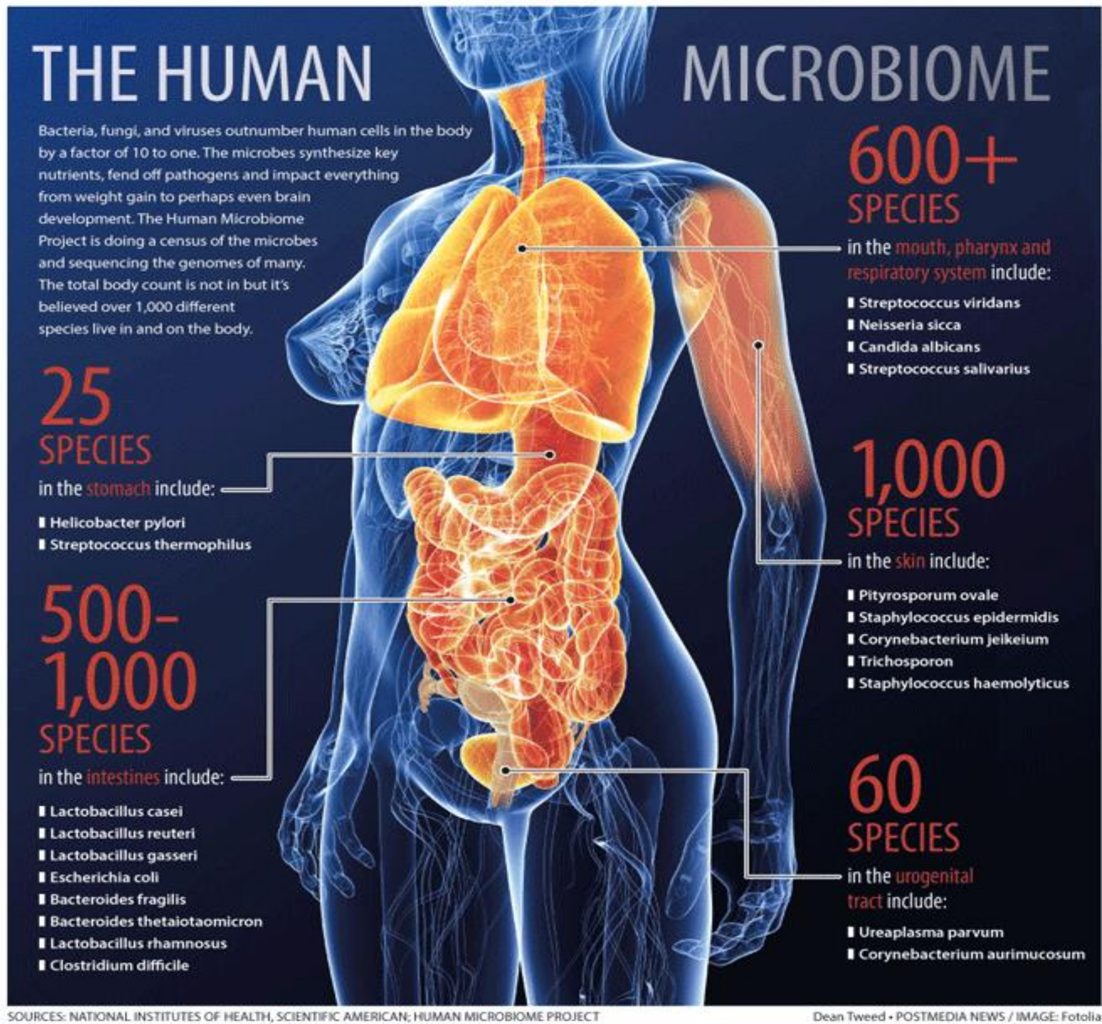

Figure 2.1. The Human Microbiome

Adapted from Human Microbiome Project Working group, 2009

bacteria lived in the gastrointestinal tract (Sender *et al*., 2016). This group studied the microbiome in four distinct sites; the gastrointestinal tracts, skin, mouth and the vagina.

Culture-dependent methods have been in used over decades. However, it is limited by inability to identify some microorganisms. Majority of the human microbiota are culture-independent. The analyses of the conserved regions of 16S rRNA genes have been a useful tool in the study of mixed microbial communities. 16S rRNA genes are used extensively to study microbial diversity in the gut, oral, vagina, and also the development of microbiome in infants and adult (Palmer *et al*., 2007; Ayeni *et al*., 2018).

2.2.2. The Gut Microbiome

The human gut is inhabited by a vast number of bacteria which tag the gut microbiome as a "super organism" (Fouhy *et al*., 2012). The gut microbiota plays an important role in the development of the immune system in infants. The host health can be related with the diversity and stability of the gut microbiota. It should be noted that the infants gut is mostly dominated by the phylum Actinobacteria (majorly *Bifidobacterium*) and Proteobacteria, (most especially the Enterobacteriaceae), while that of adult is dominated by the Firmicutes and Bacteroidetes (Jandhyala *et al*., 2015). The gut microbiota performed various functions such as metabolic; for example, amino acids synthesis, production of short-chain fatty acids, and activation of glucose homeostasis. Structure-dependent functions like gut permeability regulations, immune system and intestinal permeability regulations, and protective functions such as debarring the colonization of pathogens, enhance the ability of innate and adaptive immunity, fat-soluble vitamin absorption and others (Blandino *et al*., 2016).

2.3. Gut microbiome of infant

The infant gut microbiome is thought to develop from birth and establish its cohabitation with the host. Initially the uterine was believed to be sterile and that colonisation starts

after delivery was a dogma (Perez-Muñoz *et al*., 2017). However, newer studies using both cultivable and non-cultivable techniques have proved the presence of microbiome in the foetal sac, foetal membrane, amniotic fluid, Umbilical Cord (UC) blood and in first-pass faeces, meconium. A culture-dependent method revealed the presence of bacterial genera *Streptococcus, Enterococcus, Staphylococcus* and *Propionibacterium* in the UC blood. Culture-independent method had indicated the presence of *Bifidobacterium* and *Lactobacillus* in the foetal sac (Aagaard *et al*., 2014; Aloisio *et al*., 2016). Members of the phyla Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes and Fusobacteria were also found in the foetal sac microbiome. All these accounts for the prenatal factors which the authors believed that bloodstream could be the medium of transfer of microbiota to the infant in the uterus (Aagaard *et al*., 2014). Regardless of either the uterine is sterile or not, the mode of birth contributes greatly to the gut microbiota of infants. Those born vaginally harbour mother's vaginal microbiome, such as Lactobacilli and Enterococci while those born via caesarean section birth harbours the mother's skin microbiota such as Staphylococci and *Propionibacterium* as well as birth environment microbiota such as Clostridia (Rutayisire *et al*., 2016). When the babies began to feed on milk, Bifidobacteria becomes more prominent in their gut (Matamoros *et al*., 2013).

2.4. Phyla composition of infant gut

The human gut generally is dynamic and composed of different microbes namely archaea, bacteria, fungi and viruses. Among this list, bacteria are the most studied. Adults gut microbiota is different from infant's gut microbiota due to its maturity and stability. While that of infants is still undergoing maturation and it is less stable until 3 years of life when it begins to mimic adults microbiota (Backhed *et al*., 2015), the adult gut is composed of the following microbiota, Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Proteobacteria, Fusobacteria, Tenericutes, Spirochaetae, Verrucomicrobia and Cyanobacteria (Rinninella *et al*., 2019). In infants, the major phyla dominating their gut is the member of the phylum Actinobacteria, Firmicutes, Bacteroidetes, and Proteobacteria (Rodríguez *et al*., 2015). These phyla are discussed below.

2.4.1. Actinobacteria

Actinobacteria are Gram-positive bacteria and have a high G+C content of DNA in its genome. The phylum comprises both anaerobic and aerobic species, filamentous and spore-forming lineage. They are ubiquitous and highly diverse in nature. They can be found in the soil, water and human bodies. Above all, they have great economic importance, majority of them produce useful metabolites that have served as antibiotic, anticancer, antihelminthic and antifungal agents. Members of the phylum Actinobacteria belong to different groups based on their ecological habitat. Examples of soil Actinobacteria are *Streptomyces* and *Actinomyces* plant Actinobacteria, *Frankia.* Actinobacteria that are human and animal pathogens are *Mycobacterium*, *Corynebacterium*and Norcadia. The prominent gastrointestinal group is the *Bifidobacterium* species among others (Barka *et al*., 2016). Actinobacteria is the largest phylum that is reported to dominate the infant's gut. The genus *Bifidobacterium* belonging to this phylum predominate the gut of infants and also acts as probiotics. The human milk oligosaccharides serve as a substrate for its proliferation in the gut of infants. *Bifidobacterium* has proved to be a prominent probiotic that confers health beneficial impacts to the host (Scott *et al*., 2015). In addition, member of this genus serves as producers of vitamin, such as vitamin B and K (McNabney and Henagan, 2017). They also help in the restoration of gut homeostasis after antibiotic intake. They released acetic and lactic acids as their secondary metabolic product which protects the gut from being colonised by pathogenic organisms. The importance of Actinobacteria is vast in the human gut in general.

2.4.2. Firmicutes

Firmicutes are Gram-positive rods, with low guanine plus cyctosine (G+C) DNA content. Some species are spore-formers and some are non-spore formers. Some members are pathogens such as *Streptococcus pyogenes*, *Staphylococcus aureus*, *Bacillus subtilis*, *Clostridium tetani* e.t.c., some are beneficial as they help in the fermentation of dairies such as yoghurt and cheese (Wiley *et al*., 2017). Interestingly members of Firmicutes form

part of the largest group of bacteria found in human and other mammalian gut. The gut Firmicutes are difficult to characterise with culture-dependent method due to their anaerobic growth requirement. Culture-independent methods have made their diversity in the human gut explicit. The normal adult gut is usually dominated by Bacteroidetes and Firmicutes, their ratio have make a big difference between the obese individuals and lean persons (Turnbaugh *et al*., 2009).

In infants, Firmicutes are found in the meconium samples. Members of the genera*Enterococcus*, *Staphylococcus*, *Streptococcus* and *Lactobacillus* have been found to dominate the first passed stool of a baby (Perez-Muñoz *et al*., 2017). Firmicutes is also associated with the initiation of solid foods in infants. Due to plant-based polysaccharide contained in the weaning diet of infants, members of *Ruminococcus*, *Roseburia*, *Faecalibacterium* and *Dalister* that are known butyrate producers are found in infants gut during the weaning period. Butyrate is a short-chain fatty acid known for its energy harvest from the diet which serves as a sole energy source for the colon and also acts as an anti-inflammatory agent preventing colorectal cancer (Louis *et al*., 2014).

2.4.3. Bacteroidetes

Bacteroidetes is a phylum that is known for its diversity. They are Gram-negative bacteria which are found basically in all the earth habitat, soil, freshwater bodies, ocean, and most importantly, in the gut of mammals and sewage treatments plant. In any of the habitat where they are found, they perform important functions of degradation of complex molecules such as cellulose, proteins and polysaccharides (Thomas *et al*, 2011).

Many of the members of phylum Bacteroidetes have genera that are pigmented, orange or yellowwith flexirubin or carotenoids. Some that possess flexirubin are chlorinated, a very uncommon characteristic of biological molecules. C*ytophaga* and *Sporocytophaga* are known for the degradation of cellulose. The genu*s Flavobacterium* is mostly found in soil and invertebrate animals where they cause serious infection. This genus also belong to the

gliding motility genera, their motility differs from the flagellated motility. Gliding motility genera of the phylum Bacteroidetes can travel as far as 120 µm per minute while some are faster. This motility is driven by glide proteins found in the motors assembled in the plasma membrane of the organisms and protrude to periplasm. The important genus found in the gut of human and the rumen of ruminants is *Bacteroides*. There is a myth that Bacteroidetes associated with mammals have their origin from the environment and they co-evolved with humans (Wiley *et al*., 2017).

Generally, there are three clades that are common in the human gut, they are *Bacteroides*, *Prevotella* and *Porphyromonas* (Weaver, 2016). Each of them performs roles that are vital to man. However, in the state of immune compromise, or when they leave the gut environment and are found in the other body sites, they could cause serious diseases in the host. Genomic study of the phylum Bacteroidetes has shown that they possess polysaccharides utilization loci (PUL)**,** and they exhibit gene duplication and lateral gene transfer (LGT). These features probably contributed to its ability to adapt to different environmental niche including the human gut (Johnson *et al*., 2017). Bacteroidetes are used as a biomarker between obese and lean people. In a study conducted on obese individuals, Ley and colleagues reported a low occurrence of Bacteroidetes in the obese people compared to lean people before dietary intervention, and after the intervention, the relative abundance of Bacteroidetes increased (Ley *et al*., 2006).

In infants, Bacteroidetes have been linked with the initiation of solid foods (Fallani *et al*., 2010). However, vaginally delivered infants usually have increased abundance of the genus *Bacteroides* compared to their caesarean section delivered counterparts (Rutayisire *et al*., 2016). Usually, *Bacteroides* are found in the gut of formula-fed infants (Liu *et al*., 2019). Genus *Prevotella* has been reported most especially in African children that feed on high fibre plant-based foods (De Filippo *et al*., 2010; Ayeni *et al*., 2018). Their ability to degrade non-digestible polysaccharides of which the product is short-chain fatty acids which is beneficial to the immune development of children as well as confers improved health benefit to adult is noteworthy.

Furthermore, the clade Bacteroides are mostly associated with diets rich in animal proteins, high in fat usually common among western dwellers and clade Prevotella related to high fibre rich foods, low fat and moderate proteins common among non-westerners mainly Africans.

2.4.4. Proteobacteria

Proteobacteria is a vast phylum that consists of diverse bacterial groups that are pathogens of animal and humans and also plays essential part in the recycling of nutrients in the ecosystem (Willey *et al*., 2017). There is variation in the Guanine plus Cytosine constituent of their DNA, ranging from low to high content (Gupta *et al*., 2007). The genomic sequencing of their 16S rRNA depicts five lineages within the phylum. These classes are the *Alpha*proteobacteria, *Beta*proteobacteria, *Gamma*proteobacteria, *Delta*proteobacteria and *Epsilon*proteobacteria. The *Alpha*proteobacteria are oligotrophs, they have the ability to live in an environment with low or little nutrient. Genus *Rickettsia* and *Chlamydia* belong to this class, they areobligate intracellular pathogens. The *Beta*proteobacteria class are eutrophs, they require a large amount of organic nutrient for their survival. Some genera under this class are *Bordetella*, *Neisseria*, *Burkholderia*, *Leptothrix*, and *Thiobacillus* among others (Hutt *et al*., 2017).

The class *Gamma*proteobacteria constituted the enteric organisms that inhabit the gut of mammals, they are the biggest and most prominent group in the animal and human intestine. They are Gram-negative rods and mostly pathogens. Some of them are normal flora of the animal and human gut. However, they are potential pathogen which possesses the ability to become pathogenic in immuno-compromised individuals. Member of the family Pasteurellaecea, for example, *Pasteurella haemolytica,* is a biological agent that causes respiratory diseases in animals and members of the Genus *Haemophilus* (*Haemophilus influenza*) is the agent that causes pneumonia, meningitis and other invasive diseases in children before the advent of vaccine (Ulanova and Tsang, 2014). Prominent in this group is the genus *Legionella*, bacteria associated with warm water pool such as the

air conditioner water. The outbreak of Legionnaires diseases has been reported among the dwellers of the building where this species was found (Leoni *et al*., 2018).

Family Enterobacteriaceae is a big family of intestinal bacteria that comprises of numerous genera. They are mostly facultative anaerobic bacteria that possess the ability to ferment carbohydrates. The family Enterobacteriaceae belong to two groups namely; coliforms and noncoliforms. Coliforms are those species that are able to ferment lactose with the end product of gas and acid while the noncoliforms are those unable to ferment lactose or ferment it partially with the release of gas or acid and not both (Martin *et al*., 2016). Examples of coliforms are *Escherichia coli*, examples of noncoliforms are *Shigella* and *Salmonella*. The class *Delta*proteobacteria is a small group in the Phylum Proteobacteria but very essential in the recycling of nutrients in the environment as well as detoxification. A good example is a sulphate reducing bacteria, *Desulfovibrio*.

The last and the smallest class in the Phylum Proteobacteria is the class *Epsilon*proteobacteria. They are the microaerophilic bacteria. Genus *Helicobacter* species *Helicobacter pylori* lives in the gut of human and usually caused gastritis and the genus *Campylobacter*, species *Campylobacter jejuni* a common pathogen of chicken which could be transferred to humans through impropercooked chicken (Wiley *et al*., 2017). Proteobacteria have been implicated in dysbiosis conditions in humans especially in IBD, asthma and chronic obstructive pulmonary disease (COPD).

2.5. Factors influencing the gut microbial composition and diversity of children

Different factors influenced the gut microbiota of children. These could be intrinsic or extrinsic factors. The intrinsic (prenatal) factors include; maternal microbiota, host genetics and gestational age. The extrinsic (postnatal) factors include; delivery mode (vaginal or caesarean section birth), feeding method (breastfeeding or formula feeding), antibiotic usage, prebiotics or probiotics use, geographical location and lifestyle (Rodríguez *et al*., 2015).

2.5.1. Mother's Microbiota

The uterine was believed to be sterile and microbiota is acquired during or after birth was an accepted dogma for more than a century (Rodríguez *et al*., 2015). Cultured and non-cultured studies have shown that microorganisms are present in the amniotic fluid, foetal sac, meconium, umbilical cord blood, and foetal membrane (Perez-Muñoz *et al*., 2017). Despite the presence of microorganisms in these niches, there have not been any sign of infection or inflammation that has led to preterm labour. These investigations were carried out in healthy mothers and babies. The route of colonisation of these organisms is not well understood but possibilities of transmission through the placenta have been reported (Aagaard *et al*., 2014). Generally, gut epithelial acts as a barrier against such translocation, however, the dendritic cells are capable of piercing the gut epithelium, pick up bacteria from the gut lumen and convey it throughout the circulatory system as they moved to the lymphoid organ. Meconium microbiota is different from the faecal microbiota as reported in a study (Gosalbes *et al*., 2016). Meconium was dominated by Firmicutes while faeces were dominated by Proteobacteria.

2.5.2. Gestational age

The normal gestational age for a term baby is 38 ± 2 weeks for singleton and 32 ± 2 weeks for multiple babies. Preterm baby birth weight is 28± 2 weeks (Lanzone *et al*., 2014). The age of the baby before delivery is one of the building blocks that aids the colonisation and development of the microbiota in the gut. Studies have shown different microbial composition and diversity in the two milieus. Preterm babies are immature, and all their organs and systems are immature including their immune system. This predisposes them to infection and diseases early in life. Consequently, they are exposed to antibiotics early in life, coupled with long-time stay in the hospital which could make themsusceptible to hospital-acquired infection. The use of aided feeding such as the NG tube and inhaled oxygen to aid their respiration and other unhealthy exposures usually contribute to their altered microbial colonisation process (Milany *et al*., 2014).

Many studies have been done to differentiate between the gut microbiota of preterm and term babies. It has been discovered that *Bifidobacterium* and *Bacteroides* are usually slow in appearance in preterm gut compared with the term babies. Their gut is rather composed of more of Enterobacteriaceae, *Enterococcus*, *Staphylococcus*, *Veillonella*, *Clostridium* (Aloisio *et al*., 2016; Hill *et al*., 2017). Delay in transition from infant-hood to adult-like microbiota has been attributed to preterm birth (Groer *et al*., 2015). Metabolic dysfunction have been observed in preterm babies with a lower number of genes coding for carbohydrate metabolism and a greater number of genes for lipid metabolism which increased the incidence of inflammatory diseases in them (Louis *et al*., 2014). It has been shown that preterm babies experience rapid growth at age 2 to 8 years, this rapid growth has been linked to the later incidence of resistance to insulin and coronary artery disease (Groer *et al*., 2015).

2.5.3. Host genetics

Host genetics is still a topic of debate. The relationship between the gut microbiota and host genetics is not well established. However, some researchers have used humans, animals and comparative species studies to understand this relationship. In human studies, twins and familial relationship study that used the fingerprinting method confirmed that host genotype has an influence on the gut microbiota with monozygotic twins having higher relatedness (Zoetendal *et al*., 2001). Another human study on children under the age 10 revealed similarities in the composition of identical twins when compared with non-identical twins (Stewart, 2005). A contrasting study that used metagenomics tool to study the gut composition of parent, monozygotic and dizygotic twins did not find any significant difference in the host genotype. Nevertheless, there was resemblance in the microbiota of family members (Turnbaugh *et al*., 2009). The impact of host genetics on the gut microbiota is clearer with the genome-wide association studies.

Many studies have considered the effect of host genotype on gut microbiota in animal using genetic inbred lines and germ-free mouse. Thesespecifically knock-out some mutational genes. A good example is the “Apoe-I knockout mouse with impaired glucose

tolerance”. This was used to evaluate the relationship between diet, host genetics and metabolic disorder. In their study, molecular method was used, and they found that genetic mutation of Apoe-I led to variation in the gut microbiota composition but diet had the highest quota (Zhang *et al*., 2015). Overall, the impact of the host genetic will greatly depend on the larger cohort study. The genome-wide association study may likely give a better understanding of the relationship. A recent study ascribed the relationship between metabolic syndromes with host genetic factor. The author reported that individuals with MetS had a lower diversity compared with the control. The microbiota was characterised with *Sutterella*, *Methanobrevibacter* and *Lactobacillus* in the MetS group, whereas *Akkermansia*, *Odoribacter* and *Bifidobacterium* were abundant in the healthy group (Lim *et al*., 2016).

2.5.4. Delivery method

Mode of delivery remains one of the major factors influencing the gut microbial composition of infants. Ultimately, the route of delivery determines greatly the initial colonisers of the guts. Those born through vaginal have a different microbial composition and diversity from those born through caesarean section (CS). Those born vaginally bears the mothers vaginal microbes such as *Lactobacillus*, *Sneathia*, *Prevotella* (Dominguez-Bello *et al*., 2010) while those born through CS harbours mothers’ skin microbes such as *Streptococcus*, *Staphylococcus*, *Enterococcus, Propionibacterium* and environmental microbes such as *Clostridium*(Hill *et al*., 2017).

2.5.5. Feeding method

The feeding method is one of the major factors that impact the gut microbiota of children. The feeding method from birth can be divided into 3 broad categories, preweaning, weaning and post-weaning. The preweaning period is when the babies are fed with milk. This could be divided into exclusively breastfeeding, breastfeeding and formula-fed and formula-fed only. The weaning period is characterized by the introduction of semi-solid

foods, and gradually solid food. According to the World Health Organization's recommendation, babies are expected to be breastfed exclusively for the first 6 months of life (Lawrence, 2007). However certain conditions such as the inability of the mother to lactate, career or multiple births (twins, triplets e.t.c.) enforce some mothers to either combine formula with breast milk or formula-fed their babies only.

The choice of food babies are fed with has an outcome on the present and future health of that child. In the point view of microbiota, it makes a great difference. The gut microbiota of exclusively breastfed babies' infants is mostly dominated with the genera Bifidobacteria and *Lactobacillus*. Culture-dependent and culture-independent methods supported this fact (Tamburini *et al.*, 2016). The human breast milk is said to possess diverse microbiota up to 700 species (Cabrera-Rubio *et al*., 2012). It also contains human milk oligosaccharides which promote the growth of beneficial organisms. All these features contribute to the microbial colonisation and development of infants. The weaning period is characterised with a more diverse gut microbial composition, microbes such as *Bacteroides*, *Blautia*, *Ruminococcus*, *Roseburia* and *Clostridium* have been reported (Fallani *et al*., 2010; Koenig *et al*., 2011).

2.5.6. Antibiotic usage

Antibiotic usage is one of the factors that could imprint the gut microbiota of children. Children are often exposed to antibiotics early in life either through the parents or direct administration. Much attention was on bactericidal activities of antibiotics with less attention given to the impact of antibiotics on the gut microbial composition. The main consequence of antibiotic usage on the gut microbiotaof infants is the reduction or exclusion of taxa from the gut community (Jandhyala *et al*., 2015). Effect of a short term dosage of Clindamycin has led to exclusion of Bacteroides in the gut. Also, the use of Clarithromycin in the treatment of *Helicobacter pylori* has led to reduction of Actinobacteria (Jenberg *et al*., 2007). Antibiotic intake usually causes disruption in taxonomic diversity of an ecological community. This disruption could result in the

exclusion of certain taxa which could persist up to 2 years or more (Ng *et al*., 2013). Antibiotic usage could necessitate the gut microbiota to serve as a reservoir for multi-drug resistance genes, thereby creating environment for horizontal gene transfer among gut residents. The pool of antibiotic resistance genes in infant's gut therefore resulted from either vertical transfer from mother to infants or horizontal transfer from the environment. Regardless of the means of acquisition of these resistant genes, accumulation in the gut has a devastating effect on the health of the infants.

2.5.7. Geographical location

Geographical location is another factor considered to influence the gut microbiota of infants. Different geographical locations have a different lifestyle, diets and culture. For example, western mothers may not breastfeed their babies at all, while some may breastfed for 3 months and consequently introduced baby formula. The infants weaning diets is usually a commercial-prepared cereal and by the culture, they do not feed their infants with bolus foods. This is in contrast with African cultural practices. Most African mothers breastfed their babies exclusively for 6 months in accordance with the World Health Organization guidelines. However, work demand or multiple babies usually reduce the breastfeeding period for some African mothers and infants. Weaning diets for African infants are usually plant-based cereals highly rich in carbohydrates and fibre (Abeshu *et al*., 2016).

Moreover, in addition to plant-based cereals, infant milk is added to supply the nutritional value the baby needs. The rural mothers only give their babies cereals without adding infant milk. African solid foods, especially Nigerian diets are typically bolus. Examples include yam, plantain, and wheat and also cassava flour made into a bolus. These are fed to some babies during weaning.

This is in contrast to what is obtained in the western world. Study on 6-month old Malawian infants with their counterpart of the same age from Finland showed a distinct

gut microbiota. In the study, *Bacteroides-Prevotella* was higher in Malawian infants compared to Finnish infants (Grzeskowiak *et al*., 2012). Also, study from six different geographical locations of Europe revealed a clear difference between the gut microbiota of 6-weeks old infants.Infants from Northern European countries had higher proportions of Bifidobacteria, whereas Southern European infants gut microbiota were dominated by*Bacteroides*(Fallani *et al*., 2010).

African children within the age of 1-6 years have been compared with European children of the same age. Children from Burkina Faso gut were enriched with Bacteroidetes and had a reduced Firmicutes, at the genus level *Prevotella* and *Xylanibacter* are dominant in Burkina Faso infants and totally absent in European children (De Filippo *et al*., 2010). All these emphasised the impact of fibre-rich diet which is typical to African but completely different from the European diet. Again, these help in delineating the cultural practices peculiar to different geographical location.

2.6. Functions of human gut microbiota

The human gut microbiota performs various functions. The functionality of the human gut microbiota is partly based on microbial metabolism. Gut microbiotais a ‘metabolic organ’ that play essential role in nutrient metabolism. They help in dietary degradation of carbohydrates such as resistant starch, plant-based polysaccharides and non-digestible oligosaccharides in the colon. Members of the genera *Prevotella*, *Ruminococcus*, *Roseburia* and others are involved in these activities. (Flint *et al*., 2012). They complement the vitamins derived from foods by producing vitamins K and B. Bifidobacteria and Lactobacilliperformed this role (LeBlanc *et al*., 2013).

Furthermore, gut microbiota uses indigestible fibre from diet as a substratum to produce SCFAs. These metabolites are transferred from the intestine to different tissues where they serve as an energy source, signal molecules that assist in the breakdown of lipids, cholesterol and glucose pathways (Kim *et al*., 2017). Also, SCFAs lower the pH of the

intestinal environment. As such, they serve a protective function against the proliferation of pathogens (Wopereis *et al*., 2014).

GM plays an essential part in the advancement of the immune system. Animal and human studies have validated this fact. GM encourage the development and maturation of B cells that produce IgA, assist gut-associated lymphoid tissues (GALT) proliferation, improves the function of the epithelial mucus layer in the gut and influences both pro-inflammation and anti-inflammation responses (Jakobsson *et al*., 2014).

Neurologically, the GM is believed to play a vital part. The evolution of an adequate composition of early life microbiota is said to be essential for different features of behaviours and physiology. The congregate of appropriate gut microbiota is associated with the development of the cognitive and emotional status of an individual. The consequence of impairment of the functional gut microbiota has led to anxiety, depression and other autoimmune diseases (Wiley *et al*., 2017).

2.7. Birthing methods

2.7.1. Caesarean section birth in Nigeria

Caesarean section birth is the process by which an offspring is born through the laceration of the lower abdominal part into the uterine where the baby is located. The rate at which caesarean section birth is on the increase globally is a great concern. The indication for caesarean section birth is divided into two, absolute indication and relative indication. The absolute indications for CS are maternal pelvic deformity, umbilical cord prolapsed, placenta previa, prolonged labour, uterine rupture, abnormal presentation among others. The relative indication is previous caesarean section birth, prolonged labour (especially for mothers carrying pregnancy for the first time, primigravida) and others (Begum *et al*., 2017).

In Nigeria, the incidence of caesarean section differs from one region to another. For instance, a five-year study of CS incidence in the University of Abuja teaching hospital had a rate of 21.4% (Isah *et al*., 2018). Another 2- year study conducted in UDUTH, Sokoto, NorthWest, Nigeria gave an incidence of 11.3% (Nnadi *et al*., 2016). Similarly, a six years retrospective study from OOUTH, Sagamu, South West, Nigeria gave a rate of 32.9% (Akadri and Odelola, 2017). According to the general trend reported regionally, nationally and globally from 1990 – 2014, the incident of CSB in Nigeria seems to have declined from 2.9% to 2% (Betrán *et al*., 2016) but the above report showed a higher rate years after the study. Although not all regions in the country submitted their report, however, WHO and UNICEF did not give a concurrence rate on CS incidence in Nigeria. Retrospective studies from tertiary teaching hospitals indicate a rise in the rate of CS in the country which is gradually tending above the threshold of CS incidence which is between 10-15% as proposed by World Health Organization (Nnadi *et al*., 2016).

2.7.2. Caesarean section birth in other Countries

The trend of caesarean section birth (CSB) is on the increase globally, this poses a serious concern to the health care settings generally. The procedure is actually meant to rescue the lives of mothers and their newborns from complications which could lead to the death of either the mother or newborn or both in some cases. However, caesarean section birth has been abused, mothers now opt for CSB without any history of medical indication, and this contributes to the increase in CSB experienced globally in decades. Starting from African countries, the poorest countries had less than 1% rate probably because of lack of access to medical facilities even when there is indication for CS.

CSB has a low incidence in the sub-Saharan African countries (less than 2%), but Rwanda, Ghana, Lesotho, Uganda and Kenya had CS rate greater than 5%. Also among the richest countries, the CS rate out shoots the WHO threshold of 15%, most especially in Indian, Pakistan and Bangladesh (Cavallaro *et al*., 2013). A global report of CS rate from 1990-2014 also gave the incidence of CS in Africa to be 7.3%, Asia 19.2%, Europe 25%,

Oceania 31.1%, North America 32.3%, North American and the Caribbean had the highest incident of CS rate (Figure 2.2). These data was gotten from a 24-year survey of CS rate in 151 countries sectionalised regionally and nationally (Betrán *et al*., 2016). The fact is that caesarean section birth is on the high increase globally most especially in the western world and this is gradually coming down to Africa which in some cases has a negative health implication in infants.

The negative implication of CS involves increased mortality and morbidity rate of mothers following complication from surgical procedures. Also, caesarean section birth babies have a different bacterial composition, hormonal make-up, physical and medical encounter that are different from their vaginal counterparts. Hence these features could perturb the infant's physiology.

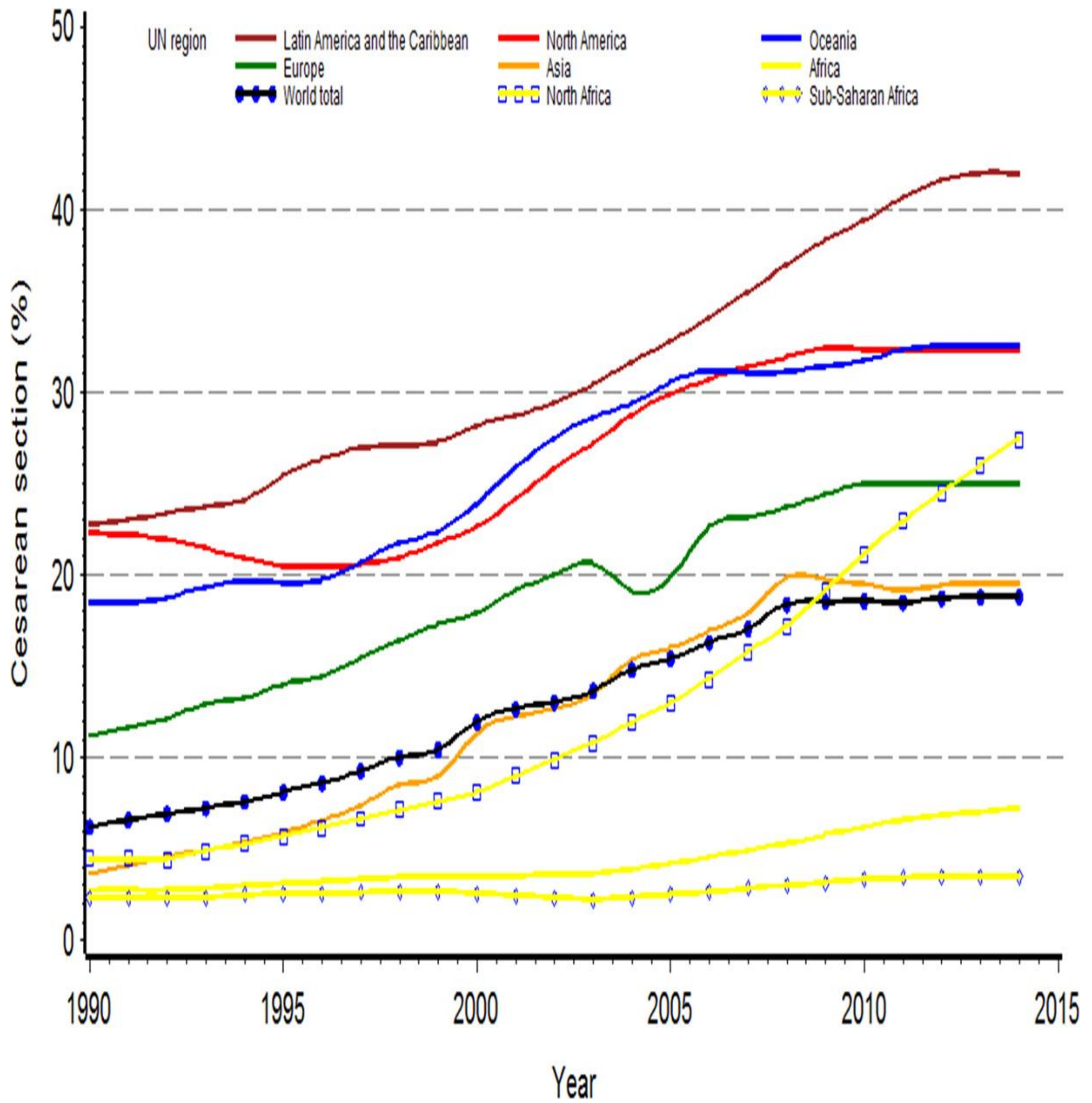

Figure 2.2. Global and regional rate of caesarean section between 1990 – 2014 (Betrán *et al*., 2016)

2.7.3. Vaginal birth in Nigeria

Vaginal birth is the process of giving birth through the birth canal. However, vaginal birth is not without complication especially among girls and women in the Northern part of the country where early marriage is legalised and traditional believes on use of orthodox medicine is a dogma. One of the complications associated with vaginal birth is obstetric fistula. This is a condition in which a hole is created in between the vagina, the rectum and the bladder leading to incontinence in urination or defecation through the vagina.

The rate of "obstetric fistula" is high in Asia and Sub-Saharan African. More than 2 million young women are suffering from this ailment without treatment (WHO, 2016). Despite the stand of UNICEF against marriage of females under 18 years, Nigeria still had 43% under age marriage incidence and this is common in the Northern region of the country. This account for the high prevalence of obstetric fistula among this ethnic group (Amodu *et al*., 2017). This condition is so peculiar to girls and women in this region that they have a praise song for females experiencing the condition in the Hausa language. It seems this is the only complication associated with vaginal birth in Nigerian women. This problem usually resulted into social stigmatisation.

Vaginal birth is associated with a significant microbial signature which perhaps seems to select for colonisation by members of *Lactobacillus*. Infants born vaginally harbour the mothers'vaginal microbiome. However, if the mother harbours unhealthy vaginal microbiome, the baby can pick it up from the mother and this could lead to dysbiosis condition. The birth method is one of the fundamental factors that contribute to infant gut microbial colonisation (Hansen *et al*., 2015; Rutayisire *et al*., 2016).

2.7.4. Vaginal birth in other countries

Vaginal birth is the method of delivery through the birth canal. However, due to the stress of labour and "pains" experienced during labour. Women from the western world found caesarean section convenient, the vast health care provider and the competence in CS

procedure also contributed to the confidence these women have in caesarean section birth. This and other factors have led to an increase in the incidence of caesarean section birth and decrease in vaginal birth in the western countries (Nilsson *et al*., 2017).

Moreover, in other to reduce CSB, vaginal birth after previous CS is now considered safe. Previous CS automatically predisposed to subsequent CS. Recently Gynaecologists have improved on handling VB after previous CS, this avail women the opportunity to have vaginal birth after CS. The rate of VB after previous CS in the United State of America is 32%, United Kingdom 26%, Australian 32%, European countries (45-55) % (Schemann *et al*., 2015). This practice greatly reduced the rate of CS in some countries; however, some women do not agree with vaginal birth or trial after previous CS. More studies need to be carried out to ascertain how vaginal birth after previous CS thrives in these countries.

2.8. Feeding methods among infants in Nigeria

Nigerian infants' feeding is very unique in its design. The method varies based on geographical location. The feeding practice in one region differs from another region. The World Health Organisation (WHO) endorsement for infants feeding is that baby should be breastfed exclusively (without any other food or liquid even water) for the first 6 months and continued up to 24 months of life alongside complementary foods (WHO, 2017). The outset of complementary feeding should start at 6 months and should be given two to three times daily up to 9 months and up to 5 times with alternation with snacks in between meals for the rest of infancy, as recommended by WHO (World Health Organisation, 2016). Despite this guideline, not all Nigerian women adhere to it. While some are compliant, some start complementary feeding from birth especially in the rural area. This disparity could select divergence in the gut microbiota of Nigeria infants. In most cases, there are reasons for non-compliance with exclusive breastfeeding, such conditions are lactation deficiency, work demand, infectious diseases and child adoption from infancy.

In a study conducted on 684 infants, record from the "Nigeria Demographic and Health Survey" an average rate of exclusive breastfeeding for less than 6 months was 16.4% and 7.1% in 5 months old babies. This is suggestive of low EBF rate in the country. It is evident that this rate is not substantive enough to prevent infectious diseases according to child survival Millennium Development Goal in 2015 (Agho *et al*., 2011). The World Bank Development indicator reported a slight increase in the rate of breastfeeding from 15.4% in 2000 to 23.3% in 2017 (WDI, 2017). The premium news from the Ministry of Health in 2018 reported a 24% incidence (Ministry of Health report, 2018). However, this rate is considerably low to 50% target in 2018. Effort should be put in place to achieve this goal.

2.8.1. Exclusive breastfeeding (EBF)

Exclusive breastfeeding is essential for the total well-being of infants. It is the method by which infants are fed with only breast milk without any other food, fluid and water. Studies have shown that feeding infants exclusively with breast milk for the first 6 months of life is helpful for the prevention of diarrhoea in infancy, especially in the developing countries (Lamberti *et al*., 2011). Other diseases such as respiratory diseases, otitis media, and obesity are well controlled with breastfeeding practice. Exclusive breastfeeding is beneficial to the growing infants. It helps psychomotor development, immune system progression, and prevention of cancer and restoration of amenorrhoea in mothers (Tromp *et al*., 2017).

Breast milk is enriched with a lot of nutrients and bioactive compounds that encourage the growth and development of infants. An important content, HMO present in the breast milk is a good substrate for the proliferation of *Bifidobacterium* a good probiotic that keep infants from infection, prevent inflammation, restore homeostasis and other important functions (Aloisio *et al*., 2016). Breastfed infants have shown the dominance of the members of the genus *Bifidobacterium* (Timmerman *et al*., 2017).

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2.8.2. Formula feeding

Formula feeding is the method by which infants are fed with commercially prepared milk only. The infant formula is usually prepared with essential nutrients needed for the proliferation and progression of infants, most especially with some components of breast milk to mimic it.

Randomized clinical trial on starter formula that examined the safety and effectiveness of infant formula with bovine milk oligosaccharides (BMO) and *Bifidobacterium animalis* subsp *lactis* reported that these supplements were well tolerated in infants and also showed a sign of enhancement of intestinal markers. It simulates the prebiotics and probiotics present in breastmilk (Radke *et al*., 2018). A study on the analyses of the faecal microbiome of 164 infants revealed an increased *Bacteroides fragilis*, *Clostridium*, *Escherichia coli* and *Bifidobacterium lactis* in formula-fed infants and associated this with the risk of coeliac disease (De Palma *et al*., 2012). Paediatricians believe that formula feeding alone has some clinical consequences especially the anti-inflammatory properties. Some of the formula constituents are not needed by the infants and they do not possess the essential system or their organs are not matured enough to metabolize them.

2.8.3. Mixed feeding

Mixed feeding practice regarded as complementary feeding by WHO is intended for 6-24 months infants (WHO, 2016). It indicates that the infant must be on exclusive breastfeeding until this period. Mixed feeding is the method by which infants are fed with additional milk other than the breast milk for the first 6 months of life.

Mixed fed infants GM has been reported to be more diverse than their exclusively breastfed counterpart (Timmerman *et al*., 2017). The formula is being fortified with prebiotics, especially fructooligosaccharide to imitate the breast milk, this prebiotic serves as a substrate for the growth of Bifidobacteria. Combination of breastfeeding with formula

feeding could possess synergistic effect on the gut microbiota of infants and, therefore, imprint it.

A clinical trial carried out oncaesarean section and antibiotic-treated infants to understand the effect of probiotics on the gut microbiota showed a decrease in Proteobacteria and Clostridia and increased abundance of Bifidobacteria. These infants were fed with *Bifidobacterium breve* Bb99, *Lactobacillus rhamnosus* GG, *Lactobacillus rhamnosus* Lc705 and *Propionibacterium freundenreichii* subsp. *hermanii*. The breastfed infants had higher *Bifidobacterium breve* and *Lactobacillus rhamnosus* recovered from their faecal samples, whereas the formula-fed did not show any increase in these species (Korpela *et al*., 2018).

2.9. Antimicrobial resistance

Antimicrobial resistance is defined as the ability of an organism to resist the effect of antibiotics that it was originally susceptible to (Li and Webster, 2018). It is a universal menace that ravages the human, animal and the ecosystem at large. It affects all cadres of population age, infants, children, adults and animals the same way. Effort to curb this menace remains a challenge. There is optimism that if the environment is free from microorganisms, then antibiotic resistance genes would be eradicated. But, no microorganism, no functional environment. Antibiotics were a solution to the primitive age when people were dying of infectious diseases.

The advent of the first antibiotic penicillin in 1928 and the first synthetic antibiotics sulphonamide in 1935 gave a total knock out to Streptococcus infection. However, not long after this breakthrough, microorganisms began to develop different resistance mechanism to subvert the efficacy of the existing antibiotics (Ferri *et al*., 2017). Also, noncompliance of patients to antibiotic regimen cum uncontrolled use of antibiotics in humans and livestock compounded the antimicrobial resistance problem. The immediate environment is not left out, soil serves as a reservoir for microorganisms well-known for their metabolites production as well as those that maintain the ecosystem. There is a

possibility of vertical transfer of antibiotic resistance gene within the ecosystem. This, in turn, affects the plantation on the soil and then onward transfer of these resistance genes to humans become possible. The cycle begins from the environment to humans or from the environment to animals and then to human or vice versa (Martínez, 2008). Unfortunately, children are not exempted in this antibiotic resistance menace.

2.9.1. Antimicrobial resistance in infants

Antimicrobial resistance affects all age groups. Ordinarily, one would have thought that children especially the newborn should not have carried these genes. However, the reverse is the case. Children are generally exposed to antibiotics than any other drug due to their vulnerability to infections. Recent studies have revealed that infants carried more AMR genes in their gut when compare with adult (Gibson *et al.*, 2016). The origin of these genes is likely to be from the mother. Accumulation of resistance gene in mothers could be acquired by the infants via vertical transfer whereas the environment also contributes to this acquisition, and their interaction with their immediate environment after birth could possibly select these resistance genes (Gosalbes *et al.*, 2016).

The Phylum Proteobacteria of the class Gammaproteobacteria are usually part of the early colonisers of infants gut and are hypothesized to be the cause of the higher AMR genes in infants gut (Gibson *et al.*, 2016). Also, study on resistome of mothers gut and that of the baby's gut revealed similarities in the carriage of AMR genes (Zhang *et al.*, 2015). However, another study did not find any difference in the AMR gene profile between mothers and babies in comparison with unrelated familial individuals (Gosalbes *et al.*, 2016). The interaction that occurs between the GM makes it easier for transmission of antimicrobial resistance genes across each other in that community as well as the mobile genetic elements.

2.9.2. Antimicrobial resistance genes

2.9.2.1. Tetracycline resistance (*tet*) gene

Tetracycline is among the first group of antibiotics discovered after penicillin in 1946; they have been effectively explored in the care of infectious diseases both in human and animal. Tetracycline is a broad-spectrum antibiotic that has activity against both Gram-positive and Gram-negative bacteria. This enabled it to gain more interest in the treatment of infections. It is also used at sub-therapeutic dose as additives in animal feed for improving the growth of animals in some countries (Granados-Chinchilla and Rodríguez, 2017).

The mechanism of action of tetracycline is inhibition of protein integration exhibited by debarring the joining of aminoacyl-tRNA to the ribosomal acceptor (A) site in the 30S ribosomal subunit (Kahn, 2017). Tetracycline mode of resistance is mediated by two processes, the energy-dependent efflux protein and protection of ribosome by large cytoplasmic proteins. Less common is the enzymatic inactivation of tetracycline (Markley and Wencewicz, 2018). The genes coding for efflux protein (*tet* efflux gene) major in the exportation of proteins from the cell are facilitators. Export of this drug from the cell reduces the concentration of drug in the ribosome thereby keeping the ribosome intact within the cell. The RPP protects the ribosome from the effect of tetracycline thus preventing the penetration.

The *tet* efflux gene are the *tet* A, *tet* B, *tet* C, *tet* D, *tet* E, *tet* Y, *tet* I and oxytetracycline, otr B. The tetracycline ribosomal protective gene that have been studied and sequenced till date are: *tet* M, *tet* O, *tet* B P, *tet* Q, *tet* S, and otrA. The resistance of tetracycline has extended to almost all bacterial gene. Tet M was first expounded in Streptococci (Martin *et al*., 1986) and also in different Gram-positive and Gram-negative bacteria. Tet S determinant was found on plasmids in food contaminanted with *Listeria monocytogenes*, *Enterococcus faecalis* and also on *Lactococcus lactis* extracted from raw milk (Zycka-Krzesinska *et al*., 2015). Other Tet genes have been recovered from human and animal isolates, suggesting that RPP *tet* genes are ubiquitous.

Studies on the infant gut microbiome have shown that tet resistance genes are prevalent in the infant's gut without selective pressure. Karami and colleagues analysed the faecal samples from 128 Swedish infants over a year and found that *tet* A and *tet* B which belong to *tet* efflux gene were prevalent in the gut of these infants without exposure of these infants to tetracycline antibiotic (Karami *et al*., 2006).

Children under the age of eight can no longer use tetracycline as well as pregnant women. A recent study conducted on 16 Finnish mother-infant pair analysed the faecal sample of the mother at thirty-two weeks gestation and one month postpartum and that of the infants at one and six months of age. It is interesting to know that these infants were not exposed to antibiotics. The colostrums and breast milk of the mothers were also gotten during 1 week postpartum and 1 month after delivery respectively. The gut microbiome, resistome and mobilome were studied. The result demonstrated a relationship between the mothers gut microbiota, infant gut microbiota and breast milk microbiota and argued that *E. coli* and other Gammaproteobacteria correlated with antimicrobial resistance gene buttressing the hypothesis that Gammaproteobacteria are responsible for the carriage of resistance genes in infants (Pärnänen *et al*., 2018).

2.9.2.2. Aminoglycoside modifying enzyme *(aac* (6')) gene

Aminoglycosides antibiotics are bactericidal in action and are used mainly in the treatment of infections caused majorly by Gram-negative organisms. It is commonly used in the treatment of infections associated with infants. Members of these antibiotics include gentamicin, tobramycin, amikacin, neomycin and streptomycin. Aminoglycosides pharmacokinetic is very certain and usually used in conjunction with other antibiotics, a property that makes it more effective. Members of this class are known for their selective toxicity. Their mode of action is by interference with the protein synthesis in the bacterial cells. It is concentration-dependent and based on their affinity with specific protein in the 30S ribosomal subunit.

Aminoglycosides resistance is very common. The resistance mechanismhas been well studied in *E. coli*, the interplay of aminoglycosides with the 16S rRNA in this organism and its outcome on the translation of mRNA into polypeptide has been well studied (Mir *et al*., 2016). Enzyme inhibitions, such as the interaction that occurs between the enzyme inhibitors and the target site of enzymes are common among the aminoglycoside. The major activity and most popular mechanism of resistance by aminoglycosides is the inactivation of antibiotics by plasmid or transposon-mediated aminoglycosides modifying enzymes.

Aminoglycosides modifying enzyme (AME) initiate the alteration of the hydroxyl or the amine, the effect of this alteration could then be acetyltransferases (AACs), nucleotidyltranferases (ANTs), or phosphotransferases (APHs). The mutation that occurs at different point of the enzymes enabled variants of these enzymes to emerge; as such it gives an opportunity for different antibiotics to utilize them. The ease of transfer on mobile genetic elements both at the cellular and molecular level makes this resistance mechanism apply to many bacteria such as *Staphylococcus aureus* or other *Staphylococci*, *E. coli*. The most confronted enzyme is the AAC (6')/APH (2') encoded by the *aac* (6')/*aph* (2').

Aminoglycosides resistance in infants are reported in Staphylococci from blood culture gotten from neonates (Klingenberg *et al*., 2004). Another study reported a high prevalence of AME in neonatal Gram-negative bacteria such as *E.coli* and *Klebsiella* spp from different specimen including blood, cerebrospinal fluid, urine e.t.c. (Lam *et al*., 2017). A faecal microbiota study on infants and children revealed a predictive determinant of a possible transfer of AME through members of Firmicutes in their gut. Some contig resembled part of the AME encoded Clostridium *difficile* genome and also identical to AME found on *Enterococcus faecium*(Moore *et al*., 2015).

A fosmid metagenomic analysis conducted on 6 month old Irish infants showed that aminoglycoside acetylation *aac* (3) genes were not discovered in the cloned or the metagenomic DNA that is the un-cloned DNA, whereas all the 13 Gent^R clones were all

positive for *aac* (6) genes which are homologous to *Pseudomonas fluorescens* gene but no amplification when challenged with metagenomics DNA. Likewise, for aminoglycoside adenylation, 9 out of 13 clones were resistant and resembled *Pasteurella multocida* gene while others look like the *E. coli* gene. For the aminoglycosides phosphorylation gene, 10 out of 13 clones were resistant and was similar to *Clostridium difficile* and *Enterococcus faecium* gene as well as another unknown source (Fouhy *et al.,* 2014). This is indicative of possible carriage of these genes by the infant GM without necessary exposure to aminoglycoside antibiotics.

2.9.2.3. β- Lactamase (*bla*Z) gene

β- Lactam antibiotics are the group of antibacterial agent that possess β- lactam bound in their molecular structure. The usage of this group of antibiotics constitutes up to 60%. Examples are penicillins, cephalosporins, carbapenem, imipenem and monobactam. Report shows the alarming rate of Gram-negative β- lactamase resistance and concluded that three out of six notorious pathogens are the β- lactam resistance bacteria (Boucher *et al*., 2009). Newer modified antibiotics are emerging, also different β- lactamase enzymes are encountered, which has greater resistive ability than the previous ones. Some of these successive β- lactamase enzymes are CTX-M extended-spectrum β-lactamases (ESBLs), plasmid-mediated AmpC β-lactamases and KPC carbapenemases, and the recent ones OXA and Metallo carbapenemase. *Bla*Z gene has only be reported in *Staphylococcus aureus* and coagulase-negative *Staphylococci*. Four different variants of β-lactamases exist, β-lactamases A, B, C and D. These are established by immunologic and substrate profile methods (Pereira *et al*., 2014).

The mechanism of resistance to β- lactamases are described in four categories: release of β-lactamase enzymes that hydrolyse the ring, Penicillin Binding Proteins (PBP) that stabilize the peptidoglycan structure in bacterial cell wall, distortion of porin channels, and induction of efflux exporter proteins (Tooke *et al*., 2019).

The GM is a reservoir for resistance genes and infants are not exempted. A resistome study of Singaporean infants at week 3, month 3, 6 and 12 revealed a pool of antibiotic resistance genes with high prevalence of *Bla*Z , *fos*A *tet*(M) and mef(A) in that order and observed that some of these resistance genes are present as early as week 3 and some persisted till month 12 (Zain *et al*., 2018). Another study on 11 preterm infants identified 39 resistance genes in the metagenomic sample of these infants and blaZ was among the resistance gene discovered with the majority of the genes associated with the abundance of Staphylococciin their gut (Rose *et al*., 2017).

2.9.2.4. Macrolide resistance gene

Macrolide antibiotics are bacteriostatic in nature. They are made up of different big molecular size lactone rings substituted with or amino or neutral Members of the class macrolides are erythromycin, clarithromycin and azithromycin. Macrolide mode of resistance is coined in its ability to inhibit protein integration by binding to the large 50S subunit of the bacterial ribosome and distort protein elongation by disconnecting the peptidyl-tRNA.

The process of resistance is in two major forms: the inducible expression of Erm methyltransferase and the peptide-mediated resistance. Studies have shown that *erm*(B) gene is very common and most encountered in *Streptococcus pneumonia* while *erm(*A) and *erm*(TR) are less common. Macrolide efflux have been encountered in *Streptococcus pneumonia* encoded by *mef*A and *mel* Operon (Schroeder *et al*., 2019). A study on 153 Bangladeshi children revealed a prevalence of *erm*B and *mef*A in non-susceptible pneumococcal isolates (Hasanuzzaman *et al*., 2017). Report revealed the incidence of Macrolide resistance gene in the faecal microbiome (Islam *et al*., 2019).

2.9.3. Health implication of AMR genes in children

Resistance to antimicrobials is a global exigency ravaging the health sector and making the treatment of infectious ailments to have a poor outcome. To worsen the case, not only those that are exposed to antibiotics are at risks, even infants who have not received antibiotics are also at risk. The accumulation of resistance genes in the gut of infants or older children would definitely lead to poor treatment outcome, where infectious agents would be resistant to virtually all available antibiotics and the resultant effect is death. The gut microbiome is a dynamic community, carriage of a resistant gene by an organism are easily transmissible through the mobile genetic elements present on these organisms in the community via horizontal gene transfer. If the gut residents are carriers of antibiotic resistance genes, it then means the possibility of transfer to the exogenous infectious agent is made easy. The health implication of resistance genes in the gut of children is dire, both to present and future life.

2.10. Short -Chain Fatty Acids (SCFAs)

SCFAs are less than 6 carbon ring molecules. They are the product of fermentation of indigestible complex carbohydrate that escaped digestion in the gastric and reached the colon. SCFAs are formate (C1), acetate (C2), propionate (C3), butyrate (C4), and valerate (C5). The most prominent of these acids are the propionate, acetate and butyrate. They are present in ratio 1:3:1 and are easily assimilated by the epithelial cells (Thursby and Juge, 2017). They occur as a natural ligand, which acts as a signalling substance that links GM and the host. SCFAs are the main bacterial commensal metabolite produced in the intestine.

Moreover, each of the SCFAs has their own niche in the gut. Acetate and propionate mainly abundant in the small and large intestine while butyrate can be found in the colon and caecum (Sun *et al*., 2017). Approximately 400-800 mmol SCFAs are released when an individual feed daily on high-fibre foods, which is equivalent to the the release of approximately 100 mMol SCFA from the fermentation of 10 g dietary fibre (Canfora *et*

al., 2015). Both dietary intervention and microbial activity of residents' bacteria in the gut contributes to the production of SCFAs. Mostly, acetate, propionate, formate and butyrate are the main end products of the activity of GM in the host.

However, lactate is one of them but it is usually produced from selected non-digestible carbohydrates. Other products are from biological degradation of protein-derived branched-chain fatty acids. The products of these are usually, valerate and its derivatives (Demehri *et al*., 2016; McNabney and Henagan, 2017). The importance of formate in the gut is not well known. It is usually implicated in a case such as inflammation and has also been associated with methanogenesis (Morrison and Preston, 2016). The type of food consumed determines the quantity of SCFAs in the gut. Breast milk contained human milk oligosaccharides which for example encourages the proliferation of *Bifidobacteria* in infants gut (Murugesan *et al*., 2017). Infant formula is composed of fructooligosaccharides and oligosaccharides which reinforced the abundance of *Bifidobacteria* in infants gut. The end product of this fermentation is acetate and lactate. Other GM also produces acetate. Genera belonging to *Bacteroides, Prevotella, Ruminococcus, Blautia, Clostridium* and *Streptococcus* are principal producers of acetate (Flint *et al*., 2012). Initiation of solid foods introduces plant-based polysaccharide into the infant's gut. This involves the metabolism of complex molecules, the resultant end products are butyrate, propionate and acetate (Canfora *et al*., 2015).

2.10.1. Acetate

Aceate is a 2-carbon molecule. It is mostly encountered in the gut through different pathways. They are also produced by acetogenic bacteria such as *Blautia hydrogenotrophica* or from formate through Wood–Ljungdahl pathway as well as from pyruvate via Acetyl Co-A. Acetate plays essential part in stimulating colonic blood flow and also assist ileal movement (McNabney and Henagan, 2017).

2.10.2. Propionate

Propionate is usually produced through the succinate pathway. Bacteroidetes and few of Firmicutes such as Negativicutes, example are *Phascolarctobacterium succinatutens*, *Dialister* and *Veillonella* are good producers of proipionate. It plays an essential role in the regulation of diet, reduction of lipogenesis and cholesterol in the circulation (Canfora *et al*., 2015; Sun *et al*., 2017).

2.10.3. Butyrate

Butyrate production in the gut is usually associated with plant-based polysaccharide intake. The production is based mainly on dietary intervention and microbial metabolism. The phylum Firmicutes are known producers of butyrate (Thursby and Juge, 2017a). Butyrate stimulation in the gut has been helpful in increasing butyrate in the gut. Studies have shown that the resistant starch from potatoes, inulin in man and resistant starch from maize have proved efficient in increasing the butyrate level in man and mice (Baxter *et al*., 2019). Butyrate is an excellent energy source point for the colon cells. They act against the inflammation of the colon, which in turn, helps to prevent colonic cancer (Chambers *et al*., 2018)

2.10.4. Importance of SCFAs in the gut

The SCFAs are easily absorbed in the ileum. The absorption of SCFAs usually lead to the stimulation of sodium, resulting in its anti-diarrhoeal activity which it accomplished by restoring the fluid lost as a result of diarrhoea. Also, lactate and acetate are highly acidic in nature; these create a growth barrier to the growth of pathogens. Some of the gut microbiota, such as members of the genus Bifidobacteria and Lactobacilli produce these acids (Morrison and Preston, 2016; Murugesan *et al*., 2017). SCFAs help in glucose homeostasis, in the liver, propionate produces glucose (gluconeogenesis) while butyrate and acetate produce lipids (lipogenesis). The daily intake of dietary is not enough for the host requirement of propionate.

The role of SCFAs in the gut is very important. One major role they play is the regulation of the immune system and inflammation processes. They augment cytokine production by stimulating the release of interleukin-18, which participate in the repair and maintenance of epithelial integrity (Belkaid and Hand, 2014). SCFAs are capable of regulating the level of lipid and glucose homeostasis through different mechanisms. In the liver, propionate can activate gluconeogenesis, acetate and butyrate can activate lipogenesis. They also modulate appetite regulation and energy intake through some receptors; all these functions promote a healthy life (Chambers *et al*., 2018).

2.11. Studies on infants gut microbiota

2.11.1. Studies on infants gut microbiota (GM) in the World

The field of GM gained appreciable attention in the last two decades. The help of a thorough-puttechnique, next-generation sequencing helped the field to thrive and inference could be made to relationship with healthy and diseased states. Studies on the human gut microbiota have been in existence. Majority of them used culture-dependent methods (Hiergeist *et al*., 2015).

Infant gut microbiota study gained appreciable interest around the year 2000 (Satokari *et al*., 2003). Molecular approach was used in studying these super organisms aimed at targeting the 16S rRNA which is conserved in all bacteria. Methods previously employed before the next generation sequencing involves fluorescence in situ hybridization (FISH), DNA-DNA hybridization, PCR-based technique characterization with 16S rRNA sequencing and Denaturing Gradient Gel Electrophoresis (DGGE) or Temperature Gradient Gel Electrophoresis (TGGE) (Satokari *et al*., 2001; Heilig *et al*., 2002).

Different studies have been done in the USA, UK, Canada, Bangladesh, Japan and Haiti reporting the microbial composition and diversity of infants gut at different age in relation to different factors. Biasucci and colleagues investigated the gut composition of 3-day old infants based on delivery mode using DGGE and TGGE method, they targeted 10

Bifidobacterium spp, 3 *Ruminococcus* and *Bacteroides* and found a delay in the colonization of *Bifidobacterium* in CS delivered infants (Biasucci *et al*., 2010). Recent studies employed 16S rRNA sequencing methods. Not only did these studies describe the factors influencing the gut composition of infants but also the gut microbiota associated with diseased states. Different studies have reported the microbiome of diabetic patients, inflammatory bowel diseases and autoimmune diseases (Kostic *et al*., 2014; Forbes *et al*., 2016).

2.11.2. Studies on infants gut microbiota in Africa

Despite the accelerating studies on the importance of gut microbiome in the world, metagenomics studies are still underrepresented in African. Majority of these studies focused on adult with their subsistence agricultural lifestyle, examples are studies on the hunter-gatherer of Tanzania (Schnorr *et al*., 2014), A study compared three population; BaAka hunter-gatherer Bantu agriculturalist from the Central African Republic and Americans. The study reported a drastic difference between the gut microbiota of African and American population (Gomez *et al*., 2016).

Other studies focused on the gut microbial composition of children in Africa. De Filippo and colleagues compared the GM of children age 1-6 years in the rural area of Burkina Faso with the same age of children in Europe (Italy) and found that diet is a strong drive that made the difference between the two groups. They found Bacteroidetes (*Prevotella* and *Xylanibacter*) to be distinctively present in Burkina Faso children and not in Italian children, also Enterobacteriaceae was reduced in Burkina Faso chidren than in Italian children. (De Filippo *et al*., 2010). Same authors also reported the gut microbiota of children (2-8 years) from both rural and urban area of Burkina Faso and compared their gut microbiota with Italian children of the same age. They observed abundance of Bacteroidetes in the rural and town settlers as seen in their previous studies and higher Firmicutes in urbanized children and European children. It was concluded that

urbanization brought about the loss of some microbial signature typical to the rural lifestyle and tend towards Westernized life (De Filippo *et al*., 2017).

There are very few studies on infants GM in Africa. A comparative study between 6 months old Malawian and Finish infants indicated abundance of Bifidobacteria in the two different groups but higher in Malawian infants. *Bacteroides-Prevotella* and *Clostridium* were also overrepresented in Malawian infants when compared with Finnish infants while other signatures are present in Finnish infants but not in Malawian infants. This study used FISH and qPCR methods (Grześkowiak *et al*., 2012). Another study from Gabon and Democratic Republic of Congo on 1-month old infants revealed abundance of *Prevotella*, *Streptococcus*, *Enterococcus*, *Acinetobacter* and *Bradyrhizobium* in the meconium samples. Vaginal birth had a dominance of *Collinsella* and *Bacteroides* while the caesarean section birth had a dominance of *Klebsiella* and *Sarcina*. This study employed 16S rRNA sequencing method. They also investigated enteric viruses and found adenovirus; rotavirus and enterovirus in some of the infants gut (Brazier *et al*., 2017).

Another study conducted on South African infants, investigated the effect of EBF on postpartum HIV transmission and observed a reduction in the transfer of HIV among exclusive breastfeeding infants but an increased in CD_4^+ T cell activation in the non-exclusive breastfed infants (Wood *et al*., 2018).

2.11.3. Studies on infants gut microbiota in Nigeria

There is a dearth of information on metagenomics study in Nigeria. However, in the last two years, there are few studies reported. A study investigated the oral microbiome of three females designated by postmenopausal, premenopausal and prepubertal and found *Streptococcus thermophilus* to be dominant in postmenopausal females and *Haemophilus parainfluenzae* dominant in the premenopausal and prepubertal females (Anukam and Agbakoba, 2017). Okoli and colleagues reported the vagina and gut microbiome of 5 females, all in their reproductive ages that were positive for bacterial vaginosis among the sampled participants. Firmicutes, Actinobacteria and Proteobacteria were predominant in

vagina and in the gut. They also observed that some of the taxa were both present in the vagina and oral environment, examples are *Aerococcus*, *Atopobium*, *Corynebacterium*, *Dialister*, *Megasphaera*, *Mycoplasma* and *Prevotella* (Okoli *et al*., 2019).

Ayeni and colleagues are the first to report infant and adult gut microbiota in Nigeria. They compared the gut microbiota of Bassa adults that practise subsistence farming with urban adults as well as infants from the two groups. The infants gut microbiota of Bassa infants resembled that of the adults. Firmicutes and Bacteroidetes dominated the gut of both rural and urban populations. *Blautia*, *Coprococcus*, *Lachnospira*, *Faecalibacterium*, and *Oscillospira* were the discriminating genera, which were less abundant in Bassa adults than rural adults. They also analysed the short-chain fatty acids production and found that acetate and valerate were higher in Bassa infants (Ayeni *et al*., 2018).

CHAPTER THREE

MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Supplies and reagents

The reagents for preservation of faecal samples, DNA extraction, PCR, agarose gel electrophoresis, Illumina sequencing sample preparation, and SCFAs determination are listed in appendices I-II.

3.1.2. DNA positive control

The DNA of *Bifidobacterium adolescentis* strain was obtained from Rowett Institute of Nutrition and Health, University of Aberdeen and used as the positive control for the verification of Bifido in NAN milk.

3.2. METHODS

3.2.1. Definition of terms as used in this study

Caesarean Section (CS): Process of giving birth through surgical procedures that involves the incision of the abdominal wall and the uterus. Thirteen infants were born through CS in this study.

Vaginal Birth (VB): Process of giving birth naturally through the birth canal, from the uterus. Fifteen infants were born through VB in this study.

Exclusive Breastfeeding (EBF): Method of feeding babies solely with milk from the mothers' breasts. Fifteen infants were exclusively breastfed.

Formula Feeding: Formula feeding is a replacement for breastfeeding; it is a method of feeding infants with formulated milk. The milk formula used in the present study is NAN 1 (Nestle foods, Nigeria). None of the babies was formula fed only.

Mixed Feeding (MF): Combination of breast milk and formula milk. Method of feeding infants with breast milk and formula milk interchangeably, especially when the breast milk cannot cater for the nutritional needs of the baby. Eight babies were mixed fed in this study

Preweaning: A process of feeding babies with milk only: breast milk, formula milk or mixed feeding. The preweaning diet for the study infant were breast milk only or breast milk combined with NAN 1 milk. All the babies (n=28) were preweaned

Weaning: A process by which milk is gradually withdrawn from babies and the babies are exposed to adult or family food, such as semi-solid food or solid food. During this time babies depend less on milk for nutrition. The weaning diet for the study infants were "*Ogi*" made from maize, millet, sorghum only or combination of two or the three cereals. *Ogi* was also combined with NAN 2formula milk. The solid foods consumed by these babies were rice, *amala* (reconstituted cooked yam flour) and *ewedu* (cooked jute leaf), *moimoi* (steamed bean puddy) and proteins such as egg and fish. Twenty-three babies got to the weaning group.

3.2.2. Ethical considerations

Ethical approval was obtained from the Federal Teaching Hospital, Ido-Ekiti, Ekiti State, South West Nigeria, with protocol number: ERC/2016/09/29/44B. Mothers of the babies provided written informed consent for their infants to participate in the study.

3.2.3. Subject enrolment

This was a longitudinal study of convenience sampled participants. The enrolment began at the postnatal ward immediately after the birth. A total of 30 babies were initially enrolled in this study, 2 were lost due to attrition. The enrolment was left with 28 babies (9 males and 19 females), seven (7) of these either provided a single sample or participated for few months, and 23 babies completed study for > 7 months.

The inclusion criteria were:

- Caesarean Section Birth Babies (CSB) - 13 Babies
- Normal / Vaginal Birth Babies (VB) - 15 Babies
- Full term and Preterm Babies (FT and PT) - 26 FT and 2 PT Babies
- Exclusively Breastfed Babies (EBF) - 15 Babies
- Mixed Breastfed and formula fed Babies (MF) - 8 Babies

While the exclusion criterion was

- Newborn with any infectious diseases such as HIV, Tuberculosis, Pneumonia among others

3.2.4. Sample collection and storage

Faecal samples were collected from recruited babies monthly within 12 months with median age of 7 months at the Department of Obstetrics and Gynaecology, Federal Teaching Hospital, Ido-Ekiti. One hundred and seventy-two (172) faecal samples were collected. The weight of faeces collected ranged from 0.4 – 3 g per sample depending on the age of the subject. The number of faecal samples gotten from the recruited babies is shown in Table 3.1.

Faecal samples were obtained from the diapers of babies immediately after defecation and transferred into a sterile 20 mL sample bottle. Absolute ethanol (15 mL) was added unto the faecal bolus to dry, and allowed to stay in room temperature for 24 h, after which the ethanol was decanted carefully, ensuring the bolus was intact. The faecal bolus was air dried for few seconds and transferred unto another sterile tube containing 15 mL of 3 mm-sized silica gel beads (Guangdong Guanghua Sci-Tech Co. Ltd, Guangzhou city, China)

with a cotton wool plug on top of the silica gel bead to prevent contact between the silica gel and the faecal bolus (Schnorr *et al*., 2014). The samples were then stored at room temperature for up to 12 months. The faecal bolus were then shipped to Rowett Institute of Nutrition and Health where they were kept at -80°C until further analyses.

Table 3.1. Number of faecal samples collected from each baby

Sample code	Number of faecal samples
Baby 1	13
Baby 2	3
Baby 3	9
Baby 4	7
Baby 5	9
Baby 6	4
Baby 7	7
Baby 8	11
Baby 9	7
Baby 10	10
Baby 11	10
Baby 12	1
Baby 13	1
Baby 14	5
Baby 15	7
Baby 16	6
Baby 17	7
Baby 18	1
Baby 19	-
Baby 20	6
Baby 21	8
Baby 22	1
Baby 23	5
Baby 24	7
Baby 25	4
Baby 26	6
Baby 27	8
Baby 28	8
Baby 29	-
Baby 30	1
Total	**n = 172**

3.2.5. Total DNA extraction from faecal samples

Total DNA was extracted from stored faecal samples with FastDNA^{TM} SPIN kit (MP Biomedicals, USA) following the manufacturer's instructions as previously described by Walker *et al*. (2015). In summary, faecal sample (0.3 g) was weighed into a lysing Matrix E tube containing varying sizes of lysing beads. Sodium phosphate (978 µL) and MT buffer (122 µL) were added, the resulting mixture was shaken vigorously and vortexed for 10 s. The mixture was homogenized in the Fast Prep instrument for 30 s at 6000 x g. This step was repeated 4 times. To remove all debris, centrifugation at 14,000 × g for 5 mins was done. The supernatant was transferred to a clean 2.0 mL microcentrifuge tube, 250 µL of Protein Precipitation Solution (PPS) was added and flicked to mix for 10 mins and incubated at room temperature for 10 mins. To pellet precipitate, centrifugation at 14,000 × g for 5 mins was done. The supernatant was transferred to a clean 15 mL falcon tube and 1 mL of binding matrix was added and inverted by hand for 3 mins to allow binding of the DNA to the matrix.

To resuspend the binding matrix, the upper layer was discarded, 800 µL of the solution was transferred to a SPIN^{TM} filter tube, centrifuged at 14,000 × g for 1 min, and the catch tube was emptied. Mixing, transferring and centrifuging of the solution were repeated to remove the remaining supernatant. To resuspend the pellet, 500 µL of prepared SEWS-M was added to the SPIN^{TM} filter tube. The tube was then flicked to mix and centrifuged at 14, 000 × g for 3 mins to dry the matrix of any residual wash solution. The catch tube was discarded and SPIN^{TM} filter tube was placed in a new clean catch tube and air dried at room temperature for 5 mins. The binding matrix was resuspended in 50 µL of DES. To increase the yield, the resultant mixture was incubated for 5 mins at 55°C in a heat block, centrifuged at 14, 000 × g for 2 mins to elute the DNA into the Eppendorf tube and the pure DNA obtained was stored at -20°C for further analysis.

3.2.6. Polymerase chain reaction amplification of V1-V2 region of 16S rRNA gene

The V1-V2 region of the 16S rRNA gene was amplified from the extracted DNA using forward primer fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′ positions 7 to 26 in the *Escherichia coli* 16S rRNA gene and reverse primer rP2 (5′-ACGGCTACCTTGTTACGACTT-3′, positions 1513 to 1494. The reaction mixture comprised of 2.5 µL of 10X buffer, 1 µL of 25mM $MgCl_2$, 2.5 µL 2mM dNTP, 0.5 µL of 10pmol/mL of primer, 0.125 µL *Taq* polymerase, 17.375 µL DNase free water and 0.5 µL of diluted DNA template, in a final volume of 25 µL. The PCR reaction was set at 95ºC for 5 mins, 95ºC for 1 min, 56ºC for 1 min, 72ºC for 2 mins in 29 cycles, and 72ºC for 10 mins in 1 cycle and then held at room temperature to achieve the cooling step. The amplicons were visualised under UV light by loading 5 µL volume of DNA with 2 µL of 6X Gel loading dye (Sigma, Aldrich) and ran on 1% agarose gel in Tris-borate EDTA buffer (TBE) at 120 V for 30 mins (Walker *et al*., 2015).

3.2.7. Library preparation for Illumina sequencing

The library preparation for the Illumina MiSeq sequencing was done using fusion barcodedprimer.27f_Miseq 5′**AATGATACGGCGACCACCGAGATCTACAC***TATGGTAATTCC*AGMGTTYGAT .YMTGGCTCAG-3′ and 338R_MiSeq 5′-**CAAGCAGAAGACGGCATACGAGAT** nnnnnnnnnnnn*AGTCAGTCAGAA*GCTGCCTCCCGTAGGAGT-3′, where the bold type indicates the adaptor sequences, italicized are the linkers and the (n) string correspond to the sample-specific molecular identifier barcodes. The primer sequence annealing to the 16S rRNA gene is in plain text. The barcoded primer sequences are listed in Appendix X. The PCR amplification was carried out using Q5 High-Fidelity PCR kit (New England Biolabs, Ipswich, Massachusetts, USA). The reaction condition was set at 20 cycles of 98ºC for 2 mins, 98ºC for 30 s, 50ºC for 30 s, 72ºC for 1min 30 s, 72ºC for 5mins and holding temperature at 10ºC. PCR was done in quadruplicate.

Following amplification, the four PCR products were pooled together for each sample. The amplicons were visualised under UV light by loading 10 µL of amplified DNA with 2 µL of 6X Gel loading dye (New England Biolabs, Ipswich, Massachusetts, USA) and run on 1% agarose gel in Tris-borate EDTA buffer (TBE) at 120 V for 30 mins. Sample clean-up was done through ethanol precipitation and quantification of DNA samples was performed using a Qubit 2.0 Fluorometer (Life Technologies). The subsequent sequencing was performed on the Illumina MiSeq platform using 2×300 bp cycles (Walker *et al*., 2015).

3.2.8. Bioinformatic analysis of the sequence reads

The quality of the sequences was assessed using FastQC (version 0.11.3) (Andrews, 2015). The generated raw sequences underwent downstream bioinformatic analysis with DADA2 (version 1.3.1) (Callahan *et al*., 2016) using default parameters to quantify sequence variants and assign taxonomy. This software differs from classical OTU clustering methods as it infers sequence accuracy based on the error profiles of all reads and uses this information to decipher sequence variants and abundances. This methodology has several advantages over classical OTU clustering methods including increased accuracy and increased resolution. DADA2 analysis categorizes Amplicon Sequence Variants (ASV) instead of the classical Operational Taxonomic Units (OTU). This is because DADA2 has an increased resolution as it infers exact ASV from sequenced data. It also has the ability to detect biological differences as little as 1 or 2 nucleotides in a sequence with increased accuracy, as it computes lesser false positive sequence variants than other reported false operational taxonomic units (OTUs)

The DADA2 pipeline encompasses read filtering and trimming, dereplication, error profiling, sample inference, merging of paired end reads, construction of the sequence table, removal of chimeras and assignment of taxonomy based upon the GreenGenes 13.8 database (Callahan *et al*., 2016). The outcome after analysis was a sequence table, analogous to an OTU table from previous methods. Singleton sequence variants (those

present in only a single sample at a single count) were removed. The sequence table was converted to Biom format using Biomformat (version 2.1.3). To assess sequence variant abundances, Biomformat (version 2.1.3) was used to summarise the Biom table, producing counts for each sample.

The infants groups compared were Caesarean Section Birth versus Vaginal Birth (CSB: VB), Exclusively Breast Fed versus Mixed Fed (EBF: MF), Exclusively Breast Fed versus Local/Solid Fed (EBF: LF/SF), Mixed Fed versus Local/Solid Fed (MF: LF/SF) and Preweaning versus Weaning or Local/Solid Fed (PW: W/LF: SF).

Comparisons between the gut microbiota at different age range (0-4, 5-8 and 9-12 months), caesarean section birth (CSB) (n=13) and vaginal birth (VB) (n=15), exclusive breastfed (EBF) (n=15) and mixed fed (MF) (n=8) and then preweaning and weaning (n=23) groups were performed. Diversity analysis was performed using the core_diversity_analyses.py script from QIIME (version 1.9.0) (Caporaso *et al*., 2010) with a subsampling level of 1993, allowing for all samples to be kept for analysis.

Five alpha diversity metrics were calculated: observed species, Chao (Chao, 1984), Shannon Index (Shannon, 1948), Simpson Index (Simpson, 1949) and Good's coverage. Observed species is the count of unique operational taxonomic units present in a given sample or community. Chao is the estimate of all the taxa present in a sample. Shannon Index is the evenness of these taxa in a sample; this metrics calculates both richness and evenness of the taxa present. Simpson Index calculates the evenness and distribution; it provides more information on the taxa present in a sample or community. The Good's coverage measures or calculates the distance from each taxon to another.

Rarefaction calculates the number of taxa present in ecological samples. It is usually applied to Operation taxonomy Units (OTU) analysis. Rarefaction analysis plots the value of a measured quantity (Rarefied) against the number of observations used in the calculation. Two beta diversity measures were used (Bray and Curtis, 1957) and Binary Jaccard (Jaccard, 1912). The Beta diversity metrics calculate the distance between or

similarity of samples based upon the taxonomic identity and abundance of sequences. The Principal Coordinate Analysis (PCoA) plots display the results of the Bray Curtis diversity metric. Statistical testing of stratification of samples by meta data category was performed using the adonis statistical test on the Bray Curtis diversity metrics, implemented by the compare categories script from QIIME (version 1.9.0) (Caporaso *et al*., 2010). Differential abundance testing of sequence variants between groups was done by converting the biom file to a PhyloSeq object (McMurdie *et al*., 2013) and testing differential abundance with DESeq2 (version 1.14.1) (Love *et al*., 2014). LEfSe analysis (Segata *et al*., 2011) identifies taxonomic biomarkers that drive differences between sample groups. It is used to identify particular bacterial signatures that clearly differentiate between groups. This analysis was done with the Huttenhower Galaxy server (http://huttenhower.sph.harvard.edu/galaxy/).

3.2.9. Denaturing Gradient Gel Electrophoresis (DGGE) analyses

3.2.9.1. Amplification of the V3 region of the 16S rRNA gene for DGGE

The V3 region of the 16S rRNA gene was amplified, with primers 518r (5'-ATTACCGCGGCTGCTGG-3') and F357-gc 2.5 µL of 10X buffer, 1 µL of 25mM $MgCl_2$, 2.5 µL 2mM dNTP, 0.5 µL of 10pmol/mL of primer, 0.125 µL *Taq* polymerase, 17.375 µL DNase free water and 0.5 µL of diluted DNA template, in a final volume of 25 µL. The reaction condition was as follows, initial heating set at 94°C for 5 mins, 94°C for 20 s, 55°C for 15 mins, 72°C for 1 min in 30 cycles and 72°C for 7 mins. The gel was run on 1.2 % 1X TBE, Quick load 1kb DNA ladder (Biolabs) was used as a standard and viewed under the UV light (Satokari *et al*., 2003).

3.2.9.2. Preparation of the denaturing solution

Denaturing solutions were prepared in 100% and 0% stock. For the preparation of the 100% stock, 10 ml of 40% acrylamide, 20 ml of 99.5% of formamide, 42 g of 7M urea

and 50X Tris-Acetate-EDTA (TAE) buffer were used. For 0% stock, 10 ml of 40% acrylamide and 90 mL of 50X TAE were used. The low and high percentage solutions were calculated (Appendix IV) (Cocolin *et al*., 2001).

3.2.9.3. Preparation of the cassettes and gel loading

The gel cassette was prepared by placing the two glass plates (20×20 cm) on the clamp. The gel plates were coupled together with a rubber gasket and spacers. For the prepared high and low solution, 80 µL of APS (Ammonium Persulphate) and 10 µL of TEMED (N, N, N', N' – tetramethylethylenediamine) (Sigma, Aldrich) were added and mixed gently. The gradient marker placed on an elevated magnetic stirrer was filled with the solution. The right chamber was filled with the high percentage solution and the left chamber with the low percentage solution. The valve was gently opened with the hose connected to the gel cassette, (this was done to allow the liquid to flow into the plate in a gradient manner with the high solution going down the cassette and low solution coming up).

After filling the gel to the marked point, the hose was removed and the solution was flooded with water saturated butanol. The gel was left to polymerise for 45 mins. The gel was set and the flooded butanol removed by rinsing under a running water. The gel comb was gently inserted and stacking gel made up of 5 ml of 0% stock in addition with 80 µL and 10 µL APS was added on top of the set gel. The cassette with the plate was placed into a preheated (60°C) 20 L of 50X TAE buffer in DGGE 2001 tank (Appendix V).
To load the samples, 2 µL of 6X loading dye and 5 µL of DNA product was loaded into the interface and run on 85 V for 16 hours, and then viewed under UV light (Carmona *et al*., 2012).

3.2.9.4. Gel removal and visualisation

The gel cassette was removed from the tank and the clamps dissembled, the yellow gasket and spacers were all removed. One of the plates was carefully removed by placing a sheet of acetate on the thin-layer gel. A 1 in 100 dilutions of 50X TAE was prepared in a tray, 20 µL of SYBR gold was added and the thin gel was gently released into the stain buffer, this was left in the dark room for 30 mins, and thereafter viewed under the UV light, to observe the bands (Cocolin *et al*., 2001).

3.2.10. Verification of manufacturer's claim on NAN 1 formula milk

The manufacturer claimed that they fortified NAN 1(Nestle, Nigeria) with Bifid LAL. This was verified by PCR. DNA was extracted from 0.6 g of NAN 1 formula milk using the FastDNA^{TM} SPIN kit (MP Biomedicals, USA) following the manufacturer's instructions and as previously described in section 3.2.5. The negative control was the molecular graded water. DANO milk (Arla foods amba, Denmark) was used as the negative control. DNA was extracted from 0.6 g of the milk.
Bifidobacterium generic primer set, Bif 164-f and Bif 662-GC-r, was used for the amplification with the forward primer Bif 164f (CCTATCCCCTGTGTGCCT TGGCAGTCTCAGGGGTGGTAATGCCGGATG, and the reverse primer Bif 662r (CCATCTCATCCCTGCGTGTCTCCGACTCAG-CCACCGTTACACCGGGAA)
(Satokari *et al*., 2003).

The extracted DNA was used as template in the PCR reaction with the following conditions, 94°C for 5 mins, 35 cycles each of 94°C for 30 s, 62°C for 20 s and 68°C for 40 s, followed by 68°C for 7 mins and finally held at room temperature to achieve the cooling step. The size of the product was estimated on 1.2% agarose gel in Tris-acetate buffer (TAE) at 120 V for 30 mins and visualised under the UV light.

3.2.11. Short-chain fatty acids analyses

3.2.11.1. Sample preparation for short-chain fatty acids

Short-chain fatty acid analysis was conducted for all the 172 faecal samples. Preparation of samples for SCFA was done by weighing 0.15 g of dried faecal sample into a 1.5 mL Eppendorf tube, 600 µL of sterile distilled water was added at first, and final volume increased to 1.25 mL. This was refrigerated overnight for proper dispersion. The sample was then vortexed and 250 µL of the supernatant added to 250 µL of sterile distilled water in a wide mouth sorval tube. This was done in duplicate.

3.2.11.2. Preparation of internal and external standard (IS and ES)

The internal standard was prepared by adding 1.258 mL of 2-ethyl butyric acid to 98 mL of SDW to make a stock solution of 0.1 M 2- ethyl butyric acid and stored at 4°C. The external standard was prepared by mixing specific amounts of salts and acids (Table 3.3) into a flask and making the volume up to 100 mL with distilled water. Twenty-five microliter (25 µL) of 0.1 M 2- ethyl butyric was used as the internal standard and 50 µL of the SCFAs mix was used as external standard (Niccolai *et al*., 2019).

3.2.11.3. Extraction of short-chain fatty acids from the samples

Each run of the SCFA experiments used two standard solutions. Standard solution 1 contained 0.1 M 2- ethyl butyric (internal standard) and standard solution 2 contained the SCFA standard solution as shown in Table 3.2. (external standard). These solutions were first weighed and the value recorded for chromatography bias.

For the extraction process, all work was performed within fumehood. A 25 µL of the internal standard was added to all the sample tubes, 250 µL of conc. HCl and 1 mL of ether was added, vortex for 1 min, and centrifuged at 3,000 rpm for 10 mins to separate the ether from the water layer. The ether portion was carefully removed with the aid of

Pasteur pipette into a clean sorval tube. This step was repeated, then 400 µL of the ether extract was pipetted into a Wheaton vial (Sigma-Aldrich, USA) and 50 µL of MTBSTFA (N-tert-Butyldimthylsilyl-N-methyl-trifluoroacetamide) (Sigma-Aldrich, USA) was added. All the extracts were transferred into Agilent crimp cap vials, screwed tightly to avoid evaporation of ether during heating. The extracts were heated at 80°C for 20 mins, and the vials were left in room temperature for 48 h to derivatize lactate. This procedure was repeated for all extracts. Thereafter, analysis was performed on GC machine to estimate the amount of SCFAs produced in each sample (Granado-Serrano *et al.*, 2019).

Table 3.2. Constituent of short chain fatty acids standard solution

Acids	Volume (µL)
Acetic	172
Propionic	149
Iso-Butyric	47
n-Butyric	184
iso-Valeric	55
n-Valeric	54
Salts	**Weight (g)**
Sodium formate	0.068
Lithium lactate	0.096
Sodium succinate	0.270

*The constituent were make up to 100 mL with sterile distilled water

3.2.12. Selected Antimicrobial Resistance Gene (AMRG) Screening

Classes of antibiotics used by the infants enrolled in this study were most frequently the β-lactams, macrolides, aminoglycosides and fluoroquinolones. The presence of six AMRG was investigated by PCR. Degenerated primers targeted at the Ribosomal Protection Proteins Tetracycline resistance gene (RPP), β- lactamase (*blaZ*), macrolide (*ermA*, *ermB*, *mef*A/E), and aminoglycoside modifying enzymes $gent^R$ (aac(6′)/aph(2″)) were used (Table 3.3). The PCR reaction mixture contained 2.5 µL of 10X buffer, 2.5 µL of 2mM dNTP, 0.125 µL of Hotstar Taq, 1 µL of 25mM $MgCl_2$, 0.25 µL of 10pmol/mL of each primer, 17.875 µL DNase free water and 0.5 µL of diluted DNA template making a total volume of 25 µL. The reaction condition set for the primers outlined in Table 3.3 as followed, initial denaturation at 95°C for 5 mins followed by 29 cycles of denaturation at 95°C for 1 min, annealing at 59°C for 1 min, extension at 72°C for 1 min 30 s and a final extension at 72°C for 10 mins. All the genes (except RPP) used the same PCR conditions. The PCR condition for degenerate RPP primer was set at initial denaturation at 95°C for 15 mins, 35 cycles of denaturation at 95°C for 1 min, annealing at 61°C for 1 min, extension at 72°C for 1 min 30 s and a final extension at 72°C for 10 mins. The PCR condition were modifications to Malhotra-kumar *et al*., (2005) methods.

Table 3.3. The AMR genes investigated and the primers used

Genes	Primer	Oligonucleotide sequence (5′-3′)	Amplicon size (bp)	Reference
aac(6′)	*aac*(6′)-F	GAAGTACGCAGAAGAGA	491	(Choi *et al*., 2003)
	aph(2″)-R	ACATGGCAAGCTCTAGGA		
mef(A/E)	*mef*(A/E)-F	CAATATGGGCAGGGCAAG	317	(Malhotra-kumar *et al*., 2005)
	mef(A/E)-R	AAGCTGTTCCAATGCTACGG		
ermA	*erm*(A)-F	CCCGAAAAATACGCAAAATTTCAT	590	(Malhotra-kumar *et al*., 2005)
	erm(A)-R	CCCTGTTTACCCATTTATAAACG		
ermB	*erm*(B)-F	TGGTATTCCAAATGCGTAATG	745	(Malhotra-kumar *et al*., 2005)
	erm(B)-R	CTGTGGTATGGCGGGTAAGT		
blaZ	*blaZ*-F	ACTTCAACACCTGCTGCTTTC	173	(Martineau *et al*., 2000)
	blaZ-R	TGACCACTTTTATCAGCAACC		
RPP *tet*	Degenerate			
	RPP-F	CCIGGVCAYATGGAYTTYH TDGC	1.3	(Warburton *et al*., 2009)
	RPP-R	CKRAARTCIGMIGGIGTRCTIA CHGG		

* Degenerate oligonucleotides: D= A, G, or T; H= A, C, or T; I =A, C, G, or T; K =G or T; M= A or C; R= A or G; V =A, C, or G; Y =C or T.

CHAPTER FOUR

RESULTS

4.1. Metadata of the infants

All the information about the gender, delivery mode, feeding method, gestational age, weight at birth, antibiotic treatment for the babies are shown in Table 4.1. Additional information about the babies for subsequent months including periods of sickness during study, date of birth, date of sample collection, age of the babies at sample collection, type of formula milk used, herbal treatments administered to the babies and timing of introduction of complementary food can be found in Appendix III. There were 9 males and 19 females in the studied group. Fifteen babies (53.6%) were delivered by vaginal birth and thirteen babies (46.4%) were delivered through caesarean section birth. The gestational ages of full-term babies (n=26) ranged between (34-42) weeks. There were two preterm babies (babies 15 and 16), with gestational ages of 30 and 32 weeks respectively. Overall, the average gestational age was 37.6±2.8 weeks. The birth weight for full term babies ranged 2.2-3.6 kg, while the weight for preterm babies was 1.3 and 1.9 kg respectively. The average birth weight for all the babies at birth was 2.9±0.6 kg.

After birth, 13 babies were exclusively breastfed and 15 babies were formula fed either for 24 hours or within hours after birth due to a delay in the mother's lactation, but formula feeding was stopped immediately lactation commenced. The period of introduction of solid foods varied among the babies. About 17.8% of the babies were mixed fed for the first 3 months of life and introduction of complementary food started earlier, at 4 months. Another 17.8% of the babies were exclusively breastfed for the first 4-5 months and complementary foods started thereafter. Other babies about 64.4% were exclusively breastfed for the first 6 months of life and introduction of solid foods started at 7 months. All of these different timings were taken into consideration during bioinformatic analysis. All babies in the exclusively breastfed and mixed fed groups were classified as the preweaning group while those in the category of complementary foods (local/solid foods) were classified as the weaning group. Nine babies (32.1%) had antibiotic treatment at

birth, and subsequently 23 babies (82.1%) received antibiotic treatment, 7 babies (25%) received herbal treatment either for topical application (bathing only) or as oral treatment. The complementary foods provided to all babies were the local cereals; yellow corn, millet and sorghum called "*Ogi*'. Depending on the choice of the parents and food tolerance by the infants, *Ogi* was prepared either from yellow corn or millet, or combination of the two or the three cereals. Irrespective of the preweaning diet, formula milk NAN 2 was added to *Ogi* to complement the breast milk.

Table 4.1. Metadata of babies at birth

Sample code	Gestational age (week)	Mode of delivery	Gender	Weight at birth (kg)	Feeding method	Antibiotic treatment
Baby 1	37 wks 5 d	CS	Male	3.45	MF	No
Baby 2	39 wks 5 d	CS	Female	3.8	MF	No
Baby 3	39 wks 4 d	VB	Female	3.0	MF	No
Baby 4	38 wk 5 d	VB	Female	2.8	EBF	No
Baby 5	37 wks 3 d	VB	Male	3.25	EBF	No
Baby 6	39 wks 6 d	CS	Male	2.9	EBF	No
Baby 7	42 wks	CS	Female	3.2	MF	Yes (Cefotaxime and Cefixime)
Baby 8	38 wks	VB	Female	2.8	EBF	Yes (Ampiclox)
Baby 9	38 wks 2 d	VB	Female	3.2	MF	No
Baby 10	34 wks 5 d	CS	Female	2.5	MF	No
Baby 11	34 wks 5 d	CS	Male	2.6	MF	No
Baby 12	38 wks 5d	CS	Female	2.6	MF	Yes (Cefuroxime, Gentamicin and Cefixime)

Table 4.1. Cont'd

Sample code	Gestational age (week)	Mode of delivery	Gender	Weight at birth (kg)	Feeding method	Antibiotic treatment
Baby 13	38 wks 5d	CS	Female	2.4	MF	Yes (Cefuroxime, Gentamicin and Cefixime)
Baby 14	40 wks 4d	VB	Female	2.6	EBF	No
Baby 15	30 wks 6d	VB	Female	1.3	MF	Yes (Cefotaxime and Gentamicin)
Baby 1	40 wks 2d	VB	Female	2.54	MF	No
Baby 20	38 wks	VB	Male	3.7	MF	No
Baby 21	40 wks	VB	Female	2.2	EBF	No
Baby 22	39 wks	CS	Male	3.0	EBF	Yes (Cefotaxime and Gentamicin)
Baby 23	33 wks 2 d	VB	Female	2.25	EBF	Yes (Cefotaxime and Gentamicin)
Baby 24	40 wks 3 d	VB	Female	3.1	EBF	No

Table 4.1. Cont'd

Sample code	Gestational age (week)	Mode of delivery	Gender	Weight at birth (kg)	Feeding method	Antibiotic treatment
Baby 25	34 wks 3 d	VB	Male	3.4	EBF	No
Baby 26	37 wks 4 d	VB	Female	3.5	EBF	No
Baby 27	37 wks 2 d	CS	Female	2.5	MF	No
Baby 28	37 wks 2 d	CS	Male	2.55	MF	No
Baby 30	34 wks	VB	Female	2.9	EBF	No

*wks- weeks, d- day, CS- Caesarean Section, MF- Mixed Fed, EBF- Exclusive Breast Fed

*Metadata for subsequent months (Appendix III)

4.2. The gut microbiota composition of the infants

The quality of the sequenced data was assessed by FastQC and the statistics of the resultant sequence reads are shown (Appendix IV). In all, between 3,251 and 41,103 raw sequences were produced per sample with an average read count of 15,405. Following bioinformatics analysis, it was determined that each of the sample had between 1,993 and 22,328 sequence variant counts with an average of 10,159.34 variants per sample. These sequences were deposited in the European Nucleotide Archive (ENA) under the Accession number: PRJEB31073.

The outcome of 16S rRNA sequence analysis provided the gut microbiota composition of the infants studied with assignment of taxonomy performed by DADA2. At the phylum level, 11 phyla were identified namely: Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, TM7, Tenericutes, Verrucomicrobia, Cyanobacteria and Lentisphaerae. The most differential abundant phyla were the Actinobacteria, Firmicutes, Proteobacteria, Bacteroidetes and Fusobacteria, and the least abundant phyla were TM7, Tenericutes, Verrucomicrobia, Bacteria, Cyanobacteria and Lentisphaerae, which were grouped together and classified as others. The phylum Actinobacteria was the most abundant across all babies at all time-points (45.95 ± 2.12%; Mean ± SEM), followed by Firmicutes (36.73 ± 1.78%), Proteobacteria (12.96 ± 1.47%), Bacteroidetes (4.30 ± 0.57%), Fusobacteria (0.05 ± 0.03%) and Others (0.01 ± 0.00%) respectively. The mean percentage of each phylum for all babies at all time points is shown in Figure 4.1. Only one baby provided sample at 11 and 12 months.

Taxonomic profiling of individual samples grouped by time point at the level of phylum showed a general decrease in Proteobacteria and a slight increase in Bacteroidetes as time points progressed as shown by the R-ggplot (Figure 4.2).

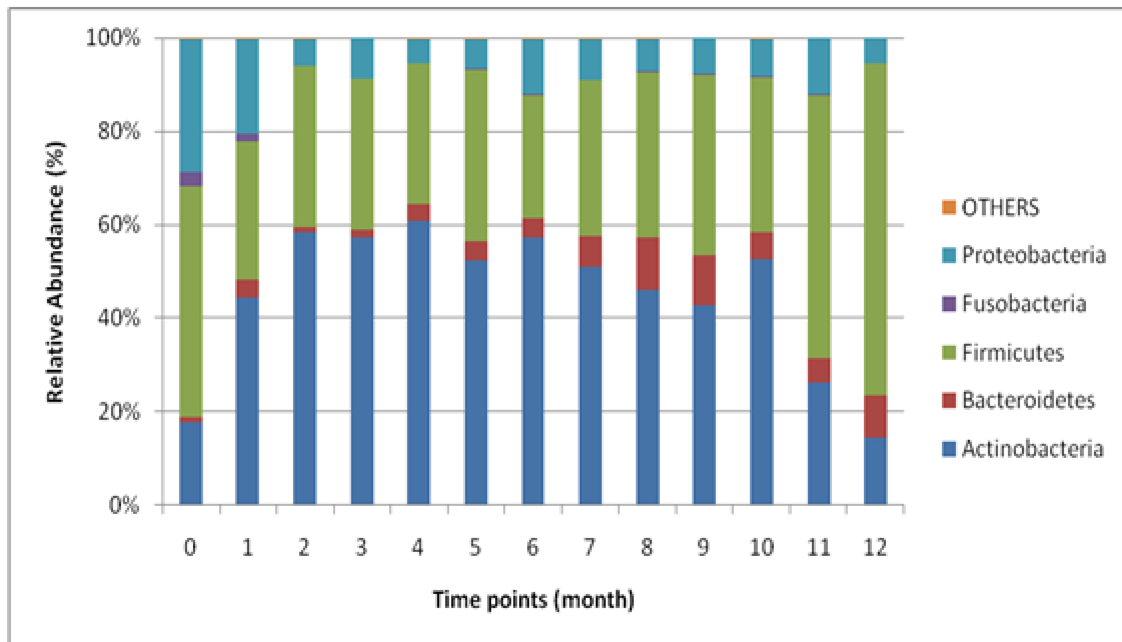


Figure 4.1. Mean proportion of the major phyla in the gut of infants from birth till 10 months

*11 and 12 months are data from single sample

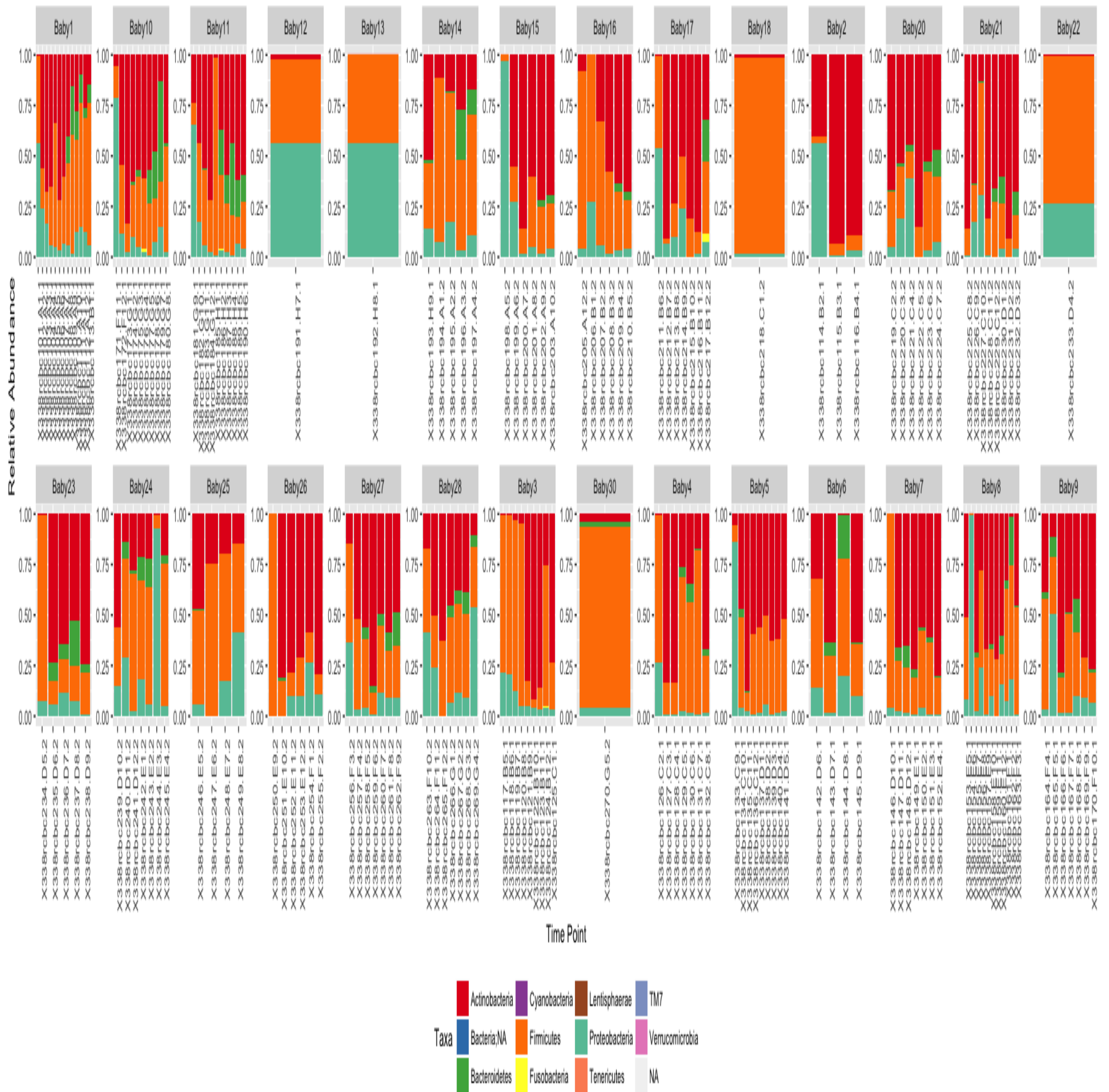


Figure 4.2. The phylum level gut composition of infants

At genus level, the gut microbiota composition of the infants comprised of these bacteria in decreasing order of abundance (% proportion): *Bifidobacterium* (41.07%), *Streptococcus* (15.79%), *Trabulsiella* (5.44%), *Klebsiella* (4.62%), *Bacteroides* (3.47%), *Enterococcus* (3.22%), *Veillonella* (2.73), *Collinsella* (2.45%), *Lactobacillus* (1.92%), *Ruminococcus* (1.84%), *Blautia* (1.39%), *Clostridium* (1.38%), *02d06* (1.02%), *Staphylococcus*(0.93%), *Faecalibacterium* (0.65%), *Megasphaera* (0.65%), *Megamonas* (0.63%), *Prevotella* (0.61%), *Propionibacterium* (0.58%), *SMB53* (0.58%), *Eubacterium* (0.47%), *Haemophilus* (0.44%), *Sutterella* (0.43%), *Dorea* (0.32%), *Coprococcus* (0.28%), *Catenibacterium* (0.28%), *Rothia* (0.27%), *Parabacteroides* (0.16%), *Dialister* (0.12%), *Roseburia* (0.11%), *Eggerthella* (0.11%), *Acinetobacter*(0.08%), *Oscillospira* (0.07%), *Lachnospira* (0.07%), *Pediococcus* (0.06%), *Peptostreptococcus* (0.06%), *Proteus* (0.06%), *Peptoniphilus* (0.06%), *Pseudoramibacter_Eubacterium* (0.06%), *Corynebacterium* (0.05%), *Lactococcus* (0.05%), *Atopobium* (0.04%), *Pseudomonas* (0.04%), *Cronobacter* (0.04%), *Fusobacterium* (0.03%), *Dysgonomonas* (0.03%)and *Butyricicoccus* (0.03%).

The low abundance (< 5% total seqs) genera were: *Neisseria, Finegoldia, Bacillus, Varibaculum, Mitsuokella, Phascolarctobacterium, Granulicatella, Lachnobacterium, Weissella, Stenotrophomonas, Erwinia, Leuconostoc, Leptotrichia, Actinobacillus, Sarcina, Campylobacter, Anaerococcus, Slackia, Aggregatibacter, Bilophila, YS2, RF39, Ralstonia, S24-7, Geobacillus, Ureibacillus, Robinsoniella, Dermabacter, Akkermansia, Morganella*, *Epulopiscium, Succinivibrio, Acidaminococcus, Anaerotruncus, Brevibacillus, Candidatus_Arthromitus, Peptococcus, Bulleidia, TM7-3, Anaerostipes, Enhydrobacter, Sporosarcina, Gardnerella, WAL_1855D, Aerococcus, Odoribacter, Alloiococcus, Adlercreutzia, RF32, Providencia, Gemella, Tessaracoccus, Alistipes, Succinatimonas, Desulfovibrio, Helicobacter, Paraprevotella, Facklamia, Tepidimicrobium, ph2, Alkanindiges* and *Citrobacter*. The mean percentage for all babies at the level of genus overtime is shown in Figure 4.3.

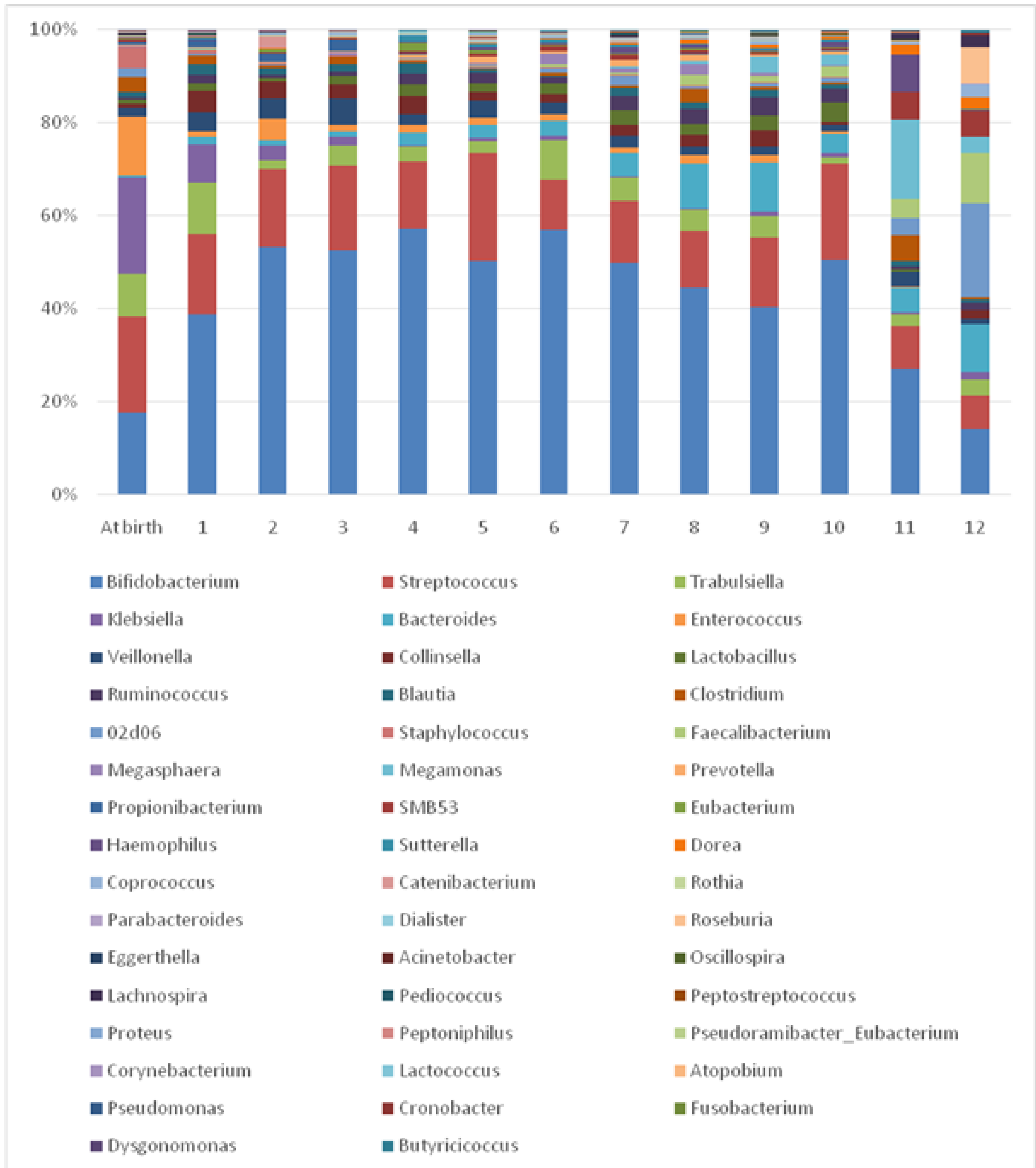


Figure 4.3. Mean proportion of the major genera in the gut of infants from birth till 10 months

*11 and 12 months are data from single sample

The genus level gut composition of individual babies across time points are shown in (Figures 4.4(1) to 4.4(30)). The low abundance and unassigned groups are not included in the bar charts.

Baby 1 represent the caesarean section delivered and mixed fed baby. It was observed that the faecal microbial composition lacked *Bifidobacterium* in the first sample, was dominated by *Klebsiella* 47.2%, followed by *Velloinella* 20.5%, *Streptococcus* 9.74%, *Enterococcus* 6.62%, and *Trabusiella* 4.86%. The other genera had a relative abundance below 3.0%. At months 1-3, *Bifidobacterium* (56.2-66.4%) predominated, while at month 4, a decline in the relative abundance of *Bifidobacterium* was observed and an increase in *Streptococcus* (53.5%). However, from the obtained metadata, the subject/baby was neither sick nor received antibiotics. The homeostasis was restored at month 5 up to 6 months with increased *Bifidobacterium*.

A drastic change occurred in the faecal composition at month 7 with a sharp decrease in *Bifidobacterium*, while *Bacteroides, Lactobacillus, Clostridium* and *Butyricoccus* appeared. Between months 8-10, different genera began to appear and dominate.The new genera were exemplified by *Faecalibacterium, Roseburia, Dorea* and *Blautia* (Figure 4.4 (1)).

Baby 2 to Baby 7 gut composition were majorly dominated by *Streptococcus* except baby 2 that was dominated by *Klebsiella* at birth. From month 1-6, *Bifidobacterium*, *Streptococcus*, *Trabulsiella*, *Enterococcus*, *Collinsella* and *Veillonella* were present but their relative abundance fluctuated. At 7-10 months the microbial diversity increased with new microbes appearing such as *Lactobacillus*, *Prevotella*, *Rothia*, *Ruminococcus*, *Balutia* and others (Figure 4.4 (2) - (7)).

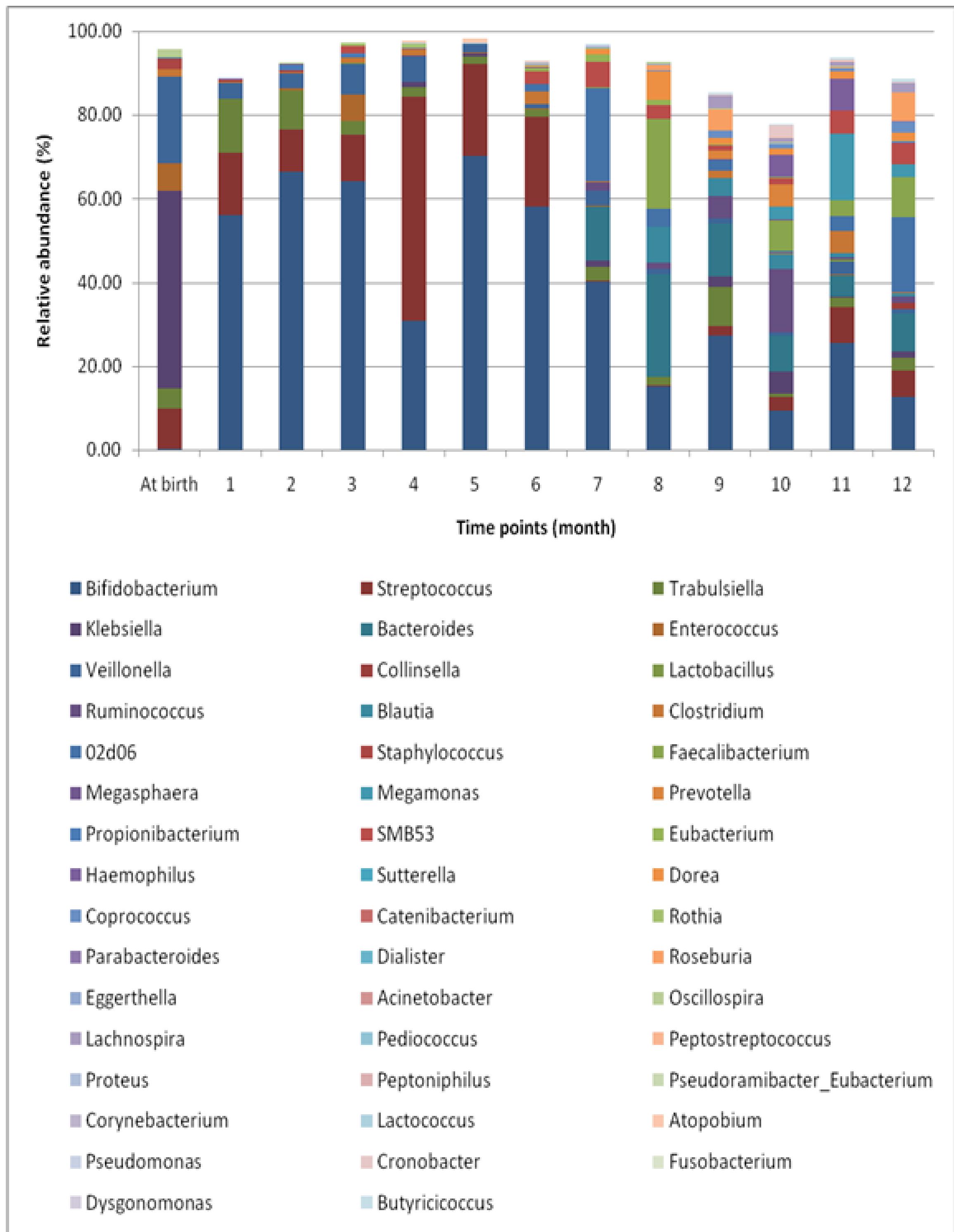


Figure 4.4(1): The genus level gut composition of Baby 1

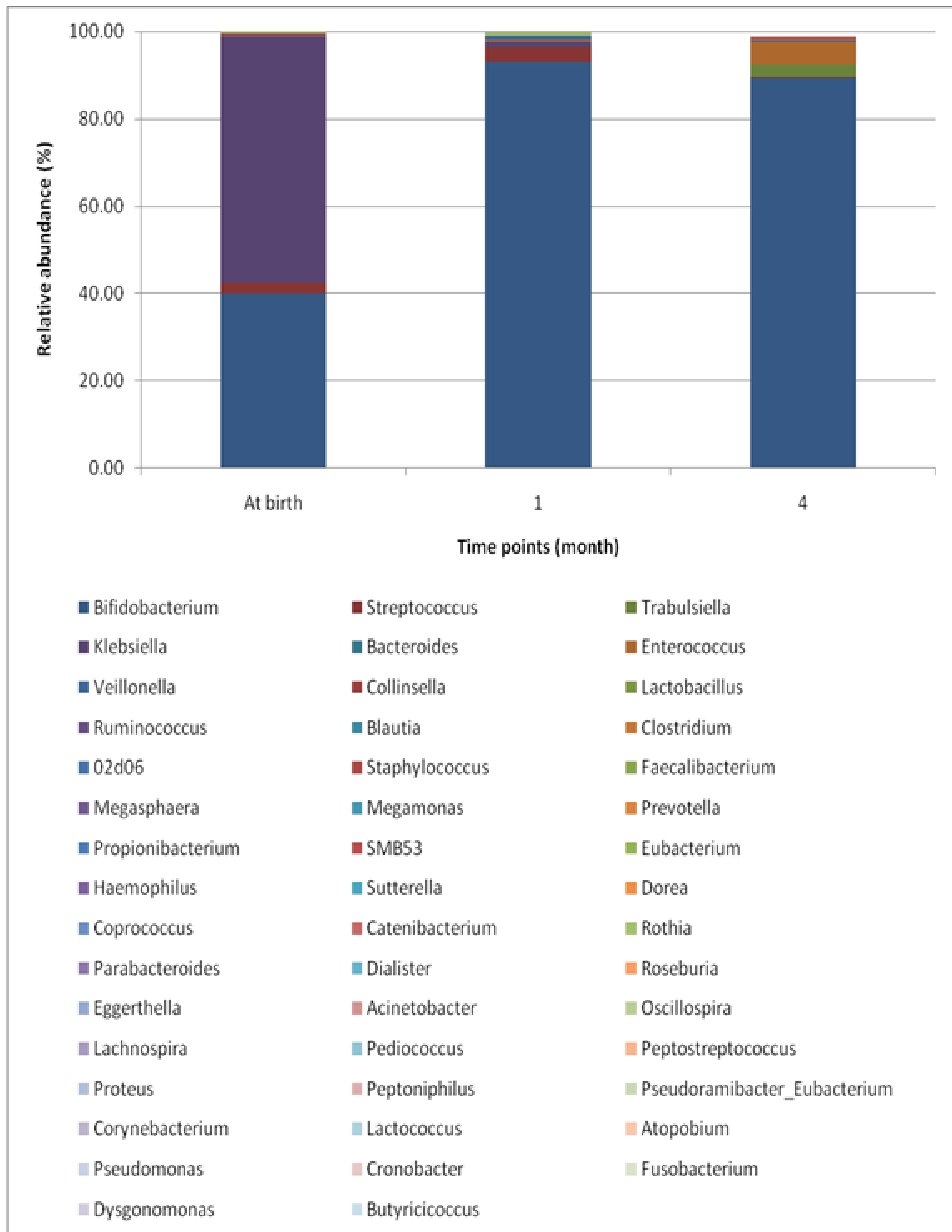


Figure 4.4(2): The genus level gut composition of Baby 2

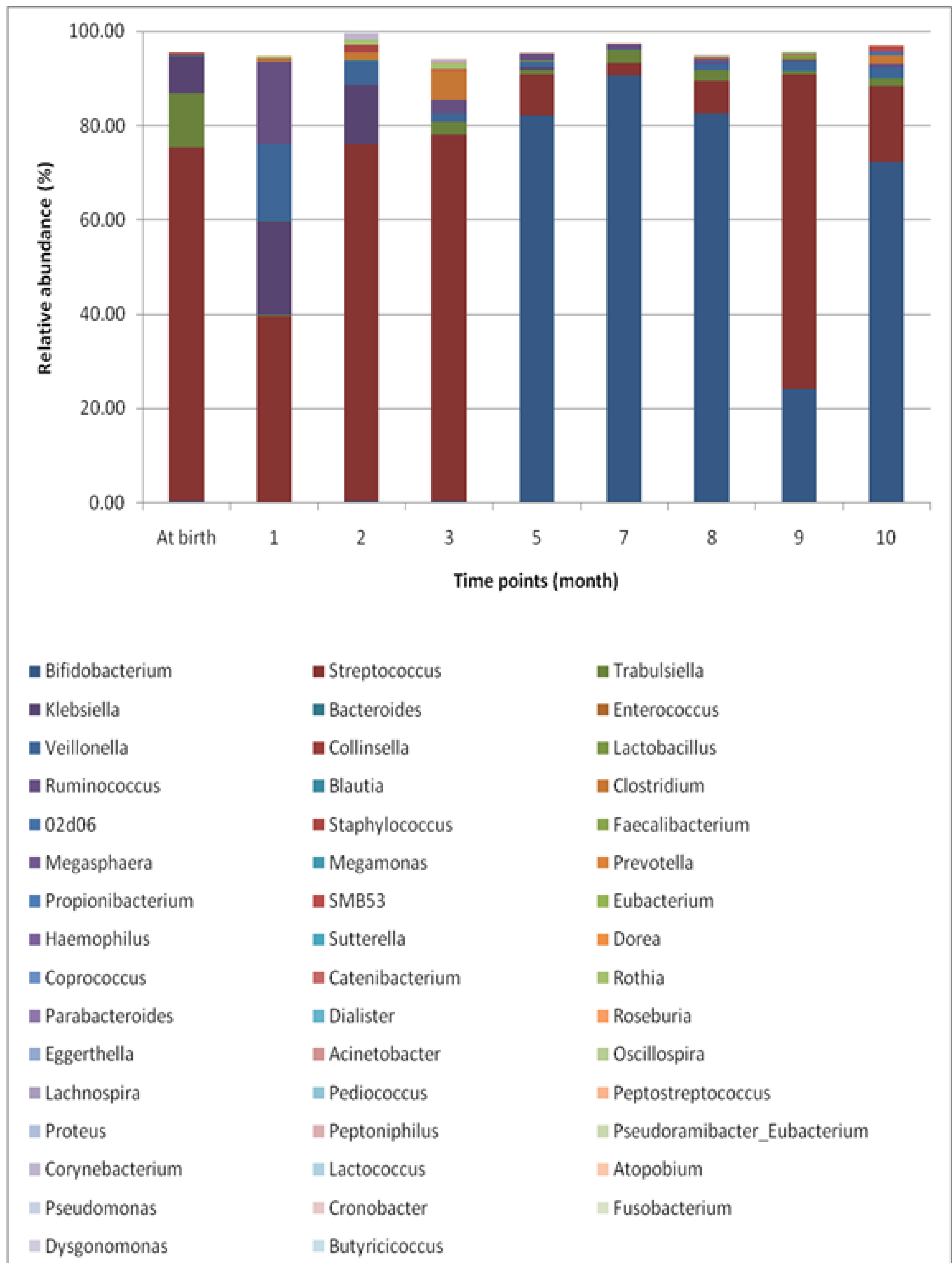


Figure 4.4(3): The genus level gut composition of Baby 3

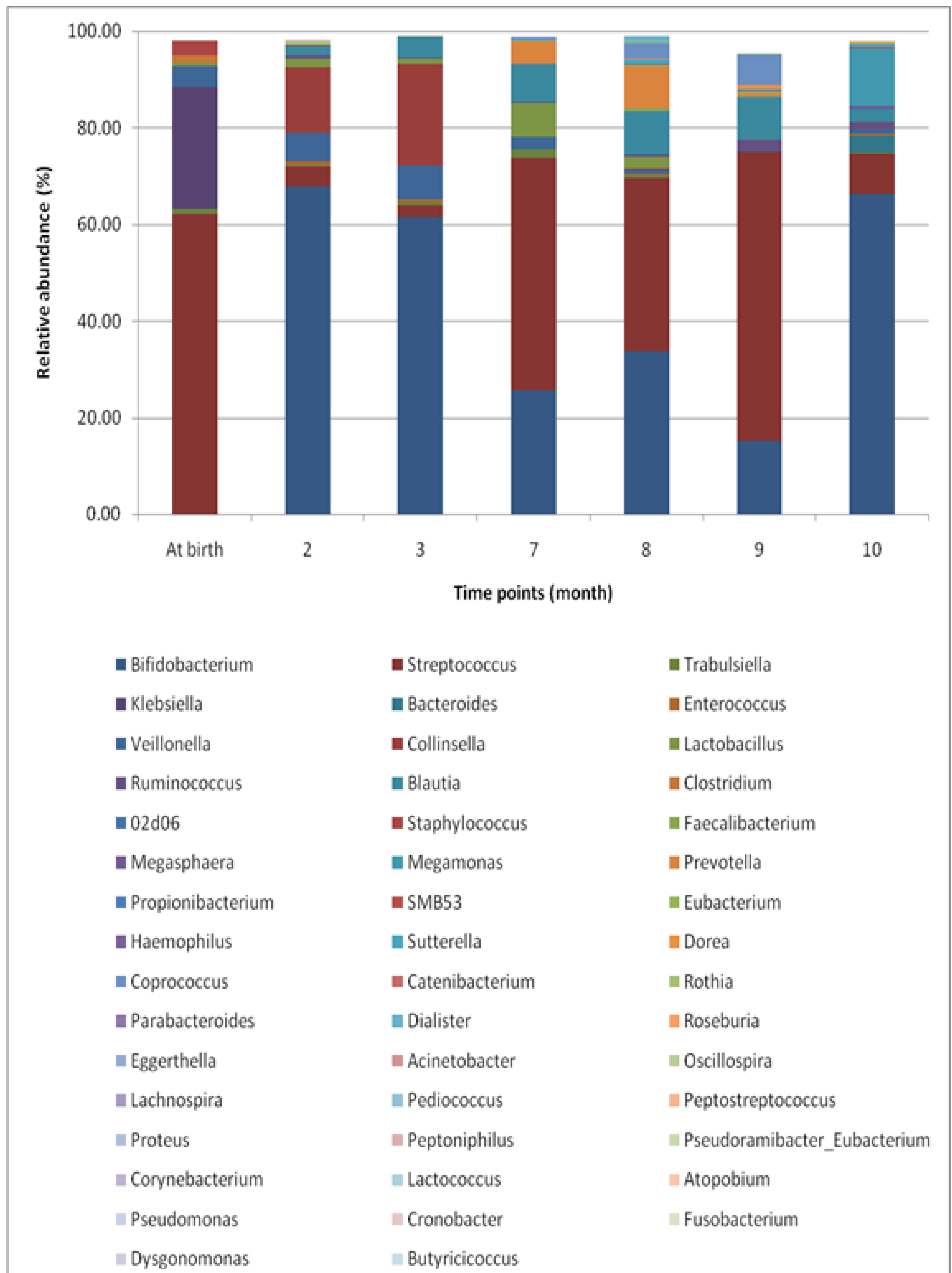


Figure 4.4(4): The genus level gut composition of Baby 4

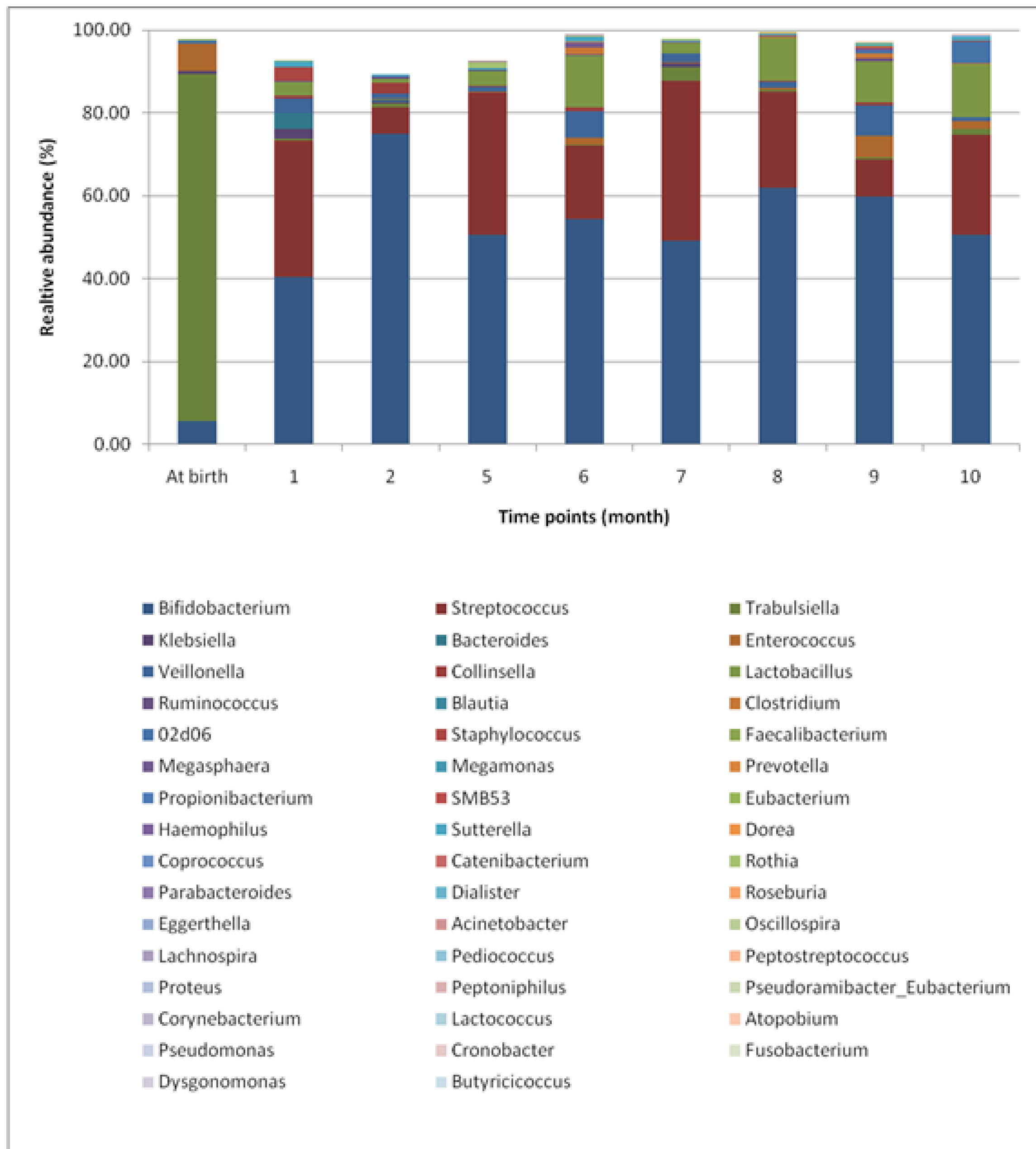


Figure 4.4(5): The genus level gut composition of Baby 5

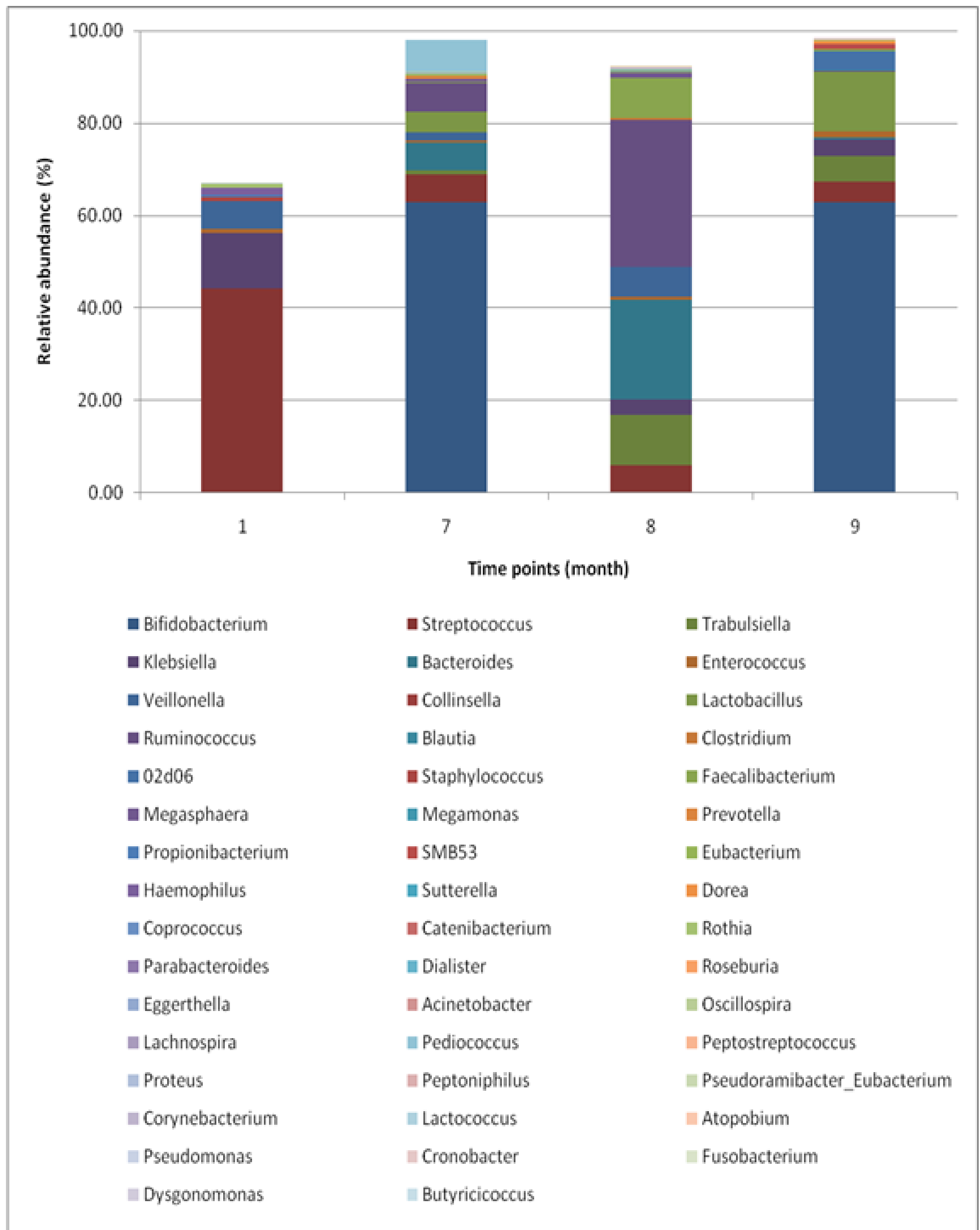


Figure 4.4(6): The genus level gut composition of Baby 6

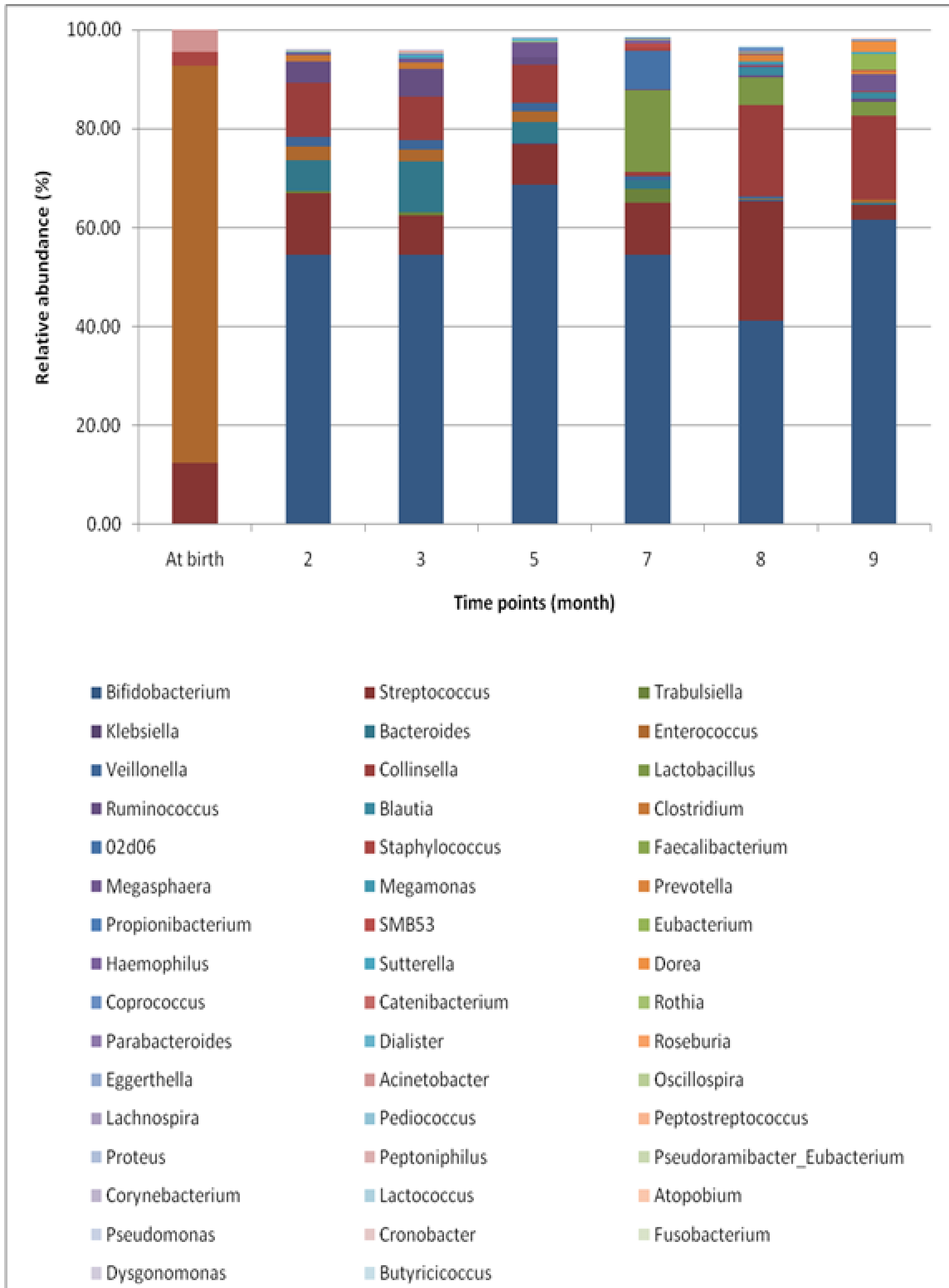


Figure 4.4(7): The genus level gut composition of Baby 7

Baby 8 is a representative of vaginally delivered and exclusively breastfed baby. The faecal microflora was dominated by *Bifidobacterium* (50.3%), *Streptococcus* (37.2%), and *Trabusiella* 5.27%, while other genera had a relative abundance below 3.0% at birth. At month 1, this baby had fever, received antibiotic (amoxicillin) treatment and was on herbal treatment (bathing). Species belonging to *Bifidobacterium* and *Streptococcus* disappeared and the faecal microbiota was dominated by *Trabulsiella* (81.6%), while other microbiota was unassigned species. At month 2, homeostasis was restored and *Bifidobacterium* was restored; however, the relative abundance of this genus fluctuated till the 6th month.

At month 7, the diversity was not as obvious as found in CSB; however, genera such as *Faecalibacterium*, *Butyricoccus*, *Enterococcus*, *Bacteroides* and *Parabacteroides* appeared. At month 9, *Bifidobacterium* disappeared again and was restored at month 10. The baby had discontinued herbal treatment, no sickness and no antibiotic usage at this time point. The relative abundance of *Bifidobacterium* seemed not to experience drastic reduction overtime but only appeared and disappeared at some point (Figure 4.4(8)).

Baby 9 is another representative of vaginally delivered and exclusively breastfed infant. *Bifidobacterium* was about 38.0% in abundance and Streptococcus (52.0%) dominated the gut at birth. At month 1, the relative abundance of *Bifidobacterium* declined to 8.0%, *Trabulsiella* (42.0%) dominated the gut and *Bacteroides* was about 8.0%. Across the remaining months *Bifidobacterium* was high and much diversity was not observed from 7-9 months rather *Bifidobacterium* was 75.0% at month 9 (Figure 4.4(9)).

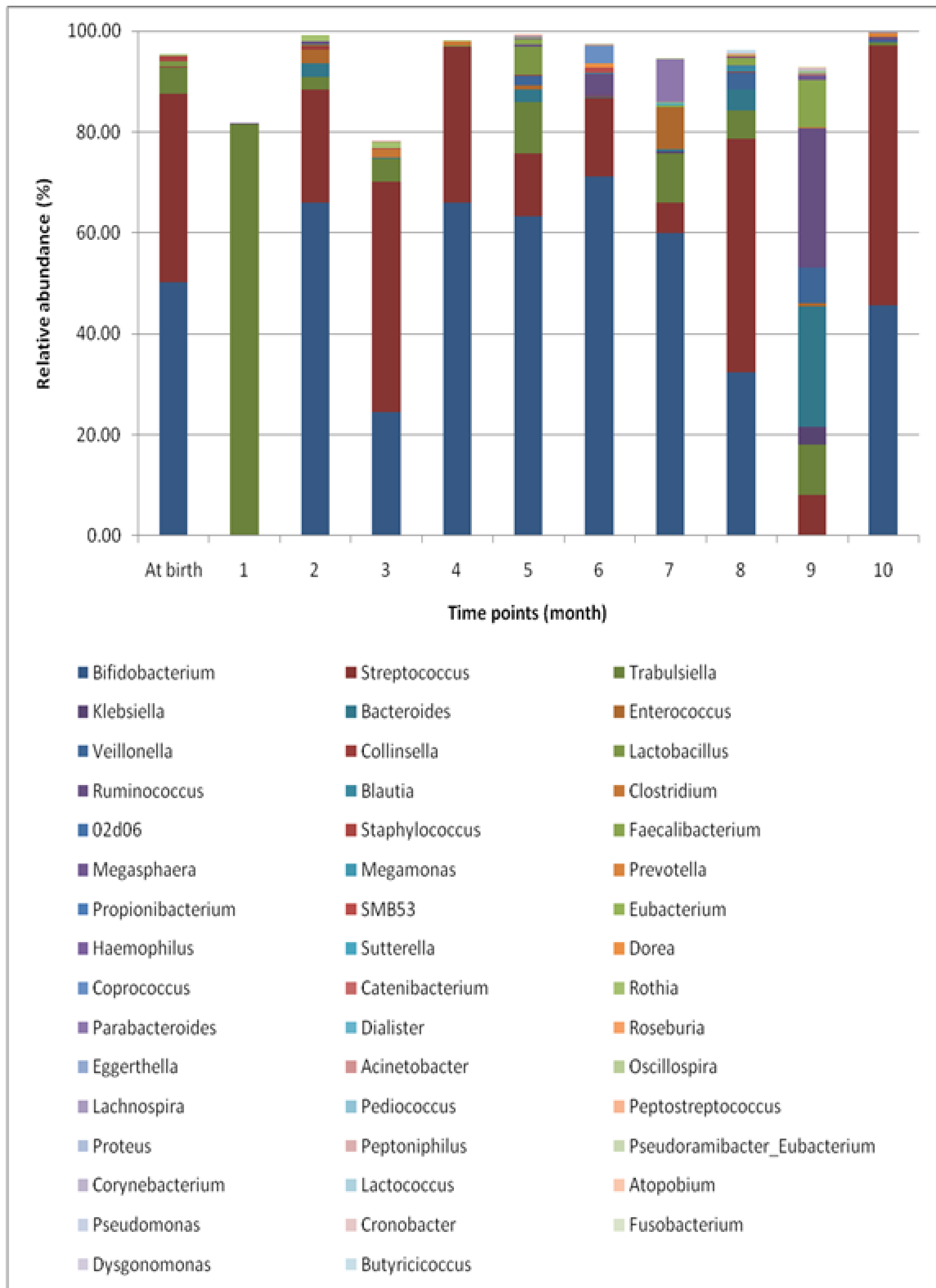


Figure 4.4(8): The genus level gut composition of Baby 8

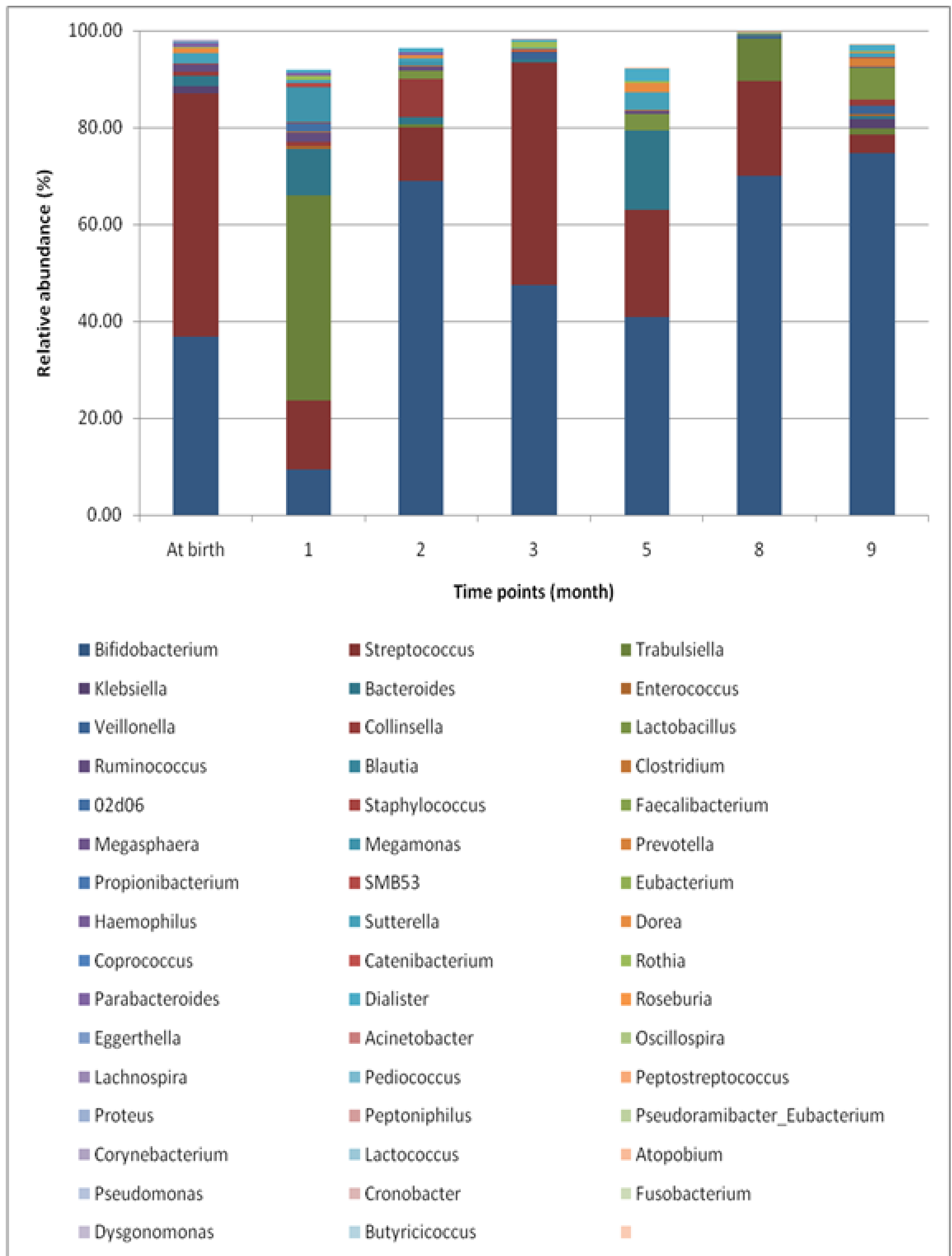


Figure 4.4(9): The genus level gut composition of Baby 9

The mode of birth for the 3 sets of twin in this study was caesarean section. Baby 10 and 11 (Figures 4.4(10) and 4.4(11)) are twins and mixed fed babies. Although they are twins, their faecal microbial compositions were not the same, emphasising the variation in the gut microbial composition among individuals. The relative abundance of *Bifidobacterium* (1.3%) was low in Baby 10, twin 1. The gut was dominated by *Trabulsiella* (41.8%), *Klebsiella* (34.9%), *Streptococcus* (9.76%), *Staphylococcus* (4.68%), *Propionibacterium* (3.38%) and other microbiota were below 3.0% in abundance. The relative abundance of *Bifidobacterium* (17.5%) was higher in Baby 11, twin 2. The gut was dominated by *Klebsiella* (36.52%), *Trabulsiella* (17.6%), *Propionibacterium* (6.02%), *Streptococcus* (5.80%) and other microbiota were below 3.0% in abundance at birth. At month 1, *Bifidobacterium* increased in the gut of both babies and fluctuated overtime. Introduction of solid foods started earlier at month 4 and diversity was observedat month 5 (Figure 4.4(10)), with *Blautia, Eubacterium, Ruminococcus, faecalibacterium, Bacteroides* appearing. However, baby 11 (Figure 4.4(11)) had microbiota comprised of 96.9% of *Streptococcus*at month 5. This baby was neither sick nor used antibiotics. The homeostasis was restored and microbial diversity was obvious at month 6 with genera such as *Bacteroides, Enterococcus, Lactobacillus* and *Clostridium* appearing, such that *Bacteroides*comprised as high as 35.9% at month 8. *Bifidobacterium* did not show any drastic reduction in these twin babies either. Generally, there was a low microbial diversity in all the first samples collected, and gradual increase as time points increased with drastic change when *Ogi* and solid foods were introduced such that between months 8-12, there was higher diversity.

Baby 12 and Baby 13 were another set of twin. These babies provided only the birth sample. At birth baby 12 (twin 1) had Proteobacteria (56.2%), Firmicutes (41.7%), Actinobacteria (2.1%), Bacteroidetes (0.0%) and Fusobacteria (0.0%) at phylum level and *Klebsiella* (53.3%), *Streptococcus* (28.0%), *Clostridium* (10.5%), *Enterococcus* (3.1%) and *Bifidobacterium* (2.1%) at the genus level while baby 13 (twin 2) had Proteobacteria (56.7%), Firmicutes (43.2%), Actinobacteria (0.1%), Bacteroidetes (0.0%) and Fusobacteria (0.0%) at phylum level with *Klebsiella* (54.7%), *Clostridium* (28.5%) *andStreptococccus* (13.9%), *Bifidobacterium* and *Enterococcus* were absent at the genus

level and (Figure 4.4 (12) and Figure 4.4 (13)).

Baby 27 and Baby 28 are the third set of twin in the present study. The gut microbiota of these infants differs, the most abundance microbes in the first sample collected from baby 27 (twin 1) are *Blautia* (31.9%), *Klebsiella* (31.8%), *Enterococcus* (5.49%) and *Bifidobacterium* (5.45%) at genus-level composition and Firmicutes (48.7%),Proteobacteria (36.7%) and Actinobacteria (14.6%) for phylum-level composition subsequently, *Bifidobacterium* increased gradually up to 75.2% at month 4 and declined to 39.9% at month 7 while *Bacteroides* and *Faecalibacterium* increased from 0.0% at birth to 16.7% and 7.0% at month 7 respectively.Baby 28 (twin 2) possessed Firmicutes (41.7%), Proteobacteria (41.5%) and Actinobacteria (16.8%) at phylum-level composition while *Clostridium* 27.3%, *Trabusiella* (25.4%), *Bifidobacterium* (16.0%), *Enterococcus* (3.9%) and *Klebsiella* (3.1%) were the most abundant in the first sample collected at genus level. *Bifidobacterium* increased to 56.9% at month 2 and declined gradually to 8.9% at month 7 while *Trabulsiella*, *Streptococcus*, *Bacteroides* and *Veillonella* increased to 36.8%, 14.3%, 6.1% and 5.9% respectively. Microbial diversity started earlier in these twins at month 5 (Figure 4.4(26) and Figure 4.4(27)).

Comparison between the gut microbiota of the three set of twins are outlined. The mode of birth was caesarean section for all the twins. At birth, Twin 1 (Baby 10) had dominance of Proteobacteria (78.4%) and Firmicutes (15.7%) as phylum composition and *Trabulsiella* (41.8%), *Klebsiella* (34.9%), *Streptococcus* (9.8%) and *Staphylococcus* (4.7%) as genus composition while Twin 2 (Baby 11) had Proteobacteria (65.6%) and Actinobacteria (23.9%) as the phylum composition and *Klebsiella* (36.5%), *Trabulsiella* (17.6%), *Bifidobacterium* (17.5%) and *Propionibacterium* (6.0%) as the genus composition.

At month 5, the sample collected after the introduction of solid foods, baby 10 had Actinobacteria (56.8%) and Firmicutes (34.6%) and *Bifidobacterium* (56.2%), *Eubacterium* (6.8%), *Enterococcus* (5.6%), *Streptococcus* (5.2%) and *Ruminococcus* (3.4%). While baby 11 had dominance of Firmicutes (97.6%), Actinobacteria (1.2%), *Streptococcus* (96.9%) and *Trabulsiella* (1.0%) at phylum and genus level respectively.

At month 10, the last weaning sample collected from this set of twin, baby 10 possessed Firmicutes (52.7%), Actinobacteria (43.2%), *Bifidobacterium* (36.3%), *Streptococcus*

(29.3%), *Lactobacillus* (13.1%) and *Veillonella* (3.2%) while baby 11 possessed Actinobacteria (59.7%), Firmicutes (22.8%) and *Bifidobacterium* (49.7%), *Bacteroides* (13.0%), *Faecalibacterium* (7.5%) and *Dorea* (3.6%) at genus and phylum level respectively.

Twin 2 (Baby 12 and 13) provided birth samples only. Baby 12 had abundance of Proteobacteria (56.2%), Firmicutes (41.7%) and *Klebsiella* (53.3%), *Streptococcus* 28.0%, *Clostridium* (10.5%) and *Enterococcus* (3.0%) while Proteobacteria (56.7%) Firmicutes (43.2%) and *Klebsiella* (54.7%), *Clostridium* (28.5%), *Streptococcus* (13.9%) and *Propionibacterium* (6.0%) dominated the gut at both phylum and genus level respectively.

Twin 3 (Baby 27 and 28) provided samples up to 7 months. For Baby 27, the birth sample comprises of Firmicutes (48.7%) and Proteobacteria (36.7%) and *Blautia* (31.9%), *Klebsiella* (31.8%), *Propionibacterium* (8.9%), *Enterococcus* and *Bifidobacterium* (5.5%) while Baby 28 comprises of Firmicutes (41.7%) and Proteobacteria (41.5%) and *Clostridium* (27.3%), *Trabulsiella* (25.3%), *Bifidobacterium* (16.0%) and *Enterococcus* (3.9%) at phylum and genus level respectively.

Sample collected after introduction of solid food was in month 4, Baby 27 had Actinobacteria (84.7%), Firmicutes (9.9%) and *Bifidobacterium* (75.2%), *Collinsella* (9.5%), *Bacteroides* (4.1%) and *Lactobacillus* (3.3%) while Baby 28 had Actinobacteria (45.0%), Firmicutes 42.5% and *Bifidobacterium* (35.3%), *Lactobacillus* (13.6%), *Collinsella* (9.2%), *Streptococcus* (7.0%) and *Blautia* (5.9%) at phylum and genus level respectively.

At month 7, the last sample collected from this set of twin, Baby 27 possessed Actinobacteria (48.5%), Firmicutes (25.6%) and *Bifidobacterium* (39.9%), *Bacteroides* (16.7%), *Collinsella* (8.3%) and *Faecalibacterium* (7.0%) while Baby 28 possessed Proteobacteria (54.1%), Firmicutes (29.6%) and *Trabulsiella* (36.8%), *Streptococcus* (14.3%), *Bifidobacterium* (8.9%) and *Bacteroides* (6.1%) at phylum and genus level respectively.

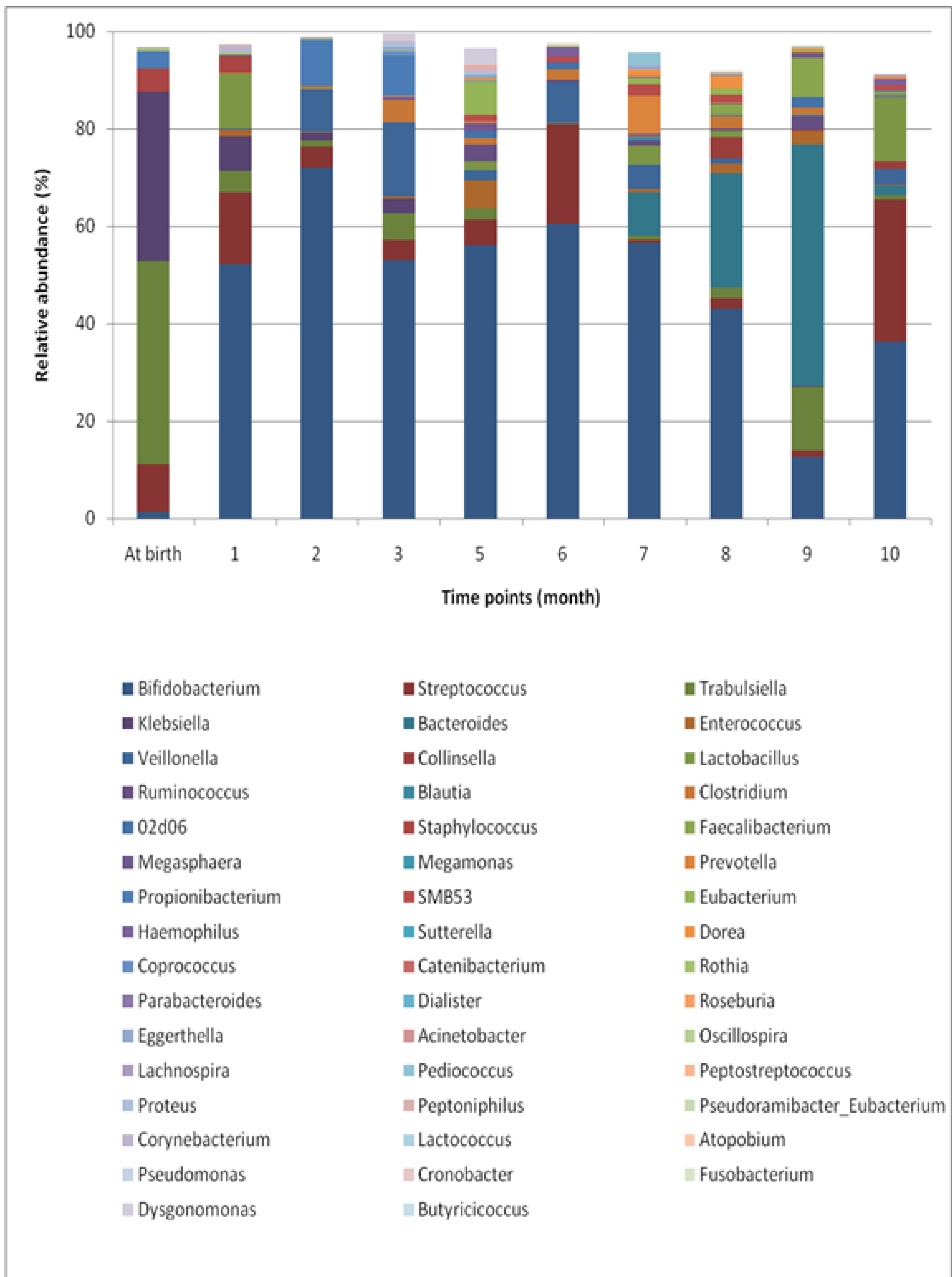


Figure 4.4(10): The genus level gut composition of Baby 10

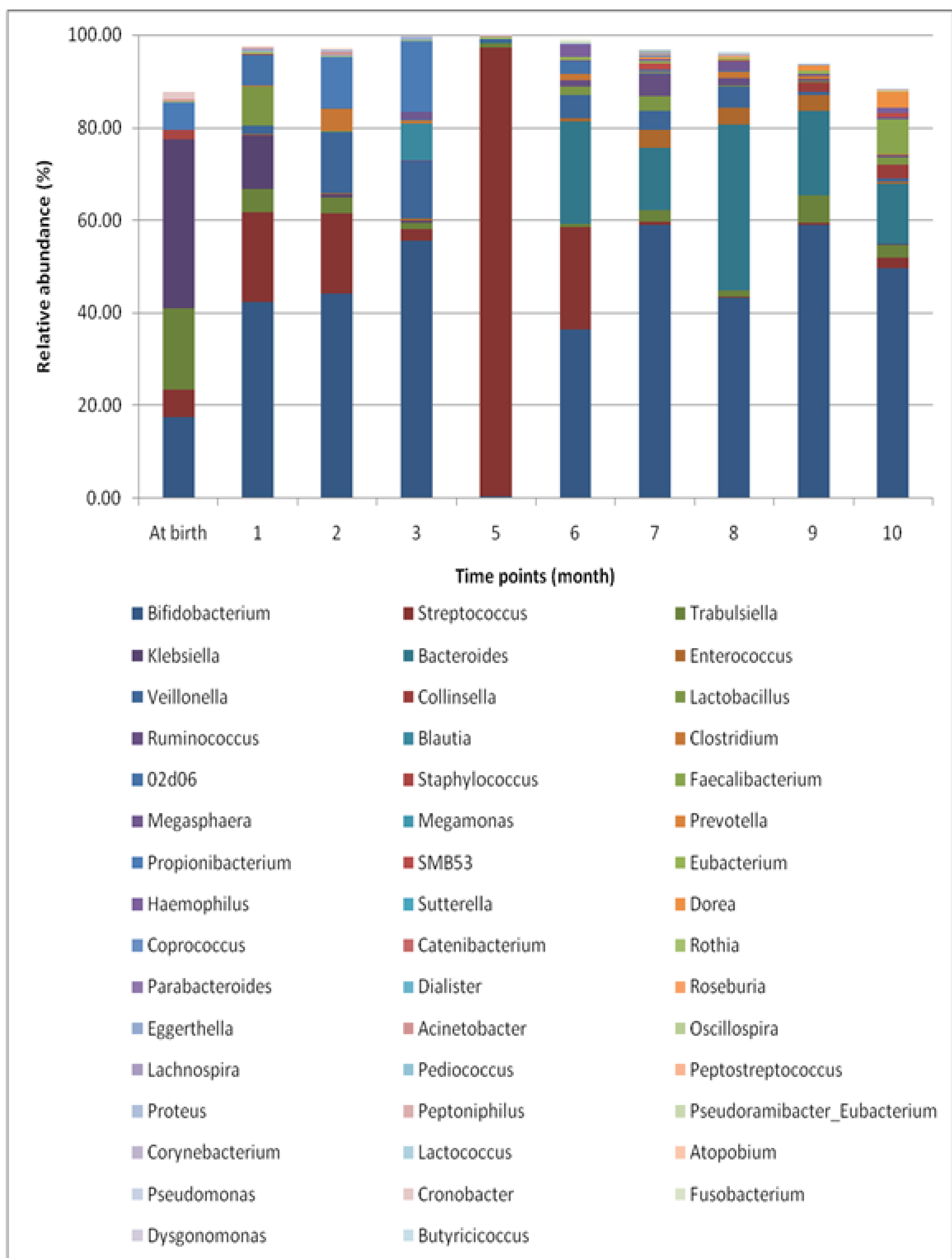


Figure 4.4(11): The genus level gut composition of Baby 11

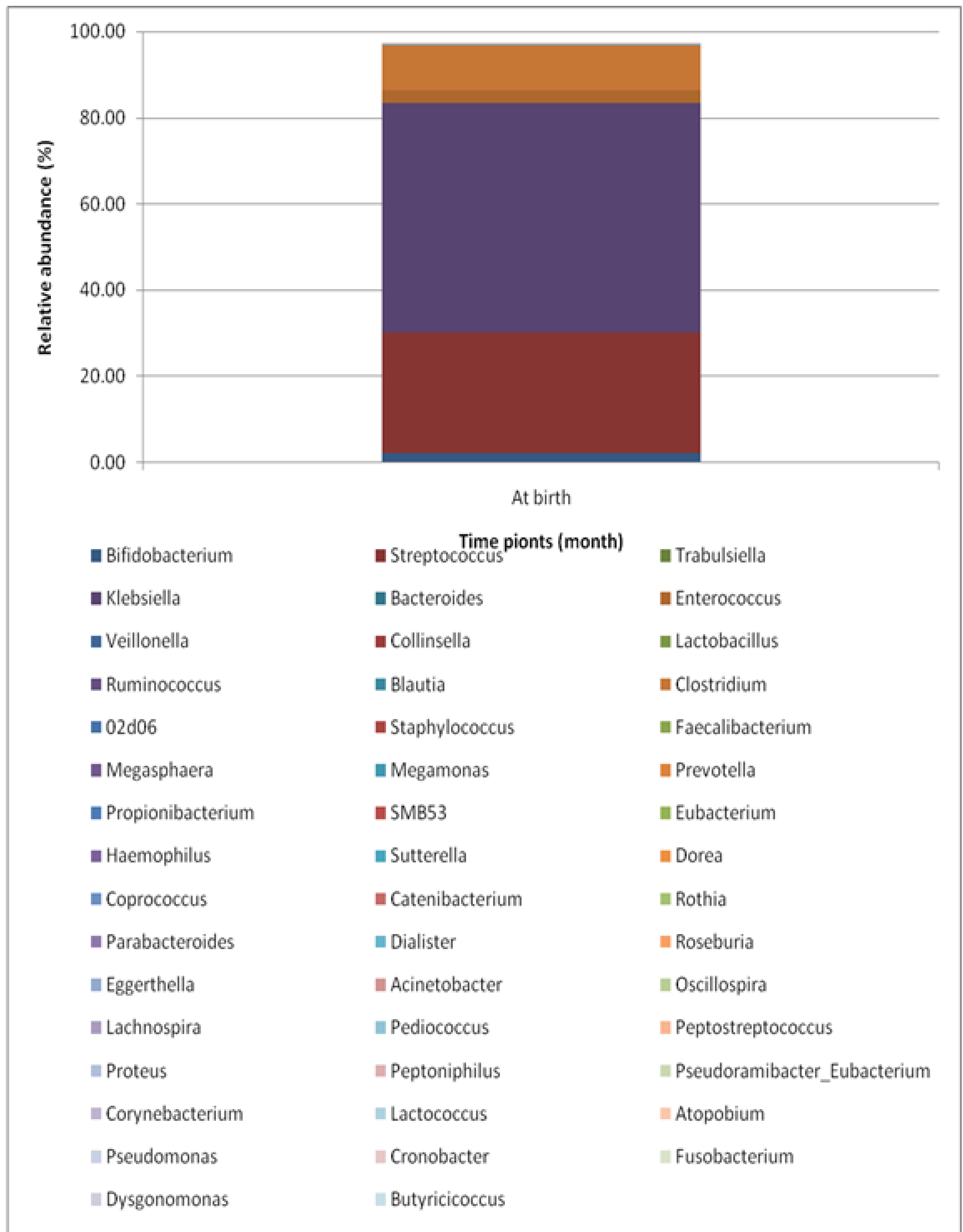


Figure 4.4(12): The genus level gut composition of Baby 12

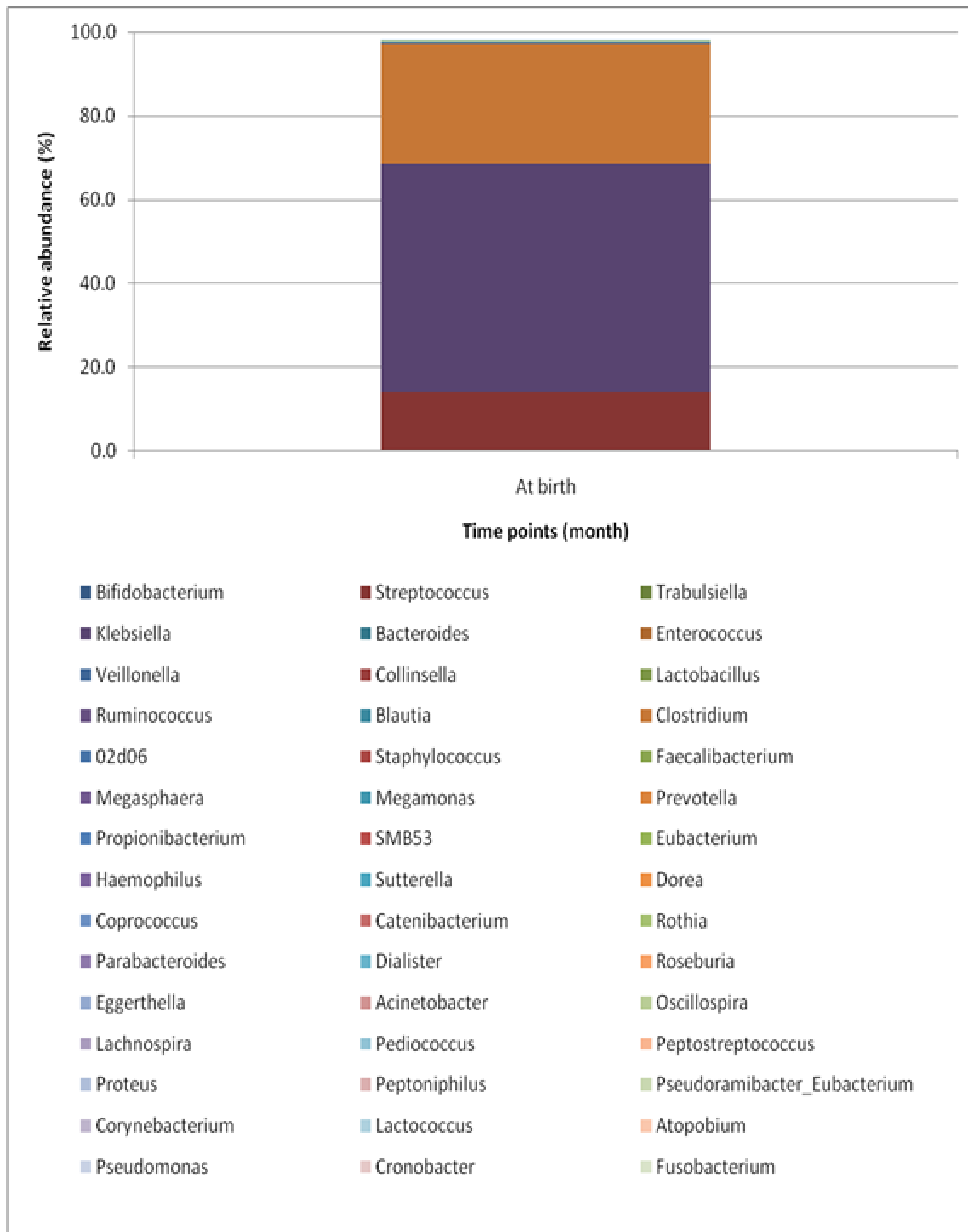


Figure 4.4(13): The genus level gut composition of Baby 13

For Babies 14-17, 20-25 and 30, individual differences are observed in the relative abundance of genera present in these infants. A low and unstable gut microbial composition in the early time points from birth to 6 months and higher diversity from months 7 - 9. The most abundant gut microbiota within the first 6 months of life in these infants was *Bifidobacterium* and *Streptococcus*. At the later time points from 7-9 months *Bacteroides*, *Dorea*, *Rothia, Eggerthella*, *Pediococcus* appeared (Figures 4.4 (14) - (17), (19) - (24) and (28)).

A noteworthy faecal microbial composition is that of meconium samples (first pass stool). The only faecal samples collected from babies 18 and 26 at birth were meconium (Figure 4.4(18)) and (Figure 4.4(25)) respectively. They both had different birth mode. Baby 18 was delivered by caesarean section and baby 26 by vaginal birth. The faecal microbiota of baby 18 was dominated by Firmicutes (97.3%) at phylum level and *Streptococcus* (97.3%) at genus level while that of baby 26 was dominated by Firmicutes (99.9%) at phylum level and *Enterococcus* (99.9%) at genus level. Both genera are members of the phylum Firmicutes they are facultative aerobes and early colonisers of infant gut.

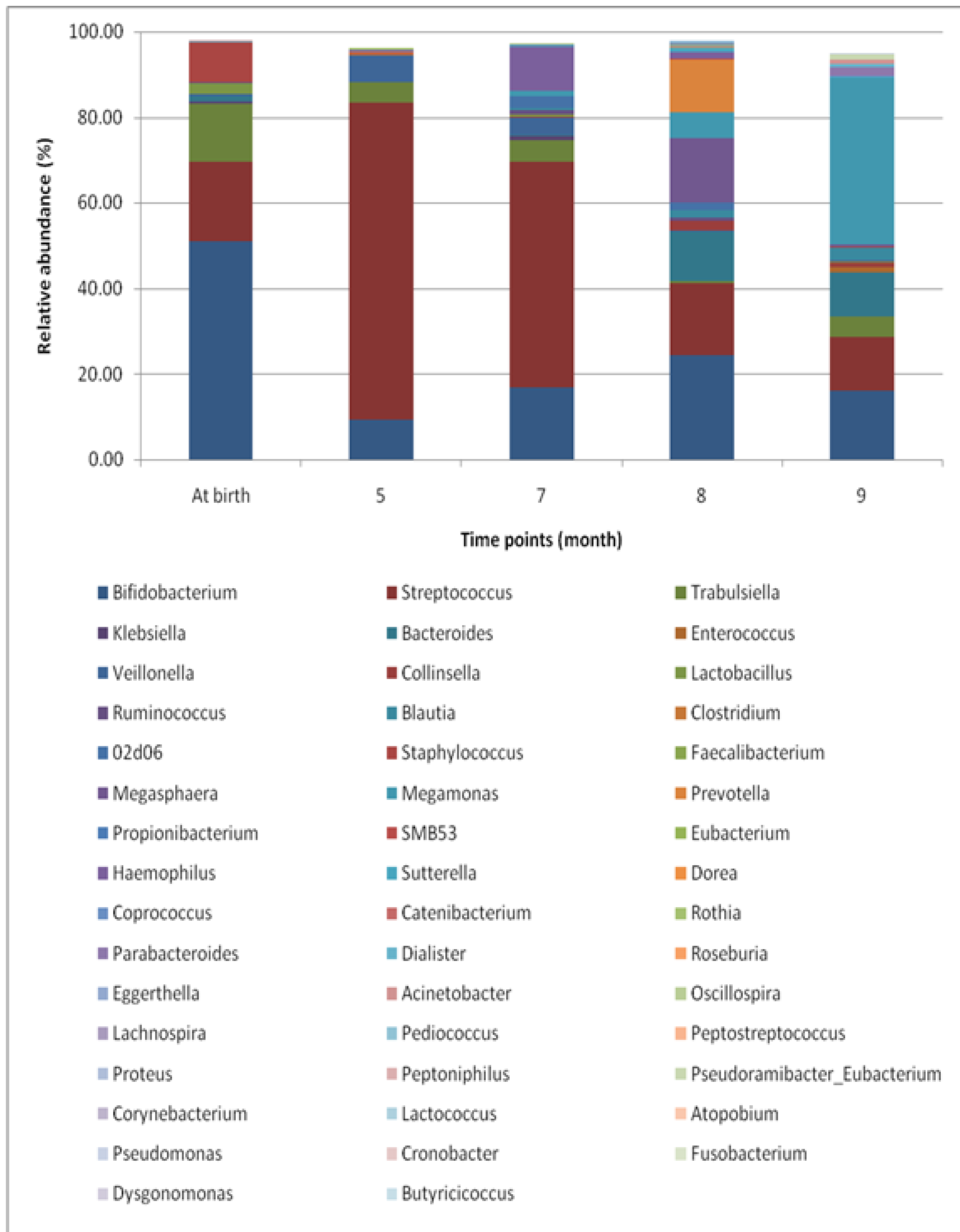


Figure 4.4(14): The genus level gut composition of Baby 14

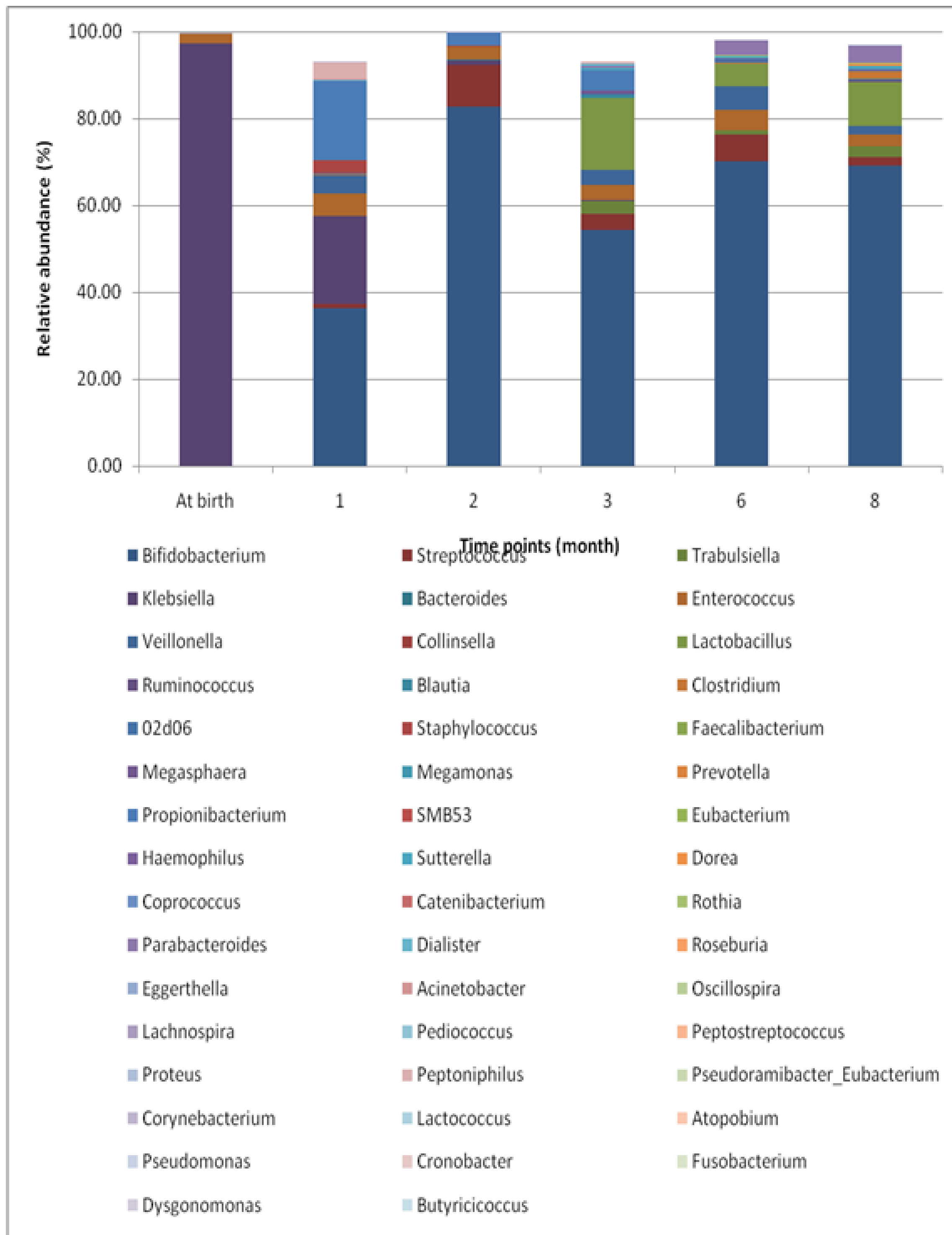


Figure 4.4(15): The genus level gut composition of Baby 15

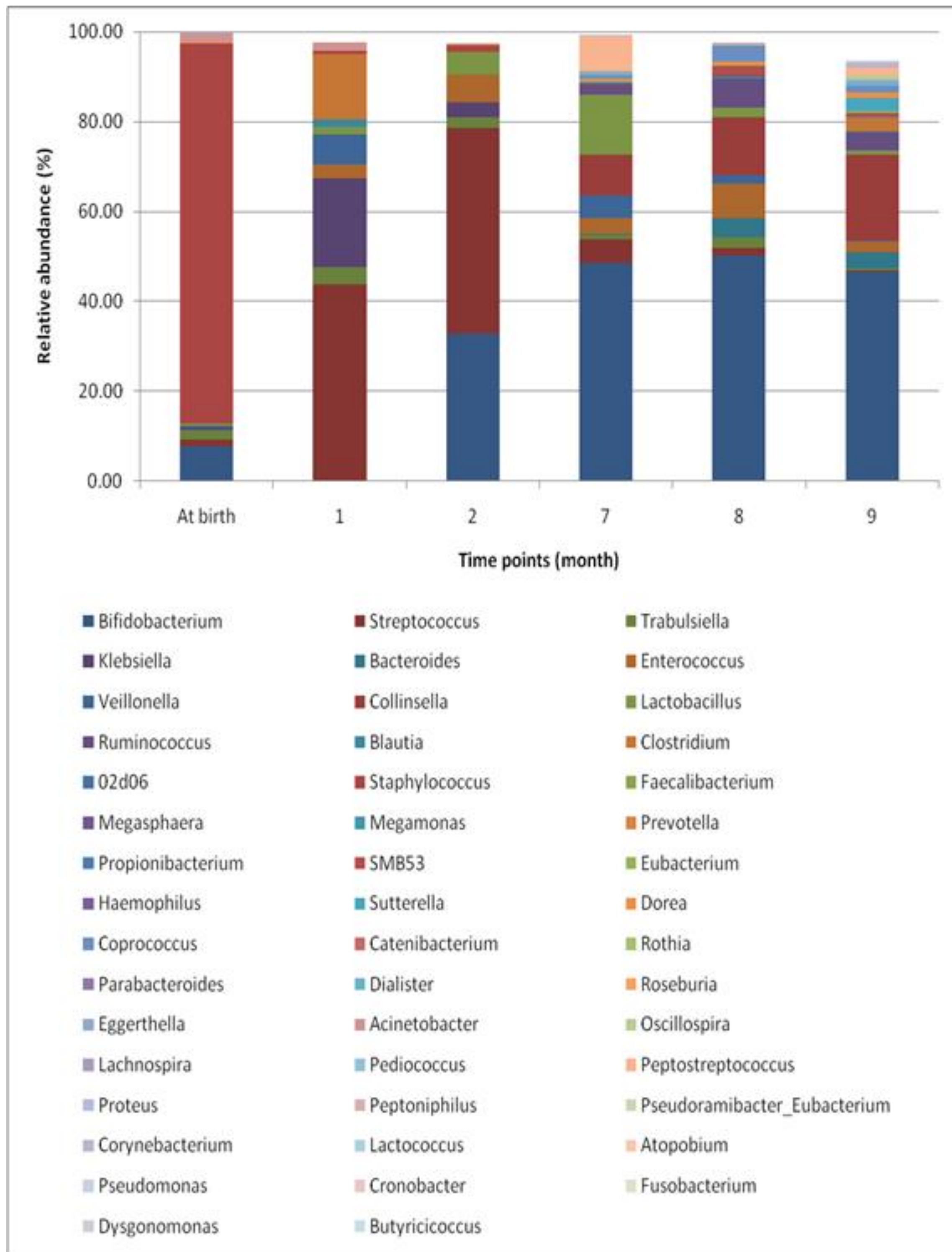


Figure 4.4(16): The genus level gut composition of Baby 16

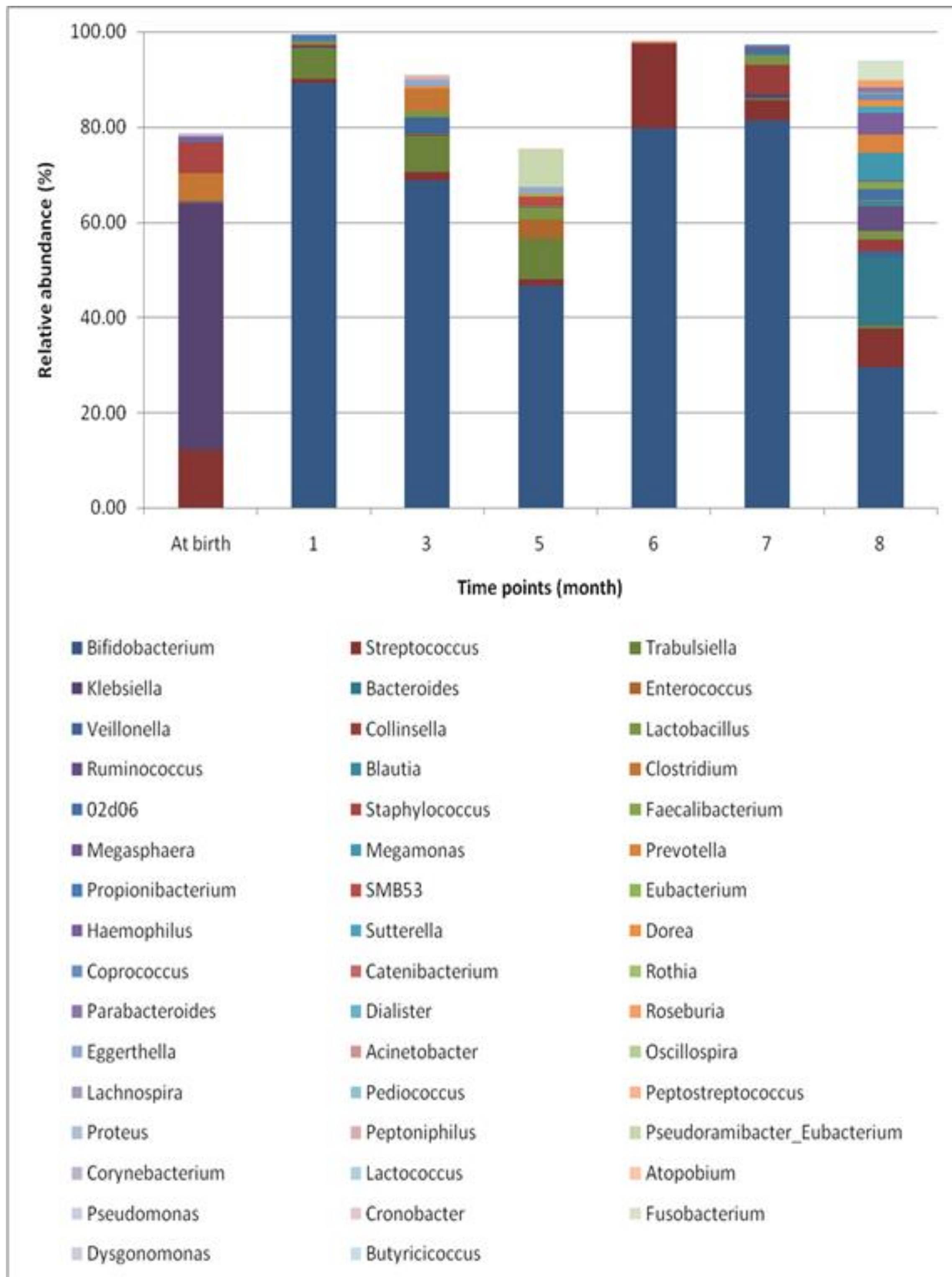


Figure 4.4(17): The genus level gut composition of Baby 17

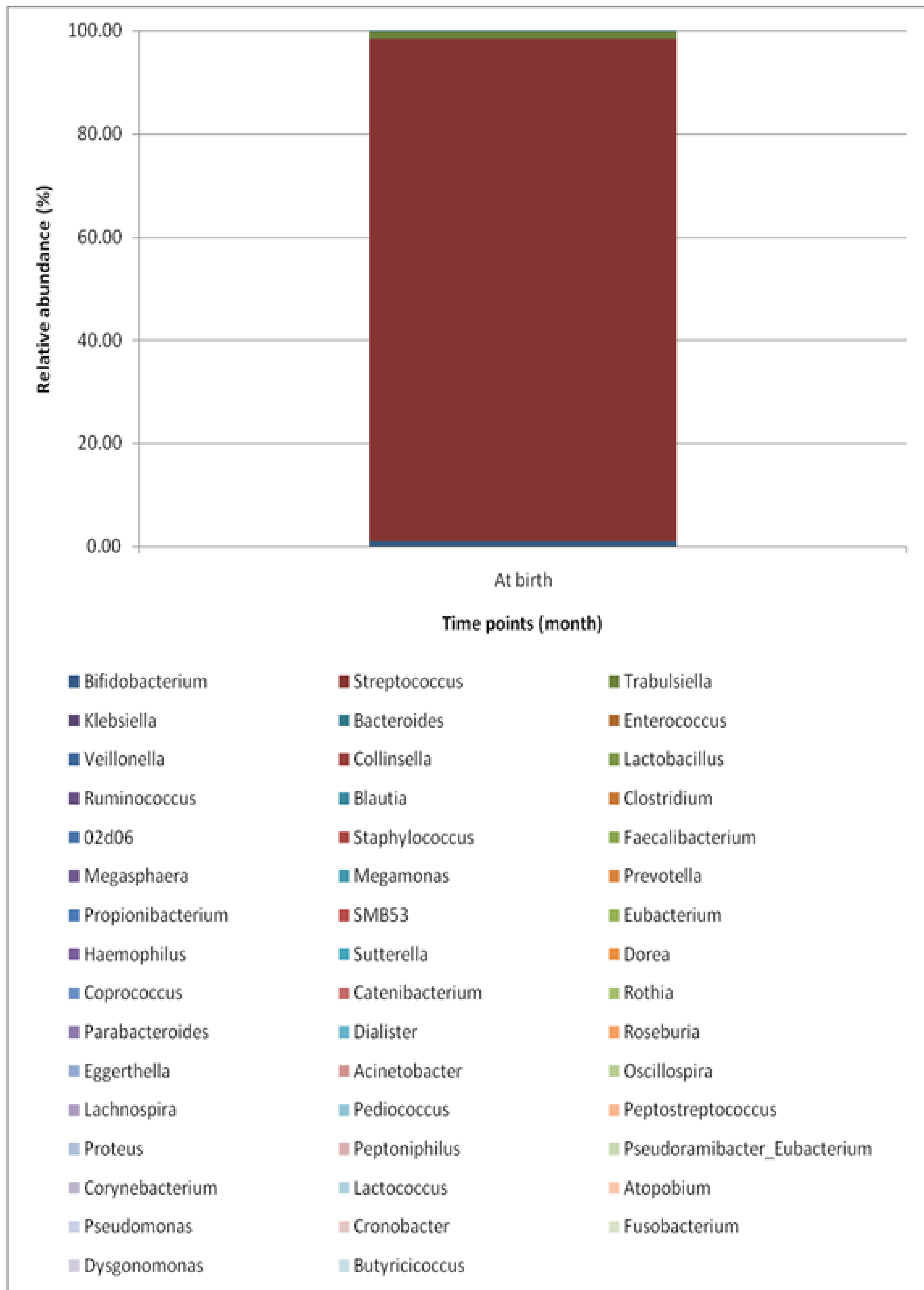


Figure 4.4(18): The genus level gut composition of Baby 18

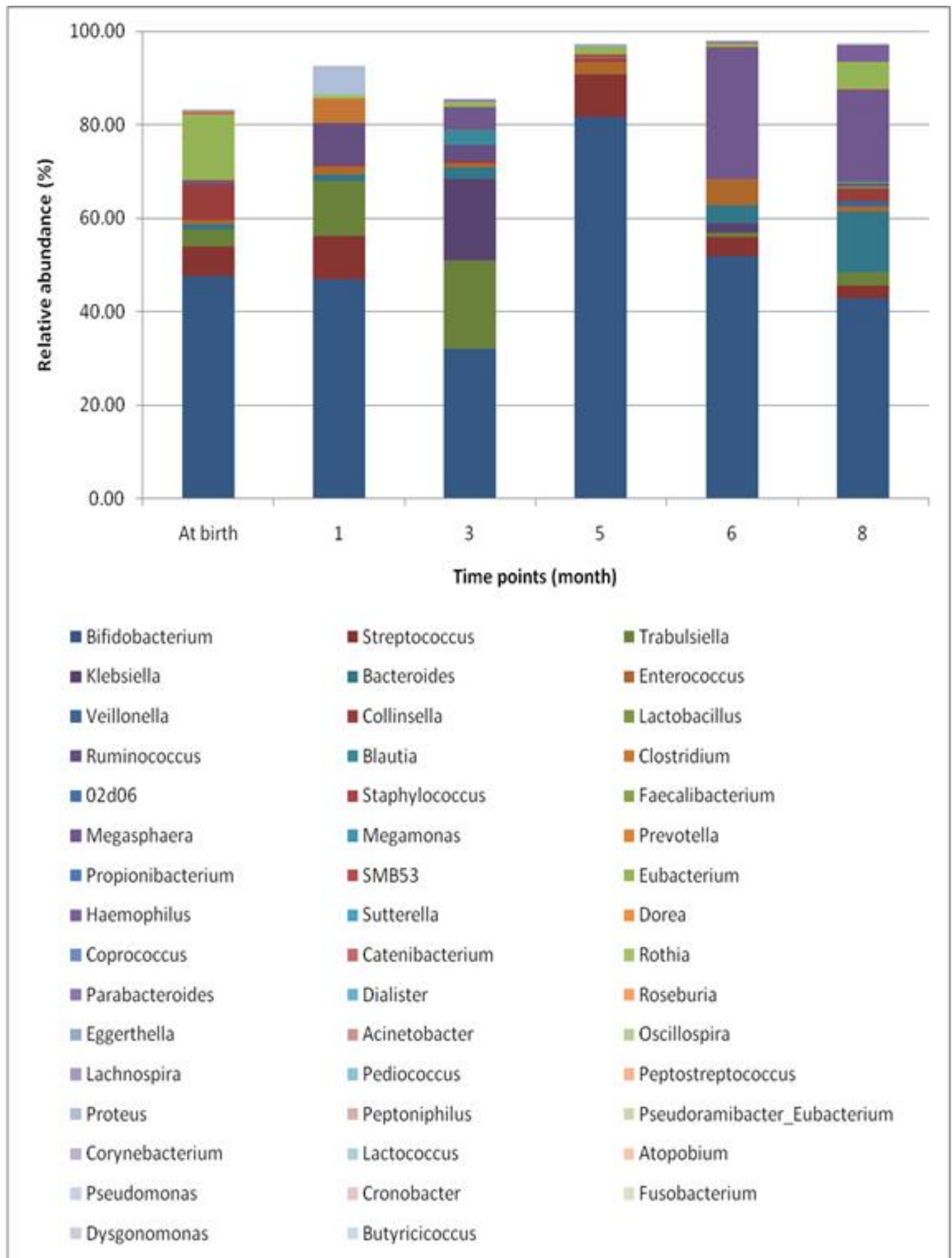


Figure 4.4(20): The genus level gut composition of Baby 20

Figure 4.4(21): The genus level gut composition of Baby 21

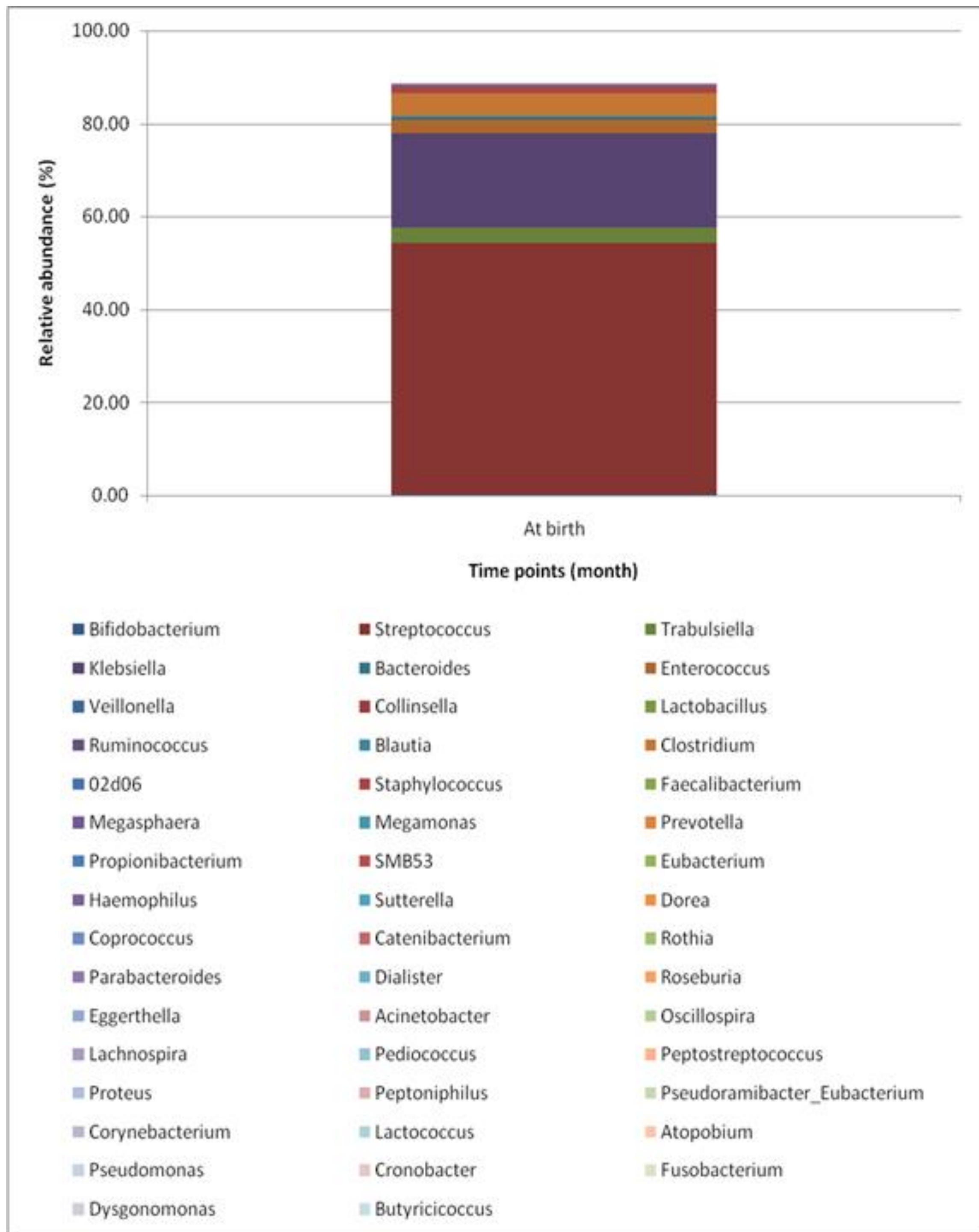


Figure 4.4(22): The genus level gut composition of Baby 22

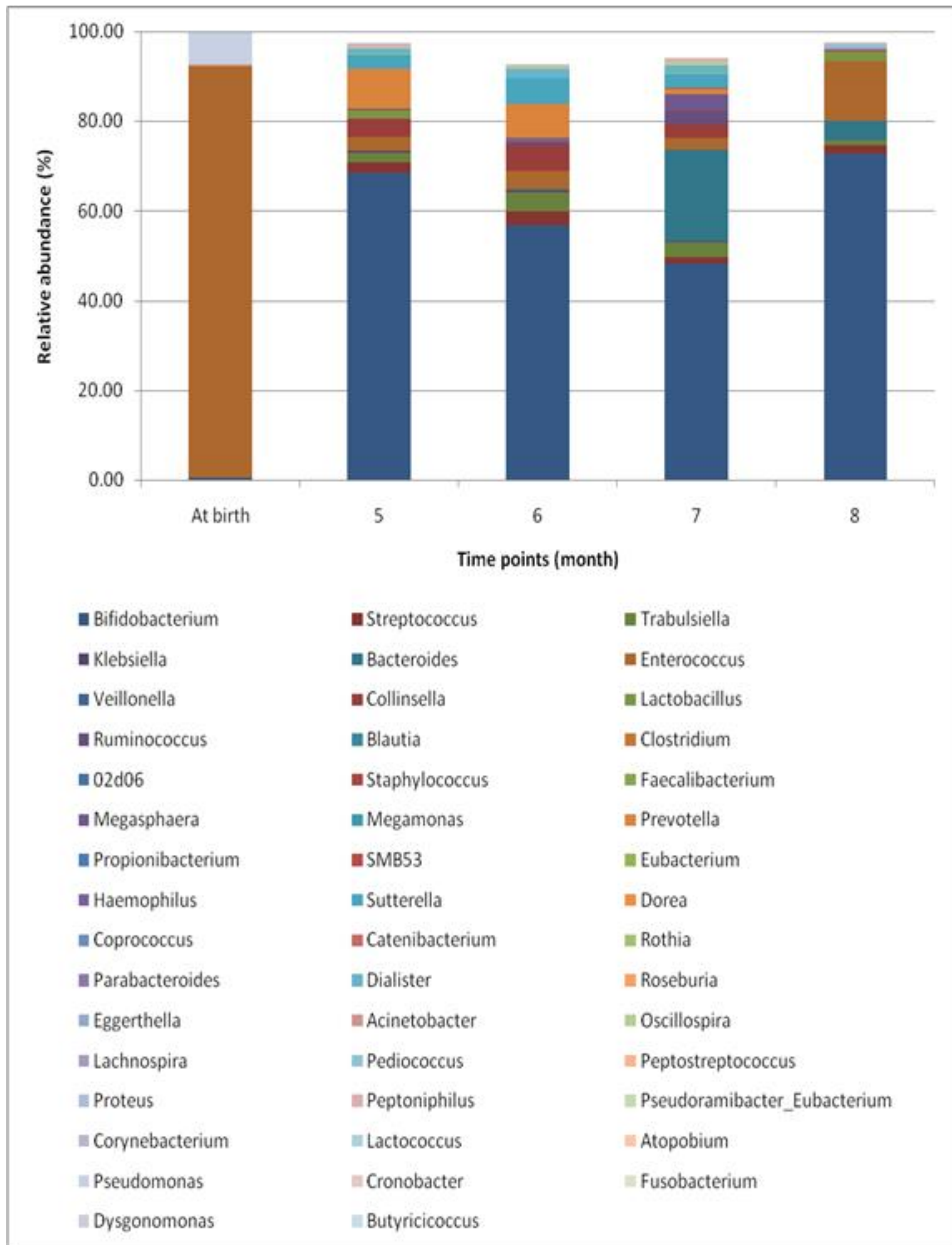


Figure 4.4(23): The genus level gut composition of Baby 23

Figure 4.4(24): The genus level gut composition of Baby 24

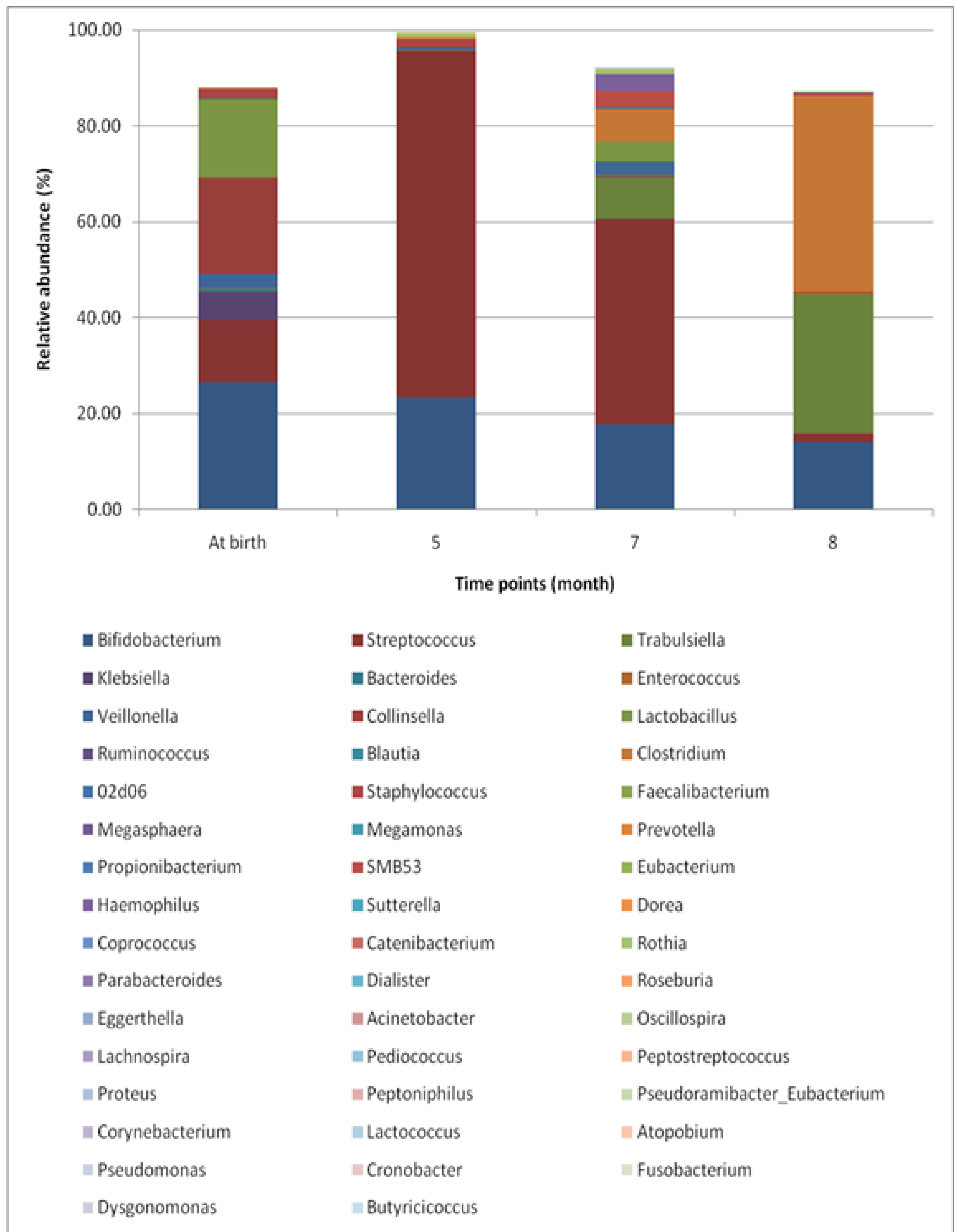


Figure 4.4(25): The genus level gut composition of Baby 25

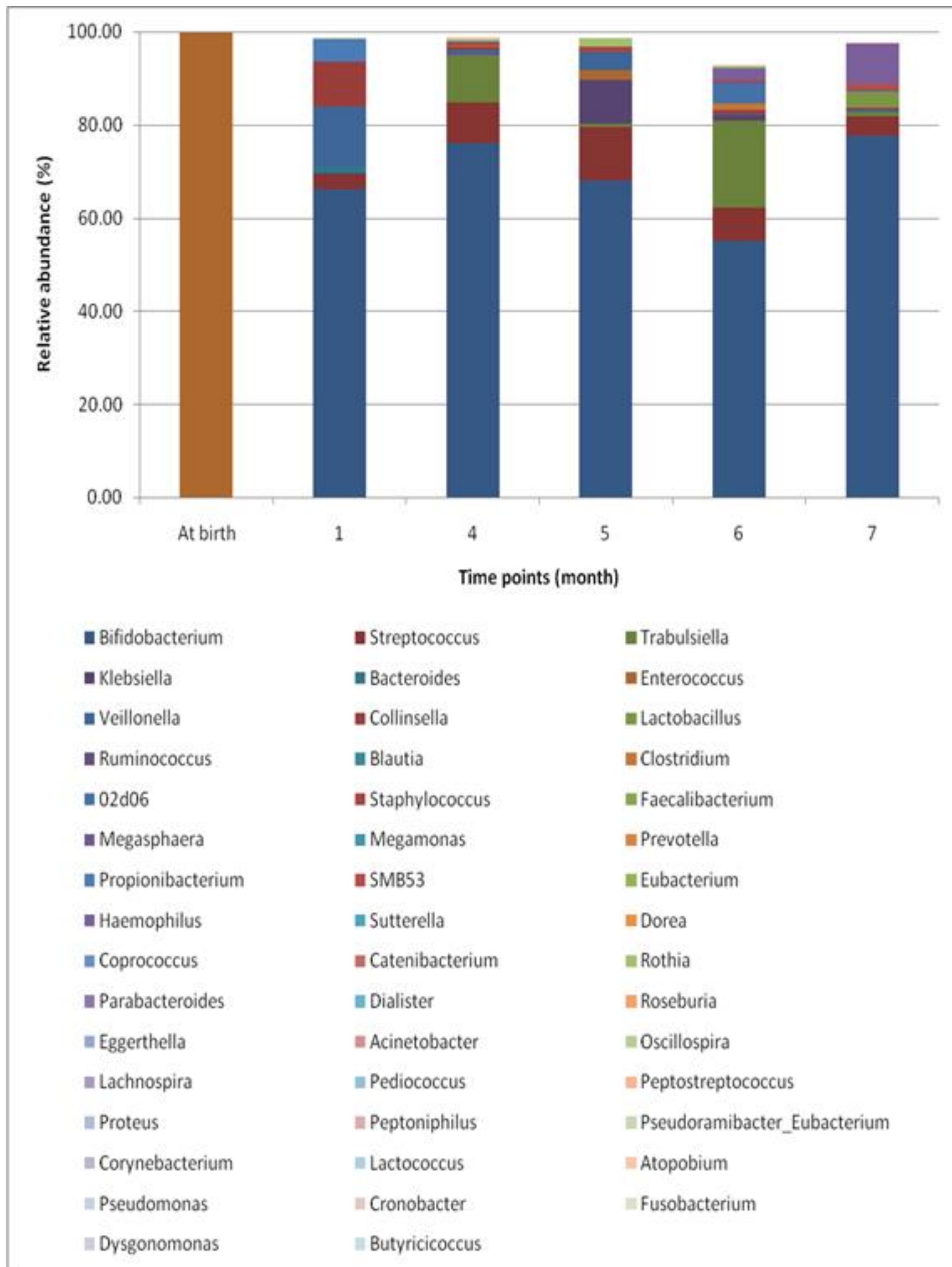


Figure 4.4(26): The genus level gut composition of Baby 26

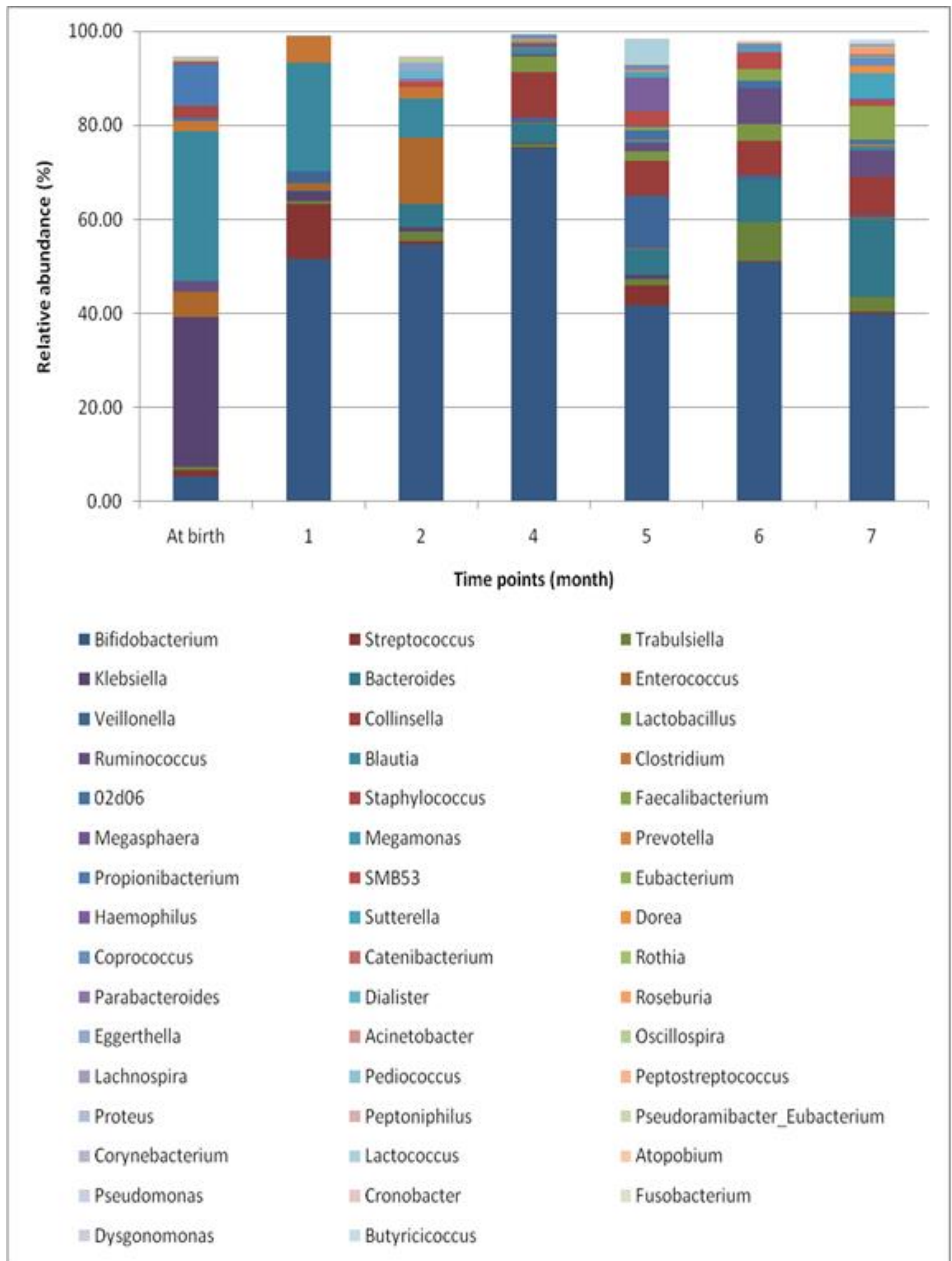


Figure 4.4(27): The genus level gut composition of Baby 27

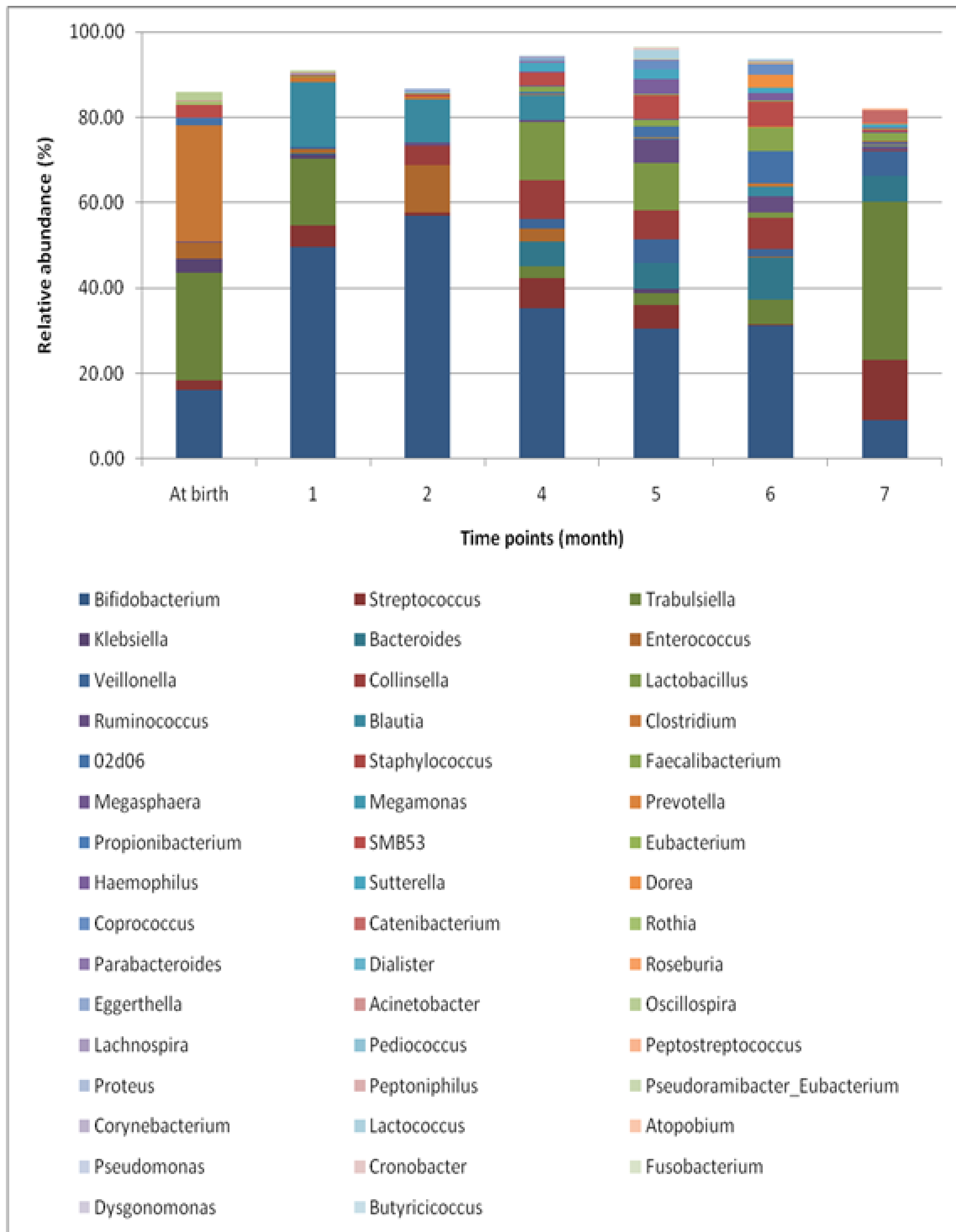


Figure 4.4(28): The genus level gut composition of Baby 28

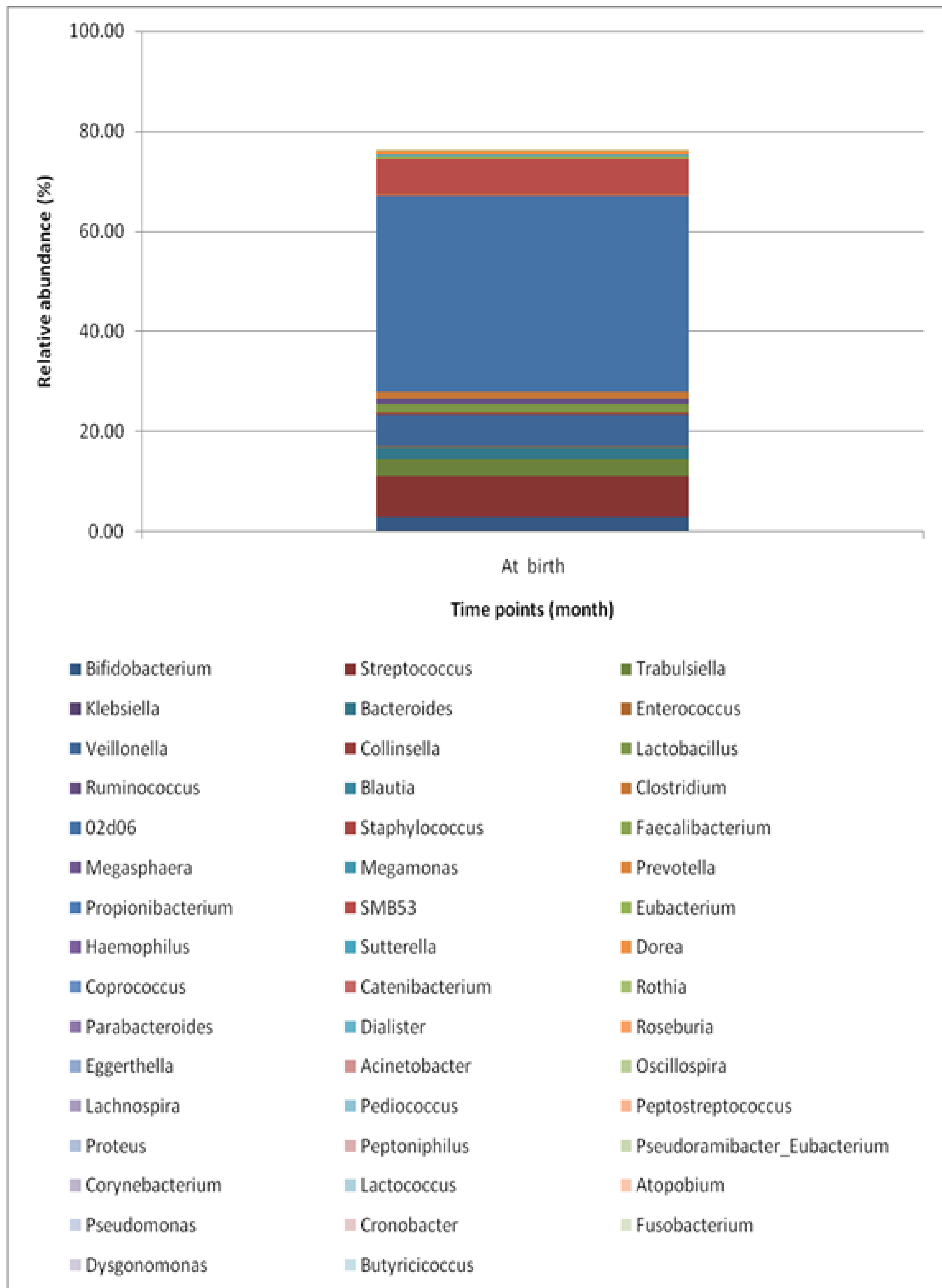


Figure 4.4(30): The genus level gut composition of Baby 30

4.3. Bacterial diversity of the samples

The alpha diversity scores showed a rise in the sample richness as the time points progressed from birth to > 7 months, when all the samples were grouped by time points (Figure 4.5). Both the distribution and evenness of the taxa showed the increased diversity. The rarefaction curves for each of the alpha diversity metrics indicate that the samples were similar along the gradients with little divergence (Figure 4.6).

The aftermath of antibiotic treatment on observed species was also investigated. For each infant, the time points associated with antibiotic administration and the names of antibiotic used were marked with coloured bars (Figures 4.7). It was observed that in the majority of cases, the number of observed species decreased either during, or at the time point after antibiotics administration. No differences were seen in the alpha diversity metrics based on the sex of the infant or birthing method at birth.

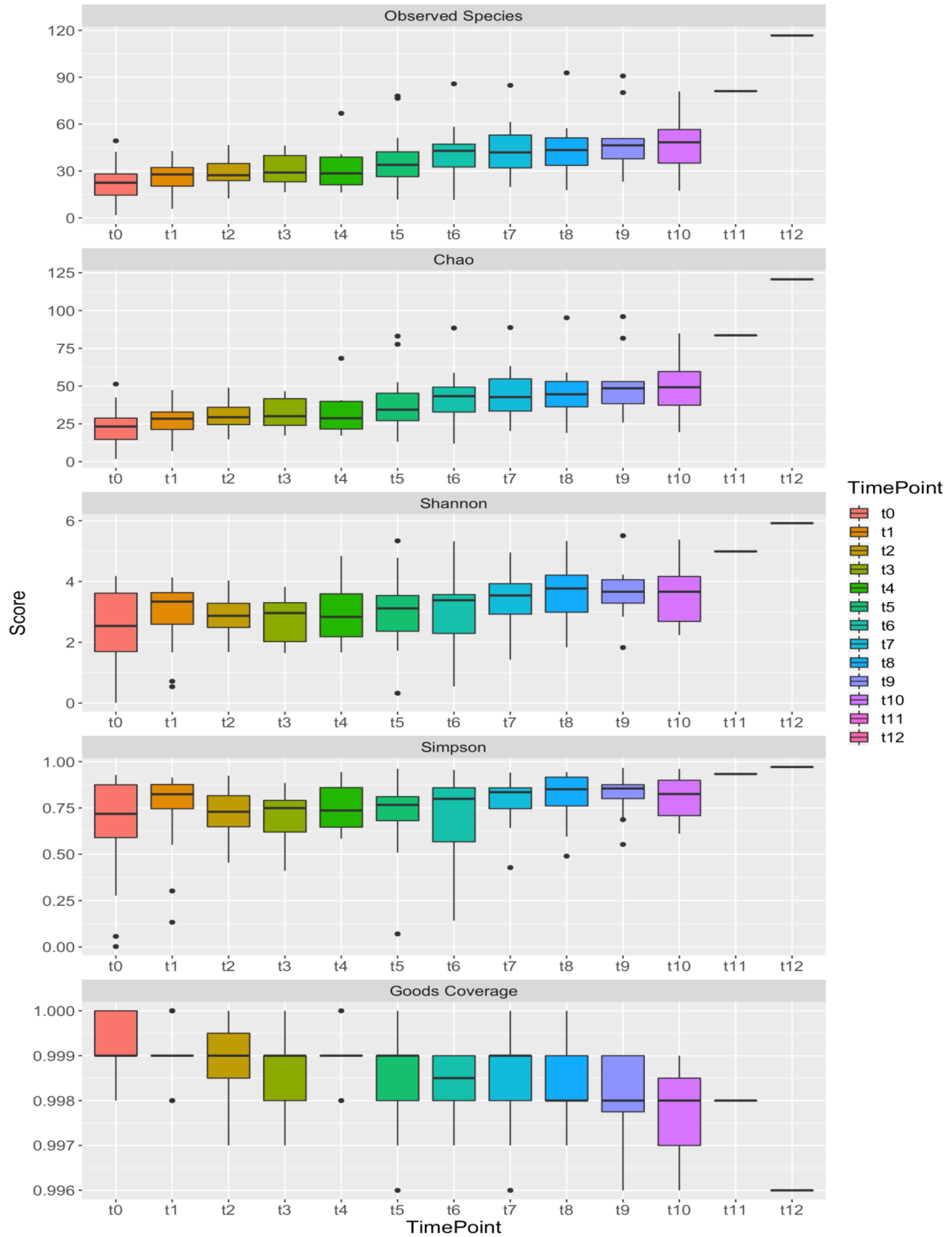
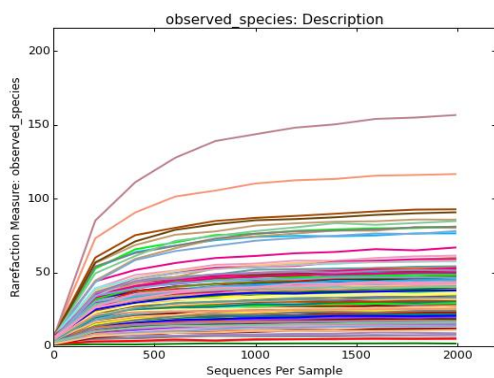
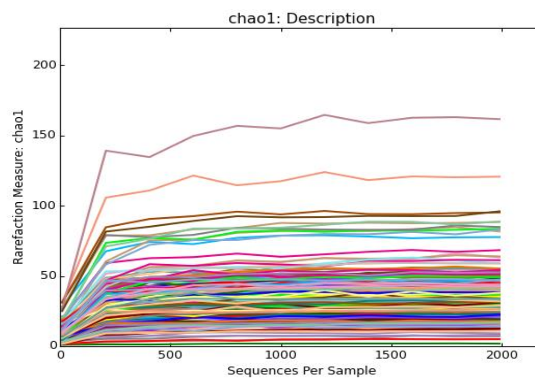


Figure 4.5. Alpha diversity metrics of samples grouped by time point

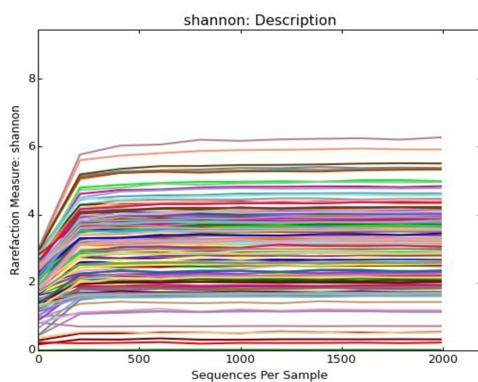
A



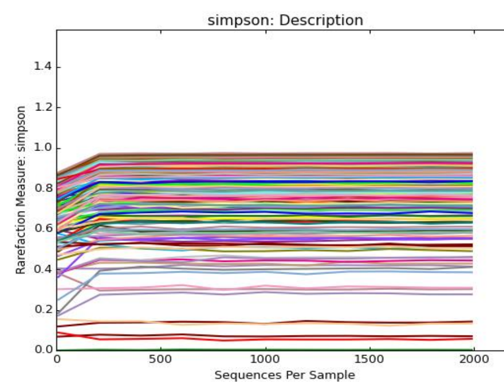
B



C



D



E

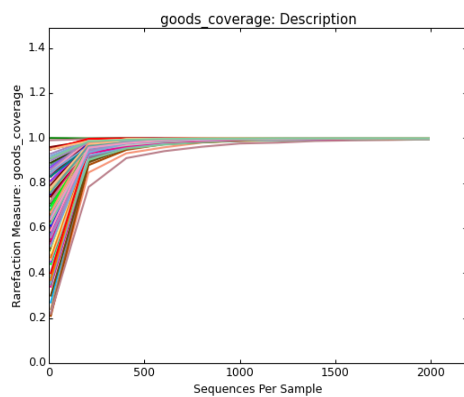


Figure 4.6. Rarefaction plots of every sample for the alpha diversity metrics.

(A) Observed Species, (B) Chao, (C) Shannon Index (D) Simpson Index

(E) Good's Coverage

Figure 4.7. Observed numbers of taxa from each infant across time points dependent on the antibiotics used

4.4. Beta diversity metrics of the samples

The analysis was done using time point as a categorical variable, with samples classified as early (t0-t4), mid (t5-t8) and late (t9-12). The majority of early time points formed a tight cluster, illustrating relatedness in the taxa present in all the samples at the early time points as seen in Figure 4.8a. All of the early time points correspond to samples collected before the introduction of any solid foods. More scattering was observed in the later time points. Further stratification of the data was carried out to know the actual time the clustering (similarity of samples) occurred. This was done based on the "age" of the infants with groupings set at 0-1 months, 2-6 months, 7-10 months (plus any earlier time points with local food/solid food), and 11-12 months (Figure 4.8b). This reflects the separation based on categorized time point with the majority of 0-1 month time points clustered closely and other time points appearing more dispersed. In addition, the PCoA containing the first weaning sample (Figure 4.8c) in green was also distinct as part of the green was seen in the early time point. The PCoA for the simplified feeding regime EBF, MF and LF were overlapping (Figure 4.8d).

Adonis statistical testing on the sample clustering was done. Statistical testing of the stratification of samples by metadata category showed that the baby ($p = 0.001$), time point ($p = 0.001$), birthing method ($p = 0.006$), birthing method and feeding regime ($p = 0.001$), simplified feeding regime ($p=0.001$), and age ($p = 0.001$) all significantly contributed towards stratification of the data (adonis; $p< 0.05$).

Principal coordinate only considered small percentage of the sample variance (<15%). Samples were additionally stratified based on birthing method, feeding regime, and gender, and no significant clustering of the samples was observed. The results of the Jaccard diversity metrics showed similar results.

A

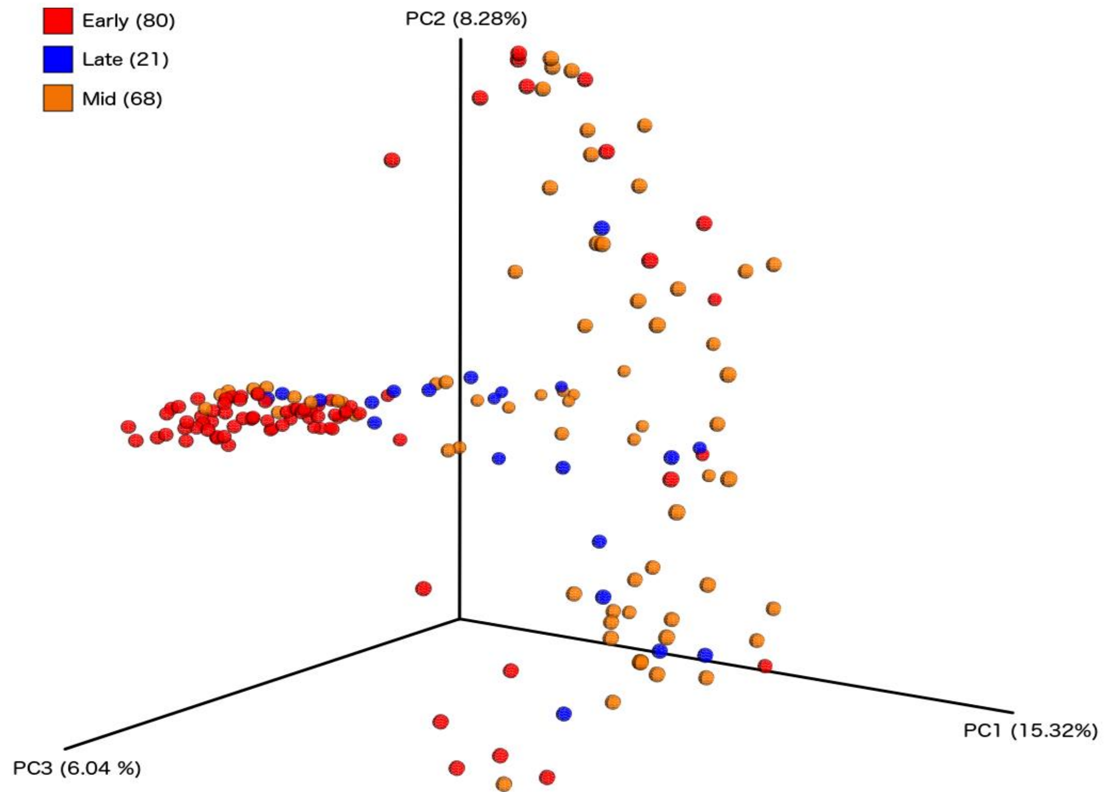


Figure 4.8. PCoA plots and clustered trees based upon Bray Curtis Diversity metrics

(A) Time point as a categorical variable

B

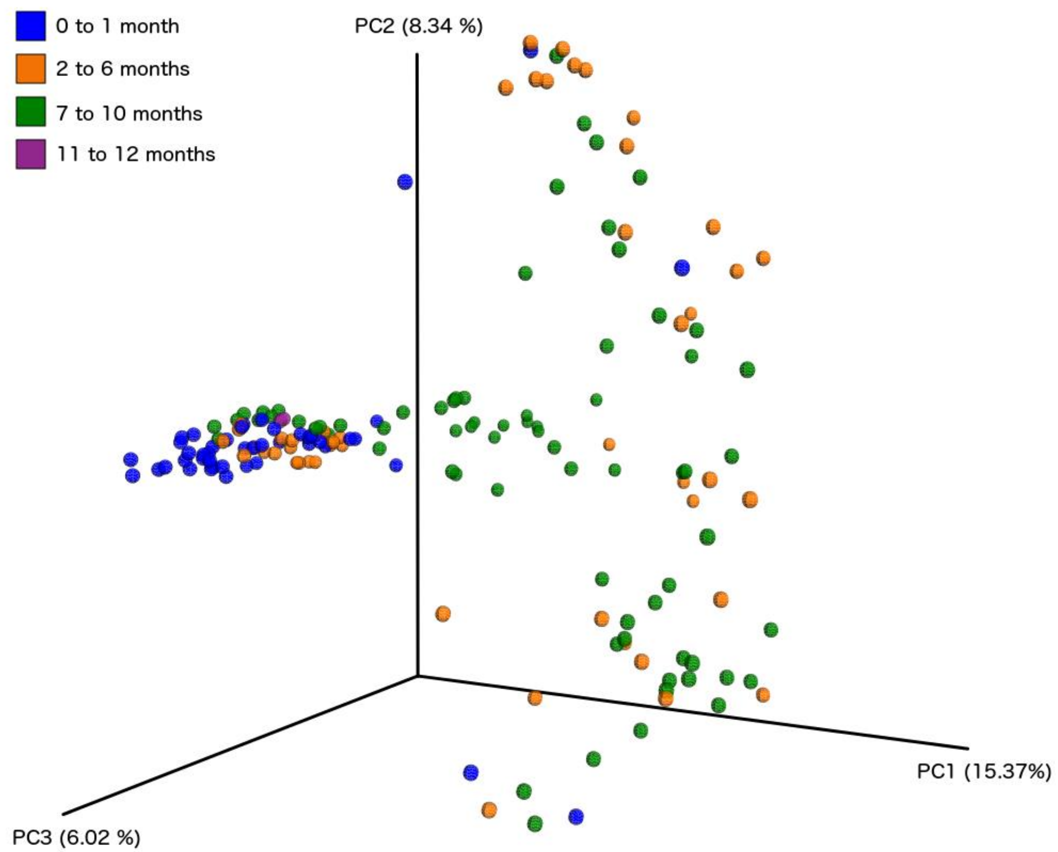


Figure 4.8. PCoA plots and clustered trees based upon Bray Curtis Diversity metrics

(B) Age as a categorical variable

C

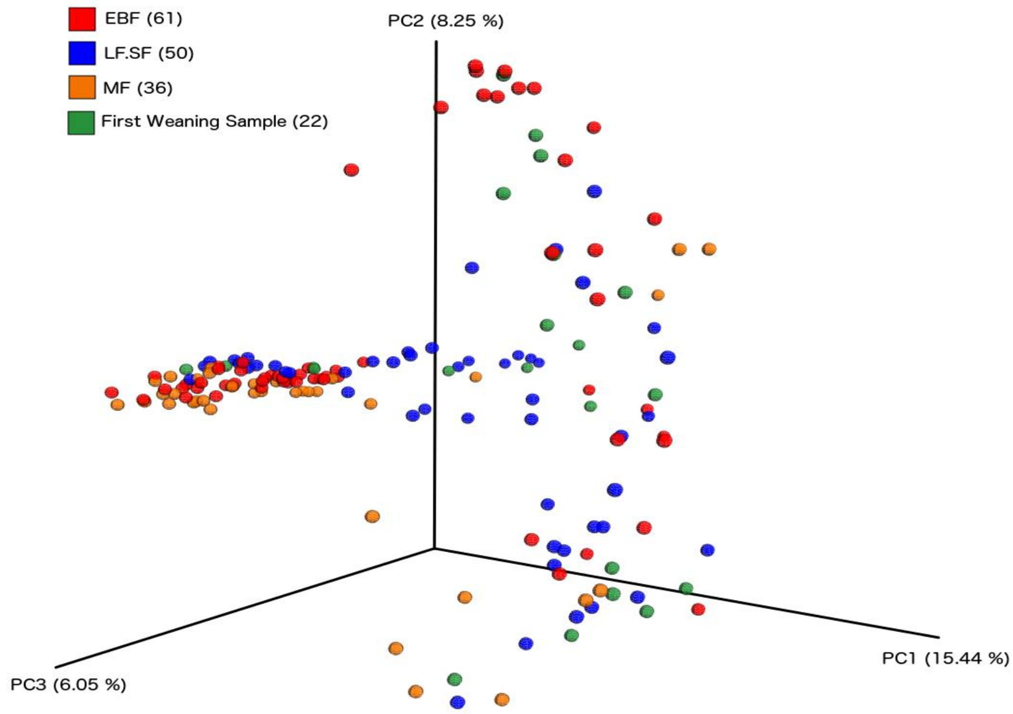


Figure 4.8. PCoA plots and clustered trees based upon Bray Curtis Diversity metrics

(C) Simplified feeding regime with first sample of weaning

D

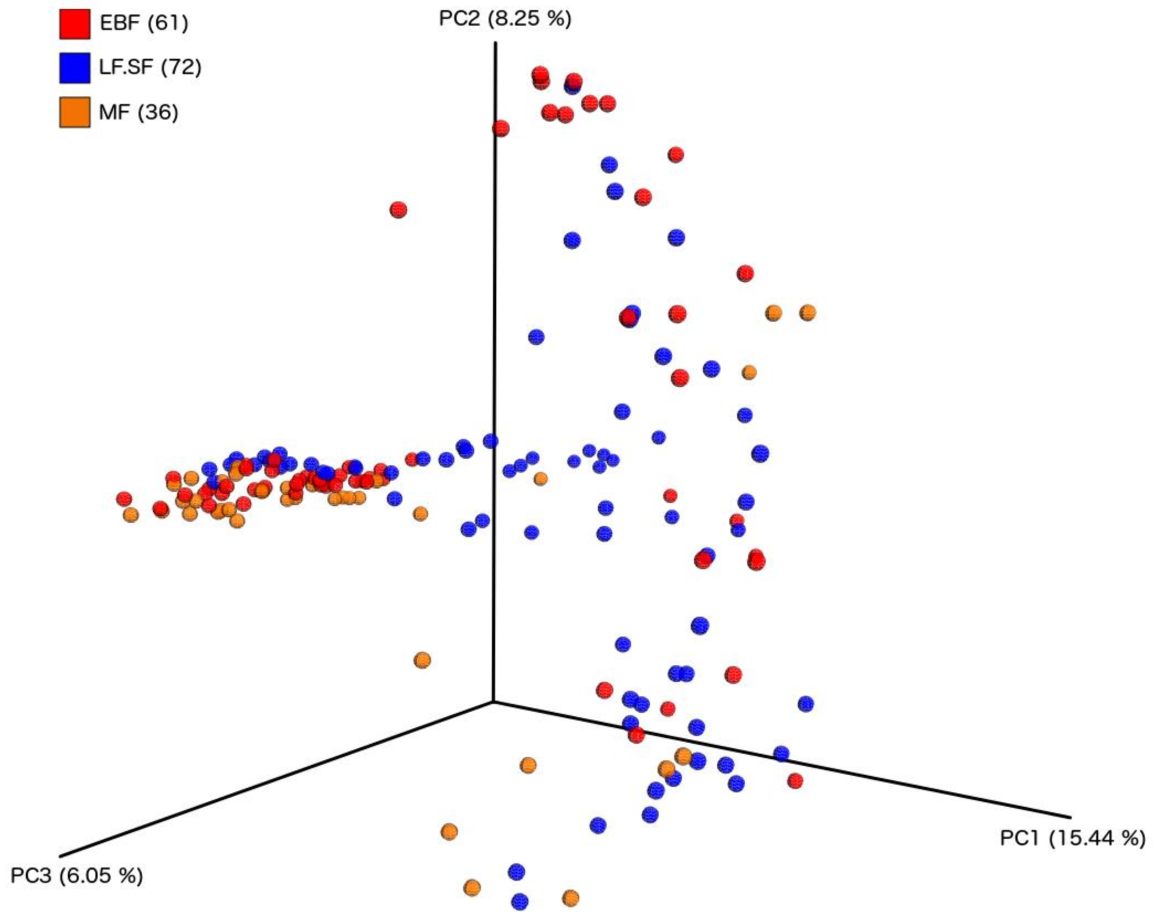


Figure 4.8. PCoA plots and clustered trees based upon Bray Curtis Diversity metrics

(D) Simplified feeding regime

4.5. The infants gut microbial composition based on birthing andfeeding method

For the birthing method (vaginal birth versus caesarean section birth) groups, no significant difference was observed in the alpha diversity between the infants birthing methods. Beta diversity grouped by age for all babies showed a tight cluster between 0-1 months (Figure 4.8b). However, there were differences in the relative abundance of taxa present in each group at birth and the percentage of the most abundant taxa was calculated. Members of the genus *Klebsiella* (33.79%), *Staphylococcus* (13.46%), *Enterococcus* (11.97%), *Streptococcus* (10.58%) and *Bifidobacterium* (9.78%) were the most abundant inCSB group, while *Streptococcus* (29.92%), *Enterococcus* (20.17%), *Klebsiella* (13.25%), *Trabulsiella* (10.44%) and *Bifidobacterium* (9.55%) were most abundant genera in VB group.

In order to compare data from the feeding methods (exclusively breastfed versus mixed fed, EBF vs MF), the taxonomic profile of the EBF versus MF at phylum level were plotted using ggplot2-R package (Figure 4.9). From the result, Proteobacteria were more abundant in the mixed fed group at phylum level, compared to EBF group. On the other hand, Bacteroidetes were more abundant in breastfed compared to mixed fed and almost not visible in the mixed fed group. The alpha diversity metrics were calculated to test for any significant differences between the groups. Samples from the exclusively breastfed and mixed fed groups had similar diversity with variation in the EBF ($p < 0.01$; Kruskal Wallis followed by Dunn Post-Hoc; Figure 4.10).

Further investigation into the differences observed was examined using DESeq2 (version 1.14.1) which identified the sequence variants with significant differential abundance in the groups. When comparing EBF and MF samples, 7 sequence variants had a significant difference in abundance (adjusted p value < 0.05). Samples from the mixed fed babies had increased *Bifidobacterium* species while samples from babies who were exclusively breastfed had increased *Collinsella*, *Bacteroides*, *Sutterella*, *Actinomyces* and Erysipelotrichaeceae (Table 4.2). Further investigation into the differences was tested with LEfSe. The outcome reveals clear differences in the infants' microbiota based on feeding

regime, similar to the results observed by DESeq2 in Table 4.2. Only taxa that had Linear Discriminate Analysis score (LDA > 2) were considered as significantly different between the groups.

Fifteen taxa were identified as significant biomarkers between exclusively breastfed and mixed fed infants (Figure 4.11; Appendix VII (a)). *Bifidobacterium* and related taxa had higher abundance in mixed fed samples while members of Bacteroidales had higher abundance in breastfed samples. The significant biomarkers in the MF group are *Bifidobacterium*species, *Bifidobacterium bifidum*, *Bifidobacterium animalis*, *Bifidobacterium* NA, *Corynebacterium*species and *Corynebacterium kroppenstedtii* while biomarkers in the EBF group are Bacteroidetes, Bacteriodales, Bacteroidia and Firmicutes (LefSe did not identify them to genus level).

The gut microbiota of those babies who are exclusively breastfed for the first 4-6 months of life before complementary foods was introduced from 5-12 months were examined. This revealed a drastic shift between preweaning and weaning period. Proteobacteria reduced in LF/SF group compared to EBF group, while Bacteroidetes increased in LF/SF group compared to EBF group. The appearance of Fusobacteria was observed in the LF/SF group (Figure 4.9). The alpha diversity revealed increased sample richness in the LF/SF group. The alpha diversity metric was not calculated for this group separately, and the sample richness in LF/SF group was estimated for both exclusively breastfed and mixed fed infants. The beta diversity metrics did not show distinct separation (Figure 4.8f).

Differential abundance testing (DesSeq) revealed that 15 sequence variants had significant changes in abundance between these groups. Some genera were absent during the exclusive breastfeeding period but appeared during local/solid feeding (weaning). Members of the genus *Coprococcus*, *Clostridium*, 02d06 and SMB53 appeared in LF/SF samples (Table 4.3). Exclusively breastfed infants had increased *Enterococcus*, *Staphylococcus epidermidis* and Gemellales while LF/SF had *Bifidobacterium*, *Bifidobacterium bifidum*, *Streptococcus*, *Haemophilus parainfluenzae*, *Coprococcus*, *Clostridium*, 02d06 and SMB53.

LefSe analysis also identified 19 taxa as significant biomarkers between breastfed and local food/solid fed group (Figure 4.12; Appendix VII (b)) and showed dominance of members of the family lactobacillaceae in LF/SF group especially *Enterococcus casseliflavus* and Clostridia. Significant biomarkers in EBF samples are *Streptococcus* and *Staphylococcus* while LF/SF had *Bifidobacterium* NA, *Pediococcus*, *Enterococcus*, *Enterococcus casseliflavus* Clostridia and members of family Lachnospiraceae as significant biomarkers.

The gut microbiota of mixed infants between the first 4-6 months of life and introduction of complementary foods thereafter was also examined. Notable changes were observed at phylum level (Figure 4.9). Increased Proteobacteria in MF was observed with reduction in LF/SF while Bacteroidetes was low in MF or almost not visible but relatively abundant in LF/SF. From the R-ggplot analysis, it seems Firmicutes greatly increased in LF/SF than in MF. The Alpha diversity of the LF/SF group showed sample richness compared to MF group ($p < 0.01$; Kruskal Wallis followed by Dunn Post-Hoc; Figure 4.10). Beta diversity did not reveal a clear distinction between these groups.

DEseq2 revealed 29 significant sequence variants when compared MF and LF/SF (Table 4.4), with some overlap with the EBF vs. LF/SF comparison. Mixed fed samples were enriched with *Klebsiella*, *Enterococcus*, *Propionibacterium*, *Trabulsiella*, *Staphylococcus epidermidis* and LF/SF had *Bifidobacterium*, *Bifidobacterium bifidum*, *Bacteroides fragilis*, *Collinsella aerofaciens*, *Bacteroides*, *Lactobacillus ruminis*, *Ruminococcus gnavus*, *Enterococcus casseliflavus*, *Coprococcus* and SMB53. LefSe identified 28 taxa as significant biomarkers that distinguished mixed fed from local/solid fed group (Figure 4.13; Appendix VII(c)). Significant biomarkers in MF are *Staphylococcus* NA, *Streptococcus*, *Bifidobacterium*, *Bifidobacterium bifidum*, *Bifidobacterium animalis*, *Corynebacterium kroppenstedtii* and LF/SF had *Bacteroides*, *Lactobacillus*, *Enterococcus casseliflavus* and Clostridia.

Both exclusively breastfed and mixed fed infants were combined to form the preweaning group while the local/solid fed group were the weaning group. Taxonomic profile at the

level of phylum revealed a clear change from preweaning to weaning with a decrease in Proteobacteria in the weaning samples and increase in preweaning samples. There was also a slight increase in Bacteroidetes abundance relatively and Firmicutes in the weaning infant groups compared to preweaning group (Figure 4.9). The observed changes from preweaning to weaning at the level of phylum was a shift from Proteobacteria in preweaning sample to Bacteroidetes in weaning sample as well as increased Firmicutes. Alpha diversity indicated greater sample richness, higher diversity in weaning group than in preweaning group ($p < 0.01$; Kruskal Wallis followed by Dunn Post-Hoc; Figure 4.10). Beta diversity also revealed a more diverse microbiota with the weaning samples showing more dispersion suggestive of a more enriched microbiota in the weaning group (Figure 4.8c and 4.8f).

DEseq2 identified 20 sequence variants with significant changes in abundance between preweaning and weaning group. The differential variants found in preweaning samples are*Streptococcus*species, *Klebsiella*, *Enterococcus*, *Streptococcus luteciae*, *Propionibacterium*, *Staphylococcus*and weaning samples had *Bifidobacterium* species, *Bifidobacterium bifidum*, *Bacteroides fragilis*, *Megamonas*, *Coprococcus*, 02d06 and SMB53 as the differential variants (adjusted p value < 0.05) (Table 4.5).
LefSE identified 39 taxa that were significant biomarkers between all preweaning and weaning samples (Figure 4.14; Appendix VII (d)). The significant biomarkers in the preweaning samples are *Bifidobacterium*, *Staphylococcus* and *Streptococcus*. In the weaning samples, specific biomarkers were seen which were not identified when comparing EBF and MF with LF/SF, these biomarkers are *Roseburia*, *Blautia*, *Cronobacter*, *Bacteroides caccae* and *Sporosarcina*. *Enterococcus*, Clostridia, *Pediococcus*, members of Lachnospiraceae and the Firmicutes were also identified and present in the aforementioned comparison.

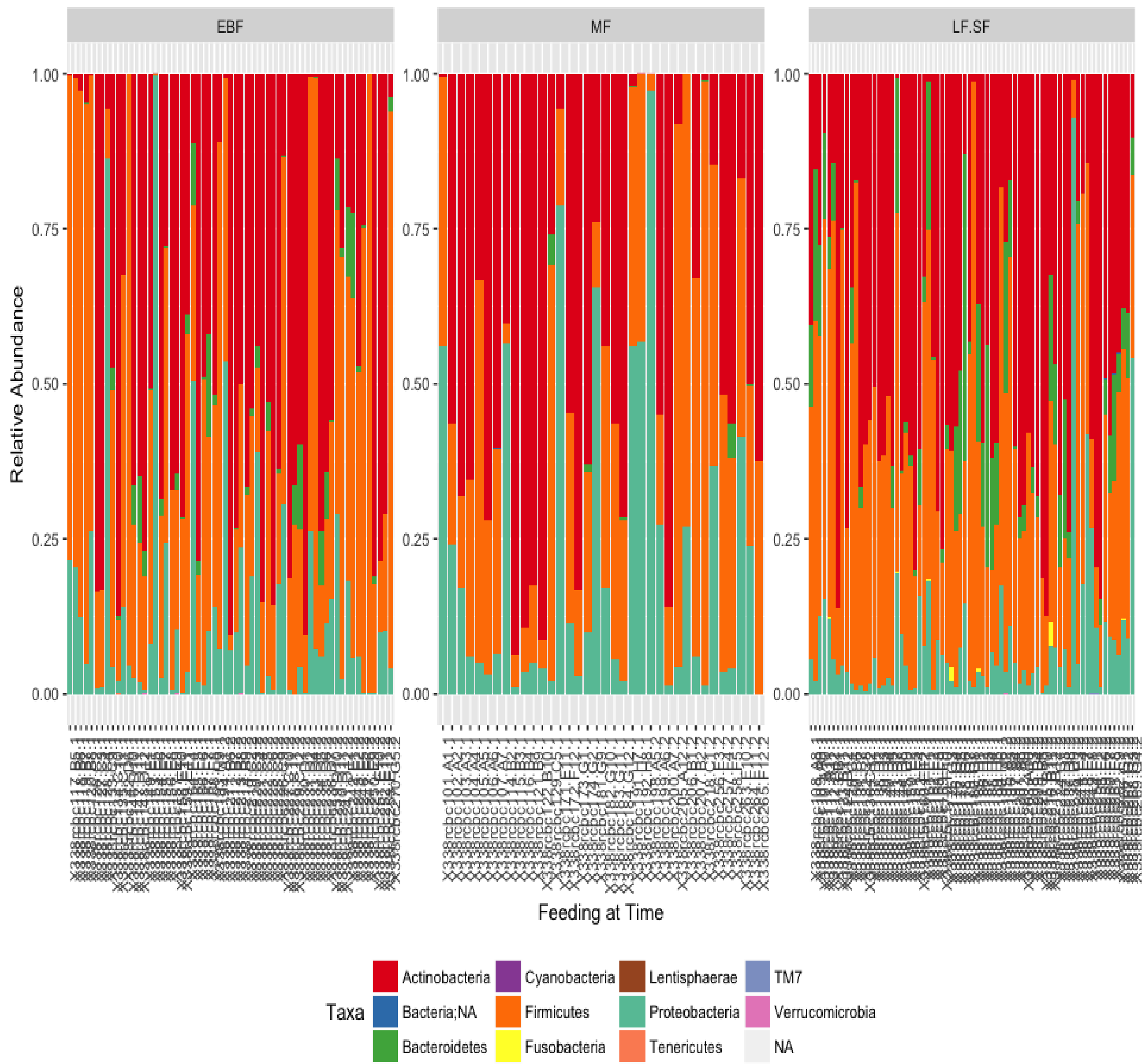


Figure 4.9. The phylum level gut composition of infants grouped by simplified feeding regime

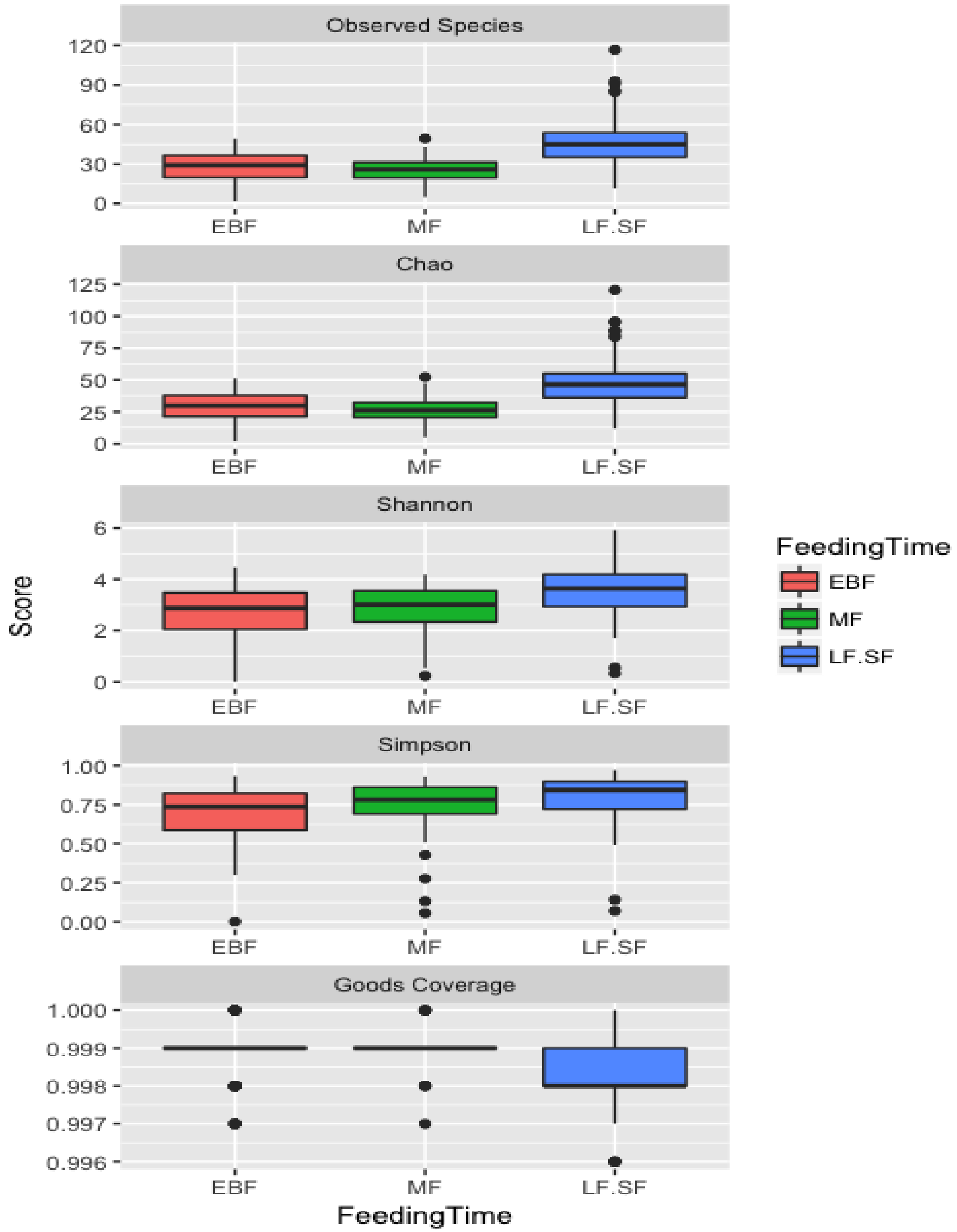


Figure 4.10. Alpha diversity metrics with samples grouped by simplified feeding regime

Table 4.2. Sequence variants with significant differential abundance (DeSeq) betweenexclusive breast-fed and mixed fed samples

Sequence Variant ID	LogFC	Adjusted P Value	Taxonomy
Seq18	-8.53	6.25e-04	Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *Bifidobacterium bifidum*
Seq24	6.33	2.06e-02	Bacteria; Actinobacteria; Coriobacteria; Coriobacteriales; Coriobacteriaceae; *Collinsella aerofaciens*
Seq26	-10.34	2.12e-04	Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *Bifidobacterium*
Seq38	6.01	3.71e-03	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; *Bacteroides*
Seq39	4.21	3.71e-03	Bacteria; Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaeceae
Seq63	5.30	3.48e-02	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Alcaligenaceae; *Sutterella*
Seq91	5.22	2.79e-02	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Actinomycetaceae; *Actinomyces*

* Negative log fold change (LogFC) indicates higher expression in Mixed fed samples, Positive log fold change indicates higher expression in Exclusively Breastfed samples (adjusted p value < 0.05)

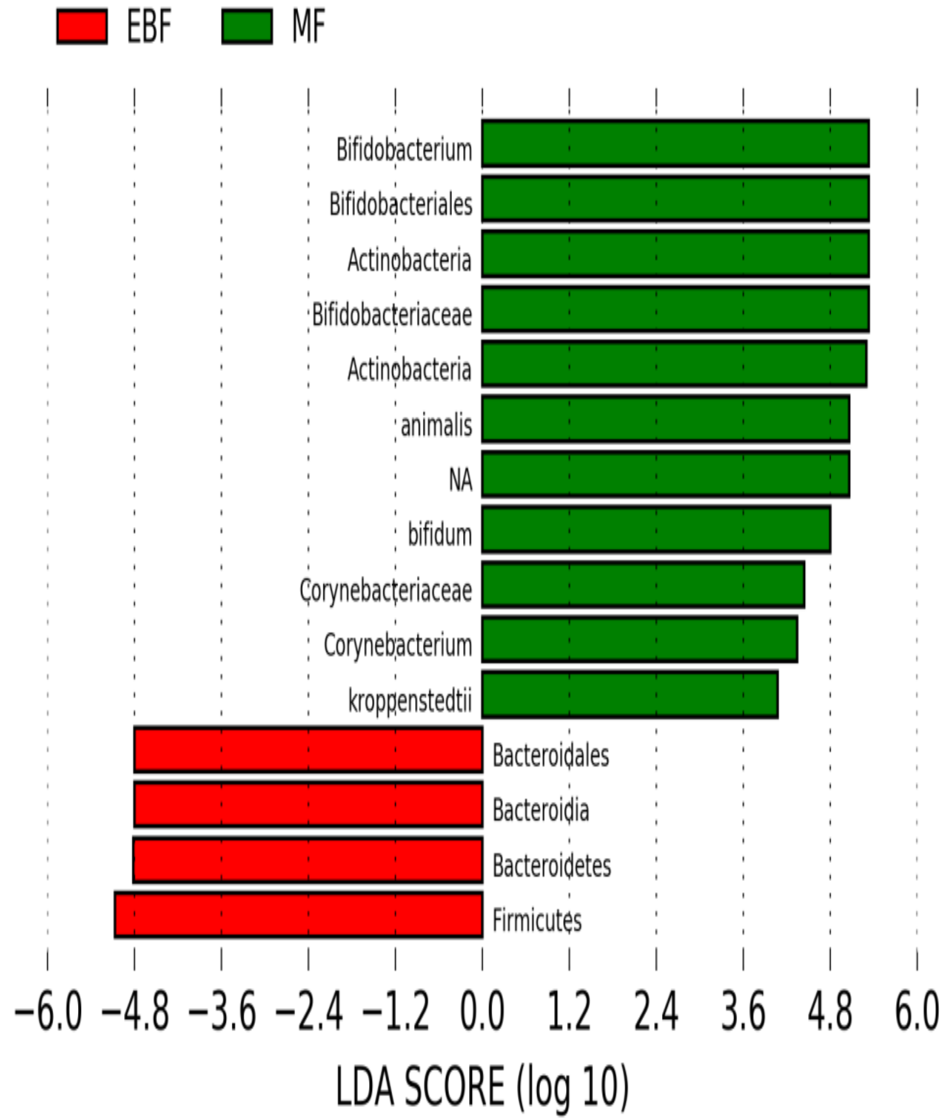


Figure 4.11. Significant biomarkers identified by LEfSe between exclusively breastfed and mixed fed samples

Table 4.3. Sequence variants with significant differential abundance between exclusive breastfed and local food/solid fed samples

Sequence variant ID	LogFC	Adjusted P value	Taxonomy
Seq 14	-4.56	6.98e-03	Bacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *Bifidobacterium*
Seq15	3.30	9.34e-03	Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; *Enterococcus*
Seq16	-6.96	1.92e-04	Bacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *Bifidobacterium bifidum*
Seq18	-7.93	8.79e-05	Bacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *Bifidobacterium bifidum*
Seq23	-7.53	2.40e-03	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; *Bacteroides fragilis*
Seq25	-7.97	4.76e-04	Bacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *Bifidobacterium*
Seq26	-6.17	1.51e-02	Bacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *Bifidobacterium*
Seq41	-5.73	3.80e-02	Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; *Streptococcus*

*Negative log fold change (LogFC) indicates higher expression in Local/solid fed samples, Positive log fold change indicates higher expression in Exclusively breastfed (adjusted p value < 0.05)

Table 4.3. Cont'd

Sequence variant ID	LogFC	Adjusted P value	Taxonomy
Seq45	-6.67	5.23e-04	Bacteria; Firmicutes; Clostridia; Clostridiales; Clostridiaceae; 02d06
Seq55	6.60	5.64e-05	Bacteria; Firmicutes; Bacilli; Gemellales
Seq65	5.08	2.16e-06	Bacteria; Firmicutes; Bacilli; Bacillales; Staphylococcaceae; *Staphylococcus epidermidis*
Seq73	-6.60	5.40e-06	Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; *Haemophilus parainfluenzae*
Seq77	-4.53	1.16e-03	Bacteria; Firmicutes; Clostridia; Clostridiales; Clostridiaceae; 02d06
Seq109	-5.89	2.36e-04	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; *Coprococcus*
Seq114	-6.32	2.08e-06	Bacteria; Firmicutes; Clostridia; Clostridiales; Clostridiaceae; SMB53

*Negative log fold change (LogFC) indicates higher expression in Local/solid fed samples, Positive log fold change indicates higher expression in Exclusively breastfed (adjusted p value < 0.05)

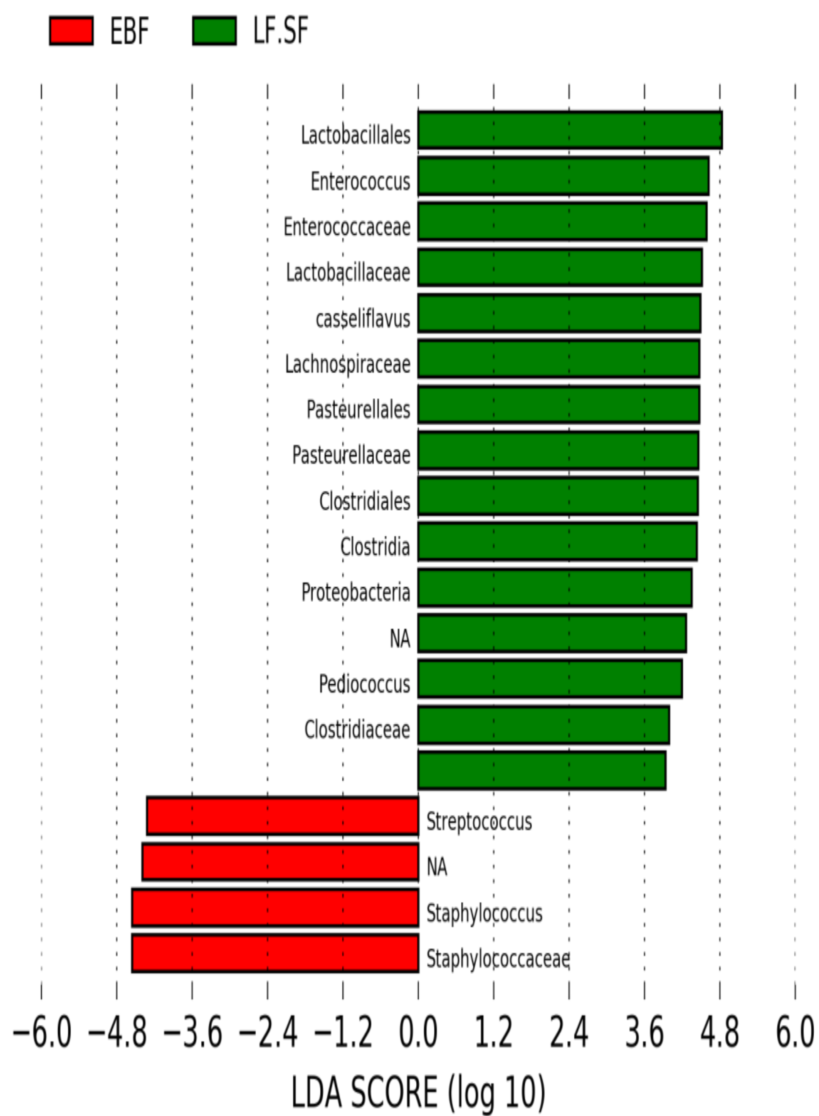


Figure 4.12. Significant biomarkers identified by LEfSe between exclusively breastfed and local food/solid food samples.

Table 4.4. Sequence variants with significant differential abundance between mixed fed and local food/solid food samples

Sequence variant ID	LogFC	Adjusted P value	Taxonomy
Seq9	-6.98	4.02e-04	Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *Bifidobacterium*
Seq12	3.20	1.95e-02	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; *Klebsiella*
Seq13	2.90	4.47e-02	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; *Klebsiella*
Seq14	-8.02	4.03e-06	Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *Bifidobacterium*
Seq15	3.42	1.06e-02	Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; *Enterococcus*
Seq16	-9.12	4.03e-06	Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *Bifidobacteriumbifidum*
Seq23	-7.07	6.15e-03	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; *Bacteroides fragilis*
Seq24	-5.87	4.40e-03	Bacteria; Actinobacteria; Coriobacteria; Coriobacteriales; Coriobacteriaceae; *Collinsella aerofaciens*

Table 4. 4. Cont'd

Sequence variant ID	LogFC	Adjusted P Value	Taxonomy
Seq25	-6.15	1.22e-02	Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *Bifidobacterium*
Seq27	4.77	1.81e-02	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Propionibacteriaceae; *Propionibacterium*
Seq29	-6.12	1.17e-02	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; *Bacteroides fragilis*
Seq38	-6.83	3.20e-05	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; *Bacteroides*
Seq39	-5.28	4.03e-06	Bacteria; Firmicutes; Erysipelotrichi; Erysipelotrichales; *Erysipelotrichaceae*
Seq40	-4.95	4.74e-02	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; *Lactobacillus ruminis*
Seq42	-3.18	2.81e-02	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; *Ruminococcus gnavus*
Seq45	-6.53	2.51e-03	Bacteria; Firmicutes; Clostridia; Clostridiales; Clostridiaceae; 02d06
Seq51	-5.90	8.79e-03	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; *Veillonella dispar*
Seq52	-5.47	2.81e-04	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; *Trabulsiella*
Seq55	5.68	1.46e-03	Bacteria; Firmicutes; Bacilli; Gemellales

*Negative log fold change (LogFC) indicates higher expression in local/solid fed samples, Positive log fold change indicates higher expression in mixed fed samples (adjusted p value < 0.05)

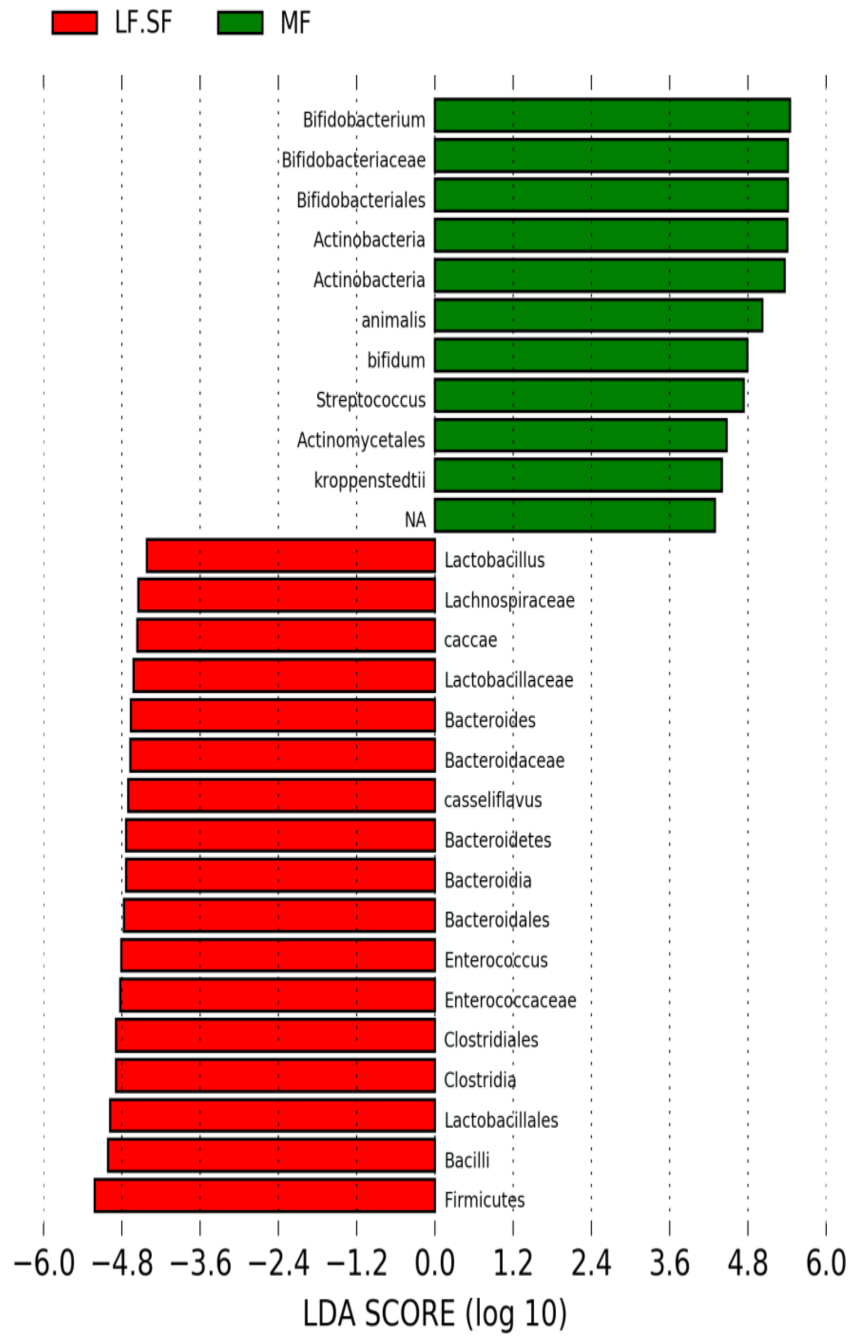


Figure 4.13. Significant biomarkers identified by LEfSe between mixed fed and localfood/solid food samples

Table 4.5. Sequence variants with significant differential abundance between preweaning and weaning samples

Sequence variant ID	LogFC	Adjusted P value	Taxonomy
Seq4	2.38	1.64e-02	Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; *Streptococcus*
Seq12	2.63	1.64e-02	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; *Klebsiella*
Seq13	2.42	3.35e-02	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; *Klebsiella*
Seq14	-5.07	1.46e-04	Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *Bifidobacterium*
Seq15	4.49	2.41e-06	Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; *Enterococcus*
Seq16	-6.95	8.94e-06	Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *Bifidobacterium bifidum*
Seq17	4.84	9.30e-03	Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; *Streptococcus luteciae*
Seq23	-7.53	1.05e-04	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; *Bacteroidesfragilis*

Table 4.5. Cont'd

Sequence variant ID	LogFC	Adjusted P Value	Taxonomy
Seq25	-7.15	1.16e-04	Bacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *Bifidobacterium*
Seq27	5.80	2.23e-04	Bacteria; Actinobacteria; Actinomycetales; Propionibacteriaceae; *Propionibacterium*
Seq45	-6.68	1.81e-05	Bacteria; Firmicutes; Clostridia; Clostridiales; Clostridiaceae; 02d06
Seq54	4.15	3.38e-02	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; *Klebsiella*
Seq55	5.80	1.61e-05	Bacteria; Firmicutes; Bacilli; Gemellales
Seq61	-5.13	1.88e-02	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; *Megamonas*
Seq65	5.04	7.30e-06	Bacteria; Firmicutes; Bacilli; Bacillales; Staphylococcaceae; *Staphylococcus*
Seq73	-6.48	8.94e-06	Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurallales; Pasteurellaceae; *Haemophilus*
Seq79	4.11	2.91e-02	Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; *Streptococcus*
Seq109	-4.64	1.12e-02	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; *Coprococcus*
Seq114	-6.11	7.30e-06	Bacteria; Firmicutes; Clostridia; Clostridiales; Clostridiaceae; SMB53
Seq132	-4.26	3.38e-02	Bacteria; Firmicutes; Clostridia; Clostridiales; Clostridiaceae; SMB53

*Negative log fold change (LogFC) indicates variants were more abundant in the preweaning group, Positive log fold change (LogFC) indicates variants were more abundant in the weaning group (adjusted p value < 0.05)

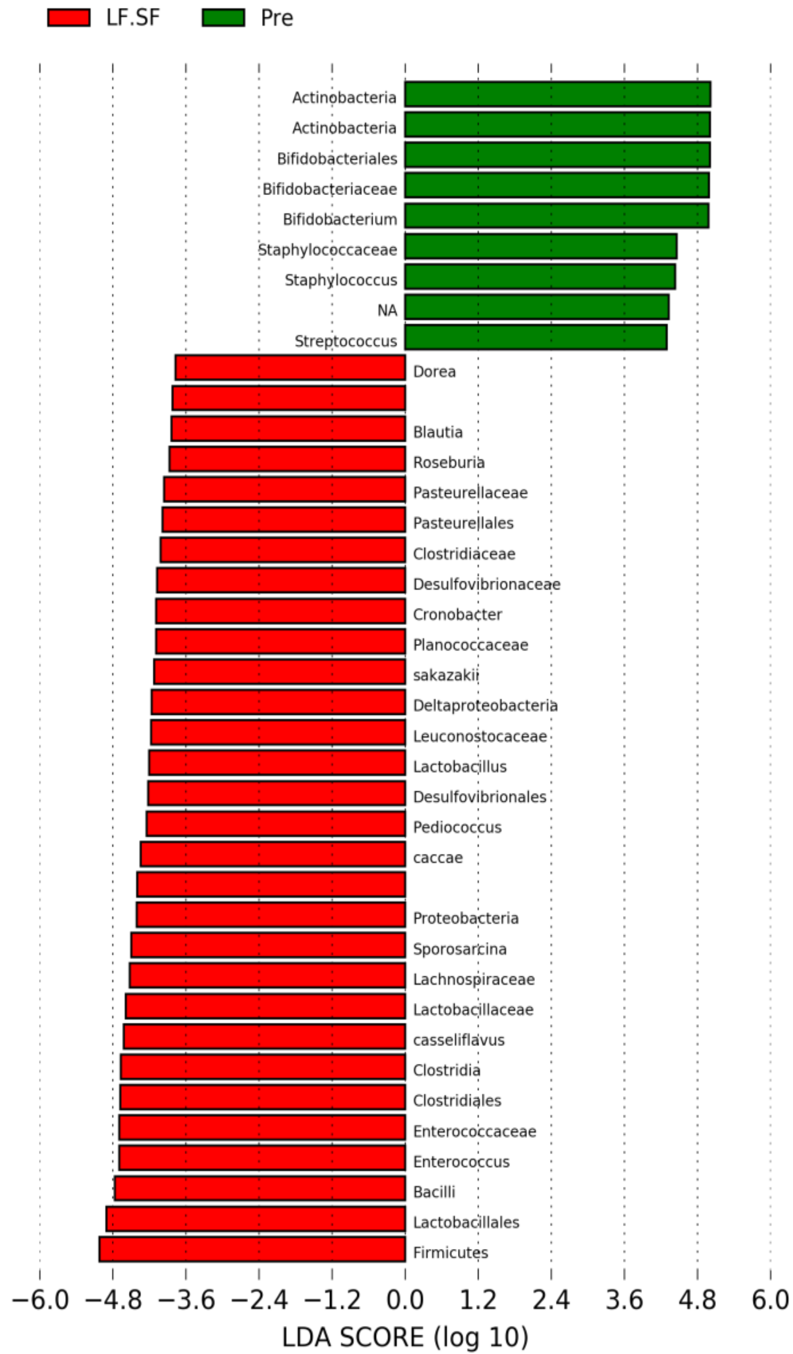


Figure 4.14. Significant biomarkers identified by LEfSe between preweaning and weaning samples.

4.6. Notable changes between preweaning and weaning groups based on fingerprinting technique

Denaturing gradient gel electrophoresis was done to further compare the changes between preweaning and weaning groups. The results clearly showed that differences exist between these two groups. Some bands appeared in the preweaning and disappeared during weaning, inferring that some taxa that were present during preweaning disappeared after weaning. However, some of the bands persisted till weaning (Figures 4.15 - 4.16).

The representative of mixed fed group is shown in Figure 4.15 the fingerprinting revealed that different taxa appeared in the weaning period indicated by emergence of bands at the upper lane which was not observed in preweaning lane. Also, at the lower lane there was a notable difference between the preweaning and weaning lanes.

Also, in the representative of exclusively breastfed infant (Figure 4.16), there was observed low diversity both in the preweaning and weaning group. However, some taxa appeared at the lower lane of preweaning that was not seen in the weaning, illustrating the disappearance of the taxa overtime. Diversity that was obviously seen in mixed fed infants was not visible in the exclusively breastfed group.

The twin babies that showed variation in their gut composition also exhibited similar differences in the fingerprinting analyses. Twin 1 (Figure 4.17) showed higher diversity in the weaning group than twin 2 (Figure 4.18) and different taxa emerged at the top and lower lane in the weaning stage which was not in the preweaning, this was also observed in twin 2.

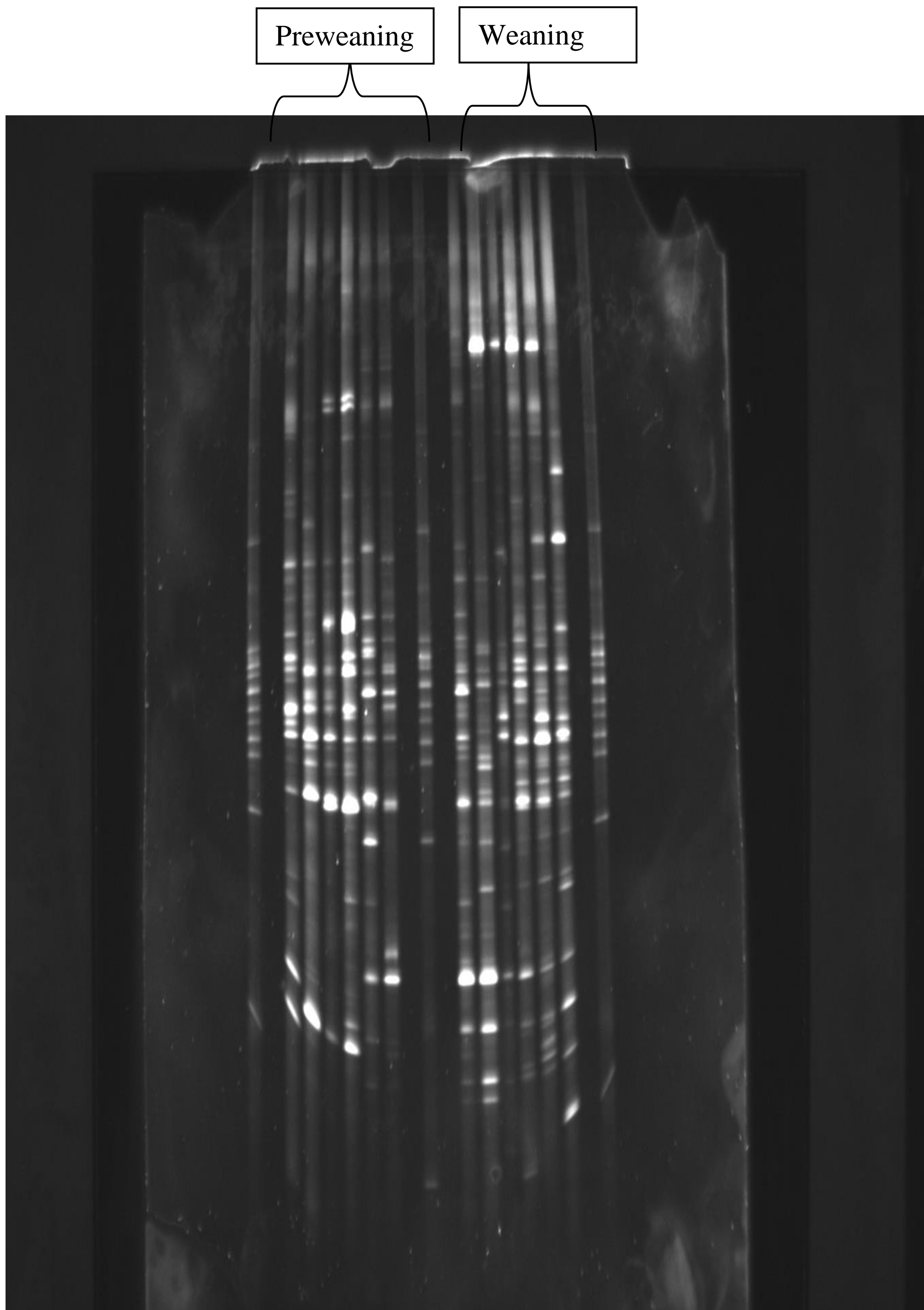


Figure 4.15. Preweaning and weaning fingerprinting profile of mixed fed baby

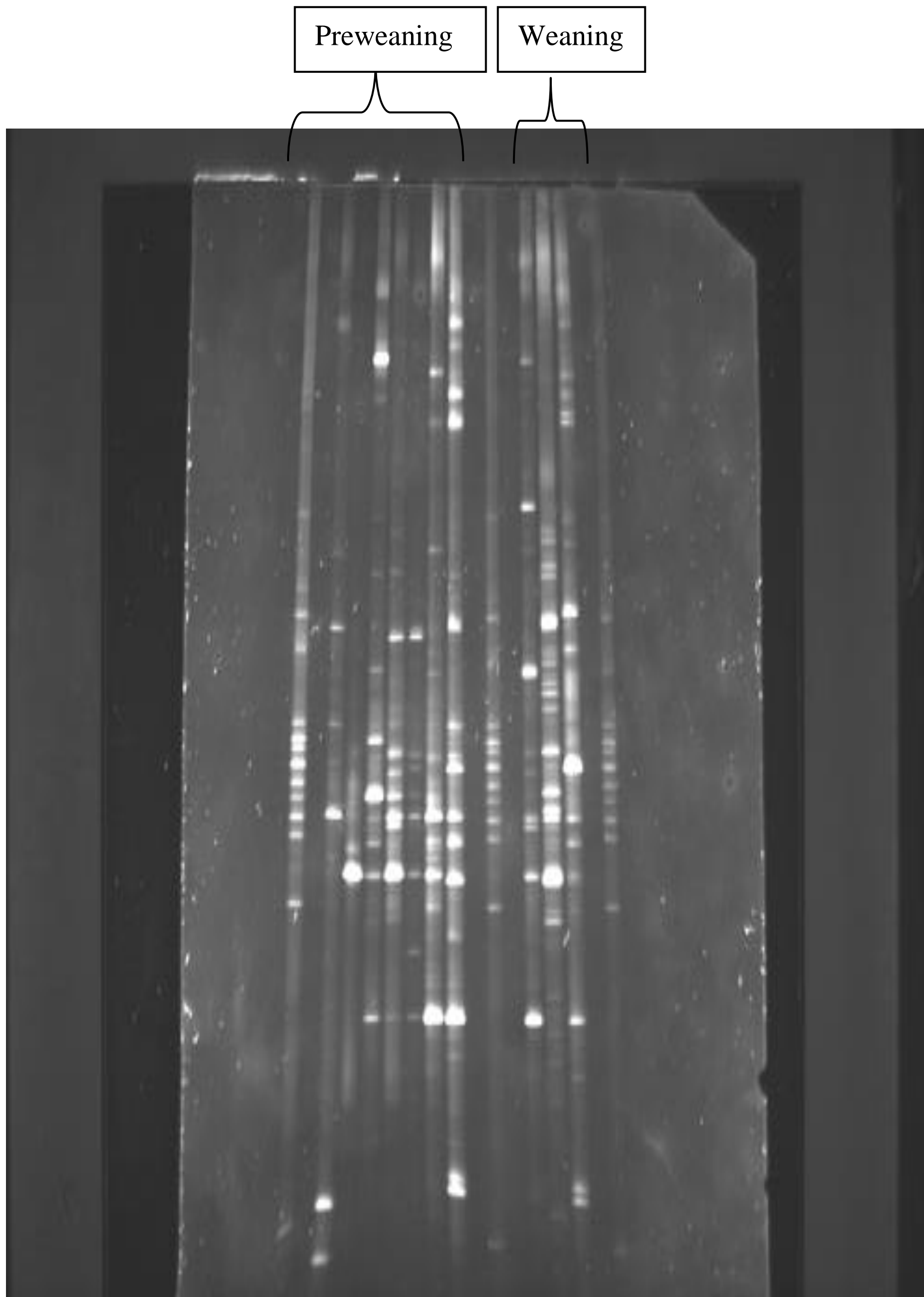


Figure 4.16. Preweaning and weaning fingerprinting profile of exclusively breastfed baby

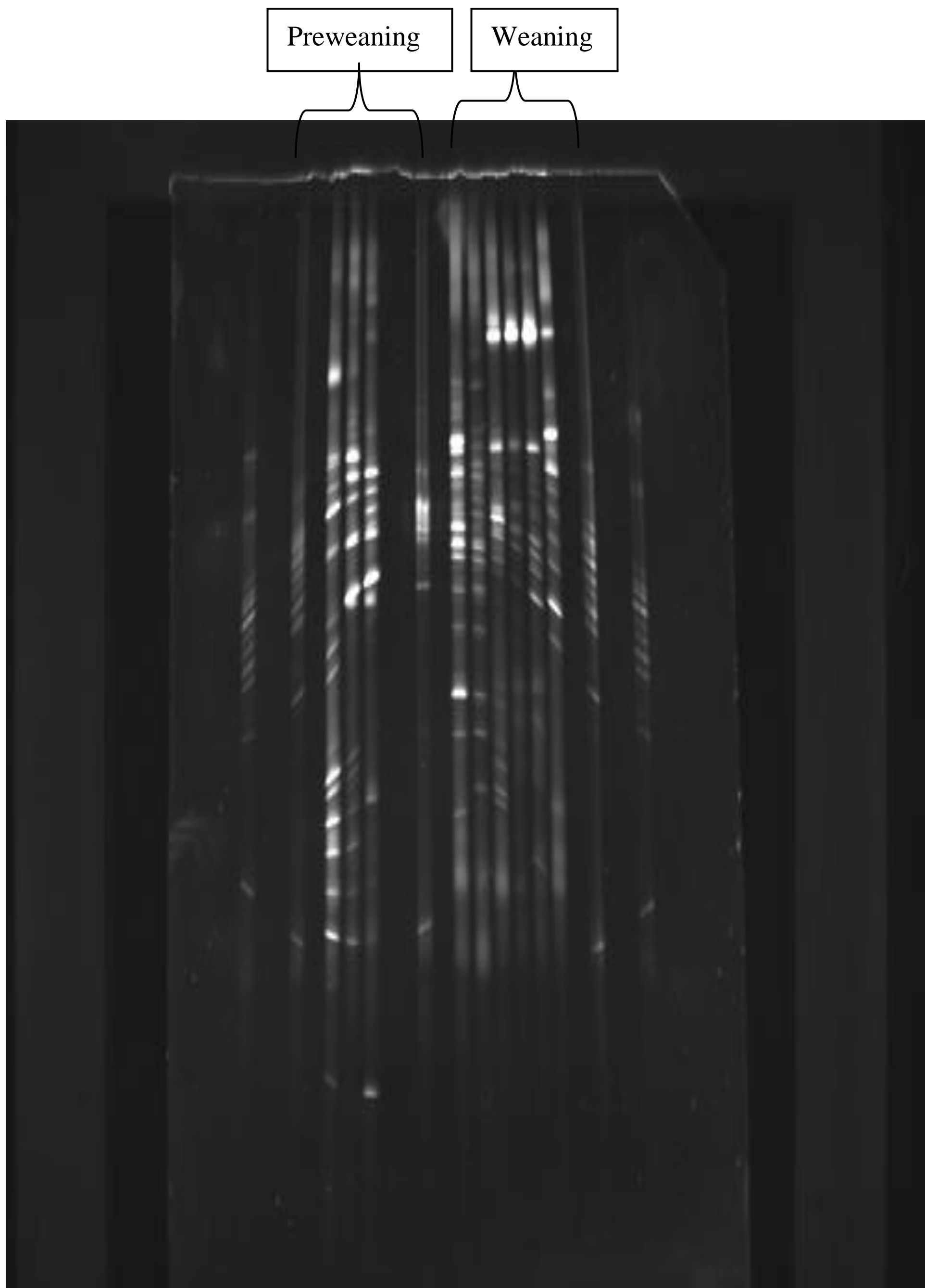


Figure 4.17. Preweaning and weaning fingerprinting profile of twin baby (twin 1)

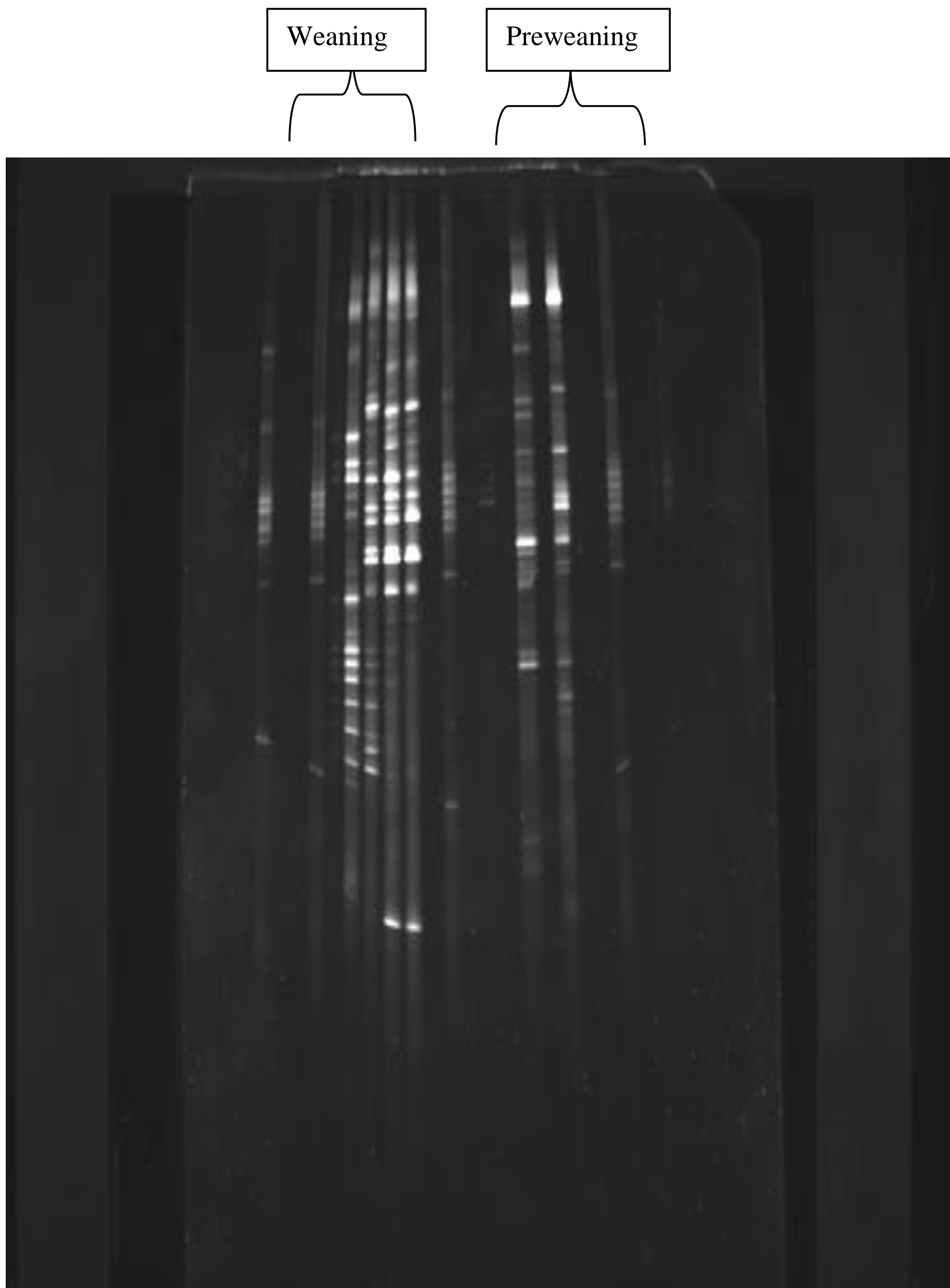


Figure 4.18. Preweaning and weaning fingerprinting profile of twin baby(twin 2)

4.7. Characterisation of *Bifidobacterium* in formula consumed by the infants

The presence of *Bifidobacterium* in the milk formula was demonstrated by PCR using genus specific primers. There was amplification of *Bifidobacterium* in NAN 1 formula milk from different container designated by S1 and S2 as revealed by a clear band size of 0.481 kb as compared with the positive control *Bifidobacterium adolescenti*s (Figure 4.19). The negative control did not yield an amplicon; neither did the adult milk (DANO milk).

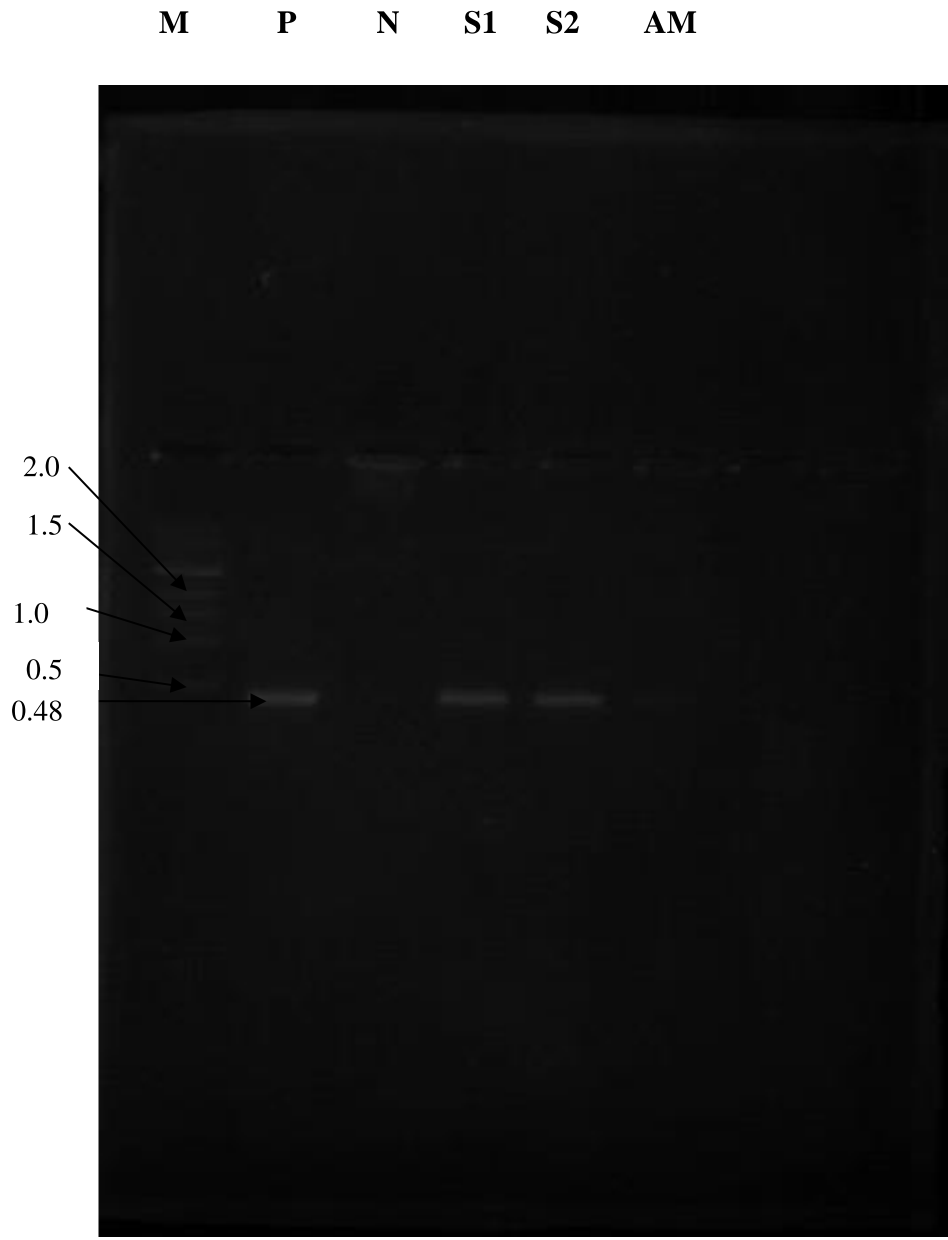


Figure 4.19. The presence of *Bifidobacterium* in NAN 1 formula milk

M = Marker (1 kb ladder), P = Positive control (*Bifidobacterium adolescentis*),

N = Negative control, S1 = NAN1, S2 = NAN, AM = Adult Milk

4.8. Prevalence of antibiotic resistance genes in samples

The antibiotic exposure was observed to elicit a decrease in the number of taxa either during antibiotic usage or the time point after antibiotic usage in majority of these infants. However, few of the infants did not show any decrease in the abundance of taxa after antibiotics administration (Figure 4.7). The presence or absence of antibiotic resistance genes were determined (*tet*, *aac*(6'), *mef(*A/E), *erm*A, *erm*B, *bla*Z) in all the samples (Appendix VIII). The *tet* gene had the highest prevalence (27%) in the study population, followed by *mef*(A/E) (24%), *erm*B (22%), *bla*Z (11%), aac (13%) and *erm*A (3%) (Figure 4.20).

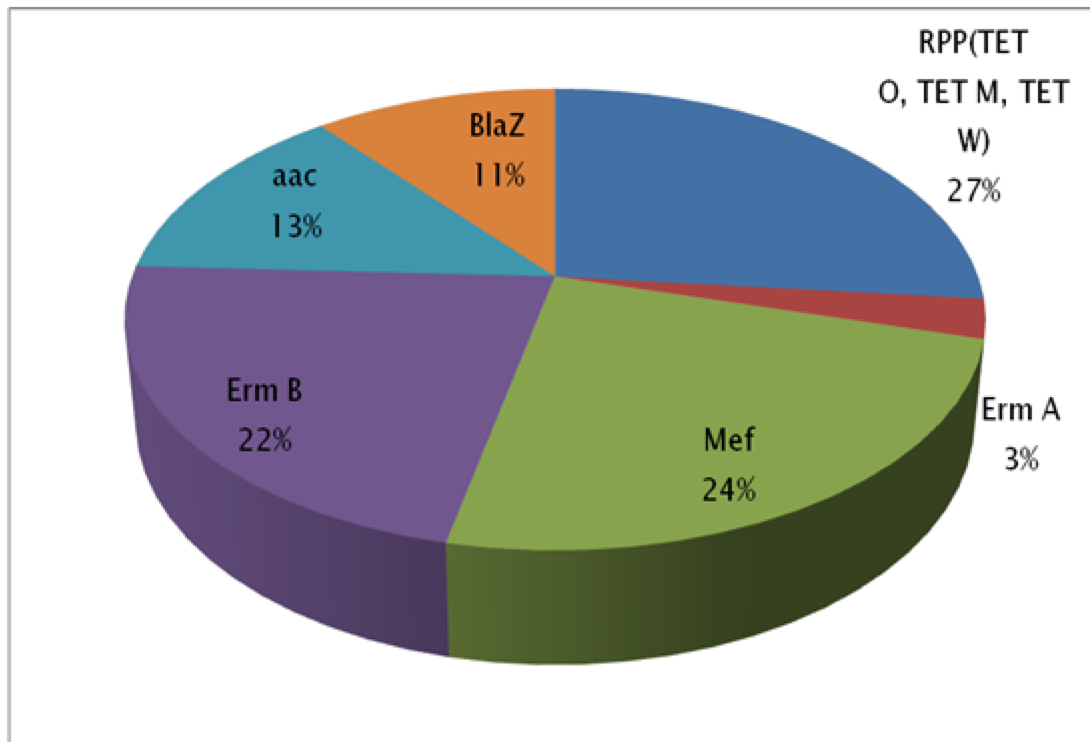


Figure 4.20. Prevalence of antibiotic resistance gene in the study population

RPP (TET) = Ribosomal Protective Protein tetracycline gene, *erm*A, *erm*B and MefA/E = Macrolide resistance gene, *bla*Z = β- lactamase gene, aac = Aminoglycosides modifying enzymes gene

4.9. Presence of short-chain fatty acids in samples

The presence of all the shortchain fatty acids in the faecal samples of all the infants across time points was analysed. Acetate and lactate were present across all time points. Propionate, succinate and formate were low at early time points (1-4 months); it however increasedat the later time points (7-12 months). Most importantly, butyrate appeared when weaning started. For those babies that started weaning as early asthe 4th month and later at the 7th month respectively (Figure 4:21).

In addition, the average of the SCFAs in all preweaning samples was compared with weaning samples. Acetate, propionate lactate and butyrate were higher during weaning. Acetate had a doubled increase during weaning compared to preweaning, followed by lactate. Propionate was slightly higher during weaning compared to preweaning. Only succinate was lower during weaning in contrast to other SCFAs (Figure 4.22).

The concentrations of each of the short chain fatty acids present in the faecal samples of individual babies are given in Appendix VIII (1) – VIII (28).

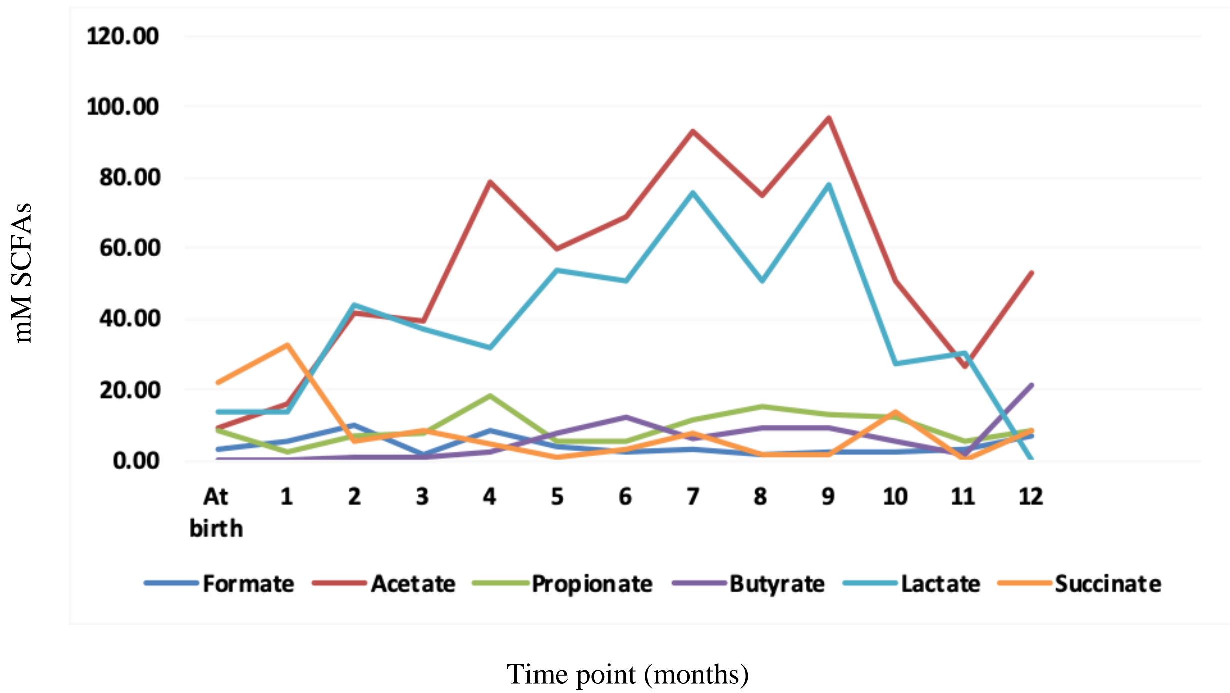


Figure 4.21. Trends of SCFAs in babies across time points

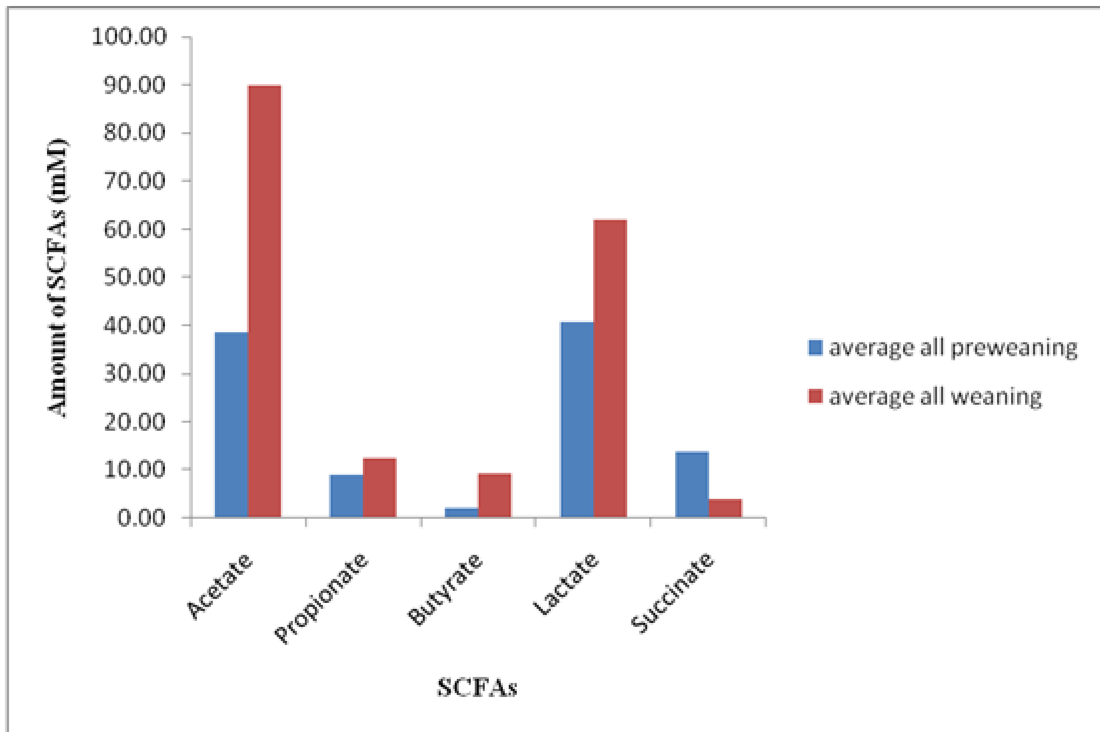


Figure 4.22. AverageSCFAs production between preweaning and weaning samples for all the babies

CHAPTER FIVE

DISCUSSION

5.1. The characteristics of the study infants

The number of females in this present study outnumbered the males. The enrollment was based on the number of babies that were delivered at the Department of Gnaecology and Obstetrics, Federal Teaching Hospital, Ido-Ekiti at the period of study. The percentage of CS birth was almost equal to that of vaginal birth. Retrospective studies from tertiary teaching hospitals indicate a rise in the incidence of CS in the country which is gradually tending above the CS threshold of 10-15% as proposed by World Health Organization (Nnadi *et al*., 2016; Akadri and Odelola, 2017; Isah *et al*., 2018).

Some of the infants in this study were exclusively breastfed while few were mixed fed. The probable reason for mixed feeding in this study was multiple birth and delay in lactation. The main source of complementary food for these infants is the locally prepared "*Ogi*" from cereals such as maize, millet and sorghum. It is usually prepared by soaking the cereal in clean water for three days either singly or in combination e.g. (maize or millet or sorghum; maize + millet; maize + millet + sorghum). *Ogi* seems to be a common weaning diet for infants in Nigeria (Bolaji *et al*., 2015; Adeyemo and Onilude, 2018; Ezenduka *et al*., 2018). In contrast to this study, Bassa infants consumed "*Ogi"* from birth and not as weaning diet (Ayeni *et al*., 2018).

Majority of babies to whom antibiotics were administered in this study were caesarean section delivered. Antibiotic usage has been reported to be higher in infants born through caesarean section birth than vaginal delivered infants (Yasmin *et al.,* 2017).

5.2. The nature of the gut microbiota composition of the infants within the first year of life

Irrespective of feeding method, the gut of infants is rapidly colonised by microorganisms shortly after birth. At phylum level, five phyla namely Actinobacteria, Firmicutes, Proteobacteria, Bacteroidetes and Fusobacteria were represented in the infants gut. The early microbial residents of infants gut are the facultative anaerobes, after exhaustion of the host oxygen they give room for strict anaerobes to strive (Timmerman *et al*., 2017).

The dominance of *Bifidobacterium* in the gut of these infants in the first year of life could be due to the presence of oligosaccharides in the breast and formula milk the infants consumed which act as a substrate for the growth of the bacterium. Bifidobacteria are heterofermenters, increase in viability of *Bifidobacterium* when grown in the presence of glucose have been described (Waddington *et al*., 2009). *Streptococcus* is a facultative anaerobes, the existence as the second highest in the gut of the infants in this study could be due to its initial colonisation from birth.

Moreover, *Bifidobacterium*as the dominant genus in terms of composition in this study is beneficial to the infants. The impact of Bifidobacteria in the gut continues from infancy to adulthood (Arboleya *et al*., 2016). Bifidobacteria are excellent producers of acetic and lactic acids. These compounds create an acidic environment for the inhibition of pathogenic organisms such as the *E. coli*, and other Enterobacteriaceae, thus, enabling the gut to fight against infections (Vieco-Saiz *et al*., 2019). It also modulates the immune system (Markowiak *et al*., 2017). Another importance is its ability to utilize fucosylated oligosaccharide in human milk; this helps the proliferation of this bacterium in the gut. Bifidobacteria and *Lactobacillus* have been used as probiotics agent. These bacteria act as precursors in the synthesis of vitamins, by converting the dietary compounds into vitamin B and vitamin K. Vitamins are essential nutrients needed for biochemical reactions in all living cells. It should be noted that vitamins synthesized by the gut bacteria and those from the dietary sources, are absorbed via different route. Vitamins from gut bacteria are absorbed via the large intestine while those from dietary sources are absorbed via the proximal small intestine (McNabney and Henagan, 2017). Bifidobacteria also restores gut

homeostasis especially after antibiotic usage. They are used both for prophylaxis and therapeutic activity against colorectal cancer where they act as anti-carcinogens (Drago, 2019). "*B. longum* subsp. *Infantis* CECT 7210 *and B. breve* K-110" were used in the treatment of diarrhoea where the combination inhibited rotavirus – the number one cause of diarrhoea disease in children (Chenoll *et al*., 2015). It has also been used in the treatment of inflammatory bowel disease, colon regularity, and necrotizing enterocolitis (Scott *et al*., 2015). *Bifidobacterium* is indeed a beneficial organism. The presence of this species in the guts of Nigeria infants could confer a health benefits to them in their early and later life.

Generally, individual differences were observed in the nature of the infants gut microbial composition. The differences are likely due to the effect of age and changes in diet, the duo and other factors may best explain the changes observed. However, from the beta diversity metric, early time point samples clustered tightly between 1- 4 months. This is suggestive of similarity among the organisms present in the gut of the infants at this period. Hill and colleagues described similar clustering on the INFANTMET cohort study conducted on infants from birth up to 24 weeks, the tight cluster was explicit at 1 and 24 weeks respectively (Hill *et al*., 2017). The divergence of the gut microbiota between 5 - 8 and 9 - 12 months exemplified the emergence of new microbes and their genetic variation. In addition to increase in age and changes in diet, different environmental factors could contribute to the diversity of gut microbiome during these periods. At this stage, infants begin to crawl, walk and get closer to the ground rather than being curdle, this may likely contribute to increase diversity. Reports have shown that various factors contribute to microbial diversity during these periods (Rutayisire *et al*., 2016; Tamburini *et al*., 2016).

Two sets of twin participated in the longitudinal studies while one set provided only the birth sample. The three sets of twin were delivered through caesarean section. Twin 1 (Baby 10 and 11) were heterozygotic twins, a male and a female. The abundance of Proteobacteria in both twins may likely be due to the fact that early colonisers are usually facultative anaerobes or inoculation into the gut from the birth environment. The twins are exposed to the same environment, diet and geographical location. Genetics could be

the counfounding factor. The composition differs at birth, although Proteobacteria was the dominating phylum in both. The abundance of Proteobacteria has been hypothesized to capture more functional variation in the human gut microbiome (Bradley and Pollard, 2017). *Trabulsiella* seems to be dominant in this set of twin. *Trabulsiella* was named after a Brazilian Bacteriologist L. R. Trabulsi,. It is one of the enteric pathogenss often confused with *Salmonella* (McWhorter *et al*., 1991). *Klebsiella* could also be gotten from the birth environment because the twins were healthy at birth. Presence of *Bifidobacterium* in baby 10 and absence in baby 11 probably suggest the maternal secretory ability due to sucking reflex of male child that predisposed enough substrate for the proliferation of *Bifidobacterium* in the gut of baby 11 more than his female counterpart. The differences were still prominent at month 5. The gut microbiota was enriched with Firmicutes and Actinobacteria with divergence in their relative abundance. Baby 10 that lacks *Bifidobacterium* at birth was dominated with the genus and baby 11 was dominated with *Streptococcus*. This dissimilarity stressed the impact of some intrinsic factors that could influence the GM composition variation in individuals but are not clearly understood. At month 10, various factors has come to play, the feeding pattern have changed with combination of breast milk, *Ogi*, formula and solid foods as well as more familiarity with the environment through crawling process. Conversely, baby 11 GM tends toward an adultlike type with signatures (*Bacteroides*, *Faecalibacterium*, *Dorea*) that participate in degradation of complex carbohydrates with resultant short chain fatty acids production being prominent. These signatures were lacking in baby 10. The changes or differences in the twin pair could probably be gender dependence. Sex-diet related differences have been reported in fish microbiome while the same effect was seen in human and mice with family belonging to Fusobacteriaceae exhibiting more sex specific effect (Bolnick *et al*., 2014). At this point also the level of *Bifidobacterium* was almost equal in the gut of the twin pair which may likely be the influence of the formula.

Twin 3 (baby 27 and 28) were another set of heterozygotic twins. The same differences observed in twin 1 were almost the same with this set of twins. Firmicutes and Proteobacteria were the dominating phyla at birth but the genus composition were different. *Blautia* was the dominating genus in baby 27, this was the only infant that has

this organism at birth. Others possessed it when on solid foods, the reason for this is unclear. However, *Blautia* and other taxa were dominant in preterm baby born with extrauterine growth restrictiona common condition with low birth weigh infant (Li *et al*., 2019). All other genera are environmental and skin related except *Bifidobacterium* that was higher in the male child than female. Again aforementioned reason may apply here too. The dominance of Actinobacteria, specifically *Bifidobacterium* in the twin pair in month 4 after introduction of *Ogi* was influenced by formula while *Lactobacillus* may likely be due to the effect of *Ogi*. Many authors have reported the enrichment of *Ogi* with *Lactobacillus* based on culture dependent methods (Sunmola *et al*, 2019, Kwasi *et al*, 2019). The gut microbiota of this twin pair was almost similar at the genus level; three genera were similar in both of them with divergence in other two among the most abundance taxon. This similarity seems to associate with diet. Again the gut microbiota was completely different at month 7 when solid foods were included in their diet.

Twin 2 (baby 12 and 13) were homozygotic twins, female but were lost due to attrition. The GM of this set of twins was similar at birth both at phylum and genus level composition. Both were enriched with Proteobacteria and Firmicutes, *Klebsiella* and *Clostridium*. This twin had sepsis at birth. *Klebsiella* has been implicated in neonatal sepsis (Ghaith *et al*., 2019). The overall observation in the set of twins is in concordance with studies that have examined the effect of genetics on a large sample size of monozygotic and dizygotic twin pairs. Their result showed that monozygotic twins had slightly more similar microbiota compare to dizygotic twins (Goodrich *et al*., 2016). Host genetics is akey factor that could confound for the differences. The authors reported that members of the family Christensenellaceae have the heritability features and the UniFrac distance between the monozygotic twins are less than that of dizygotic twins (Goodrich *et al*., 2016).

5.3. Effect of birth mode on the gut microbiota composition of the infants

Birthing method is one of the essential factors that influenced the infant gut microbial composition. Few studies have reported a lack of significant difference between the birthing methods (Chu *et al*., 2017; Stewart *et al*., 2017). In the present study, there was no statistically significant difference between the vaginal and caesarean section births. Contrary to our result, some studies found differences between caesarean section birth and vaginally delivered infants (Jakobsson *et al*., 2014; Rutayisire *et al*., 2016; Hill *et al*., 2017). The disparity in the aforementioned studies and the present study could have been due to the sample size in this study; thus, a large cohort study from Nigerian infant is needed to substantiate this result. In the present study, microbial colonisation at birth differs between the birthing methods; this revealed the effect of the route of delivery on microbiota abundance. At birth, higher abundance of *Klebsiella*, *Staphylococcus*, *Enterococcus*, *Streptococcus* and *Bifidobacterium* were observed in caesarean section birth (CSB) compared to VB which did not show the presence of Staphylococci. *Staphylococcus* could be derived from mother's skin while the environment of birth could probably be the source of *Klebsiella*. This result is in accordance with the report of Biasucci *et al*. (2010) and Martin *et al*. (2016) who found similar taxa in caesarean section birth.

The VB also had abundance of *Streptococcus*, *Enterococcus*, *Klebsiella, Trabusiella* and *Bifidobacterium*. *Streptococcus* and *Enterococcus* are vaginal colonisers. Mother's faecal microbiota has been reported to be similar to that of infants at day 3 and 7 of infants' life (Makino *et al*., 2011). Environment is the likely source of *Trabulsiella* and *Klebsiella*. Regardless of the mode of delivery, infants are exposed to hospital staff and the environment at birth, it is therefore not unlikely that the vaginal delivered infants acquired these genera from the environment. This result is in concordance with previous report (Milani *et al*., 2017). The overall result of the birthing method in this study agrees with other studies that found similar genera in the two birthing methods (Aagaard *et al*., 2012; Liu *et al*., 2019).

The first colonisers in the study infants were the facultative anaerobes. Members of group belonging to the genera *Streptococcus* and *Enterococcus* dominated the meconium samples and persisted overtime while *Staphylococcus, Trabulsiella, Klebsiella* were found in the first sample collected that were not meconium. These genera belong to the phyla Proteobacteria and Firmicutes. Members of these phyla have been reported as early infants gut colonisers (Jandhyala *et al*., 2015; Perez-Muñoz *et al*., 2017).

5.4. The Impact of feeding on the gut microbiota composition of the infants

Diet has a strong influence on the composition of human gut. Many reports have examined the effect of diet on the gut composition of both infants and adults (Rios-Covian *et al*., 2016; Korpela *et al*., 2018; Quin *et al*., 2018). Food is a substrate for microbial degradation. Certain foods help the proliferation of specific organisms especially the beneficial ones. Food rich in fibre such as maize, sorghum, millet, wheat, oat, vegetables and fruits support the growth of the beneficial organisms such as *Ruminococcus, Faecalibacterium, Prevotella and Roseburia* (Rios Covian *et al*., 2016).

The developmental stage of the microbiota from infanthood to adulthood is partly dependent on diet. Higher Proteobacteria in the mixed fed (MF) infants observed in this study may probably have been due to the handling/preparation of formula milk. Most mothers used feeding bottles; improper cleaning of the feeding utensils may seed the infant gut with members of this group. The family Enterobacteriaceae which belong to γ-Proteobacteria have been reported in MF infants (Bäckhed *et al*., 2015; O'Sullivan *et al*., 2015). In contrast, Bacteroidetes washigher in the EBF group in the present study. Abundance of Bacteroidetes was reported in exclusively breastfed infants (Wang *et al.* 2015).

At the genus level, *Bifidobacterium* was the discriminator as it was higher in the MF infants compared to EBF. There was a positive relationship between the fortified infant formula and the higher abundance of *Bifidobacterium* in the gut of infants that consumed the milk. In contrast to this study, a clinical trial done on infants that were mixed fed and

breastfed revealed higher *Bifidobacterium* in the EBF than the mixed fed (Bazanella *et al*., 2017). Many studies are in agreement with higher abundance of *Bifidobacterium* in breastfed infants (Azad *et al*., 2014; Wopereis *et al*., 2014;Hill *et al*., 2017), which was not the case in the present study in Nigeria. This may likely due to some environmental factors that select for reduced colonisation by Bifidobacteria in the infantsB gut. For instance, Bifidobacterial speceies are endowed with special ability to utilize human milk glycans present in breastmilk differently (Garrido *et al*., 2013). Likewise, the rate of maternal secretion differs, for instance the type and concentration of fucosylated oligosaccharides in the breast milk could be influenced by the secretory status which in turn impact the microbial community structure (Lewis *et al*., 2015). Geographical location may likely contribute to low or no Bifidobacteria in Nigeria infants as reported in the Bassa infants study, Bassa infants are fed with *Ogi* from birth (Ayeni *et al*., 2018). The exclusively breastfed (EBF) infants had higher abundance of *Collinsella*, *Sutterella*, *Actinomyces* and *Bacteroides* compared with MF. *Collinsella aerofaciens* and *Sutterella* dominance in the breastfed infants is in agreement with previous studies (Lazar *et al.,* 2018; Srikantha and Mohajeri 2019). *Actinomyces* are colonizers of the oral cavity, their presence in the gut may be due to transit from the oral cavity to the gut during feeding (Li *et al*., 2018). Human Oral Microbiome Database (http://www.homd.org/) reported 18 species of unnamed *Actinomyces* taxa in the human oral cavity. *Bacteroides* is usually associated with formula fed infants (Penders *et al*., 2006; Azad *et al*., 2013) or vaginal birth infants (Tanaka and Nakayama 2017). However, the presence of *Bacteroides* in the EBF infants in this study is similar to the report of Wang *et al.* (2015). The higher abundance of *Bacteroides* in the current study may probably be due to substrate specificity. Whole genome transcriptional profile have shown that some members of the genera *Bifidobacterium* and *Bacteroides* possess the ability of expressing glycoside hydrolase and gut membrane transporters that are vital for the breaking down of human milk oligosaccharides (Marcobal *et al*., 2011). Thus, the presence of the two genera in exclusively breastfed infant may result in competition for HMO as the metabolic substrate. This probably may give preference to utilization by *Bacteroides* more than *Bifidobacterium* in these infants.

The introduction of "*Ogi"* and solid foods made a big difference in both initially exclusively breastfed and mixed fed infants gut microbiota. However, the mixed fed infants gut microbiota had higher diversity and enrichment than their solely breastfed counterparts when complementary foods were consumed. The reason for more diversity in the initially mixed fed infants could simply be explained by the fortified formula that enhanced the breast milk and "*Ogi"*, therefore increasing the number of taxa or encouraging the growth of other microbes. Significant biomarkers during this period are *Bifidobacterium*, *Bacteroides fragilis*, *Ruminococcus gnavus*, *Veillonella dispar*, *Lactobacillus ruminis*, *Enterococcus casseliflavus* and Clostridia. *Bacteroides fragilis* is an obligate anaerobic organism that inhabits mammalian lower GIT, the organism serves a beneficial purpose while in the gut but could cause serious infection outside the gut environment (Wexler, 2007). Polysaccharide A produced by *Bacteroides fragilis* has the potential of inhibiting inflammatory response in the gut by stimulating interlukin-10 producing CD_4^+ $Foxp_3^+$ T-regulatory cells as reported in a mice model (Mazmanian *et al*., 2008)

Ruminococcus gnavus participates in mucin degradation in the gut. The role of this organism is relatively unclear in infants (Sagheddu *et al*., 2016). However, Blanton *et al*. (2016) suggested a putative role of *R. gnavus* in promoting protein synthesis and ameliorating growth and metabolic abnormalities in recipient germ-free mice after faecal transplantation from healthy and malnourished Malawian infants aged 6 and 18 months.

For the initially breastfed infants, decrease in Proteobacteria was observed when *Ogi* and solid foods were introduced. This could be as a result of the activity of lactic acid bacteria that have been reported in "*Ogi*" (Afolayan and Ayeni, 2017; Omemu *et al*., 2018). There was an increase in Bacteriodetes at the period when the babies were eating solid foods. This is likely due to plant-based polysaccharide derived from cereals the infants consumed. Also, Fusobacteria which was not present during the exclusively breastfed period appeared when *Ogi* and solid foods were introduced. The probable reason for the appearance of this phylum explains the paradigm shift from milk to solid foods. These changes were at the level of phylum and it further explained increased diversity that was exemplified during complementary feeding.

At the level of genus, the relative abundance of *Enterococcus* increased when "*Ogi*" and solid foods were introduced to babies in this group. Species such as *Enterococcus casseflavus* appeared during this period. LefSeanalysis indicated more significant biomarkers during the period of "*Ogi*" and solid foods consumption with genera such as *Pediococcus,* Clostridia and members of Lachnospiraceae family appearing distinctively. These organisms play key role in maintaining the structure of the gut. Additional observation was the abundance of *Bifidobacterium*at this period. It should be noted that this microorganism was not significantly present when the babies were on exclusive breastfeeding, but the presence was apparent whenthe infants consumed *"Ogi"* with fortified formula. It could then be deduced that the fortification of NAN with Bifid LAL as proved in the present study may be responsible for the higher abundance of *Bifidobacterium* during complementary feeding in the initially exclusively breastfed infants. About 98% of infants in the present study used NAN milk during complementary feeding period.

Furthermore, the differences between the preweaning and weaning stages explained the paradigm shift from milk to solid foods. This includes those infants that were initially exclusively breastfed or mixed fed and later introduced to *Ogi* and solid foods. An indication of the shift is the emergence of specific signatures that were not present in the preweaning and appeared in the weaning or those that were less abundance in the preweaning stage but increased in abundance during weaning. These signatures usually participated in active carbohydrate metabolic activity. Examples of biomarkers present in the infants during weaning include *Coprococcus*, *Clostridium*, 02d06, SMB53, members of the family Lachnospireaceae, Pasteurellaceae, and Lactobacillaceae most especially *Enterococcus casseliflavus*. Other biomarkers were also present ut do not participate in carbohydrate metabolism. Group belonging to Clostridia and Enterococci have been reported to increase during weaning period (Adlerberth and Wold, 2009). Members belonging to Lachnospireaceae family are known for their degradation of complex carbohydrates with the resultant short chain fatty acids. The abundance of Lachnospireaceae family during weaning in this study is in concordance with Danish

infants who had increased Lachnospireaceaeduring weaning. *Coprococcus* is one of the genera in this family (Bergstrom *et al*., 2014). Pasteurellaceae was also found in the gut of infants during weaning (Lozzo and Sanguinetti 2018).

The local food consumed by the infants in this study is highly rich in fibre, vitamins and contained an abundance of *Lactobacillus* spp. Extensive work has been done on the indigenous Nigerian weaning diet (*Ogi*) and it is an established fact that it is enriched with *Lactobacillus* spp which is potent against many pathogenic strains (Afolayan and Ayeni, 2017, Afolayan et al. 2017, Sunmola et al, 2019, Kwasi et al, 2019)). *Enterococcus casseliflavus* is involved in the biodegradation of complex plant-derived polysaccharide as well as in the bioconversion of polyphenols to the absorbable form in the colon (Braune *et al*., 2016). Since Bacteroides are endowed with polyssacharide utilization loci in their genome, which gives them the ability to utilize plant based polysaccharides, increased in the abundance of *Bacteroides*during weaning could still be due to the consumption of fibre-rich foods which is usually typical during weaning in Africa. These local cereals help in supplying substrate for metabolic activities of the gut microbes. The increased level of *Bacteroides* observed in the study infants during weaning agrees with the findings of Fallani *et al*. (2011) that found higher *Bacteroides* in European infants during weaning. Members of *Bacteroides* possess genes that code for enzymes which are capable of degrading complex carbohydrates (Flint *et al*., 2012). The fermentation of the undigested carbohydrates usually yields short-chain fatty acids such as butyrate, which is a good energy source for colonic health. Butyrate helps in stimulating mucosal proliferation, being the chief source of energy supply to the colonocytes, thereby acting as cell growth stimulant. It also acts as an anti-inflammatory agent (Zhang and Davies, 2016).

Likewise, some signatures were not present at all in the preweaning stage but are represented in the weaning stage. The biomarkers include *Roseburia*, *Blautia*, *Pediococcus*, *Ruminococcus gnavus* and *Dorea*. These genera also differentiate these periods, thus, exemplifying the maturation of the gut microbiota and development towards an adult gut microbiota. These taxa are capable of different complex metabolic activities and help in maintenance of gut structure and permeability (Crost *et al*., 2018). These

biomarkers have been reported in infants during weaning (Laursen *et al.,* 2016). The overall changes that occur in the gut microbiota of these infants in the preweaning and weaning stage followed reported transit from milk-dependent food to solid food such as dominance of Actinobacteria during milk-based feeding to Bacteroidetes during solid food period as well as increased metabolic products in the later period (Koenigt *et al*., 2011; Fallani *et al*., 2011). In contrast, infants from a northern settlement, Bassa, in Nigeria possessed a more diverse and adult-like gut microbiota at infancy because these infants consumed *Ogi* usually called "*koko*" from birth (Ayeni *et al*., 2018). More diverse microbial community are usually involved in catabolism of complex molecules derived from food and indicate that the infant gut microbiota is undergoing maturation to adult-like microbiota.

There was a positive correlation between the results obtained from the 16S rRNA sequences and the DGGE. The changes between the preweaning and weaning stages were also obvious in the fingerprinting analysis. Some taxa appeared in the preweaning stage, and was absent in the weaning stage whereas some microbes were consistently present throughout the two periods as revealed by each band. Others disappeared during weaning while new species appeared at this period. This observation revealed that the infants gut microbiota were influenced by diet. The main purpose of the fingerprinting analysis was for visualisation. Originally, each band was supposed to be cut-out from the gel and extracted in appropriate buffer, re-amplified, to be certain and then sequence to characterise the particular taxa. However in this study, 16S rRNA sequencing gave the nomenclature of the taxa present in all the samples and there was no need for further assessment of bands from DGGE analysis.

5.5. Impact of shortchain fatty acids in the gut of the study infants

Shortchain fatty acids are secondary metabolites produced as a result of microbial activity on undigested dietary fibres. Interlink exist between diet, gut microbiota and shortchain fatty acid production. Diet influences the gut microbiota, this was revealed in the amount and types of metabolites (short-chain fatty acids) produced. In this study, lactate and

acetate were predominant overtime, these findings corroborate the results of 16S rRNA sequences which found that infant guts were enriched with *Bifidobacterium*. Higher occurrence of acetate and lactate in the infant guts may be attributed to the presence of Human Milk Oligosaccharide (HMO) in the breast milk. The galacto-oligosaccharides are partially digested in the small intestine and usually get to the colon where they are fermented by Bifidobacteria to produce acetate and lactate. This fact was supported by a study that observed an increase in concentration of acetate and lactate in relation to reduction in the level of HMO in infants' faeces, meaning that *Bifidobacterium* had utilized the oligosaccharide thereby aiding the proliferationof the genus in infant guts (Tanaka and Nakayama, 2017). This result is also consistent with the dominance of acetate in Swedish and Estonian infants after 1 month of life (Norin *et al*., 2004). Lactate and acetate serve a protective function against the proliferation of pathogenic organisms. It should be noted that acetate production pathways are common within most bacterial groups in the gut (Louis *et al*., 2014).

Bacterial species belonging to *Akkermansia municiphilla* are known producers of propionate in adult guts. However, not many genera make this short chain fatty acids but they may be produced as a subset of other gut residents from a different pathway which is usually conserved and specific (Morrison and Preston, 2016).The presence of propionate in the present study may likely due to production through other SCFAs pathway and probably due to the effect of *Bacteroides* and *Veillonella* that are proospective propionate producers (Shimizu *et al*., 2018).

Notable in this study is the appearance of butyrate when weaning starts (between 4-6 months). It suggests or supports a positive association between production of butyrate and fermentation of non-digestible carbohydrates from plant-based polysaccharides that was present in *Ogi* and other solid foods consumed by these infants. Only 5 babies started weaning earlier at 4 month, yet the effect was obvious at the period and the little effect could be due to different time when solid food was introduced into the infant's diet.The production of SCFAs also correlates with the significant biomarkers seen in the weaning samples. As earlier discussed, the abundance of *Roseburia* and *Coprococcus*, during

weaning may likely be responsible for the increased butyrate. These genera are excellent producers of butyrate in adult gut (Flint *et al*., 2015). Moreover, the increase in abundance of Bacteroidetes during weaning was also suggestive of the breakdown of plant polysaccharides which could result in the proliferation of members of this phylum. The association between Bacteroidetes and diet was once tested in obese individuals who usually had low plant-based food regime. The study discovered that these obese individuals had low occurrence of Bacteroidetes in their gut emphasising the metabolic activity associated with plant polysaccharide (Ley *et al*., 2006). Likewise, *Bacteroides* belonging to this phylum was also observed to be high during weaning and in exclusively breastfed infants. Members of the genus *Bacteroides* areorganismsknown for carbohydrate metabolism through enzymatic expression with resultant butyrate production (Jandhyala *et al*., 2015). The increase in acetate and lactate may likely be due to the duration of breastfeeding. All the infants' in the present study were still on breast milk as at the last sample collected. Combination of breast milk, formula milk, *Ogi* and solid foods during weaning may probably increased the metabolic activity of the resident microbes resulting in increased acetate and lactate production. Propionate can be formed through the succinate pathway (Sun *et al*., 2017). However, Bacteroidetes and few of Firmicutes usually produce this SCFA in the gut. The increase in the aforementioned phyla during weaning could contribute to the increase of propionate during weaning. Succinate production is usually an intermediate metabolite in the microbial production of SCFAs (Louis and Flint, 2016) thus its activity is unclear. The increase in all the SCFAs during weaning (except succinate) supports Koenig *et al*. (2011) who reported increments of shortchain fatty acids and bacterial diversity after the introduction of solid foods. Increased shortchain fatty acids production has also been reported in African children with distinct production of butyrate different from their European counterparts (De Filippo *et al*., 2010).

5.6. Effect of antibiotic usage on the gut microbiota of the infants

Antibiotic usage usually has a short- and long-term effect on the ecology of the normal gut microbiota. Antibiotics used by the study infants were the β-lactams, macrolides, aminoglycosides and fluoroquinolones. Multi-drug resistant genes have been in existence before the advent of antibiotics, pointing to the facts that the environment is occupied with small molecules of some growth inhibitory compounds (Jandhyala *et al*., 2015). Selected genes were screened in this study. Infant gut is said to harbour antibiotic resistance genes more than the adults even without exposure (Pärnänen *et al*., 2018). Being colonized by opportunistic carriers of resistant genes pose a threat to these infants as the gut microbiota and immune system are fairly unstable and are still in the developing stage compared to adults. Among the first gut colonizers are the Proteobacteria; the Gammaproteobacteria usually carry resistance genes, their abundance in the gut was hypothesised to have caused higher level of antibiotic resistance genes in infants (Hu *et al*., 2013).

Antibiotic resistance may or may not correlate with usage. In this study the use of β-lactam antibiotics (Penicillin) correspond to the presence of β-lactamase genes as observed that sudden appearance of this gene was apparent at the period when penicillins was administered and disappearance when not in used. Kahlmeter and colleagues experiment the effect of antibiotic usage in 14 European countries and found that antibiotic usage correlate with resistance to ciprofloxacin, nitrofurantoin, nalixidic acid and gentamicin while correlation with the consumption of broad spectrum penicillin did not reach a significant level (Kahlmeter *et al*., 2003). β-lactamase genes are common among Gram-negative organisms, however some Gram-positive organisms are carriers of these genes especially *bla*Z which is carried by Staphylococci. This genus has also been associated with *mec*A, *aac*(6')-*aph*(2'), *erm*A, *erm*B, *erm*C, and *msr*A resistance genes (Martineau *et al*., 2000). The presence of Staphylococci in the gut of these babies could be possible carriers of *bla*Zgene. β-lactam antibiotics are commonly used and prescribed, infants might have acquired the resistance genes as a result of usage. Correlation between consumption of penicillins and resistance to ampicillin has been described (Kahlmeter *et al*., 2003). Likewise, since this gene is plasmid-borne, there is likelihood that it may be transient.

Tetracyclines usage in the treatment of infectious diseases have been explored in humans and animals; however, in recent times the usage has decreased and no longer in use for pregnant women and children under 8 years. Some countries still use tetracycline as growth promoters in animal feeds (Milani *et al*., 2017). In the current study infants did not use tetracycline antibiotic. However, infants carried *tet* genes, this stressed the fact that carriage of resistance gene does not necessarily relate to usage in all cases. In a previous study performed on 16 healthy infants and their mothers, with no prior exposure to tetracycline antibiotic, *tet*M was found to be dominant in both mothers and infants, while *tet*O was found in the mother only (Gueimonde *et al*., 2006).

The general effect of antibiotic usage on the gut microbiota has been the reduction in abundance of taxa or total loss (Milani *et al*., 2017), as observed in the alpha diversity metrics on antibiotic usage. Correlation between antibiotic usage and the abundance of gut microbiota could not be established in this study. The reason for this is hinged on observation that at the time points when antibiotic was used, there were reductions in abundance and diversity of the gut microbiota composition in some babies, whereas in others no changes were seen or rather homeostasis was regained. Various factors could result in reduction of taxon at a particular time point. Since a culture-independent method was used, the exact taxon that was depleted or the organism that carries the resistant genes could not be ascertained.

The changes in taxonomic diversity with antibiotic intake have been reported: there was a 2-year non-recovery of *Bacteroides* after a short-term usage of clindamycin (Jernberg *et al*., 2007). Similarly, a short-term treatment of *Helicobacter pylori* with clarithromycin has resulted in decrease in abundance of Actinobacteria and also increase in *erm*B resistance gene in the gut (Jakobsson *et al*., 2014). The likelihood of horizontal transfer among gut microbiota is another point, *Bifidobacterium longum*isolated from the faecal sample has been reported to harbour *tet* gene (Scott *et al*., 2000). Bacteria resistance to more than one antibiotic is common. There is possibility of a bacterium to harbour multiple resistance genes. Enteric isolates resistant to ampicillin had been reported to harboured *tet* genes (Murray *et al*., 2018)

Ribosomal protective tetracycline gene was the most prevalence in the study infants. Macrolide resistant *mef* gene was the second in prevalence, followed by *erm*B, aac 6', *bla*Z, and then the least was *erm*A. The prevalence of resistance genes could also be as a result of horizontal gene transfer among gut microbiota and as earlier mentioned, as well as vertical transfer from mothers. This was evident in a study that found that resistance genes in the mothers' gut were similar to those found in the infants' gut (Gosalbes *et al*., 2016). A similar study also compared breast milk, faecal samples of infants and the mothers and found that resistance genes and mobile genetic elements in breast milk and infants' faecal samples were similar (Zhang *et al*., 2015). On the other hand, a study did not found correlation between the resistome in mothers' gut and infants' gut (Moore *et al*., 2015).

In the last decades, macrolide resistance determinants such as *erm* and *mef* have been reported in Streptococci. The genetic elements on which these genes are transferred (transposons) also carries other resistance genes conferring resistance to other antibiotics such as tetracyclines, aminoglycosides and chloramphenicol. Abundance of Streptococci and Enterococci in the gut of the infants during the preweaning period could be responsible for the high proportion of *erm*B and *mef*A/E resistance genes (Varaldo *et al*., 2008).

5.7. Conclusion

This study showed the microbial compositional diversity of some Nigerian infants within the first year of life. From the present study, microbial diversity increases with age, a low diversity within the first 6 months of life and higher diversity from 7 to 12 months. Dominance of the genera *Klebsiella* and *Staphylococcus* in the caesarean section birth infants and abundance of *Streptococcus* and *Enterococcus* in vaginal birth infants depict that route of delivery influences the composition of infant gut microbiota.

The gut microbiota of Nigerian infants is influenced by diet. The mixed fed infant guts has enrichment of *Bifidobacterium* than the exclusively breastfed infants due to the fortification of the infant formula with Bifid LAL. Abundance of *Bacteroides* in exclusively breastfed infants contrary to literature report. In addition, the local/solid fed infants typifying the weaning period, had a more diverse gut microbiota composition with appearance of new taxa such as *Roseburia*, *Coprococcus*, *Blautia* and *Dorea* suggestive of active carbohydrate metabolism.

Acetate and lactate were the highest SCFAs produced by the gut microbiota in this study, appearance and increased butyrate during weaning were impact of diet and it's indicative of complex metabolic activities in the gut of the infants.

Presence of antibiotic resistance genes in the gut of the studied infants indicate the fact that antibiotic resistance is not only acquired by exposure but is influenced by different environmental factors contributing to the spread. Tetracycline's ribosomal protection protein (*tet*) gene was the most prevalent in the study population

The observed gut microbiota taxonomic differences between preweaning and weaning in these infants as well as butyrate production were influenced by diet. Introduction of solid foods encouraged the colonisation and adaptation of specific marker organisms associated with carbohydrate metabolism helpful for a healthy life.

5.8. Contributions to knowledge

- This is the first study in Nigeria looking at the composition and longitudinal changes in the gut microbial composition of infants. This study provided information on the gut microbiota composition of some Nigeria infants which serves as a reference point for other studies, and also for comparison with infants from Western countries.

- Mixed feeding encourages the growth and the proliferation of *Bifidobacterium* more than exclusive breastfeeding. Hence, mixed feeding is more beneficial from the perspective of beneficial gut microbiota. While exclusively breastfeeding encourages the growth of *Bacteroides*.

- Early initiation of local/solid food is helpful for the maturation of the gut microbiota as evident by selection of microbial biomarkers essential for metabolism of complex molecules, good for present and future healthy life.

- Dominance of acetate and lactate in the infants' guts within the first year of life showed that Nigerian infants could be protected from pathogenic colonisation in the gut. Also butyrate in the gut of these infants during weaning could also help colonic health.

- Antibiotic resistance is a global problem, unexposed infants, are not left out as observed in this study. Transmission of resistance genes is common and easier within the gut community as a result of horizontal gene transfer.

5.9. Recommendations

Building a good and beneficial microbiota helps the development of the immune system of infants which in turn improves their present and future healthy living. Formula feeding is not bad in the point view of gut microbiota; provided it is fortified with *Bifidobacterium* and the feeding utensils could be kept hygienic.

Introduction of local/solid foods imprint significant biomarkers that helps in the biological degradation of complex plant-based polysaccharides in foods. Earlier introduction of solid foods could be helpful for infants gut health. Healthy foods promote healthy living, foods that are rich in fibre select for butyrate production in the gut. Consumption of fibre-rich food is recommended.

A large cohort study is needed to further understand the effect of birth mode on the gut composition of Nigerian infants. Studies covering the 6 geopolitical zones in Nigeria are required to be able to get a consensus on Nigerian infants gut microbiota composition and to know the impact of weaning diet in different geographical location of the country.

5.10. Future directions

Follow-up on the present study infants gut microbiota between 3 – 5 years, to compare the infants gut microbiota with adults as well as the functionality of the gut microbiota with their metabolite production.

A large cohort studies is required to ascertain that delivery mode does not have significant effect on Nigerian infants gut microbiota.

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APPENDIX I

Equipments and other materials

FastPrep instrument

Microcentrifuge

Vortexer

Weighing balance

PCR machine

S1000 Thermal cycler

Electrophoresis tank and power pack

UV Transilluminator

Photo gel documentation system

Microwave oven

Refrigerated centrifuge

Heating block

Qubit 2.0 Fluorometer

DGGE tank

Gas Chromatography machine

Automatic pipettes.

Sterile tips.

Sterile eppendorf tubes

Rack.

Disposable gloves.

Parafilm

Crimper

Microcentrifuge tubes

Falcon tubes

Volumetric flasks

APPENDIX II

Reagents and Buffers

Absolute ethanol

10X reaction buffer.

MgCL2 [25 mM].

dNTPs 2mM

DNA Taq polymerase

Agarose powder

Tris-Borate-EDTA (TBE)

Tris-Acetate EDTA (TAE)

Tris-EDTA (TE)

Gel red Nucleic Acid stain

Gel Loading dye

Sodium chloride

2-Ethyl butyric acid

Concentrated hydrochloric acid

Ether

Acetic acid

Propionic acid

Iso-Butyric acid

N-Butyric acid

Iso-Valeric acid

O-Valeric acid

Sodium formate

Lithium lactate

Sodium succinate

APPENDIX III

Metadata of infants' overtime

Sample code	Sex	Gestational age	Mode of delivery	Weight (kg)	Feeding method	Age of baby	Antibiotic treatment	Herbal treatment	Complementary food	Sickness during the study
Baby1 t0	Male	37 wk, 5 d	CSB	3.45	FF/BF	3 wk	-	-	-	-
Baby1t1				4.4	BF	1 mo	-	-	-	-
Baby1t 2				6.3	FF/BF	2 mo	-	-	-	-
Baby1 t 3				6.7	FF/BF	3 mo	-	-	-	-
Baby1 t 4				7.9	FF/BF	4 mo	-	-	-	-
Baby1 t5				NA	FF/BF	5 mo	-	-	-	-
Baby1 t6				NA	FF/BF	6 mo	-	-	-	-
Baby1 t7				8.5	FF/BF/CF	7 mo	Amoxicillin	-	Fortified ogi & friscogold	Rashes/fever
Baby 1 t8				9.5	FF/BF/CF	8 mo	-	-	Fortified ogi & friscogold	-
Baby 1 t9				NA	FF/BF/CF	9 mo	-	-	Unsieved ogi, solid foods, fruits	-
Baby 1 t10				NA	FF/BF/CF	10 mo	-	-	Unsieved ogi, solid foods, fruits	-
Baby 1 t11				10.5	FF/BF/CF	11 mo	Amoxicillin	-	Unsieved ogi, solid foods, fruits	Cough & catarrh
Baby 1 t12				NA	FF/BF/CF	12 mo	-	-	Unsieved ogi, solid foods, fruits	-
Baby 2 t0	Female	39 wk 5 d	CSB	3.8	FF/BF	6 d	-	-	-	-

APPENDIX III: Cont'd

Sample code	Sex	Gestational age	Mode of delivery	Weight (kg)	Feeding method	Age of baby	Antibiotic treatment	Herbal treatment	Complementary food	Sickness during the study
Baby 2 t1				NA	BF	1 mo	Erythromycin	-	-	Catarrh and cough
Baby 2 t4				NA	BF	4 mo	-	-	-	-
Baby3t1				NA	EBF	1 mo	-	-	-	-
Baby3t 2				5.6	EBF	2 mo	-	-	-	-
Baby3 t 3				5.7	EBF	3 mo	-	-	-	-
Baby3 t 4				6.2	EBF	4 mo	-	-	-	-
Baby3 t5				NA	FF/BF/CF	5 mo	-	-	Ogi- Millet only	-
Baby3 t6				NA	FF/BF/CF	6 mo	-	-	Ogi	-
Baby3 t7				NA	FF/BF/CF	7 mo	-	-	Ogi and custard	-
Baby3 t8				8.0	FF/BF/CF/SF	8 mo	-	-	Ogi , introduce solid	-
Baby3 t9				7.2	FF/BF/CF/SF	9 mo	-	-	Ogi + solid foods	-
Baby3 t10				8.0	FF/BF/CF/SF	10 mo	-	-	Ogi + solid foods	-
Baby4 t0	Female	38 wk 5d	VB	2.8	EBF	7 d	-	-	-	-
Baby4 t1				4.0	EBF	1 mo	Cefuroxime and Chloramphenicol eye drops	-	-	Skin rashes and eye discharges
Baby4 t2				4	EBF	2 mo	-	-	-	-
Baby4 t3				4.5	EBF	3 mo	-	-	-	-

APPENDIX III. Cont'd

Sample code	Sex	Gestational age	Mode of delivery	Weight (kg)	Feeding method	Age of baby	Antibiotic treatment	Herbal treatment	Complementary food	Sickness during the study
Baby4 t4				NA	EBF	4 mo	-	-	-	-
Baby4 t5	Female	38 wk 5 d	VB	NA	EBF	5 mo	-	-	-	-
Baby4t6				NA	EBF	6 mo	-	-	-	-
Baby4t 7				7.0	BF/FF	7 mo	-	-	Golden morn	-
Baby4 t 8				7.5	BF/FF/SF	8 mo	-	-	Introduce solid foods	-
Baby4 t 9				7.0	FF/BF/SF	9 mo	-	-	Solid foods	-
Baby4 t10				8.0	FF/BF/SF	10 mo	-	-	Solid foods	-
Baby5 t0	Male	37 wk 3 d	VB	3.3	EBF	1 d	-	-	-	-
Baby5 t1				NA	EBF	1 mo	Chloramphenicol Eye drop	-	-	Eye discharge
Baby5 t2				NA	EBF	2 mo	-	-	-	-
Baby5 t3				NA	EBF	3 mo	-	-	-	-
Baby5 t4				NA	EBF	4 mo	-	-	-	-
Baby5 t5				8.0	BF/FF/CF	5 mo	-	-	Ogi - Maize only	-
Baby5 t6				NA	BF/FF/CF	6 mo	-	-	Ogi	-
Baby5 t7				NA	BF/FF/CF/SF	7 mo	-	-	Ogi, introduce solid foods	-
Baby5 t8				9.5	BF/FF/CF/SF	8 mo	-	-	Ogi + Solid foods	-
Baby5 t9				9.0	BF/FF/CF/SF	9 mo	-	-	Ogi + Solid foods	-

APPENDIX III. Cont'd

Sample code	Sex	Gestational age	Mode of delivery	Weight (kg)	Feeding method	Age of baby	Antibiotic treatment	Herbal treatment	Complementary food	Sickness during the study
Baby5 t10				10.0	BF/FF/CF/SF	10 mo	-	-	Ogi + Solid foods	-
Baby6 t0	Male	39 wk 6 d	CSB	2.9	EBF	3 wk	-	-	-	-
Baby6 t1				4.6	EBF	1 mo	-	-	-	-
Baby6 t2				5.2	EBF	2 mo	-	-	-	-
Baby6 t3				NA	EBF	3 mo	-	-	-	-
Baby6 t4				NA	EBF	4 mo	-	-	-	-
Baby6 t5				NA	EBF	5 mo	-	-	-	-
Baby6 t6				NA	EBF	6 mo	-	-	-	-
Baby6 t7				7.0	BF/FF/CF/SF	7 mo	-	-	Ogi, cerelac, introduce solid foods	-
Baby6 t8				7.5	BF/FF/CF/SF	8 mo	-	-	Ogi + Cerelac + Solid foods	-
Baby6 t9				8.0	BF/FF/CF/SF	9 mo	-	-	Ogi + Cerelac + Solid foods	-
Baby7 t0	Female	42 wk	CSB	3.2	BF	4 d	Cefotaxime and Cefixime	-	-	Presumed sepsis
Baby7 t1				NA	EBF	1 mo	-	-	-	-
Baby7 t2				5.2	EBF	2 mo	-	-	-	-
Baby7 t3				5.5	EBF	3 mo	-	Yes	-	-
Baby7 t4				6.7	EBF	4 mo	-	Yes	-	-

APPENDIX III. Cont'd

Sample code	Sex	Gestational age	Mode of delivery	Weight (kg)	Feeding method	Age of baby	Antibiotic treatment	Herbal treatment	Complementary food	Sickness during the study
Baby7 t5	Female	42 wk	VB	7.3	EBF	5 mo	-	Stopped	-	Fever
Baby7 t6				NA	EBF	6 mo	-	-	-	-
Baby7 t7				9.0	BF/CF/SF	7 mo	-	Yes	Ogi, introduce solid foods	-
Baby7 t8				9.5	BF/FF/SF	8 mo	-	Yes	Ogi + Solids foods	-
Baby 7 t9				8.2	BF/FF/SF	9 mo	-	-	Solid foods	-
Baby8 t0	Female	38 wk	VB	2.8	EBF	8 d	Ampiclox	-	-	Fever
Baby8 t1				3.5	EBF	1 mo	-	Yes	-	-
Baby8 t2				6.0	EBF	2 mo	-	Yes	-	-
Baby8 t3				6.5	EBF	3 mo	-	Yes	-	-
Baby8 t4				7.1	EBF	4 mo	-	Yes	-	-
Baby8 t5				NA	EBF	5 mo	-	-	-	-
Baby8 t6				NA	EBF	6 mo	-	-	-	-
Baby8 t7				8.0	BF/SF	7 mo	-	-	Introduce solid foods	-
Baby8 t8				8.5	BF/SF	8 mo	-	-	Solid foods	-
Baby8 t9				8.1	BF/SF	9 mo	-	-	Solid foods	-
Baby8 t10				8.2	BF/SF	10 mo	-	-	Solid foods	-
Baby9 t0	Female	38 wk	VB	3.2	BF	3 d	-	-	-	-
Baby9 t1	-	-	-	4.5	EBF	1 mo	-	-	-	-
Baby9 t2	-	-	-	4.7	EBF	2 mo	-	-	-	-
Baby9 t3	-	-	-	-	EBF	3 mo	-	-	-	-
Baby9 t4	-	-	-	-	EBF	-	-	-	-	-
Baby9 t5	-	-	-	-	-	5 mo	-	-	-	-
Baby9 t6	-	-	-	-	-	-	-	-	-	-

APPENDIX III. Cont'd

Sample code	Sex	Gestational age	Mode of delivery	Weight (kg)	Feeding method	Age of baby	Antibiotic treatment	Herbal treatment	Complementary food	Sickness during the study
Baby9 t7	-	-	-		-		-	-	-	-
Baby9 t8				7.5	BF/SF	8 mo	-	-	-	-
Baby9 t9				7.0	BF/SF	9mo	-	-	-	-
Baby10 t0	Female	34 wk 5 d	CSB	2.5	BF/FF	1 d	-	-	-	-
Baby10 t1				2.9	BF/FF	1 mo	-	-	-	-
Baby10 t2				5.0	BF/FF	2 mo	-	-	-	-
Baby10 t3				6.9	BF/FF	3 mo	Augumentin	-	-	Cough
Baby10 t4				NA	BF/FF/CF	4 mo	-	-	Sorghum and millet	-
Baby10 t5				NA	BF/FF/CF	5 mo	-	-	Ogi	-
Baby10 t6				NA	BF/FF/CF	6mo	-	-	Introduce solid foods	-
Baby10 t7				8.0	BF/CF/SF	7 mo	-	-	Ogi + solid foods	-
Baby10 t8				8.0	BF/CF/SF	8 mo	Augumentin	-	Ogi + solid foods	Cough
Baby10 t9				7.4	BF/CF/SF	9 mo	Augumentin	-	Ogi + solid foods	Cough
Baby10 t10				7.5	BF/CF/SF	10 mo	-	-	Ogi + solid foods	-
Baby11 t0	Male	34wk, 5d	CSB	2.6	BF /FF	2 d	-	-	-	-
Baby11 t1	-			3.2	BF /FF	1 mo	-	-	-	-
Baby11 t2				5.4	BF/ FF	2 mo	-	-	-	-
Baby11 t3				7.9	BF/FF	3 mo	Augumentin	-	-	-
Baby11 t4				NA	BF/FF	4 mo	-	-	-	Ogi-Sorghum + millet

APPENDIX III. Cont'd

Sample code	Sex	Gestational age	Mode of delivery	Weight (kg)	Feeding method	Age of baby	Antibiotic treatment	Herbal treatment	Complementary food	Sickness during the study
Baby11 t5				NA	FF/BF/CF	5 mo	-	-	Ogi -Sorghum + millet	-
Baby11 t6				NA	FF/BF/CF	6 mo	-	-	Introduce solid foods	-
Baby11 t7				9.0	FF/BF/CF	7 mo	-	-	Ogi + solid foods	-
Baby11 t8				9.0	BF/CF/SF	8 mo	Augumentin	-	Ogi + solid foods	Cough
Baby11 t9				8.6	BF/CF/SF	9 mo	Augumentin	-	Ogi + solid foods	Cough
Baby11 t10				9.0	BF/CF/SF	10 mo	-	-	Ogi + solid foods	-
Baby12 t0	Female	38wk, 5 d	CSB	2.6	BF/FF	6 d	IV Cefuroxime, gentamicin, cefexime	-	-	-
Baby13 t0	Female	38wk, 5 d	CSB	2.4	BF/FF	4 d	Cefuroxime, gentamicin, cefexime	-	-	-
Baby14 t0	Female	14wk, 4 d	VB	3.4	EBF	2 wk	-	-	-	-
Baby14 t1				3.6	EBF	1 mo	-	-	-	-
Baby14 t2				4.2	EBF	2 mo	-	-	-	-
Baby14 t3				5.2	EBF	3 mo	-	-	-	-
Baby14 t4				6.0	EBF	4 mo	-	-	-	-

APPENDIX III. Cont'd

Sample code	Sex	Gestational age	Mode of delivery	Weight (kg)	Feeding method	Age of baby	Antibiotic treatment	Herbal treatment	Complementary food	Sickness during the study
Baby14 t5				NA	EBF	5 mo	-	-	-	-
Baby14 t6				8.0	EBF	6 mo	-	-	-	-
Baby14 t7				8.0	BF/CF/SF	7 mo	-	-	Ogi /solid foods	-
Baby14 t8				10.0	BF/CF/SF	8 mo	-	-	Ogi /solid foods	-
Baby14 t9				11.0	BF/CF/SF	9 mo	Amoxyil	-	Ogi /solid foods	Fever
Baby15 t0	Male	30wk,6 d	VB	1.3	BF/FF	3 d	Cefotaxime, gentamicin	-	-	Premature, Presumed sepsis
Baby15 t1				1.4	BF	1 mo	Amikacin, IV cipro, IV flagyl	-	-	-
Baby15 t2				1.7	BF	2 mo	-	-	-	-
Baby15 t3				3.5	BF/FF	3 mo	-	-	-	-
Baby15 t4				5.5	BF	4 mo	-	-	-	-
Baby15 t5				NA	BF	5 mo	-	-	-	-
Baby15 t6				7.0	BF	6 mo	-	-	-	-
Baby15 t7				NA	BF/CF	7 mo	-	-	Ogi	-
Baby15 t8				7.5	BF/CF	8 mo	Amoxicillin	-	Ogi	Cough and catarrh
Baby15 t9				8.0	BF/CF/SF	5 mo	-	-	Ogi, Introduce solid foods	-

APPENDIX III. Cont'd

Sample code	Sex	Gestational age	Mode of delivery	Weight (kg)	Feeding method	Age of baby	Antibiotic treatment	Herbal treatment	Complementary food	Sickness during the study
Baby16 t0	Male	32 wk	CSB	1.9	BF	7 d	Cefepime, gentamicin, crystal penicillin	-	-	Respiratory distress and sepsis
Baby16 t1				1.75	EBF	1 mo	IV Ciprofloxacin	-	-	Pretermature baby
Baby16 t2				2.7	EBF	2 mo	-	-	-	-
Baby16 t3				3.5	EBF	3 mo	Zinnat	-	-	Bleeding due to circumcision
Baby16 t4				NA	EBF	4 mo	-	-	-	-
Baby16 t5				5.5	EBF	5 mo	-	Yes	-	Cough
Baby16 t6				5.5	BF/CF	6 mo	Amoxicillin	Yes	Ogi	-
Baby16 t7				6.3	BF/CF/SF	7 mo	-	Yes	Introduce solid food+Ogi	-
Baby16 t8				6.5	BF/CF/SF	8 mo	-	Yes	Ogi+solid foods	-
Baby16 t9				7.0	BF/CF/SF	9 mo	-	-	Ogi+solid foods	-
Baby17 t0	Female	40 wk 2 d	CSB	3.6	EBF	2 wk	-	-	-	-
Baby17 t1				3.6	EBF	1 mo	-	-	-	-
Baby17 t2				4.9	EBF	2 mo	-	-	-	-
Baby17 t3				5.5	EBF	3 mo	-	-	-	-

APPENDIX III. Cont'd

Sample code	Sex	Gestational age	Mode of delivery	Weight (kg)	Feeding method	Age of baby	Antibiotic treatment	Herbal treatment	Complementary food	Sickness during the study
Baby17 t4				6.5	EBF	4 mo	-	-	-	-
Baby17 t5				6.0	EBF	5 mo	Amoxycillin	-	-	Cough and Catarrh
Baby17 t6				6.0	BF//SF	6 mo		-	-	-
Baby17 t7				6.0	BF//SF	7 mo	-	-	-	-
Baby17 t8				7.0	BF/SF	8 mo	-	-	Introduce solid foods	-
Baby18 t0	Female	40 wk 2d	VB	2.54	BF	1 d	-	-	-	-
Baby18 t1				2.6	BF		Ampicillin	-	-	Cough and Catarrh
Baby18 t2					BF		-	-	-	-
Baby20 t0	Male	38 wks	VB	3.7	EBF	5 d	-	-	-	-
Baby20 t1				4.5	EBF	1 mo	-	-	-	-
Baby20 t2				6.0	EBF	2 mo	-	-	-	-
Baby20 t3				7.7	EBF	3 mo	Amoxicillin	-	-	Cough and Catarrh
Baby20 t4				7.7	EBF	4 mo	-	-	-	-
Baby20 t5				10.5	EBF	5 mo	-	-	-	Rashes
Baby20 t6				11.0	BF/CF/SF	6 mo	Amoxicillin	-	Ogi, Introduce solid foods	Cough
Baby20 t7					BF/CF/SF	7 mo	-	-	Ogi+solid foods	-
Baby20 t8				12.0	BF/CF/SF	8 mo	-	-	Ogi+solid foods	-
Baby21 t0	Female	40 wk	VB	2.2	EBF	2 wk	-	-	-	-

APPENDIX III. Cont'd

Sample code	Sex	Gestational age	Mode of delivery	Weight (kg)	Feeding method	Age of baby	Antibiotic treatment	Herbal treatment	Complementary food	Sickness during the study
Baby21 t1				2.8	EBF	1 mo	-	-	-	-
Baby21 t2				5.3	EBF	2 mo	-	Yes	-	-
Baby21 t3				6.0	EBF	3 mo	-	Yes	-	-
Baby21 t4					EBF	4 mo	-	Yes	-	-
Baby21 t5					EBF	5 mo	-	Yes	-	-
Baby21 t6				8.0	EBF	6 mo	-	-	-	-
Baby21 t7				7.0	BF/CF	7 mo	-	-	-	-
Baby22 t0	Female	39 wk	CSB	3.0	BF	22 d	IV cefotaxime, gentamicin	-	-	
Baby23 t0	Female	33 wk	VB	2.25	EBF	2 d	IV cefotaxime, gentamicin	-	-	Presumed sepsis
Baby23 t1				NA	EBF	-	-	-	-	-
Baby23 t2				NA	EBF	-	-	-	-	-
Baby23 t3				NA	EBF	-	-	-	-	-
Baby23 t4				NA	EBF	-	-	-	-	-
Baby23 t5				9.0	EBF	5 mo	-	-	-	-
Baby23 t6				9.0	EBF	6 mo	-	-	-	-
Baby23 t7				7.5	BF/CF/FF	7 mo	-	-	-	-
Baby23 t8				7.0	BF/CF/FF	8 mo	-	-	-	-
Baby24 t0	Female	40wk	VB	3.1	EBF	10 d	Cefixime	-	-	-
Baby24 t1				NA	EBF	1 mo	-	Yes	-	-

APPENDIX III. Cont'd

Sample code	Sex	Gestational age	Mode of delivery	Weight (kg)	Feeding method	Age of baby	Antibiotic treatment	Herbal treatment	Complementary food	Sickness during the study
Baby24 t2				5.0	EBF	2 mo	-	-	-	-
Baby24 t3				5.7	EBF	3 mo	-	-	-	-
Baby24 t4				6.3	EBF	4 mo	-	-	-	-
Baby24 t5				7.5	EBF	5 mo	-	-	-	-
Baby24 t6				7.0	BF/CF/SF	6 mo	Amoxicillin	-	-	Cough/fever
Baby24 t7				7.8	BF/CF/SF	7 mo	-	-	-	-
Baby25 t1	Male	34wk	VB	3.4	EBF	17 d	-	-	-	-
Baby25 t5				7.0	EBF	5 mo	-	-	-	-
Baby25 t7				8.5	BF/CF/FF	7 mo	Amoxicillin	-	-	Cough
Baby25 t8				9.0	BF/CF/FF	8 mo	-	-	-	-
Baby26 t0	Female	37wk	VB	3.5	BF	1 d	-	-	-	-
Baby26 t1				NA	EBF	1 mo	-	-	-	-
Baby26 t2				NA	EBF	2 mo	-	-	-	-
Baby26 t3				NA	EBF	3 mo	-	-	-	-
Baby26 t4				9.0	EBF	4 mo	-	-	-	-
Baby26 t5				9.0	EBF	5 mo	-	-	-	-
Baby26 t6				8.0	BF/CF/FF	6 mo	Amoxicillin	-	-	Fortified ogi
Baby26 t7				9.0	BF/CF/FF	7 mo	-	-	-	Fortified ogi
Baby27 t0	Female	37 wk	CSB	2.5	BF/FF	17 d	-	-	-	-
Baby27 t1				3.0	BF/FF	1 mo	-	-	-	-
Baby27 t2				4.0	BF/FF	2 mo	-	-	-	-
Baby27 t3				6.0	BF/FF	3 mo	-	-	-	-

APPENDIX III. Cont'd

Sample code	Sex	Gestational age	Mode of delivery	Weight (kg)	Feeding method	Age of baby	Antibiotic treatment	Herbal treatment	Complementary food	Sickness during the study
Baby27 t4				6.2	BF/CF/FF	4 mo	-	-	Sorghum+ millet+ soyabean	-
Baby27 t5				7.0	BF/CF/FF	5 mo	Augumentin	-	Sorghum+ millet+ soyabean	Cough
Baby27 t6				8.0	BF/CF/FF	6 mo	-	-	Sorghum+ millet+ soyabean	-
Baby27 t7				8.5	BF/CF/FF	7 mo	Ampliclox	-	Sorghum+ millet+ soyabean	Catarrh/fever
Baby28 t0	Male	37 wk	CSB	2.55	BF/FF	15 d	-	-	-	-
Baby28 t1				4.0	BF/FF	1 mo	-	-	-	-
Baby28 t2				6.2	BF/FF	2 mo	-	-	-	-
Baby28 t3				4.2	BF/FF	3 mo	-	-	-	-
Baby28 t4				6.2	BF/CF/FF	4 mo	-	-	-	-
Baby28 t5				7.0	BF/CF/FF	5 mo	Augumentin	-	Sorghum+ millet+ soyabean	Cough
Baby28 t6				8.5	BF/CF/FF	6 mo	-	-	-	-
Baby28 t7				9.8	BF/CF/FF	7 mo	Ampliclox	-	-	Catarrh and fever
Baby30 t0	Female	34 wk	VB	2.9	EBF	1 d	-	-	-	-

*VB – Vaginal birth, CS – Caesarean section birth,EBF – Exclusively breastfed, FF – Formula fed, CF – Complementary food, wk – week, mo –month, d –day, t –time point

APPENDIX IV

Gradient calculation for DGGE

100% denaturant stock solution required (Y) was calculated as follows.

For the low % solution required:

$$Y = \frac{X}{100} \; x \; 11.5ml$$

(e.g. 35% for low) $Y = \frac{35}{100} \; x \; 11.5ml$

= 4.025 ~ 4ml

= 4 ml of 100%

0% denaturant stock solution required was calculated as follows:

Z = 11.5 ml – Y

Z = 11.5 ml – 4 ml = 7.5 ml

Z = 7.5 ml

For the high % solution required:

$$Y = \frac{X}{100} \; x \; 11.5ml$$

(e.g. 60%) Y = 60/100 × 11.5 ml

Y = 6.9 ml

0% denaturant stock solution required was calculated as follows:

Z = 11.5 ml - Y

Z = 11.5 ml – 6.9 ml = 4.6 ml

Z = 4.6 ml

From the concentration calculation above, the resultant denaturant stock solution was used for low and high percentage solution:

Low concentration:

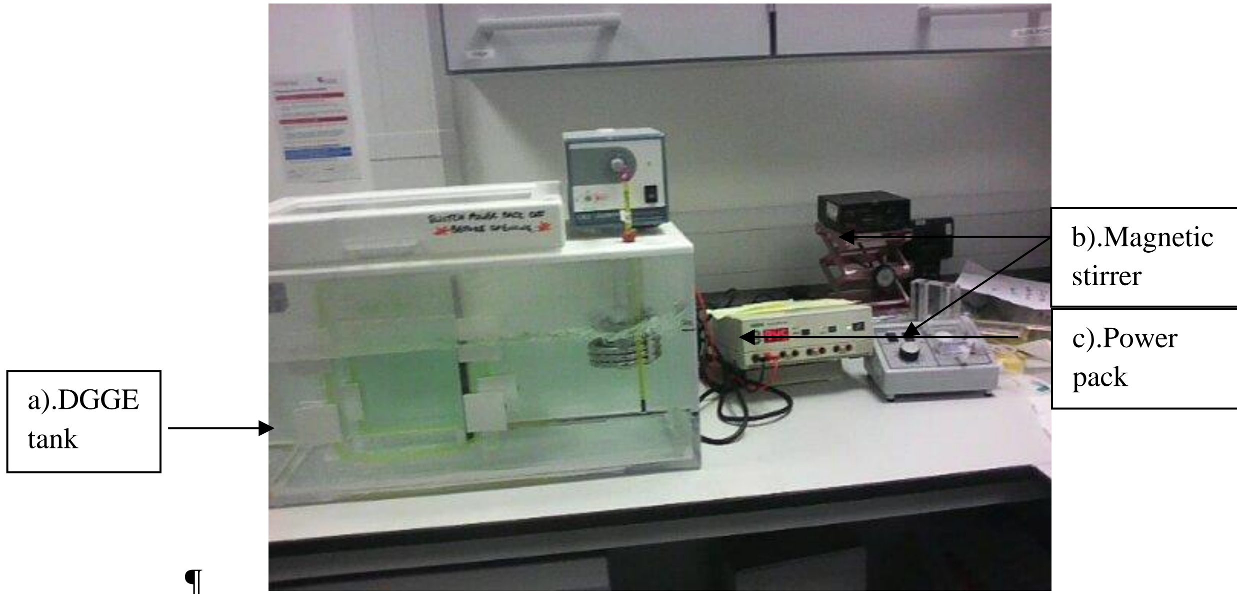
100 % stock - 4 ml and 0% stock -7.5 ml

High concentration:

100% stock – 6.9 ml and 0% stock -4.6 ml

APPENDIX V

DGGE set up



APPENDIX VI

Sequence read statistics

Sample ID	Number of raw reads	Number filtered reads
Baby1 t0	13548	11147
Baby1 t1	15642	12810
Baby1 t2	13599	11200
Baby1 t3	18792	15691
Baby1 t4	18323	15107
Baby1 t5	17999	14829
Baby1 t6	16217	13264
Baby1 t7	17762	14372
Baby1 t8	3251	2599
Baby1 t9	18817	15003
Baby1 t10	22057	17738
Baby1 t11	17829	14554
Baby1 t12	20315	16342
Baby2 t0	10318	8623
Baby2 t1	11094	9191
Baby2 t4	8749	7131
Baby3 t0	16488	13813
Baby3 t1	19291	15733
Baby3 t2	10428	8861
Baby3 t5	18206	15119
Baby3t7	14188	11737
Baby3t8	12322	10179

APPENDIX VI: Cont'd

Sample ID	Number of raw reads	Number filtered reads
Baby3 t9	19306	16274
Baby3 t10	17708	14654
Baby4 t0	17513	14894
Baby4 t2	16170	13260
Baby4 t3	17074	13622
Baby4 t7	15495	12868
Baby4 t8	22803	19002
Baby4 t9	18744	15551
Baby4 t10	17451	14472
Baby5 t0	17476	14301
Baby5 t1	25824	21437
Baby5 t2	9764	7978
Baby5 t5	20696	17066
Baby5 t6	19170	16010
Baby5 t7	16671	14009
Baby5 t8	13612	11389
Baby5 t9	18198	14929
Baby5 t10	11202	9367
Baby6 t1	18131	15381
Baby6 t8	15088	12210
Baby6 t9	8970	7281
Baby7 t0	17931	14920
Baby7 t2	15975	12971

APPENDIX VI: Cont'd

Sample ID	Number of raw reads	Number filtered reads
Baby7 t3	22074	17647
Baby7 t5	18974	15707
Baby7 t7	9212	7632
Baby7 t8	22237	17883
Baby7 t9	16479	13160
Baby8 t0	20959	17443
Baby8 t1	12220	9883
Baby8 t2	15436	12534
Baby8 t3	16171	13227
Baby8 t4	17447	14485
Baby8 t5	16676	13747
Baby8 t6	24329	20119
Baby8 t7	18476	15103
Baby8 t8	16840	13926
Baby8 t9	15941	12833
Baby8 t10	14458	12060
Baby9t0	10653	8780
Baby9 t1	7259	5743
Baby9 t3	13398	11196
Baby9 t5	15473	12811
Baby9 t8	15816	13164
Baby9 t9	17591	14431

APPENDIX VI: Cont'd

Sample ID	Number of raw reads	Number filtered reads
Baby10 t0	21492	17683
Baby10 t1	20978	17216
Baby10 t2	13677	11123
Baby10 t3	17874	14513
Baby10 t5	14655	12018
Baby10 t6	11981	9987
Baby10 t7	15995	13028
Baby10 t8	13747	11137
Baby10 t9	17358	14234
Baby10 t10	19317	15718
Baby11 t0	14438	11768
Baby11 t1	19432	14387
Baby11 t2	12578	15948
Baby11 t3	8800	10152
Baby11t5	16818	7371
Baby11 t6	16384	13874
Baby11 t7	15019	13545
Baby11 t8	13730	12354
Baby11 t10	14703	13850
Baby12 t0	17843	12286
Baby13 t0	15894	14698
Baby14 t0	15748	13161
Baby14 t5	11961	13172

APPENDIX VI: Cont'd

Sample ID	Number of raw reads	Number filtered reads
Baby14 t7	16995	9933
Baby14 t8	9598	13746
Baby14 t9	9045	7832
Baby15 t0	16480	7525
Baby15 t1	14030	13261
Baby15 t2	16812	11624
Baby15 t3	11928	13748
Baby15 t6	18500	9830
Baby15 t8	21025	15029
Baby16 t0	9298	4681
Baby16t1	15379	7443
Baby16t2	15864	12857
Baby16 t7	14526	12619
Baby16 t8	15894	11603
Baby16 t9	13964	12698
Baby17 t0	17618	11506
Baby17 t1	16914	14525
Baby17 t5	11887	14614
Baby17 t6	11893	9894
Baby17 t7	19045	9611
Baby17 t8	4753	15680
Baby18 t0	12459	4069
Baby20 t1	17404	13200

APPENDIX VI: Cont'd

Sample ID	Number of raw reads	Number filtered reads
Baby20 t3	17774	14324
Baby20 t5	8891	14626
Baby20 t6	15364	7343
Baby20 t8	4085	12651
Baby21 t0	13944	3315
Baby21 t1	15684	10527
Baby21t2	7847	12617
Baby21t3	8224	6318
Baby21 t4	16137	6734
Baby21 t5	11434	13114
Baby21 t6	8066	9291
Baby21 t7	17659	6624
Baby22 t0	7149	14922
Baby23 t0	19209	5747
Baby23 t5	16011	15663
Baby23 t7	17081	13218
Baby23 t8	12616	14159
Baby24 t0	16765	10420
Baby24 t1	17407	13763
Baby24 t2	21065	14184
Baby24 t4	18195	16617
Baby24 t5	13123	14471
Baby24 t6	9323	10777
Baby24 t7	11029	7452

APPENDIX VI: Cont'd

Sample ID	Number of raw reads	Number filtered reads
Baby25 t0	20344	8984
Baby25 t5	16919	17125
Baby25t7	12887	13913
Baby25t8	6587	10559
Baby26 t0	9849	5439
Baby26 t1	14831	8020
Baby26 t4	6306	12254
Baby26 t5	14293	5211
Baby26 t6	11360	11604
Baby26 t7	17003	9377
Baby27 t0	13876	13615
Baby27 t1	13774	11571
Baby27 t2	13512	11316
Baby27 t5	9829	16657
Baby27 t6	26693	7848
Baby27 t7	13553	21478
Baby28 t0	14142	10940
Baby28 t1	5867	11625
Baby28 t2	23753	4834
Baby28 t4	16953	19138
Baby28 t5	16126	13685
Baby28 t6	16753	12725
Baby28t7	16566	13782
Baby30 t0	13548	13308

APPENDIX VII

Comparison of feeding groups with LefSe

Table VIIa. Significant biomarkers identified by LEfSe between exclusive breast fed and mixed fed samples

Taxa	Sample	LDA	P value
Bacteroidetes	EBF	4.81	0.023
Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Corynebacteriaceae	MF	4.44	0.046
Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae	MF	5.32	0.00013
Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *Bifidobacterium*	MF	5.33	5.20e-05
Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *Bifidobacterium bifidum*	MF	4.80	0.0030
Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Corynebacteriaceae; *Corynebacterium kroppenstedtii*	MF	4.07	0.023
Firmicutes	EBF	5.07	0.022
Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales	MF	5.33	0.00013
Bacteria; Actinobacteria; Actinobacteria	MF	5.33	0.00034
Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *BifidobacteriumNA*	MF	5.06	0.00038
Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Corynebacteriaceae; *Corynebacterium*	MF	4.35	0.046
Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales	EBF	4.80	0.023
Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *Bifidobacterium animalis*	MF	5.06	2.94e-05
Bacteria; Bacteroidetes; Bacteroidia	EBF	4.80	0.023
Bacteria; Actinobacteria	MF	5.30	0.0014

*EBF - Exclusive breast fed, MF – Mixed fed (LDA > 2)

Table VIIb. Significant biomarkers identified by LEfSe between exclusive breast fed and local food/solid food samples.

Taxa	Sample	LDA	P value
Bacteria; Firmicutes; Bacilli; Bacillales; Staphylococcaceae; *Staphylococcus* NA	EBF	4.39	0.028
Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; *Streptococcus*	EBF	4.32	0.028
Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae	LS.SF	4.52	0.022
Bacteria; Proteobacteria	LF.SF	4.35	0.030
Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; *Enterococcus casseliflavus*	LF.SF	4.49	0.028
Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	LF.SF	4.47	0.0040
Bacteria; Firmicutes; Bacilli; Bacillales; Staphylococcaceae; *Staphylococcus*	EBF	4.55	0.014
Bacteria; Firmicutes; Clostridia; Clostridiales; Clostridiaceae	LF.SF	3.99	0.034
Bacteria; Firmicutes; Bacilli; Bacillales; Staphylococcaceae	EBF	4.56	0.014
Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *Bifidobacterium* NA	LF.SF	4.26	0.022
Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales	LF.SF	4.47	0.022
Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae	LF.SF	4.59	0.016
Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; *Pediococcus*	LF.SF	4.20	0.022

TableVIIb. Cont'd

Taxa	Sample	LDA	P value
Bacteria; Firmicutes; Clostridia; Clostridiales	LF.SF	4.45	0.011
Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; *Pediococcus* NA	LF.SF	3.94	0.037
Bacteria; Firmicutes; Bacilli; Lactobacillales	LF.SF	4.83	0.033
Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae	LF.SF	4.46	0.022
Bacteria; Firmicutes; Clostridia	LF.SF	4.43	0.011
Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; *Enterococcus*	LF.SF	4.62	0.016

EBF – Exclusive breast fed, LF.SF –Local food/solid food fed, (LDA > 2)

Table VIIc. Significant biomarkers identified by LEfSe analysis between mixed fed and local food/solid food samples

Taxa	Sample	LDA	P value
Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; *Bacteroides*	LF.SF	4.66	0.030
Bacteria; Bacteroidetes	LF.SF	4.74	0.034
Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; *Lactobacillus*	LF.SF	4.42	0.020
Bacteria; Firmicutes; Bacilli; Bacillales; Staphylococcaceae; *Staphylococcus* NA	MF	4.29	0.013
Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae	MF	5.42	1.02e-06
Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; *Streptococcus*	MF	4.73	0.045
Bacteria; Actinobacteria; Actinobacteria; Actinomycetales	MF	4.47	0.034
Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *Bifidobacterium*	MF	5.45	1.02e-06
Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *Bifidobacteriumbifidum*	MF	4.79	0.0069
Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae	LF.SF	4.62	0.014
Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae	LF.SF	4.67	0.030
Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Corynebacteriaceae; *Corynebacterium kroppenstedtii*	MF	4.40	0.013
Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; *Enterococcus casseliflavus*	LF.SF	4.70	0.025

MF- Mixed fed (breast fed and formula fed), LF.SF – Local food/solid food fed, (LDA > 2)

Table VIIc. Cont'd

Taxa	Sample	LDA	P value
Bacteria; Firmicutes	LF.SF	5.22	8.16e-05
Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	LF.SF	4.55	0.0011
Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales	MF	5.41	1.02e-06
Bacteria; Actinobacteria; Actinobacteria	MF	5.41	1.84e-06
Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroidescaccae	LF.SF	4.57	0.0099
Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales	LF.SF	4.77	0.034
Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae	LF.SF	4.82	0.017
Bacteria; Firmicutes; Bacilli	LF.SF	5.01	0.0064
Bacteria; Firmicutes; Clostridia; Clostridiales	LF.SF	4.89	0.00027
Bacteria; Firmicutes; Bacilli; Lactobacillales	LF.SF	4.98	0.0060
Bacteria; Firmicutes; Clostridia	LF.SF	4.89	0.00027
Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; *Enterococcus*	LF.SF	4.81	0.016
Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; *Bifidobacterium animalis*	MF	5.02	0.00011
Bacteria; Bacteroidetes; Bacteroidia	LF.SF	4.74	0.034
Bacteria; Actinobacteria	MF	5.36	9.72e-06

*MF- Mixed fed (breast fed and formula fed), LF.SF – Local food/solid food fed, (LDA > 2)

Table VIId. Significant biomarkers identified by LEfSe between preweaning and weaning

Taxa	Sample	LDA	P value
Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; *Roseburia*	Weaning	3.87	0.043
Bacteria; Firmicutes	Weaning	5.02	0.0019
Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; *Blautia*	Weaning	3.84	0.043
Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae	Weaning	4.53	6.15e-05
Bacteria; Firmicutes; Bacilli; Bacillales; Staphylococcaceae; *Staphylococcus*	Pre-wean	4.43	0.020
Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales	Pre-wean	5.01	0.0035
Bacteria; Firmicutes; Clostridia; Clostridiales; Clostridiaceae	Weaning	4.02	0.014
Bacteria; Firmicutes; Bacilli; Bacillales; Staphylococcaceae	Pre-wean	4.46	0.020
Bacteria; Proteobacteria; Deltaproteobacteria	Weaning	4.17	0.043
Bacteria; Actinobacteria; Actinobacteria	Pre-wean	5.02	0.0043
Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroidescaccae	Weaning	4.34	0.0072
Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales	Weaning	3.98	0.020
Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae	Weaning	4.69	0.0027

Table VIId. Cont'd

Taxa	Sample	LDA	P value
Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; *Pediococcus*	Weaning	4.25	0.0039
Bacteria; Firmicutes; Bacilli	Weaning	4.77	0.040
Bacteria; Firmicutes; Clostridia; Clostridiales	Weaning	4.68	0.00021
Bacteria; Proteobacteria; Deltaproteobacteria; Desulfovibrionales; Desulfovibrionaceae	Weaning	4.08	0.043
Bacteria; Firmicutes; Clostridia	Weaning	4.67	0.00021
Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; *Cronobacter*	Weaning	4.09	0.019
Bacteria; Firmicutes; Bacilli; Bacillales; Planococcaceae; *Sporosarcina*	Weaning	4.50	0.043
Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; *Enterococcus*	Weaning	4.69	0.0026
Bacteria; Firmicutes; Bacilli; Bacillales; Planococcaceae; Sporosarcina NA	Weaning	4.40	0.043
Bacteria; Actinobacteria	Pre-wean	5.01	0.0056

APPENDIX VIII

Presence or absence of antibiotic resistance genes in infants gut

Faecal samples	Antimicrobial Resistance Genes					
	RPP (*Tet*)	***Erm* A**	***Erm* B**	***Aac***	***Bla*Z**	***Mef***
Baby1 t0	-	-	-	-	-	-
Baby1 t1	+	-	-	+	+	+
Baby1 t2	+	-	-	+	+	-
Baby1 t3	+	-	-	-	+	-
Baby1 t4	+	-	-	-	+	+
Baby1 t5	+	-	-	-	-	+
Baby1 t6	+	-	+	+	+	+
Baby1 t7	+	-	+	+	-	+
Baby1 t8	-	-	-	-	-	-
Baby1 t9	+	-	-	-	-	+
Baby1 t10	+	-	+	+	-	+
Baby1 t11	+	-	+	+	-	+
Baby1 t12	+	-	+	-	-	+
Baby2 t0	-	-	-	-	-	+
Baby2 t1	+	-	-	-	-	+
Baby2 t4	-	-	-	-	-	-
Baby3 t0	-	-	+	+	+	+
Baby3 t1	+	+	-	+	+	+
Baby3 t2	-	+	+	+	+	+
Baby3 t3	+	+	+	+	+	+
Baby3 t5	+	-	+	-	-	+
Baby3 t7	+	-	-	-	-	+
Baby3t8	+	-	+	-	-	+
Baby3 t9	+	-	-	-	-	+
Baby3 t10	+	-	-	-	-	+
Baby4 t0	-	-	-	-	-	+
Baby4 t2	+	-	+	+	+	+
Baby4 t3	+	-	+	-	+	+
Baby4 t7	+	-	+	-	-	+
Baby4 t8	+	-	-	-	-	+
Baby4 t9	+	-	-	+	+	+
Baby4 t10	+	-	+	-	-	+
Baby5 t0	-	-	+	+	+	-

Appendix VIII: Cont'd

	Antimicrobial Resistance Genes					
Faecal samples	**RPP (*Tet*)**	***Erm* A**	***Erm* B**	***Aac***	***BlaZ***	***Mef***
Baby10 t0	-	-	+	-	-	+
Baby10 t1	-	-	-	-	-	-
Baby10 t2	-	-	-	-	-	+
Baby10 t3	-	-	+	-	-	+
Baby10 t5	-	-	+	-	-	-
Baby10 t6	+	-	-	-	-	+
Baby10 t7	+	-	+	+	-	+
Baby10 t8	-	-	+	-	-	-
Baby10 t9	-	-	+	-	-	-
Baby10 t10	-	-	-	-	-	-
Baby11 t0	+	-	-	-	-	-
Baby11 t1	-	-	-	+	-	-
sBaby11 t2	-	-	+	+	-	-
Baby11 t3	-	-	-	-	-	-
Baby11 t5	-	-	+	-	-	-
Baby11 t6	-	-	+	-	-	-
Baby11 t7	+	-	+	-	-	-
Baby11 t8	+	-	+	-	-	+
Baby11 t9	+	-	+	-	-	-
Baby11 t10	+	-	+	-	-	-
Baby12 t0	+	-	+	-	-	+
Baby13 t0	-	-	+	+	-	-
Baby14 t0	-	-	+	-	-	-
Baby14 t5	+	-	-	-	-	-
Baby14 t7	-	+	+	-	-	+
Baby14 t8	-	-	+	-	-	+
Baby14 t9	-	-	-	+	-	+
Baby15 t0	-	-	+	-	-	-
Baby15 t1	-	-	+	-	-	-
Baby15 t2	-	-	+	-	-	-
Baby15 t3	-	-	-	-	-	-
Baby15 t6	-	+	-	-	-	-
Baby15 t8	-	-	-	-	-	-

Appendix VIII. Cont'd

	Antimicrobial Resistance Genes					
Faecal samples	**RPP (*Tet*)**	***Erm* A**	***Erm* B**	***Aac***	***BlaZ***	***Mef***
Baby16 t0	-	-	-	-	-	+
Baby16 t1	-	-	-	+	-	+
Baby16 t2	+	-	-	-	-	+
Baby16 t7	+	-	+	-	-	+
Baby16 t8	-	-	-	-	-	-
Baby16 t9	-	-	+	-	+	+
Baby17 t0	+	-	-	+	+	+
Baby17 t1	+	-	+	+	-	-
Baby17 t3	+	-	+	+	-	-
Baby17 t5	-	-	+	+	+	-
Baby17 t6	+	-	+	+	-	-
Baby17 t7	-	-	-	-	-	-
Baby17 t8	+	-	-	+	+	+
Baby18 t0	-	-	-	+	+	-
Baby20 t0	+	-	-	+	+	-
Baby20 t1	+	-	-	-	-	+
Baby20 t3	-	-	+	-	-	-
Baby20 t5	+	-	+	-	-	+
Baby20 t6	-	-	+	+	+	-
Baby20 t8	+	-	+	+	-	+
Baby21 t0	-	-	-	-	-	-
Baby21 t1	-	-	-	-	-	-
Baby21 t2	-	-	-	-	-	-
Baby21 t3	-	-	-	-	-	-
Baby21 t4	-	-	+	-	-	-
Baby21 t5	-	-	-	-	+	+
Baby21 t6	-	-	-	+	+	+
Baby21 t7	-	-	-	+	+	+
Baby22 t0	-	-	+	-	-	+
Baby23 t0	-	-	-	-	+	-
Baby23 t5	+	+	+	-	-	+
Baby23 t6	+	+	-	-	-	+
Baby23 t7	+	+	-	-	-	+

Appendix VIII. Cont'd

	Antimicrobial Resistance Genes					
Faecal samples	**RPP (*Tet*)**	***Erm* A**	***Erm* B**	***Aac***	***Bla*Z**	***Mef***
Baby23 t8	+	+	-	+	+	+
Baby24 t0	-	-	-	+	+	-
Baby24 t1	-	-	+	-	-	-
Baby24 t2	+	-	-	-	-	+
Baby24 t4	+	-	-	-	-	-
Baby24 t5	-	-	-	-	-	+
Baby24 t6	+	-	+	-	-	+
Baby24 t7	-	-	+	+	+	-
Baby25 t0	+	-	-	-	-	+
Baby25 t5	+	-	+	+	-	+
Baby25 t7	-	-	+	+	-	-
Baby25 t8	-	-	+	+	-	-
Baby26 t0	+	-	+	+	-	+
Baby26 t1	+	-	+	+	-	+
Baby26 t4	+	-	+	-	-	+
Baby26 t5	+	-	+	-	-	-
Baby26 t6	+	-	-	+	-	-
Baby26 t7	+	-	+	+	-	-
Baby27 t0	+	-	+	+	-	-
Baby27 t1	+	-	-	-	-	+
Baby27 t2	+	-	-	-	-	-
Baby27 t4	+	-	+	-	-	+
Baby27 t5	+	-	+	-	-	+
Baby27 t6	-	-	+	-	-	-
Baby27 t7	-	-	-	-	-	-
Baby28 t0	-	-	-	-	-	-
Baby28 t1	-	-	-	-	-	-
Baby28 t2	-	-	+	-	-	-
Baby28 t4	-	-	-	-	-	-
Baby28 t5	-	-	-	-	-	-
Baby28 t6	-	-	-	-	-	-
Baby28 t7	-	-	-	-	-	-
Baby30 t0	-	-	-	-	-	-

APPENDIX IX

The short-chain fatty acids present in all the infants

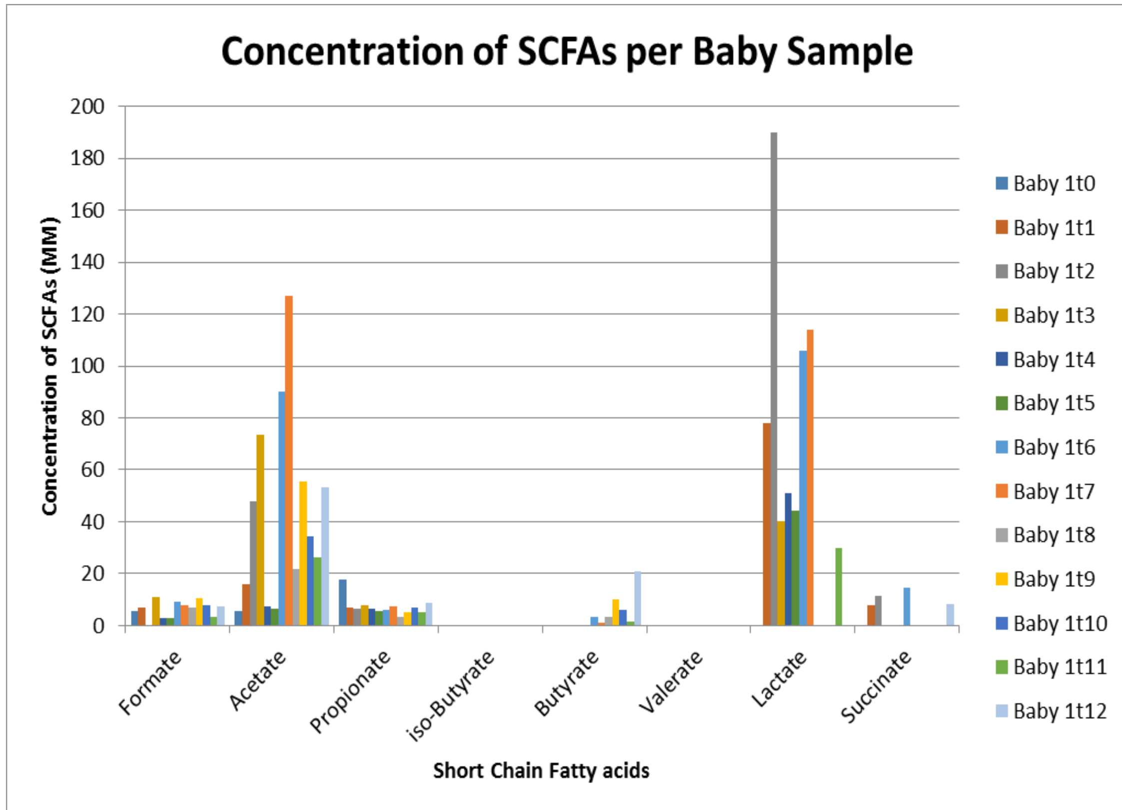
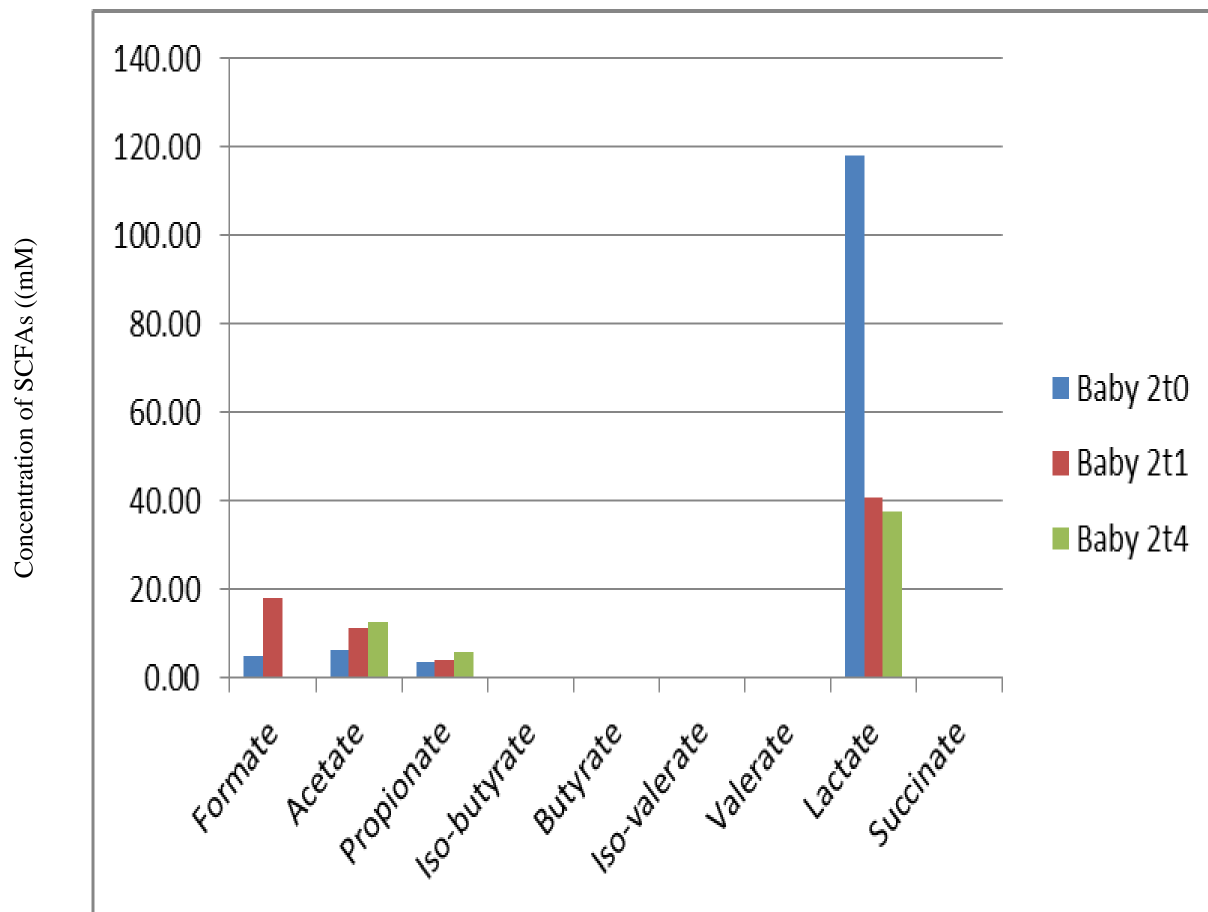


Figure VIII(1). Presence of Short Chain Fatty Acids in Baby 1 Sample



Short chain fatty acids

Figure VIII(2). Presence of SCFAs in Baby 2 Sample

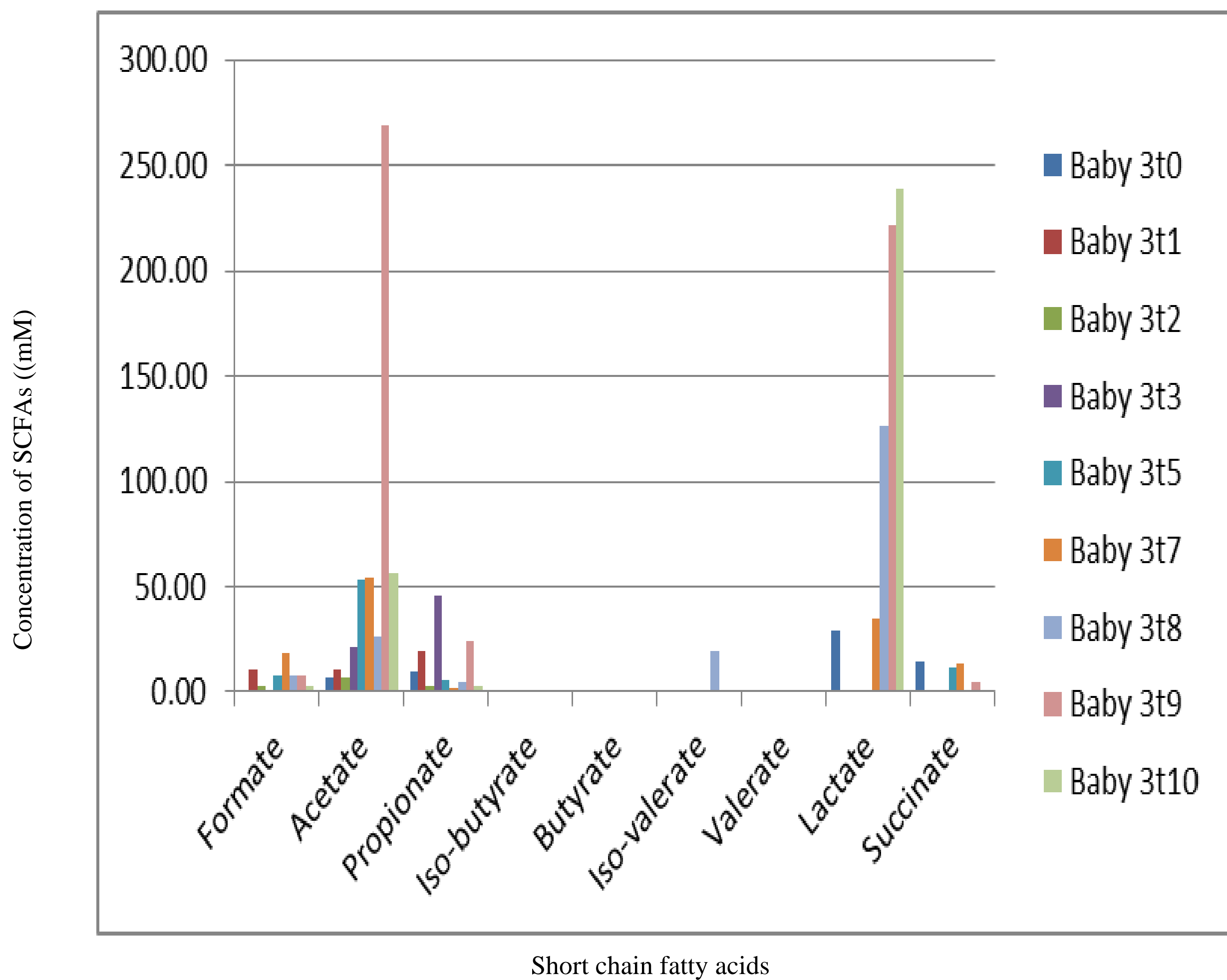


Figure VIII(3). Presence of SCFAs in Baby 3 Sample

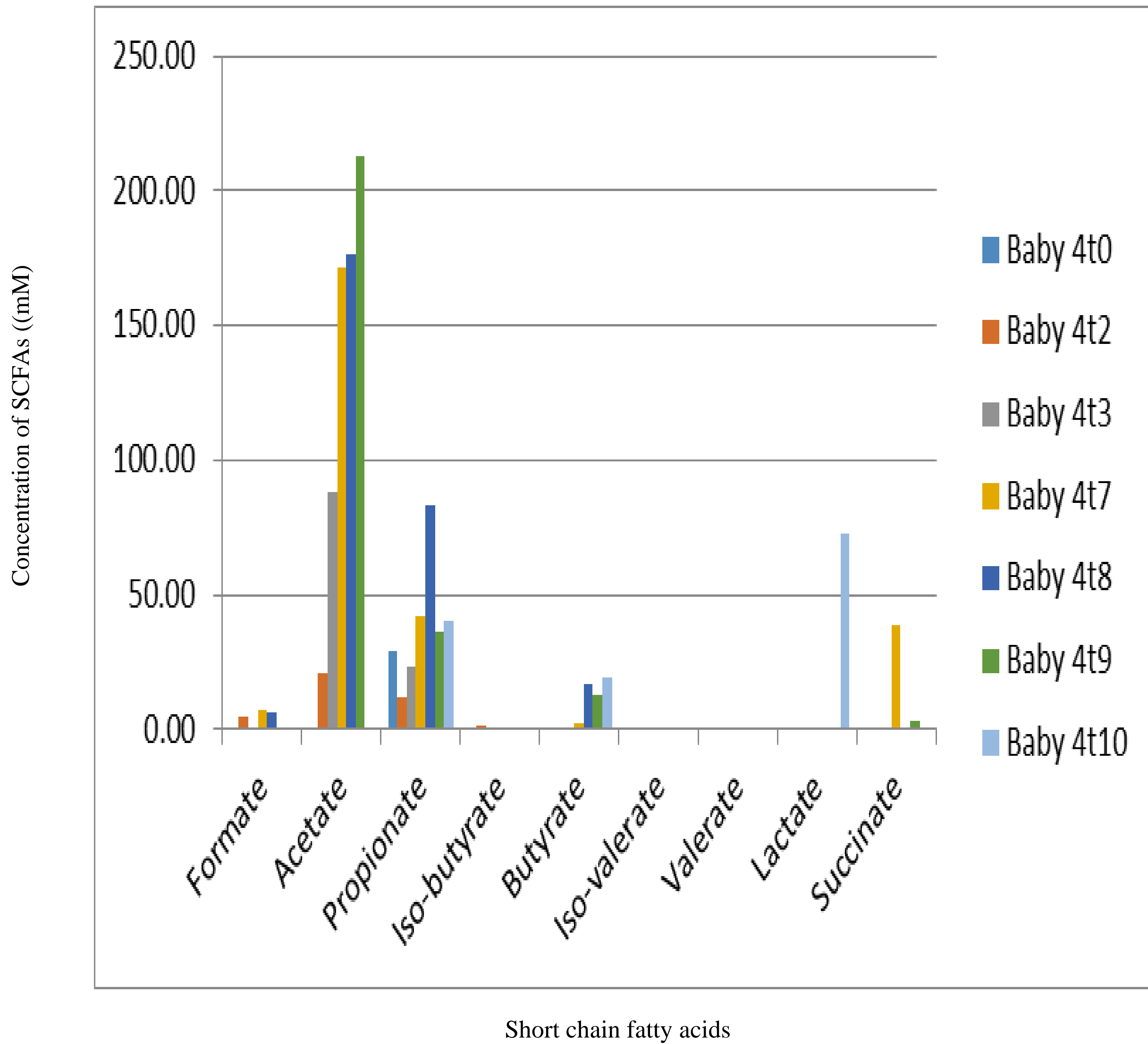


Figure VIII(4). Presence of SCFAs in Baby 4 Sample

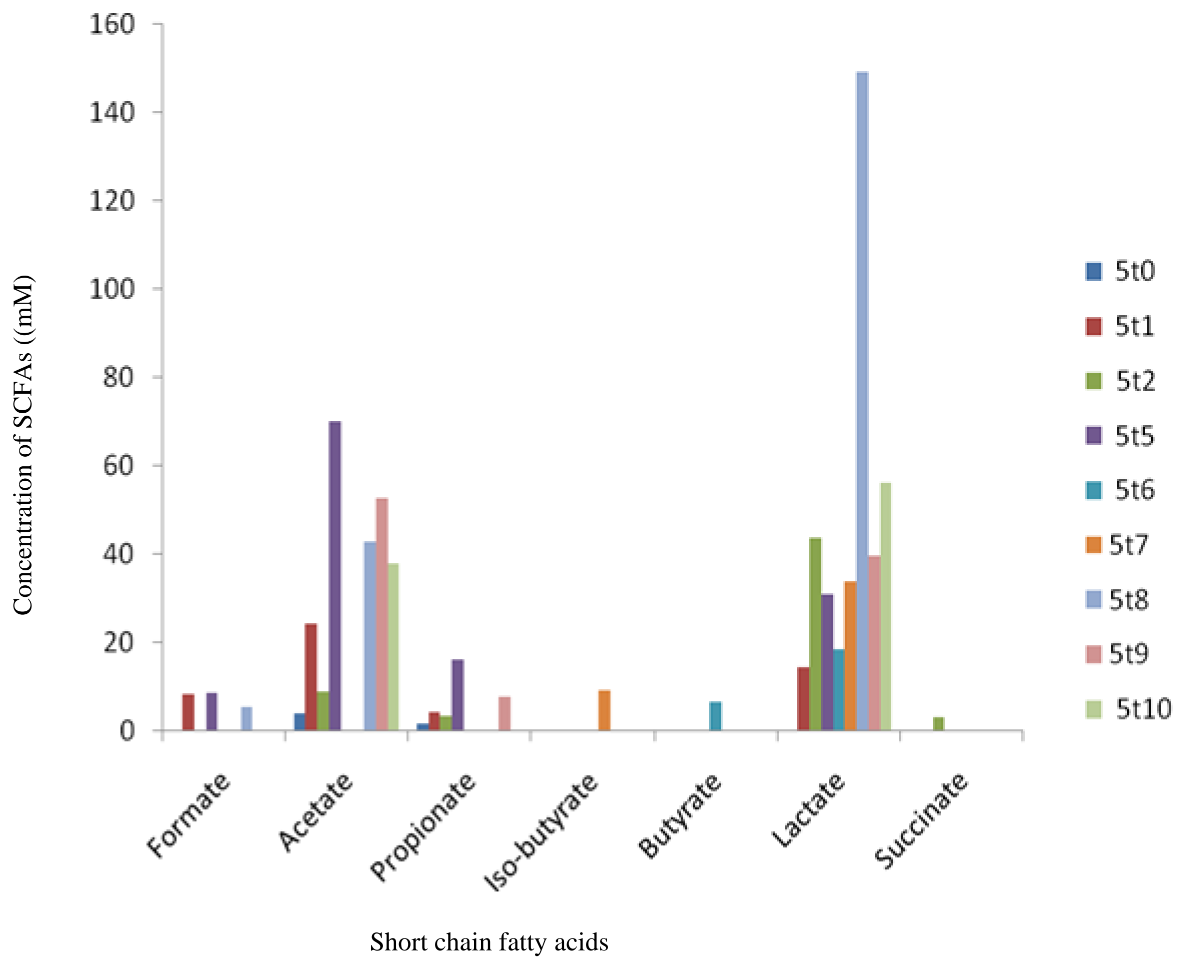


Figure VIII(5). Presence of SCFAs in Baby 5 Sample

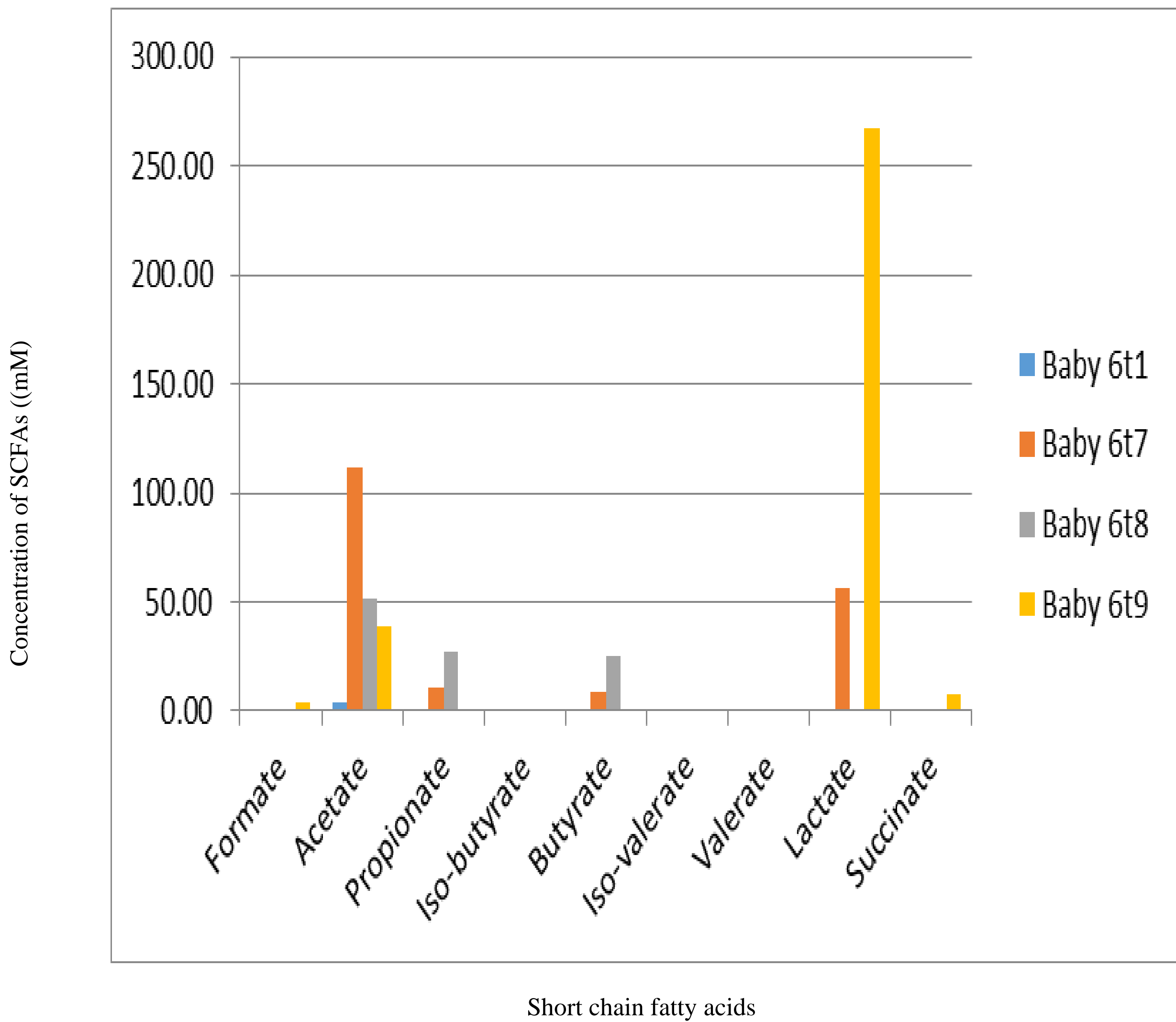


Figure VIII(6). Presence of SCFAs in Baby 6 Sample

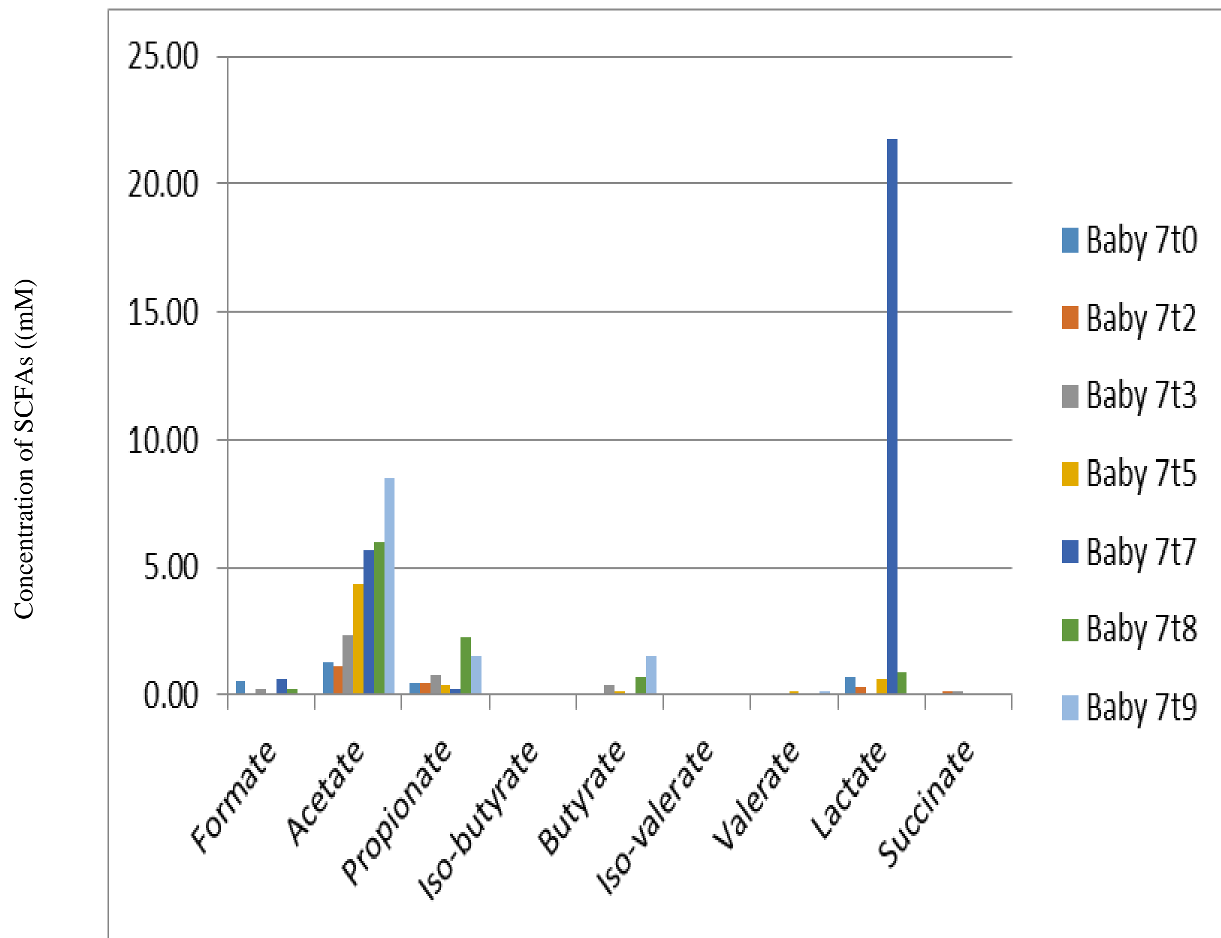


Figure VIII(7). Presence of SCFAs in Baby 7 Sample

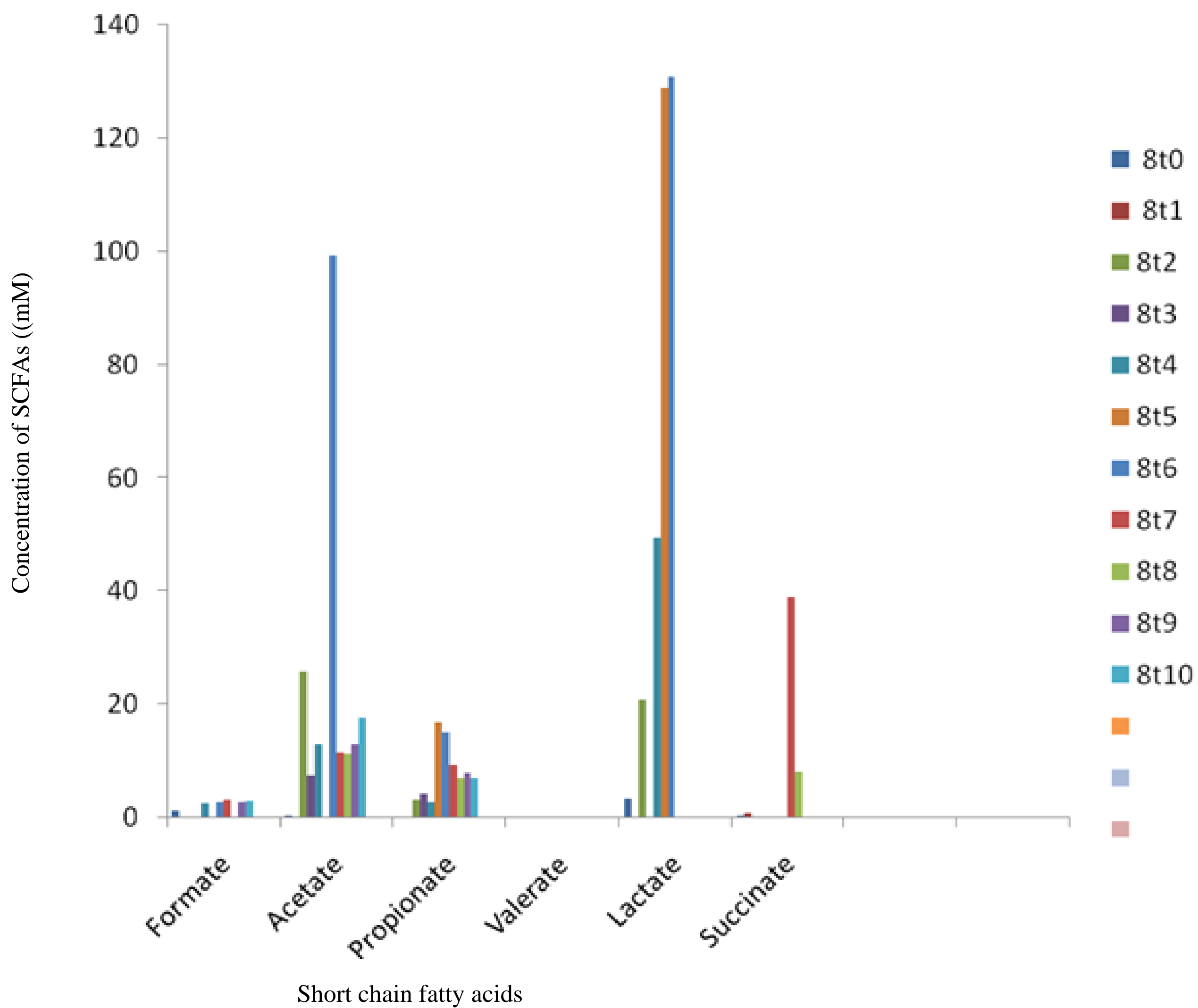


Figure VIII(8). Presence of SCFAs in Baby 8 Sample

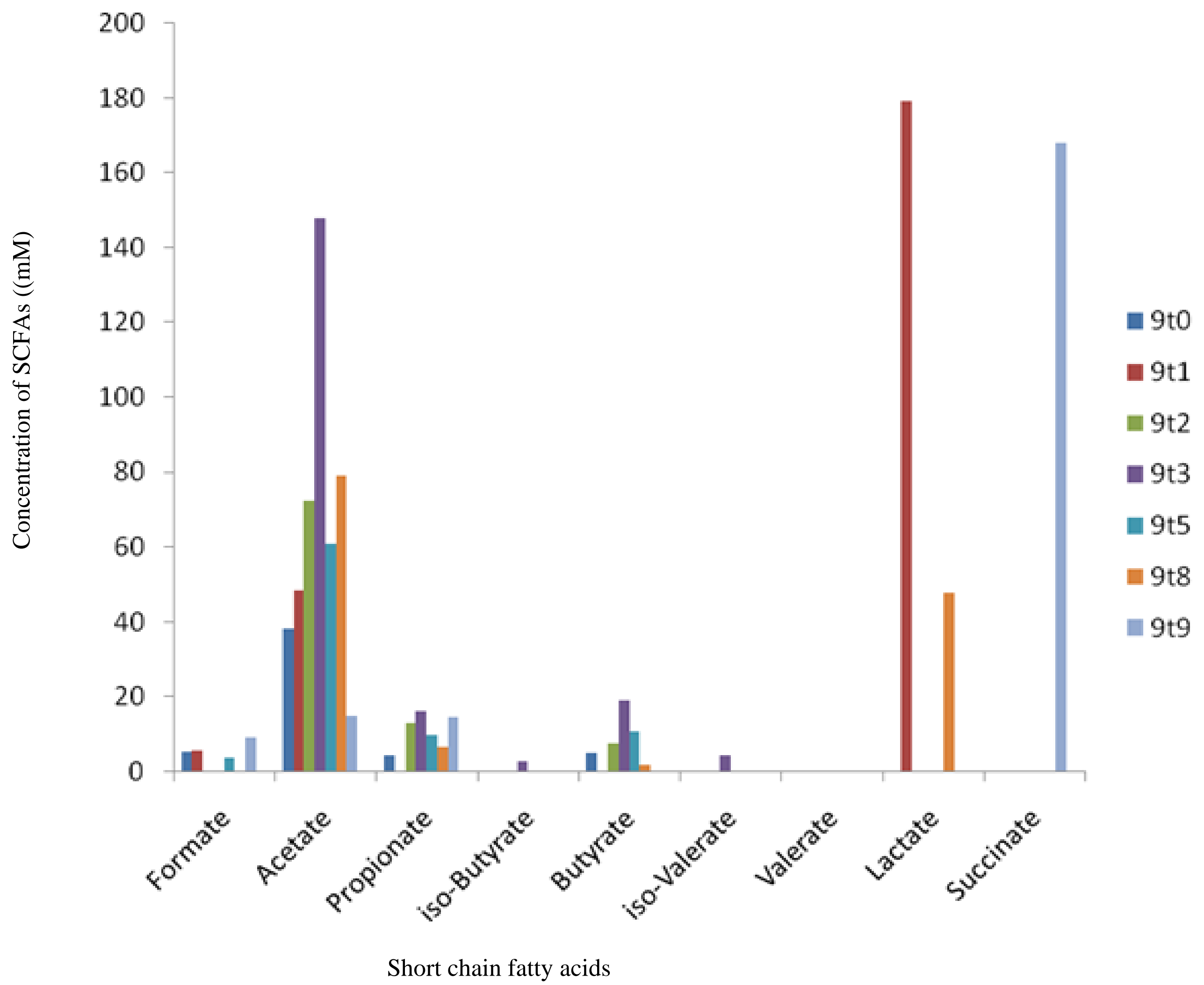


Figure VIII(9). Presence of SCFAs in Baby 9 Sample

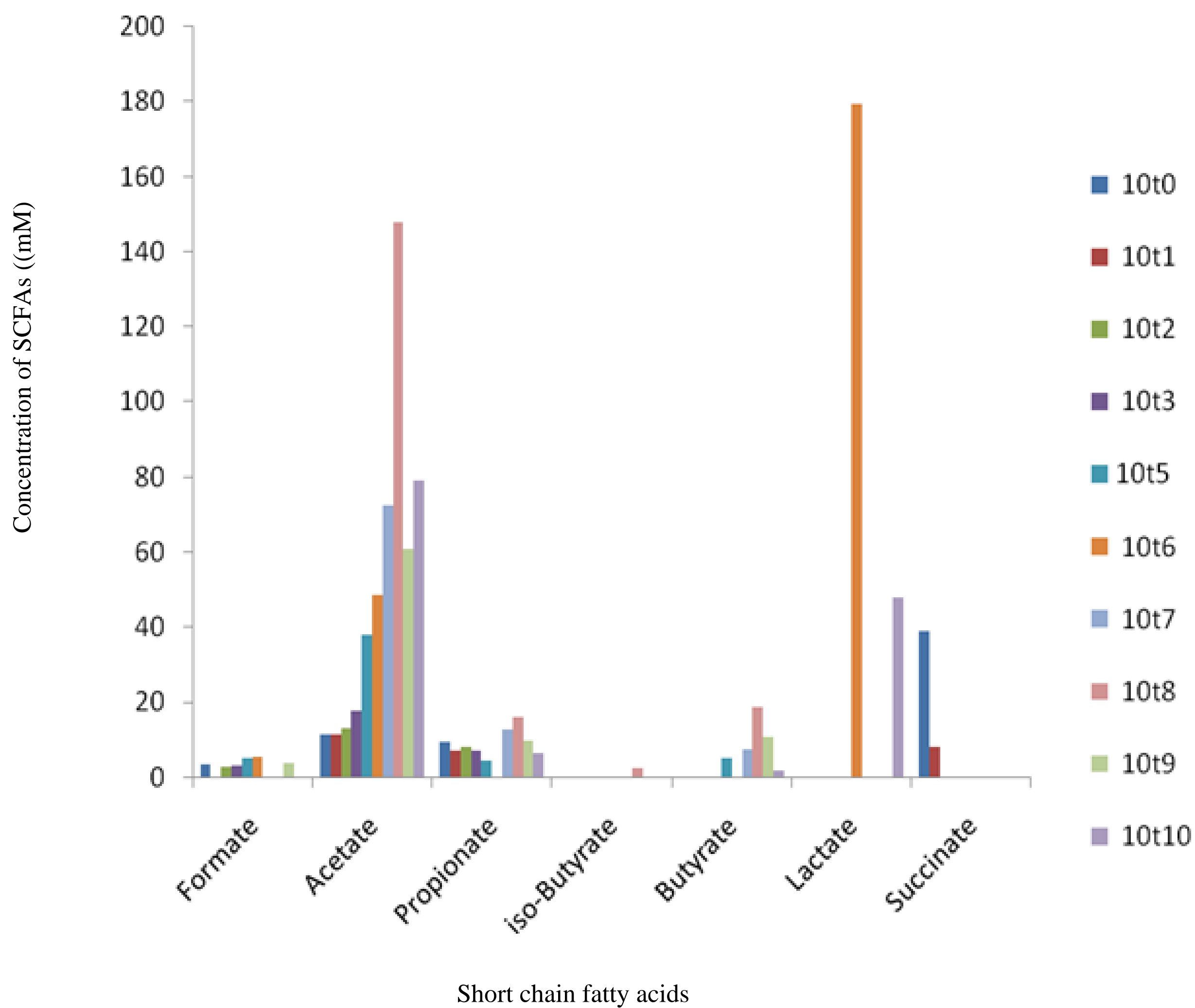


Figure VIII(10). Presence of SCFAs in Baby 10 Sample

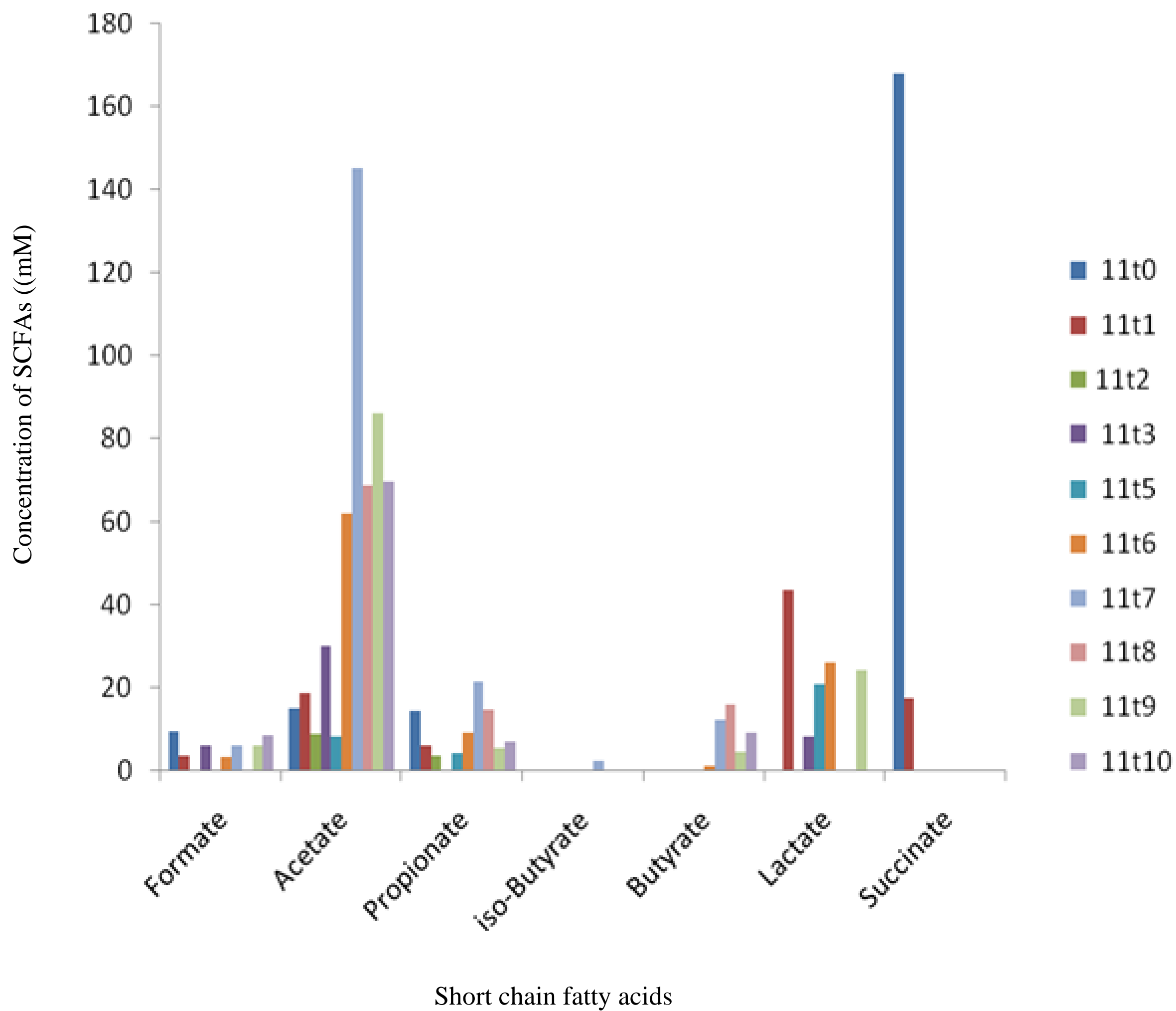
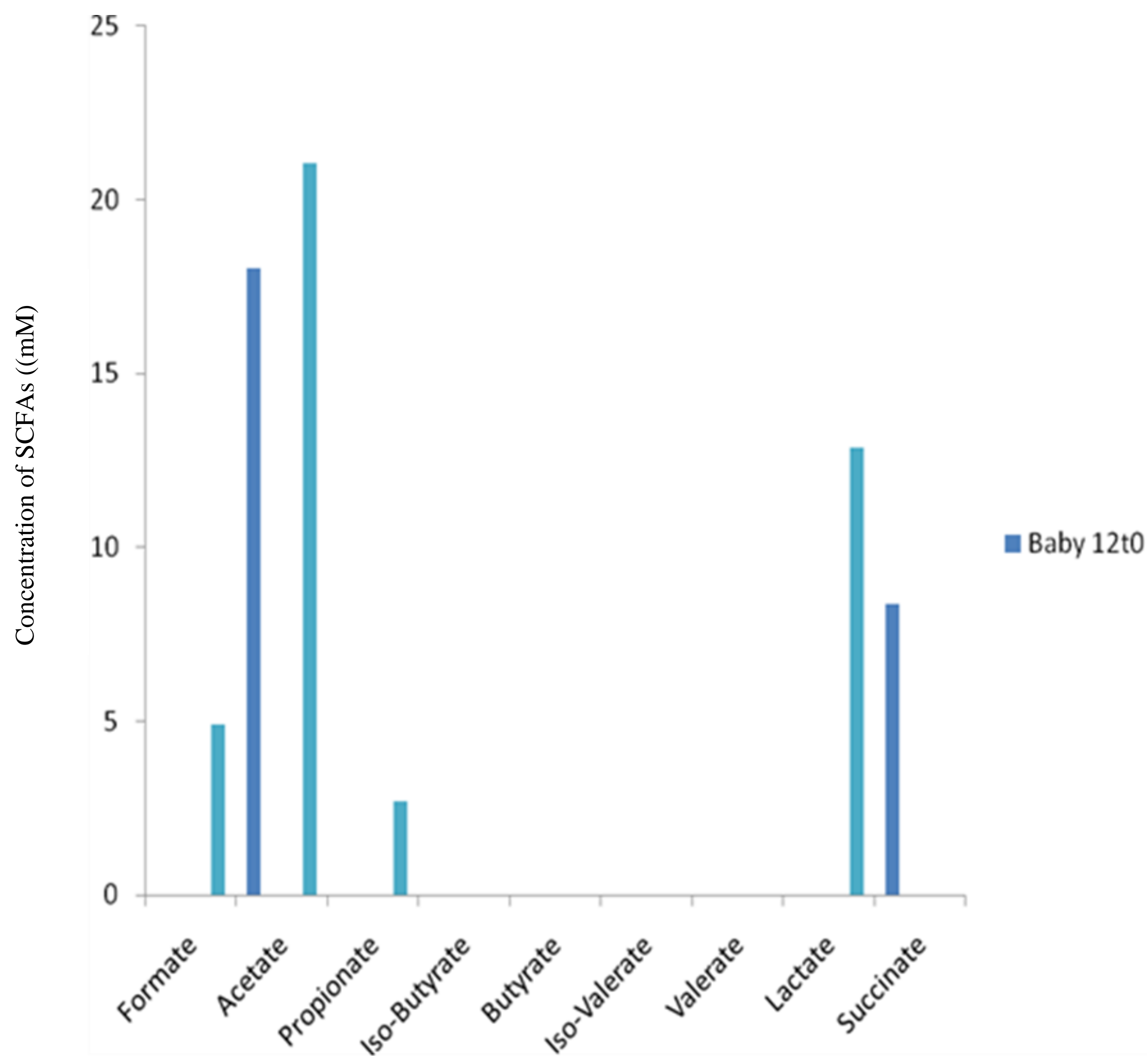


Figure VIII(11). Presence of SCFAs in Baby 11 Sample



Short chain fatty acids

Figure VIII(12). Presence of SCFAs in Baby 12 Sample

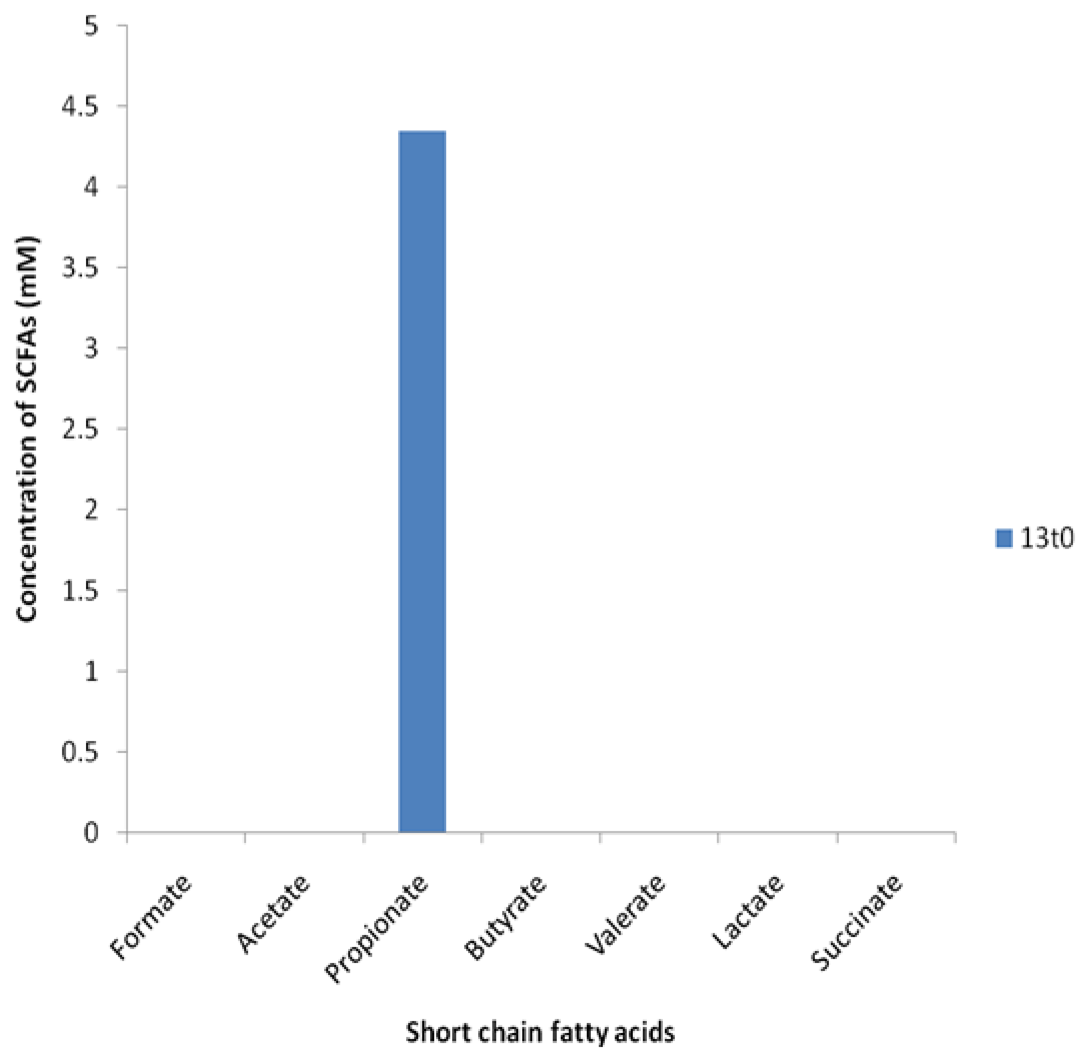


Figure VIII(13). Presence of SCFAs in Baby 13 Sample

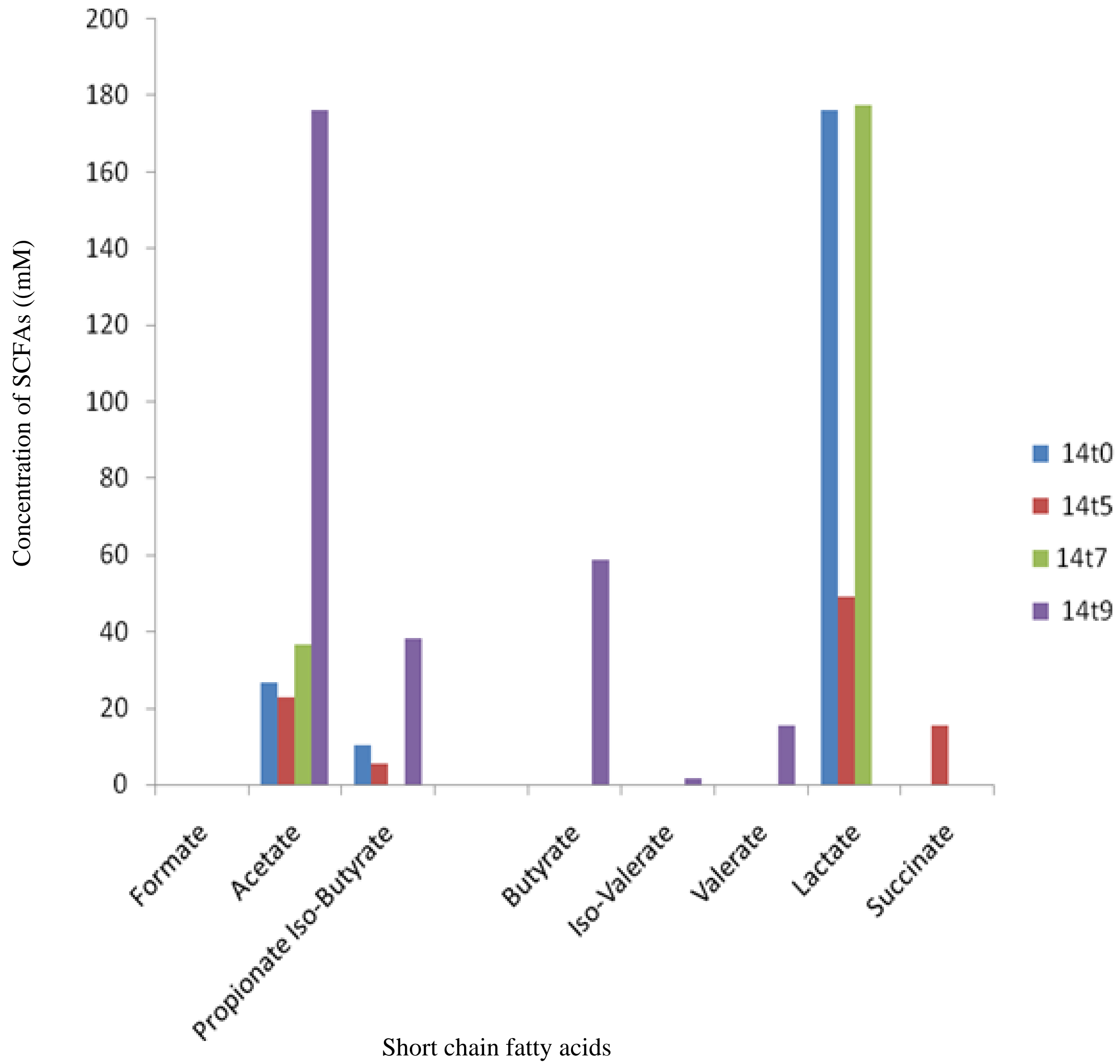
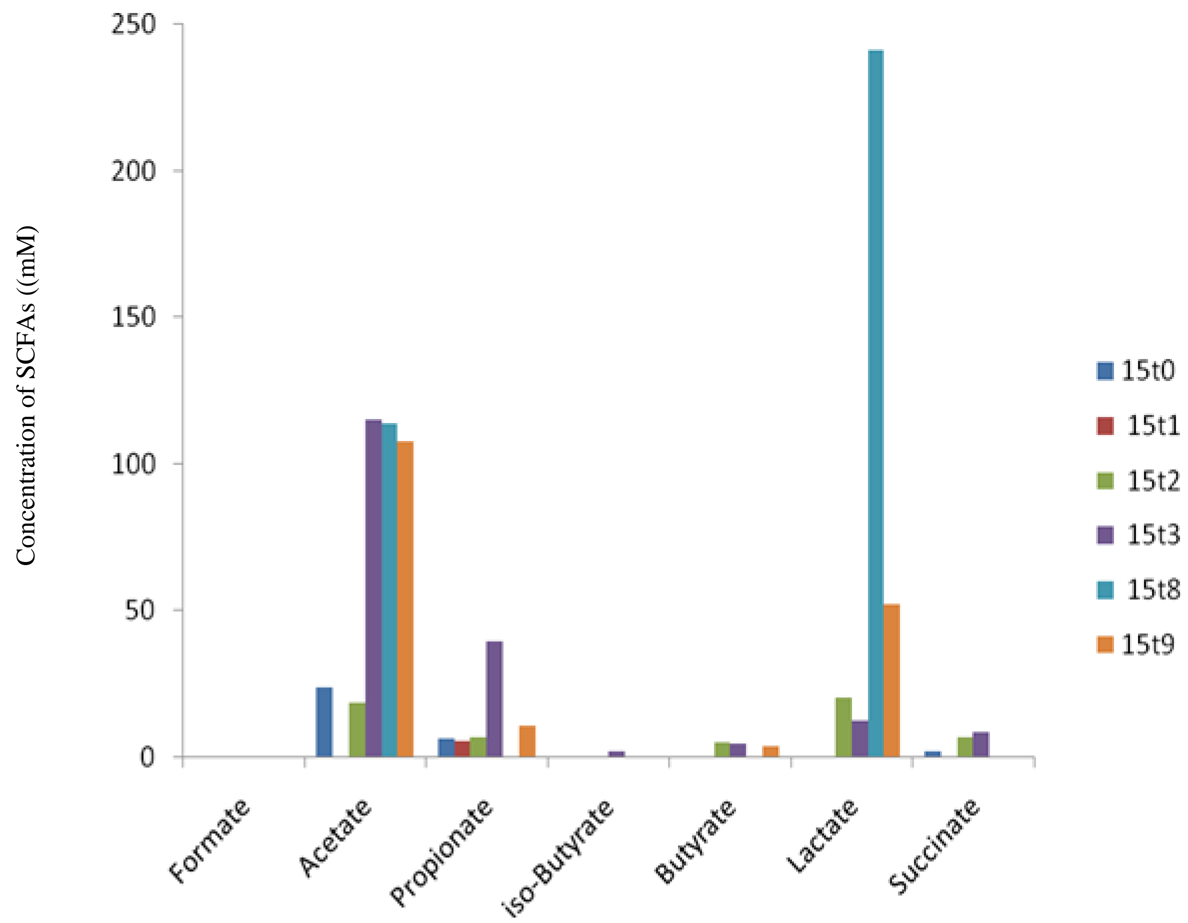


Figure VIII(14). Presence of SCFAs in Baby 14 Sample



Short chain fatty acids

Figure VIII(15). Presence of SCFAs in Baby 15 Sample

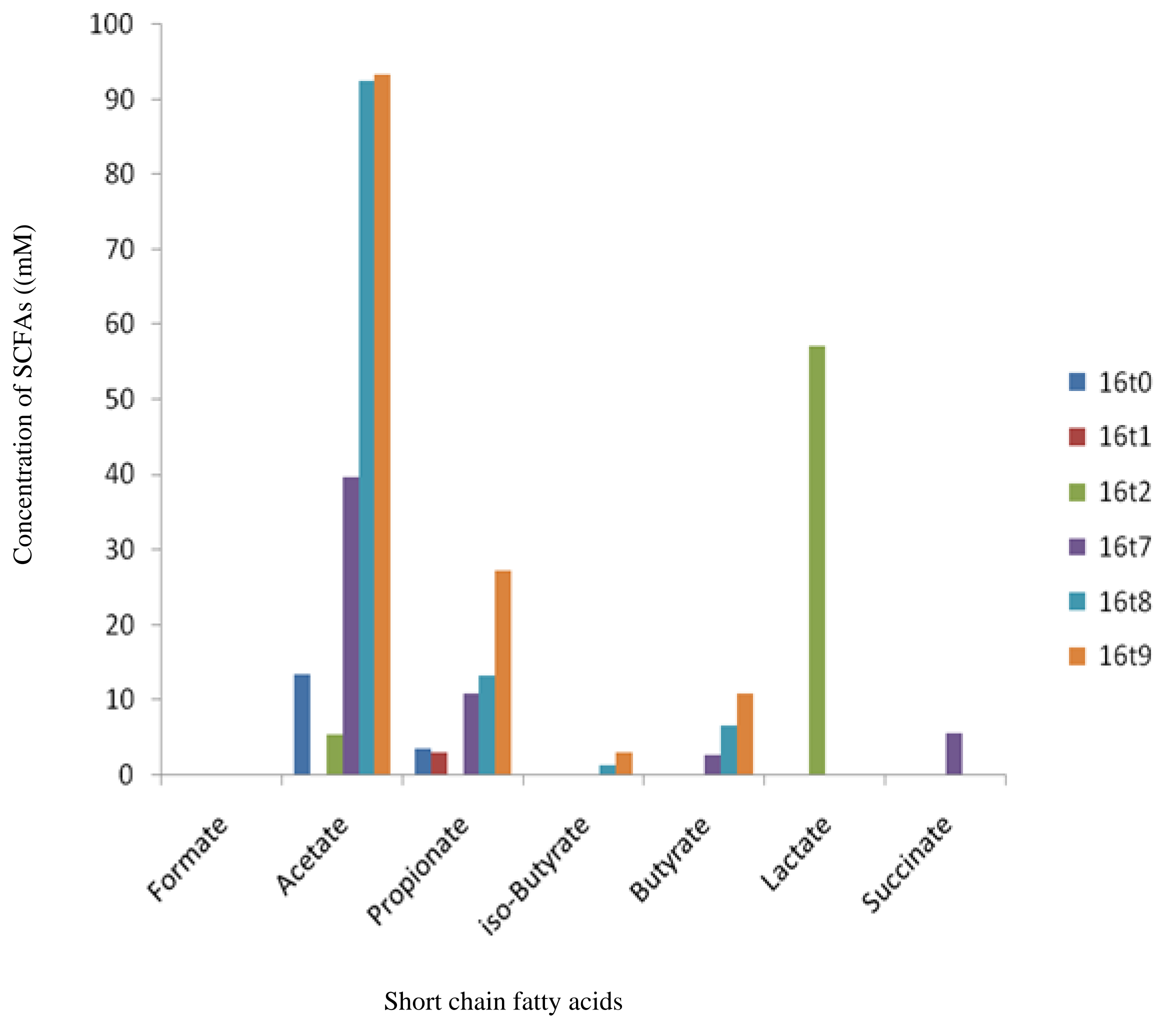


Figure VIII(16). Presence of SCFAs in Baby 16 Sample

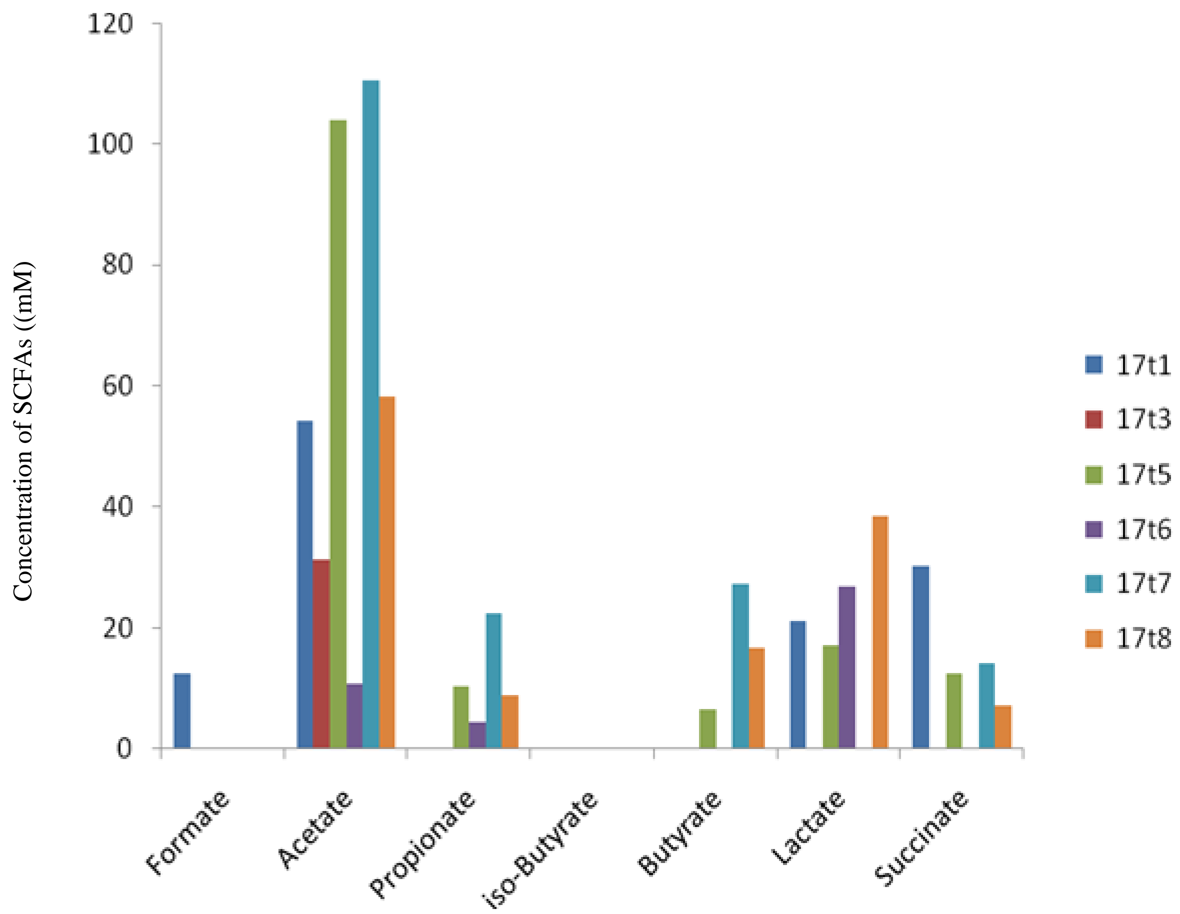


Figure VIII(17). Presence of SCFAs in Baby 17 Sample

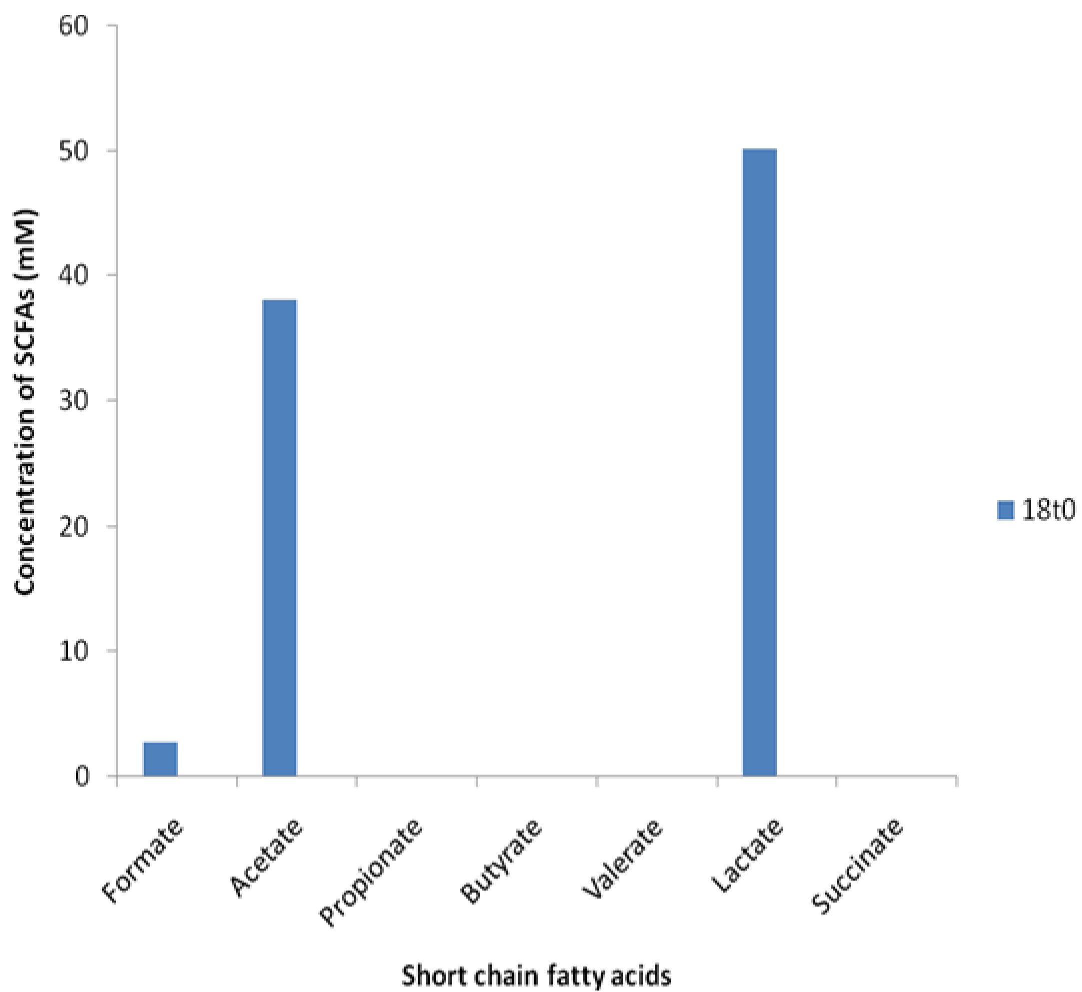


Figure VIII(18). Presence of SCFAs in Baby 18 Sample

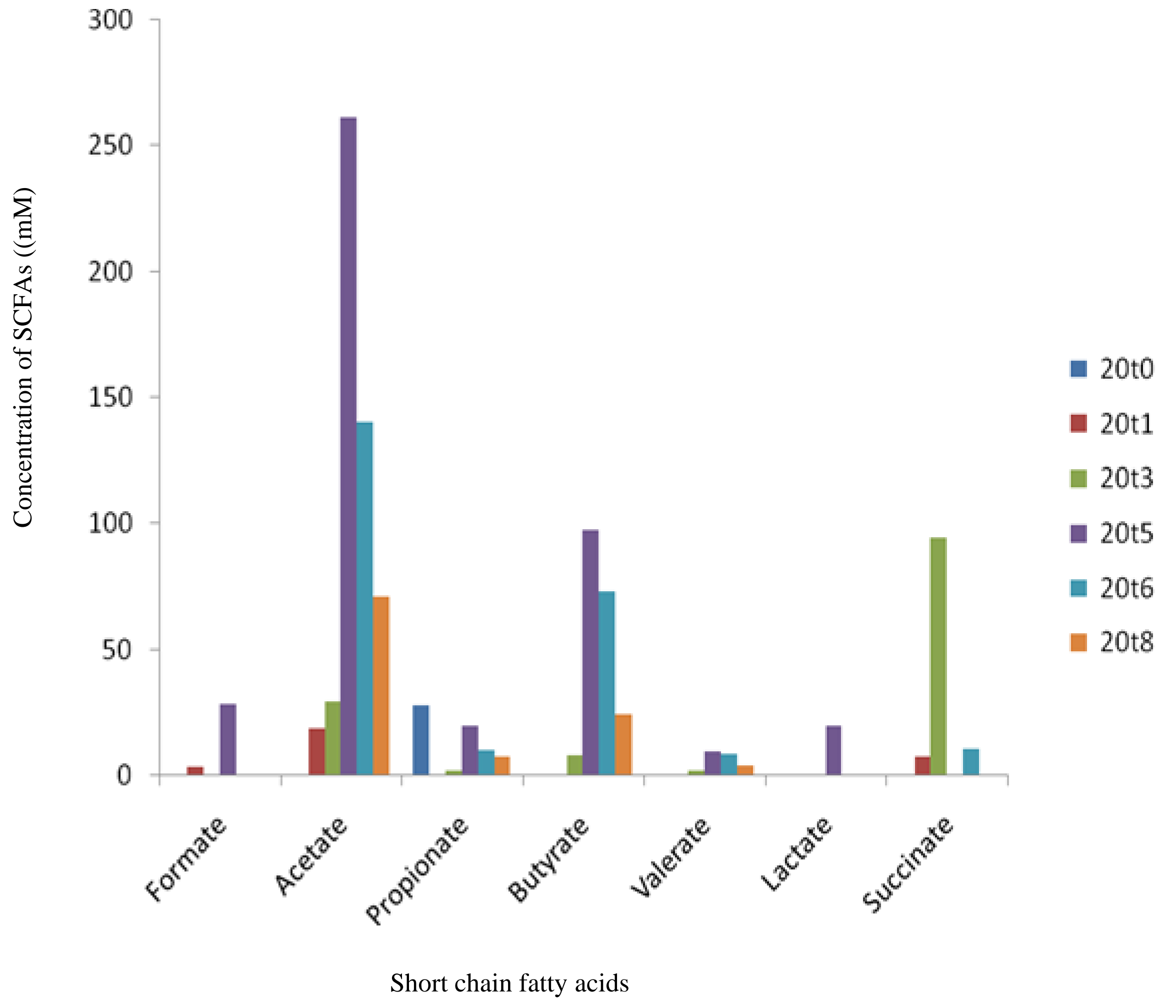


Figure VIII(19). Presence of SCFAs in Baby 20 Sample

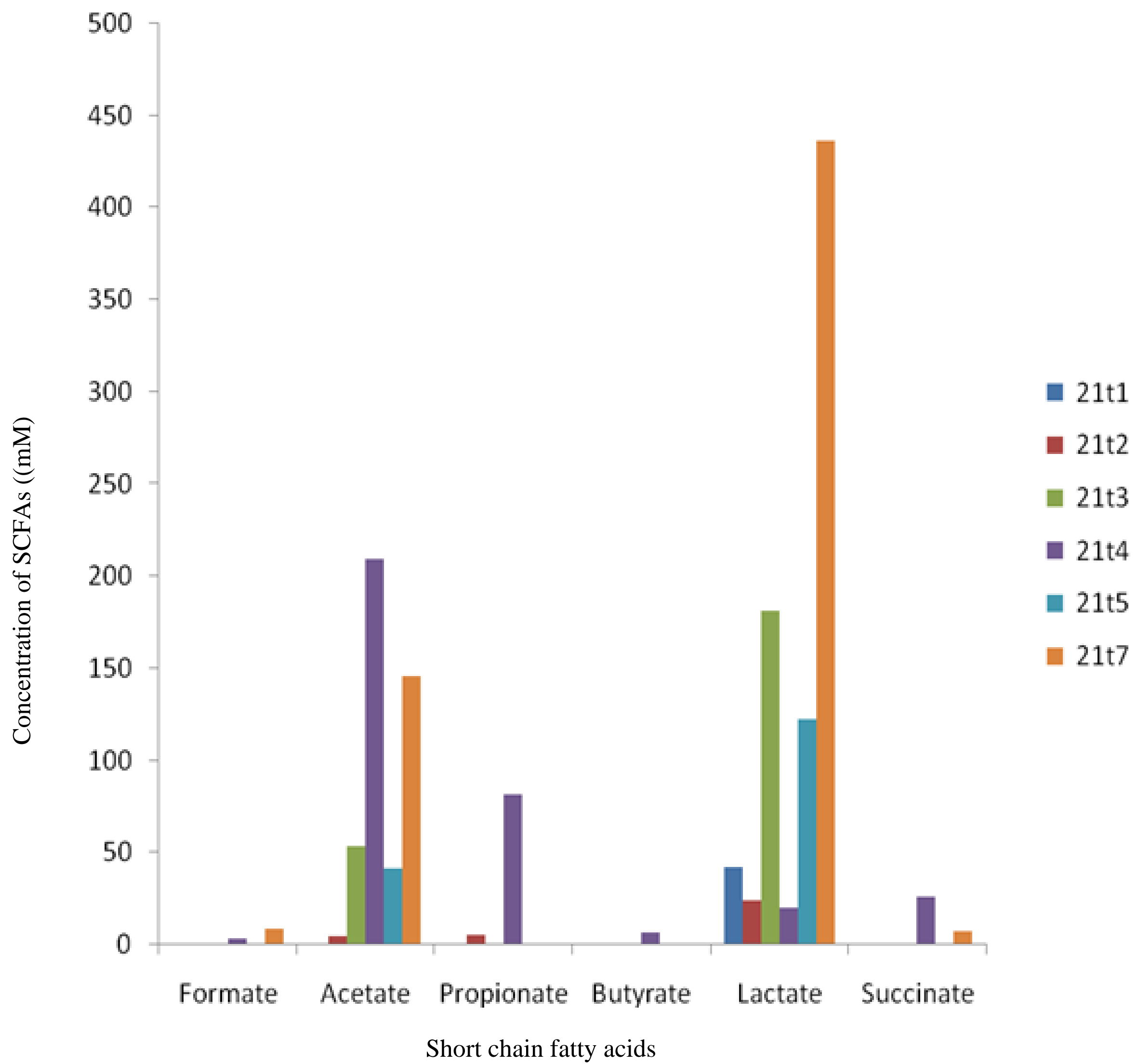


Figure VIII(20). Presence of SCFAs in Baby 21 Sample

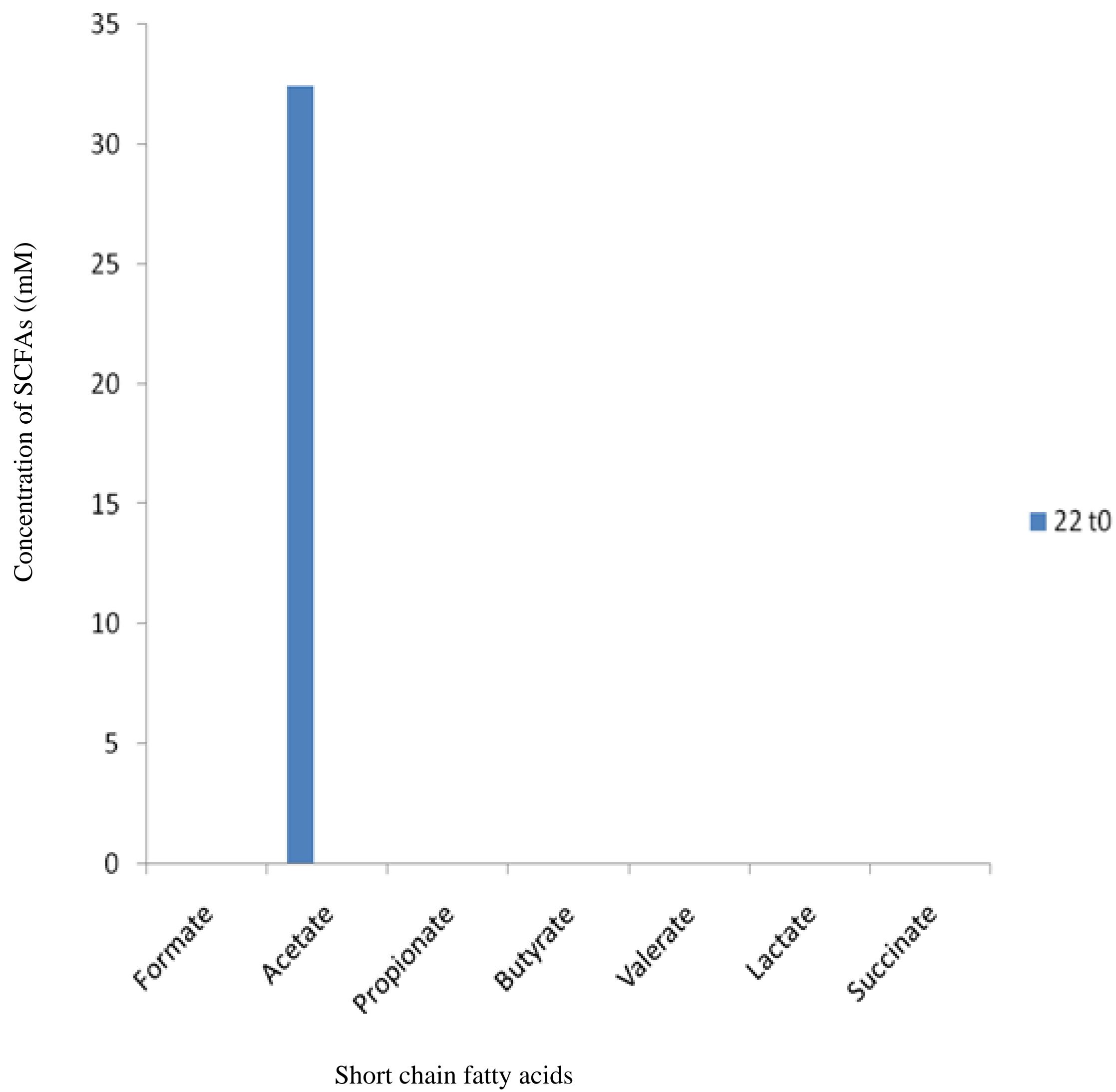


Figure VIII(21). Presence of SCFAs in Baby 22 Sample

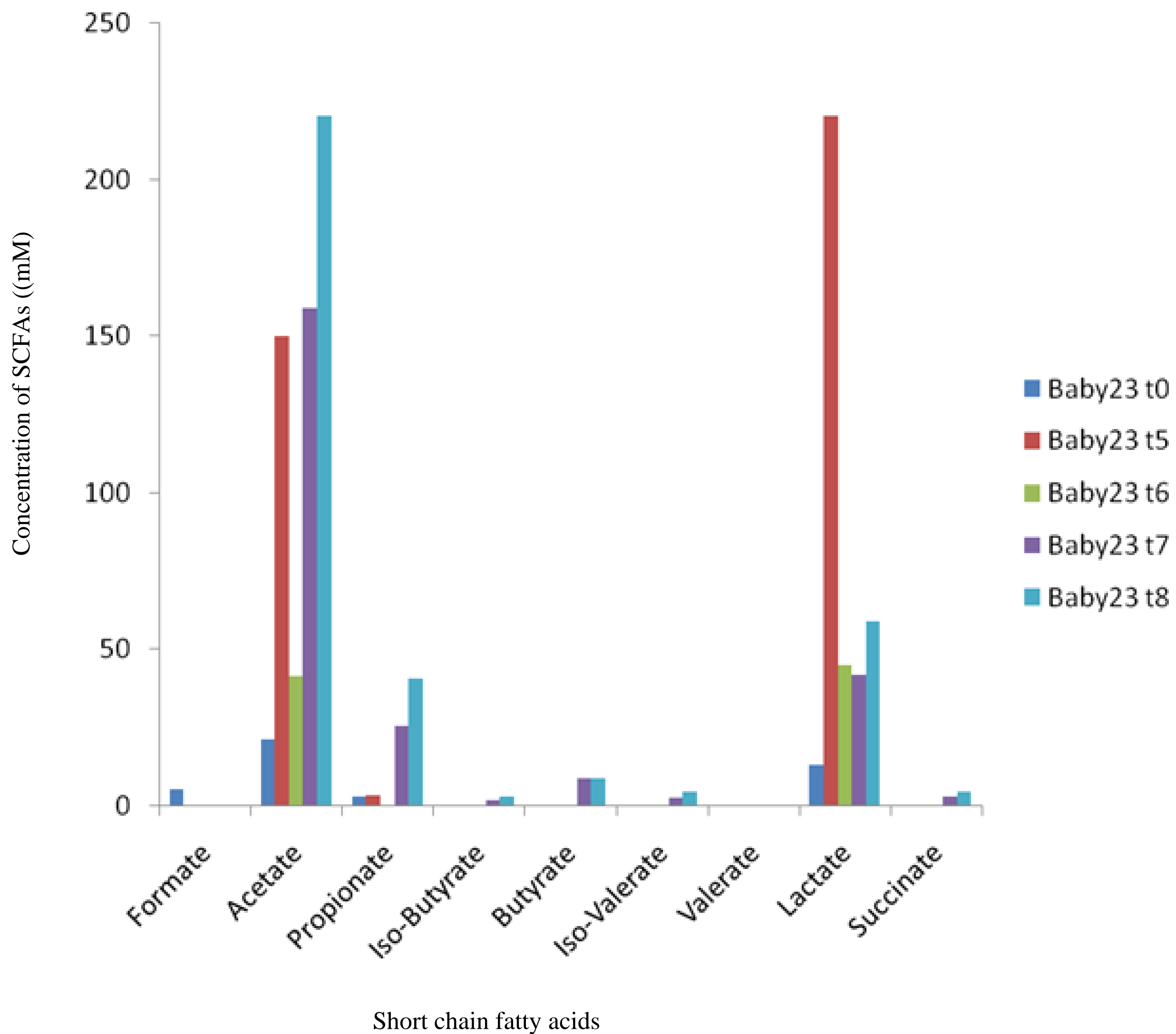


Figure VIII(22). Presence of SCFAs in of Baby 23 Sample

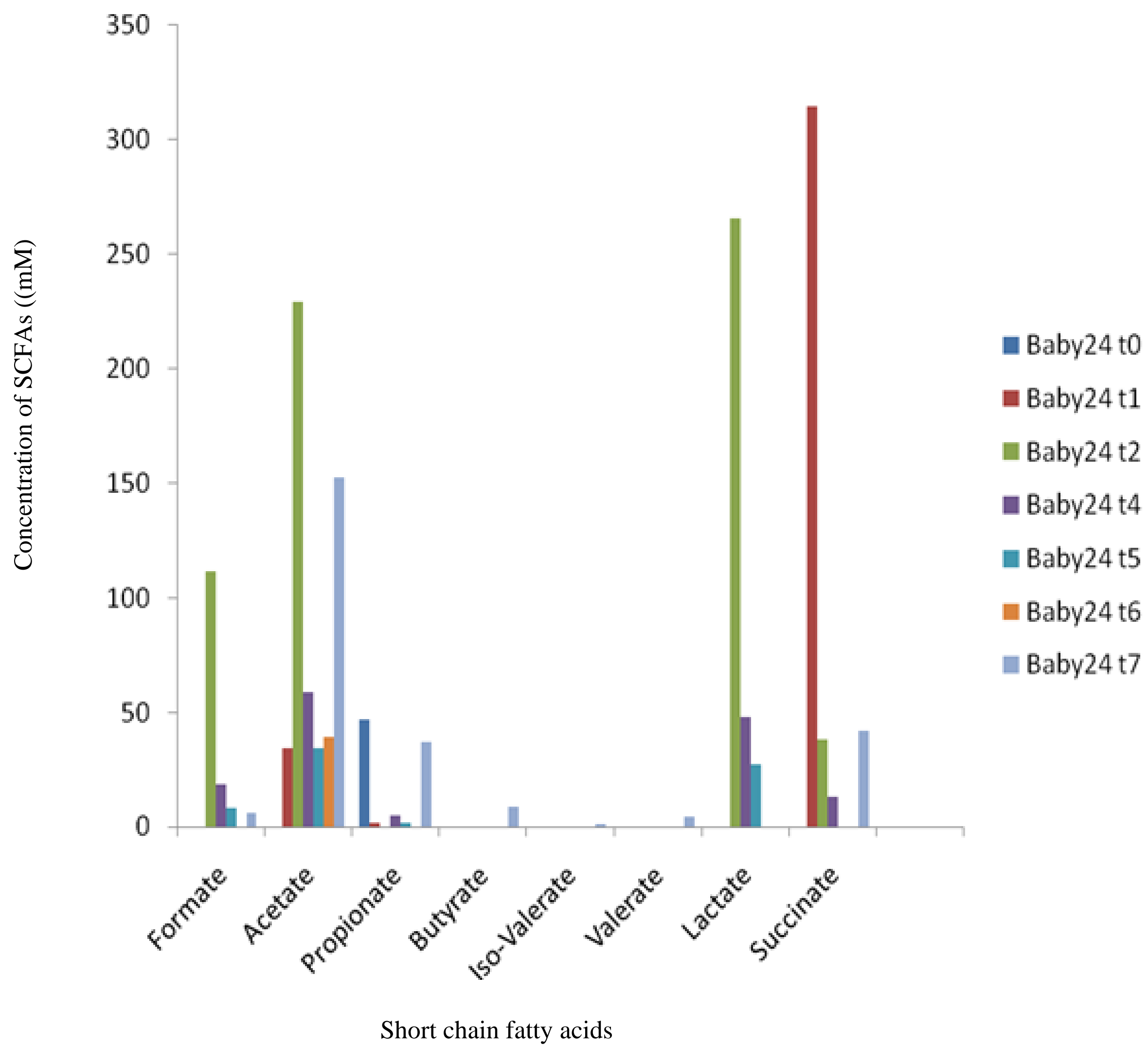


Figure VIII(23). Presence of SCFAs in Baby 24 Sample

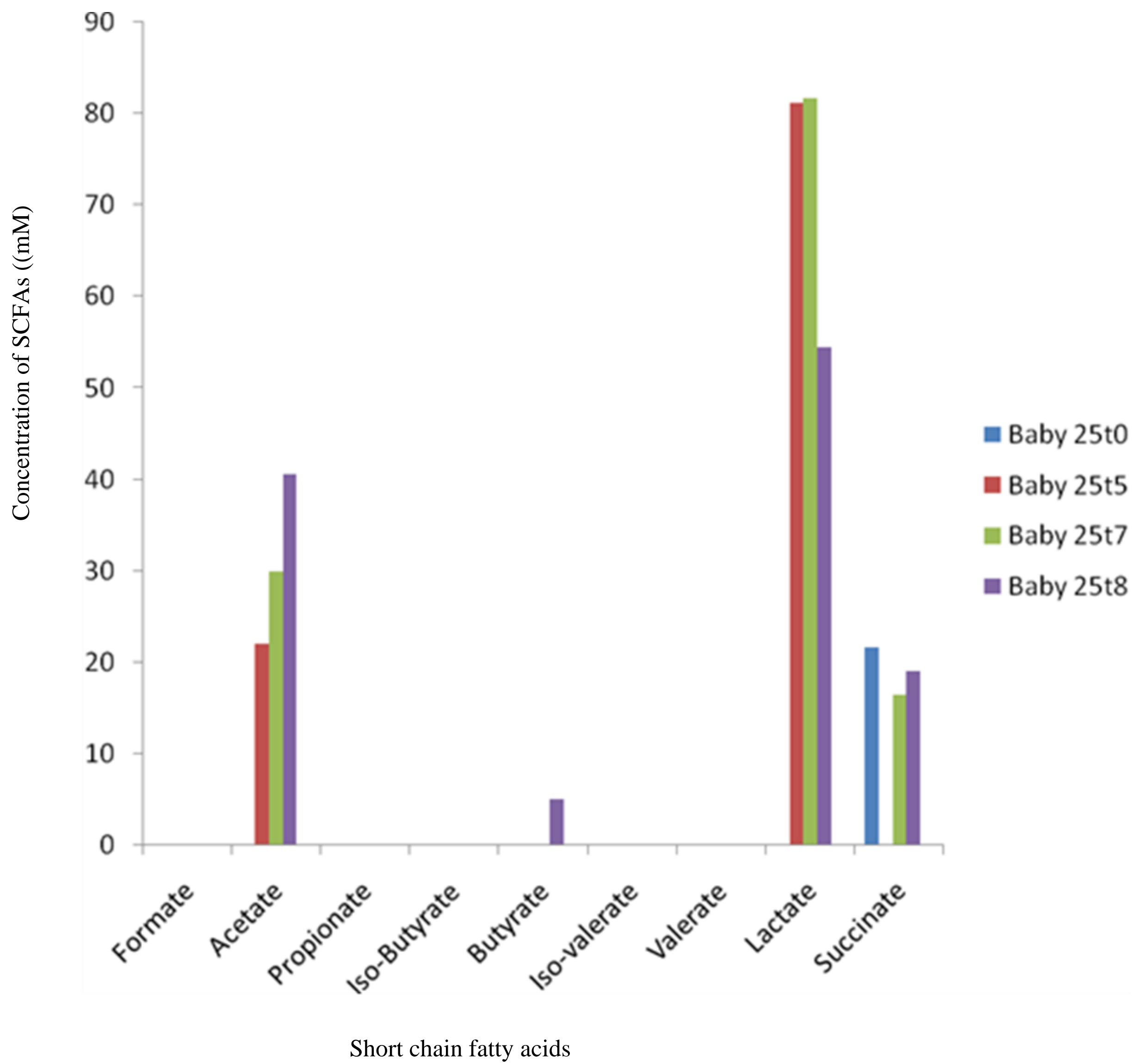


Figure VIII(24). Presence of SCFAs in Baby 25 Sample

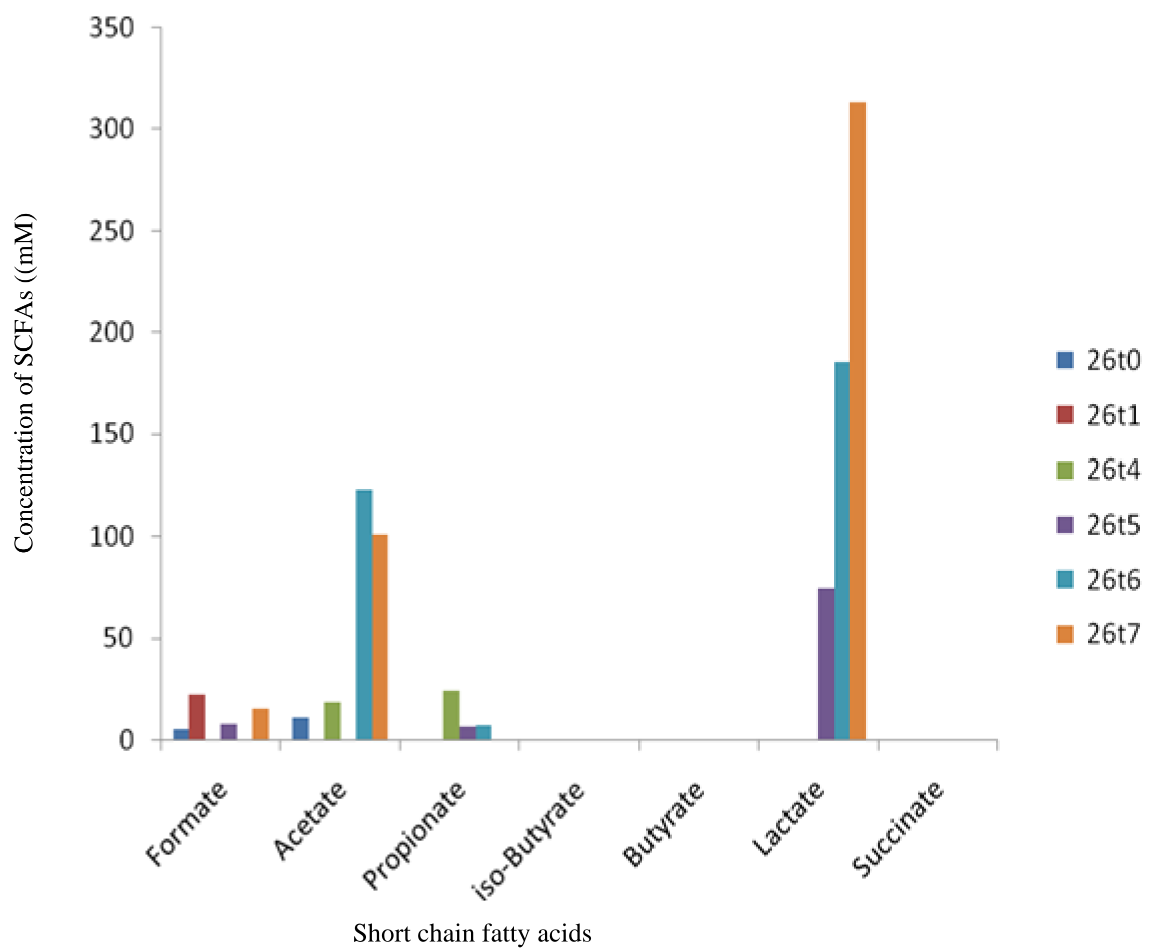


Figure VIII(25). Presence of SCFAs in Baby 26 Sample

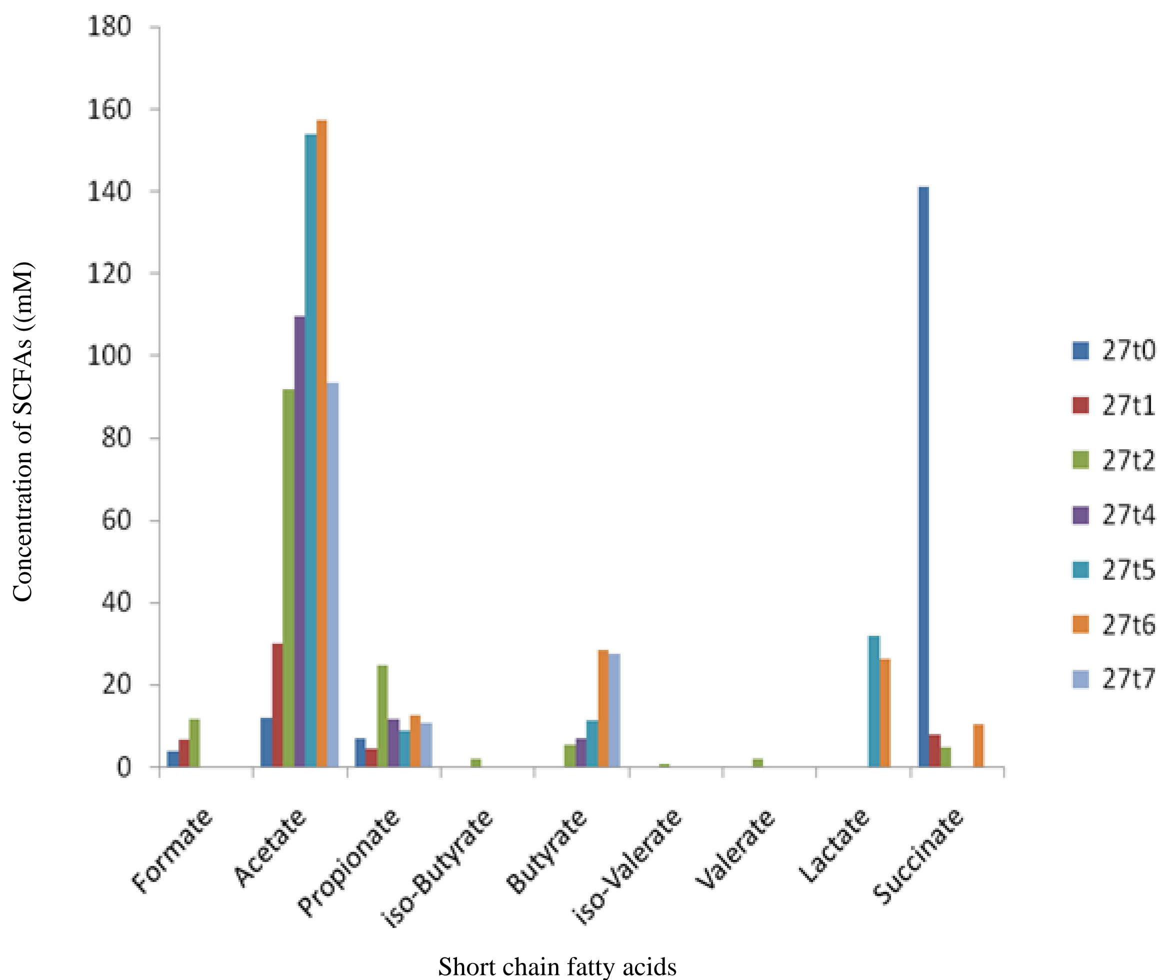


Figure VIII(26). Presence of SCFAs in Baby 27 Sample

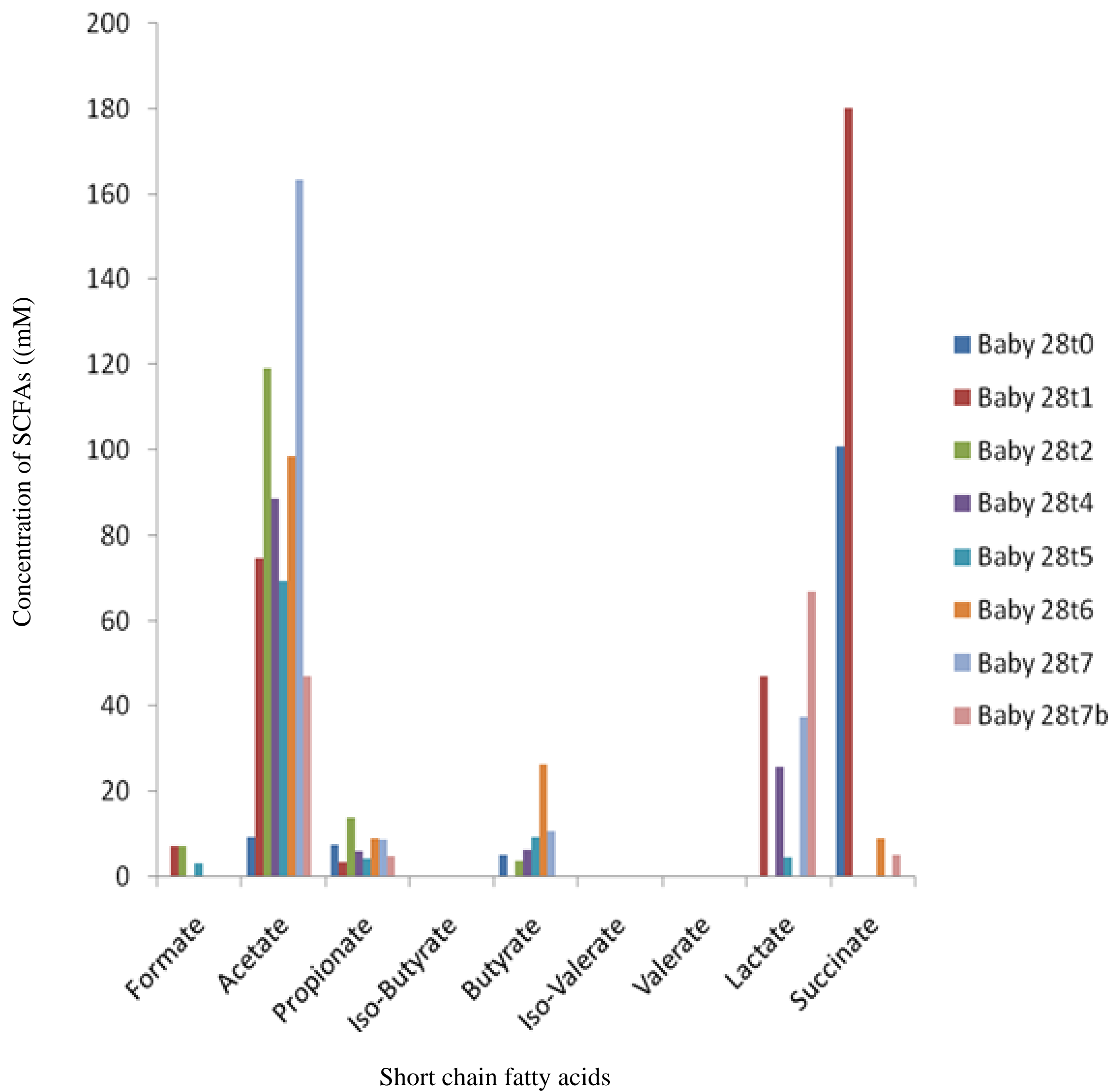


Figure VIII(27). Presence of SCFAs in Baby 28 Sample

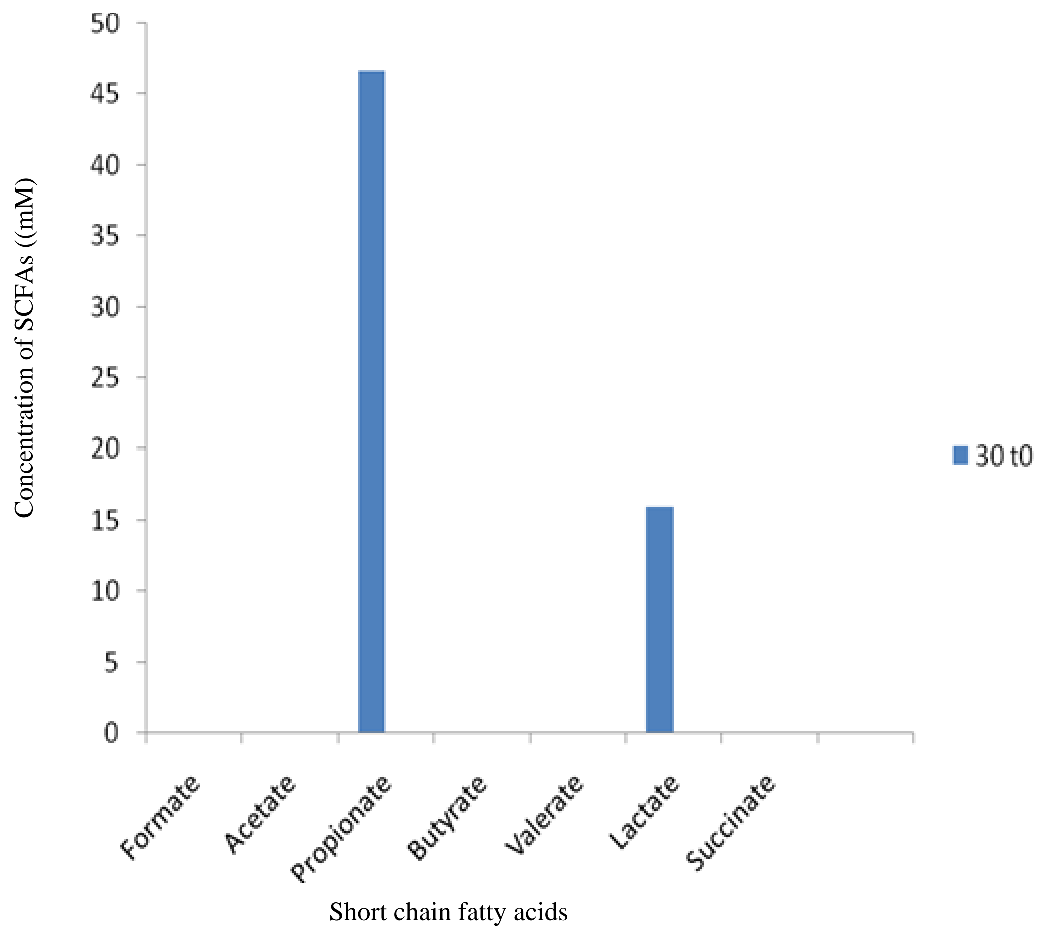


Figure VIII(28). Presence of SCFAs in Baby 30 Sample

APPENDIX X

List of primer sequences used during the PCR amplification of V1-V2 region of 16S rRNA.

<u>Barcoded Primer Sequences</u>

Forward primer:

27F_Miseq AATGATACGGCGACCACCGAGATCTACACTATGGTAATTCCAGMGTTYGATYMTGGCTCAG

Reverse primers:

338rcbc1	CAAGCAGAAGACGGCATACGAGATACGAGACTGATTAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc2	CAAGCAGAAGACGGCATACGAGATGCTGTACGGATTAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc3	CAAGCAGAAGACGGCATACGAGATATCACCAGGTGTAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc4	CAAGCAGAAGACGGCATACGAGATTGGTCAACGATAAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc5	CAAGCAGAAGACGGCATACGAGATATCGCACAGTAAAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc6	CAAGCAGAAGACGGCATACGAGATGTCGTGTAGCCTAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc7	CAAGCAGAAGACGGCATACGAGATAGCGGAGGTTAGAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc8	CAAGCAGAAGACGGCATACGAGATATCCTTTGGTTCAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc9	CAAGCAGAAGACGGCATACGAGATTACAGCGCATACAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc10	CAAGCAGAAGACGGCATACGAGATACCGGTATGTACAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc11	CAAGCAGAAGACGGCATACGAGATAATTGTGTCGGAAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc12	CAAGCAGAAGACGGCATACGAGATTGCATACACTGGAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc13	CAAGCAGAAGACGGCATACGAGATAGTCGAACGAGGAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc14	CAAGCAGAAGACGGCATACGAGATACCAGTGACTCAAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc15	CAAGCAGAAGACGGCATACGAGATGAATACCAAGTCAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc16	CAAGCAGAAGACGGCATACGAGATGTAGATCGTGTAAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc17	CAAGCAGAAGACGGCATACGAGATTAACGTGTGTGCAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc18	CAAGCAGAAGACGGCATACGAGATCATTATGGCGTGAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc19	CAAGCAGAAGACGGCATACGAGATCCAATACGCCTGAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc20	CAAGCAGAAGACGGCATACGAGATGATCTGCGATCCAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc21	CAAGCAGAAGACGGCATACGAGATCAGCTCATCAGCAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc22	CAAGCAGAAGACGGCATACGAGATCAAACAACAGCTAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc23	CAAGCAGAAGACGGCATACGAGATGCAACACCATCCAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc24	CAAGCAGAAGACGGCATACGAGATGCGATATATCGCAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc25	CAAGCAGAAGACGGCATACGAGATCGAGCAATCCTAAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc26	CAAGCAGAAGACGGCATACGAGATAGTCGTGCACATAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc27	CAAGCAGAAGACGGCATACGAGATGTATCTGCGCGTAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc28	CAAGCAGAAGACGGCATACGAGATCGAGGGAAAGTCAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT

338rcbc29 CAAGCAGAAGACGGCATACGAGATCAAATTCGGGATAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc30 CAAGCAGAAGACGGCATACGAGATAGATTGACCAACAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc31 CAAGCAGAAGACGGCATACGAGATAGTTACGAGCTAAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc32 CAAGCAGAAGACGGCATACGAGATGCATATGCACTGAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
338rcbc33 CAAGCAGAAGACGGCATACGAGATCAACTCCCGTGAAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
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338rcbc36 CAAGCAGAAGACGGCATACGAGATCACTACGCTAGAAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
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338rcbc38 CAAGCAGAAGACGGCATACGAGATACCATAGCTCCGAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
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338rcbc44 CAAGCAGAAGACGGCATACGAGATTAATACGGATCGAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
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338rcbc46 CAAGCAGAAGACGGCATACGAGATTGTGAATTCGGAAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
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338rcbc49 CAAGCAGAAGACGGCATACGAGATGGCCAGTTCCTAAGTCAGTCAGAAGCTGCCTCCCGTAGGAGT
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