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The Oral Microbiota of Irish Children in Health and Disease:

A Longitudinal and Cross Sectional Study.

A thesis presented to the National University of Ireland for the Degree of

**Doctor of Philosophy**

By

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## List of Abbreviations

16S rRNA	16 Svedberg ribosomal Ribonucleic acid
BP	Base pair
BLAST	Basic Local Alignment Search Tool
CAC	Caries-active caries
CAS	Caries-active saliva
CFS	Caries-free saliva
CS	Caesarean Section
CSTs	Community State Types
CUH	Cork University Hospital
CUMH	Cork University Maternity Hospital
DNA	Deoxyribonucleic Acid
dmft	decayed, missing (due to caries), or filled tooth in primary teeth
dmfs	decayed, missing (due to caries), or filled tooth surfaces in primary teeth
ECC	Early childhood caries
FFQ	Food Frequency Questionnaire
FLASH	Fast Length Adjustment of SHort reads to improve genome assemblies
FP	Forward Primer
FT	Full-term
FISH	Fluorescence in situ hybridisation
GIT	Gastrointestinal Tract
HMP	Human Microbiome Project
HMO	Human Milk Oligosaccharide
HOMD	Human Oral Microbiome Database



HOT	Human Oral Taxon
IAP	Intrapartum Antibiotic Prophylaxis
MID	Multiplexing Identifier
NICU	Neonatal Intensive Care Unit
OTU	Operational Taxonomic Unit
PCoA plot	Principle Co-ordinate Analysis plot
PCR	Polymerase Chain Reaction
PT	Preterm
PPROM	Preterm premature rupture of membranes
QIIME	Quantitative Insights Into Microbial Ecology
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RP	Reverse Primer
S- ECC	Severe Early Childhood Caries
STIRRUPS	Species-level Taxon Identification of rDNA Reads using a USEARCH Pipeline Strategy)
SVD	Spontaneous Vaginal Delivery
$\alpha$ -Diversity	Alpha Diversity
$\beta$ -Diversity	Beta Diversity

## Declaration

Data described in this thesis is the result of my work, except where explicitly stated otherwise. This work has been carried out in the School of Microbiology, University College Cork, Cork University Dental School and Hospital and at Cork University Maternity Hospital between March 2012 and September 2017. The bioinformatic analysis was carried out by Mr. Hugh Harris, a postgraduate scientist and Mr. David Mullins, a research bioinformatic assistant in Prof. Paul O'Toole's Lab. The material is not substantially the same as that has been submitted for any degree or qualification at this or any other University.

The research undertaken in this thesis was funded by a research award from the Health Research Board (HRA\_POR/2012/123) in 2012. The work undertaken was entitled 'ORAL-MET'. 'ORAL' highlighting the research focus on the oral cavity, and 'MET' symbolising the 'metagenomic' work which would be undertaken to investigate the microbiome of the oral cavity of both adults and infants.

This ORALMET study involved two separate studies. One longitudinal study and one cross sectional study. The grant application was designed and written by the PhD Candidate, with various inputs by the candidate's PhD supervisors.

Signed \_\_\_\_\_

Eimear Hurley, September 2017

# Aims and Objectives

## Aims

The overall objectives of this ORALMET study were to understand the role of birth modality on the oral microbiota of neonates from birth, and to observe the oral microbiota of these infants from birth to 3 years of age (Cohort study/Longitudinal study), and to describe the oral microbiota of children with and without caries (Cross sectional study). All studies are illustrated in Figure 1.1.

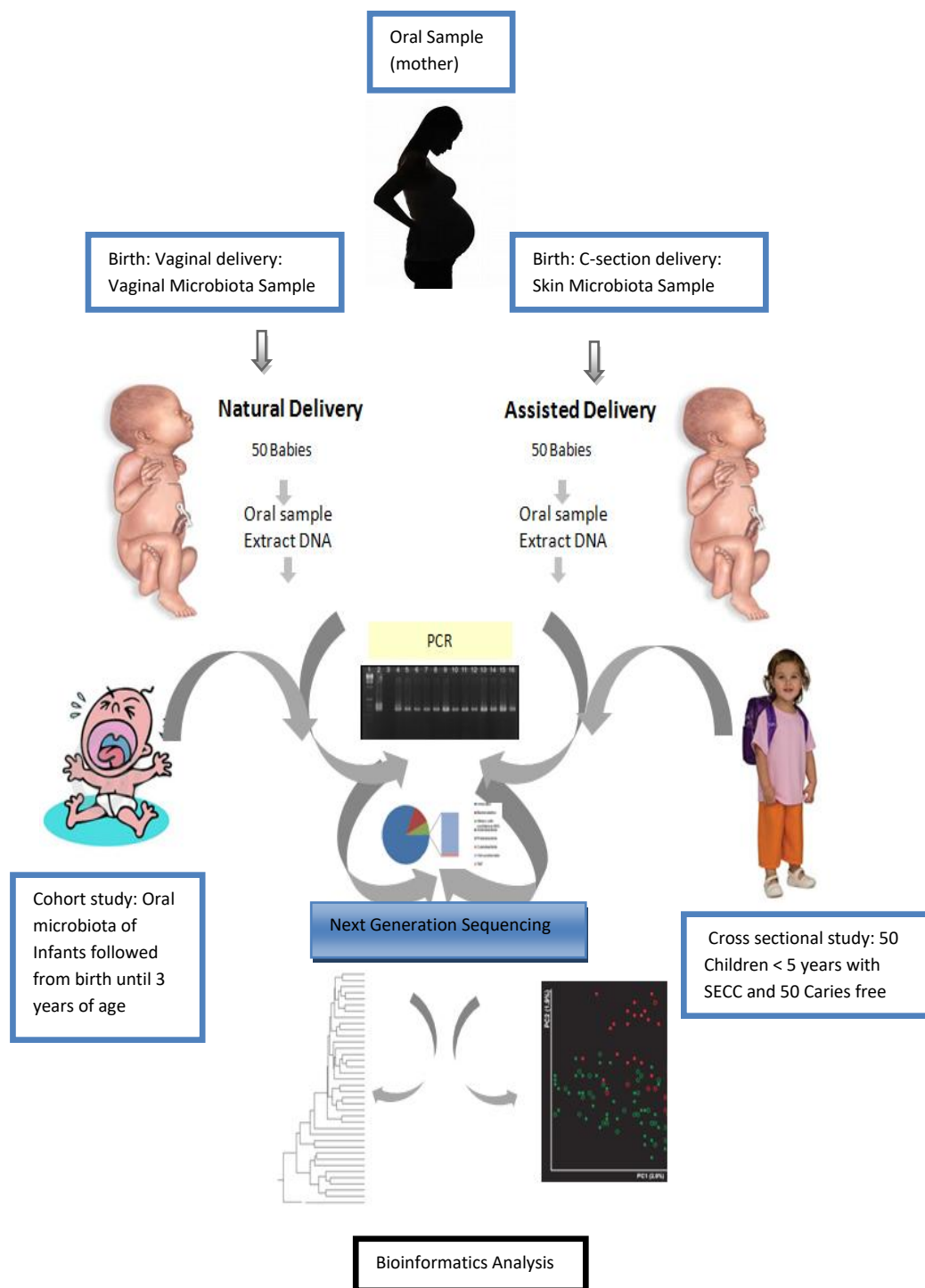
## Objectives

### Cohort/Longitudinal study

- 1) To determine if birth mode (Spontaneous Vaginal delivery (SVD) v C-section (CS) delivery) would have an influence on the composition and development of the oral microbiota.
- 2) To describe the changes in the oral microbiota from birth to 3 years of age among breast fed infants.

### Cross sectional study

- 3) Compare the oral microbiota (saliva/dentinal microbiota) of children aged less than 5 years with high levels of caries (S-ECC) to caries-free children (saliva).



**Figure 1.1:** Study overview-schematic overview of the ORALMET study including Cross sectional study & Cohort/Longitudinal study.

**Aims:**

The specific aims of the two studies (Cohort study & Cross-sectional study).

**Cohort/ Longitudinal Study:**

A prospective three-year cohort study was designed to follow the evolution of the oral microbiome of neonates from birth to 3 years of age. There were two cohorts: one cohort included infants delivered by Spontaneous Vaginal Delivery (SVD) and the second included infants delivered by Caesarean Section (CS). In total 100 infants would be followed longitudinally from birth to three years of age, collecting the salivary oral microbiota of these infants at different time points until 3 years of age (1 week, 4 weeks, 8 weeks, 6 months/24 weeks, 1 year, 2 years and 3 years of age). At the time of delivery, a saliva sample would be collected from the mother along with a skin (CS) or vaginal sample (SVD), dependent on birth mode.

**Aims**

- 1) To describe and compare the microbiota of the different body sites of the mother (vagina, skin, oral cavity) to their infants microbiota (oral cavity).
- 2) To identify if there is any influence on mode of delivery on the oral microbiota of the infant within 1 week of birth, at 4 weeks, 8 weeks, 6 months, 1 year, 2 years and 3 years of age.
- 3) To describe the oral microbiota changes over time as the infant develops and grows from 1 week to 3 years of age (1 week, 4 weeks, 8 weeks, 6 months/24 weeks and 1, 2 and 3 years of age).

- 4) To identify if breast feeding has an influence on the oral microbiota of the neonate, and also to identify if duration of breast feeding has an impact on the oral microbiota.

### **Cross Sectional Study:**

A cross sectional study was designed to compare the oral microbiota of children affected by severe early childhood caries (S-ECC) to that of caries-free children, by high-throughput sequencing of 16S rRNA amplicons. This study aimed to describe the oral microbiota (saliva and dentinal microbiota) of children less than 5 years old, with high caries levels in their primary dentition (S-ECC) to those that are caries free (saliva).

### **Aims**

- 1) To describe and compare the salivary microbiota of Irish children with caries (S-ECC) to children who are caries-free.
- 2) To investigate the dentinal microbiota of deep caries and to identify taxa that may be advancing the progression of the disease.
- 3) To explore the differences in the oral microbiota of the two groups and to investigate potential explanatory variables (including diet analysis).

The attainment of these aims would lead to the identification of microbiota associated with health and disease. While much of the outcome of this project is necessarily descriptive, there is a core of hypothesis driven research:

### **Hypotheses to be tested**

- There is no difference in the oral microbiome of children
  - a) who are delivered by Caesarean section and vaginally
  - b) from birth to 3 years of age
  - c) with high caries and those who are caries free

### **Structure of the Thesis**

This thesis is structured in accordance with the guidelines set out by University College Cork. Literature review (Chapter 2) is followed by a Methodology Chapter (Chapter 3). The research results are presented in Chapter 4 with discussion of the research findings in Chapter 5 and a final discussion in Chapter 6. All work has been completed while the candidate has been registered as a PhD student.

### **Candidate's contribution to PhD.**

The role of the candidate is described in this section. The candidate is the first author of all manuscripts under review in this thesis (Chapter 4). The candidate provided the following contributions:

- Concept of ORALMET study
- Grant Application
- Study design
- Patient recruitment
- Patient Sampling for both Cross sectional and longitudinal study
- Compilation of patient questionnaires

- Laboratory work
- Manuscript preparation

The concept and study design of the ORALMET study was completed by the PhD candidate and assisted by Professor. Paul O'Toole, Professor. Helen Whelton and Professor. Martin Kinirons. Bioinformatic support was provided by Hugh Harris and David Mullins. Patient recruitment and sample collection was assisted by Carol-Anne O'Shea, a research nurse on the Infantmet project. All authors contributed feedback on manuscripts prior to submission.



## Thesis Abstract

The oral cavity harbours a very rich and diverse microbial community. In the last decade, the oral microbiota of children and adults has been studied in detail using continuously developing DNA sequencing methods. In particular focusing on the oral microbiome changes in the presence of diseases such as dental caries, periodontal disease and the relationship of the oral microbiome with oral health and disease states. The overall aim of these studies was to unravel the complexity of the oral ecosystem and the driving forces behind the imbalance of this ecosystem which leads to increased risk of disease and poor oral health. The oral microbiota of the edentulous neonate and infant is poorly characterised particularly the dynamics of the oral microbial changes of the neonate as it grows from birth to an older age. This is important as it would give researchers that study the oral health of children a better understanding of the foundation of the oral microbiome. An important addition would also be to explore the influence feeding modality (breast or formula), introduction of solid foods, eruption of teeth, antibiotic intake, fluoridation intake and oral hygiene habits all have on the infant oral microbiota. Gaining a better understanding of the dynamics and maturation of the oral microbiome of neonates from birth is important to identify any microbial factors which increase the risk of dental diseases in these children.

In this ORALMET study, we recorded the oral microbial changes from birth to 2 years of age of infants (n=382) (year 3 data is not included in this thesis) born via C-section or Vaginal delivery. Factors included in our analysis of the oral microbiota of these infants included breast feeding duration and the influence of the maternal microbiota (saliva/skin or vaginal microbiota). We observed that breast feeding

duration does not have an effect on the oral microbiota of infants, and birth mode has an effect on the oral microbiota of infants up to 4 weeks of age only, but not beyond that. The oral microbiota changes in composition are most apparent from 6 months of age to 2 years of age, with less microbial changes within the oral cavity from birth to 8 weeks of age. The oral microbiota composition is continually changing and developing up to 1 year of age and at 2 years of age, illustrating continuous maturation, with no evidence of stability by 2 years of age. The second part of the ORALMET study (cross-sectional study) compared the oral microbiota of children between 3-5 years with S-ECC (n=68) and without caries (n=70). We observed distinct microbiota differences in the caries microbiota of children with active ECC, and the presence of species in the salivary microbiota of children with active caries, but not in the salivary microbiota of children without caries.

ORALMET is the first in depth longitudinal study of the oral microbiome from birth of the infant, together with the influence of the maternal microbiota and also the first microbiota study of Irish children with and without caries. Together these studies provide us with information of the infant oral microbiome and a better understanding of the microbial dynamics within the oral cavity of neonates, infants and children.

## **Study Abstracts**

1. Longitudinal study: Birth to 1 year of age.
2. Longitudinal study: Birth to 2 years of age.
3. Cross Sectional Study.

## **Abstract:**

### **1. Longitudinal Study: Birth to 1 year of age.**

#### **Background**

The oral cavity is a rich and diverse microbial niche. While the microbiology of the infant gut has been studied in detail, the acquisition of microbial communities and the influence of delivery mode on the oral microbiota of the newborn infant remains poorly characterised. Studies of the influence of delivery mode accompanied by longitudinal sampling of the infant oral cavity from birth, while assessing confounders such as feeding modality, would increase our knowledge of this under explored area, and may also facilitate early prediction of the risks of developing oral childhood diseases such as severe early childhood caries (S-ECC).

#### **Methods**

A cohort of pregnant women in their second or early third trimester were enrolled in the study (n=81). All infants were born full-term, without complications, by Spontaneous vaginal delivery (SVD) or by Caesarean section (CS). At delivery, vaginal samples were taken from the mother if the infant was born by SVD, along with a saliva sample (n=37). If the infant was delivered by CS, a skin swab was taken from the mother within 1 hour of delivery along with a saliva sample (n=37). To investigate the role of delivery mode on the oral microbiota of their infants, oral saliva samples were taken from the infant within one week of birth, and by longitudinal sampling again at week 4, week 8, 6 months and 1 year of age (Total samples n=340). We used high-throughput sequencing of V4-V5 region 16S rRNA

amplicons to compare the microbiota of all samples from mother (vaginal, skin or saliva) and infant (oral/saliva).

## **Results**

The vaginal, skin and oral microbiota of the mother taken at birth were significantly different ( $p < 0.001$ ) and when compared to the infant oral microbiota at the various time points, by two established metrics, Bray Curtis dissimilarity and UniFrac distances. The vaginal microbiota had a lower alpha diversity than the skin microbiota of the mother, while the infant oral microbiota diversity remained relatively stable from birth to 8 weeks of age. Birth mode had a significant effect on the oral microbiota composition of the newborn infant within 1 week of age ( $p < 0.05$ ), but not at 1 year of age. The influence of the duration of breastfeeding on the early infant oral microbiota was not significant, when birth modes were combined or separated.

## **Conclusions**

We conclude that birth mode does not have an effect on the infant oral microbiota from 4 weeks of age, and the oral microbiota of infants continues to mature and develop after 8 weeks until 1 year of age.

## **Abstract:**

### **2. Longitudinal study: Birth to 2 years of age.**

#### **Background**

The human oral microbiome is a diverse and rich environment. While caesarean section has been linked with disruption of the microbiome of the infant gut, the impact of birth modality (CS v SVD) on the infant oral microbiome has received less attention. In addition, how the oral microbial ecosystem develops and matures from birth to an older age remains under-explored. In this ORALMET study, we continue our investigation to analyse the impact of birth mode on the infant oral microbiota and observe the oral microbiota changes from birth until 2 years of age.

#### **Methods**

A cohort of pregnant women in their second or third trimester was enrolled in the study. Depending on birth modality of their newborn infant (SVD or CS), a maternal vaginal sample or maternal skin sample was collected after birth, together with a saliva sample from the mother. Neonates born by Spontaneous vaginal delivery (SVD) or Caesarean Section (CS) were then followed longitudinally by sampling of the saliva to measure the infant oral microbiota collected at birth (1 week) and at 4 weeks, 8 weeks, 6 months and 1 year and 2 years of age. We used high-throughput sequencing of V4-V5 region 16S rRNA amplicons to assess the microbiota of all samples from mother and their infant.

#### **Results**

We found that the neonatal oral microbiota immediately after birth was influenced by birth mode, but by 2 years of age there was no effect. The infant oral microbiota

at 2 years of age is distinct, as demonstrated by its composition and beta diversity separation when compared alongside the earlier ages of the infant oral microbiota.

## **Conclusion**

We conclude that the infant oral microbiota still continues to undergo change and development from 1 year to 2 years of age.

## **Abstract:**

### **3. Cross sectional study.**

#### **Objectives**

The main objectives of this study were to describe and compare the microbiota of 1) deep dentinal lesions of deciduous teeth of children affected with S-ECC and 2) the unstimulated saliva of these children and 3) the unstimulated saliva of caries-free children, and to compare microbiota compositional differences and diversity of taxa in these sampled sites.

#### **Methods**

We compared the oral microbiota of children affected by severe early childhood caries (S-ECC) to that of caries-free children, by high-throughput sequencing of 16S rRNA amplicons. We compared the salivary microbiota of those with S-ECC (n=68) and without caries (n=70), and also investigated the caries microbiota of deep dentinal lesions of those children with S-ECC.

#### **Results**

Using two established metrics (Bray Curtis dissimilarity and UniFrac distance); we found that the caries microbiota was distinct from that of either of the saliva groups (caries-free & caries-active) when bacterial abundance was taken into account. However, when the comparison was made by measuring only presence and absence of bacterial taxa, we found that all three microbiota types separated. While the alpha diversity of the caries microbiota was lowest, the diversity difference between the caries samples and saliva samples was statistically significant ( $p < 0.001$ ). The major phyla of the caries active dentinal microbiota were Firmicutes (median abundance value 33.5%) and Bacteroidetes (23.2%), with *Neisseria* (10.3%) being the most



abundant genus, followed by *Prevotella* (10%). The caries-active salivary microbiota was dominated by Proteobacteria (median abundance value 38.2%) and Bacteroidetes (27.8%) with the most abundant genus being *Neisseria* (16.3%), followed by *Porphyromonas* (9.5%). Caries microbiota samples were characterized by high relative abundance of *Streptococcus mutans*, *Prevotella*, *Bifidobacterium* and *Scardovia* species

## **Conclusions**

We identified distinct differences between the caries microbiota and saliva microbiota, with separation of both salivary groups (caries-active and caries-free) whereby rare taxa are highlighted.

# **Chapter 1**

## **Introduction**

1. Longitudinal study: Birth to 1 year of age.
2. Longitudinal study: Birth to 2 years of age.
3. Cross Sectional Study.

## **Introduction:**

### **1. Longitudinal study: Birth to 1 year of age.**

The human microbiome is made up of a rich environment of microbes and has been described as ‘a forgotten organ’ (O’Hara and Shanahan, 2006). These microbes play an important role in modifying human health and disease (Ma et al., 2012; Wade, 2013; Xu and Gunsolley, 2014). Advances in molecular methods analysing the microbiome have allowed us to study various body sites in great depth, with the most extensively studied microbiota site in infants being the gut microbiota (Azad et al., 2013; Hill et al., 2017; Penders et al., 2006). As the infant grows the gut microbiota develops and matures from birth until 2 to 3 years of age, to establish a more adult-like ‘stable’ microbiota compositional state (Blaser, 2014; Yatsunenko et al., 2012). In recent years there has been a surge of interest studying the effect of birth mode and feeding on the infant microbiota from birth until 2 to 3 years of age, to gain an understanding of the importance this period plays in programming the microbiota and innate immune system for later life. While the establishment of the gut microbiota composition has been demonstrated to be affected by birth mode, cessation of breastfeeding and weaning onto solids (Azad et al., 2014, 2013; Bergstrom et al., 2014; Bokulich et al., 2016; Grönlund et al., 1999; Hill et al., 2017; Huurre et al., 2008; Penders et al., 2006), there are fewer studies demonstrating these effects on the oral microbiome of the infant (Boustedt et al., 2015; Dominguez-Bello et al., 2010; Lif Holgersson et al., 2011).

The initial microbial exchanges between mother and infant at birth and shortly thereafter are fundamental to the infant microbiota, as these early colonizers play an important role in the development of the neonate’s immune system and long term in

the activity and function of the microbiota (Mueller et al., 2014; Scholtens et al., 2012). During vaginal delivery, the newborn infant is colonised by the mothers vaginal fluid microbiota and intestinal microbiota, whereas the microbiota of those infants born via C-section share a close resemblance to the skin microbiota of the mother (Dominguez-Bello et al., 2010; Huurre et al., 2008; Lupp and Finlay, 2005; Mueller et al., 2017). C-sectioned delivery has been shown to have an effect on the microbiota composition and this change is associated with medical complications such as coeliac disease, asthma, autism and obesity (Biasucci et al., 2008; Curran et al., 2015; Decker et al., 2010; Kero et al., 2002; Neu and Rushing, 2011). C-sectioned delivery is often medically necessary due to maternal or fetal complications and can be accompanied by antibiotic administration before and after birth, which can also impact the microbiota (Bokulich et al., 2016; Gyte et al., 2014; Ledger and Blaser, 2013). While the C-section rates worldwide are increasing (Vogel et al., 2015) and because of the increased risk of complications associated with C-section, there is increased focus investigating the effect birth mode has on the newborn microbiota with studies exploring the possibility of re-colonising the newborn born by C-section, with swabs of the mothers vaginal fluid, in an effort to mitigate any disruption in the loss of these important early microbial colonizers of the infant intestine (Dominguez-Bello et al., 2016).

Birth mode is known to influence the oral microbiota in infants at three months of age (Lif Holgerson et al., 2011) and infants born by vaginal delivery have a higher oral microbial diversity than C-sectioned infants in the first 6 months post birth (Boustedt et al., 2015). A higher abundance of health associated species of *Streptococcus* and *Lactobacillus* were detected in the oral cavity of vaginally delivered infants (Nelun Barfod et al., 2011), while infants delivered by C-section

acquired *Streptococcus mutans* nearly 12 months earlier than vaginally delivered infants (Li et al., 2005). The influence of birth mode on the oral microbial composition of infants has been demonstrated, however saliva samples were taken from infants after birth at mean age of 8.25 months (Barfod et al., 2012), 3 months (Lif Holgerson et al., 2011), and at 1, 3 and 6 months (Boustedt et al., 2015) without taking vaginal or skin samples from the mother, based on mode of delivery. There are some limitations in these studies, particularly with sampling of the infant at only one time point (Lif Holgerson et al., 2011; Nelun Barfod et al., 2011), while the strength of the study by Boustedt et al. (Boustedt et al., 2015) was increased by longitudinal sampling within 2 days of birth to 6 months, which allowed examination of change in the oral microbiota over time. Studies investigating the impact of feeding modality (breast or formula) on the oral microbiome have also demonstrated microbiota compositional changes (Holgerson et al. 2013; Romani Vestman et al. 2013). *Lactobacillus* species such as *L. grasseri* were detected at higher abundance in breast fed infants, compared to formula fed infants (Lif Holgerson et al., 2013) and these oral lactobacilli have antimicrobial properties and probiotic qualities (Vestman et al., 2013).

Despite the increased work studying the oral microbiome of children and adults (Aas et al., 2005; Jiang et al., 2013; Lazarevic et al., 2009; Nasidze et al., 2009), there is need for more in depth research to investigate the oral microbiota from birth of the edentulous neonate, where to our knowledge there are only a few studies using high throughput sequencing to examine the neonatal oral microbiota (Cephas et al., 2011; Chu et al., 2017; Dominguez-Bello et al., 2010). In addition to this, the use of high throughput sequencing is needed to examine the oral microbiota by longitudinal

sampling of the neonate from birth through to older age, taking into account factors such as eruption of teeth, feeding modality at birth (breast or formula) and introduction of solid food. Understanding the oral microbiota at birth, prior to eruption of teeth and longitudinal sampling may provide us with vital information on the evolution of the oral microbiome, birth mode influence and potential early indicators of disease risk (caries) in these infants.

In this prospective study, we employed 16S rRNA gene amplicon Illumina sequencing to investigate the microbiota of the mother across different body sites at birth (vagina/skin and oral) and the oral microbiota of their infants at birth forming mother-infant pairs, followed by longitudinal sampling at five time points until 1 year of age (1 week, 4 weeks, 8 weeks, 6 months/24 weeks and 1 year of age). All infants were from a single geographical area (Cork, Ireland), full-term delivery, initially breastfed for 4 weeks minimum and born by either Spontaneous Vaginal delivery (SVD) or Caesarean section delivery (CS). The main objectives of this study were to describe and compare the microbiota of 1) the different body sites of the mother (vagina, skin, oral cavity) with and that of their infant (oral cavity), 2) to identify if there is any influence on mode of delivery on the oral microbiota of the infant, 3) to identify if breast feeding and the length of breast feeding has an impact on the oral microbiota of the infant and finally 4) to describe the oral microbiota changes over time as the infant develops and grows from birth to 1 year of age (1 week, 4 weeks, 8 weeks, 6 months/24 weeks and 1 year of age).

## **Introduction:**

### **2: Longitudinal study: Birth to 2 years of age.**

Detailed information on the development of the infant oral microbiota from birth to an older age is scarce. In this study we extend our previous analysis of the infant oral microbiota changes from 1 year of age to 2 years of age, in the same cohort of infants. While others studies have discussed the infant oral composition at a mean age of 8.25 months (Barfod et al., 2012), 3 month old infants (Lif Holgerson et al., 2011), and at 1, 3 and 6 months (Boustedt et al., 2015), we are unaware of studies investigating the oral microbiota development from birth until 2 years of age, together with birth mode influence of the maternal microbiota.

From 6 months of age, solid foods are typically introduced, deciduous teeth on average begin to erupt, and oral hygiene practices begin. By 2 years of age, the infant is now a toddler and milestones which are under development include walking, speech and ability to self feed, while weaning from bottle or breastfeeding has typically ceased (CDC, 2016; WHO, 2016). Within the first few years of life, these developmental milestones can affect the microbiota of the infant by introduction of objects into the mouth, hand contact with floor and outside surfaces especially during the crawling and walking stages.

The influences of these factors on the gut microbiota of the infant have been studied in great detail (Fallani et al., 2011; Koenig et al., 2011; Laursen et al., 2016, 2017). The infant gut microbiota reaches stability similar to an adult- like microbiota by 3 years of age (Yatsunenko et al., 2012). However, there are limited studies investigating the oral microbiota of an infant and if stability in the oral microbiota is

reached at a similar age. Other factors which could be potential influencers of the infant oral microbiota changes include the introduction of solid foods, development of dentition and oral hygiene practices, and is another area which is underexplored (Johansson et al., 2010; Lif Holgerson et al., 2015; Milgrom et al., 2000; Zaura et al., 2014). Studies investigating the oral microbiota of toddlers younger than 3 years of age mainly include studies determining the oral microbiota composition of children with and without caries (Barfod et al., 2012; Hughes et al., 2012; Kanasi et al., 2010; Lif Holgerson et al., 2015; Milgrom et al., 2000; Xu et al., 2014). These studies include comparisons between those with caries and without caries. Enabled by advanced sequencing methods, there has been analysis of a ‘healthy’ oral microbiome of children aged 2-6 years (Kanasi et al., 2010), 3-6 years old (Z. Ling et al., 2010), 3-4 years old (Jiang et al., 2016; Ma et al., 2015), 3-7 years old (Jiang et al., 2014) and less than 30 months old (Xu et al., 2014), all performed as cross sectional studies to compare against the oral microbiota of children with caries.

Species associated with a ‘healthy’ oral microbiome in children include *Streptococcus oralis*, *Haemophilus parainfluenzae*, *Neisseria* species, *Fusobacterium* species (Ma et al., 2015), including *Fusobacterium periodonticum* (Jiang et al., 2016). Genera associated with a ‘healthy’ oral microbiome include *Fusobacterium*, *Capnocytophaga*, *Leptotrichia* and *Porphyromonas* (Xu et al., 2014). Species richness and diversity has been shown to increase in the oral cavity of infants as they age from 3 months to 3 years of age (Lif Holgerson et al., 2015). This study by Lif Holgerson et al. (Lif Holgerson et al., 2015) supported previous research findings that the oral microbial composition changes in richness and diversity with increased age, with increased frequency of anaerobes in the oral cavity



(Könönen et al., 1999). The ‘healthy’ salivary microbiome of children with deciduous dentition was found to be composed mainly of Firmicutes, with a higher abundance of Proteobacteria (Crielaard et al., 2011). The salivary microbiome at 3 years of age demonstrated complexity, with the salivary microbiota continuing to undergo maturation until adulthood (18 years of age) (Crielaard et al., 2011), unlike the intestinal microbiota where stability is already reached by 3 years of age (Yatsunenko et al., 2012). The studies noted above have investigated the oral microbiota of children on average between 3-7 years of age, but as mentioned previously there are no studies that we are aware of that have assessed the oral microbiome of infants from birth until 2 years of age, with their maternal samples (saliva/skin/vagina) from birth.

We conducted a large cohort study and employed 16S rRNA gene amplicon Illumina sequencing to investigate the microbiota of the mother across different body sites at birth (vagina/skin and oral). The development of the infant oral microbiota was observed from birth until 2 years of age (at 6 time points), born full-term by separate birth modes (CS & SVD). We hypothesised that oral microbiota changes would be evident as the infant grows from birth, until two years of age, and the influence of birth mode would not be evident by 2 years of age. The main objectives of this study were thus to describe the development and changes in the infant oral microbiota from birth to 2 years of age and to identify if there was any influence of mode of delivery and the maternal microbiota (vagina/skin and saliva) on the oral microbiota of the infant at 2 years of age.

## **Introduction:**

### **3. Cross sectional study.**

Dental caries is the “single most common chronic disease of childhood” (National Call to Action to Promote Oral Health. Rockville, MD: U.S. Department of Health and Human Services, Public Health Service, 2003) and affects 60-90% of all school children (World Health Organisation, 2012). Severe early childhood caries (S-ECC) is an aggressive form of dental caries and is defined by any sign of smooth surface caries in children younger than 3 years of age. It is also classified as any smooth surface of an antero-posterior deciduous tooth that is decayed, missing (due to caries), or filled smooth surfaces in children between 3 and 5 years old, and the presence of a dmft index score of  $\geq 4$  (age 3),  $\geq 5$  (age 4), or  $\geq 6$  (age 5) (American Academy of Pediatric Dentistry (AAPD), 2008).

S-ECC is a destructive and common dental infection (Gussy et al., 2006), and when it progresses, it can cause acute pain and sepsis, and potential tooth loss when left untreated (Finucane, 2012). Poor dental health in early childhood can influence growth and cognitive development by interfering with quality of life, nutrition, concentration and school participation (Gussy et al., 2006; Martins-Júnior et al., 2013). S-ECC can have an impact on the primary dentition (Tanner et al., 2011b) and in turn is a risk factor for caries of permanent teeth (Alm et al., 2008; Dülgergil and Colak, 2012; Li and Wang, 2002). Because of the young age of the children, S-ECC is difficult to treat successfully in the dental chair (Almeida et al., 2000; Graves et al., 2004; Tanner et al., 2011a). These children frequently require treatment under general anaesthesia (Albadri et al., 2006), which increases treatment costs (McAuliffe U, Kinirons M, Woods N, 2017), and these affected children are at a

higher risk of developing recurrent caries (Almeida et al., 2000; Berkowitz et al., 2011; Ng et al., 2014).

Dental caries has been investigated for many years using selective culture-based methods, and the role of *Streptococcus* species and the presence of *Lactobacillus* species have both long been recognized as playing substantial roles in dental caries (Beighton, 2005; Borgström et al., 1997; Hughes et al., 2012; Pearce et al., 1995; Rosebury, 1932; Van Houte, 1994; Yang et al., 2010). However, recent advances in molecular methods have allowed scientists to study the microbiology of oral disease with greater power, with technologies (Nyvad et al., 2013) such as 16S rRNA gene amplicon sequencing showing that the microbiology of dental caries is much richer than previously believed.

The oral cavity has been shown to harbour more than 700 bacterial taxa, with one third of these described as non-culturable *in vitro* (Paster et al., 2006; Paster and Dewhirst, 2009), and two thirds belonging to cultivable species (Dewhirst et al., 2010). Although there is compositional variation between sample sites taken from the oral cavity, a ‘core’ microbiome in health has been identified (Zaura et al., 2009). Movement away from this core and the associated alteration in microbiota composition and its role in the development of oral disease is a key area of oral health research. Such studies have also demonstrated that oral disease is not due to an isolated organism such as *Streptococcus mutans* causing caries, but is more polymicrobial in nature (Dewhirst et al., 2010; Gross et al., 2012; Nyvad et al., 2013; Paster et al., 2006; Paster and Dewhirst, 2009; Rosebury, 1932; Zaura et al., 2009). Studies have identified *Scardovia wiggsiae*, *Bifidobacterium*, *Veillonella*, *Granulicatetta*, *Scardovia*, *Fusobacterium*, *Prevotella* and *Actinomyces* as potential contributors to ECC evidenced by their altered abundance in the caries microbiota

(Almeida et al., 2000; Li et al., 2007; Li and Wang, 2002; Z. Ling et al., 2010; Nasidze et al., 2009; Nyvad et al., 2013; Obata et al., 2014; Paster et al., 2006; Xu et al., 2014).

To understand the microbiology of dental caries, it is helpful to use the combined findings from molecular and culture-based studies (Aas et al., 2008; Corby et al., 2005; Z. Ling et al., 2010; Munson et al., 2004; Tanner, 2015; Tanner et al., 2011b) because molecular methods, when compared to culture-dependent methods have been shown to underestimate the proportions of certain phyla such as *Actinobacteria* (Munson et al., 2004; Schulze-Schweifing et al., 2014). Studies have shown differences in the oral microbiota in children with caries and those that are caries free (Beighton, 2005; Li et al., 2007; Z. Ling et al., 2010; Tanner et al., 2011b). *Streptococcus mutans* is found at higher levels at early stages of caries (Gross et al., 2010), while *Lactobacillus* spp. are associated with disease progression of caries and *Scardovia* species have been isolated from dentinal caries and have previously been associated with having a role in the advancement of deep caries in S-ECC (Becker et al., 2002; Mantzourani et al., 2009; Munson et al., 2004; Tanner et al., 2011b).

Limited data are available by molecular methods on the microbiota of advanced deep dentinal caries and pulpal infections of deciduous teeth, knowledge of which could aid in the development of anti-bacterial medicaments in pulp therapy of these affected teeth. Gram-negative species have been identified in deep dentinal caries of ECC affected teeth (Aas et al., 2008; Corby et al., 2005), and are present in deep pulpal infections of primary teeth (Gomes et al., 2013; Hoshino, 1985; Ruvierre et al., 2007; Tavares et al., 2011; Topcuoglu et al., 2013; Triches et al., 2014). Chalmers and colleagues (Chalmers et al., 2015) studied the microbiota of exposed vital pulp chambers and carious lesions of deciduous teeth, and found that these vital pulps

were dominated by phylum Firmicutes and phylum Actinobacteria. Rôças et al. (Rôças et al., 2016) used next-generation sequencing approaches to study the microbiota of deep dentinal caries of permanent teeth with irreversible pulpitis, and showed that Firmicutes was the most abundant phylum, followed by phyla Actinobacteria and Proteobacteria.

In the present study, we employed 16S rRNA gene amplicon sequencing to compare the microbiota of the deep dentinal lesions of S-ECC affected deciduous teeth, and saliva of these caries-active children, with the saliva of caries-free children. The main objectives were to describe and compare the microbiota of 1) deep dentinal lesions of deciduous teeth of children affected with S-ECC and 2) the unstimulated saliva of these children and finally, 3) the unstimulated saliva of caries-free children and compared compositional differences and diversity of taxa in these sampled sites.

## **Chapter 2: Literature Review**

The oral microbiome of children in health and disease and the maternal influence before, during and after birth on the microbiome of the neonate.

## 2.1 Abstract

The presence of an extra organ ‘the microbiome’ throughout life has been shown to have a major influence on human health and response to disease risk. The initial microbiota transfer does not begin when we are born, but prior to this, during pregnancy when the maternal microbiota can have an impact on the growing fetus before seeding the newborn at birth. Until recently, it was believed that the environment *in utero*, which encompasses the growing fetus was sterile, and during birth the fetus is first exposed to a wave of microbial contamination. In this review I present multiple studies which have examined the microbiota compositions of the placenta, vagina and skin and their influences on microbiota acquisition of the newborn before and during birth. I also discuss current research studies of pregnancy and birth mode impact on the oral microbiota of newborns and of edentulous infants. Furthermore, I discuss factors which can influence the oral microbiota after birth such as feeding mode (breast feeding or formula feeding), antibiotic intake and finally discuss the microbiota associated with dental diseases such as Early Childhood Caries (ECC).

## 2.2 Introduction

The human body is a complex frame of internal and external cells, which make up the unique structure of tissues (epithelia, connective tissue, nervous system, and muscles) and different organs. There are approximately 79 organs of the human body and the human body consists of 10 to 100 trillion microorganisms, (Cani and Delzenne, 2011), with the vast majority of these microorganisms residing in the gastrointestinal tract (Sender et al., 2016). Previous estimates of bacteria cells to human cells were in the order of 10:1, while recent analysis have shown that the number of bacterial cells to human cells is found to be in the same ratio of 1:1, with a total weight of about 0.2kg (Sender et al., 2016). The term ‘microbiome’ was initially given by Joshua Lederberg “to signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease” (Lederberg and Mccray, 2001), and more recently the ‘microbiome’ has been labelled as ‘the forgotten organ’ (O’Hara and Shanahan, 2006).

With the launch in 2007 of the ‘Human Microbiome Project’ (HMP) by the US National Institute of Health (NIH) (Human Microbiome Project Consortium, 2012; Turnbaugh et al., 2007) there has been increased interest in studying this ‘superorganism’ within all sites of the human body, both in health and disease. The HMP studied two distinct geographic locations in the United States, with 242 healthy males and females screened, in 15 and 18 body habitats respectively. This large study showed that the each body habitat was unique (Human Microbiome Project Consortium, 2012) and the bacterial diversity was much more varied than previously believed.



Following on from this study, there have been a large number of studies researching the microbiome with projects focused initially on identifying a 'healthy' microbiome, and secondly studying the role of the microbiome in disease (Blaser, 2014; Lloyd-Price et al., 2016). Disturbances of the microbiome known as 'dysbiosis', has been linked to diseases such as type II diabetes (Qin et al., 2012), obesity (Cani, 2013; Devaraj et al., 2013; Turnbaugh et al., 2006), colorectal cancer (Tremaroli and Bäckhed, 2012), inflammatory bowel disease (Qin et al., 2010), irritable bowel syndrome (IBS) (Kassinen et al., 2007) and dental caries (Jiang et al., 2014).

The human intestine is intensively studied and is colonised with an estimated  $10^{13}$  to  $10^{14}$  microorganisms, with the most abundant phyla being *Bacteroidetes* and *Firmicutes* (Eckburg et al., 2005; Gill et al., 2006). Research has focused on looking at the causative factors of disease, with factors such as age, diet and antibiotic intake all shown to have an impact on microbial compositional (Claesson et al., 2012, 2011; Francino, 2016). Other areas of microbiota research include body sites such as the oral cavity (Dewhirst et al., 2010; Zaura et al., 2014), the placenta (Aagaard et al., 2014; Cao et al., 2014) and the role of the birth mode in shaping the microbiome of the developing neonate (Chu et al., 2017; Dominguez-Bello et al., 2010; Lif Holgerson et al., 2011; Rutayisire et al., 2016). With birth mode influencing the neonatal microbiome (Dominguez-Bello et al., 2010; Lif Holgerson et al., 2011), each body habitat is distinct in its composition (i.e. skin, gut, oral cavity).

Within the oral cavity there appears to be high microbial diversity, which is to a great degree site and individual specific (Aas et al., 2005; Nasidze et al., 2009). The Human Oral Microbiome Database currently includes 772 microbial species,

with 57% of these species named and 13% unnamed but cultivated ([www.homd.org](http://www.homd.org)). The use of high-throughput sequencing of 16S rRNA amplicons and other culture independent methods has generated a deeper understanding of the microbiota composition in the oral cavity. In this review the placental, amniotic fluid, vaginal, skin microbiome are discussed, along with their influences on the growing fetus, and their role during birth. Post birth I discuss the oral microbiome development together with the influence of feeding modality (breast or formula feeding), antibiotic intake on the oral microbiome of infants. I also review the oral microbiome maturation from the newborn edentulous oral cavity through to childhood as the deciduous dentition emerge, with a focus on health and disease risk factors. While bacteria, viruses, fungi, archaea and protozoa are habitants of the oral microbiome (Wade, 2013), viruses, fungi, protozoa and archaea will not form part of this review.

## 2.3 Methods for studying the Oral Microbiome

Research into the microorganisms within the oral cavity began back in 1663, when Antoni van Leeuwenhoek examined plaque taken from teeth under the microscope. He identified ‘little animals’ representing the first identification of oral bacteria (van Leeuwenhoek, 1683). Until the advent of culture independent methods in the early 1980s a variety of methods were used to study the microbiota composition of the oral cavity. These included culture, microscopy, biochemical analysis and immunofluorescence staining (Beighton et al., 2004; Hoshino, 1985; Kanasi et al., 2010; Maiden et al., 1997; Nyvad et al., 2013; Paster and Dewhirst, 2009; Wade, 2011). Bacterial culture was the main method for studying bacterial communities in the oral cavity, with particular focus on dental diseases such as dental caries (Beighton and Lynch, 1995; Marchant et al., 2001; Marsh, 1994; Rosebury, 1932).

Before the launch of The Oral Microbiome project, around 280 species were identified by culture and with the development of culture independent methods, there is now estimated to be around 1000 species in the oral cavity (Dewhirst et al., 2010; Olsen, 2015; Paster and Dewhirst, 2009). One study proposed that there may be several thousand species in the oral cavity (Keijser et al., 2008), second to the colon in complexity (Human Microbiome Project Consortium, 2012). It must be taken into account that some species in the oral cavity may be transient in such an open environment and some microbes may not be permanent residents. The oral microbiota composition includes a diverse range of obligate aerobes and facultative and obligate anaerobes (Aas et al., 2005; Paster et al., 2006). Certain oral bacteria have strict growth requirements and are slow growing, strict

anaerobes and therefore when sample taking and growing these cultures, strict procedures must be followed to avoid any bias and a misunderstanding of oral microbiome composition (Aas et al., 2005; Paster et al., 2006).

The development of culture independent methods has supported the identification of microbes that may have been missed by culture, through the strict and complex growth requirements of certain oral bacteria. As of late 2017, 32% of taxa in the oral cavity are uncultivable ([www.homd.org](http://www.homd.org)) (Chen et al., 2010), and this figure would be even lower if we had a better understanding of their strict growth requirements. . While anaerobic culture plays a role in detection of microbes which instigate and propel dental caries, molecular methods (cloning and sequencing analysis) have unfortunately been shown to under represent their presence (Munson et al., 2004). This highlights the combined need for dual analysis in some studies, in combination with more advanced techniques to fully dissect the oral microbiome in health and disease.

The main methods of molecular identification of bacteria are PCR based methods (quantitative PCR), DNA-DNA hybridization methods (16S RNA based microarrays) and 16S rRNA amplicon sequencing methods (Paster and Dewhirst, 2009). The use of high-throughput sequencing of the 16S rRNA gene amplicons, which has both conserved parts and variable regions (n=9), with length of around 1500bp is a major advancement on this technique. These regions are used for identification of the bacteria present, using PCR primer sets, which target and anneal to these variable regions on the 16S rRNA gene (Claesson et al., 2010; Schloss et al., 2011). The most recent platforms after the original 454 pyrosequencing technology used for sequencing of 16S rRNA amplicons are the Illumina MiSeq and HiSeq platforms (Caporaso et al., 2012; Lin Liu et al., 2012).

The most critical step for accurate sequencing of 16S rRNA amplicons is the decision on primer choice (Schloss et al., 2011). Primer choice is important as bias can be introduced by amplifying one area, whilst aiming to get maximum coverage of the targeted bacteria, and under amplifying or missing other targets as a result of primer design (Claesson et al., 2010; Schloss et al., 2011). A large study of 175 primers and 512 primer pairs found that only 10 could be recommended as broad range primers (Klindworth et al., 2013) while use of primers targeting the V4-V5 hyper variable region has been shown to be most comparable, with the use of platforms Illumina MiSeq and HiSeq platforms (Fouhy et al., 2016).

With the availability of increased read length of 300bp using Illumina MiSeq sequencing of the amplifiable 16S V1-V3 region, researchers have identified a richer and more diverse oral microbiota, using a more time efficient and cost effective method (Zheng et al., 2015). A study of periodontal disease in adults focussed on the V1-2 and V4 regions of the 16S rRNA gene (Griffen et al., 2012). An increasing number of studies are targeting the V3-V4 region, with increased read length of up to 460bp using Illumina Miseq sequencing (Zheng et al., 2015). The computer analyses for managing the data from the 16S rRNA amplicon gene sequencing are done using software including QIIME, Mothur, USEARCH, SPINGO with statistical analysis completed using R (Allard et al., 2015; Caporaso et al., 2010; Schloss et al., 2009; Team RDR, n.d.).

The HMP examined the microbiome composition of nine intraoral sites (buccal mucosa, hard palate, keratinized gingiva, palatine tonsils, saliva, subgingival and supragingival plaque, throat and tongue dorsum) from around 200 individuals and identified over 600 taxa, with a high diversity within the oral cavity sampling sites

(Dewhirst et al., 2010; Human Microbiome Project Consortium, 2012; Zhou et al., 2013). The large data compiled from this work facilitated the development of the Human Oral Microbiome Database (HOMD) (Chen et al., 2010; Dewhirst et al., 2010). This database was developed in 2010, using 16S rRNA to identify all genome sequences (HOMD, [www.homd.org](http://www.homd.org)) and provides a resource for the identified oral taxa which are given a unique Human Oral Taxon (HOT) number, along with a taxonomic design for any unnamed oral bacteria (Chen et al., 2010; Dewhirst et al., 2010).

Although there has been many studies examining the oral cavity both in health and disease (dental caries, plaque, saliva), using 16S rRNA gene sequencing to identify bacteria, the use of meta-transcriptomics (RNA) to assess bacterial activity and metabolism of the oral bacteria is limited, unlike the gut samples (Jorth et al., 2014; Turnbaugh et al., 2010). Using both methods could be complementary to one another in the study of the oral microbiota in health and disease states (Jorth et al., 2014). Metagenomic sequencing of the whole community DNA by shotgun sequencing avoids the PCR step that may introduce bias and sequences the whole pool of microbial genomic DNA in the sample which allows one to study specific genes. However this requires specific tools and detailed computer analysis to analyse the data and is more expensive (Gilbert et al., 2011; O'Toole and Flemer, 2017; Sharpton, 2014). While studies are continuing to use these methods to study the oral microbiota, the technology is constantly evolving, with studies always striving to adapt to these technical improvements and explore the dynamics within the oral cavity.

## 2.4 Pregnancy and microbiome changes *in utero* on the growing fetus.

During pregnancy, the body goes through a number of major anatomical, physiological, hormonal, and metabolic and biochemical changes. As early as the turn of the 20<sup>th</sup> century (Tissier H, 1900) it was believed that the womb was a sterile environment which housed and protected the growing fetus, with the newborn first exposed to microbes at birth directly from the mother's birth canal (Funkhouser and Bordenstein, 2013). It was thought that for a healthy pregnancy to develop it was essential for the fetus to grow in a sterile environment and the presence of bacteria internally was a serious risk to the fetus. If bacteria were to enter the body, it was believed that the only way bacteria entered was by ascending up from the vagina (Goldenberg et al., 2000). The hypothesis of a fetus born germ-free has since been questioned (Perez-Muñoz et al., 2017) with studies reporting microbes present in the umbilical cord blood, amniotic fluid (DiGiulio, 2012; DiGiulio et al., 2008; Mendz et al., 2013), foetal membranes (Steel et al., 2005) and the placenta (Aagaard et al., 2014).

Furthermore, the mode of delivery of the infant, C-section or vaginal delivery, has an influence on the microbiota of the newborn child (Biasucci et al., 2010; Dominguez-Bello et al., 2010). A change from a vaginal delivery to a C-sectioned delivery of a newborn has been shown to be hugely influential by disturbing the microbiota transfer from mother to infant, and this disturbance is associated with medical complications such as coeliac disease, asthma, autism and obesity (Biasucci et al., 2008; Curran et al., 2015; Decker et al., 2010; Kero et al., 2002; Neu and Rushing, 2011). The discovery of these complications associated with C-sectioned delivery has increased research on the influence of birth mode on the

neonatal microbiota with researchers exploring the area of re-colonising the newborn born by C-section, with swabs from the mothers vaginal fluid, in an effort to replenish any important early microbes potentially lost by this disruption (Dominguez-Bello et al., 2016). In the next section, I discuss early colonisation and exchanges between mother and infant during gestation and the influences of birth mode, feeding practices and early microbial exchanges on the microbiome of the newborn.

#### 2.4.1 Early colonisation of the fetus.

The hypothesis that the growing fetus is protected in a sterile environment has been challenged by studies examining the stool of infants born to mothers whom have been taking probiotics during pregnancy (Rautava et al., 2012a; Schultz et al., 2004). Schultz et al. administered probiotics containing *Lactobacillus rhamnosus* to six pregnant mothers and by morphological and molecular analysis, the probiotic was found in the stool samples of all 6 infants at 1 and 6 months of age (Schultz et al., 2004). Supporting this evidence of vertical transmission from mother to the fetus, was a study by Rautava et al. (Rautava et al., 2012a). Probiotics *Bifidobacterium lactis*, *Bifidobacterium lactis* with *Lactobacillus rhamnosus* GG (LGG) or placebo were administered to 43 mothers during pregnancy. The microbial DNA of probiotic origin was identified in the amniotic fluid and placenta of these women (Rautava et al., 2012a). The data provided by both of these studies and supported by animal models (Jiménez et al., 2008) indicate vertical transmission from mother to fetus and shared exchanges between the growing fetus and its mother.



#### 2.4.2 Placental Microbiome

The placenta has two main components; the foetal placenta (chorion frondosum), and the maternal placenta (Decidua basalis) (Wassenaar and Panigrahi 2014). The placenta connects the growing fetus to the uterine wall, to allow the fetus to develop by providing nutrients and oxygen, and removing waste products from the fetus via the mother's blood supply. Studies of the placental organ are limited, however since the development of the advanced sequencing of 16S rRNA amplicons and the launch of the HMP, there has been surge in researching the microbiology of the placenta, its surrounding environments, and the influence of these microbial communities on pregnancy outcome.

A healthy pregnancy was believed to begin with a sterile placenta and sterile encasement of the growing fetus, but recent research has suggested the placenta to be rich and diverse in its microbiota composition (Aagaard et al., 2014; Stout et al., 2013). Microbes have been identified in the placenta and in the foetal membrane of both full-term and preterm subjects sampled (Steel et al., 2005; Stout et al., 2013). Before the launch of metagenomic studies, a study in 2013 by Stout et al. found evidence of bacteria localising in the placental samples. They performed a large cross sectional morphological study of 195 pregnant women to identify bacteria within samples from the basal plate of the placenta of each patient, in term and preterm deliveries (Stout et al. 2013). Stout et al. hypothesized that the cells in the basal plate of the placenta, at the maternal foetal interface, may be a source of microbes associated with negative pregnancy outcomes. Using histopathology and histochemical analysis they found that 27% of the placentas studied had evidence of bacteria within the cells of the placenta sampled; both gram negative and gram positive with all ranges of morphology. The percentage

of intracellular bacteria in found in the placental basal plates of spontaneous preterm deliveries before 28 weeks was 54.5%, and was more than double than that of term spontaneous deliveries (26.7%). However beyond 28 weeks delivery, this difference was not significant (Stout et al., 2013).

The presence of oral microbes in the placenta were also identified by Steel et al. with bacteria evident in over two thirds of foetal membranes sampled after preterm premature rupture of membranes (PPROM) or after a preterm labour, using Fluorescence in situ hybridisation (FISH) (Steel et al., 2005). Sweeney et al. identified a high abundance of *Ureaplasma* spp. associated with chorioamnionitis in both preterm and term placental samples (Sweeney et al., 2016). Chorioamnionitis is inflammation of the foetal membranes (amnion and chorion) due to an intra-amniotic infection (IAI). Prince et al., found that those preterm subjects with severe chorioamnionitis had high abundances of *Ureaplasma parvum*, *Fusobacterium nucleatum*, and *Streptococcus agalactiae* within the placental membranes (Prince et al., 2016). Using whole genome shotgun sequencing, Prince et al. compared the placental membrane microbiome and cord blood cytokine levels of term gestations, with and without chorioamnionitis and preterm with and without chorioamnionitis. They found increased levels of inflammatory cytokines in the cord blood samples of preterm subjects, and high levels of oral and urogenital bacteria (*Ureaplasma parvum*, *Fusobacterium nucleatum* and *Streptococcus agalactiae*) they hypothesise could be a factor that increases risk of preterm birth (Prince et al., 2016).

*Ureaplasma* species are the most common pathogens associated with preterm birth and have been identified in the amniotic fluid and vagina of patients with preterm labours (DiGiulio, 2012; DiGiulio et al., 2015; Kacerovsky et al., 2015;

Kasper et al., 2010; Kikhney et al., 2017; Romero et al., 2014b; Yoon et al., 1998). Using high-throughput sequencing a study of the placental microbiome by Aagaard et al. (Aagaard et al., 2014) characterized the placenta as harbouring a 'unique microbiome'. However the presence of a 'unique' microbiome in the placenta has recently been challenged due to high levels of bacterial contamination in the DNA extraction kits (Lauder et al., 2016). (See Table 2.1 for summary of placental metagenomic studies).

Placental biopsies from healthy mothers (n=320) of full-term births were examined by 16S ribosomal DNA-based and whole-genome shotgun (WGS) metagenomics (Aagaard et al., 2014). The taxa and the genes identified were compared to previous microbiome analysis of other body sites (oral, skin, nasal airway, vaginal and intestinal microbiomes) from the HMP database (Aagaard et al., 2014; Human Microbiome Project Consortium, 2012). This comparison demonstrated that the placenta harboured taxa Firmicutes, Proteobacteria, Bacteroides and Fusobacteria, and found similarities between these the microbes within the placenta to those isolated from the oral cavity (Aagaard et al., 2014; Human Microbiome Project Consortium, 2012; The Human Microbiome Project Consortium, 2012). The similarity of the placental microbiome to that of the oral cavity further supports the strong link between the oral microbes of periodontal disease with preterm birth, and poor pregnancy outcomes (Ercan et al., 2013; Goldenberg et al., 2008; Offenbacher et al., 1998). This suggests spread of periodontal pathogenic bacteria to the fetus (Fardini et al., 2010; Han et al., 2006) while a blood borne route is the most likely pathway for bacteria to travel to the placenta (Goldenberg and Culhane, 2006; Han, 2011; Amanda L Prince et al., 2014).

If these periodontal microbes have the ability to travel in the bloodstream and reach the amniotic fluid and placenta of the growing fetus, the question remains as to why these oral bacteria are the main causative pathogens to accelerate preterm birth, and how these microbes avoid the immune system and target the developing fetus. The oral microbe *Fusobacterium nucleatum* has been located in the amniotic fluid (Bearfield et al., 2002) and the placenta (Aagaard et al., 2014; Blanc et al., 2015) of pregnant women, and this bacterium has been shown to cause increased vascular permeability, allowing for the growth of other pathogenic bacteria such as *Escherichia coli* (Fardini et al., 2010).

**Table 2.1.** Recent microbiome studies investigating the placenta, amniotic fluid, vagina, oral microbiome and birth mode influence on the neonatal microbiota.

Study	Sampling site	Pregnancy status	Outcomes
(Han et al. 2006)	Amniotic fluid	Amniotic fluid	<i>Bergeyella</i> spp. was detected in the sub gingival plaque of the patient and amniotic fluid but not in her vaginal tract.
(Zhou et al. 2007)	Vagina	Vaginal communities Caucasian v Black women (non-pregnant)	Vaginal communities different among these racial groups: lactobacilli lower in black women v Caucasians
(DiGiulio et al. 2008)	Amniotic fluid	Amniotic fluid	The amniotic cavity of women in preterm labour harbours DNA of a greater diversity including microbes: <i>Sneathia sanguinegens</i> , <i>Leptotrichia amnionii</i> and an, uncultivated bacterium
(Ravel et al. 2011)	Vagina	Non pregnant	African-American women have a vaginal microbiome more likely not dominated by <i>Lactobacillus</i> .
(Human Microbiome Project Consortium 2012)	Vagina, skin, oral cavity and other body sites	Non Pregnant	All body sites characterised.
(Aagaard et al. 2012)	Vagina	Pregnant	Diversity and species richness reduced in pregnancy with dominance of <i>Lactobacillus</i> .
(Stout et al., 2013)	Placenta	Term & preterm Placental basal plates	Intracellular bacteria at higher levels in placental basal plate associated with preterm deliveries. No difference was noted between placental basal plate of preterm and term deliveries.
(Aagaard et al. 2014)	Placenta	Pregnant v Human microbiome data base (non pregnant)	Placenta microbiome most similar to Oral microbiome (containing <i>F. nucleatum</i> )
(Hyman et al. 2014)	Vagina	Pregnant; preterm birth	Change in microbial composition did not correlate with PTB

(Romero, Hassan, Gajer, Tarca, Fadrosh, Bieda, et al. 2014)	Vagina	Pregnant women with Preterm births. Case control study.	The composition of the vaginal microbiota changes as pregnancy advanced, with differences were not noted in those that had a spontaneous preterm delivery
(Walther-Antônio et al., 2014)	Vagina	Pregnant: longitudinal	Vaginal microbiome is one of low diversity and high stability during pregnancy
(Romero, Hassan, Gajer, Tarca, Fadrosh, Nikita, et al. 2014)	Vagina	Pregnant and non-pregnant	Difference in the stability and composition between Non Pregnant and Pregnant vaginal microbiome.
(DiGiulio et al. 2015)	Vagina, gut, saliva	Pregnant: samples taken before and after delivery. Included preterm deliveries. Case control study	Microbiota composition and diversity during pregnancy remained stable. There was an abrupt change in vaginal composition after birth,
(MacIntyre et al. 2015)	Vagina	Pregnant: longitudinal study: during pregnancy	The vaginal microbiome changes dramatically after birth, during pregnancy the vaginal microbiome shows low diversity.
(Prince et al. 2016)	Placenta	Pregnant :preterm	Placental microbiome changed with the alteration in severity of chorioamnionitis, and this in PT subjects there was higher abundance of urogenital and oral bacteria.
(Bokulich et al. 2016)	Stool, vaginal, rectal swab from mothers	During Pregnancy and after birth	Birth mode, feeding practice and antibiotic intake influence the newborns acquisition and structure in the first 2 years of life.
(Dominguez-Bello et al. 2016)	Mode of delivery (vaginal, skin, oral microbiome)	Pregnant: samples taken from mother (skin, vaginal, saliva) before birth and from newborn infant after birth (saliva, fecal)	Birth mode influences the initial microbiota of infant between multiple sites of infant (skin, saliva, fecal).
(Chu et al., 2017)	Mode of delivery (at delivery and 6 weeks after birth:Nares skin, saliva, vagina, fecal)	Pregnant women at delivery and their newborn at birth and 6 weeks after	Microbiota structure in infant driven by body site, not by delivery

## 2.5 Influence of birth mode and maternal microbiota on the neonatal microbiota.

### 2.5.1 Vaginal Microbiome

The vaginal microbiome in pregnancy plays a very important role in both the maternal health and also that of the growing fetus health outcomes. In the early nineteenth century the presence and importance of *Lactobacillus* species was noticed within vaginal secretions (Thomas, 1928). Thomas described what he saw as “a gram positive non-spore forming rod, sometimes quite long and slender, with square or very slightly tapering ends occurring singly or in chains” (Thomas, 1928).

Studies of the bacteria present in the vagina, similar to other body sites have been limited because many taxa are unculturable and certain bacteria remained undetected. With recent advances in culture independent approaches, there has been a shift and improvement in our understanding of the vaginal microbiome both in health and disease, and particularly during pregnancy (Fettweis et al., 2012a; Huang et al., 2014). (See Table 2.1 for summary of metagenomic studies). The HMP along with other studies have identified microbiota variance within the sample location points of the vagina itself (lower, upper vagina and cervix) (Kim et al., 2009). Other studies are focusing on longitudinal analysis to record this diversity within the vagina over time, with a particular focus on any evidence for any dynamics within the vaginal microbiota (Fredricks 2011; Xiao et al. 2016).

Using high-throughput sequencing of 16S rRNA amplicons and whole genome shotgun (WGS) sequencing, studies of the vaginal flora of healthy pregnant (gravid) and of non-pregnant (non-gravid) women have advanced significantly.

These studies have coincided with the increase of research focusing on vaginal flora with preterm and PPROM (preterm pregnant rupture of the membranes) affected mothers (Fettweis et al., 2012a). In the following sections I discuss the vaginal microbiome of non-pregnant (non-gravid) women, and pregnant (gravid) women and also discuss the changes within the vaginal flora during pregnancy which can lead to preterm births (see Figure 2.1). The importance of the vaginal microbiota is highlighted, in particular for women who suffer from pregnancy complications as a result of imbalances of their vaginal microbiota.

#### 2.5.1.1 Vaginal Microbiota: Non-gravid

The vaginal microbiome is unique and undergoes changes in its community from birth to puberty to menopause (Hickey et al., 2012). There are changes associated with both menstrual cycles and sexual activity and hormones are said to play a role in these changes (Brotman et al., 2014; Hickey et al., 2012; Johnson et al., 1985; Lorenz et al., 2015). The vaginal microbiome was one of the body sites examined as part of the Human Microbiome Project (HMP) (Human Microbiome Project Consortium, 2012). Following on from this initial work by the HMP was the development of ‘The Vaginal Human Microbiome Project’ at Virginia Commonwealth University (VCU) (Fettweis et al., 2012a, 2012b). This project studied the vaginal microbiome of women and focused on the microbiota changes within the vaginal microbiome when exposed to infection or physiological conditions such as the menopause (Fettweis et al., 2012a). Similar to the Human Oral Microbiome Database (HOMD), a 16S rRNA reference database of taxa found in the vaginal microbiome has been developed, along with a method developed to classify species present termed STIRRUPS (Species-level Taxon



Identification of rDNA Reads using a USEARCH Pipeline Strategy) (Fettweis et al., 2012b).

The HMP established that the vaginal microbiome is of lower diversity compared to other body sites such as the oral cavity (Dewhirst et al., 2010; Human Microbiome Project Consortium, 2012). The vaginal microbiota is inhabited by more than 200 bacterial species, with a dominance of *Lactobacillus* which outweighs other microbes during the reproductive years of the female. *Lactobacillus* were first cultivated and identified from vaginal secretions in the late 19<sup>th</sup> century, and believed to play a role in protecting the vaginal environment from harmful microorganisms (Doderlein, 1928). These bacteria produce lactic acid, lowering the pH to an average pH of 4 +/- 0.5 (Tevi-Benissan et al., 1997) and producing bacteriocins (Dover et al., 2008) playing a role in protecting the vagina, and also protecting the unborn fetus by inhibiting pathogenic bacteria growth (Goplerud et al., 1976; Moberg et al., 1978).

Culture independent methods have identified different five vaginal microbial communities evident in healthy non-pregnant women of reproductive age, four of which are dominated by one of the following *Lactobacillus* species: *Lactobacillus iners*, *Lactobacillus crispatus*, *Lactobacillus jensenii*, and *Lactobacillus gasseri* (Ravel et al., 2011). The five groups labelled as ‘community state types (CST’s)’ (CST I to CST IV). The fifth group is characterised by low levels of *Lactobacillus* species, with increased presence of anaerobic microbes including *Prevotella*, *Gardnerella*, and *Sneathia*. The predominant species in a healthy vagina has been identified as *L. crispatus* (Antonio et al., 1999), but with the availability of culture independent methods, studies have found *L. iners* present in very high abundance although previously undetectable through culture studies (Falsen et al., 1999;

Zhou et al., 2004). Ravel et al. found that *L. iners* was detectable in nearly 85% of 396 healthy women sampled, followed by *L. crispatus*, *L. gasseri* and *L. jensenii* in descending order. They also detected that women whose lower genital tract microbiota was dominated by *Lactobacillus*, the pH was typically 4–4.5 (Ravel et al., 2011). When the genital tract microbiota was not dominated by *Lactobacillus*, the pH was >5.5 (Ravel et al., 2011). The low pH together with high *Lactobacillus* levels have an important role to play in resistance of HIV transmission (Borgdorff et al., 2014; Mirmonsef and Spear, 2014). Bacteriocins are antimicrobial peptides, produced by *Lactobacillus* species, causing breakdown of the membranes of antagonistic microbes. *Lactobacillus* species do not cause vaginal irritation and have a role to prevent bacterial infections of the vagina such as Bacterial Vaginosis (BV) (Dover et al., 2008; Klaenhammer, 1988).

Bacterial vaginosis (BV) is an infection of the vagina as a result of change in the vaginal microbiota composition, which affects millions of women every year, and is associated with a two-fold increased risk of preterm delivery (Hillier et al., 1995; Leitich et al., 2003). BV is associated with a shift from high abundances of *Lactobacillus* species to a more polymicrobial community (Onderdonk et al., 2016). When *Lactobacillus* species are in low abundance other anaerobic bacteria can thrive in a slightly higher pH (5.3-5.5). Genera such as *Prevotella*, *Gardnerella*, *Sneathia*, *Corynebacterium*, *Anaerococcus* and anaerobic species such as *Gardnerella vaginalis* and *Mycoplasma hominis* have been isolated at this higher pH level and this finding highlights the important protective role *Lactobacillus* plays (Hillier et al., 1995). BV in non-pregnant women is associated with the acquisition of sexually transmitted infections and other chronic health problems, while during pregnancy it is associated with increased risk of preterm

birth (Hillier et al., 1995; Leitich et al., 2003), preterm premature rupture of membranes (PPROMS) and miscarriage (Lamont et al., 2011). Zhou et al, (Zhou et al., 2007) found differences in the composition of vaginal communities in different racial groups (Caucasian and black women), with *Lactobacillus* less dominant in black women compared to Caucasian women. These findings suggest that this vaginal microbiota difference may partly account for the known susceptibility of women in these racial groups to bacterial vaginosis (BV) and sexually transmitted diseases. Ling et al. (Ling et al., 2010) studied the vaginal microbiota diversity and richness of 50 women with BV and compared these to 50 healthy women. They found that there was a higher number of phylotypes associated with BV and three phyla (Bacteroidetes, Actinobacteria and Fusobacteria) were strongly associated with BV and with BV, there is an increase in richness and diversity, compared to the healthy vaginal communities. (Ling et al., 2010; Srinivasan et al., 2012). These BV bacteria are labelled as BV-associated bacteria (BVAB) (Onderdonk et al., 2016).

There is supporting evidence to suggest that BV is caused by a change in bacterial communities from a *Lactobacillus* rich community to a more polymicrobial community with greater species richness and diversity and a higher abundance of anaerobic bacteria (Onderdonk et al., 2016; Srinivasan et al., 2012). What is not fully clear is whether these microorganisms associated with BV are opportunistic causing an increase in the pH or are pathogenic (Ma et al., 2012).

#### 2.5.1.2 Vaginal Microbiota: gravid

Understanding the diversity and composition of the vaginal microbiome in non-pregnant women is an important foundation to have before comparing the role of the vaginal microbiota in pregnant women, in both health and disease states. Studies have found differences between the vaginal microbiome diversity and stability in gravid and non-gravid women (Aagaard et al., 2012; Huang et al., 2014; Prince et al., 2014a; Prince et al., 2014b; Romero et al., 2014b; Walther-António et al., 2014). Of importance to our understanding of the vaginal microbiome is observing these changes and the effect the changes have on the imbalance within the vaginal microbiome community, especially when imbalance can have a negative impact on the growing fetus during pregnancy. Also, because the passage of the newborn through the vaginal birth canal has been shown to have an impact on the neonatal microbiome (Dominguez-Bello et al., 2010; Grönlund et al., 1999; Penders et al., 2006; Turnbaugh et al., 2009) a detailed overview of the vaginal microbiota is discussed, before discussing the role of the vaginal microbiota during birth.

During pregnancy, there are many changes in hormone levels, such as oestrogen and progesterone, along with physiological changes in the vaginal epithelium. It is thought that oestrogen has a role to play in changing the composition of the vaginal microbiome by providing a source of energy for *Lactobacillus* to increase in abundance (MacIntyre et al., 2015). During pregnancy glycogen is deposited within the epithelium of the vaginal wall and when oestrogen levels rise this glycogen is metabolised to lactic acid, maintaining a low pH (<4.5) (Boskey et al., 2001). This low pH prevents the growth of other bacteria (Nasioudis et al., 2015), while after birth oestrogen levels fall 100-1000 fold within the first week after

giving birth (Nott et al., 1976). This illustrates the role *Lactobacillus* has during pregnancy in providing protection to prevent ascending infections, leading to preterm birth (Aagaard et al., 2012).

There have only been a few studies using culture-independent methods to study the vaginal microbiome during pregnancy (Aagaard et al., 2012; Romero et al., 2014c; Verhelst et al., 2004). Some studies have focused on the vaginal microbiome of the mother during gestation, close to delivery and after birth (DiGiulio et al., 2015; MacIntyre et al., 2015; Romero et al., 2014c). Their findings have illustrated the low diversity and high stability of the vaginal microbiome during pregnancy (Aagaard et al., 2012; Hyman et al., 2014; Romero et al., 2014b) and compositional differences between vaginal sampling site (lower, upper vagina and cervix) (Kim et al., 2009).

It is believed that pregnancy causes a shift in the vaginal microbiota to a microbiota of lower diversity and of increased stability dominated by one or two *Lactobacillus* species (Aagaard et al., 2012; Romero et al., 2014c). Aagaard et al. (2012) defined a 'pregnant vaginal microbiome' at 3 sites; posterior fornix, mid vagina & vaginal introitus (Aagaard et al. 2012b). Similar to their methodology with their study of the placental microbiome (Aagaard et al. 2014), Aagaard et al., (Aagaard et al. 2012b) demonstrated that the vaginal microbial community changed as the growing fetus increased in gestational age and also by closeness to the cervix (Prince et al. 2014a; Aagaard et al. 2012b). The microbial communities at the preterm stage (<32 weeks) were found to be less diverse and less complex than those sampled beyond 32 weeks. During pregnancy, both diversity and richness were reduced, with a dominance of *Lactobacillus* species (*L. iners*, *crispatus*, *jensenii* and *johnsonii*). The increase in *Lactobacillus* species

abundance in gravid subjects furthermore suggests a distinct community in gravid versus non-gravid women, and a classification of the vaginal microbiota into different community state types (CSTs) as previously described (Ravel et al. 2011). Walther et al., also showed that during pregnancy microbiota diversity was reduced with higher stability and a high abundance of *L. crispatus* (Walther-António et al., 2014). Romero et al. found that the vaginal microbiota of pregnant women had a higher abundance of *Lactobacillus vaginalis*, *L. crispatus*, *L. gasseri* and *L. jensenii* (Romero et al., 2014c). The dominance of *Lactobacillus* species varies according to ethnic groups with *L. gasseri* absent in black women, and *L. jensenii* present in Asian and Caucasian women (MacIntyre et al., 2015).

To investigate the vaginal microbiota changes before and after pregnancy, studies have included pre-delivery and post-partum samples, and have identified that after birth there is a change and restructuring of the vaginal microbiome community. MacIntyre et al. sampled the vaginal microbiome of 42 British women during pregnancy and again 6 weeks post birth and found Bacilli dominated during pregnancy but after birth, the vaginal microbiome shifted with increased Clostridia and Bacteroidia, and decreased *Lactobacillus* species (MacIntyre et al., 2015). Huang et al. also identified a change in the microbiota diversity levels during the different trimesters and after birth (Huang et al., 2015), while DiGiulio et al. illustrated a decrease in *Lactobacillus* species levels after birth, with an increased abundance of anaerobes like *Prevotella* and *Anaerococcus* species (DiGiulio et al., 2015).

### 2.5.2 Skin Microbiome

The skin is the largest organ of the body and because of its external exposure, it can be colonised by a huge variety of microorganisms, which demonstrate differences in colonisation depending on skin site i.e. retroauricular creases, antecubital fossa, anterior nares (Human Microbiome Project Consortium, 2012). An estimated 1 million bacteria, with hundreds of distinct species, inhabit each square centimetre of skin (Grice et al., 2008). The primary role of the skin is to act as a physical barrier, protecting the body, by renewing itself every 3-4 weeks. The bacterial ecology of the skin can be affected by such factors such as environment, immune system, lifestyle and underlying conditions such as diabetes (Grice and Segre, 2011).

Metagenomic analysis has shown that the profile of skin bacteria is different in moist areas of the skin (navel, groin, behind the knee, inner elbow) versus dry areas of the skin (forearm, buttock, various parts of the arm) (Grice et al., 2009; Grice and Segre, 2011). *Staphylococcus* and *Cornebacterium* species are most abundant in the moist habitats, whereas in dry areas, gram negatives are in abundance, and there is a mixed abundance of phyla Proteobacteria, Firmicutes and Bacteroidetes (Grice et al., 2008). Studies have found variability within habitats and between people (Costello et al., 2009; Grice et al., 2009).

*In utero*, the skin of a fetus is surrounded and protected by amniotic fluid, but minutes after birth the skin of the newborn is colonised by increased microbial numbers by the surrounding microbe-rich air (Capone et al., 2011; Dominguez-Bello et al., 2010; Sarkany and Gaylarde, 1968). As mentioned, delivery mode, environment, hospital workers and equipment can influence the bacteria that

colonise the infant. Early skin-to-skin contact from mother to infant evokes neuron-signals between mother and their newborn infant (Moore et al., 2012) and early skin-to-skin contact of a mother and her preterm neonate, has been shown to have a positive effect on the oral microbiota development of the neonate, with increased *Streptococcus* levels in infants where skin-to-skin contact was undertaken, while levels of *Corynebacterium* and *Pseudomonas* were at increased levels in the oral cavity of those infants whom did not partake in early skin-to-skin contact (Hendricks-Muñoz et al., 2015). During the first few days of life the newborn baby comes into contact with many microbes and matures from a skin microbiota of high numbers of *Staphylococcus* species to one of increased diversity and composition (Capone et al., 2011).

It has been shown that vaginally delivered infants are colonised with bacterial communities most like the vaginal communities of their mothers, whereas infants delivered via C-section have bacterial communities most like their mothers skin microbiota (Dominguez-Bello et al., 2010). This influence was observed in the first 24 hours, although Capone et al. did not see any clear separation with the mode of delivery influence on the microbiota of infants tested, in the youngest group of 1-3 months of age (Capone et al., 2011). The authors suggested that the skin microbiome in early life is very unstable and evolves over the first year to one of increased diversity and perhaps beyond one year of age (Capone et al., 2011).

During the first year of life, the skin undergoes maturation and becomes more adult-like after one year of age (Nikolovski et al., 2008). The infant skin has a thinner epidermis and stratum corneum until at least the second year of life (Stamatas et al., 2011). The infant skin and preterm skin differs from adult skin in



structure and function, and these characteristics poses the preterm skin at higher risk of pathogen attack (Stamatas et al., 2011, 2010). Infant skin is dominated mainly by the phylum Firmicutes, followed by Actinobacteria, Proteobacteria and Bacteroidetes (Capone et al., 2011). Adult skin is dominated by Proteobacteria, followed by phyla Actinobacteria and Firmicutes (Costello et al., 2009; Grice et al., 2009).

A study of preterm skin illustrated an increased abundance of Firmicutes and decreased Proteobacteria compared to term neonates, whom presented with increased *Staphylococcus*, *Corynebacterium* and *Prevotella* levels (Pammi et al., 2017). The high abundance of *Staphylococcus* species on the skin are believed to increase the immunity of the skin, preventing the growth and colonisation of pathogenic species (Iwase et al., 2010).

While birth mode did not seem to affect the diversity of the skin microbiota at birth of preterm infants (Salava et al., 2017) and of term infants (Capone et al., 2011), the influence of birth mode has been identified with C-section infants acquiring the maternal skin microbiota including *Staphylococcus* species (Dominguez-Bello et al., 2010). Because of the interruption in vertical transfer of vaginally microbiota caused by a C-section delivery, this may lead to a different pathway in the succession of bacteria to the newborn infant. As a result, efforts and studies are now focused into how one can restore the beneficial maternal vaginal microbiota to a C-sectioned delivered infant. One such study demonstrated that partial restoration of the maternal vaginal microbes can be accomplished artificially by replenishing the newborn born by C-section with these vaginal microbes in the first month of life (Dominguez-Bello et al., 2016). By studying the skin microbiota newborns, born preterm or full-term, and

examining the changes from birth over time this will aid in development of beneficial skin probiotics and prebiotics for those with skin conditions or poor skin health (Al-Ghazzewi and Tester, 2014; Krutmann, 2009), similar to the increased focus aiming to improve gut health with such beneficial bacteria (Neu, 2014). Because C-sectioned infants have been shown to have a lower diversity within their oral microbiota (Lif Holgerson et al., 2011), it would be important to investigate the potential impact the skin microbiota has on the oral microbiota of the newborn and infant, in particular if any disturbances would lead to a poorer oral health outcome (Barfod et al., 2012; Nelun Barfod et al., 2011).

### 2.5.3 Preterm birth: the role of the oral, placental and vaginal microbiome in Preterm birth.

Preterm births are defined as “babies born alive before 37 weeks of pregnancy are completed”. One quarter of preterm births have been linked with a bacterial invasion into the amniotic fluid and sac (DiGiulio et al., 2008; Goldenberg et al., 2008; Romero et al., 2014a). In 2005, the World Health Organisation estimated that 12.9 million births worldwide were preterm, and in 2014 this figure rose to an estimated 15 million births per year (World Health Organisation report November 2014). Factors contributing to infants delivering less than 37 weeks gestational age are (1) spontaneous labour with intact membranes, (2) preterm premature rupture of the membranes (PPROM) and (3) labour induction or caesarean delivery due to pregnancy complication such as pre-eclampsia (Bobetsis et al., 2006; Goldenberg et al., 2008). Preterm birth is the leading cause of infant mortality and morbidity and linked to 5 to 18% of pregnancies (Li Liu et al., 2012; Romero et al., 2014a).

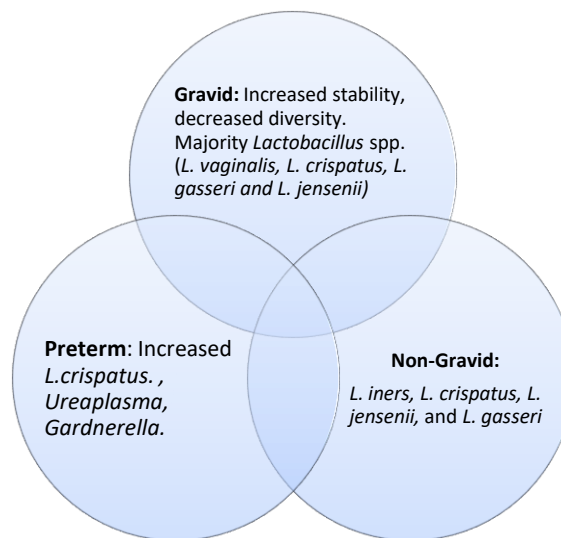
Bacterial infection is associated with pregnancy complications, along with other factors such as diet and excess gestational weight gain (Antony et al., 2015; DiGiulio et al., 2008; Donders et al., 2009; Hillier et al., 1995). Studies have found a strong link between intrauterine infection and preterm labour. It is thought that ascending bacterial infection is a cause of 40-50 % of preterm births (Witkin, 2015), with other correlations linking oral infection and amniotic/placental infection with pregnancy complications, illustrating a bloodborne route for bacterial invasion (Fardini et al., 2010; Hill, 1998).

Understanding the role of changes in the levels of *Lactobacillus* species such as those found in healthy pregnancy vaginal microbiome (*L. iners*, *crispatus*, *jensenii* and *johnsonii*) (Aagaard et al., 2012) are important in understanding the affect this has on the vaginal microbiome and adverse pregnancy outcomes. These decreased levels of *Lactobacillus* species are replaced by high abundance of *Streptococcus*, *Staphylococcus*, *Prevotella*, *Gardnerella* and vaginal candidiasis and is associated with preterm births and inflammatory diseases (Holst et al., 1994; Onderdonk et al., 2016; Wijgert and Jespers, 2017). This change in the structure of the vaginal microbial community has been shown to be linked with preterm birth through culture studies (Fredricks et al. 2005; Hillier et al. 1995), but there are few studies using metagenomics to illustrate this link.

Recently, Hyman et al. (2014) looked to investigate if preterm birth was linked with the vaginal microbiome during pregnancy (Hyman et al. 2014). They found that changes in the vaginal microbiome were linked with preterm birth, and found a higher alpha diversity in those born preterm. A higher alpha diversity was also found in the vaginal community by Romero et al. (Romero et al., 2014b). Hyman et al. (2014) did however find a dominance of *Lactobacillus* in the vaginal communities of all women, with no difference in that of term and preterm mothers, but identified *Bifidobacterium* and *Ureaplasma* in high abundance in two mothers with preterm births.

To identify the risks posed to mothers of early delivery of their newborn baby, it is important to understand the bacterial community in pregnant women during a non-complicated pregnancy and in non-pregnant women. Digiulio et al. identified very low abundances of *Lactobacillus* species in the vaginal microbiota of mothers whom delivered preterm infants, while overall the diversity was higher

in their vaginal microbiome (DiGiulio et al., 2015). The altered vaginal composition early in gestation was a early sign of poor pregnancy outcome for these mothers and linked with preterm birth (DiGiulio et al., 2015). Hyman et al., demonstrated a link between high alpha diversity in the vaginal microbiome and preterm birth (Hyman et al., 2014). Romero et al., did not find such an association (Romero et al., 2014c).



**Figure 2.1. The Vaginal Microbiota composition of non-pregnant (non-gravid), pregnant (Gravid) and vaginal microbiome of mothers whom delivered preterm neonates.** References: (Aagaard et al., 2012; DiGiulio et al., 2015; Fettweis et al., 2014; Huang et al., 2014, 2015; Lamont et al., 2011; MacIntyre et al., 2015; Romero et al., 2014b; Walther-António et al., 2014)

It is believed that oral microbes can travel to the placenta and amniotic fluid via a blood borne route (Fardini et al., 2010; Han et al., 2006), and these findings have challenged the concept of the ‘sterile womb’ (Mueller et al., 2014; Perez-Muñoz et al., 2017). The identification of oral bacteria in the amniotic fluid of patients with preterm labour (Madianos et al., 2013) and in the placenta of pregnant women (Aagaard et al., 2014), have increased the energy of focus to study both the placenta and amniotic fluid with the advancement of technologies. Aagaard et al. showed that the placenta harbours taxa Firmicutes, Proteobacteria, Bacteroides and Fusobacteria, with the bacteria most similar to microbes located in the human oral cavity (Aagaard et al., 2014). Using more simplified methods (Histology & FISH), two research groups have shown that the placental samples were more likely to harbour intracellular bacteria, in women whom have shorter gestations, compared to those that have longer full-term gestations (Steel et al., 2005; Stout et al., 2013). These intracellular bacteria were at a higher level in preterm deliveries (Stout et al., 2013).

Prince et al. detected a high abundance of oral bacteria in placental membrane tissue from preterm subjects with chorioamnionitis (Prince et al., 2016). These bacteria included *Streptococcus* and *Fusobacterium* species while preterm subjects with more severe chorioamnionitis had higher abundances of *Ureaplasma parvum*, *Fusobacterium nucleatum*, and *Streptococcus agalactiae*. Interestingly, *Ureaplasma* species were isolated from chorioamnion tissue and cord blood samples, and found to be associated with chorioamnionitis (intra-amniotic infection) in moderate/late preterm and term placenta (Sweeney et al., 2016).

The finding of oral species in the placental samples is in concordance with isolation of similar oral species from the amniotic fluid examined (Bearfield et al., 2002). Han et al. identified *Bergeyella* species in the amniotic fluid and from the subgingival plaque of the same subjects examined, and when the vaginal cavity was examined this species was not identified suggesting the transmission from the oral cavity to the amniotic fluid (Han et al., 2006). The oral microbe *Fusobacterium nucleatum* has been located in the amniotic fluid (Bearfield et al. 2002) and in the placenta (Blanc et al. 2015; Aagaard et al. 2014) of pregnant women, and this species has been shown to cause increased vascular permeability (Fardini et al. 2010).

There is a longstanding link between periodontal disease and preterm birth (Goldenberg and Culhane, 2006) and it is thought that the presence of periodontal disease can facilitate the transport of microbes from the periodontal site within blood to the placental-fetal interface; stimulating an inflammatory response which can trigger preterm delivery. Gram negative bacteria are the main causes of chronic periodontal disease. Bacteria such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans* (Norskov-Lauritsen and Kilian, 2006) are the main culprits, which trigger significant amounts of proinflammatory mediators, mainly IL-1 $\beta$ , IL-6, PGE2, and TNF- $\alpha$  (Pihlstrom et al., 2005). It is believed that the initiation of an immune response to these bacteria in the placenta and fetal circulation may stimulate preterm birth or cause structural changes in the placenta, which then leads to early birth (Madianos et al., 2013). These periodontal bacteria along with *Tannerella forsythia*, *Tannerella denticola*, and *Capnocytophaga* species have been identified at higher levels in the saliva of mothers who deliver preterm infants, which

suggested a role for these microbes in pregnancy outcome (Chen et al., 2012; Ide and Papapanou, 2013; Santa Cruz et al., 2013).

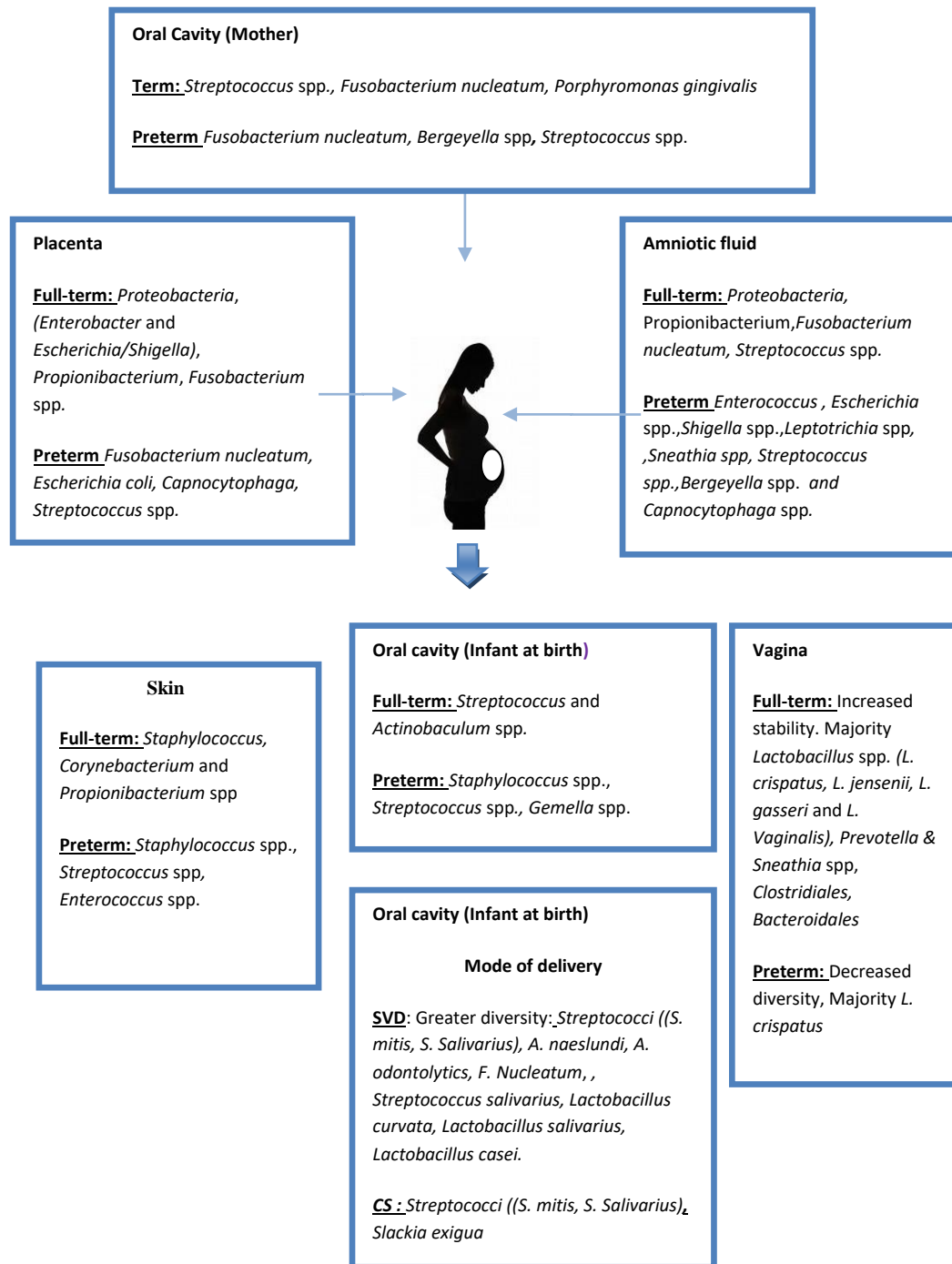
Animal studies have shown that infections with periodontal pathogens increase levels of circulating Interleukin (IL) IL-1 $\beta$ , IL-6, IL-8, IL-17, and TNF- $\alpha$  to induce preterm birth (Ren and Du, 2017). Han et al., have identified pro-inflammatory cytokine (e.g. IL-6) at increased levels in the amniotic fluid of preterm mothers, produced as a response to interuterine infection. Elevated levels of pro-inflammatory cytokines IL-6, IFN- $\gamma$  and TNF- $\alpha$  have been observed in the umbilical cord blood of preterm subjects compared to full-term subjects (Zhong et al., 2016). Contrary to this finding, increased levels of anti-inflammatory cytokines (IL-10) were found in the amniotic fluid of preterm subjects, suggesting a role in dampening the inflammatory response (Greig et al., 1995).

The stimulation of an immune response during pregnancy has been suggested to act as a protective measure to prevent rejection of the fetus and prepares the fetus for life outside of the womb. In addition, antimicrobial peptides (AMPs) are released into the amniotic fluid during pregnancy and act as a chemical barrier for ascending infections. These AMPs are found in the amniotic membranes in the placenta and it is believed that their presence may be the reason why researchers have not found viable bacteria in the placentas that they have examined (Perez-Muñoz et al., 2017).

We have widened our thinking from the concept of sterility within the womb, to infection ascending up from the vagina, to the additional source of haematogenous spread of microbes, in particular from the oral cavity. While studies have identified differences in abundances of the placental bacterial communities in



preterm versus full-term births (Romero et al., 2014c), no significant differences were seen between preterm and term placentas using histology staining of placental samples in another study (Stout et al., 2013). Avoidance of sampling error is critical to prevent any contamination. This is especially important to keep in mind when collecting ‘sterile’ samples from an *in utero* environment is extremely difficult, immediately after delivery of a newborn in a busy hospital setting. Also there has been evidence of contamination of samples from DNA extraction kits, and controls are seen as a necessity alongside samples tested (Lauder et al., 2016). Along with using culture, immunological and morphological studies, the use of current and advancing technologies in microbiome research are needed to validate the microbial signatures in the placenta, oral cavity, amniotic fluid and vagina of pregnant women, to help with the understanding of possible triggers or risk factors for preterm birth.



**Figure 2.2. The microbiota compositional differences in each body site of mother and infant.** The microbiota compositional differences are described for each body site of the mother (placenta, amniotic fluid, oral cavity, skin, vagina) and of the infant after birth (oral). Birth mode influence and microbiota differences between full-term and preterm infants are also illustrated.

**References: Placenta:** (Collado et al., 2016), (Aagaard et al., 2014), (Stout et al., 2013). **Amniotic fluid:**(Collado et al., 2016), (Han et al., 2006), (DiGiulio, 2012; DiGiulio et al., 2008), (Bearfield et al., 2002), (Hill, 1998). **Oral (mother):** (Bearfield et al., 2002), (Dominguez-Bello et al., 2010), (Costello et al., 2013). **Oral (Infant) :**(Boustedt et al., 2015), (Lif Holgerson et al., 2015), (Li et al., 2005), (Lif Holgerson et al., 2011), (Holgerson et al. 2013), (Lif Holgerson et al. 2011a), (Nelun Barfod et al. 2011b). **Skin:** (Dominguez-Bello et al., 2010), (Capone et al., 2011), (Costello et al., 2013). **Vagina:**(Aagaard et al., 2012; Dominguez-Bello et al., 2010; Hyman et al., 2014; MacIntyre et al., 2015; Ravel et al., 2011; Romero et al., 2014b, 2014c; Walther-António et al., 2014).

#### 2.5.4 Influence of birth mode on the neonatal microbiota

Birth is a transition from sterile intrauterine life to the extra uterine existence with continuous exposure to microorganisms. Mother to infant exchange of microbiota plays an essential role in the future of this infant, in terms of immunity and metabolic health (Mueller et al., 2014). The first exposure to micro-organisms in vaginally delivery infants is during the passage through the birth canal, while with Caesarean section (C-section) the micro-organisms are acquired from the skin of the parents (Moore et al., 2012) workers and even the medical equipment (Adlerberth & Wold., 2009). The initial development and maturation of the neonatal microbiome is hugely determined by exchanges at birth, and shortly after. Shortly after birth, the population of early colonizers stabilizes, and their presence plays a very important role in host defence, and in the development of the neonatal immune system, and these early colonisers may play an important long term role in the activity and function of the microbiota (Mueller et al., 2014; Scholtens et al., 2012). See Figure 2.2.

The delivery mode has been shown to also have an effect on the bacterial composition of the gut and oral cavity of the newborn (Biasucci et al., 2010; Dogra et al., 2015; Dominguez-Bello et al., 2010; Lif Holgerson et al., 2011; Rutayisire et al., 2016). During vaginal delivery the newborn infant is colonised by the mothers vaginal fluid microbiota and intestinal microbiota, whereas the microbiota of those infants born via C-section share a close resemblance to the skin microbiota of the mother (Dominguez-Bello et al., 2010; Huurre et al., 2008; Lupp and Finlay, 2005; Mueller et al., 2017). Up to twenty-four hours after birth, infants born via Spontaneous vaginal delivery (SVD) have acquired bacteria most similar to their mother's vaginal community (*Lactobacillus*, *Prevotella* and

*Sneathia* species), while C-sectioned infants have acquired bacteria most similar to their mother's skin community microbiota (*Staphylococcus*, *Corynebacterium*, and *Propionibacterium* species) (Dominguez-Bello et al., 2010). This study also showed that at twenty-four hours after birth, there is no differentiation between the microbiota compositions in the following body sites of the newborn: oral cavity, skin and intestine.

The vertical transmission of microbes from mother to neonate is vital in developing the newborns immunity and metabolic development, but when vaginal delivery cannot occur for medical reasons, C-section delivery is required. There is increasing evidence that highlights how altering or interrupting this microbiota transmission with C-section delivery at birth may be associated with diseases such as coeliac disease (Decker et al., 2010), obesity (Barros et al., 2012; Mueller et al., 2015), asthma (Kero et al., 2002; Thavagnanam et al., 2008), type 1 diabetes (Algert et al., 2009; Cardwell et al., 2008) and allergies (Kristensen and Henriksen, 2016). A systematic review has recently supported the concept that C-section delivery is linked with autism (Curran et al., 2015), and disruption of the vertical transmission of beneficial vaginal microbes to the neonate can have a negative impact, with strategies developed to diminish this impact (Dominguez-Bello et al., 2016; Moya-Pérez et al., 2017).

The effects of birth mode on intestinal bacterial acquisition can be detected in the early weeks and also up to the early years of childhood (Dogra et al., 2015; Grönlund et al.; Hill et al., 2017; Penders et al., 2006; Salminen et al., 2004). Birth mode influence was identified in mouse models after a few days with the gut microbiota of the newborn having a high abundance of *Lactobacillus* from the mothers vaginal canal (Pantoja-Feliciano et al., 2013). C-section delivery has been

shown to have an effect on the development of newborn gut with lower intestinal counts of bacteria and lower diversity in the gut (Azad et al. 2013; Grönlund et al. 1999). The intestinal microbiota can be affected for the first two years of life (Jakobsson et al., 2014). *Bifidobacterium* species have been found a higher levels in the intestinal microbiota of infants born by vaginal delivery (Biasucci et al., 2010; Fanaro et al., 2003; Scholtens et al., 2012), while *Clostridium* and *Lactobacillus* species been found at higher levels in the intestinal microbiota of C-sectioned infants (Rutayisire et al. 2016; Biasucci, Benenati, Morelli, Bessi & Gunther Boehm 2008; Penders et al. 2006). Disturbances to the gut microbiota was evident up to six months after birth in C-Sectioned infants (Grönlund et al. 1999)

In a recent study, the effects of birth mode on the gut microbiota was evident by one week of age in newborns (Hill et al., 2017). Hill et al. identified an increase in Firmicutes levels and lower abundance of Actinobacteria at one week post birth in C-section neonates. They also found that the gut microbiota diversity by 8 weeks post birth of infants born via C-section, became more stable and was more similar to those infants born via vaginal delivery, and this stability was maintained at 6 months post birth (Hill et al., 2017).

While birth mode has an effect on the gut microbiota, the early acquisition of certain microbes via mode of delivery, and the timing of this acquisition can have an effect on weight gain as early as 18 months of age (Dogra et al., 2015). Within the early weeks of life the C-sectioned delivery has been associated with increased risk of childhood obesity (Blustein et al., 2013; Li et al., 2014; Mueller et al., 2017). Interestingly, a high maternal pre-pregnancy weight is also associated with differences in the neonatal colonisation of its gut microbiota in vaginally delivered

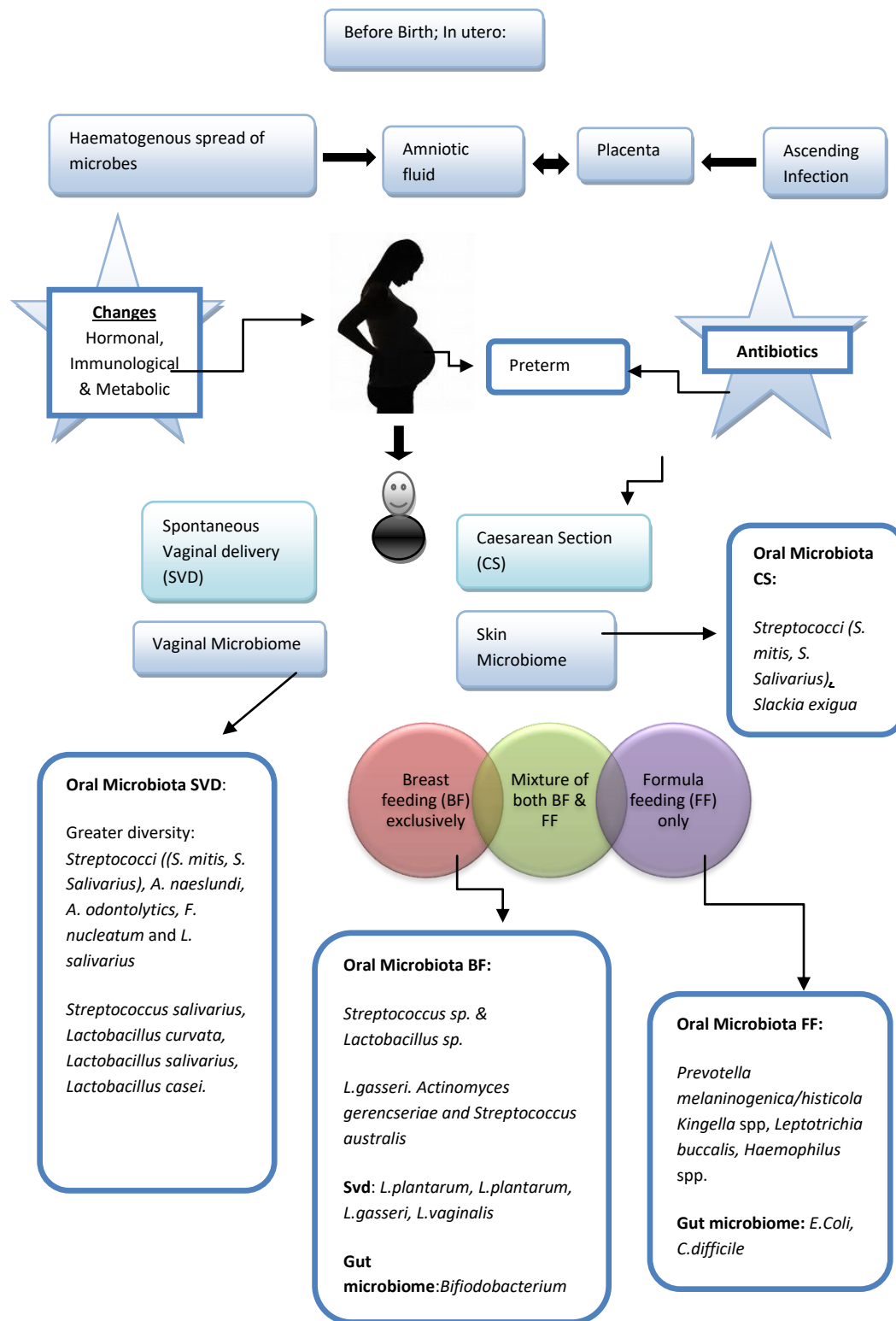
infants but not in those infants born via C-section (Mueller et al., 2016). By inoculating newborn infants born by C-section with vaginal fluid from their mother, these infants can acquire microbes lost via C-sectional delivery (Dominguez-Bello et al., 2016).

The typical age for introduction of solids and weaning from breastfeeding is around 5-6 months. Bergström et al. found changes in the infant gut microbiome typically between 9 to 18 months when breastfeeding stopped, and this was followed by an increase in microbiota diversity, with increased levels of *Lactobacillus*, *Bifidobacterium* and *Enterobacteriaceae* (Bergstrom et al., 2014). At 2-3 years of age, the composition of the gut microbiome during infancy becomes more stable, and more similar to the adult microbiota (Power et al., 2014). Yatsunenko et al. reported differences in the composition of the gut microbiome depending where they lived (Yatsunenko et al., 2012). However all these subjects showed increased diversity with a more adult-like microbiota by 3 years of age (Yatsunenko et al., 2012). While changes in the intestinal microbiota are evident with geographical location, the oral microbiota does not appear to be affected by geographical location (Nasidze et al., 2009).

The influence of birth mode on the infant oral microbiota can be seen as early three months of age (Lif Holgersson et al., 2011). Infants born via vaginal delivery were seen to have more of a similar oral composition to their mothers and a greater diversity than C-sectioned infants in the first 6 months post birth (Boustedt et al., 2015). A higher abundance of health associated species *Streptococcus* and *Lactobacillus* were detected in the oral cavity of vaginally delivered infants (Nelun Barfod et al., 2011), while infants delivered by C-section acquired *Streptococcus mutans* nearly 12 months earlier than vaginally delivered infants

(Li et al., 2005). It is believed that when *Streptococcus mutans* levels are diminished, *Streptococcus sanguinis* colonises around 9 months of age during a discrete "window of infectivity". This colonisation has potential health benefits as this species acts as an antagonist to the cariogenic species *Streptococcus mutans* (Caufield et al., 2000, 1993). The influence of birth mode on the newborns oral microbiota has been demonstrated with the oral microbial communities of the C-section and vaginally delivered infants resembling that of their mothers skin and vaginal communities respectively (Dominguez-Bello et al., 2010). Contrary to these studies, Chu et al. found that the infants gut and oral cavity undergoes change during the first 6 weeks of life, and not until 6 weeks does each body site show uniqueness (Chu et al., 2017). These changes did not seem to be affected by delivery mode (Chu et al., 2017). Studies investigating the influence of birth mode on the oral microbiota are limited. Larger cohorts studying the infant oral microbiota are required to gain further knowledge of the influence of pregnancy, delivery mode, maternal microbiota and early feeding mode on the oral microbiota of the newborn.





**Figure 2.3. Factors influencing the acquisition and development of the infant**

**oral microbiota.** References: (Lif Holgerson et al., 2013, 2011; Nelun Barfod et al., 2011)

## 2.6 Antibiotics and feeding mode (breast or formula feeding) and their influence on the microbiome of the newborn.

The human body and its microbiota are obviously affected by both external and internal influences. The human body is influenced by the environment, diet, age, antibiotic usage, presence of disease, host genetic factors and the immune and stress response. The establishment of the microbiota begins at birth, and is strongly influenced by birth mode (Bokulich et al., 2016; Dominguez-Bello et al., 2010; Madan et al., 2016; Song et al., 2013). After birth, antibiotic usage, geographic location and feeding mode (formula fed or breast fed) can all have a major impact on the developing microbiome. I discuss each of these influences on the microbiome of the newborn, focusing on their impact on the oral microbiome.

### 2.6.1 Antibiotics: during pregnancy and post birth

A single dose of antibiotics can disturb the human microbiota with some evidence to show that the original microbial ecosystem does not return to normal following this course of treatment (Bokulich et al., 2016; Cho et al., 2012; Francino, 2016; Zaura et al., 2015). The use of broad spectrum antibiotics reduces bacterial diversity in the intestine (Greenwood et al., 2014; Modi et al., 2014), although the human microbiome does demonstrate a degree of resilience to antibiotic intake (Relman, 2012). If antibiotics are taken early in life, their effect can be disruptive, and lead to an increased risk of development of diseases such as obesity, inflammatory bowel disease and diabetes later in life (Cho et al., 2012; Cox and Blaser, 2014; Shaw et al., 2010). Mueller et al. found that early exposure to antibiotics in the second or third trimester in C-sectioned infants, posed these children to a 84% higher risk of obesity later in childhood (Mueller et al., 2015).

Increased asthma risk is also associated with a lower diversity as a result of antibiotic intake (Abrahamsson et al., 2014; Goksör et al., 2013).

While one study demonstrated recovery of the intestinal microbiota by 89% within 60 days after stopping the antibiotic, the microbial community did not return to the starting configuration (De La Cochetière et al., 2005). From birth up to 3 years of age, the infant microbiota undergoes maturation and increases in diversity and stability, and if antibiotics are administered from birth or within these early years, the microbiota at this stage is much more vulnerable to disruption (Cox and Blaser, 2014).

Antibiotics may be administered prophylactically to the mother during pregnancy, before birth, during a C-section delivery and also after birth during nursing. Antibiotics are routinely administered to the mother during pregnancy if the mother tested positive with Group B Strep (GBS). GBS can lead to early onset neonatal sepsis after premature rupture of membranes with no sign of labour. If the mother presents with fever, antibiotics are administered to prevent any risk of infection to the growing fetus (Chapman et al., 2014; de Tejada, 2014; Flenady et al., 2013; Wynn, 2016). Newborn infants are more than likely to be given antibiotics when born prematurely, and their mother may be administered antibiotics during delivery. Broad spectrum antibiotics are commonly administered as a prophylactic measure to reduce the risk of infectious diseases during C-Section delivery (Smaill and Grivell, 2014).

Antibiotics account for 80% of all medication prescribed during pregnancy (Bookstaver et al., 2015) and these antibiotics when administered during pregnancy are termed Intrapartum antimicrobial prophylaxis (IAP). Antibiotics

most commonly administered are penicillin and cephalosporin, and both target gram positive and gram negative bacteria (Gyte et al., 2014). With over 40% women receiving antibiotics during pregnancy there is concern about its overuse and its health affects long term (Blaser, 2016; Cho et al., 2012; Ledger and Blaser, 2013). The long term effect of early antibiotic use during pregnancy and at birth has not been studied in great detail, and because of the lack of research, there is concern about the long-term consequences of antibiotic use (Kuperman and Koren, 2016).

Mothers that received antibiotics during pregnancy had a different vaginal microbiota characterised by an increased abundance of *Staphylococcus* species and potential interruption of transfer of beneficial vaginal microbes which were depleted (Stokholm et al., 2014). Infants born to mothers that had received antibiotics during pregnancy had perturbations of their gut microbiota and the effect was not seen until 10, 30 and 90 days after the birth (Arboleya et al., 2015). The preterm infants harboured a reduced abundance in Bacteroidetes and a higher abundance of Proteobacteria (mainly Enterobacteriaceae) (Arboleya et al., 2015). Increases in Enterobacteriaceae abundance in the gut microbiota have been linked with increased risk of diabetes type 1 in children (Soyucen et al., 2014). Early use of antibiotics has been shown to decrease the diversity with lower abundances of Bifidobacteria, and higher abundances of Enterobacteria and Proteobacteria (Fouhy et al., 2012; Greenwood et al., 2014). Interestingly the gut microbiota composition at 8 weeks was different to the composition recorded at 4 weeks, where Fouhy et al. noted a recovery of the reduced *Bifidobacterium* numbers (Fouhy et al., 2012).

While there has been a lot of work exploring the effect of antibiotics on the intestinal microbiome, the impact on the oral microbiome has not received as much attention. As discussed, the majority of women receiving a C-section receive antibiotics prophylactically to prevent per partum infections.

A study by Holgerson et al. reported differences in the infant oral microbiota associated with mode of delivery, but antibiotic intake was not taken into account (Lif Holgerson et al., 2011; Smaill and Grivell, 2014). Of the mothers included in this study, 15 of the 41 mothers received intravenous antibiotics, and this may have had a significant factor based on previous research discussed, but this did not form part of the results or discussion (Lif Holgerson et al., 2011). A recent study, by Gomez-Arango et al. investigated the effect of intrapartum antibiotic exposure on the oral microbiota of the newborn infant (Gomez-Arango et al., 2017). They investigated the source of the initial infant oral microbiome from maternal placental, oral and gut samples. The neonatal oral microbiota was composed of 65.35% of microbes of maternal oral microbiome origin, 3.09% of placental origin, 31.56% of unknown and 0% of maternal gut origin. Intrapartum antibiotics were administered to 64% of all women. The authors found that these antibiotics had an effect on the initial oral microbiome. Infants whose mothers were administered antibiotics had an oral microbiota with a higher abundance of phylum Proteobacteria, while infants whose mothers were not exposed to antibiotics had an oral microbiota higher in *Streptococcaceae*, *Gemellaceae* and order *Lactobacillales* (Gomez-Arango et al., 2017).

The increased presence of the phylum Proteobacteria the gut is seen as a marker of disease and dysbiosis (Shin et al., 2015). This phylum is most abundant in the oral cavity of the deciduous dentition (Crielaard et al., 2011). *Streptococcus* is

viewed as an early colonizer of the oral cavity, with *Streptococcus mutans* evident almost 1 year earlier (17.1 months of age) in C-sectioned infants compared to vaginally born infants (Caufield et al., 1993; Li et al., 2005).

Costello et al. studied the microbiome of the stool, skin and oral saliva samples of six low birth weight infants, of which were 5 born prematurely, at days 8, 10, 12, 15, 18 and 21 days after birth (Costello et al., 2013). All except one preterm infant had received antibiotics only during the first week of life and their oral microbiota demonstrated high levels of Firmicutes, with high abundance of *Staphylococcus* and *Streptococcus*. The preterm infant on antibiotics for the duration of sampling demonstrated a much higher abundance in the oral microbiome of the genera *Mycoplasma* (a novel *Mycoplasma* species) and *Pseudomonas* (*P. aeruginosa*) in comparison to infants whom did not have continued antibiotic treatment (Costello et al., 2013). The peaking of these genera between days 15 to 21 coincided with antibiotic treatment for suspected necrotizing enterocolitis. The presence of these pathogenic microbes in the oral cavity of this infant warrants further study as to its health significance and the possible pathogenic role of these microorganisms (Costello et al., 2013). While research investigating the oral microbiome post antibiotics is limited in infants, in adults when the microbiota of saliva and faecal samples were examined from the same adult, at baseline after exposure to antibiotic and again at 1, 2, 4 and 12 months post treatment, they found that while there was changes in the faecal microbiota composition and oral cavity remained stable throughout those time points mentioned (Zaura et al., 2015).

So overall research investigating the antibiotic effect on the oral cavity in infants has received very little focus and with antibiotic use increasing, along with antibiotic resistance, increased energy on researching this area is needed.

### 2.6.2 Feeding mode (Breast feeding v Formula feeding) impact on the Oral microbiome of the newborn.

An exclusively breastfeeding infant is seen as the gold standard method for feeding the newborn infant. Ireland's breast feeding rates are the lowest compared to other countries, with 46% breastfeeding on leaving hospital in a recent report (HSE: Health Service Breastfeeding Action Plan, 2016). This is compared to rates of 90% in Australia and 81% in the United Kingdom (HSE: Health Service Breastfeeding Action Plan, 2016; McAndrew et al., 2010; Public Health Association of Australia (PHAA), 2010)

Breast milk is rich in beneficial bacteria that colonise the gut of the newborn, increasing diversity of the gut microbiota compared to infants that are formula fed (Azad et al., 2013; Fallani et al., 2010). The bacteria of breast milk mainly includes species of *Lactococcus*, *Enterococcus* and *Lactobacillus* while there is increased interest in the probiotic bacteria isolated from breast milk which includes *Lactobacillus salivarius*, *Lactobacillus gasseri* and *Lactobacillus fermentum* (Lara-Villoslada et al., 2018; Martín et al., 2007; Olivares et al., 2006). Breast milk is made up of Prebiotic oligosaccharides (OS), which stimulate the growth and activity of the microbes in the gut to aid with gut health (Vandenplas et al., 2014). Oligosaccharides that can be difficult to digest and species *Bifidobacterium infantis* (Yoshida et al., 2012) and *Bifidobacterium breve* (James et al., 2016) are required to help break down these oligosaccharides. The oligosaccharide are a source of energy for lactate producing microbes such as *Bifidobacterium*, *Streptococcus*, *Staphylococcus* and *Enterococcus* and these milk oligosaccharides prevent growth of pathogenic microbes by acting as decoy receptors (Bode, 2012). *Bifidobacterium* spp. play a role in protecting against

pathogens, and keep the immune response active in the epithelial cells of the gut. The benefits to the mother and infant have been seen after administering these species to mothers while pregnant and to their infants after birth, with prevention of allergies in the infant (Enomoto et al., 2014; Mikami et al., 2012, 2009).

The aim of formula manufacturing companies is to bring artificially made formula as close to the gold standards of breast milk, by adding OS such as galacto-oligosaccharides, fructo-oligosaccharides and/or probiotics (Vandenplas et al., 2014). Exclusively breast fed infants have a gut microbiota dominated by *Bifidobacterium* (Bezirtzoglou et al., 2011; Harmsen et al., 2000; Penders et al., 2006; Power et al., 2014; Tannock et al., 2013; Turrone et al., 2009). Formula fed infants demonstrate a more varied gut microbiota with lower *Bifidobacterium* levels (Coppa et al., 2006).

It is thought that breastfeeding enhances lactic acid producing bacteria acquired from the mothers vaginal microbiota (Coppa et al., 2006). Previous molecular studies suggested that the *Lactobacillus* present in the infant gut were acquired from the mothers vaginal canal or direct from breast milk (Martín et al., 2007). Recent studies did however find the common gut bacteria *Lactobacillus johnsonii* at higher abundances in the vaginal microbiome of pregnant women than non-pregnant women, suggesting a role for the vaginal community of the mother in shaping the infant gut community (Aagaard et al., 2012). *L. johnsonii* has been suggested to aid with digestion of breast milk and is acquired by the neonate from the birth canal during delivery (Abee et al., 1994; Prince et al., 2014). While these studies have shown the influence of vaginal delivery on acquiring beneficial microbes, a recent study demonstrated the positive effects of breastfeeding



exclusively over four months on the gut microbiome of C-sectioned infants (Hill et al., 2017).

Formula supports the infant gut of development in a much more diverse microbiota than breast feeding (Azad et al., 2013). Levels of *Escherichia coli*, *Clostridium difficile* and *Bacteroides fragilis* are at much lower levels in breast fed, compared to formula fed infants (Penders et al., 2006; Yoshioka et al., 1983). Formula fed infants have a gut microbiota which comprises *Streptococcus*, *Bacteroides*, *Clostridium*, and *Bifidobacterium* (Bezirtzoglou et al., 2011; Harmsen et al., 2000; Yoshioka et al., 1983). Azad et al. studied faecal samples taken at 4 months of age from 24 healthy infants and these formula fed infants showed an increased species richness and increased levels of *Clostridium difficile* in their gut microbiome (Azad et al., 2013). While C-section can lower gut diversity and richness (Azad et al., 2013), breastfeeding has the ability to modify and improve some of these affects (Azad et al., 2016). Allergy risk can be reduced further by administration by breastfeeding and by taking probiotics during pregnancy (Enomoto et al., 2014; Rautava et al., 2012b).

Changes in the gut microbiome based on feeding modality have been noted. The oral microbiome also demonstrates microbiota influences (Holgerson et al. 2013; Romani Vestman et al. 2013).(Summarised in figure 2.3). Both these studies found that the oral microbiota varied if the infant was formula fed versus breast fed, with *Lactobacillus* (*L. gasseri*) detected and at a higher abundance in breast fed infants only (Lif Holgerson et al., 2013). Breast fed infants also demonstrate oral *Lactobacillus* by 3 months of age within the oral cavity, which are not found in the oral cavity of formula fed infants (Lif Holgerson et al., 2013). These oral *Lactobacillus* (*L. gasseri*) have antimicrobial properties and probiotic qualities

and are found at much higher levels in the oral cavity of breast fed infants (Vestman et al., 2013). *L. gasseri* attaches itself to epithelial cells and is present in saliva, and has strong probiotic benefits by inhibiting growth and attachment of pathogens such as *Streptococcus mutans* (Kotzamanidis et al., 2010; Vestman et al., 2013). *L. gasseri* presence in the vaginal microbiome is variable between ethnic groups (MacIntyre et al., 2015). When *L. gasseri* is present in low abundance in the vaginal microbiota of certain ethnic groups, there is a stronger need for these mothers to breastfeed, to counteract the poor levels of these species which may be acquired during birth.

While exclusively breastfeeding is recommended up to 6 months of age, studies have reported that prolonged breastfeeding is a risk factor for ECC (Chaffee et al., 2014; Hong et al., 2014; Kato et al., 2015). Two recent systematic reviews and meta-analysis however both concluded that breastfeeding can protect against dental caries, while both studies recommended that further research is needed in children whom are breastfed after 12 months of age (Avila et al., 2015; Tham et al., 2015). While breast feeding up to 12 months is not associated with an increased risk of caries, a meta-analysis of infants breast fed beyond 12 months found that these children were at an increased risk of dental caries (Richards, 2016). This increased risk of dental caries if breast feeding is continued beyond 12 months of age may be due to other factors such as dentition, poor oral hygiene, frequency of cariogenic food and drink intake and breastfeeding during the night. Nocturnal breastfeeding causes increased risk of caries because of the pooling of milk and thus milk remains in the mouth for a longer duration to attack the dentition and also the protective measure of saliva is depleted as salivary flow is lower at night time (Dowd, 1999).

There has been controversy for many years as to the cariogenic effects of substitute formulas (Bowen et al., 1997), while further studies reported the need for further evaluation to assess the cariogenicity of formula (Chaudhary et al., 2011). There also seems to be lack of evidence supporting the idea that breast milk is cariogenic. It has been found that some breast milk components have an anti-cariogenic effect, inhibiting growth by production of peptides and by interrupting adhesions of *Streptococcus mutans* (Aimutis, 2004; Wernersson et al., 2006), supporting the oral health benefit of breast feeding.

## 2.7 The Oral Microbiome.

### 2.7.1 The oral microbiome of the newborn edentulous infant from birth to childhood.

The oral microbiota of the newborn infant is initially acquired at birth, from the mother, the surrounding hospital environment and its workers, and also from parents, siblings and carers (Cephas et al., 2011; Stahringer et al., 2012; Tanner et al., 2002).

In the early months of life, little is known about the arrival of oral microbes. During the first few days of life, Pearce et al. found that *Streptococcus oralis*, *Streptococcus mitis* and *Streptococcus salivarius* colonize the oral cavity (Pearce et al., 1995). Colonisation by *Streptococcus mutans* was found to occur at the median age of 26 months whilst *Streptococcus sanguinis* colonised during a discrete “window of infectivity” at the median age of 9 months (Caufield et al., 2000, 1993). A study by Wan et al. (Wan et al., 2001) showed the presence of *Streptococcus mutans* in edentulous infants as young as 3 months of age and also demonstrated that preterm infants are 4.4 times more likely to be colonized by *Streptococcus mutans* than are full-term infants (Wan et al., 2003).

Relatively little is known about the timing of arrival of the key microbes found in the oral cavity of children and the key factors involved in the establishment of the oral microbiota, unlike the gut microbiota where these phenomena are well understood (Claesson et al., 2012, 2010). The seeding of the gut microbiota in infants has been shown to be affected by birth mode and feeding practices (Dominguez-Bello et al., 2010; Hill et al., 2017; Penders et al., 2006; Song et al.,

2013), but there is less research looking at the establishment of the oral microbiota from birth.

As mentioned previously, acquiring the oral microbiome by the newborn infant begins in the first few seconds of birth. The mode of delivery has an impact on the acquiring of oral microbiomes from the vaginal birth canal and from skin of the mother when delivered by C-section (Boustedt et al., 2015; Dominguez-Bello et al., 2010; Li et al., 2005; Lif Holgerson et al., 2011; Nelun Barfod et al., 2011). (See Figure 2.3).

Examining the saliva samples of 149 infants (SVD n=96; C-section n=53) within 2 days of birth and again at 1, 3 and 6 months, Boustedt et al. found that birth mode had an effect on the saliva microbiota, with a greater diversity in vaginally delivered infants (Boustedt et al., 2015). Microbes *A. naeslundi*, *A. odontolytics*, *F. nucleatum* and *L. salivarius* were only found among vaginally delivered infants (Boustedt et al., 2015). Dominguez-Bello et al. illustrated the effect birth mode has on the neonatal oral microbiota within twenty-four hours after birth, with transfer of the mothers vaginal microbiota (*Lactobacillus*, *Prevotella* and *Sneathia* species) and skin microbiota (*Staphylococcus*, *Corynebacterium*, and *Propionibacterium* species) to the infants oral cavity if delivered vaginally or by C-section (Dominguez-Bello et al., 2010).

Further work supporting the effects the delivery mode has on the oral microbiota was published by Lif Holgerson et al., who identified that the influence of birth mode on the oral microbiota can be seen as early three months of age (Lif Holgerson et al., 2011). Using Human Oral Microbe Identification Microarray (HOMIM), they studied the oral biofilm (cheek, tongue, alveolar ridges) in

healthy three month old infants. 38 infants were born by C-section and 25 infants were delivered vaginally. The vaginally delivered infants had higher numbers of taxa, and identification of *Slackia exigua* in the oral microbiota of C-section infants only (Lif Holgerson et al., 2011) warrants further investigation. A higher abundance of health associated *Streptococcus* and *Lactobacillus* species have been found in the oral cavity of vaginally delivered infants (Nelun Barfod et al., 2011). Species of high abundance found in vaginally delivered infants included *Streptococcus salivarius*, *Lactobacillus curvata*, *Lactobacillus salivarius*, and *Lactobacillus casei* (Nelun Barfod et al., 2011), while Li et al. found the presence of *Streptococcus mutans* in the oral cavity 16 months earlier in C-Sectioned delivered infants (Li et al., 2005).

While those research studies mentioned have shown the influence of delivery mode on the oral microbiota. Chu et al. observed the influence of birth mode immediately after birth, but this influence on the microbiota of the infant was not identified by 6 weeks after birth. At 6 weeks post birth each body site sampled demonstrated uniqueness (Chu et al., 2017). They found that the oral cavity was mainly composed of *Streptococcus*, *Propionibacterium*, *Lactobacillus* and *Staphylococcus* at birth, but by 6 weeks, there was increased abundance and dominance of *Streptococcus* and *Veillonella* (Chu et al., 2017).

There is evidence to suggest that the oral microbiome is dynamic with changes arising with the eruption of teeth, and also with the shedding of these primary teeth (Xu et al., 2015). Despite this, there are limited research studies using high-throughput sequencing to focus on the oral microbiome of the edentulous mouth of infants and following the oral microbiota of these infants as their teeth emerge

(Cephas et al., 2011; Chu et al., 2017; Costello et al., 2013; Dominguez-Bello et al., 2010).

Before teeth emerge the edentulous infant appears to have a higher abundance of *Streptococcus* (62%) in their oral microbiota compared to their mothers or carers who have a lower abundance of *Streptococcus* (20%) (Cephas et al., 2011). These adults have a higher microbial diversity compared to the infants, although the infants did demonstrate some species richness in the oral microbiota with the presence of *Streptococcus*, *Veillonella* and *Neisseria* before teeth emerge (Cephas et al., 2011). From birth to week 6 after birth, Chu et al. found that the genera in the oral microbiota changed from mixed composition of *Lactobacillus*, *Propionibacterium* and *Streptococcus* to increased abundance of predominance of *Streptococcus* (61%) followed by *Veillonella* (Chu et al., 2017). Crielaard et al. studied the salivary microbiome of children from ages 3 to 18 years, at different stages of teeth eruption and development, between deciduous and permanent dentition (Crielaard et al., 2011). They identified that the deciduous dentition had a higher proportion of Proteobacteria (Gammaproteobacteria, Moraxellaceae) (Crielaard et al., 2011). As the infant increased in age, the levels of Bacteroidetes (mainly *Prevotella*), Veillonellaceae family and Spirochaetes levels increased (Crielaard et al., 2011).

While there is limited research looking at the changes and dynamics over time in a young infant from the edentulous oral cavity until the development of deciduous dentition and permanent successors, there is more research investigating the oral microbiome of adults.

Zaura et al. found a ‘core microbiome in health’ in the oral microbiome of adults (Zaura et al., 2009). Variability within the oral cavity has been found within and among individuals (Huse et al., 2012; Li et al., 2013; Paster et al., 2006; Zhou et al., 2013). The oral cavity microbiota appears to be stable with highest alpha diversity, while the niches within the oral cavity (hard palate, buccal mucosa, gingival, alveolar ridges, subgingival pockets) all demonstrated differences in bacterial richness and diversity (Human Microbiome Project Consortium, 2012). The supragingival plaque had the highest richness, with hard palate having the lowest (Huse et al., 2012). Saliva was reported as the niche with the highest alpha diversity, but lowest beta diversity, highlighting richness and similarity between individuals (Human Microbiome Project Consortium, 2012; Zhou et al., 2013). *Streptococcus*, *Prevotella* and *Veillonella* made up 50% of the salivary microflora in adults when sampled, while 50% of plaque comprised of 6 genera including *Streptococcus*, *Prevotella*, *Veillonella* plus *Fusobacterium*, *Actinomyces*, *Corynebacterium* and *Rothia* (Keijser et al., 2008).

When the oral microbiome of adults was sampled at different time points and sampled, variability between habitats examined alongside the oral cavity was noted (Aas et al., 2005; Nasidze et al., 2009). Individuals showed more variability from the same location and did not demonstrate additional variation when from a different geographical location (Nasidze et al., 2009). Costello et al. found that all body sites demonstrated microbiota stability over time within individuals, but the oral cavity was less variable over time in microbiota than other body sites such as gut or skin (Costello et al., 2009), thus further highlighting the stability of the oral cavity (Zhou et al., 2013).



These studies have demonstrated the oral microbiota to be the most stable, compared to other body sites and at different time points (Human Microbiome Project Consortium, 2012; Lazarevic et al., 2010; Zhou et al., 2013). Temporal variability has been demonstrated when tongue samples were examined over time (Flores et al., 2014). Together, these studies illustrate the stability of oral cavity, but a deeper assessment of variation needs to be examined in detail when it occurs. This is important to see what effects this change has on the structure of the oral microbiome and also if composition of the oral microbiota is a causative factor for this shift. Furthermore, more research is needed to assess the oral microbiome of the newborn infant at birth and changes in the oral microbiota when teeth emerge and as the oral cavity matures.

#### 2.7.2 The oral microbiome & dental caries: Early childhood caries (ECC) & Severe-early childhood caries (SECC).

Dental caries is the “single most common chronic disease of childhood” and affects 60-90% of all school children, Severe early childhood caries (S-ECC) is an aggressive form of dental caries and is defined by any sign of smooth surface caries in children younger than 3 years of age. It is also classified as any smooth surface of an antero-posterior deciduous tooth that is decayed, missing (due to caries), or filled smooth surfaces in children between 3 and 5 years old, and the presence of a dmft index score of  $\geq 4$  (age 3),  $\geq 5$  (age 4), or  $\geq 6$  (age 5) (American Academy of Pediatric Dentistry (AAPD), 2008).

ECC can cause pain, lead to sepsis, potential tooth loss when left untreated, and interferes with the child’s quality of life, nutrition, concentration and school participation (Finucane, 2012; Gussy et al., 2006; Martins-Júnior et al., 2013).

Because of the young age of children when ECC presents, often they are very young to undergo any treatment in the dental chair and there is a higher need for dental treatment under general anaesthesia (Albadri et al., 2006). Unfortunately, more often than not there is an increased risk of recurrent caries at a later stage (Almeida et al., 2000; Berkowitz et al., 2011; Ng et al., 2014) with permanent teeth often affected and thus the requirement for further treatment (Alm et al., 2008; Dülgergil and Colak, 2012; Li and Wang, 2002).

Dental caries has been investigated for many years using selective culture-based methods, and the role of *Streptococcus* and the presence of *Lactobacillus* have both long been recognized as playing substantial roles in dental caries (Beighton, 2005; Borgström et al., 1997; Corby et al., 2005; Hughes et al., 2012; Pearce et al., 1995; Rosebury, 1932; Van Houte, 1994; Yang et al., 2010). *Streptococcus mutans* is seen as the main player in caries progression, with *Lactobacillus* seen as an opportunistic pathogen. Both of these are acidogenic bacteria and thrive on carbohydrate substrates by fermenting them and producing acid (Van Houte, 1994; Yang et al., 2010). Other species associated with caries include non-*mutans* *Streptococci*, *Actinomyces*, *Bifidobacteria*, *Veillonella* and *Scardovia* (Becker et al., 2002; Ma et al., 2015; Mantzourani et al., 2009; Tanner et al., 2011b).

While it has been found that the average prevalence by age at which *Streptococcus mutans* infection occurs ranges from 30% in 3 month old infants to over 80% in 24 month old children, Caufield et al., found that the median age of colonisation by *Streptococcus mutans* was 26 months, during a discrete “window of infectivity” (Caufield et al. 1993). However, a study by (Wan et al., 2001) showed the presence of *Streptococcus mutans* in edentulous infants as young as 3 months of age.

While work has been done to characterise the oral microbiome in health, the main focus of other research groups with the use of new advances in sequencing technologies is to identify any rare taxa that may be driving dental caries disease, such as Early childhood caries (ECC), and its more severe division of Severe-early childhood caries (SECC). The use of culture studies have long been in use before the advent of current sequencing methods (Nyvad et al., 2013; Pearce et al., 1995; Tanner et al., 2011b). Culture studies have focused on selected bacteria groups that are linked to caries, such as *Streptococcus*, *Lactobacillus* and *Veillonella* spp. (Loesche and Syed, 1973; Marchant et al., 2001; Van Houte, 1994). *Veillonella* species are not acidogenic, but support growth of other cariogenic species (Milnes and Bowden, 1985).

Whilst sequencing methods are constantly evolving, culture still has a role to play when there may be several species underrepresented by molecular methods (Munson et al., 2004). Munson et al. found the diversity of species detected by culture comparable to molecular methods, but also reported increased levels of Actinobacteria by culture (Munson et al., 2004). Tanner et al. used anaerobic culture to identify species *Streptococcus mutans*, *Scardovia wiggsiae*, *Veillonella parvula*, *Streptococcus cristatus*, and *Actinomyces gerensceriae* as being associated with SECC (Tanner et al., 2011b). Hughes et al. also found by anaerobic culture of plaque samples from teeth with ECC, higher *Streptococcus mutans* levels, compared to those teeth that were caries free, with lower levels after treatment compared to before (Hughes et al., 2012).

Differences in the microbiota before and after treatment of the caries has also been identified using 16S rRNA microarray and PCR, with identification of ECC associated species *Slackia exigua* and *Scardovia wiggsiae*. After treatment there

was a decrease in *S. mutans*, *Campylobacter gracilis*, *Campylobacter concisus*, *Campylobacter rectus* and *S. mitis* (Tanner et al., 2011a). Using 454-pyrosequencing of plaque samples from children with an average age of 20 months, Xu et al. found that caries active children had plaque microbiota composed of *Streptococcus* and *Veillonella*, while *Leptotrichia*, *Selenomonas*, *Fusobacterium*, *Capnocytophaga* and *Porphyromonas* were seen at higher abundances in caries free children (Xu et al., 2014).

The salivary microbiota compositional changes between caries free and caries active have also been described in older children between ages 3-4, with mixed dentition, but these studies did not find a species specific ‘core’ associated with caries or caries free children, with genera *Streptococcus*, *Prevotella*, *Veillonella*, *Neisseria*, *Rothia*, and *Haemophilus* in saliva of both groups (Jiang et al., 2016). They did identify potential biomarkers of caries associated species *Fusobacterium periodonticus* and *Rothia dentocariosa* (Jiang et al., 2016), with Crielaard et al., identifying *Porphyromonas catoniae* and *Neisseria flavescens* associated with caries free children (Crielaard et al., 2011).

The transition from a deciduous dentition to a mixed-dentition, and then to permanent dentition, is documented by changes in the oral microbiota. The deciduous dentition has high abundances of *Proteobacterium*, but as the dentition profile changes with increased age, Bacteroidetes (*Prevotella*), Veillonellaceae and Spirochaetes all become more abundant (Crielaard et al., 2011).

Although dominant species associated with caries and with a caries-free oral status in children have been noted, it is important to assess the changes that occur to drive these bacteria from health to disease states and also to understand at what

stages during this process do these bacteria come into fruition. One such study has assessed this by examining the different stages of caries progression over time, and comparing to those without caries disease progression (Gross et al., 2012). With a mean age of 24 months, plaque samples were collected at three stages of caries development (intact enamel, white spot lesion and cavitated). They found that as lesion progressed, the diversity of the microbiota decreased. An interesting finding was that not all caries samples were dominated by *S.mutans*, but this was replaced by *S. sobrinus* in one subject and by *S. vestibularis* and *S. salivarius* in the other. Other studies have found that when *S.mutans* was absent, either *Bifidobacterium* spp. (*B. dentium*, *Scardovia wiggsiae*), *Veillonella* species, *Leptotrichia* species or *Lactobacillus* species become dominant and are associated with caries (Aas et al., 2008; Corby et al., 2005; Gross et al., 2010; Z. Ling et al., 2010; Luo et al., 2012; Milnes and Bowden, 1985; Tanner et al., 2011b).

The variation of microbiota based on caries depth (i.e. white spot versus cavitation) was supported with Jiang et al. who identified variation with shifts in the oral microbiota based on caries depth (Jiang et al., 2014). Deep Caries can often progress into the pulp chamber and the bacteria associated with this are a subset of those involved in the caries lesion, with fewer taxa identified within the pulp, and increased *Veillonella* species, with *Veillonella dispar* found in high abundance in pulps chambers of vital exposed deep caries lesions (Chalmers et al., 2015), and these taxa found may be the primary stimulators of caries from dentine into pulp.

## 2.8 Conclusion

In conclusion, it is clear that there is a vast amount of knowledge and microbiota research investigating the role of the maternal microbiota (pre and post birth) and the influence birth mode has on the newborn microbiota structure. The role of the maternal microbiota during pregnancy (i.e. placental microbiome and sterility in utero) was discussed at length in this review, and although analysis of the placental microbiota and analysis of preterm birth does not form part of this ORALMET study, the impact of the oral microbiota on preterm birth, and the identification of microbes in utero, was important to discuss, as this could have a potential impact on the oral microbiota of the infants that we studied from birth.

It is obvious that the influence of birth modality on the gut microbiota of the infant has received far more attention than the oral microbiota of the infant in recent years. The studies investigating birth mode and feeding modality (breast feeding) on the infant microbiota are limited from birth, and thus our knowledge of the potential oral health effects are insufficient. This ORALMET study aims to alleviate this current lack of knowledge by observing and documenting the oral microbiota from birth until 3 years of age.

## Chapter 3

Overall Methodology: Clinical recruitment,  
sample collection and Experimental Techniques.

### **3.1. Recruitment & Sample Collection: Cohort Study (Longitudinal Study).**

#### **3.1.1. Recruitment:**

I aimed to target pregnant women who attended the CUMH for antenatal clinics, in their second and third trimester. Before the recruitment of subjects, I met with the midwives in the CUMH. I gave a presentation on the ORALMET project and the aims and objectives of the study. The logistics of the sample collections and the need for the midwives to support the study was crucial. The exact timing of birth and the need for caesarean section (CS) or spontaneous vaginal delivery (SVD) meant that planning for the spontaneity of birth meant that the sampling packs (including the swabs and consent) were attached to each chart and the contact number given. All women were informed and consented in the CUMH, while awaiting their antenatal visit. Information leaflets were given to mothers to take home, and contacted again before any subsequent visits. Appendix 7.5.

#### **3.1.2. Sample Collection.**

- 1) Depending on the delivery mode, the swab collection differed for the CS and SVD infants.
- 2) If SVD, a vaginal swab was taken within one hour of delivery by the midwives in the labour ward.
- 3) If CS, a skin swab was collected post delivery, with 12 hours.
- 4) A saliva swab was taken from the mother also within 12 hours of delivery.

Pooled unstimulated saliva sample was collected from the floor of the mouth with use of a CatchAll™ collection swab, with hard pack for storage after collection (Cambio UK) (Turnbaugh et al., 2007). This was



rotated in the floor of the mouth for 1-2 minutes, and placed back in the collection tube.

- 5) The collection of the newborn infant's saliva was collected within the first week of birth. A research nurse travelled to collect this saliva sample if living within the 50 km catchment of Cork City area.
- 6) An unstimulated saliva sample was collected from the floor of the mouth with use of a CatchAll™ collection swab, with hard pack for storage after collection (Cambio UK). This was placed on ice and transported to the Lab.
- 7) The remaining samples at 8 weeks, 24 weeks, 1 year, 2 years and 3 years were collected within their own homes by the research nurse and followed the same process as of (6).

### **3.2. Recruitment & Sample Collection: Cross-sectional Study.**

The recruitment process and sequence of sample collection is described for both the caries-free cohort and the caries-active cohorts.

#### **3.2.1. Recruitment: Caries-free Cohort.**

1. Crèches were approached in the Cork City area. Information leaflets and consent forms were given to the parent/guardians.
  - Of the 316 primary schools included nationally in the Department of Education's disadvantaged areas scheme, 31 are based in Cork City. Nine have been designated 'disadvantaged'.
  - Core areas of social deprivation in the city are: Knocknaheeny/Churchfield; Fairhill/Gurranabraher/Farranree; Blackpool/The Glen, Mayfield, Togher and Mahon.
  - The various crèches in the Cork area were identified through Cork City Childcare Company Ltd. The Cork City Childcare Company is one of 33 childcare communities set up around the country under the childcare directorate at the office of the Minister for Children.
  - Samples were taken from children less than five years of age. Children were selected at random.
  - For the caries group, I aimed to collect saliva and decayed caries tissue from the extracted tooth – max 75 samples.
  - For the caries-free group, I aimed to collect saliva only –max 75 samples.

2. After visiting the various crèches, the study aims and objectives were described to the parents and a presentation providing all the background information on the ORALMET study to the parents and guardians of the children was given.

Appendix 7.5.

3. Consent Forms were given to parents/guardians. Consent forms and diet analysis forms were both returned within one week. Appendix 7.2 and 7.3.

4. No Radiographs were taken as part of this study. If any caries was noted during the course of sampling, parents were informed and these children were excluded from the study.

### **3.2.2: Sample Collection: Caries-free Cohort.**

1. All children were consented for the study previous to the day of sample collection.
2. A method of Tell-show-do was used to demonstrate how sampling would be done.
3. Each child sat on a crèche chair, with good natural light.
4. Beginning with the upper right quadrant, and continuing clockwise to the upper left quadrant, and then the lower left and then lower right quadrant, the dentition were counted, and charted.
5. Children's teeth were wiped with a cotton wool roll and sterile gauze square to remove plaque and debris prior to examination which was carried out under natural light, using a standard size 4 mirror and ball ended CPI 'C' probe (Whelton et al., 2006).

6. The mouth was illuminated with a Promed Penlight, which consists of bright concentrated halogen light when natural light was insufficient.
7. The dmft score was recorded along with the dmfs score. Caries was recorded at the level of cavitation into dentine (cavitation level), using the WHO criteria (Whelton et al., 2006; WHO, 1997). The dmft/dmfs was measured, and sample collection was performed. All charting and sample collection was performed by the PhD Candidate.
8. Caries-free children did not show clinical evidence of early pre-cavitation of caries or white spot lesions and had no history of treatment on any tooth surfaces, as defined (Dye et al., 2007).
9. A pooled unstimulated saliva sample was collected from the floor of the mouth with use of a CatchAll™ collection swab, with hard pack for storage after collection (Cambio UK) (Turnbaugh et al., 2007). This was rotated in the floor of the mouth for 1-2 minutes, and placed back in the collection tube.

### **3.2.3. Recruitment: Caries-Active Cohort.**

All recruitment of children affected by caries was performed at a Cork University Hospital Dental Theatre.

- All children were initially visited their local Health Service Executive (HSE) Dental Treatment Centre where clinical examinations were performed.
- The majority of the children referred to General Anaesthetic were of a young age, (1- 16 years of age), dentally anxious and presented with dental

caries; notably ECC. These children were then placed on a waiting list at the Cork University Dental School and Hospital and were scheduled for extraction of their carious teeth, under general anaesthetic.

- All these children referred for general anaesthetic had radiographs taken as part of the examination prior to referral.
- Recruitment and consent were performed on the day of the general anaesthetic. Appendix 7.5.



Figure 3.1. Dental Theatre in Cork University Hospital .This is the location where all the sample collection of the caries-free cohort was performed.

#### **3.2.4. Sample Collection: Caries-Active Cohort.**

All children attended the dental theatre fasting at 7 am-8am. All were consented and checked by both the dentist and anaesthetist before the general anaesthetic. Each child/parent was given the information of the ORALMET study, and explained the objectives of the study along with the sampling that would be done. The diet analysis was also completed on the day of consent and sample collection.

A complete dental examination was completed bedside before the child went under General anaesthetic.

1. Each child sat bedside, with good natural light.
2. Beginning with the upper right quadrant, and continuing clockwise to the upper left quadrant, and then the lower left and then lower right quadrant, the dentition were counted, and charted.
3. Children's teeth were wiped with a cotton wool roll and sterile gauze square to remove plaque and debris prior to examination which was carried out under natural light; using a standard size 4 mirror and ball ended CPI 'C' probe (Whelton et al., 2006).
4. The mouth was illuminated with a Promed Penlight, which consists of bright concentrated halogen light when natural light was insufficient.
5. The dmft score was recorded along with the dmfs score. Caries was recorded at the level of cavitation into dentine (cavitation level), using the WHO criteria (Whelton et al., 2006; WHO, 1997), with the addition of visible non-cavitated dentine caries as referenced by Whelton et al. (Whelton et al., 2006).

6. A saliva sample was collected from the floor of the mouth before the general anaesthetic. If the child did not comply, a saliva sample was taken while the child underwent general anaesthetic.
7. Pooled unstimulated saliva sample was collected from the floor of the mouth with use of a CatchAll™ collection swab, with hard pack for storage after collection (Cambio UK) (Turnbaugh et al., 2007). This was rotated in the floor of the mouth for 1-2 minutes, and placed back in the collection tube.
8. The child then underwent general anaesthetic in the dental theatre (figure 3.1).
9. While the child was anaesthetised, all carious teeth were extracted by a dentist.
10. These extracted teeth were placed in sterile gauze and individually, dentine was excavated from each carious tooth with a sterile spoon excavator and pooled in a sterile eppendorf/collection tube.

### **3.3. Procedure for collection of saliva from each mother/infant.**

1. Explain the procedure
2. Wash your hands
3. Put on disposable gloves
4. Check that the outer packaging has the seal intact.
5. Break the seal.
6. Hold the swab at the upper end of the swab, ensuring that the inner shaft is not handled.
7. Insert swab into patient's mouth, letting it rest on the floor of the mouth allowing for soakage of any pooled saliva present. Leave it rest there for 30 sec.
8. The swab should not be moved around in the mouth during this time.
9. Then rub the swab on the mothers tongue, roof of mouth and left and right buccal mucosa for 10 seconds each
10. Replace the swab into the swab cover.
11. Label the sample bottles with Subject Number, Initials, Sample Number, Date of Collection and tick.
12. Remove disposable gloves and wash hands
13. Immediately after collection, storage tube stored in a cooler bag with ice pack.
14. Saliva sample to be frozen (-20°C) within two hours of collection.



### **3.4. Laboratory Methodology**

#### **3.4.1. Piloting the Methodology: DNA extraction**

DNA extraction Kits used

1. Modified Protocol uses Ready-Lyse (tm) Lysozyme Solution (Epicentre, Cat. No. R1802M) for overnight incubation and MasterPure DNA Purification Kit (Epicentre, Cat. No. MCD85201) (Forsyth Institute, Boston, USA).

2. MoBio PowerSoil™ DNA Isolation kit.

Samples were taken from children (saliva and dental caries), infants (saliva) and skin (mother) to pilot the DNA extraction protocol in the above kits (1-2).

Firstly, DNA was extracted using the above kits (1-2), and PCR was completed on the extracted DNA.

#### **Method:**

**A. Kit 1:** Modified Protocol uses Ready-Lyse(tm) Lysozyme Solution (Epicentre, Cat. No. R1802M) for overnight incubation and MasterPure DNA Purification Kit (Epicentre, Cat. No. MCD85201) (Forsyth Institute, Boston, USA).

This Kit was adapted within the Forsyth Institute (<http://mim.forsyth.org>)

## **Protocol DNA Extraction:**

### **1. Lysis**

1. Suspend sample in 150µl of TE Buffer.
2. Add 1µl of Ready-Lyse Lysozyme to each sample.
3. Incubate at 37°C overnight.
4. Add 150µl of 2X T&C Lysis Solution to each sample and pipette up and down when adding.
5. Add 1µl Proteinase K to each sample.
6. Incubate at 65°C for 30 minutes, vortexing briefly every 5 minutes.
7. Cool the sample to 37°C
8. Place the samples on ice for 3-5 minutes and then proceed to part B.

### **B. DNA Clean-up**

1. Add 175µl of MPC Protein precipitation reagent to 300µl of lysed sample and vortex mix vigorously for 10 seconds.
2. Pellet the debris by centrifugation at 4°C for 10 minutes at  $\geq 10,000 \times g$  in a microcentrifuge. Place on ice immediately after centrifugation.
3. Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.
4. Add 500 µl of isopropanol to the recovered supernatant. Invert the tube 30-40 times.
5. Place on ice for 10 minutes.
6. Pellet the DNA by centrifugation at 4°C for 10 minutes at  $\geq 10,000 \times g$  in a microcentrifuge.

7. Pour off the isopropanol, being careful not to lose the pellet. Use a pipette tip to remove the remaining propanol without dislodging the DNA pellet.
8. Rinse the pellet with 500µl 75% ethanol. Centrifuge briefly if the pellet is dislodged. Repeat rinse with additional 500µl 75% ethanol.
9. Remove residual ethanol.
10. Resuspend the DNA in 25 µl of TE buffer.

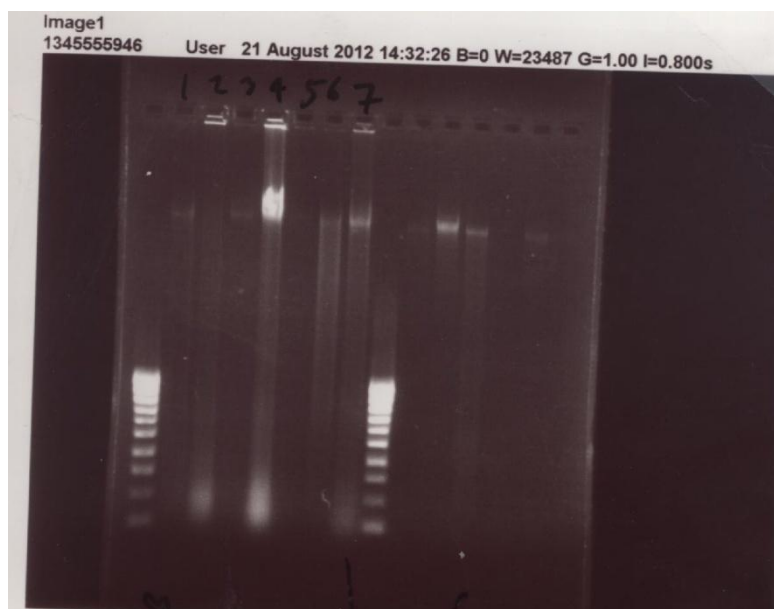
Protocol amended from Epicentre Masterpure<sup>™</sup> DNA purification kit and Epicentre Masterpure<sup>™</sup> Gram positive DNA purification kit.

#### Method:

1. This method was initially used to extract DNA from saliva (fresh, frozen), and from dental caries.
2. Following steps A& B, DNA was quantified using the Nanodrop 1000 (Table 3.1.) (Thermo Fisher Scientific, Ireland).
3. DNA was visualised on a 0.8% agarose gel (Figure 3.2). DNA was then stored at -80°C.

Sample	Date	Nanodrop		
		[DNA] ng/μl	260/280	260/230
1.Saliva swab (frozen)	20.08.2012	207.3	1.74	1.10
2.Saliva fresh (spit)	20.08.2012	245.1	1.55	0.96
3.Saliva frozen (swab)	20.08.2012	208.8	1.83	1.29
4.Dental caries (dentine)	20.08.2012	949.1	1.81	1.28
5.Saliva swab (frozen)	20.08.2012	233.6	1.69	1.20
6.Saliva spit frozen	20.08.2012	178.3	1.70	1.35
7.Dental caries (dentine)	20.08.2012	937.1	1.68	1.11

**Table 3.1.** Nanodrop Results using the DNA extraction kit A: MasterPure DNA Purification Kit



**Figure 3.2.** DNA was visualised on a 0.8% agarose gel. (Lane 1-7 (Samples 1-7(as in Table 3.1))

**B. Kit 2: MoBio PowerSoil™ DNA Isolation kit.**

**Protocol DNA Extraction:**

Extraction of DNA from all samples (vagina, skin and saliva/Oral) was carried out with the MO BIO PowerLyzer® 24 homogenizer with some initial optimisation for extraction from using a CatchAll™ collection swab, (Cambio UK) rather than a soil sample as previously described (Dominguez-Bello et al., 2010).

The sample (vagina/skin/saliva) was contained in a CatchAll™ collection swab on the end of a collection tube.

NOTE: The only deviation from the MoBio kit protocol was in step 12: The tubes are then centrifuged at room temperature for two minutes at 10,000 x g.

Gloves were worn at all times.

1. Saliva, skin and vaginal samples taken by the swabs are all stored in hard packs (Catch-All™). Aseptically remove the head of the Catch-All™ Sample Collection Swab, by pressing to side of the PowerBead Tube. For the dental caries sample, 0.2 g of dentine samples was weighed and added directly to the PowerBead Tube.

Tubes were incubated at 65°C for 10 minutes and then shaken horizontally at maximum speed for 2 minutes, using the MO BIO vortex adapter. For the dentinal caries sample, the sample was vortexed for an additional one minute when the dentine did not break-up and mix with the solution. The remainder of the protocol was followed as per manufacturer's instructions.

2. Gently vortex to mix. In the case all the swab collected specimens, aseptically cut the head 1 cm above this swab of the Catch-All™ Sample Collection Swab.

The swab end was inserted into the PowerBead tubes, to which 60µl of solution C1 had been added. Twist the swab head and press it against the side of the tube, in order to wring out as much as possible of the specimen-containing solution.

3. Check Solution C1. If Solution C1 is precipitated, heat solution to 60°C until dissolved before use.

4. Add 60 µL of Solution C1 and invert several times or vortex briefly.

5. Secure PowerBead Tubes horizontally using the MO BIO Vortex Adapter tube Holder for the vortex (MO BIO Catalog No. 13000-V1) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.

6. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x g for 30 seconds at room temperature.

CAUTION: Be sure not to exceed 10,000 x g or tubes may break.

7. Transfer the supernatant to a clean two ml Collection Tube (provided). Note: Expect between 400 to 500 µL of supernatant. Supernatant may still contain some sample particles.

8. Add 250 µL of Solution C2 and vortex for 5 seconds. Incubate at 4°C for five minutes.

9. Centrifuge the tubes at room temperature for one minute at 10,000 x g.

10. Avoiding the pellet, transfer up to, but no more than, 600 µL of supernatant to a clean two mL Collection Tube (provided).

11. Add 200  $\mu$ L of Solution C3 and vortex briefly. Incubate at 4°C for 5 minutes.
  12. Centrifuge the tubes at room temperature for 2 minutes at 10,000 x g.
  13. Avoiding the pellet, transfer up to, but no more than, 750  $\mu$ L of supernatant into a clean 2 mL Collection Tube (provided).
  14. Add 1200  $\mu$ L of Solution C4 to the supernatant and vortex for 5 seconds.
  15. Load approximately 675  $\mu$ L onto a Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow through and add an additional 675  $\mu$ L of supernatant to the Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. Load the remaining supernatant onto the spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature.
- Note: A total of three loads for each sample processed are required.
16. Add 500  $\mu$ L of Solution C5 and centrifuge at room temperature for 30 seconds at 10,000 x g.
  17. Discard the flow through.
  18. Centrifuge again at room temperature for 1 minute at 10,000 x g.
  19. Carefully place Spin Filter in a clean 2 mL Collection Tube (provided). Avoid splashing any Solution C5 onto the Spin Filter.
  20. Add 100  $\mu$ L of Solution C6 to the centre of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog No. 17000-10).
  21. Centrifuge at room temperature for 30 seconds at 10,000 x g.

22. Discard the Spin Filter. The DNA in the tube is now ready for any downstream application.

DNA was visualised on a 0.8% agarose gel and quantified using the Nanodrop 1000 (Thermo Fisher Scientific, Ireland). DNA was then stored at -80°C.

### **DNA Extraction:**

Samples: Saliva (spit), saliva swab, caries, and skin

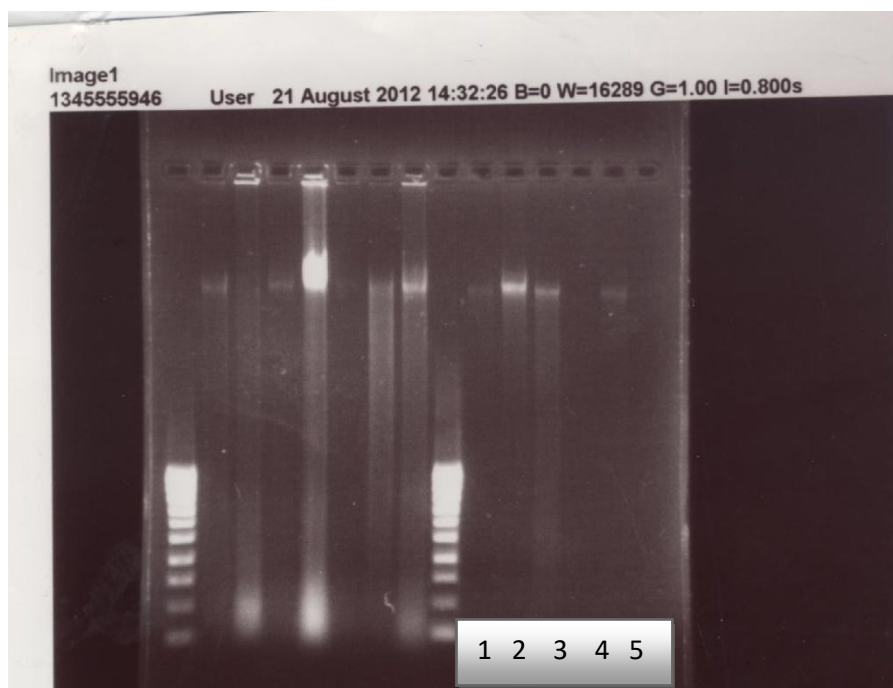
Method:

1. This method was initially adapted and piloted to extract DNA from above samples
2. DNA was quantified using the Nanodrop 1000 (Table 3.2.) (Thermo Fisher Scientific).
3. DNA was visualised on a 0.8% agarose gel (Figure 2.3). DNA was then stored at -80°C.



Sample	Date	Nanodrop		
		[DNA] ng/μl	260/280	260/230
1.Saliva Fresh (spit)	21.08.2012	207.3	1.74	1.10
2.Saliva fresh (spit)	21.08.2012	245.1	1.55	0.96
3.Saliva frozen (swab)	21.08.2012	208.8	1.83	1.29
4.Dental caries (dentine)	21.08.2012	949.1	1.81	1.28
5.Skin swab	21.08.2012	233.6	1.69	1.20

**Table 3.2.** Nanodrop Results from DNA Extraction kit B: MoBio PowerSoil™ DNA Isolation kit.



**Figure 3.3.** DNA visualised on a 0.8% agarose gel .Lanes 1-5 representing samples 1-5 as in Table 3.2.

### **3.3.2. PCR Amplification of DNA extracted from both kits (1&2).**

PCR Amplification was performed of all samples extracted, using both DNA extraction kits.

#### **PCR amplification:**

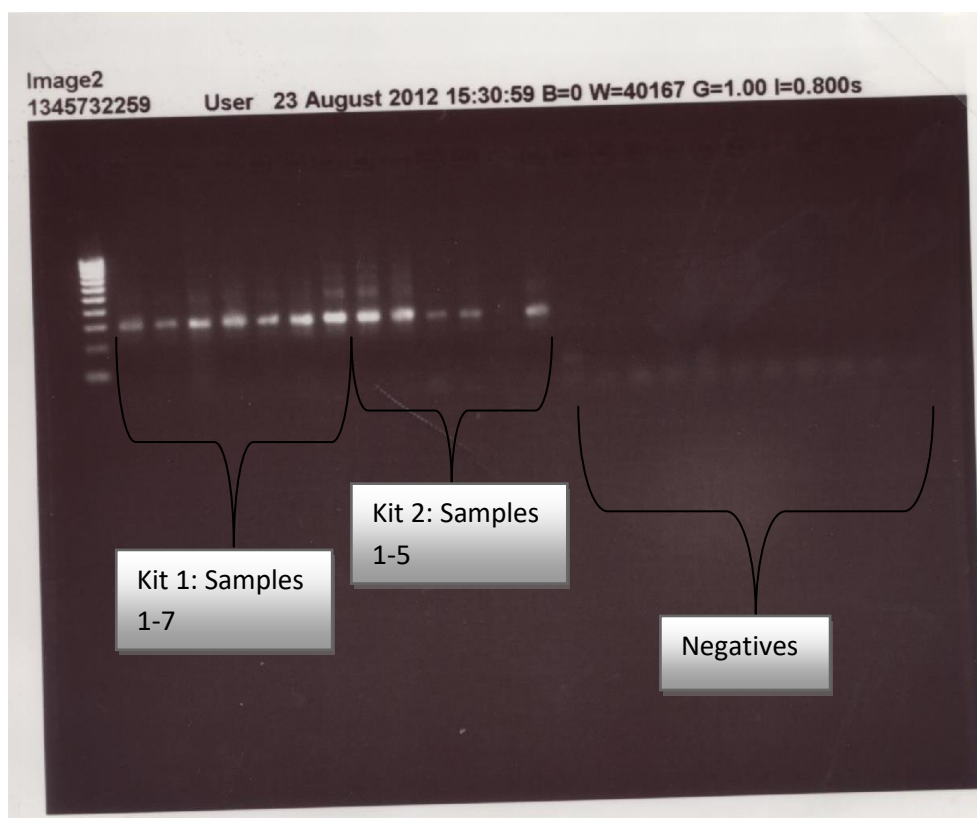
Primers used for PCR amplification were the V4 - V5 region primers 520F (AYTGGGYDTAAAGNG) and 926R (CCGTCAATTYYTTTTRAGTTT).

The V4 - V5 amplicons for Illumina sequencing were generated using a two-step amplification procedure. The first step reaction mix contained 50 µl BIO-X-ACT™ Short Mix (BIOLINE), 10 µl of 2 nM forward and reverse primers, 50 ng genomic DNA, and ddH<sub>2</sub>O to give a final volume of 100 µl.

Cycling conditions were: an initial 95°C, 5-min denaturation step; 30 cycles of 95°C for 15s, 42°C for 15s, and 72°C for 30s; and a final 10-min extension at 72°C.

PCR products ran on a 2% gel, along with equal negatives (Figure 3.4)

## Results:



**Figure 3.4.** Gel Image of PCR products ran on 2% agarose gel. PCR productions of Kit 1& Kit 2.

Both DNA extraction kits were effective as extraction DNA from all samples tested. I decided to select the MoBio PowerSoil™ DNA Isolation kit, because of the similarity of the study of mother infant pairs by Dominguez-Bello et al. to my study design (Dominguez-Bello et al., 2010), and the due to the piloted DNA extraction results for each of the samples taken. Furthermore, I continued to use the using a CatchAll™ collection swab, (Cambio UK), as used Human Microbiome Project, and due to the fact that these swabs could be used for all sample sites to be examined (vagina, skin, oral/saliva).

### **3.5. Studies Methodology:**

1. Longitudinal study: Birth to 1 year of age.
2. Longitudinal study: Birth to 2 years of age.
3. Cross Sectional Study.

#### **3.5.1 Longitudinal study: Birth to 1 year of age.**

##### **Materials and Methods**

##### **3.5.1.1 Study design, ethics and recruitment**

The study design was to recruit a cohort of mother-infant dyads for longitudinal sampling (from birth to 1 year of age) as part of the ORALMET study cohort. Mothers were approached and recruited antenatally between February 2012 and May 2014 at the Cork University Maternity Hospital (CUMH). Ethical approval was obtained from the Cork Teaching Hospitals Clinical Research Ethics Committee (reference code: ECM 3 (cc) 01/07/14). All mothers were consented for the study within 1-2 months prior to their estimated date of delivery, and two groups of mother-infants dyads (n=150) were created based on their mode of delivery (full-term spontaneous vaginally delivered infants (SVD), and full-term Caesarean section delivered infants (CS). True definition of a baby aged 28 days or less is defined as a “neonate” and aged 29 days or greater is defined as an “infant” (Chu et al., 2017), but in this study, although sampling began at birth, all neonates and infants are defined under the same label ‘infant’. Body sites included

in sampling were: vagina, skin, oral of the mother and oral of the infant. All SVD and CS deliveries were uncomplicated, and all infants were born full-term. Inclusion criteria applied to both SVD and CS delivered infants, were that all infants were born full-term (>35 weeks gestation), medically healthy, neonates did not receive antibiotic treatment, breast feeding for a minimum of 4 weeks post partum. Mothers who delivered by CS were all given prophylactic IV antibiotics prior to or during the surgery. If antibiotics were administered to the infant during the first year of life, this was recorded along with the length of duration of breast feeding. The recruitment resulted in the enrolment of 81 mothers into the study, after withdrawal of 24 mothers from the study. Skin samples from 37 mothers of CS infants were collected, and 37 vaginal samples from SVD delivered infants (Supplementary table 4.2). Information about the infants was collected at delivery using medical records at CUMH and further data was collected from each mother/infant pair, when the infant was one year old.

#### **3.5.1.2 Sample Collection**

A CatchAll™ collection swab (Cambio UK), with a hard pack for storage after collection was used to collect all samples from each of the following body sites: vagina, skin and oral cavity (saliva) (Turnbaugh et al., 2007). From the mother, a saliva sample was collected and a skin or vaginal sample depending if C-sectioned or vaginal delivery respectively. For the SVD delivered infants, using a CatchAll™ collection swab, (Cambio UK) the midwife or gynaecologist collected mid-vaginal samples from the mother within 1 hour before delivery. For CS delivered infants, the mother's skin (right and left forearm area) was swabbed after moistening a CatchAll™ collection swab in sterile water, within 1 hour before delivery. For emergency C-sectioned deliveries, the skin swabs were taken

within 1 hour after delivery. The vaginal and skin samples were placed immediately on dry ice and transported to the laboratory, where they were frozen until further analysis and stored at -80°C. Saliva samples from the mother's oral cavity of SVD and CS delivered infants were taken after the infant was born, within 1 hour. The saliva sample from the mother's oral cavity is labelled as 'Saliva' in the results section. CatchAll™ collection swab was used to collect pooled unstimulated saliva in the floor of the mouth for 1-2 minutes. This unstimulated saliva sample is recognized as a representation of the whole oral ecosystem (Aas et al., 2005; Crielaard et al., 2011; Zaura et al., 2009). For the infants, a saliva sample was collected using a CatchAll™ collection swab. The saliva sample collected from the infant's oral cavity at each time point is labelled as 'Oral week 1, Oral week 4 etc' in the results section. The first saliva sample was collected from the newborn infants within 2 days of delivery, before the mothers left hospital (n=75). This was repeated again at 4 weeks (n=60), 8 weeks (n=59), 24 weeks (6 months) (n=63) and at 1 year (n=83) of age (Supplementary table 4.1). All saliva samples were placed immediately on dry ice, transported to the laboratory, where they were frozen until further analysis and stored at -80°C.

### **3.5.1.3 Sample Extraction and Processing**

Extraction of DNA from all samples (vagina, skin and saliva/Oral) was carried out with the MO BIO PowerLyzer® 24 homogenizer with some initial optimisation for extraction from using a CatchAll™ collection swab, (Cambio UK) rather than a soil sample as previously described (Dominguez-Bello et al., 2010). The sample (vagina/skin/saliva) was contained in a CatchAll™ collection swab on the end of a collection tube. The tube was cut 1 cm above this swab, and this was inserted

into the PowerBead tubes, to which 60µl of solution C1 had been added. Tubes were incubated at 65°C for 10 minutes and then shaken horizontally at maximum speed for 2 minutes, using the MO BIO vortex adapter. The remainder of the protocol was followed as per manufacturer's instructions. DNA was visualised on a 0.8% agarose gel and quantified using the Nanodrop 1000 (Thermo Scientific, Ireland). DNA was then stored at -80°C.

#### **3.5.1.4 16S rRNA gene amplification primers**

Primers used for PCR amplification were the V4 - V5 region primers 520F (AYTGGGYDTAAAGNG) and 926R (CCGTCAATTYYTTTRAGTTT) (Supplementary table 4.2; Chapter 4). Initial primers for Illumina sequencing contain the sequencing primer binding sites, forward or reverse 16S rRNA gene specific primer, and a 10nt in-line multiplexing identifier (MID). Dual separate MIDs were attached to both ends of the PCR product (Supplementary table 4.2). The V4 - V5 amplicons for Illumina sequencing were generated using a two-step amplification procedure. The first step reaction mix contained 50 µl BIO-X-ACT™ Short Mix (BIOLINE), 10 µl of 2 nM forward and reverse primers, 50 ng genomic DNA, and ddH<sub>2</sub>O to give a final volume of 100 µl. Cycling conditions were: an initial 95°C, 5-min denaturation step; 30 cycles of 95°C for 15s, 42°C for 15s, and 72°C for 30s; and a final 10-min extension at 72°C. The products were purified using SPRI select beads (Beckman Coulter, Indianapolis IN) as per manufacturer's instructions, using a 0.9:1 volume ratio of beads to product. The purified PCR products were eluted in 40 µl of ddH<sub>2</sub>O. DNA quantity was assessed via Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen™). The samples were pooled in equimolar amounts and then sequenced by Eurofins Genomics (Eurofins

Genetic Services Ltd, I54 Business Park, Valiant Way Wolverhampton WV9 5GB, UK) using Illumina MiSeq 2x300 bp paired end technology. Nextflex Rapid library preparation was carried out by the company to attach bridge adaptors necessary for clustering. Sequencing of 16S DNA was carried out on the V4/V5 region using a Miseq (301 bp paired-end reads). Sequence data were stored on a Linux server and backed up on external hard-drives.

### **3.5.1.5 Bioinformatic analysis & statistical analysis**

#### **Sequence processing, OTU clustering and taxonomy assignment**

The software flash (v1.2.8), was used to join paired-end reads. Paired-end reads with more than 25% incorrect bases in their region of overlap were excluded from subsequent steps. Qiime (v1.9.1) was used to extract barcodes (extract\_barcodes.py) and for demultiplexing (split\_libraries\_fastq.py).

The USEARCH (v8.0.1623) pipeline was used for the following steps: de-replication of reads (identical reads are represented by a single sequence), exclusion of reads shorter than 350 bp and longer than 370 bp, exclusion of unique reads, chimera filtering, OTU clustering at 97 % identity and calculation of representative OTU sequences. Using USEARCH, all reads (including unique reads) were then mapped back to the representative OTU sequences to give the final OTU read count for each sample. The software fastQC (v0.11.3) was used after each filtering step to assess read quality. The sample number after sequence processing was 495.

Part of the mothur (v1.36.1) (Schloss, 2009) pipeline was used to run the RDP classifier using a filtered version of the RDP database in order to assign taxonomy



down to genus level. The software SPINGO (v1.3) (Allard et al., 2015), was used to assign taxonomy at species level. For both mothur/RDP and SPINGO, confidence cut-offs of 80% were used.

### **Alpha and beta diversity analysis**

Alpha and beta diversity metrics were calculated in Qiime (v1.9.1)(Caporaso et al., 2010). To calculate diversity metrics, several additional steps were carried out (also in Qiime). The OTU table was rarefied (single\_rarefaction.py) at 10,540 reads (the lowest read count in the dataset). Representative OTU sequences were aligned using pyNAST (align\_seqs.py) and filtered to remove columns that do not contribute to phylogenetic signal (filter\_alignment.py). A phylogenetic tree was generated using FastTree (make\_phylogeny.py). This tree is necessary for phylogenetic alpha and beta diversity metrics. The rarefied OTU table was used in the calculation of all diversity metrics.

The following alpha diversity metrics were calculated: chao1, Shannon (Shannon's index), Simpson (Simpson's index), Observed species (OTU count) and Phylogenetic (PD whole tree). The following beta diversity metrics were calculated: weighted and un-weighted unifrac distances, and Bray-Curtis dissimilarity.

### **Statistics and data visualisation**

All statistics and data visualisation were carried out in R (v3.2.3 and v3.4.0) (Statistical and Computing, Vienna, 2016). Alpha diversity boxplots were created using the package ggplot2. A Mann-Whitney U test was used to test whether SVD- and CS-born babies differed significantly for each time point. A Kruskal-

Wallis test was performed on alpha-diversity metrics for each baby time point, followed by Mann-Whitney U pairwise comparisons corrected using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). Dunn test was performed for the pairwise comparison of the alpha diversity for the mum samples with the baby time points. Principal Coordinate Analysis (PCoA) plots of beta diversity metrics were created using the package ade4. Statistical differences in beta diversity were tested using the adonis function from the vegan package. Taxon abundance barplots were created using the packages reshape2, ggplot2, and ggthemes. Taxon abundance was normalised to sample proportions for the barplots. Kruskal-Wallis tests and Benjamini Hochberg pairwise tests were used to test for statistical difference in particular taxa at different time points. Clustering of mum-baby pairs based on beta diversity was performed using the hclust function from base R, and the plot created using the rafalib package. The heatmaps to investigate clustering of mum-baby pairs and baby time points based on genera abundances were created using the metagenomeSeq package in R.

### **3.5.2 Longitudinal study: Birth to 2 years of age.**

#### **Materials and Methods**

##### **3.5.2.1 Study design, ethics and recruitment**

This study included recruitment of a cohort of mother-infant pairs that were observed for longitudinal sampling of the infant oral microbiota (saliva) from birth to 2 years of age. Mothers were recruited antenatally in their second or early third trimester, between February 2012 and May 2014 at the Cork University Maternity Hospital (CUMH), and all mothers were consented for the study around 2 months before their estimated date of delivery. Ethical approval was obtained from the Cork Teaching Hospitals Clinical Research Ethics Committee (reference code: ECM 3 (cc) 01/07/14). In total we aimed to recruit two groups of mother-infants pairs (n=150) based on their mode of delivery (SVD or CS). The maternal sampling sites included vagina if SVD delivered neonate or skin if CS delivery, together with oral saliva sample from all mothers. The inclusion criteria were that all deliveries were uncomplicated, that all infants were born full-term (>35 weeks gestation), medically healthy, neonates did not receive antibiotic treatment, and were exclusively breast fed for a minimum of 4 weeks. Mothers that delivered neonates by CS were all given IV prophylactic antibiotics prior to the surgery and if any antibiotics were administered to the infant during the first year of life, this was recorded. We recruited 81 mothers into the study, after withdrawal of 24 mothers from the study, with 37 skin samples and 37 vaginal samples from CS and SVD delivered infants (Supplementary table 4.6.). We collected 382 oral samples between week 1 and 2 years of age (table 4.5), with a total of n=42 infants aged year 2, and of these 18 were CS infants and 24 were SVD infants.

### **3.5.2.2 Sample Collection**

To collect all the samples from each of the body sites (vagina, skin and oral cavity (saliva)) we used a CatchAll™ collection swab (Cambio UK), with a hard pack for storage (Turnbaugh et al., 2007). From all mothers, a saliva sample was collected within 1 hour of delivery and a skin or vaginal sample depending on delivery mode. For the SVD delivered infants, the midwife or gynaecologist collected mid-vaginal samples from the mother within 1 hour before delivery using a CatchAll™ collection swab, (Cambio UK). For CS delivered infants, the mother's skin (right and left forearm area) was swabbed after moistening a CatchAll™ collection swab in sterile water, within 1 hour before delivery. For emergency C-sectioned deliveries, the skin swabs were taken within 1 hour after delivery. All samples were immediately placed on dry ice and transported to the laboratory, where they were frozen at -80°C until further analysis. For the infants, a saliva sample was collected using a CatchAll™ collection swab. The saliva sample collected from the infant's oral cavity at each age as the infant increased in age labelled as 'Oral week 1, Oral week 4 etc'. The first saliva sample was collected from the newborn infants within 2 days of delivery, before the mothers left hospital (n=75). This was repeated again at 4 weeks (n=60), 8 weeks (n=59), 24 weeks (6 months) (n=63), 1 year (n=83) and 2 years of age (n=42) (Supplementary table 4.6). All saliva samples were placed immediately on dry ice, transported to the laboratory, where they were frozen until further analysis and stored at -80°C. All saliva samples were collected by the candidate when within the hospital, or by the research nurse on the Infantmet study. All saliva samples from 4 weeks to 2 years of age were collected in the home of the infant by the research nurse, together with a stool sample (Infantmet study).

### **3.5.2.3 Sample Extraction and Processing**

Extraction of DNA from all samples (vagina, skin and saliva) was carried out with the MO BIO PowerLyzer® 24 homogenizer with some initial optimisation for extraction from using a CatchAll™ collection swab, (Cambio UK) rather than a soil sample as previously described (Dominguez-Bello et al., 2010). The tube was cut 1 cm above this swab, which contained the sample, and this was inserted into the PowerBead tubes, to which 60µl of solution C1 had been added. Tubes were incubated at 65°C for 10 minutes and then shaken horizontally at maximum speed for 2 minutes, using the MO BIO vortex adapter. The remainder of the protocol was followed as per manufacturer's instructions. DNA was visualised on a 0.8% agarose gel and quantified using the Nanodrop 1000 (Thermo Scientific, Ireland). DNA was then stored at -80°C.

### **16S rRNA gene amplification primers**

Primers used for PCR amplification were the V4 - V5 region primers 520F (AYTGGGYDTAAAGNG) and 926R (CCGTCAATTYYTTTRAGTTT) (Supplementary table 4.2; Chapter 4). Initial primers for Illumina sequencing contain the sequencing primer binding sites, forward or reverse 16S rRNA gene specific primer, and a 10nt in-line multiplexing identifier (MID). Dual separate MIDs were attached to both ends of the PCR product (Supplementary table 4.2). The V4 - V5 amplicons for Illumina sequencing were generated using a two-step amplification procedure. The first step reaction mix contained 50 µl BIO-X-ACT™ Short Mix (BIOLINE), 10 µl of 2 nM forward and reverse primers, 50 ng genomic DNA, and ddH<sub>2</sub>O to give a final volume of 100 µl. Cycling conditions were: an initial 95°C, 5-min denaturation step; 30 cycles of 95°C for 15s, 42°C for

15s, and 72°C for 30s; and a final 10-min extension at 72°C. The products were purified using SPRI select beads (Beckman Coulter, Indianapolis IN) as per manufacturer's instructions, using a 0.9:1 volume ratio of beads to product. The purified PCR products were eluted in 40 µl of ddH<sub>2</sub>O. DNA quantity was assessed via Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen™). The samples were pooled in equimolar amounts and then sequenced by Eurofins Genomics (Eurofins Genetic Services Ltd, I54 Business Park, Valiant Way Wolverhampton WV9 5GB, UK) using Illumina MiSeq 2x300 bp paired end technology. Nextflex Rapid library preparation was carried out by the company to attach bridge adaptors necessary for clustering. Sequencing of 16S DNA was carried out on the V4/V5 region using a Miseq (301 bp paired-end reads). Sequence data were stored on a Linux server and backed up on external hard-drives.

#### **3.5.2.4 Bioinformatic analysis & Statistical analysis.**

##### **Sequence processing, OTU clustering and taxonomy assignment**

The software flash (v1.2.8), was used to join paired-end reads. Paired-end reads with more than 25% incorrect bases in their region of overlap were excluded from subsequent steps. Qiime (v1.9.1) was used to extract barcodes (extract\_barcodes.py) and for demultiplexing (split\_libraries\_fastq.py).

The USEARCH (v8.0.1623) pipeline was used for the following steps: de-replication of reads (identical reads are represented by a single sequence), exclusion of reads shorter than 350 bp and longer than 370 bp, exclusion of unique reads, chimera filtering, OTU clustering at 97 % identity and calculation of

representative OTU sequences. Using USEARCH, all reads (including unique reads) were then mapped back to the representative OTU sequences to give the final OTU read count for each sample. The software fastQC (v0.11.3) was used after each filtering step to assess read quality. The sample number after sequence processing was 537.

Part of the mothur (v1.36.1) (Schloss, 2009) pipeline was used to run the RDP classifier using a filtered version of the RDP database in order to assign taxonomy down to genus level. The software SPINGO (v1.3) (Allard et al., 2015), was used to assign taxonomy at species level. For both mothur/RDP and SPINGO, confidence cut-offs of 80% were used.

### **Alpha and beta diversity analysis**

Alpha and beta diversity metrics were calculated in Qiime (v1.9.1)(Caporaso et al., 2010). To calculate diversity metrics, several additional steps were carried out (also in Qiime). The OTU table was rarefied (single\_rarefaction.py) at 10,540 reads (the lowest read count in the dataset). Representative OTU sequences were aligned using pyNAST (align\_seqs.py) and filtered to remove columns that do not contribute to phylogenetic signal (filter\_alignment.py). A phylogenetic tree was generated using FastTree (make\_phylogeny.py). This tree is necessary for phylogenetic alpha and beta diversity metrics. The rarefied OTU table was used in the calculation of all diversity metrics. The following alpha diversity metrics were calculated: chao1, Shannon (Shannon's index), Simpson (Simpson's index), Observed species (OTU count) and Phylogenetic (PD whole tree). The following

beta diversity metrics were calculated: weighted and un-weighted unifrac distances, and Bray-Curtis dissimilarity.

### **Statistics and data visualisation**

All statistics and data visualisation were carried out in R (v3.2.3 and v3.4.0) (Statistical and Computing, Vienna, 2016). Alpha diversity boxplots were created using the package ggplot2 and a Mann-Whitney U test was used to test whether the infant oral microbiota of SVD- and CS-born babies differed significantly at each age of the infant and a Dunn test was performed for the pairwise comparison of the alpha diversity for the maternal samples and the infant ages over time. A Kruskal-Wallis test was performed on alpha-diversity metrics for each baby time point (week 1 to 2 years of age), followed by Mann-Whitney U pairwise comparisons corrected using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). Principal Coordinate Analysis (PCoA) plots of beta diversity metrics were created using the package ade4. Statistical differences in beta diversity were tested using the adonis function from the vegan package. Taxon abundance barplots were created using the packages reshape2, ggplot2, and ggthemes. Taxon abundance was normalised to sample proportions for the barplots. Kruskal-Wallis tests and Benjamini Hochberg pairwise tests were used to test for statistical difference in particular taxa at different time points. The heatmaps generated to investigate clustering of the maternal samples and all of the infant oral microbiota samples at each time point were created using the metagenomeSeq package in R.



### 3.5.3 Cross-sectional study

#### Materials and Methods

##### 3.5.3.1 Study design, ethics and recruitment

The study design was to recruit two cohorts of children under the age of 60 months. These two groups were categorized into those with S-ECC (caries-active), and a caries-free cohort, all medically healthy. In total, we recruited 68 caries-active and 70 caries-free children. The deep dentinal lesion microbiota were labelled as Caries-active cavity (CAC) and the salivary microbiota of these caries-active children labelled as Caries-active saliva (CAS), while the saliva of the caries-free children were labelled as Caries-free saliva (CFS). Of the caries-active, all were S-ECC affected deciduous teeth, and the CAC and CAS are paired samples, each from the same subject. Ethical approval was obtained from the Teaching Hospitals Clinical Research Ethics Committee for the recruitment and sampling of these cohorts of children. Ethical approval reference: ECM 3 (cc) 01/07/14.

Recruiting of the children affected by S-ECC was performed at a Hospital Dental Treatment Centre. All children were referred to the Hospital Dental Treatment Centre where clinical examinations were performed and they were then scheduled for extraction of their carious teeth, under general anaesthetic. All these children referred to the Hospital Dental Treatment Centre for general anaesthetic had radiographs taken as part of the examination prior to referral. The caries-free cohort was recruited from various crèches, where a paediatric dentist travelled to each of the crèches and examined the children's teeth.

Inclusion criteria applied to both caries-free and caries-affected groups were that they were medically healthy, had no antibiotic intake in the 3 months prior to sampling, and were under the age of 60 months.

### **3.5.3.2 Diet and Lifestyle data collection**

Before undergoing the dental examination, informed consent was obtained from the parent/guardians of the children. We also collected habitual dietary data using a validated Food Frequency Questionnaire (FFQ) (Claesson et al., 2012) which was provided to each parent/guardian, to record food intake. A detailed questionnaire was given to each parent/guardian. Data collected included antibiotic treatment history, general medical history and dental history, fluoridation status of home (well/public water), feeding practices in infancy, birth mode, and oral health related quality of life.

### **3.5.3.3 Oral Examination**

Oral examinations for both caries-free and caries-active groups were performed by a trained Paediatric dentist. Children in crèches and in the Hospital Dental Treatment Centre were examined in a quiet area with their parents present. Children's teeth were wiped with a cotton wool roll and sterile gauze square to remove plaque and debris prior to examination which was carried out under natural light, using a standard size 4 mirror and ball ended CPI 'C' probe (Whelton et al., 2006). The mouth was illuminated with a Promed Penlight, which consists of bright concentrated halogen light when natural light was insufficient. For the caries-active group, caries was recorded at the level of cavitation into dentine (cavitation level), using the WHO criteria (Whelton et al., 2006; WHO, 1997), with the addition of visible non cavitated dentine caries as referenced by

Whelton et al. (Whelton et al., 2006). The dmft score was recorded along with the dmfs score, and sample collection performed. For the caries-free group, caries was recorded at the level of cavitation into dentine (cavitation level), using the WHO criteria (Whelton et al., 2006; WHO, 1997). The dmft/dmfs was measured, and sample collection was performed. Caries-free children did not show clinical evidence of early pre-cavitation of caries or white spot lesions and had no history of treatment on any tooth surfaces, as defined (Dye et al., 2007).

#### **3.5.3.4 Sampling**

The same trained paediatric dentist took all samples after the teeth were examined. All children were instructed not to brush their teeth the evening and the morning before sampling. A CatchAll™ collection swab, with hard pack for storage after collection was used (Cambio UK) (Turnbaugh et al., 2007). For the caries-active S-ECC group, both a carious lesion sample and a saliva sample were taken. After a full dental examination, and pre-general anaesthetic, the CatchAll™ collection swab was used to collect pooled unstimulated saliva in the floor of the mouth for 1-2 minutes. This unstimulated saliva sample is recognized as a representation of the whole oral ecosystem (Aas et al., 2005; Crielaard et al., 2011; Zaura et al., 2009). The swab was placed back in the collection tube, and stored at -80°C. To sample the carious lesions, while the child was under general anaesthetic, the carious deciduous tooth was extracted and under isolation, the tooth was irrigated with saline. Under care, by a paediatric dentist, the superficial carious dentin was excavated with a sterile spoon excavator and the next layer of deep dentinal caries was excavated using a new separate sterile spoon excavator and the sample was pooled in a sterile 1.5-ml micro-centrifuge tube with 1 ml of TE buffer (50Mm Tris-HCL, 1Mm EDTA). The samples were placed in a sterile 1.5-ml micro-

centrifuge tube and transported to the laboratory, where they were frozen until further analysis and stored at -80°C. For the caries-free group, after a full dental examination, the CatchAll™ collection swab was used to collect pooled unstimulated saliva in the floor of the mouth for 1-2 minutes. The swab was placed back in the collection tube, and stored at -80°C.

### **3.5.3.5 DNA Extraction**

Extraction of DNA from all samples was carried out with the MO BIO PowerLyzer® 24 homogenizer following some initial optimisation for extraction from an oral catch-all swab rather than a soil sample as previously described (Dominguez-Bello et al., 2010). The saliva sample was contained in a catch-all swab in the end of a collection tube. The tube was cut 1 cm above this swab, and this was inserted into the PowerBead tubes, to which 60µl of solution C1 had been added. Tubes were incubated at 65°C for 10 minutes and then shaken horizontally at maximum speed for 2 minutes, using the MO BIO vortex adapter. The remainder of the protocol was followed as per manufacturer's instructions. For the caries sample, the tubes were incubated at 65 °C for 10 minutes and then shaken horizontally at maximum speed for 4 minutes, using the MO BIO vortex adapter. The remainder of the protocol was followed as per manufacturer's instructions. DNA was visualised on a 0.8% agarose gel and quantified using the Nanodrop 1000 (Thermo Scientific, Ireland). DNA was then stored at -80°C.

### **3.5.3.6 16S rRNA gene amplification primers**

Primers used for PCR amplification were the V4 - V5 region primers 520F (AYTGGGYDTAAAGNG) and 926R (CCGTCAATTYYTTTTRAGTTT) (Supplementary table 4.2). Initial primers for Illumina sequencing contain the sequencing primer binding sites, forward or reverse 16S rRNA gene specific primer, and a 10nt in-line multiplexing identifier (MID). Dual separate MIDs were attached to both ends of the PCR product.

The V4 - V5 amplicons for Illumina sequencing were generated using a two-step amplification procedure. The first step reaction mix contained 50 µl BIO-X-ACT™ Short Mix (BIOLINE), 10 µl of 2 nM forward and reverse primers, 50 ng genomic DNA, and ddH<sub>2</sub>O to give a final volume of 100 µl. Cycling conditions were: an initial 95°C, 5-min denaturation step; 30 cycles of 95°C for 15s, 42°C for 15s, and 72°C for 30s; and a final 10-min extension at 72°C. The products were purified using SPRIselect beads (Beckman Coulter, Indianapolis IN) as per manufacturer's instructions, using a 0.9:1 volume ratio of beads to product. The purified PCR products were eluted in 40 µl of ddH<sub>2</sub>O. DNA quantity was assessed via Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen™). The samples were pooled in equimolar amounts and then sequenced by Eurofins Genomics (Eurofins Genetic Services Ltd, I54 Business Park, Valiant way Wolverhampton WV9 5GB, UK) using Illumina MiSeq 2x300 bp paired end technology. Nextflex Rapid library preparation was carried out by the company to attach bridge adaptors necessary for clustering. Sequencing of 16S DNA was carried out on the V4/V5 region using a Miseq (301 bp paired-end reads). Sequence data were stored on a Linux server and backed up on external hard-drives.

### **3.5.3.7 Bioinformatic analysis and Statistical analysis**

#### **Sequence processing, OTU clustering and taxonomy assignment**

The software, flash (v1.2.8), was used to join paired-end reads. Paired-end reads with more than 25% incorrect bases in their region of overlap were excluded from subsequent steps. Qiime (v1.9.1) was used to extract barcodes (extract\_barcodes.py) and for demultiplexing (split\_libraries\_fastq.py).

The USEARCH (v8.0.1623) pipeline was used for the following steps: de-replication of reads (identical reads are represented by a single sequence), exclusion of reads shorter than 350 bp and longer than 370 bp, exclusion of unique reads, chimera filtering, OTU clustering at 97% identity and calculation of representative OTU sequences. Using USEARCH, all reads (including unique reads) were then mapped back to the representative OTU sequences to give the final OTU read count for each sample. The software fastQC (v0.11.3) was used after each filtering step to assess read quality. The median read count for the samples after sequence processing was 44,400. The sample number after sequence processing was 206.

Part of the mothur (v1.36.1) (Schloss, 2009) pipeline was used to run the RDP classifier using a filtered version of the RDP database in order to assign taxonomy down to genus level. The software SPINGO (v1.3) (Allard et al., 2015), was used to assign taxonomy at species level. For both mothur/RDP and SPINGO, confidence cut-offs of 80% were used.

## **Alpha and beta diversity analysis**

Alpha and beta diversity metrics were calculated in Qiime (v1.9.1)(Caporaso et al., 2010). To calculate diversity metrics, several additional steps were carried out (also in Qiime). The OTU table was rarefied (single\_rarefaction.py) at 10,540 reads (the lowest read count in the dataset). Representative OTU sequences were aligned using pyNAST (align\_seqs.py) and filtered to remove columns that do not contribute to phylogenetic signal (filter\_alignment.py). A phylogenetic tree was generated using FastTree (make\_phylogeny.py). This tree is necessary for phylogenetic alpha and beta diversity metrics. The rarefied OTU table was used in the calculation of all diversity metrics.

The following alpha diversity metrics were calculated: chao1, Shannon (Shannon's index), Simpson (Simpson's index), Observed species (OTU count) and Phylogenetic (PD whole tree). The following beta diversity metrics were calculated: weighted and un-weighted unifrac distances, and Bray-Curtis dissimilarity.

## **Statistics and data visualisation**

All statistics and data visualisation were carried out in R (v3.2.3) (Statistical and Computing, Vienna, 2016). Paired Mann-Whitney tests were used to compare microbiota of saliva samples (CAS) (n=68) with that of caries samples (CAC) (n=68) taken from the caries group. Both CAC and CAS are paired, both samples from the same subject. Un-paired Mann-Whitney tests were used to compare the saliva and caries samples from the caries group with a control group of caries-free saliva (CFS) (n=70) of caries-free individuals. Benjamini and Hochberg

correction (Benjamini and Hochberg, 1995) was used to adjust p-values for multiple testing.



## **Chapter 4**

### **Results**

1. Longitudinal study: Birth to 1 year of age.
2. Longitudinal study: Birth to 2 years of age.
3. Cross Sectional Study.

## **Results**

### **4.1. Longitudinal study: Birth to 1 year of age.**

This chapter has been submitted for peer review in ‘Journal of Dental Research’.

Entitled:

‘The influence of birth mode and breastfeeding duration on the emerging neonatal oral microbiota from birth to 1 year of age, a cohort study.’

Authors: Eimear Hurley, Hugh M.B. Harris, David Mullins, Maurice P.J Barrett, Carol Anne O’Shea, Martin Kinirons, Helen Whelton, C. Anthony Ryan, Catherine Stanton, Paul W. O’Toole.

## 4.1 Results

### **4.1.1 The oral microbiota of the infant is similar at weeks 1, 4 and 8 but becomes more distinct by 1 year of age.**

To investigate the relatedness of the microbiome composition between samples including the maternal vagina, skin and their oral cavity (saliva) to their infants from week 1 to 1 year of age (at the following time points: 1 week, 4 weeks, 8 weeks, 6 months and 1 year) we generated PCoA (principle co-ordinate) plots showing relatedness by two established metrics, Bray Curtis dissimilarity, and UniFrac distances. The Bray Curtis plot (Fig. 4.1A), weighted unifrac (Fig. 4.1B) and unweighted unifrac (Fig. 4.1C) both demonstrate substantial overlap between each group, indicating similarity in the general composition of the microbial taxa. However, using PerMANOVA to compare the microbiota of the mother (vagina, skin and oral), with the oral microbiota of the infants at different time points, we found statistically significant differences between each group, as measured by each metric ( $p < 0.001$ ) (Table 4.1).

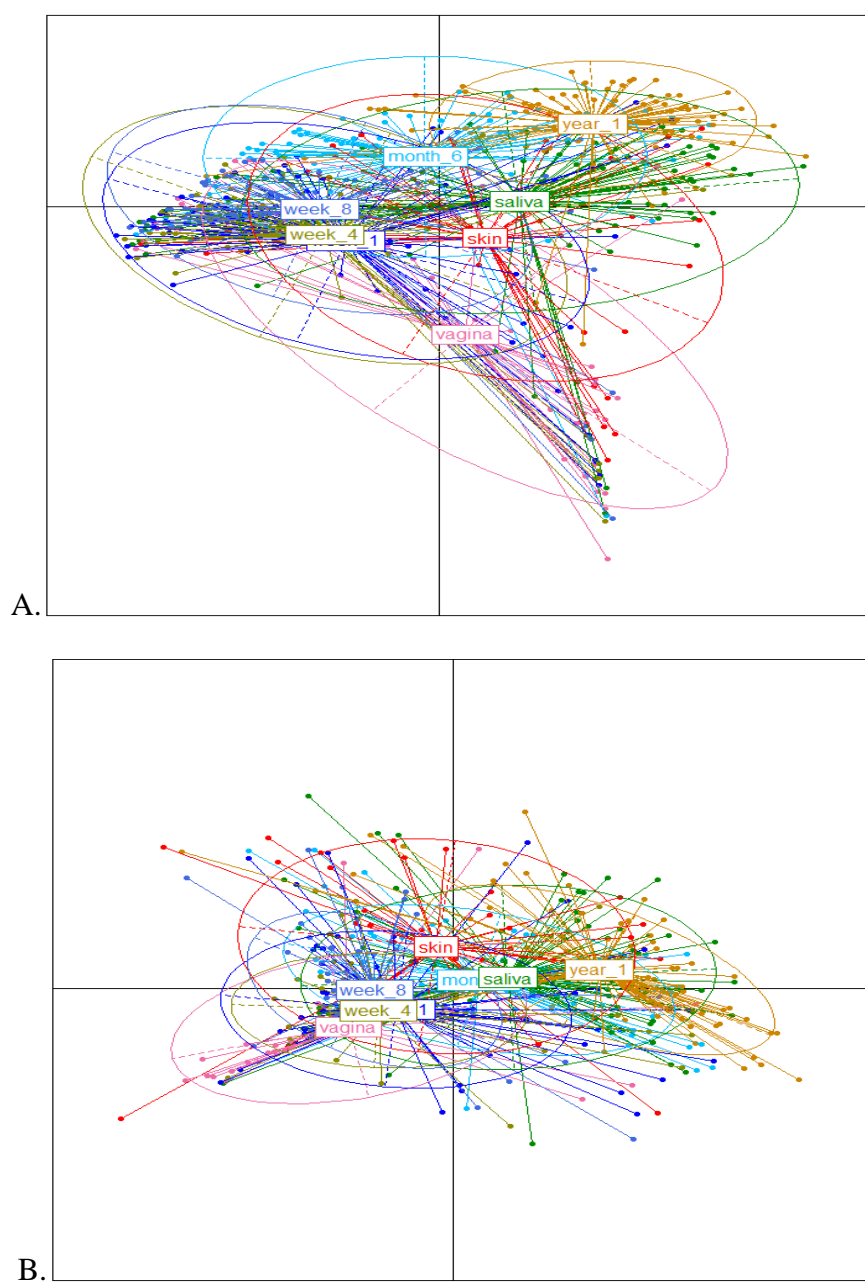
There is sharing of taxa between maternal microbiota (skin/vaginal) and infant oral microbiota independent of birth modality (SVD/CS). The Bray Curtis (Fig. 4.1A) and weighted UniFrac (Fig. 4.1B) demonstrate overlap between the earlier ages of the infant (week 1, week 4, week 8) along with the vagina of the mother in the weighted PCoA, demonstrating similarity in the composition of microbial taxa at this early age. The closeness and overlap of these early oral microbiota samples from the infant with the skin and vaginal samples from the mother illustrate sharing of taxa independent of birth mode with obvious sharing of taxa of the week 1 and week 4 infant oral microbiota with mothers vagina and skin

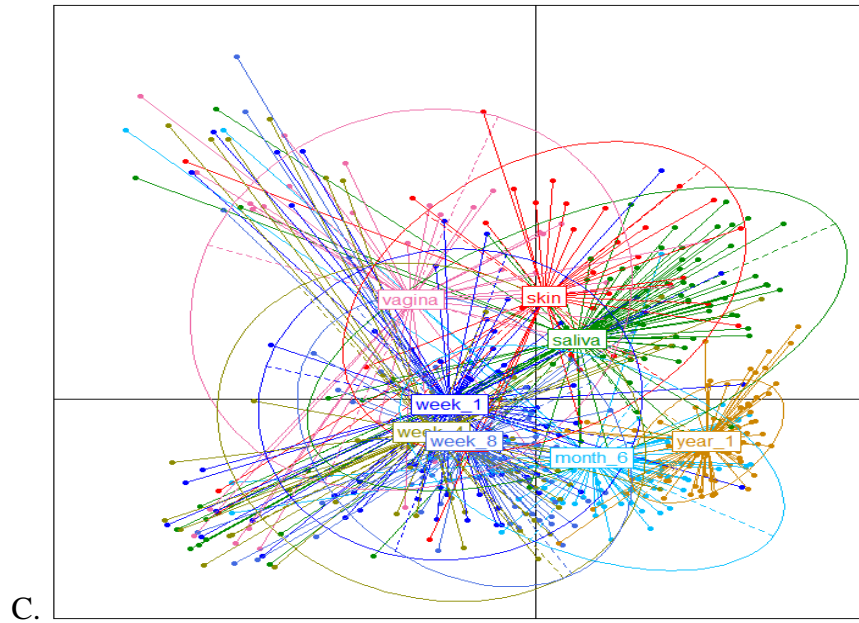
microbiota. While weighted UniFrac is sensitive to the differences in the presence/absence and abundance/proportions of OTUs, there is evidence of tight clustering of week 1, week 4 and week 8 infant oral microbiota samples along with less clustering together with 6 months and 1 year samples, demonstrating that at an early age (week 1 to week 8) the abundance of certain OTUs is shared between samples, but not as strongly as the infant increases in age, with separation of the 6 months and 1 year infant microbiota into their own distinct clusters. This longitudinal sampling of the oral microbiota of the infant demonstrates similarity of the infant oral microbiota in the first 8 weeks, and then change in the microbial oral community structure over time until 1 year, where the infant oral microbiota community becomes more distinct.

Plotting datasets using the second UniFrac metric, unweighted UniFrac distances (Fig. 4.1C), illustrates tight clustering again of the earlier age time points of the oral microbiota of the infant (week 1, week 4 and week 8), with less overlap with the increased age (6 months and 1 year). This index measures the presence and absence of taxa only and does not take taxon abundance into account. By this method there is evidence of clustering of week 1, 4 and 8 of the oral microbiota of the infant, indicating sharing of rare taxa, while year 1 has minimal overlap with these earlier age time points, indicating less sharing of rare taxa between early and later stages of age. This is further supported by the oral microbiota of infants at 6 months, positioned between week 1 to week 8 and year 1, thus highlighting a gradual change in the microbial composition from week 8 to 6 months and finally to 1 year of age, where the microbiota appears more distinct (Fig. 4.1C).

	axis 1	axis 2	PERMANOVA
un-weighted unfrac	18.4	6.7	p<0.001
weighted unfrac	39.2	11	p<0.001
Bray-Curtis	18.5	9.2	p<0.001

**Table 4.1.** Significance values of Bray Curtis dissimilarity and UniFrac distances metrics. Significance was calculated using permutational multivariate analysis of variance (PerMANOVA).  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*).

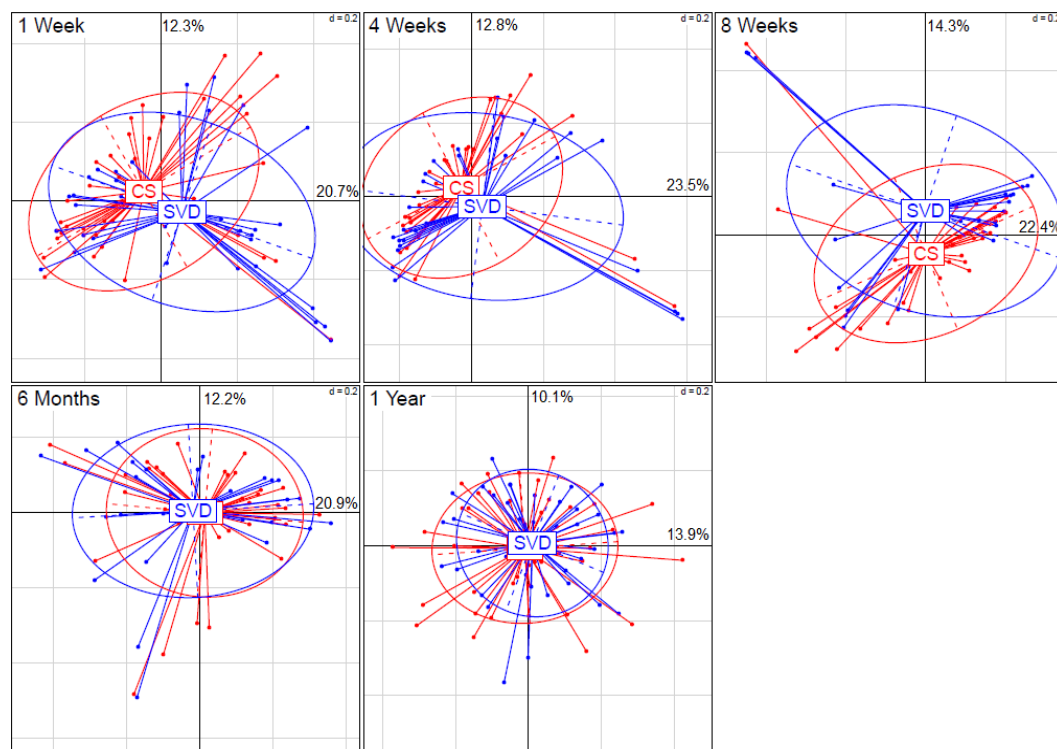




**Fig. 4.1 Principle Coordinates Analyses (PCoAs) on Spearman distance matrices of samples from mother (saliva, skin & vagina) & from infant oral at 5 time points (week 1, 4, 8, 6 months and 1 year).** A. Bray Curtis dissimilarity, B. UniFrac Weighted & C. Unweighted UniFrac between the 5 groups (mother saliva/oral, skin & vaginal & infant oral week 1,4,8,6 months and 1 year). A. Plot of principle co-ordinates using **Bray-Curtis dissimilarity**. Points are coloured according to group and ellipses describe the distribution of points for each group. Percentage variation explained: PCA 1 (18.5%) and PCA 2 (9.2%). B. Plot of principle co-ordinates using **weighted unifrac distance**. Points are coloured according to group and ellipses describe the distribution of points for each group. Percentage variation explained: PCA 1 (39.2%) and PCA 2 (11%). C. Plot of principle co-ordinates using **un-weighted unifrac distance**. Points are coloured according to group and ellipses describe the distribution of points for each group. Percentage variation explained: PCA 1 (18.4%) and PCA 2 (6.7%).

#### **4.4.2 The oral microbiota of the infant is influenced by delivery mode at 1 week of age, but not at 1 year of age.**

We investigated the influence of birth mode (SVD v CS) on the oral microbiota composition of the newborn infant and significance was calculated using permutational multivariate analysis of variance (PerMANOVA) (Table 4.2). The results were presented by PCoA (principle co-ordinates) plots (Fig. 4.2). The beta diversity of the infant oral microbiota at 1 week of age was shown to be affected by birth mode (SVD or CS). The impact of birth mode on the infant oral microbiota at 1 week of age was statistically significant ( $p < 0.05$ ). With increased age of the infant, we did not find any significant impact of birth mode on the infant oral microbiota composition (Table 4.2). Visually, the overlap/clustering between SVD and CS becomes tighter with older age, with nearly complete overlap by 6 months and 1 year, illustrating homogeneity of the oral microbiota within the oral cavity by birth modes.



**Fig. 4.2 PCoA (principle co-ordinates) plots each demonstrating the effect of birth mode (SVD v CS) on oral microbiota composition of the infant at different ages (week 1, 4, 8, 6 months and 1 year).** Significance was calculated using permutational multivariate analysis of variance (PerMANOVA) (Table 4.2).

Time point	PerMANOVA p-value
<b>1 Week</b>	0.047*
<b>4 Weeks</b>	0.31
<b>8 Weeks</b>	0.1
<b>6 Months</b>	0.324
<b>1 Year</b>	0.329

**Table 4.2.** Significance values for the difference between SVD and CS at each time point. Significance was calculated using permutational multivariate analysis of variance (PerMANOVA).  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*)



#### **4.1.3 Diversity of the oral microbiota remains relatively stable up to 6 months of age.**

To study the diversity of the microbiota from all samples, we calculated a series of alpha ( $\alpha$ ) diversity metrics: the Chao index, phylogenetic diversity (PD whole tree), observed species (OTU count), the Simpson index and the Shannon index (Fig.4.3). Alpha diversity is a measure of the diversity within a sample. The Chao diversity (Fig. 4.3A) which calculates species diversity within the sample and observed species (OTU count) index, which measures OTUs observed in a sample (Fig. 4.3B), both illustrated that the  $\alpha$ -diversity of the year 1 oral microbiota was lowest and the  $\alpha$ -diversity of the oral microbiota decreased steadily over time from week 1 to year 1. However the two remaining indices measuring  $\alpha$ -diversity; phylogenetic diversity (Fig. 4.3C) and Shannon diversity (Fig. 4.3D), demonstrate that the diversity of the oral microbiota remains stable with increasing age.

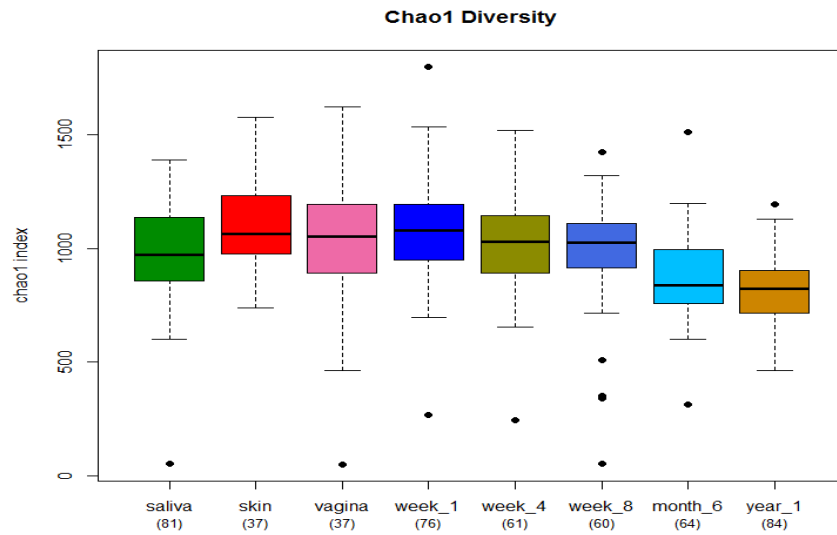
All metrics illustrated that of the samples analysed the skin microbiota diversity was highest, with high abundance of *Propionibacterium*, *Streptococcus* and *Corneibacterium*, and the diversity of the vaginal microbiota was lower than the skin microbiota, composed mainly of genera *Streptococcus*, *Haemophilus*, *Neisseria* and *Lactobacillus*, while both the maternal skin and vaginal microbiota demonstrate compositional differences ( $p < 0.001$ ). While the mother's skin diversity can vary by sample site location, skin diversity is high due to its open environment (Costello et al., 2013; Grice et al., 2008; Grice and Segre, 2011), while *Lactobacillus* species can dominate the vaginal microbiota of the mother, and makes up the majority of the composition, and the vaginal microbiota diversity was previously found to be the lowest compared to skin and oral of adults sampled (Li et al., 2012; Zhou et al., 2013). Interestingly, considering the

Chao diversity, there were no microbiota differences between the maternal skin microbiota and oral of the infant at 1 week of age, and the maternal vaginal microbiota and infant at 1 week of age, but as the infant increases in age, the microbiota difference increased in significance between the oral cavity of the infant from 6 months of age, to the maternal microbiota of the vagina and skin at birth ( $p < 0.0001$ ). This illustrates similarity of the maternal skin and vaginal microbiota at birth to the infant oral microbiota at week 1, 4 and 8 weeks and not until 6 months and beyond does the oral microbiota of the infant appear to acquire its own distinct composition, with less influence of the maternal microbiota.

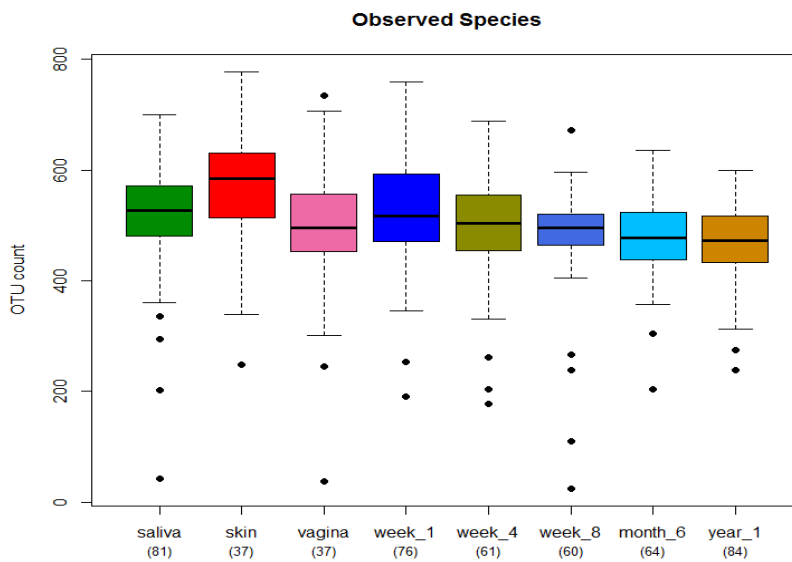
When species diversity was measured by Chao diversity, we observed that the maternal oral microbiota and the infant oral microbiota at week 1 of age are different ( $p < 0.005$ ), while at 1 year of age the  $\alpha$ -diversity difference between these two samples is still significantly different (Fig. 4.3A). By the other  $\alpha$ -diversity measures, the phylogenetic diversity measurement illustrated similar findings at week 1 ( $p < 0.05$ ), while OTU count showed difference in diversity of significance from 8 weeks ( $p < 0.05$ ). These results demonstrate that at a younger age, the differences in microbiota diversity between mother's saliva and infant saliva is less than at an older age, highlighting some relatedness at an early age between the oral microbiota of mother and infant.

The composition of the oral microbiota of the infant does not appear to change between week 1 and 8 weeks of age when measured using Chao diversity and Shannon diversity. At phylum level, Firmicutes abundances at week 1 and week 8 are similar with relative abundances at week 1 (82.3%) , week 4 (87.3%) and at 8

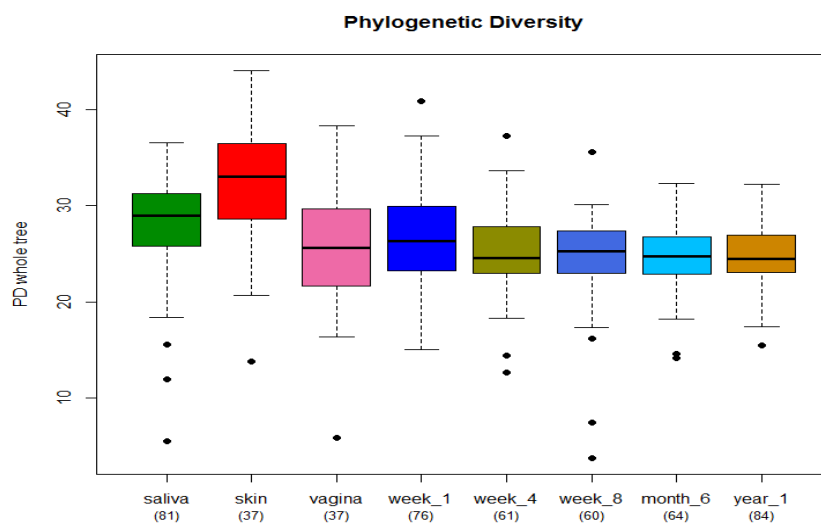
weeks (81.1%). Only after 8 weeks are changes in composition evident ( $p < 0.05$ ) (Chao diversity), with Firmicutes levels beginning to decrease from 6 months (60.2%) until 1 year (33.7%). From 6 months to 1 year, as the relative abundances of Firmicutes decreases within the oral cavity this coincides with an increase in phyla at 1 year of age Proteobacteria (25.6%) and Bacteroidetes (16.7%). Similarly, as measured by the Shannon index (Fig. 4.3D) oral microbiota diversity differences are apparent from 6 months of age, with oral microbiota differences evident between 6 months and 1, 4 and 8 weeks ( $p < 0.0001$ ) and 6 months and 1 year ( $p < 0.05$ ). These metrics demonstrate that the diversity of the oral microbiota remains relatively stable from week 1 to 8 weeks of age and it is not until the infant reaches 6 months of age and 1 year of age do we see significant changes in the diversity of the infant oral microbiota.



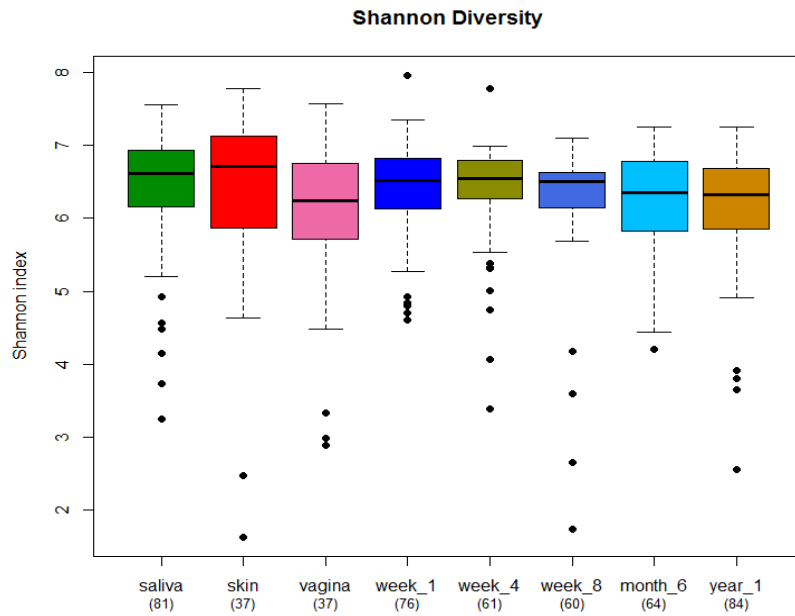
A.



B.



C.



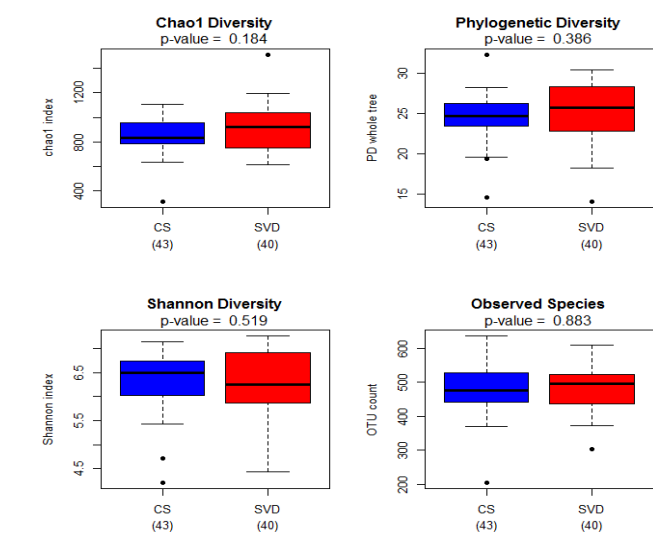
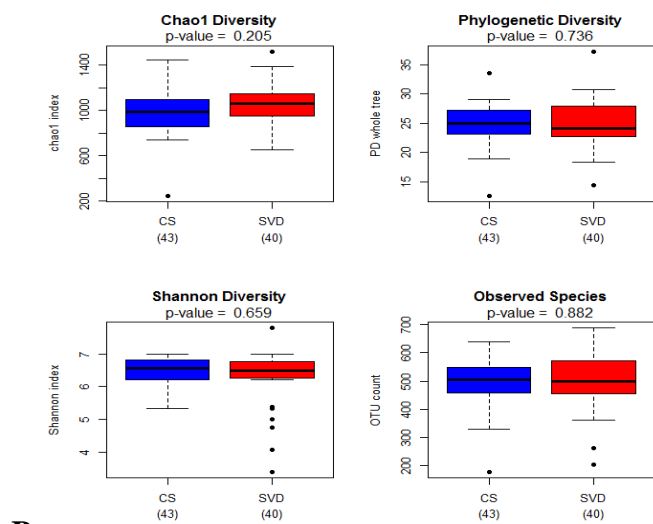
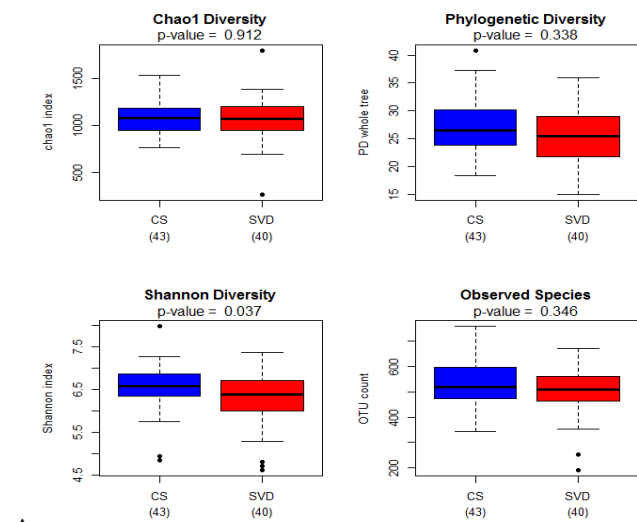
D.

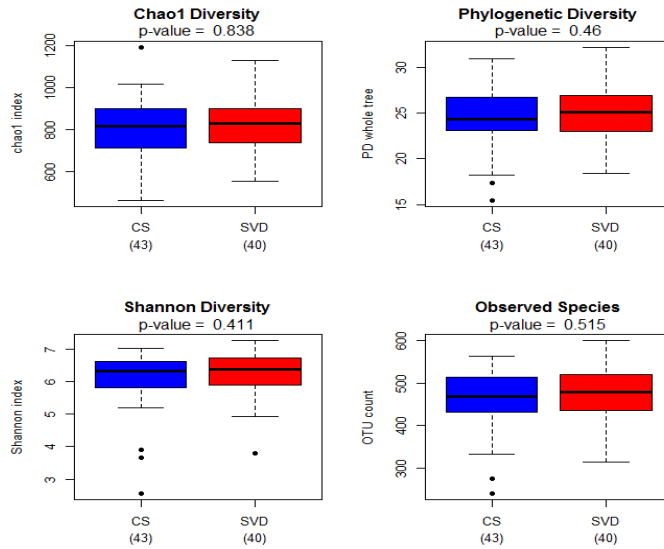
**Fig.4.3. Alpha diversity comparisons of the 5 groups (mother saliva, skin & vaginal & infant oral week 1, 4, 8, 6 months and 1 year).**

A. Boxplot of chao1 diversity in the three groups. B. Boxplot of observed species in the eight groups. C. Boxplot of Phylogenetic diversity in the eight groups. D. Boxplot of Shannon diversity in the eight groups. Outliers are represented by black points.

#### **4.1.4 The diversity of the oral microbiota of the infant is influenced by birth mode at 1 week of age, but not beyond 1 week of age.**

To investigate the influence of birth mode on the oral microbiota composition as the infant increases in age, infant oral microbiota data was separated based on birth mode (SVD or CS) at the various time points (week 1, week 4, 6 months and 1 year). Alpha diversity was used to measure the overall diversity of the community present in the sample. Four indices were used (Fig. 4.4). The alpha diversity as represented by Shannon diversity index, of the infant oral microbiota at 1 week of age was influenced by birth modality (p-value <0.037), but at an older age, there was no influence of birth mode on the oral microbiota of the infant. Shannon diversity index takes the abundance of species into account, and in this case this index indicates that the diversity of the infant oral microbiota at week 1 is lower in SVD infants, compared to CS infants, and therefore the species abundance is richer in CS infant oral microbiota.





**D**

**Fig. 4.4. Alpha Diversity measurement of the influence of mode of delivery (SVD v CS) on the infant oral microbiota at various time points (week 1, week 4, 6 months and 1 year).** A. Boxplot of the chao1 diversity, observed species, Phylogenetic diversity and Shannon diversity in the two groups (SVD & CS) at **week 1**. B. Boxplot of the chao1 diversity, observed species, Phylogenetic diversity and Shannon diversity in the two groups (SVD & CS) at **week 4**. C. Boxplot of the chao1 diversity, observed species, Phylogenetic diversity and Shannon diversity in the two groups (SVD & CS) at **6 months**. D. Boxplot of the chao1 diversity, observed species, Phylogenetic diversity and Shannon diversity in the two groups (SVD & CS) at **1 year**. Outliers are represented by black points.



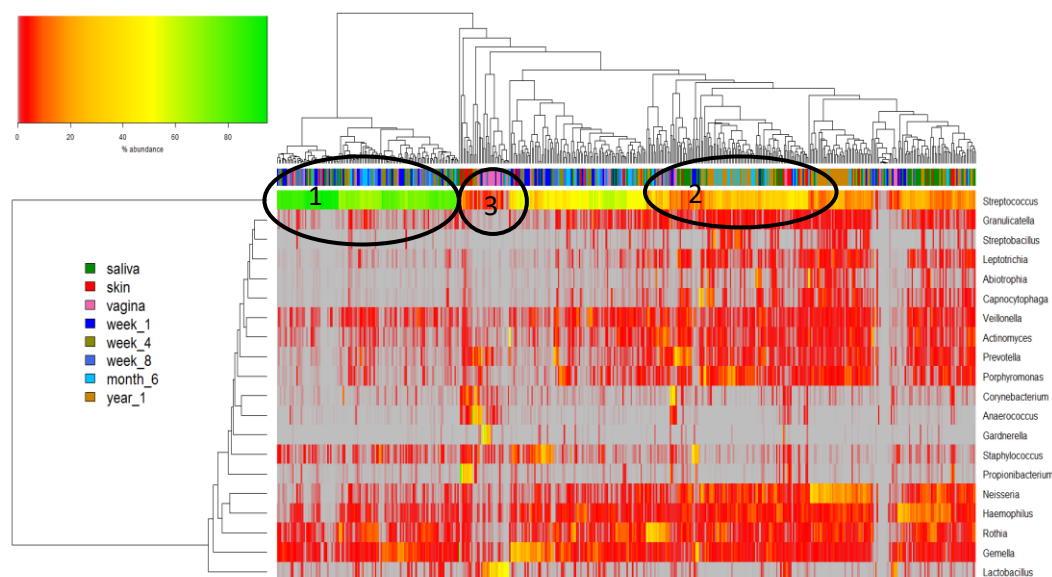
#### **4.1.5 The infant oral microbiota clusters by differential taxon abundance from week 1 to 8 weeks, with separation of year 1 infant oral microbiota.**

The abundance levels in different microbiota datasets (skin/vaginal/saliva) was graphically demonstrated by hierarchical clustering and the similarity between these samples was represented by vertical and horizontal dendrograms incorporating a “heatmap” colour scale to convey abundance levels at genus and species level (Fig. 4.5, 4.6). In this analysis, we compared the abundance levels of the taxa in the maternal (skin/vagina/saliva) datasets along with the infant oral microbiota datasets at the various ages. In figure 4.7, as illustrated by hierarchical clustering we compared the microbiota datasets from mother (saliva) and infant oral microbiota pairs, at genus level and clustering of mother-infant pairs were presented on a cluster dendrogram (Supplementary figure 4.1).

The abundance of bacterial taxa at genus level, of the maternal (skin/vagina/saliva) datasets along with the infant oral microbiota datasets at the various ages is illustrated in figure 4.5. There is evidence of two main branches in the horizontal dendrogram above the colour bar. The first branch illustrates clustering of samples, mainly of oral origin from the infant at weeks 1, 4, 8 and 6 months, with a very high abundance of the genera *Streptococcus* (Fig 4.5: labelled 1). Interestingly, the year 1 oral samples from the infant appear clustered to the right, together with some 6 month infant oral samples, with 6 month samples seen as a transition between the early infant oral microbiota (week 1 to week 8) to later ages of 1 year (Fig. 4.5: labelled 2). The genera that are abundant in year 1 infant oral microbiota include *Neisseria*, *Haemophilus*, *Porphyromonas* and *Streptococcus*. At 1 year of age, there are more diverse genera present, compared to 1 week of age of the infant, where the diversity is less, and only a few genera

dominate, such as *Streptococcus*. Although we do not see a distinct trend where the oral microbiota cluster based on increased age of the infant, we do however see that at the early ages of the infant, week 1 to week 8, there is tighter clustering, compared to the later stages where 6 months to closer to 1 year of age. This trend is supported by our alpha diversity measures (Fig. 4.3), where from 6 months the difference in diversity becomes more significant than the earlier time points.

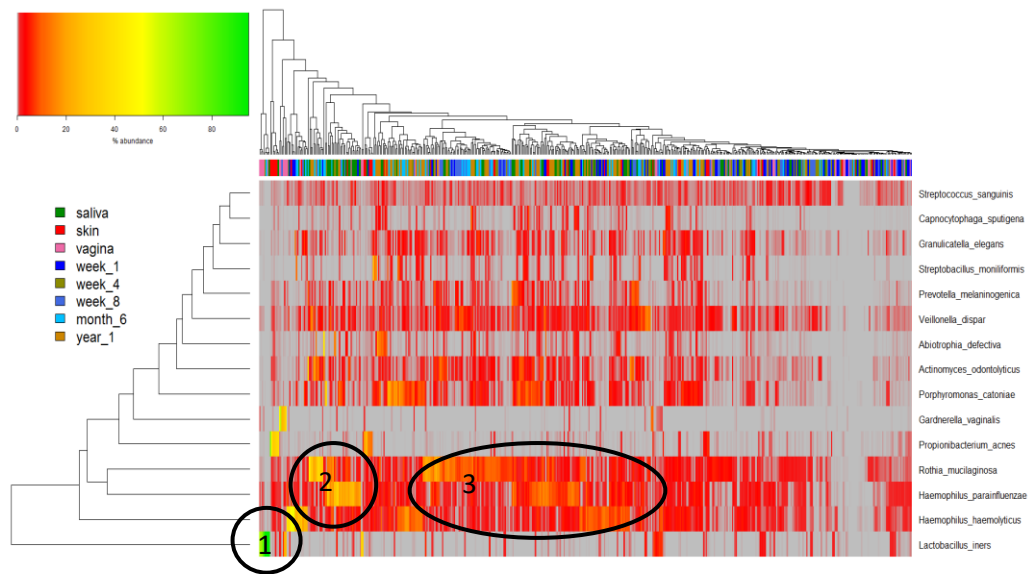
The maternal saliva microbiota data appear to be distributed throughout the horizontal dendrogram, with small areas of tight clustering, highlighting its high diversity, with increased abundances of genera *Streptococcus*, *Neisseria*, *Haemophilus* and *Prevotella*. The maternal skin and vaginal microbiota cluster at one point (Fig. 4.5: labelled 3). There are high abundances of genus *Streptococcus* numbers in both samples, with the genera *Lactobacillus* in high abundances in the vaginal samples only. The genera associated with the skin samples are more variable, with higher diversity, including *Propionibacterium*, *Haemophilus*, and *Staphylococcus*.



**Fig. 4.5. Hierarchical clustering of microbiota data at bacterial Genus level.**

Abundances are colour-coded according to the colour key on the top left with grey representing a value of zero. Euclidean distance and complete linkage were used to cluster the rows and columns of the heatmap. The colour bar on side of the heatmap corresponds to sample type. Each genus with a mean  $\geq 0.5\%$  across all samples was included. All taxa present at less than 1% in all groups are excluded from the heatmap.

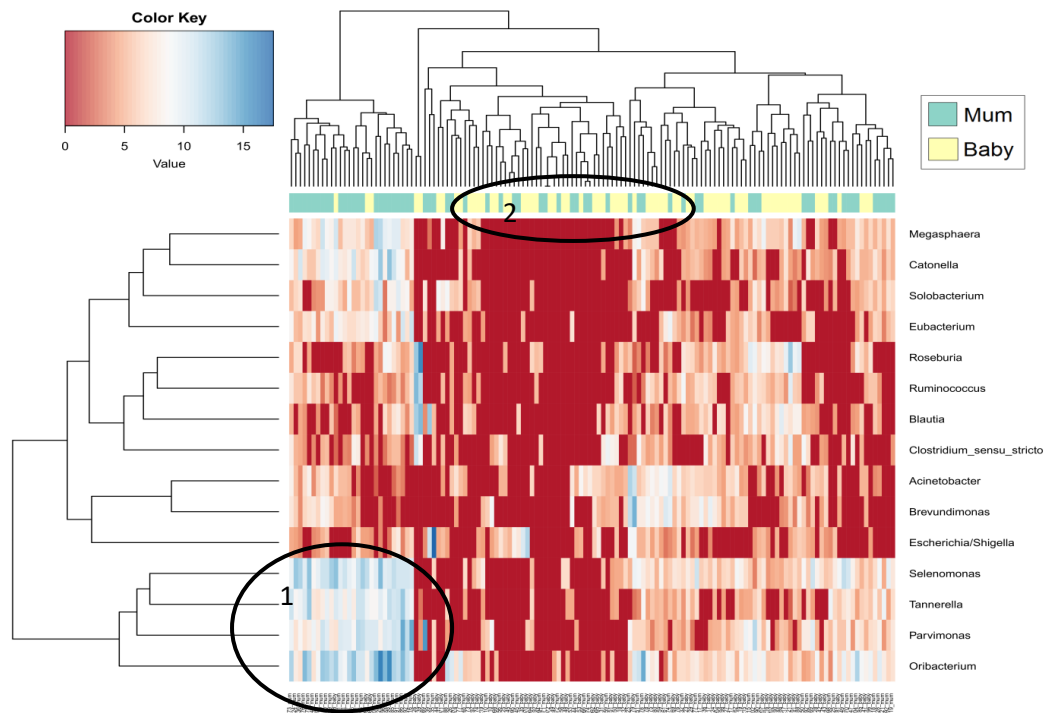
We included in this analysis all samples with at least one species present with a median value  $\geq 0.5\%$  across all samples, and this is presented in figure 4.6. There was no obvious clear separation between each group of samples evident. We did however notice tight clustering of some samples, such as vaginal samples with high abundances of *Lactobacillus iners* (Fig. 4.6: labelled 1), skin samples with high abundance of *Propionibacterium acnes* (Fig. 4.6: labelled 2) and oral saliva samples with high abundances of *Rothia mucilaginosa*, *Haemophilus parainfluenzae* and *Haemophilus haemolyticus* (Fig. 4.6: labelled 3).



**Fig. 4.6. Hierarchical clustering of microbiota data at bacterial species level.**

Abundances are colour-coded according to the colour key on the top left with grey representing a value of zero. Euclidean distance and complete linkage were used to cluster the rows and columns of the heatmap. The colour bar on top of the heatmap is coloured according to sample type. All taxa present with at least one species with a median value  $\geq 0.5\%$  across all samples are included.

We compared the saliva microbiota of the mother with the the infant oral microbiota and bacterial taxon abundance were illustrated on a vertical and horizontal dendrograms (Fig. 4.7). This analysis did not identify individual mother to infant pairs clustering together (Supplementary figure 4.1), but did identify clustering of maternal saliva microbiota and infant oral microbiota as separate groups. One subset of mothers illustrated a high abundance of genera *Selenomonas*, *Oribacterium* and *Tanerella*, (Fig. 4.7. labelled 1), and a lower abundance of genera such as *Ruminococcus*, *Escherichia/Shigella*. Infant sample clustering was evident, with genera such as *Selenomonas*, *Onbacterium*, and *Megasphaera* in higher abundance (Fig. 4.7: labelled 2).



**Fig. 4.7. Hierarchical clustering of microbiota data between maternal saliva microbiota and infant oral microbiota at bacterial Genus level.** Abundances are colour-coded according to the colour key on the top left with red representing a value of zero. Euclidean distance and complete linkage were used to cluster the rows and columns of the heatmap. The colour bar on side of the heatmap corresponds to sample type (green =mother, yellow=infant). All taxa present at less than 1% in all three groups are excluded from the heatmap.

#### **4.1.6 Broad and fine detail compositional differences distinguish between maternal and infant microbiota datasets.**

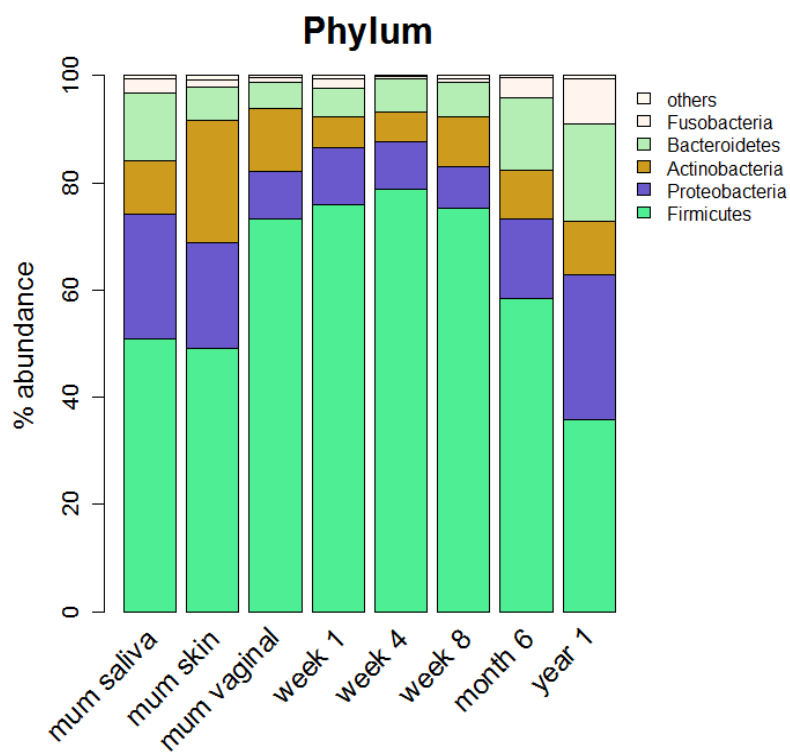
We assessed all samples in each group (mother: skin, vagina & oral/saliva; Infant Oral at week 1, 4, 8, 6 months & 1 year) for their compositional differences at Phylum, Genus and species level. The relative abundances of each group is presented at each level (Fig. 4.8) and their abundances and any significant differences between groups. The mothers oral saliva is dominated by Firmicutes, followed by Proteobacteria and Bacteroidetes. The skin and vagina both have a mixed composition also dominated by Firmicutes, while the mothers skin has increased Proteobacteria and Actinobacteria, and both body sites are statistically different in their microbiota composition ( $p < 0.001$ ) (Fig. 4.8A).

At phylum level (Fig. 4.8A), the composition of the oral microbiota between week 1 and 1 year is different ( $p < 0.001$ ), while only from 6 months do we see the significance begin to emerge. The infant oral saliva microbiota is dominated from birth to 6 months by Firmicutes and is relatively stable in its abundance from week 1 to 6 months (Fig. 4.8A). Firmicutes levels between week 1 and week 8 are similar with relative abundances at week 1 (82.3%), week 4 (87.3%) and at 8 weeks (81.1%). Only after 8 weeks does the abundance of Firmicutes begin to decrease, from 6 months (60.2%) until 1 year (33.7%), and this coincides with an increase in abundances of Proteobacteria at 1 year (25.6%) ( $p < 0.0001$ ) and Bacteroidetes (16.7%). These changes from 6 months to 1 year may be due to the influence of tooth eruption and introduction of solid foods which typically occurs at this time.

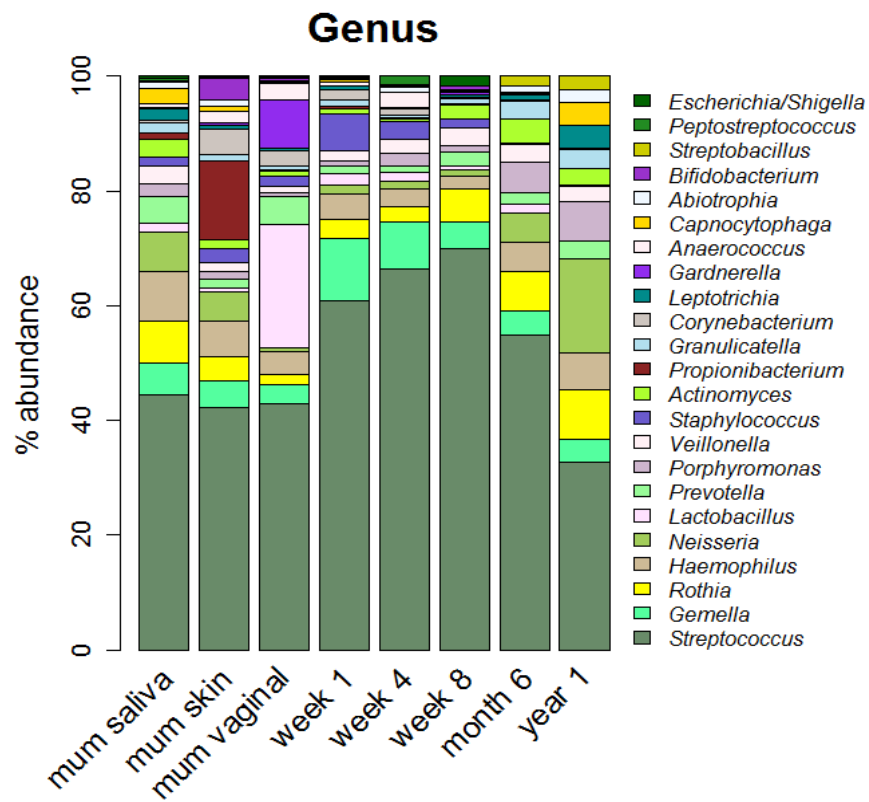
As expected, at genus level, the dominant genera of the infant oral microbiota at 1 week of age was *Streptococcus*, with *Streptococcus* levels gradually increasing in abundance, from week 1 (53.8%) to week 4 (67.6%) and week 8 (67.5%) (Fig. 4.8B). However, beyond 8 weeks, this trend ceases, with decreasing levels of *Streptococcus* at 6 months (42.8%) and again at 1 year of age (23.1%). When *Streptococcus* levels begin to decrease, this is counteracted by increased abundances by year 1 of genera such as *Neisseria* (10.3%), *Porphyromonas* (3.96%), *Rothia* (3.68%) and *Haemophilus* (3.87%).

By 1 year of age, the infant microbiota is different to the microbiota at a younger age ( $p < 0.001$ ) and there was increased presence of genera such as *Streptococcus*, *Porphyromonas*, *Neisseria* and *Haemophilus*. Although the microbial composition within the oral microbiota of an infant aged year 1 is composed of genera similar to the mother's microbiota, their microbial composition is different ( $p < 0.001$ ) (Fig. 4.8). From week 1 to year 1, species that dominate the oral microbiota include *Rothia mucilaginosa*, *Haemophilus haemolyticus*, *Haemophilus parainfluenzae*, *Porphyromonas catoniae*, *Veillonella dispar*, *Lactobacillus iners* and *Prevotella melaninogenica*.

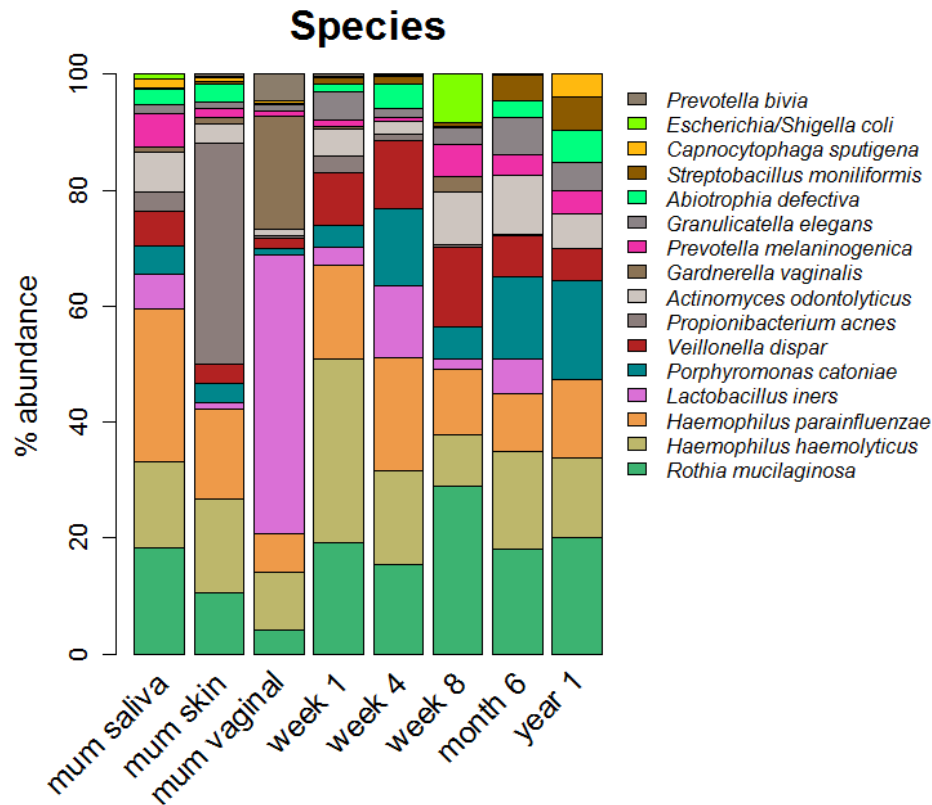




A.



B.



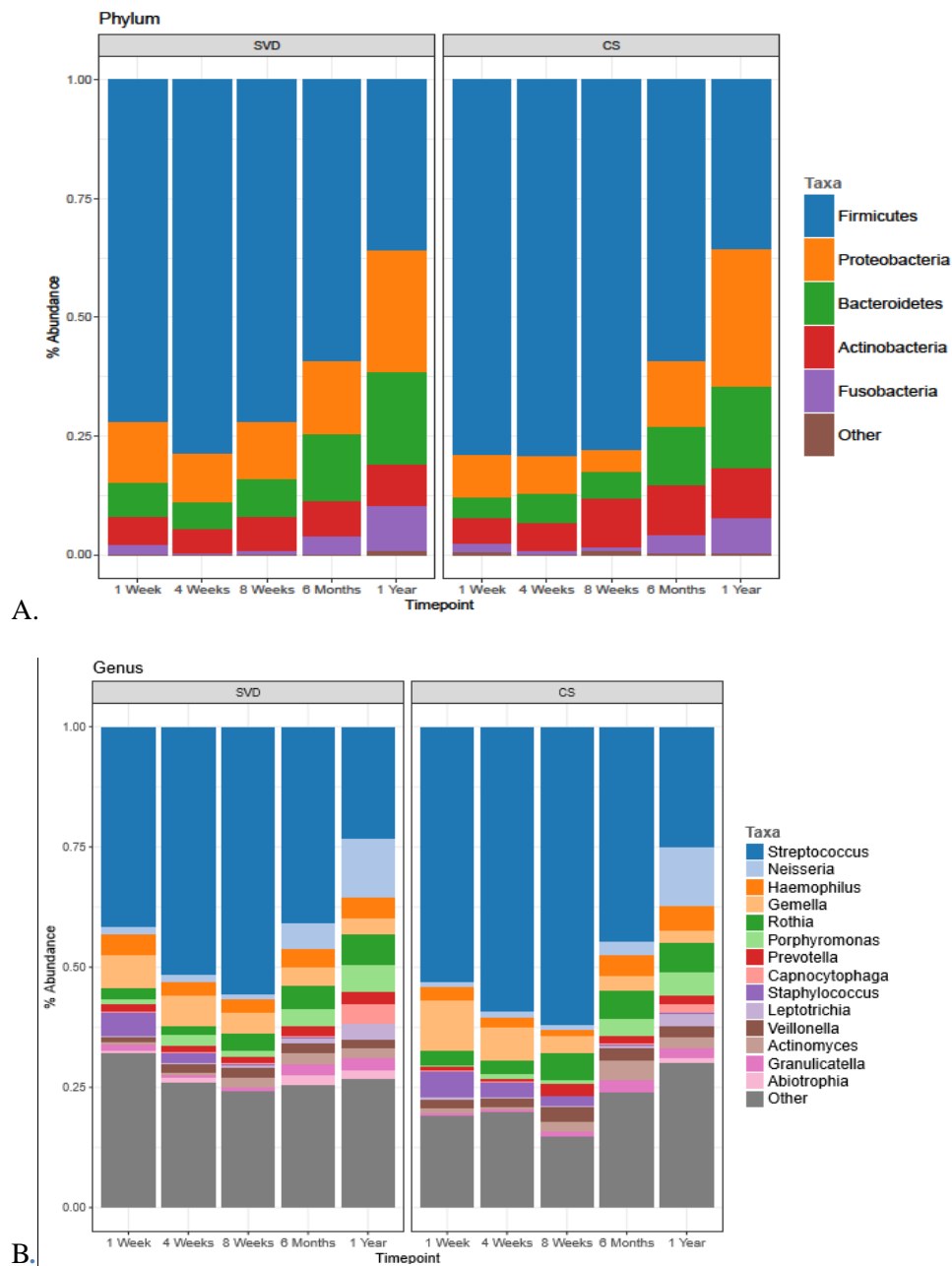
C.

**Fig. 4.8. Broad and fine detail compositional differences at Genus, phylum and species level.**

A. Microbiota composition at phylum level. Percentages for each taxon represent the median abundance values for the sample types. B. Barplot of percentage abundance at genus level. Percentages for each taxon represent the median values for the groups. C: Barplot of percentage abundance at species level. Percentages for each taxon represent the median values for the groups.

The bacterial composition of the mothers skin includes *Propionibacterium*, *Streptococcus* and *Corneibacterium*, and the species present include *Propionibacterium acnes*, *Haemophilus parainfluenzae/haempolticus* and *Rothia mucilaginosa*. The vaginal microbiota composition is made up mainly of the genera *Streptococcus*, *Haemophilus*, *Neisseria* and *Lactobacillus*, and species

*Lactobacillus iners* is the main *Lactobacillus* species identified in the vaginal microbiota of the mother, and makes up the majority of the composition, followed by *Gardnerella vaginalis* (Fig. 4.8C). The influence of birth mode on the oral microbiota from these maternal body sites (skin/vagina) is evident within 1 week of age only but not beyond (Fig 4.2). *Lactobacillus iners* is present at week 1 and surprisingly increases in abundances by week 4 in the infants oral microbiota highlighting the potential transfer from mother (vaginal) to infant, along with species *Propionibacterium acnes* from maternal skin. To assess the effect of birth mode on the oral microbiota composition of the infant, we divided the oral microbiota of infants by birth mode (Figure 4.9). At phylum level, for both CS and SVD delivered infants, there is a similar pattern in the phylum composition over time (Fig.4.9A), while at genus level, at week 1, infants born by SVD appear to have lower levels of *Streptococcus*, *Gemella* and more *Porphyromonas* and *Prevotella* than CS infants at week 1.



**Fig. 4.9. Comparison of the microbiota composition of infants born by different birth modes (SVD & CS) across five time-points from one week to 1 year of age.**

A. Microbiota composition at phylum level. Percentages for each taxon represent the median abundance values for the sample types. B. Barplot of percentage abundance at genus level. Percentages for each taxon represent the median values for the groups. Graph includes data for genera found at >1% average in total population. Genera found at <1% were grouped as “other”.

#### **4.1.7 Breast feeding duration did not influence the oral microbiota of the infant.**

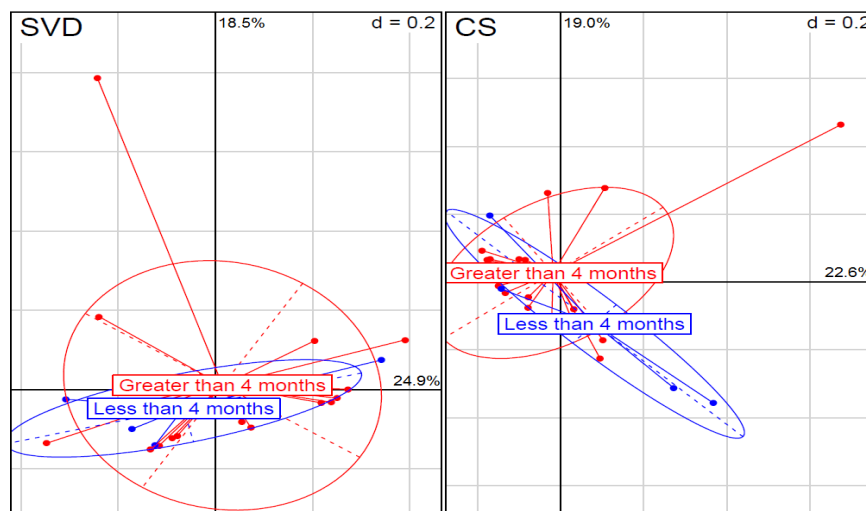
Metadata were collected for each infant from birth until 1 year of age including duration of exclusive breastfeeding. Two categories were recorded: less than 4 months and greater than 4 months duration of breastfeeding (Table 4.3). this data was recorded by during the home-visits to the Mother and Infant at 4 weeks, 8 weeks, 6 months etc. Significance of microbiota composition comparison was calculated using permutational multivariate analysis of variance (PerMANOVA) (Table 4.4) and presented by PCoA (principle co-ordinates) plots (Fig. 4.10). No differences in the oral microbiota of the infants were detected when separated by birth mode (SVD & CS) and the duration of breast feeding (Fig. 4.10). When infant microbiota data of both birth modes were combined, the duration of breast feeding did not influence the infant oral microbiota (Fig. 4.10C). Thus, this analysis indicates that breastfeeding, less than or greater than 4 months does not influence the oral microbiota of infants, independent of birth mode.

	<b>Breast Feeding Duration</b>	
<b>Birth Mode</b>	> 4 months	< 4 months
CS	18	4
SVD	18	4

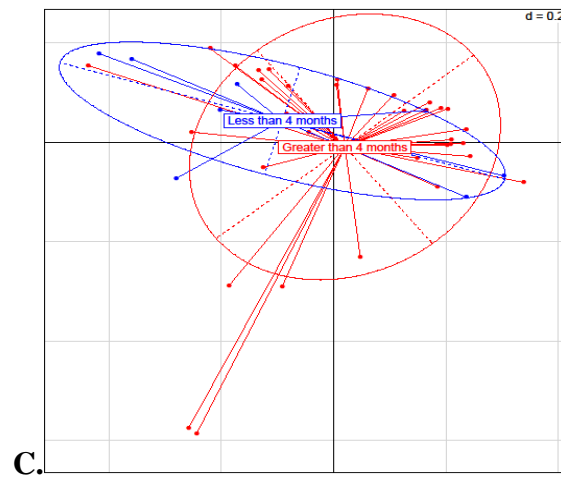
**Table 4.3.** Total sample number of exclusively breast fed infants included in the analysis whom breast fed for greater or less than 4 months.

Birth Mode	PerMANOVA p-value
CS	0.246
SVD	0.359
Combined (SVD & CS)	0.23

**Table 4.4.** Significance values (p-values) calculated using permutational multivariate analysis of variance (PerMANOVA) for the difference between ‘less than 4 months breast feeding’ and ‘greater than 4 months breast feeding’ for SVD and CS infants. Both groups (CS & SVD) were combined and tested against duration of breast feeding.



**Fig. 4.10.** Beta diversity PCoA (principle co-ordinates) plots (Bray Curtis) illustrating the influence of breast feeding duration on the oral microbiota of Spontaneous vaginally delivered (SVD) and C-sectioned (CS) infants. A. Spontaneous vaginally delivered infants (SVD). B. Caesarean sectioned infants (CS). In blue are infants that were breastfed for less than 4 months. In red are infants that were breast fed exclusively for longer than 4 months.



**C. Beta diversity PCoA (principle co-ordinates) plots (Bray Curtis) illustrating influence of breast feeding duration on the oral microbiota of Combined (CS & SVD) infants.**

#### 4.1.8 Supplementary Material

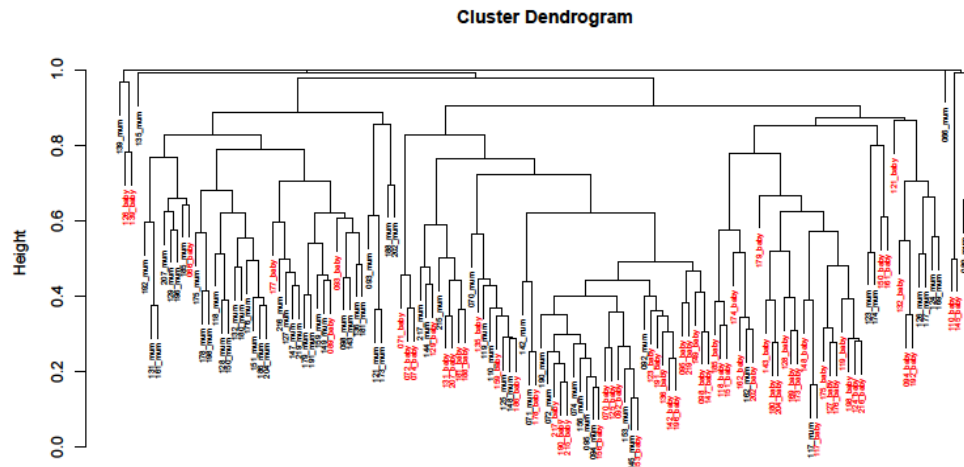
			Total number of samples
<b>Vaginal</b>			37
<b>Skin</b>			37
<b>Oral: Mother</b>			81
	<b>CS</b>	<b>SVD</b>	
<b>Oral: Infant</b>			
<b>1 Week</b>	<b>41</b>	<b>34</b>	75
<b>4 Weeks</b>	<b>31</b>	<b>29</b>	60
<b>8 Weeks</b>	<b>33</b>	<b>26</b>	59
<b>6 Months</b>	<b>37</b>	<b>26</b>	63
<b>1 Year</b>	<b>43</b>	<b>40</b>	83

**Supplementary table 4.1.** Total number of samples collected in this study from mother at birth and from infant at the various time points.



Oligoname	Barcode	Linker	Forward Primer (520F)	Whole Forward Primer	Length (bp)
V4F_7	AACCGGAA	AA	AYTGGGYDTAAAGNG	AACCGGAAAAAYTGGGYDTAAAGNG	25
V4F_78	ACACTGTG	AA	AYTGGGYDTAAAGNG	ACACTGTGAAAYTGGGYDTAAAGNG	25
V4F_139	ACGATCCA	AA	AYTGGGYDTAAAGNG	ACGATCCAAAAAYTGGGYDTAAAGNG	25
V4F_272	AGGTACGA	AA	AYTGGGYDTAAAGNG	AGGTACGAAAAAYTGGGYDTAAAGNG	25
V4F_494	CATCGTTC	AA	AYTGGGYDTAAAGNG	CATCGTCAAAAYTGGGYDTAAAGNG	25
V4F_632	CGTATTGG	AA	AYTGGGYDTAAAGNG	CGTATTGGAAYTGGGYDTAAAGNG	25
V4F_693	CTCTACAC	AA	AYTGGGYDTAAAGNG	CTCTACACAAAYTGGGYDTAAAGNG	25
V4F_948	GCTACGTA	AA	AYTGGGYDTAAAGNG	GCTACGTAAAAAYTGGGYDTAAAGNG	25
V4F_1062	GTAGTGGA	AA	AYTGGGYDTAAAGNG	GTAGTGGAAYTGGGYDTAAAGNG	25
V4F_1116	GTGTCAGT	AA	AYTGGGYDTAAAGNG	GTGTCAGTAAAYTGGGYDTAAAGNG	25
V4F_1178	TACGAAGG	AA	AYTGGGYDTAAAGNG	TACGAAGGAAAYTGGGYDTAAAGNG	25
V4F_1247	TCAGCACA	AA	AYTGGGYDTAAAGNG	TCAGCACAAAAAYTGGGYDTAAAGNG	25
V4F_1430	TGGAGTTC	AA	AYTGGGYDTAAAGNG	TGGAGTCAAAAYTGGGYDTAAAGNG	25
Oligoname	Barcode	Linker	Reverse Primer (926R)	Whole Reverse Primer	Length (bp)
V5R_22	AACGGCTT	TT	CCGTCAATTYYTTTRAGTT	AACGGCTTTTCCGTCAATTYYTTTRAGTT	30
V5R_125	ACCTTGCT	TT	CCGTCAATTYYTTTRAGTT	ACCTTGCTTTCCGTCAATTYYTTTRAGTT	30
V5R_341	ATCGAACC	TT	CCGTCAATTYYTTTRAGTT	ATCGAACCTTCCGTCAATTYYTTTRAGTT	30
V5R_452	CAGAACTC	TT	CCGTCAATTYYTTTRAGTT	CAGAACTCTTCCGTCAATTYYTTTRAGTT	30
V5R_555	CCTACGAA	TT	CCGTCAATTYYTTTRAGTT	CCTACGAATTCCGTCAATTYYTTTRAGTT	30
V5R_592	CGATATGC	TT	CCGTCAATTYYTTTRAGTT	CGATATGCTTCCGTCAATTYYTTTRAGTT	30
V5R_889	GATGCAAG	TT	CCGTCAATTYYTTTRAGTT	GATGCAAGTTCCGTCAATTYYTTTRAGTT	30
V5R_992	GGATGGTA	TT	CCGTCAATTYYTTTRAGTT	GGATGGTATCCGTCAATTYYTTTRAGTT	30
V5R_1108	GTGATCTG	TT	CCGTCAATTYYTTTRAGTT	GTGATCTGTTCCGTCAATTYYTTTRAGTT	30
V5R_1234	TCACCTGT	TT	CCGTCAATTYYTTTRAGTT	TCACCTGTTTCCGTCAATTYYTTTRAGTT	30
V5R_1463	TGTCTCAC	TT	CCGTCAATTYYTTTRAGTT	TGTCTCACTTCCGTCAATTYYTTTRAGTT	30
V5R_1526	TTGCGCTT	TT	CCGTCAATTYYTTTRAGTT	TTGCGCTTTTCCGTCAATTYYTTTRAGTT	30

**Supplementary table 4.2:** Complete list of oligonucleotides used for PCR amplification.



Clustering of Bray-Curtis beta diversity for mum and baby saliva samples

**Supplementary figure 4.1.** Cluster dendrogram of maternal saliva microbiota data and infant oral microbiota data.

Alpha diversity metric	1 Week N=75	4 Weeks N=60	8 Weeks N=59	6 Months N=63	1 Year N=83	p-value overall
chao1	1076 [948;1188]	1031 [888;1146]	1025 [913;1110]	842 [773;995]	819 [715;901]	<0.001
PD wholetree	26.2 [23.2;29.6]	24.7 [22.9;27.8]	25.3 [23.0;27.4]	24.7 [23.2;26.8]	24.5 [23.1;26.9]	0.047
Simpson	0.96 [0.95;0.97]	0.96 [0.95;0.97]	0.96 [0.95;0.97]	0.96 [0.94;0.97]	0.96 [0.94;0.97]	0.98
Shannon	6.52 [6.13;6.82]	6.53 [6.25;6.80]	6.50 [6.15;6.63]	6.35 [5.88;6.78]	6.34 [5.87;6.68]	0.162
observed species	516 [472;593]	504 [455;556]	492 [465;521]	479 [440;524]	473 [432;517]	<0.001

Alpha diversity metric	1 Week vs. 4 Weeks	1 Week vs. 8 Weeks	1 Week vs. 6 Months	1 Week vs. 1 Year
chao1	0.136	0.117	<0.001	<0.001
PD wholetree	0.159	0.159	0.14	0.139
Simpson	0.976	0.976	0.976	0.976
Shannon	0.774	0.605	0.538	0.241
observed species	0.356	0.061	0.021	0.002

Alpha diversity metric	4 Weeks vs. 8 Weeks	4 Weeks vs. 6 Months	4 Weeks vs. 1 Year
chao1	0.941	<0.001	<0.001
PD wholetree	0.881	0.98	0.881
Simpson	0.976	0.976	0.976
Shannon	0.774	0.774	0.395
observed species	0.43	0.14	0.038

Alpha diversity metric	8 Weeks vs. 6 Months	8 Weeks vs. 1 Year
chao1	<0.001	<0.001
PD wholetree	0.765	0.728
Simpson	0.976	0.976
Shannon	0.774	0.486
observed species	0.451	0.138

Alpha diversity metric	6 Months vs. 1 Year	6 Months vs. 2 Years	1 Year vs. 2 Years
chao1	0.015	0.005	0.235
PD wholetree	0.881	0.213	0.159
Simpson	0.976	0.976	0.976
Shannon	0.605	0.605	0.774
observed species	0.516	0.451	0.703

**Supplementary table 4.3:** Alpha diversity stats (p-values) between each time point of the infant oral microbiota.

Genera	1 Week vs. 4 Weeks	1 Week vs. 8 Weeks	1 Week vs. 6 Months	1 Week vs. 1 Year
<b>Streptococcus</b>	0.076	0.023	0.245	<0.001
<b>Unclassified</b>	0.248	0.14	0.193	<0.001
<b>Neisseria</b>	0.54	0.826	<0.001	<0.001
<b>Haemophilus</b>	0.358	0.217	0.017	<0.001
<b>Gemella</b>	0.543	0.091	0.069	0.018
<b>Rothia</b>	0.668	0.012	<0.001	<0.001
<b>Porphyromonas</b>	0.866	0.22	<0.001	<0.001
<b>Prevotella</b>	0.597	0.074	<0.001	<0.001
<b>Lactobacillus</b>	0.439	0.35	<0.001	<0.001
<b>Capnocytophaga</b>	0.133	0.893	0.007	<0.001
<b>Staphylococcus</b>	0.512	0.003	<0.001	<0.001
<b>Leptotrichia</b>	0.326	0.424	<0.001	<0.001
<b>Veillonella</b>	0.328	0.002	<0.001	<0.001
<b>Actinomyces</b>	0.543	0.004	<0.001	<0.001
<b>Bifidobacterium</b>	0.492	0.076	0.615	<0.001
<b>Granulicatella</b>	0.292	0.06	<0.001	<0.001
<b>Abiotrophia</b>	0.3	0.365	0.032	<0.001
<b>Fusobacterium</b>	0.956	0.462	<0.001	<0.001
<b>Streptobacillus</b>	0.087	0.002	<0.001	<0.001
<b>Corynebacterium</b>	0.323	0.272	0.002	0.117
<b>Propionibacterium</b>	0.26	0.2	0.022	<0.001
<b>Lachnoanaerobaculum</b>	0.701	0.039	<0.001	<0.001
<b>Oribacterium</b>	0.456	0.694	<0.001	<0.001

**Supplementary table 4.4:** Genera difference and associated p-values between

week 1 and older age.

Genera	8 Weeks vs. 6 Months	8 Weeks vs. 1 Year	6 Months vs. 1 Year
<b>Streptococcus</b>	<0.001	<0.001	<0.001
<b>Unclassified</b>	0.001	<0.001	0.045
<b>Neisseria</b>	<0.001	<0.001	<0.001
<b>Haemophilus</b>	<0.001	<0.001	0.014
<b>Gemella</b>	0.888	0.483	0.483
<b>Rothia</b>	0.38	0.03	0.18
<b>Porphyromonas</b>	<0.001	<0.001	0.001
<b>Prevotella</b>	0.006	<0.001	0.009
<b>Lactobacillus</b>	<0.001	<0.001	0.001
<b>Capnocytophaga</b>	0.009	<0.001	<0.001
<b>Staphylococcus</b>	0.018	<0.001	0.001
<b>Leptotrichia</b>	<0.001	<0.001	<0.001
<b>Veillonella</b>	0.328	0.736	0.215
<b>Actinomyces</b>	0.005	<0.001	0.44
<b>Bifidobacterium</b>	0.031	<0.001	<0.001
<b>Granulicatella</b>	<0.001	<0.001	0.932
<b>Abiotrophia</b>	0.005	<0.001	<0.001
<b>Fusobacterium</b>	<0.001	<0.001	<0.001
<b>Streptobacillus</b>	<0.001	<0.001	<0.001
<b>Corynebacterium</b>	0.089	0.654	0.117
<b>Propionibacterium</b>	0.26	0.002	0.022
<b>Lachnoanaerobaculum</b>	<0.001	<0.001	0.005
<b>Oribacterium</b>	<0.001	<0.001	<0.001

**Supplementary table 4.5:** Genera difference and associated p-values between 8

weeks and 6 months and 1 year of age.

## **Results:**

### **4.2. Longitudinal study: Birth to 2 years of age.**

The infant oral microbiota demonstrates continued change in microbial composition from birth to 2 years of age.

## 4.2. Results

Age of Infant	Numbers of samples		
	CS	SVD	Total
1 Week	41	34	75
4 Weeks	31	29	60
8 Weeks	33	26	59
6 Months	37	26	63
1 Year	43	40	83
2 Years	18	24	42

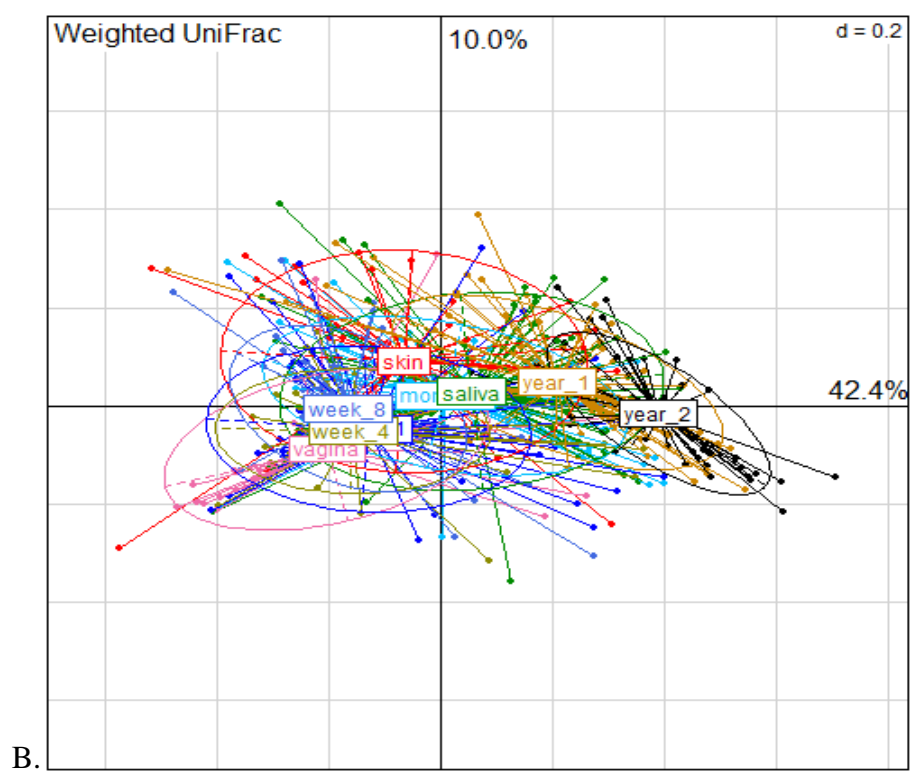
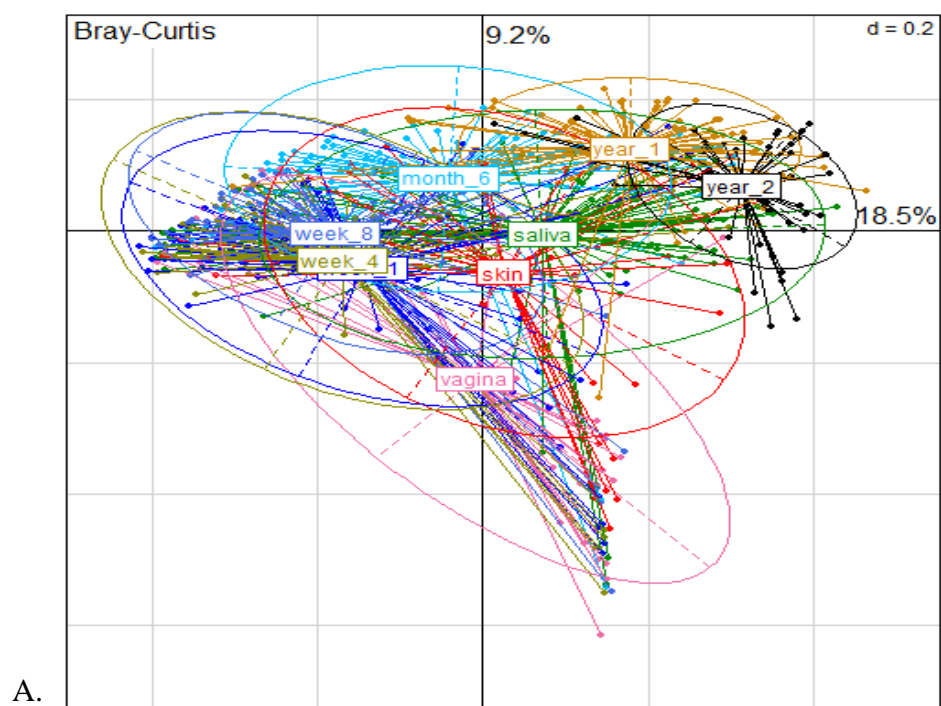
**Table 4.5.** Number of oral samples collected in this study at each of the various infant ages.

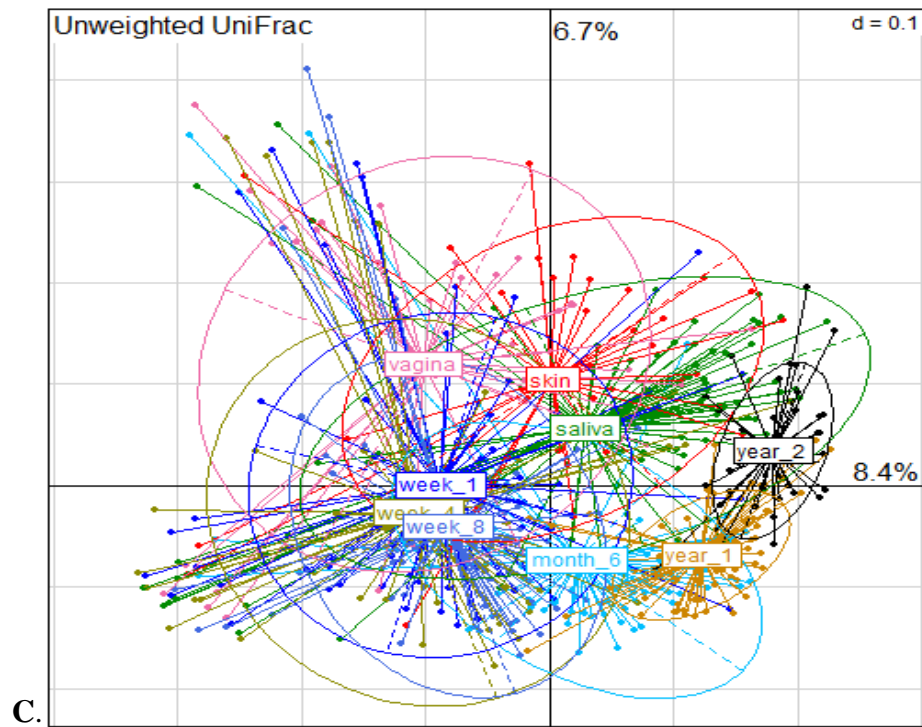
### 4.2.1 The oral microbiota of infants at 2 years of age is distinct and is not altered by birth mode.

PCoA (principle co-ordinates) plots were generated including the year 1 infant oral microbiota data and the maternal microbiota data (vagina, skin, oral) using the beta diversity measures: Bray Curtis dissimilarity and UniFrac distances metrics (Fig 4.11), which measure diversity between samples. Following on from the year 1 analysis, we found that with increased age, the oral microbiota of the infant became more distinct, demonstrated by separate clustering, with minimal overlap at year 1 and year 2 on the Bray Curtis plot (Fig. 4.11A), weighted unifrac (Fig. 4.11B) and unweighted unifrac (Fig. 4.11C). While the early time points overlap (week 1, 4 & 8), there is a transition from 6 months to year 1 and year 2 oral microbiota. The year 2 oral microbiota of the infant demonstrates sharing of

taxa with the year 1 oral microbiota and saliva of the mother in Bray Curtis and weighted analysis (Fig. 4.11A, B). However in the unweighted analysis (Fig. 4.11C), where rare taxa are measured, rather than abundance, the year 2 infant oral microbiota demonstrates less relatedness. Using Permutational Multivariate Analysis of Variance (PerMANOVA) analysis we found statistical differences between the abundance of each group at OTU level, as measured by each metric ( $p < 0.001$ ) (Table 4.6). The separation of the year 2 infant oral microbiota from the earlier ages, highlights differences in beta diversity between samples at 2 years of age, and signifies development in the oral microbiota from 8 weeks of age.







**Fig.4.11. Principle Coordinates Analyses (PCoAs) on Spearman distance matrices of samples from mother (saliva, skin & vagina) & from infant oral at 6 time points (week 1, week 4, week 8, 6 months, 1 year and 2 years).**

A. Bray Curtis dissimilarity, B. UniFrac Weighted & C. Unweighted UniFrac between the 5 groups (mother saliva/oral, skin & vaginal & infant oral week 1, week 4, week 8, 6 months, 1 year and 2 years). A. Plot of principle co-ordinates using **Bray-Curtis dissimilarity**. Points are coloured according to group and ellipses describe the distribution of points for each group. Percentage variation explained: PCA 1 (18.5%) and PCA 2 (9.2%). B. Plot of principle co-ordinates using **weighted unifrac distance**. Points are coloured according to group and ellipses describe the distribution of points for each group. Percentage variation explained: PCA 1 (42.4%) and PCA 2 (10%). C. Plot of principle co-ordinates using **un-weighted unifrac distance**. Points are coloured according to group and

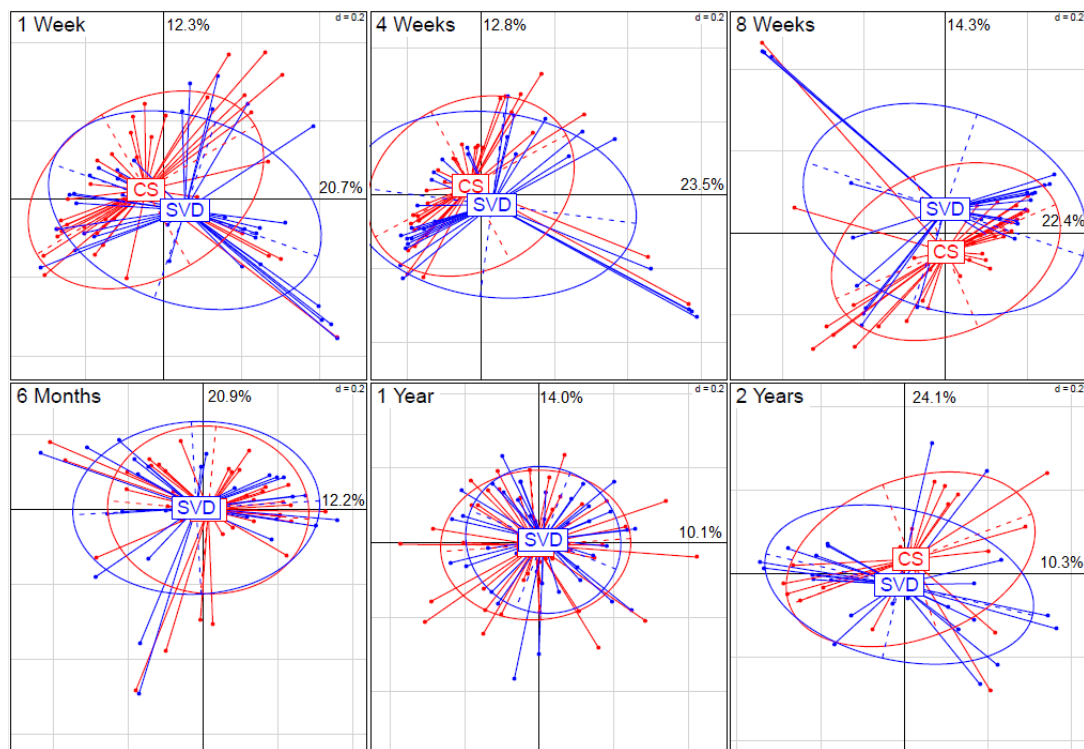
ellipses describe the distribution of points for each group. Percentage variation explained: PCA 1 (8.4%) and PCA 2 (6.7%).

	X-axis	Y-axis	PerMANOVA P-value
Unweighted UniFrac	8.4	6.7	0.001**
Weighted UniFrac	42.4	10	0.001**
Bray-Curtis	18.5	9.2	0.001**

**Table 4.6.** Significance values of Bray Curtis dissimilarity and UniFrac distances metrics. Significance was calculated using permutational multivariate analysis of variance (PerMANOVA).  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*).

#### 4.2.2 Birth mode does not impact on the infant oral microbiota by 2 years of age.

To assess the affect of birth mode on the infant oral microbiota, we compared the infant oral microbiota by age and birth mode (CS & SVD), with significance calculated by PerMANOVA. This analysis indicated that there was a significant difference by birth mode in the infant oral microbiota at week 1 (( $p < 0.05$  (\*))), but not at 2 years of age, ( $p=0.502$ ).



**Fig. 4.12. PCoA (principle co-ordinates) plots, each demonstrating the effect of birth mode (SVD v CS) on oral microbiota composition of the infant at different ages (week 1, week 4, week 8, 6 months, 1 year and 2 years).**

A. Week 1 SVD v CS; B. Week 4 SVD v CS; C. Week 8; D. 6 months (week 24); E. 1 year; F. 2 years. Significance was calculated using permutational multivariate analysis of variance (PerMANOVA) (Table 4.7).

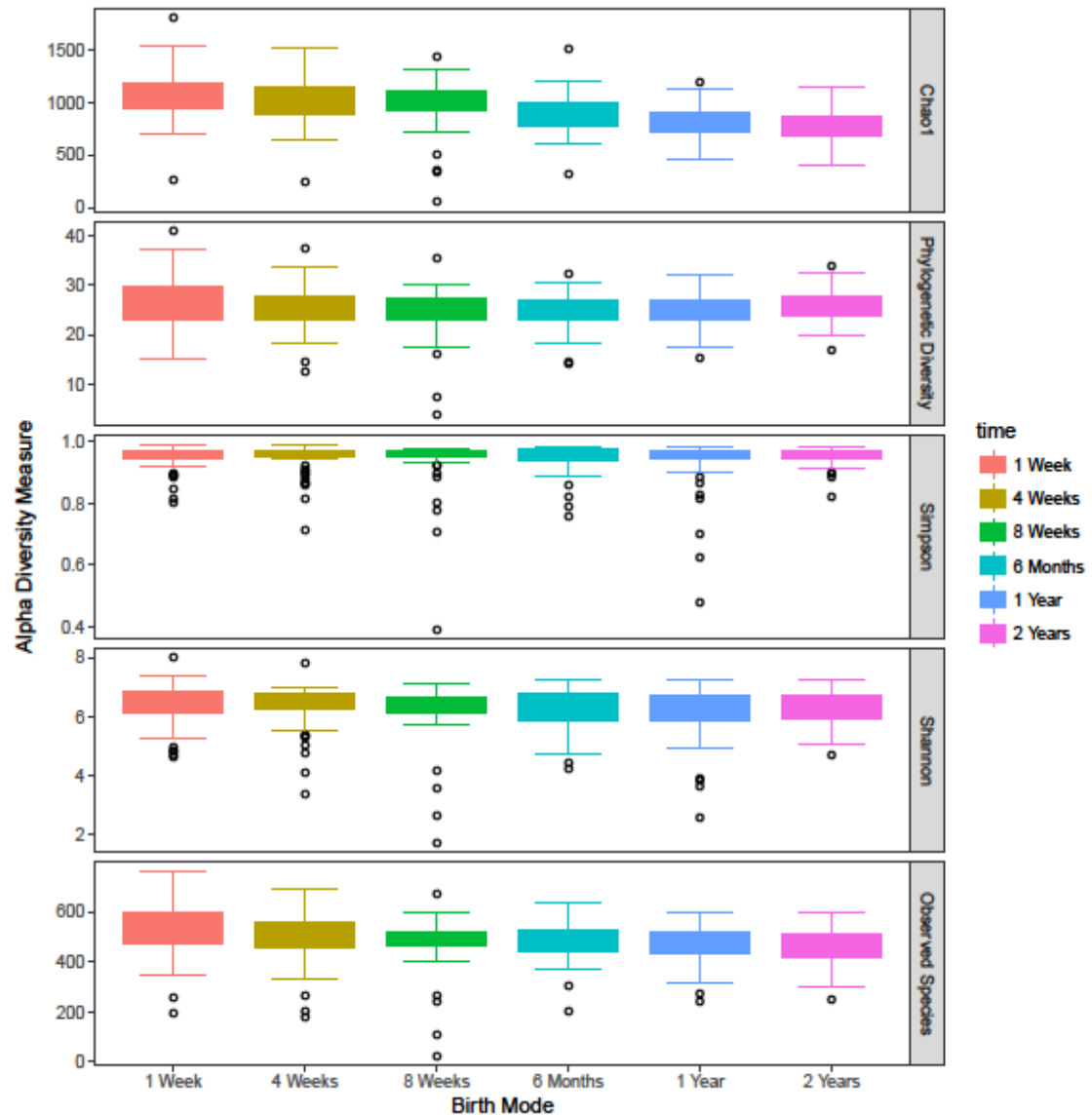
Timepoint	PerMANOVA p-value
1 Week	0.047*
4 Weeks	0.336
8 Weeks	0.077
6 Months	0.347
1 Year	0.314
2 Years	0.502

**Table 4.7.** Significance values for the comparison of the oral microbiota data difference between SVD and CS at each age of the infant. Significance was calculated using permutational multivariate analysis of variance (PerMANOVA).  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*).

#### **4.2.3 Species richness and diversity of the infant oral microbiota at 2 years of age are lower than at an early age.**

The alpha diversity measurement of species richness was lower at 2 years of age and was accompanied by microbiota composition changes from the early age data when measured by Chao diversity and OTU count (Observed species). Significance was measured using the Mann-Whitney U test ( $p < 0.001$ ), while the Phylogenetic diversity showed lower significance ( $p < 0.05$ ) (Table 4.8). The Chao diversity, which measures species richness within the infant oral microbiota, demonstrated that the oral microbiota diversity from week 1 to 2 years of age decreased as the infant increased in age. The differences in diversity between week 1 and week 8 infant oral microbiota and the year 2 infant oral microbiota was higher ( $p < 0.001$ ) than when the year 2 oral microbiota compared to the 6 months infant oral microbiota ( $p < 0.01$ ). While we identified these differences in diversity between the year 2 oral microbiota and at 1 week to 6 months of age, no difference in the diversity was found between the year 1 and year 2 infant oral

microbiota ( $p$  value = 0.235) demonstrating some relative stability with increased age.



**Fig.4.13.** Alpha diversity comparisons of the oral microbiota of infants from week 1, week 4, 8 weeks, 6 months, 1 year and 2 years). Boxplots of Chao1 diversity, Phylogenetic diversity, Simpson diversity, Shannon diversity and Observed species in the infant oral microbiota with increased age. Outliers are represented by black points.

Alpha diversity metric	p-value 1 Week vs 2 Years	p-value 8 Weeks vs 2 Years	p-value 6 Months vs 2 Years	p-value 1 Year vs 2 Years	p-value overall
chao1	<0.001	<0.001	0.005	0.235	<0.001**
PD_whole_tree	0.722	0.463	0.213	0.159	0.047*
Simpson	0.976	0.976	0.976	0.976	0.98
Shannon	0.241	0.486	0.605	0.774	0.162
observed_species	0.009	0.181	0.451	0.703	<0.001**

**Table 4.8.** Alpha diversity metrics each demonstrating individual P-values using the Mann-Whitney U test at each time point of the infant oral microbiota. Significant differences between groups are shown by the following notation:  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*)

By alpha diversity measures (chao1 diversity, observed species, Phylogenetic diversity, Simpson diversity, and Shannon diversity), we calculated the diversity of the oral microbiota datasets from infants at different ages, separated by birth mode (Fig. 4.14). It was found by Shannon diversity that birth mode had a significant influence on the diversity of the infant oral microbiota at 1 and 4 weeks of age ( $p < 0.05$ ), but not by 2 years of age (Fig. 4.14) (Table 4.9).



**Fig 4.14. Comparison of Alpha Diversity measurement of the infant oral microbiota as a function of mode of delivery (SVD v CS) at various infant ages (week 1, week 4, 6 months, 1 year and 2 years).** A. Boxplots of the chao1 diversity, observed species, Phylogenetic diversity, Simpson diversity, Shannon diversity, Observed species in the two groups (SVD & CS) at **week 1, 4 weeks, 8 weeks, 6 months, 1 year and 2 years, separated by birth mode (SVD or CS).** Outliers are represented by black points.



	1 Week	4 Weeks	8 Weeks
<b>chao1</b>	0.259266	0.233729	0.725472
<b>PD whole tree</b>	0.343536	0.216761	0.541395
<b>Simpson</b>	0.147782	0.067716	0.625151
<b>Shannon</b>	0.037*	0.022286*	0.830745
<b>observed species</b>	0.17648	0.106851	0.836693

	6 Months	1 Year	2 Years
<b>chao1</b>	0.706215	0.912915	0.611223
<b>PD whole tree</b>	0.615251	0.722269	0.252732
<b>Simpson</b>	0.219237	0.590785	0.959459
<b>Shannon</b>	0.264049	0.376689	0.721968
<b>observed species</b>	0.538987	0.887654	0.702975

**Table 4.9.** Alpha diversity comparisons and accompanying P-values using the Mann-Whitney U test on whether SVD differs from CS at each time point of the infant oral microbiota. Significant differences between groups are shown by the following notation:  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*).

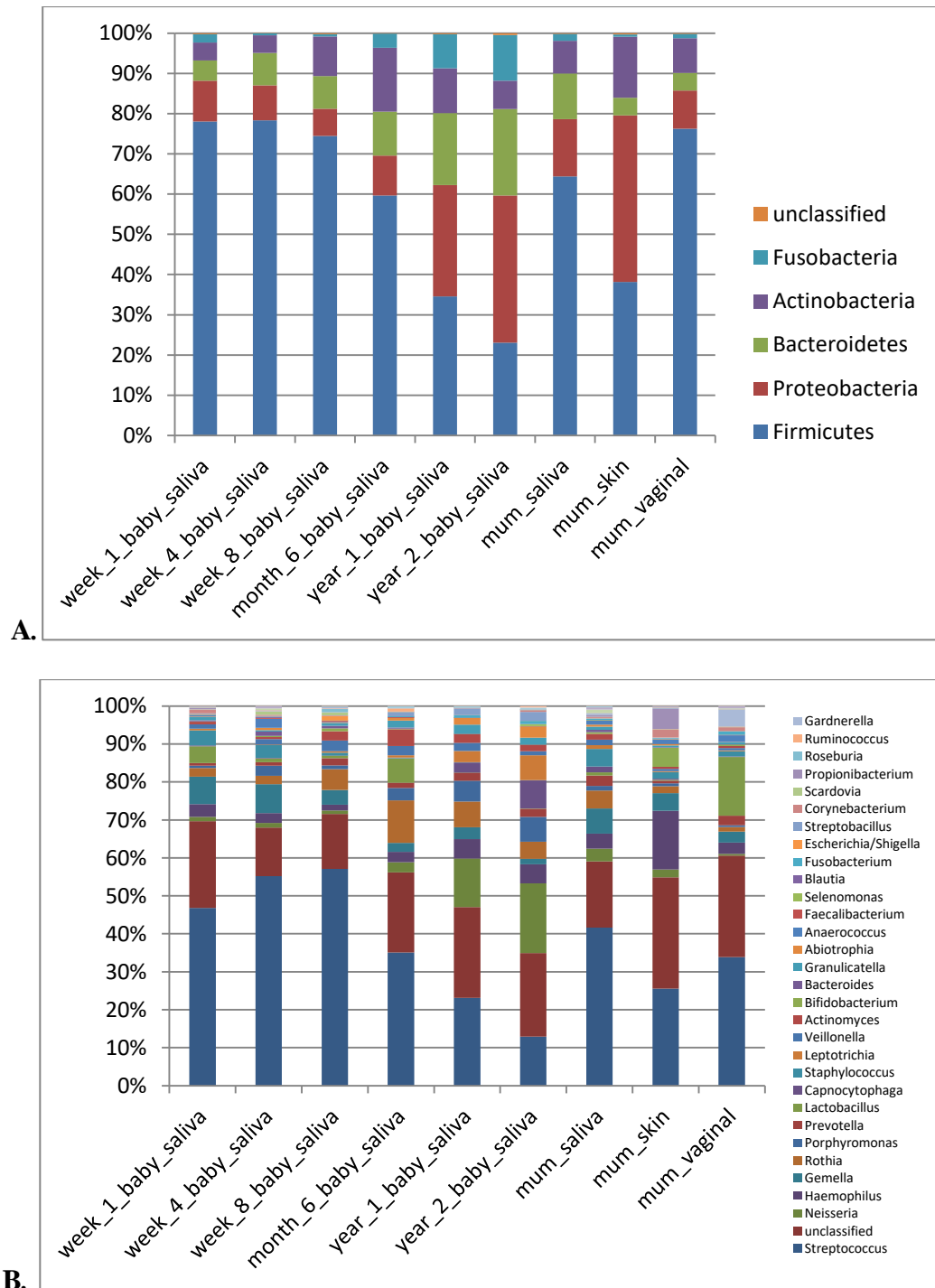
#### 4.2.4 Clear separation of the infant oral microbiota at 2 years of age.

The relatedness of microbiota datasets by bacterial taxon abundance of the infant oral microbiota datasets at different ages at genus level were represented by a vertical and horizontal dendrogram (Fig. 4.15). The infant oral microbiota at week 1, week 4 and week 8 demonstrates clustering with high abundances of *Streptococcus*, *Gemella* and *Haemophilus*. However the strongest clustering occurs at 2 years of age when the infant oral microbiota harbours a distinct oral microbiota, with high abundances of *Neisseria* (16.4%), *Haemophilus* (5.24%) and *Streptococcus* (11.2%) and very low abundances of *Lactobacillus*, *Anaerococcus*, *Staphylococcus* (0-0.01%). While we noted some fluctuations in abundances of the infant oral microbiota between week 1 and 2 years of age ( $p < 0.001$ ), only a minority of genera are different between year 1 and year 2 of age and include genera *Streptococcus*, *Gemella*, *Capnocytophaga* and *Staphylococcus* ( $p < 0.001$ ).



#### **4.2.5 Compositional analysis of the infant oral microbiota at 2 years of age.**

The infant oral microbiota composition is different across all major phyla between week 1 and 1 year and 2 years of age ( $p < 0.001$ ). While the phyla profiles appear relatively similar/stable from week 1 to week 8, compositional changes begin from 6 months onwards. The only phylum that is not statistically different in abundance between 6 months and 2 years of age is Actinobacteria. Between year 1 and year 2, the only two phyla that are statistically differentially abundant include Firmicutes and Proteobacteria ( $p < 0.001$ ). Firmicute levels decrease over time from week 1 (82.3%) to year 2 (21.1%), while Proteobacteria levels increase (3.98% at week 1 to 36.7% at year 2). Bacteroidetes (16.7%-17.7%), Actinobacteria (6.24-6.08%) and Fusobacteria (6.58-7.13%) remain consistent in abundances between year 1 and year 2, while Firmicutes decrease from year 1 (33.7%) to year 2 (21.1%) and Proteobacteria increase from year 1 (25.6%) to year 2 (36.7%). Thus at phylum level, from 1 year of age to 2 years of age there is evidence of some stability arising in the composition at phylum level (Fig 4.16A) with less change within the oral cavity of the infant.

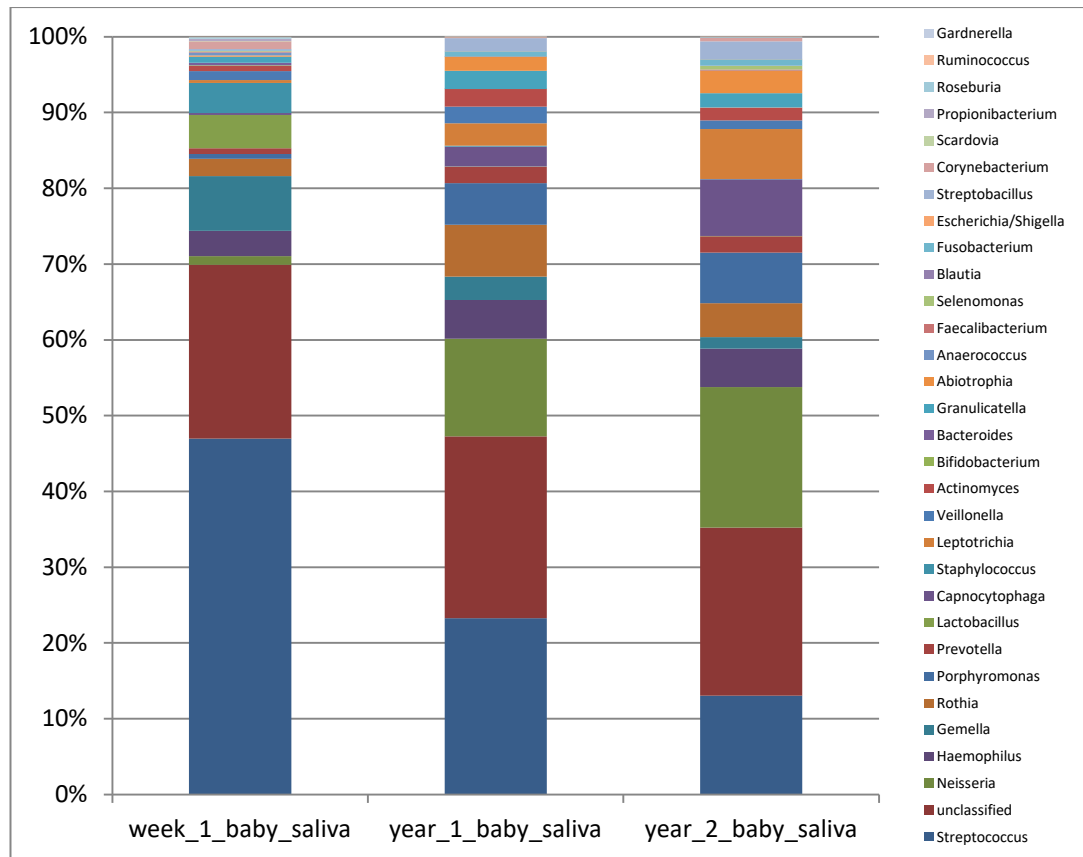


**Fig. 4.16. Broad and fine detail compositional differences at phylum and genus level.**

A. Microbiota composition at phylum level. Percentages for each taxon represent the median abundance values for the sample types. B. Barplot of percentage

abundance at genus level. Percentages for each taxon represent the median values for the groups.

There are compositional differences at genus level between the infant oral microbiota at week 1 to 2 years of age ( $p < 0.001$ ) (Fig. 4.17). The composition changes associated in this timeframe include genera *Streptococcus*, *Neisseria*, *Gemella*, *Capnocytophaga*, *Staphylococcus* ( $p < 0.001$ ) and less changes with genera *Porphyromonas*, *Leptotrichia*, *Veillonella* and *Fusobacteria* ( $p < 0.01$ ). Genera that remain similar and not significantly different in abundances between year 1 and year 2 oral microbiota include *Haemophilus*, *Rothia*, *Prevotella*, *Lactobacillus*, *Actinomyces*, *Propionibacterium* and *Oribacterium*. While no differences in alpha diversity (within samples) were noted between year 1 and year 2 (Table 4.8), we did see differences of significance with beta diversity measures. Beta diversity measures the diversity between samples and demonstrated significant differences between the infant oral microbiota at 2 years of age and the earlier ages of the infant (Fig. 4.15) (PerMANOVA:  $p < 0.001$ ). This separation between the year 1 and year 2 oral microbiota, along with the earlier ages of the infant (week 1, 4, 8) highlights developmental changes within the oral microbiota between from week 1 to 2 years of age. We therefore sought to identify differentially abundant taxa that must drive separation on these PCoAs between the infant oral microbiome at year 1 and year 2 (Fig. 4.11).



**Fig. 4.17. Compositional differences at Genus level of the infant oral microbiota at week 1, 1 year and 2 years of age.** Barplot of percentage abundance at genus level. Percentages for each taxon represent the median values for the groups.

The abundances levels of *Streptococcus* decrease dramatically from week 1 to year 2 of age, along with levels of *Gemella*, *Lactobacillus* and *Staphylococcus* ( $p < 0.001$ ) while genera such as *Haemophilus*, *Prevotella*, *Neisseria* and *Fusobacterium* increase dramatically. Between 1 year of age and 2 years of age, genera which decrease from 1 year to 2 years are highlighted in green, and those that decreased from 1 year to 2 years of age are highlighted in red (Table 4.10)

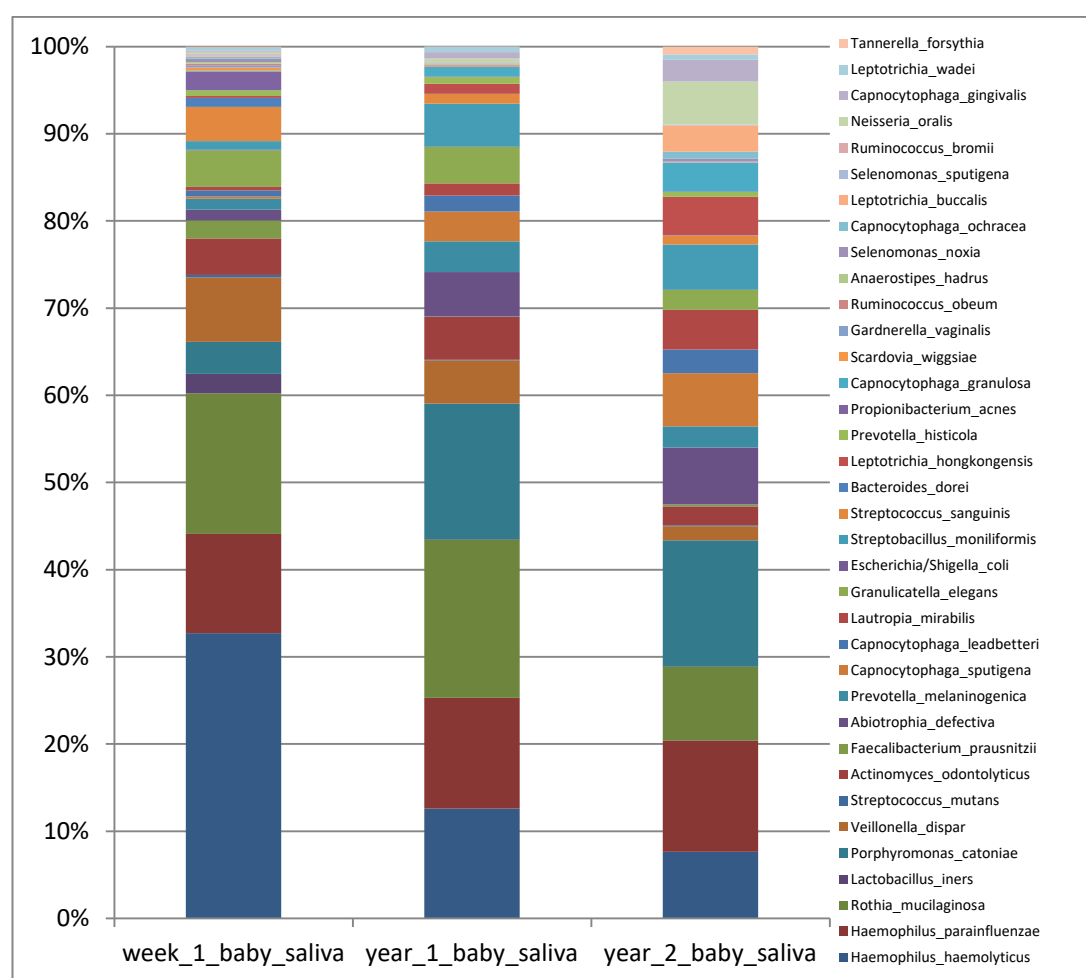
Genera	%Rel.Abundance		p-value
	1 Year N=83	2 Years N=42	1 Year vs 2 Years
<b>Streptococcus</b>	23.1 [16.1;29.5]	11.2 [7.36;17.8]	<0.001
<b>Unclassified</b>	20.5 [14.0;28.6]	21.9 [18.3;25.0]	0.503
<b>Neisseria</b>	10.3 [5.84;17.5]	16.4 [12.2;24.4]	0.002
<b>Haemophilus</b>	3.87 [2.45;6.77]	5.24 [2.93;7.92]	0.251
<b>Gemella</b>	2.18 [1.43;3.59]	1.26 [0.86;1.86]	<0.001
<b>Rothia</b>	3.68 [1.87;8.33]	2.55 [1.25;6.12]	0.161
<b>Porphyromonas</b>	3.96 [2.13;7.02]	6.50 [4.31;8.75]	0.008
<b>Prevotella</b>	1.22 [0.65;2.36]	1.33 [0.87;2.54]	0.661
<b>Lactobacillus</b>	0.00 [0.00;0.01]	0.00 [0.00;0.02]	0.637
<b>Capnocytophaga</b>	0.87 [0.29;3.25]	3.76 [2.21;11.7]	<0.001
<b>Staphylococcus</b>	0.05 [0.02;0.10]	0.01 [0.00;0.04]	<0.001
<b>Leptotrichia</b>	1.85 [0.59;3.96]	3.09 [1.77;6.02]	0.005
<b>Veillonella</b>	1.27 [0.70;2.23]	0.74 [0.29;1.45]	0.005
<b>Actinomyces</b>	1.49 [0.72;2.38]	1.23 [0.87;2.02]	0.543
<b>Bifidobacterium</b>	0.01 [0.00;0.02]	0.01 [0.00;0.05]	0.042
<b>Granulicatella</b>	2.15 [1.20;3.02]	1.58 [1.05;2.27]	0.06
<b>Abiotrophia</b>	0.49 [0.20;1.23]	1.84 [0.85;3.53]	<0.001
<b>Fusobacterium</b>	0.33 [0.16;0.86]	0.67 [0.35;1.24]	0.009
<b>Streptobacillus</b>	1.01 [0.29;2.25]	0.76 [0.36;2.12]	0.734
<b>Corynebacterium</b>	0.03 [0.01;0.12]	0.31 [0.07;0.56]	<0.001
<b>Propionibacterium</b>	0.01 [0.00;0.03]	0.01 [0.00;0.02]	0.371
<b>Lachnoanaerobaculum</b>	0.23 [0.13;0.57]	0.31 [0.22;0.78]	0.07
<b>Oribacterium</b>	0.15 [0.06;0.50]	0.15 [0.08;0.47]	0.774

**Table 4.10. Average relative abundances (%) of genera present at 1 year and**

**2 years of age and the P-Values of statistical difference between the two time points.** Highlighted in red are the names of genera that significantly increase in relative abundances from year 1 to year 2, while in green are the names of genera that significantly decrease in abundances from year 1 to year 2; both of which demonstrate statistical differences.



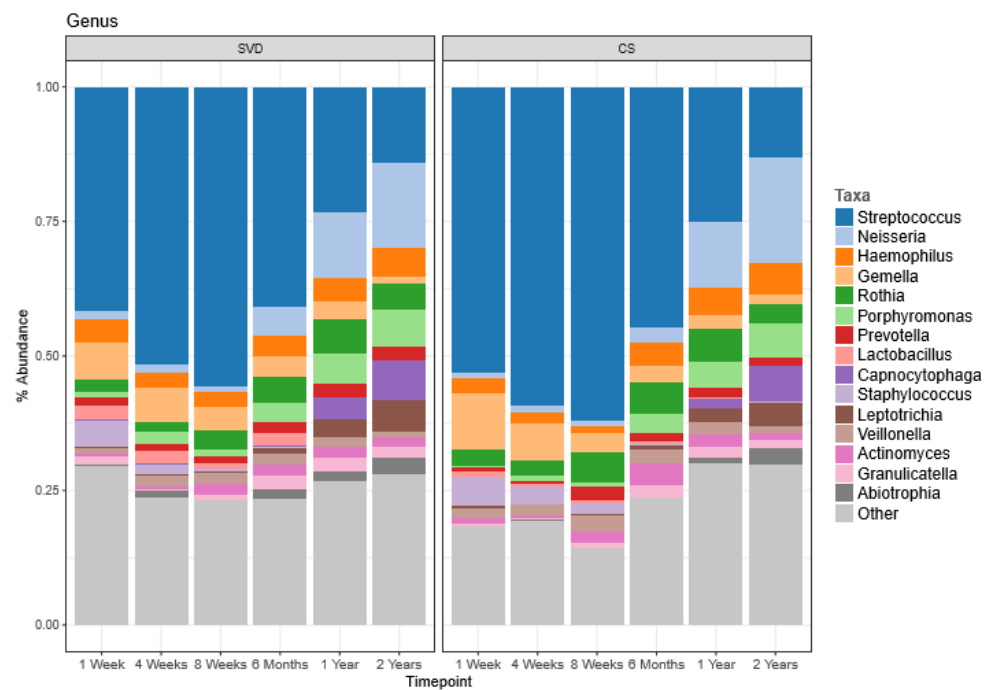
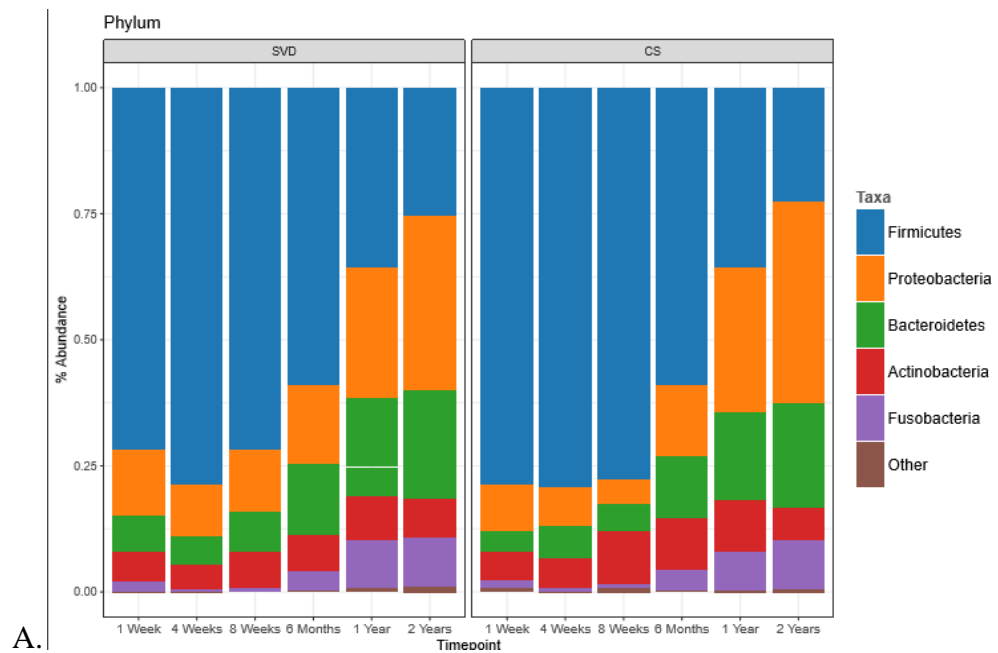
Compositional changes at species level are evident (Fig 4.18) between year 1 and year 2 infant saliva. The species that dominate the infant oral saliva at 1 and 2 years of age include *Haemophilus haemolyticus*, *Haemophilus parainfluenza*, *Rothia mucialoginosa* and *Porphyromonas catoniae*, while species which increase in abundances at 2 years of age include *Capnocytophaga sputigena*, *Capnocytophaga gingivalis*, *Leptotrichia buccalis*, *Neisseria oralis*, *Tannerella forsythia* and *Veillonella dispar*.



**Fig. 4.18. Compositional differences at species level in the infant oral microbiota at week 1, year 1 and 2 years of age. Barplot of percentage**

abundance at species level. Percentages for each taxon represent the median values for the groups.

To assess the effect of birth mode on the oral microbiota of the infant we separated the oral microbiota of infants by birth mode, and assessed the phyla and genera at each time point the oral microbiota of the infant was examined (Figure 4.19). At phylum level and genus level, for both CS and SVD delivered infants, there was no obvious impact of birth mode on the oral microbiota composition at 2 years of age, in contrast to previous observation of birth mode impact being evident at the early ages of the infant oral microbiota.



**Fig.4.19. Comparison of the microbiota composition of infants born by different birth modes (SVD & CS) across six time-points from one week to 2 years of age.**

A. Microbiota composition at phylum level. Percentages for each taxon represent the median abundance values for the sample types. B. Barplot of percentage

abundance at genus level. Percentages for each taxon represent the median values for the groups. Genera shown are those found at >1% average in total population. Genera found at <1% were grouped as “other”.

#### 4.2.6. Supplementary Material

			<b>Total</b>
<b>Vaginal</b>			37
<b>Skin</b>			37
<b>Oral: Mother</b>			81
	<b>CS</b>	<b>SVD</b>	
<b>Oral: Infant</b>			
<b>1 Week</b>	<b>41</b>	<b>34</b>	75
<b>4 Weeks</b>	<b>31</b>	<b>29</b>	60
<b>8 Weeks</b>	<b>33</b>	<b>26</b>	59
<b>6 Months</b>	<b>37</b>	<b>26</b>	63
<b>1 Year</b>	<b>43</b>	<b>40</b>	83
<b>2 Years</b>	<b>18</b>	<b>24</b>	42

**Supplementary table 4.6.** Total number of samples collected in this study from mother at birth and from infant at the various ages.

Alpha diversity metric	1 Week N=75	4 Weeks N=60	8 Weeks N=59
chao1	1076 [948;1188]	1031 [888;1146]	1025 [913;1110]
PD_whole_tree	26.2 [23.2;29.6]	24.7 [22.9;27.8]	25.3 [23.0;27.4]
Simpson	0.96 [0.95;0.97]	0.96 [0.95;0.97]	0.96 [0.95;0.97]
Shannon	6.52 [6.13;6.82]	6.53 [6.25;6.80]	6.50 [6.15;6.63]
observed_species	516 [472;593]	504 [455;556]	492 [465;521]

Alpha diversity metric	6 Months N=63	1 Year N=83	2 Years N=42	p-value overall
chao1	842 [773;995]	819 [715;901]	795 [681;868]	<0.001**
PD_whole_tree	24.7 [23.2;26.8]	24.5 [23.1;26.9]	26.3 [23.9;27.9]	0.047*
Simpson	0.96 [0.94;0.97]	0.96 [0.94;0.97]	0.96 [0.94;0.97]	0.98
Shannon	6.35 [5.88;6.78]	6.34 [5.87;6.68]	6.28 [5.89;6.69]	0.162
observed_species	479 [440;524]	473 [432;517]	488 [420;512]	<0.001**

**Supplementary table 4.7.** Alpha diversity metrics for each time point with OTU

counts and overall P-values using the Mann-Whitney U test. Significant differences between groups are shown by the following notation:  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*).

## **Results:**

### **4.3 Cross Sectional study.**

This chapter has been submitted for peer review in ‘Journal of Dental Research’.

#### **Entitled:**

‘Comparison of the salivary and dentinal microbiome of children with severe-early childhood caries to the salivary microbiome of caries-free children.’

Authors: Eimear Hurley, Maurice P.J Barrett, Martin Kinirons, Helen Whelton, C. Anthony Ryan, Catherine Stanton, Hugh M.B. Harris and Paul W. O’Toole.

### 4.3. Results

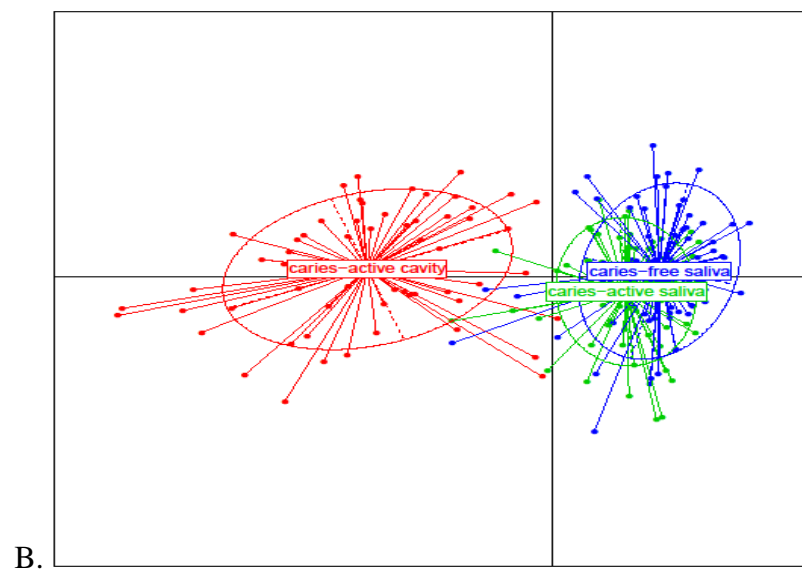
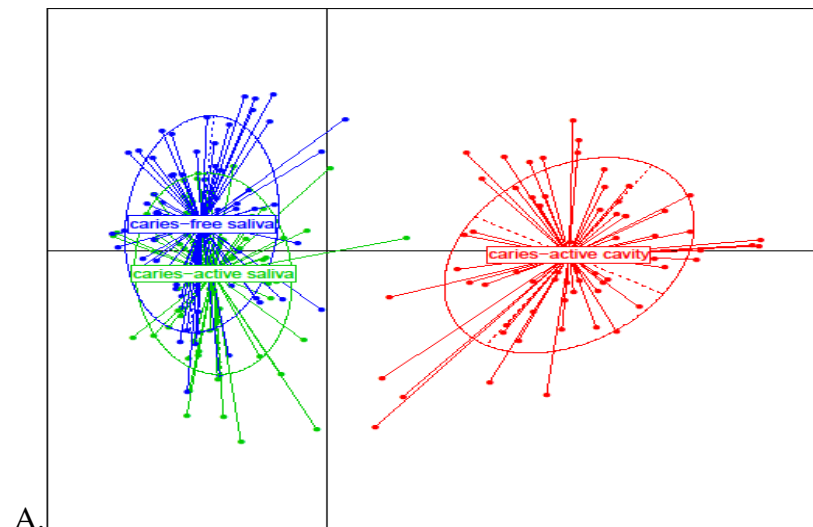
#### 4.3.1 A distinct microbiota in caries lesions but not saliva in children with S-ECC.

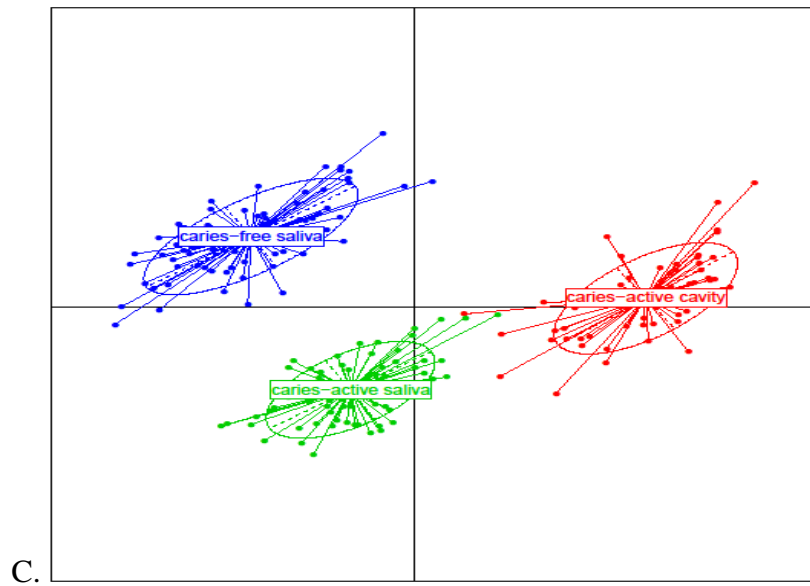
Given that S-ECC is such an acute disease, it seemed possible that it resulted from a global microbiota change in the oral cavity. To investigate the relatedness of microbiome composition in CAC (caries-active caries), CAS (caries-active saliva) and CFS (caries-free saliva), we generated PCoA (principle co-ordinates) plots showing relatedness by two established metrics, Bray Curtis dissimilarity, and UniFrac distances. The Bray Curtis plot (Fig.4.20A) shows separation of the caries lesion samples (CAC) and the two saliva sample types (CFS & CAS) based on PCoA axes 1 and 2. The microbiota of the two saliva groups (CAS & CFS) considerably overlap, indicating a similarity in the general composition of microbial taxa. For weighted UniFrac, (Fig.4.20B) the caries microbiota group is again separated from the two saliva microbiota groups, with only minimal overlap of some samples. Combined with the Bray Curtis analysis, this shows convincingly that there is no major separation between the microbiota of caries-active saliva (CAS) and caries-free saliva (CFS) groups, even though this metric is very sensitive to the differences in the presence/absence and abundance of OTUs/samples.

Plotting the second UniFrac metric, unweighted UniFrac distances (Fig.4.20C), illustrates separation between the three groups (CAC, CAS & CFS). This index measures the presence and absence of taxa only and does not adjust the distance metric according to taxon abundance, so unlike weighted analysis, it reflects the



contribution of rare taxa (that get overwhelmed in a weighted analysis). When presence/absence of taxa is the dominant parameter used to calculate distance, separation between all three groups occurs. The CAS microbiota was closer to the CAC microbiota than the CFS microbiota, suggesting rare taxa are shared between the former two samples.



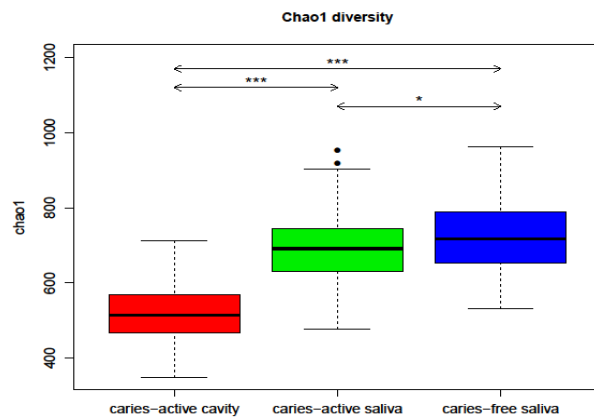


**Fig.4.20. PCoA (principle co-ordinates) plots showing relatedness by two established metrics, Bray Curtis dissimilarity, and UniFrac distances, while unweighted UniFrac illustrates separation between the three groups (CAC, CAS & CFS).**

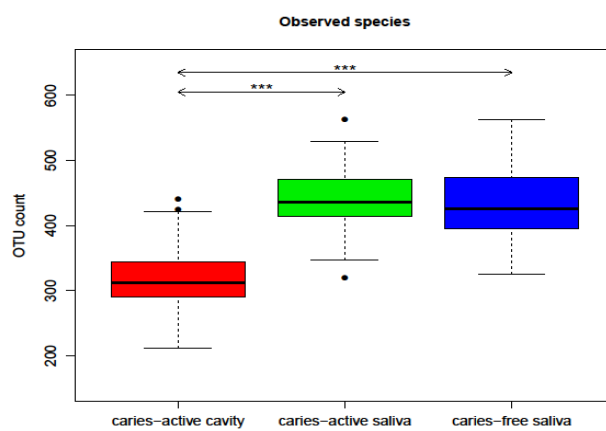
- A. Plot of principle co-ordinates using **Bray-Curtis dissimilarity**. Points are coloured according to group and ellipses describe the distribution of points for each group. Percentage variation explained: PCA 1 (22.3 %) and PCA 2 (7.7 %).
- B. Plot of principle co-ordinates using **weighted unifrac distance**. Points are coloured according to group and ellipses describe the distribution of points for each group. Percentage variation explained: PCA 1 (45 %) and PCA 2 (11.7 %).
- C. Plot of principle co-ordinates using **un-weighted unifrac** distance. Points are coloured according to group and ellipses describe the distribution of points for each group. Percentage variation explained: PCA 1 (16.6 %) and PCA 2 (4.9 %)

#### **4.3.2 Microbiota diversity in caries lesions is lower than that of saliva from children with or without S-ECC.**

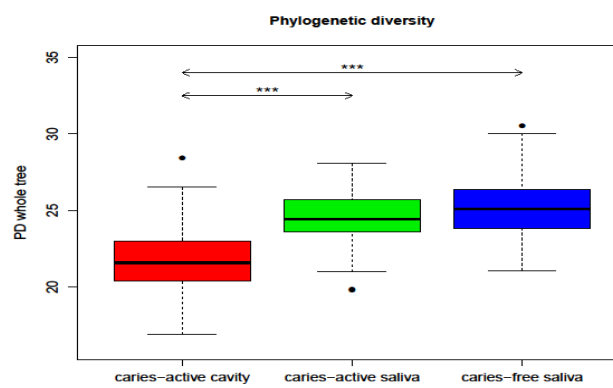
To study the diversity of the microbiota from the caries lesions (CAC) and saliva samples of both caries-free children (CFS) and caries-active children (CAS), we determined a series of alpha diversity metrics: the Chao index, phylogenetic diversity (PD whole tree), observed species (OTU count), the Simpson index and the Shannon index (Fig.4.21). All metrics illustrate that the diversity of caries microbiota was the lowest of these sample types. The difference in the diversity values of the caries samples and saliva samples was significant ( $p < 0.001$ ). The Chao diversity measurement, as illustrated in Fig.4.21A, estimates the number of species from observed data, and the diversity of the low abundance taxa, and according to this metric, the CFS versus CAS comparison was significantly different with a p-value of  $< 0.05$ . All other alpha diversity comparisons (Fig.4.21.B,C,D) indicated that the diversity difference between the caries samples and saliva samples was significant ( $p < 0.001$ ).



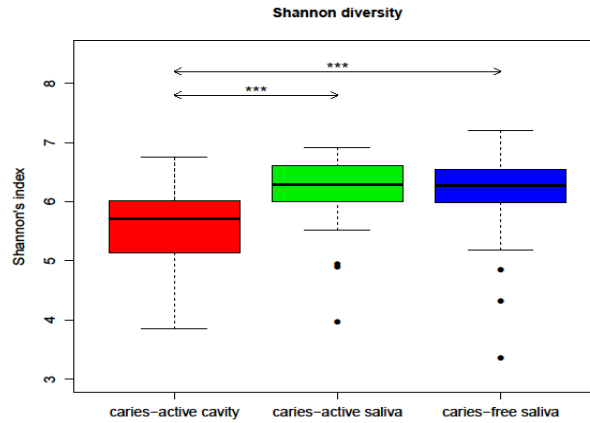
. A.



B.



C.



D.

**Fig 4.21. Pairwise alpha diversity comparisons of saliva and caries microbiota.**

A. Boxplot of chao1 diversity in the three groups. Outliers are represented by black points. Significant differences between groups are shown by arrows and the following notation:  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*). B. Boxplot of observed species in the three groups. Outliers are represented by black points. Significant differences between groups are shown by arrows and the following notation:  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*). C. Boxplot of Phylogenetic diversity in the three groups. Outliers are represented by black points. Significant differences between groups are shown by arrows and the following notation:  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*). D. Boxplot of Shannon diversity in the three groups. Outliers are represented by black points. Significant differences between groups are shown by arrows and the following notation:  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*).

### **4.3.3 Habitual diet is not significantly different in children with or without S-ECC.**

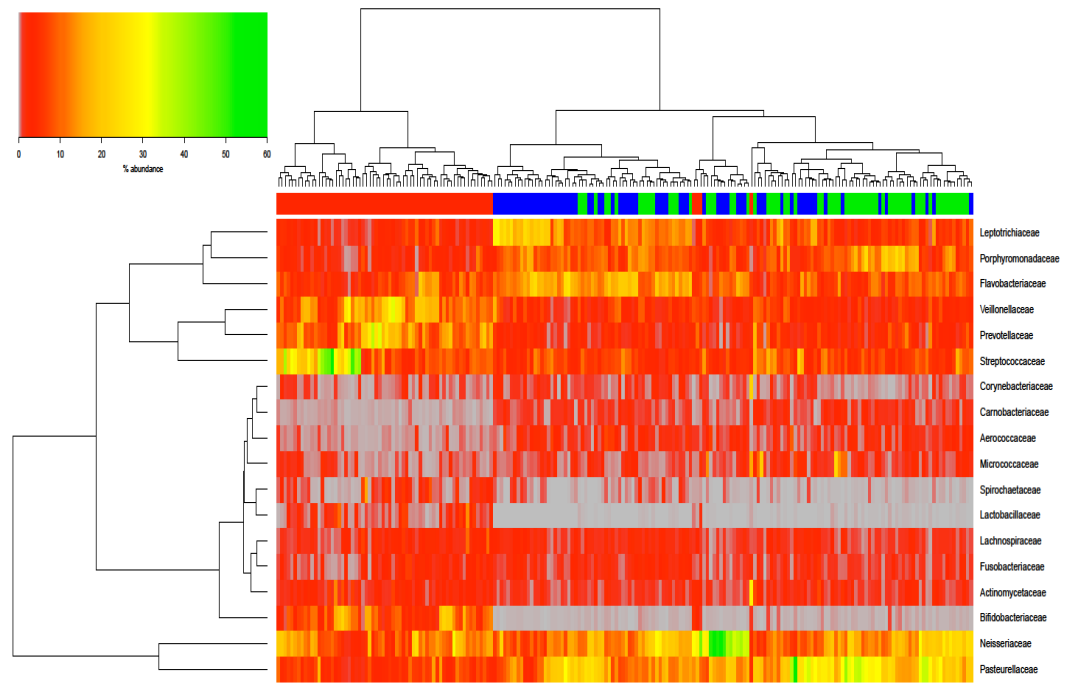
Diet can have a profound impact on oral health and caries risk. We found no significant differences when we compared the habitual intake of each food group from the FFQ data derived from the caries-active and caries-free subjects. No food group was consumed at significantly different frequency when we tested either for unequal presence/absence of food groups in the diet using the Fisher's test, or different frequencies of food groups in the diet using the Mann-Whitney test. Any trends were not supported by significant p-values ( $<0.05$ ).

### **4.3.4 Differentially abundant taxa in CAC compared to saliva microbiota of both CAS & CFS**

Differential bacterial taxon abundance in compared microbiota datasets may be graphically demonstrated by hierarchical clustering, whereby samples are grouped based on similarity of the taxa in their microbiota. These relatedness levels between samples, and their constituent microbial taxa, are represented by vertical and horizontal dendrograms incorporating a “heatmap” colour scale to convey abundance levels. Fig.4.22 illustrates the abundance of bacterial taxa at family level. The three groups are split into two main branches visible in the horizontal dendrogram above the colour bar in Fig.4.22. CAC clusters on one branch (red bar) and the two saliva groups (CAS in green & CFS in blue) cluster on the other. There is clustering of the CAS and the CFS within this branch, showing considerable similarity between the two groups at family level. When compared

against the patient metadata, there was no obvious variable that convincingly separates CAS from CFS (data not shown).

The microbiota of the CAC samples was characterized by high relative abundance of *Prevotellaceae*, *Veillonellaceae*, *Bifidobacteriaceae* and *Streptococcaceae*, and by low relative abundance of *Corynebacteriaceae*, *Carnobacteriaceae*, *Aerococcaceae* and *Micrococcaceae*. Both saliva sample types (CAS & CFS) showed higher abundances than caries samples of *Leptotrichiaceae*, *Porphyromonadaceae* and *Flavobacteriaceae* and of *Neisseriaceae* and *Pasteurellaceae*, while illustrating a very low abundance compared to CAC of *Spirochaetaceae*, *Bifidobacteriaceae* and *Lactobacillaceae*.



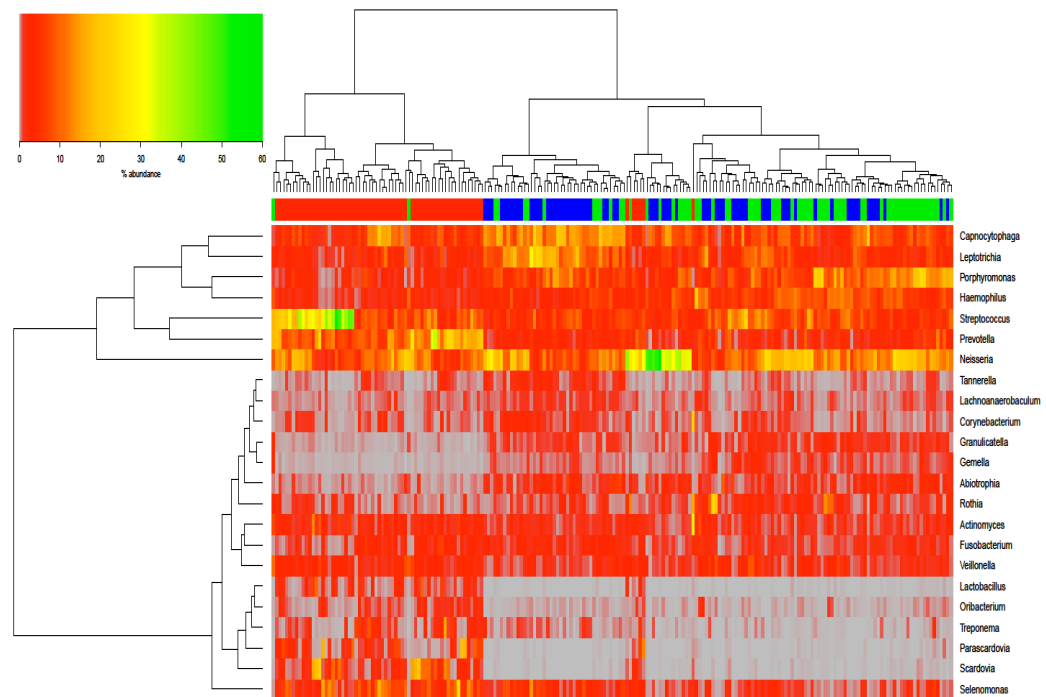
**Fig. 4.22. Hierarchical clustering of microbiota data at bacterial family level.**

Abundances are colour-coded according to the colour key on the top left with grey representing a value of zero. Euclidean distance and complete linkage were used to cluster the rows and columns of the heatmap. The colour bar on top of the heatmap corresponds to sample type: CAC red, CAS green and CFS blue. All taxa present at less than 1 % in all three groups are excluded from the heatmap.



A more nuanced picture emerges when differentially abundant taxa were analysed at genus level (Fig.4.23). The samples again separate laterally into caries versus saliva (with both caries-active and caries-free clustering together). The microbiota content appears to be split vertically in two groups of differentially abundant genera as revealed by the dendrogram on the Y-axis based on bacterial abundance. The top branch is divided, with CAS and CAC showing higher abundance of *Streptococcus* and *Prevotella*, and with *Neisseria* at a higher abundance in both. In the saliva samples (CAS & CFS), *Leptotrichia*, *Porphyromonas* and *Haemophilus* are in higher abundance, with *Leptotrichia* at higher abundance in CFS than CAS.

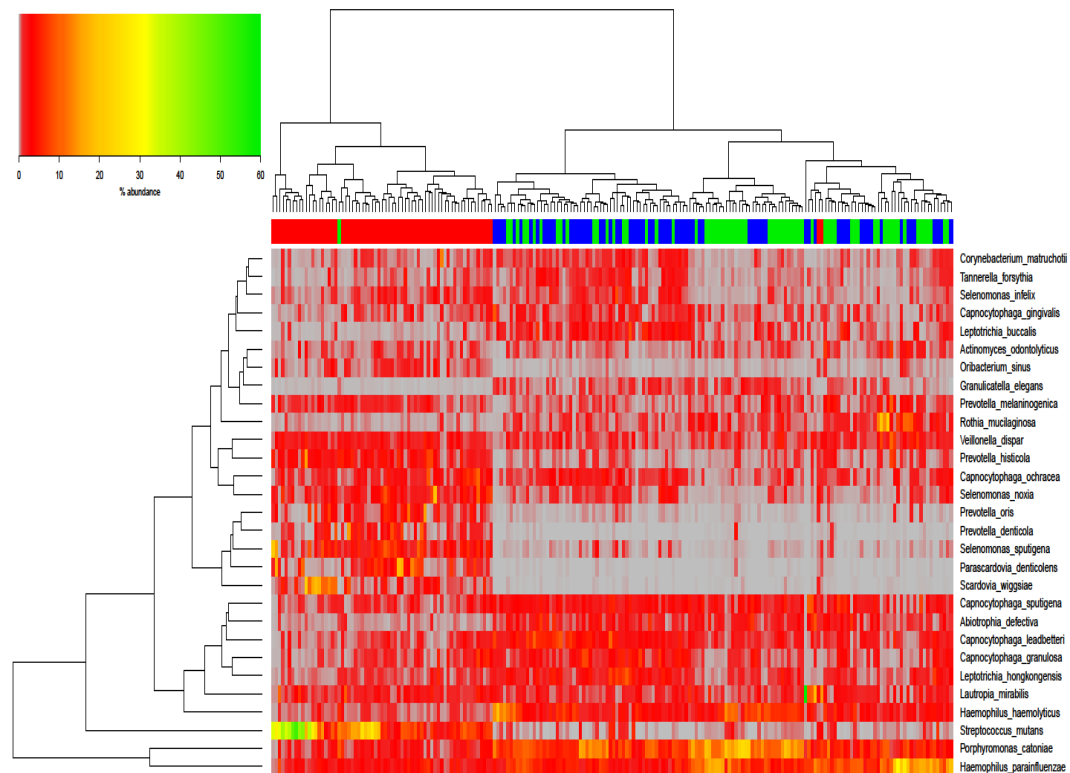
The lower branch shows clear low-abundance of the genera *Lactobacillus*, *Treponema*, *Scardovia* and *Parascardovia* in the CFS & CAS. (Fig.4.23), while low abundance taxa in CAC are *Gemella* and *Granulicatella*.



**Fig.4.23. Hierarchical clustering of microbiota data at bacterial genus level.**

Abundances are colour-coded according to the colour key on the top left with grey representing a value of zero. Euclidean distance and complete linkage were used to cluster the rows and columns of the heatmap. The colour bar on top of the heatmap is coloured according to sample type: CAC red, CAS green and CFS blue. All taxa present at less than 1 % in all three groups are excluded from the heatmap.

When we included samples in the analysis with at least one species with a median value of  $\geq 0.5\%$ , we identified fewer outliers, with only one CAS sample within the CAC branch, and two CAC within the second branch of the saliva samples. There was clearer separation of the samples, with CAC on the first branch, with high abundance of *Streptococcus mutans*, compared to the saliva samples. The two saliva samples split into two branch points, with CFS illustrating clustering within the first branch, with higher abundance of *Tannerella forsythia*, *Capnocytophaga gingivalis*, and *Leptotrichia buccalis*. Species with a clear low abundance in the majority of saliva samples included *Scardovia wiggsiae*, *Parascardovia denticolens*, *Prevotella denticola* and *Prevotella oris*, where these were present at higher abundance in CAC.

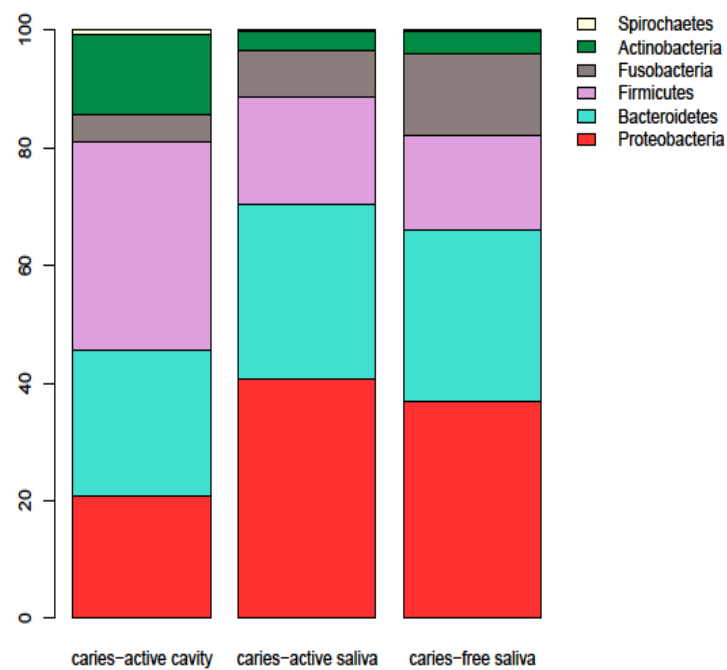


**Fig.4.24. Hierarchical clustering of microbiota data at bacterial species level.**

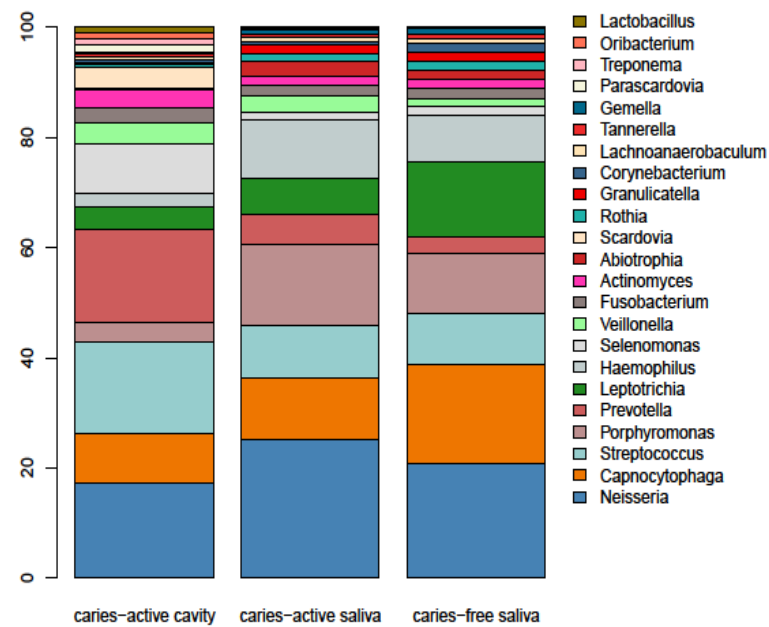
Abundances are colour-coded according to the colour key on the top left with grey representing a value of zero. Euclidean distance and complete linkage were used to cluster the rows and columns of the heatmap. The colour bar on top of the heatmap is coloured according to sample type: CAC red, CAS green and CFS blue. All taxa present with at least one species with a median value  $\geq 0.5$  % in all three groups are included.

#### **4.3.5 Broad and fine detail compositional differences distinguish caries microbiota from paired and healthy control saliva samples.**

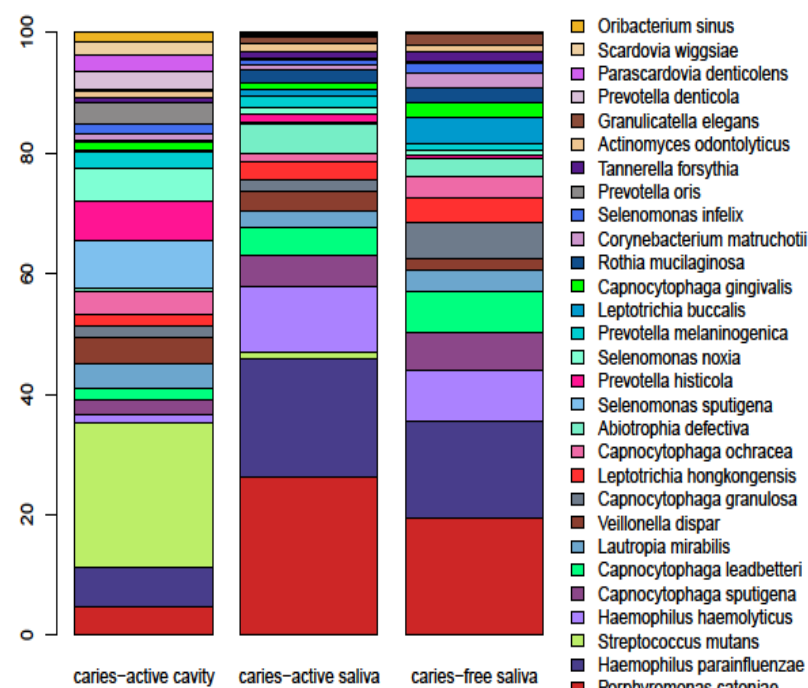
At phylum level (Fig.4.25), the CAC microbiota is dominated by the Firmicutes (median abundance value 33.45%), while the CAS microbiota was dominated by Proteobacteria (median abundance value 38.18%;  $p < 0.0001$ ). At phylum level, both the CAS and CFS microbiota composition is quite similar (Fig.4.24A) , with the main phylum difference between saliva of CAS versus CFS children being Fusobacteria. Its abundance has a median value of 13.4% in the saliva microbiota of CFS children, but 7.37% in CAS ( $p < 0.0001$ ). Both the CAS and CFS microbiota are dominated by Proteobacteria at 38.2% and 36.1% respectively.



A



B.



C.

**Fig.4.25. Broad and fine detail compositional differences at Genus, phylum and species level.**

A. Microbiota composition at phylum level. Percentages for each taxon represent the median abundance values for the sample types. B. Barplot of percentage abundance at genus level. Percentages for each taxon represent the median values for the groups. C: Barplot of percentage abundance at species level. Percentages for each taxon represent the median values for the groups.

Excluding the “other” and “unassigned” categories, the three microbiota types (CAC, CFS & CAS) are dominated by taxa *Neisseria*, *Capnocytophaga*, *Porphyromonas*, *Streptococcus*, *Prevotella*, *Leptotrichia*, and *Haemophilus*. *Streptococcus*, *Neisseria*, *Prevotella*, *Capnocytophaga*, dominate the CAC sample microbiota and at lower levels, taxa *Scardovia*, *Parascardovia*, *Selenomonas* and *Lactobacillus*. CAC sample microbiota include numerous species of higher relative abundance: *Streptococcus mutans*, *Alloprevotella denticola*, *Prevotella histicola*, *Scardovia wiggsiae*, *Parascardovia denticolens*, *Prevotella tanneriae* and *Bifidobacterium dentium*.

The CFS microbiota composition differs significantly from the saliva of CAS group by the presence of the following microbial genera: *Leptotrichia*, *Bifidobacterium*, *Corynebacterium*, *Alloprevotella*, *Cardiobacterium* and *Veillonella* ( $p < 0.0001$ ). The abundance of all six genera was significantly higher in CFS ( $p < 0.0001$ ). The CAS microbiota was dominated by *Neisseria*, *Porphyromonas*, *Streptococcus* and *Haemophilus*, and species included *Streptococcus mutans*, *Prevotella histicola*, *Prevotella melaninogenica*, *Porphyromonas catoniae* and *Prevotella salivae*. CFS samples were dominated by *Leptotrichia*, *Capnocytophaga*, *Neisseria*, *Haemophilus*, *Streptococcus* and *Porphyromonas* at genus level, while at species level, the CFS group include species *Haemophilus haemolyticus*, *Haemophilus parainfluenzae*, *Rothia mucilaginosa*, *Porphyromonas catoniae* and *Streptococcus sanguinis*.

Microbial taxa showing statistically significant differential abundance between CFS and CAS children included *Streptococcus mutans*, *Haemophilus parainfluenzae*, *Prevotella histicola*, *Leptotrichia buccalis*, *Veillonella dispar*, *Alloprevotella tanneriae* and *Prevotella salivae*. Interestingly, *Streptococcus*



*mutans*, *Prevotella histicola* and *Veillonella dispar* were present at higher abundance in CAS than the saliva of CFS.

## **Chapter 5**

### **Discussion**

1. Longitudinal study: Birth to 1 year of age.
2. Longitudinal study: Birth to 2 years of age.
3. Cross Sectional Study.

## 5.1. Discussion:

### 1. Longitudinal study: Birth to 1 year of age.

In this large cohort study we investigated the oral microbiota of infants from birth to 1 year of age in infants that were born full-term, via by Spontaneous vaginal delivery (SVD) or by Caesarean section (CS). All infants were initially breastfed for a minimum of 4 weeks and the oral microbiota of these infants were sampled at five time points in the first year of life (1 week, 4 weeks, 8 weeks, 6 months and 1 year of age).

At birth, we found that the mother's skin, vagina and oral microbiota communities are distinct, and for each body habitat (vagina, skin, oral) they demonstrate a unique composition (Fig. 4.1). The diversity of the vaginal microbiota was lower than the skin microbiota of the mother as measured by alpha diversity metrics (Fig. 4.3), similar to previous observations (Li et al., 2012; Zhou et al., 2013). This low diversity within the vaginal samples is most likely due to the dominance of genus *Lactobacillus* with *Lactobacillus iners* and *Gardnerella vaginalis* in high abundances, as represented by CST III and CST IV (Romero et al., 2014c). These species have been previously identified in the vaginal microbiota of healthy women of reproductive age (Ravel et al., 2011; Zhou et al., 2007, 2004) and of pregnant women (Romero et al., 2014c) particularly in the last trimester (Walther-António et al., 2014).

In agreement with previous studies (Chu et al., 2017; Dominguez-Bello et al., 2010), we found that birth mode had an influence on the neonatal oral microbiota for a short window after birth. We found that the oral microbiome of the infant was influenced by birth mode within 1 week of age only, but from 4 weeks of age

to 1 year the effect of birth mode was not apparent. The overlap and tight clustering presented by PCoA plots (Fig 4.2) of the infant oral microbiota by birth mode is obvious by 6 months and 1 year of age, while the beta diversity between samples (Fig 4.1) demonstrates sharing of taxa between the maternal skin and vaginal microbiota and the oral microbiota of the infant at week 1, week 4 and week 8, with less overlap and less effect of the maternal microbiota with increased age (6 months and 1 year). This early stage impact highlights transfer of maternal microbiota, respective to the mode of delivery (CS or SVD), and this was further reinforced by the identification of *Lactobacillus iners* and *Propionibacterium acnes* of vaginal and skin origin in the infants oral microbiota within 1 week of birth. While the impact of the maternal vagina and skin are apparent, the maternal salivary oral microbiota did not have any significant effect on the infant oral microbiota, with no clustering of mother-infant pairs when compared by their oral microbiota composition only (Fig 4.8) (Supplementary figure 4.1). We identified that there was no impact of the maternal oral microbiota on their own infant oral microbial community, as previously demonstrated (Dominguez-Bello et al., 2010), and also found no direct similarity between the mothers salivary microbiota and their infants salivary microbiota, consistent with previous findings (Cephas et al., 2011; Chu et al., 2017), reflecting compositional differences between the edentulous neonate and the established and mature adult oral microbiome.

Previous studies have reported increased diversity associated with SVD delivered infants (Boustedt et al., 2015; Lif Holgerson et al., 2011). However, we did not identify such diversity difference by alpha diversity measures. While mode of delivery and age have both been shown to affect the microbial composition of the

infant gut microbiota up 6 months (Hill et al., 2017), this influence was not seen in the oral microbiota of the infants from 4 weeks to 6 months of age (Fig. 4.4). This supports previous research findings that as the infant ages, the influence of mode of delivery on the oral microbiota of the infant diminishes (Boustedt et al., 2015). We did however demonstrate that the diversity of microbes in the oral cavity of the infant decreased slightly with increased age as measured by Chao diversity and Shannon diversity, while stability within the oral cavity was demonstrated from week 1 to 1 year of age by the remaining alpha diversity measures (Phylogenetic and Shannon diversity) (Fig 4.3). While one would expect to see increasing diversity with increased age, we found the alpha diversity to be relatively stable over time, perhaps symbolising low inter-variation differences between the oral microbiota of the infant over time. While the diversity did not dramatically change from 1 week to 8 weeks and again to 1 year, there were significant compositional changes which signify the role of factors such as diet, dentition, salivary flow rate, oral health, siblings etc have to play. However, we did observe a lower diversity at all ages in the infant oral microbiota compared to the maternal ‘adult’ salivary microbiota, with similar compositional differences previously identified between adult and infant salivary microbiome (Aas et al., 2008; Crielaard et al., 2011; Foxman et al., 2016; Ling et al., 2013). The diversity gap between the mother and infant oral microbiota increased as the infants aged, demonstrating closer relatedness at a younger age. Both findings highlight that at 1 year of age, the diversity is stable, potentially influenced by a myriad of factors (diet, oral health, dental factors, social status, salivary flow rate, fluoridation status and education).

The infant oral microbiota illustrates tight overlap by beta diversity measures (Fig 4.1) at 1 week of age to 8 weeks of age, but from 6 months of age the infant oral microbiota develops its own distinct composition with a gradual transitioning away from this early age taxa (week 1-8), with no overlap or clustering by 1 year of age as presented on the PCoA plots (Fig 4.1). This highlights the continued development and maturation of the infant oral microbiota with its distinct composition, with no influence of birth mode at this age, while other studies illustrate that oral microbiota composition can continue to change and develop into adulthood (Crielaard et al., 2011).

At phylum level, the infant oral microbiota of infants from week 1 to week 8 was dominated by Firmicutes with *Streptococcus* the predominant genus at week 1 consistent with previous studies of the oral microbiome of infants (Cephas et al., 2011) and of children (Z. Ling et al., 2010). Only after 8 weeks does the abundance of Firmicutes begin to decrease, from 6 months (60.2%) until 1 year (33.7%), while *Streptococcus* levels gradually decrease in abundance from week 1 (53.8%) to 1 year of age (23.1%). When *Streptococcus* levels begin to decrease, this is counteracted by increased abundances by 1 year of age with genera such as *Neisseria*, *Porphyromonas*, *Rothia* and *Haemophilus*, highlighting compositional changes within the oral microbiota from the neonatal period into toddlerhood. These compositional changes, in particular are most apparent from 6 months of age, co-inciding with the average eruption of the first deciduous teeth (typically averaging around 4-6 months for lower primary incisors) and the introduction of solid foods to the infant. Previous studies have revealed changes in the oral microbial composition with eruption of teeth (Aas et al., 2008) and in the infant

gut microbiota after introduction of solid foods (Fallani et al., 2011; Bokulich et al., 2016). Eruption of teeth increases the surface area for biofilm attachment, enhancing bacterial attachment and allowing bacterial compositional change. Sampling of the infants in this study was done in their own home. However, due to personnel constraints we were unable to do dental examinations at each stage of sampling. More frequent examinations would have pinpointed the exact timing of eruption of the deciduous dentition, which would have benefited this study.

Long term breastfeeding has been linked with increased risk of dental caries (Avila et al., 2015; Bahuguna et al., 2013; Kato et al., 2015) and feeding modality (breast fed or formula fed) has been shown to have an effect on the oral microbial composition of the infant, with increased levels of oral *Lactobacillus* species in breast fed infants (Lif Holgerson et al., 2013; Vestman et al., 2013). We assessed whether breast feeding for a minimum of 4 weeks had an effect on the oral microbiota of the infant and did not observe any significant effect on the oral microbiota of infants born by SVD or CS, or when birth modality breast feeding duration was combined (Fig. 4.10). We did however identify species within the oral cavity of the newborn at 1 week of age previously found to be associated with breastfeeding (*Actinomyces gerencseriae*) and species more frequently detected in the oral cavity of formula fed infants (*Granulicatella elegans*, *Prevotella melaninogenica* and *Neisseria* species) (Lif Holgerson et al., 2013), but no impact of breastfeeding on the oral microbiota. Perhaps a longer duration of breastfeeding may have an impact on the oral microbiota, but all infants in this study were breastfed for a minimum of 4 weeks only, some with introduction of mixed-

feeding (formula and breast) after this stage, and this data was not collected in this cohort

### 5.1.1. Conclusion

This study is the first longitudinal study to date that we are aware of using culture-independent methods to analyse the influence of birth mode on the oral microbiota of infants from birth at five time points until 1 year of age. We observed the influence of birth mode on the oral microbiota of the infant by 1 week of age, and both diversity and compositional changes in the oral microbiota of the infant over time. We also demonstrated that breast feeding duration did not have a significant effect on the oral microbiota of the infant, while sudden compositional changes were evident at 6 months and again at 1 year of age, when both teeth begin to erupt and solid foods are introduced. Our findings provide a closer insight into the oral microbiota development from birth, and the influence of birth mode together with the documented changes in diversity and composition will aid us to get a better understanding of the long term health impact within the oral cavity for the infant, and provide a platform for additional studies to establish how early life disturbances can impact the oral health outcome of these infants.



## 5.2. Discussion:

### 2. Longitudinal study: Birth to 2 years of age.

This study documents the oral microbiota changes that occur when the infant grows from birth into toddlerhood at 2 years of age, and a continuation of our previous analysis of the ORALMET cohort. At 2 years of age, we found that the infant oral microbiota was not influenced by birth modality and there is no effect of birth mode on the infant oral microbiota except up to 4 weeks of age. We identified taxa of maternal skin and vaginal microbiota origin within the oral microbiota of the infant immediately after birth, with some relationship between these maternal body sites and the infant oral microbiota up to 8 weeks of age (Fig 4.11). These maternal microbes of vaginal and skin origin included *Lactobacillus iners* and *Propionibacterium acnes* in the infants oral microbiota after birth. The maternal salivary oral microbiota however does not have any significant affect on the infant oral microbiota at birth or at any age.

The diversity of the oral microbiome has been shown to increase with increased age (Könönen et al., 1994; Lif Holgerson et al., 2015), with the salivary microbiota demonstrate continued change in the oral microbiota composition until adulthood (18 years of age) (Crielaard et al., 2011). We did not detect any increased diversity with the increased age from 1 year to 2 years of age. However, we noted that the alpha diversity difference at 2 years of age was greater when compared to the neonatal period (1 week to 6 months) ( $p < 0.001$ ), than the diversity difference when compared to 1 year of age, highlighting less diversity differences with increased age.

While the alpha diversity within samples demonstrated stability, the beta diversity difference between samples at 2 years of age illustrates clear separation, and uniqueness (Fig.4.11). We observed continued development and maturation of the oral microbiota from the neonatal period and as the infant aged from 1 to 2 years of age. The oral cavity is 'open' to a wide range of microbes from the surrounding environment, sibling influence and is the first entry point to the body with food and fluid intake. All these factors have an impact on the infant oral microbiota dynamics and influence a continuous change of microbial composition within the oral cavity. The presence of this distinct separation demonstrates that the taxa present at 2 years of age are different to that at an earlier age, in particular the neonatal period (week 1, week 4, week 8). This microbial composition difference at 2 years of age is further supported by clear separation of the year 2 infant oral microbiota based on abundances at bacterial genus level on a vertical and horizontal dendrogram (Fig. 4.15).

By 3 years of age, the salivary microbiome has been shown to be composed mainly of Firmicutes and a higher abundance of Proteobacteria (Crielaard et al., 2011). We found the oral microbiome at 2 years of age to have decreased Firmicute levels. These levels decreased from week 1 (82.3%) to year 2 (21.1%), while Proteobacteria levels increased (3.98% at week 1 to 36.7% at year 2). The abundances of Bacteroidetes (16.7%-17.7%), Actinobacteria (6.24-6.08%) and Fusobacteria (6.58-7.13%) remain consistent between year 1 and year 2. Thus at phylum level, from 1 year to year 2 there is evidence of some stability arising in the composition at phylum level with less change within the oral cavity of the infant.

At phylum level there is evidence of stability between groups, the diversity of the oral microbiota is becoming more distinct and less diverse. *Streptococcus* is the most abundant genera at 1 week of age in the infant microbiota (53.8%) and there is a stepwise decrease *Streptococcus* levels from week 1 to year 2 of age (11.2%) (Figure 4.17). The initial dominance of *Streptococcus* at a young age is consistent with *Streptococcus* levels found in previous studies where levels of *Streptococcus* in the salivary microbiota of infants at 5 months was 62.2% (Cephas et al., 2011) and at 6 weeks was found to be 60.7% (Chu et al., 2017). The low level of *Streptococcus* at 2 years of age (11.2%) is counterbalanced with increased abundances of genera including *Haemophilus*, *Prevotella*, *Neisseria* and *Fusobacterium*.

Ling et al., (Z. Ling et al., 2010) identified similar genera in the oral microbiome of children aged 3-6 years, with *Streptococcus* (25%) and increased levels of genera including *Neisseria* (10.3%), *Porphyromonas* (3.96%), *Rothia* (3.68%) and *Haemophilus* (3.87%). In 3-4 year olds, 75% of the salivary microbiota was composed of genera including *Streptococcus*, *Prevotella*, *Neisseria*, *Rothia*, *Haemophilus*, *Gemella* and *Veillonella*, highlighting a similar maturation pathway the 2 years olds in this study are developing towards (Jiang et al., 2016). Stability of the infant gut microbiota reaches an ‘adult-like’ stability by 2-3 years of age and we identified that at 2 years of age *Streptococcus* levels to be at 11.2%. *Streptococcus* levels in the adult salivary microbiota have been shown to be at 20.4% (Cephas et al., 2011) and 19.68% (Keijser et al., 2008). Our findings demonstrate a trend towards and a more adult like microbiota, by the decreasing levels of *Streptococcus*. However, statistically significant differences in diversity

and composition between the maternal oral microbiota and the infant oral microbiota at 2 years of age were demonstrated, questioning if the stability milestone is reached at 2 years. Reinforcing this observation is the finding that the maternal salivary microbiota sampled in this cohort was dominated by Firmicutes, and much higher abundances of *Streptococcus* than the infant oral microbiota at 2 years of age, and thus does not confirm to us that an ‘adult-like’ oral microbiota is developing in the infant oral cavity, but reinforces that the infant oral microbiota is under constant change at 2 years and perhaps will continue beyond this age.

To identify if the infant oral microbiota continues to mature and diversify, it would be necessary to continue sampling the oral microbial profiles of this cohort from 2 years of age to 3 years of age, and to an older age as the child experiences development of the deciduous dentition and mixed-dentition phase of eruption. Profiling of the oral microbiota to an older age from birth would give us a better understanding if the oral cavity microbiota reaches stability or because of the exposed open environment of the oral cavity does this ecosystem ever reach such stability.

Overall we found that as the infant grows from 1 year of age into a toddler at 2 years of age, there is corresponding development and maturation of the oral microbiota, with distinct compositional changes. It is important to study the oral microbiota development from birth to an older age, to give us a better understanding of the dynamics within this rich ecosystem, and in particular the role birth mode has to play. This foundational work will supplement previous research that have focused their attention on the oral microbiome of children (e.g.

with dental diseases such as caries) and also complement future studies, with the ultimate aim to improve and maintain the oral health of children.

### 5.3. Discussion:

#### 3. Cross Sectional study.

In this study, we used Illumina MiSeq sequencing to explore the oral microbiota of deep dentinal carious lesions and saliva of Irish children affected with S-ECC and the salivary microbiota of those that are caries-free. S-ECC is an aggressive form of caries, and we analyzed the microbiota of the deep dentinal caries of deciduous teeth to determine if the salivary microbiota was a reservoir or source of taxa linked with this form of caries.

Using two well established metrics (Bray Curtis dissimilarity and UniFrac distance) we found that the caries dentine microbiota was distinct from that of either CFS or CAS, illustrating, when abundance is taken into account, that CAC has considerably different proportions of certain high-abundance taxa. However, when measured using unweighted UniFrac, which measures presence and absence of taxa only, we found that all three groups are distinct, showing that each group is different in terms of rare or low-abundance taxa. Of interest was the closer microbiota relatedness of CAS to CAC, with the presence of certain CAS outliers overlapping with CAC. This suggests that some samples share similar low-abundance taxa between CAC and CAS or that CAS harbours some taxa which may have stimulated the increased caries rate compared to CFS, or there is some shedding of taxa from CAC into the CAS. When we implemented a leave-one-out strategy with dominant taxa such as *Neisseria* and *Streptococcus*, these outliers changed position, sometimes clustering within their own group and a few samples that clustered within their group in the full dataset became outliers with the reduced dataset. When we studied these outliers in detail, we found that these samples were not influenced by any metadata, suggesting that factors not

accounted for in this study are responsible for unusual taxon composition in a subset of samples.

Focusing on the dominant taxa and their abundance illustrated graphically by hierarchical clustering on the heatmap, we identified samples grouped based on microbiota similarity, but also of interest was the partial agreement between the outliers identifiable in the PCoA plots and in the heatmaps. The identity of these outliers could not be readily correlated with patient metadata and when we looked in detail at the taxon abundance level, at genus and at family level there is splitting of CAC with both the saliva groups (CFS & CAS), with the CFS samples clustering mostly together, with some intermixing of both saliva microbiota types. There is clear clustering of CAC in branch one to left, with CFS at next branch in blue (Fig.4.22, 4.23), with CAS lastly split on a third branch. This is more apparent at species level by hierarchical clustering, with less intermixing of both salivary microbiota types (Fig.4.24).

This incomplete separation of samples between saliva groups suggests that the salivary microbiota is not specific enough to be used as an identifier for caries risk in children. The oral cavity is an entry point for colonisation of microbial species and saliva is a reservoir for a multitude of bacteria, with its microbial and nutritional composition being shaped by food intake, reflux, environment and other influences (Crielaard et al., 2011; Lazarevic et al., 2010; Nasidze et al., 2009). In this study, we did not find an association between factors like habitual diet, brushing habits or fluid intake with microbiota composition, although it is possible these meta data are not sufficiently granular in the FFQ data and patient questionnaire. Furthermore, previous studies have shown variability between sites in the oral cavity itself, with niches among the tongue, soft and hard palates,

supra- and sub-gingival surfaces of teeth and saliva each demonstrating microbiota variability (Aas et al., 2005; Dewhirst et al., 2010). The flow rate, buffering capacity, and molecules within saliva which can aid attachment of bacterial cells, all play a role in both the compositional balance of the oral microbiome (Marsh et al., 2016). While some studies have also found an association between the microbiota and disease in plaque samples, but not within the saliva samples (Huang et al., 2011; Z. Ling et al., 2010) and our findings support these findings, with saliva and caries representing two distinct habitats.

As caries lesions progress and become more severe, the diversity of caries microbiota decreases (Gross et al., 2012, 2010; Li et al., 2007). In this study, alpha diversity of caries microbiota was lowest, and differences in the diversity of the caries samples and saliva samples were significant ( $p < 0.001$ ). However the Chao diversity index for CFS was significantly higher than CAS with a  $p$ -value of  $< 0.05$ , again supporting the previous data, that when low-abundance or rare taxa are given an equal weighting to higher-abundance taxa, differences in diversity between the two saliva groups become apparent. This suggests that even at low abundance, certain taxa such as the acid-producing lactobacilli can play a strong role in caries progression. This highlights an important possibility, that taxon abundance of cavity-causing microbes may not be strongly correlated with progression of caries; low-abundance taxa at abundance levels that typically do not feature in microbiome summary data might be the main indicator of future tooth decay because, for instance, a small number of acid-producing or biofilm-producing species may have a disproportionate impact on oral health. In addition, the potential affect that the removal or exclusion of these rare taxa may have on



caries prevention and general oral health makes a solid case for their identification.

As caries progresses to a more advanced state, the bacteria that dominate this cavity are less diverse, because aciduric organisms have been selected and enriched, and we found that the main genera that dominated the CAC lesion were *Neisseria*, *Streptococcus* and *Prevotella*, while the species that dominate the caries lesion (CAC) include of *Streptococcus mutans*, *Prevotella* species, *Scardovia* species and *Bifidobacterium dentium*. *Neisseria*, *Streptococcus*, *Prevotella* and *Porphyromonas* have all been strongly associated with caries in past studies (Aas et al., 2008; Jiang et al., 2013; Li et al., 2007; Munson et al., 2004; Nasidze et al., 2009; Nyvad et al., 2013; Tanner et al., 2011b). *Neisseria* and *Streptococcus* produce acid which lowers the pH of the mouth and leads to increased demineralisation of enamel (Gross et al., 2010) while *Prevotella* has a known role in caries progression and endodontic infections (Tanner et al., 2011a; Tavares et al., 2011). The high level of *Streptococcus mutans* in the carious lesion is consistent with previous studies, and its presence is a strong indicator for caries (Jiang et al., 2013). *Streptococcus mutans* aids in caries initiation by adhering to the enamel, forming a biofilm on the surface and producing acid, and because it is aciduric, it can often be part of a more complex community of microorganisms working together (Aas et al., 2008; Gross et al., 2010; Lif Holgerson et al., 2015; Tanner et al., 2011b), and a risk factor for caries progression (Gross et al., 2012).

*Scardovia* is documented as having a role as a cariogenic bacterium involved in the later stages of S-ECC (Tanner et al., 2011b). *Scardovia wiggsiae* is significantly associated with S-ECC, based on a culture study of plaque from children (Xu et al., 2014), and in adults with caries (Gomar-Vercher et al., 2014).

*Prevotella* species have been shown to play an important role in endodontic infections (Tavares et al., 2011), and *Prevotella tanneriae*, *Prevotella histicola* (isolated from human oral mucosa (Z. Ling et al., 2010)) and *Alloprevotella denticola* (Lazarevic et al., 2010; Munson et al., 2004) have all been shown to be associated with dental caries. *Lactobacillus*, which is notably associated with caries progression (Aas et al., 2008; Becker et al., 2002; Fabris et al., 2014; Obata et al., 2014; Yang et al., 2010) was found at very low levels compared to other genera (0.675% (CAC) and 0.031% (CAS)). These low levels were also reported in previous studies (Gross et al., 2012, 2010; Nasidze et al., 2009) and this interesting finding supports the idea that when certain acid producers are at low levels, their acidogenic properties may nevertheless be strong enough to allow other acid producers to take their place, such as *Neisseria*, *Selenomonas* and *Streptococcus mitis* (Gross et al., 2010). *Neisseria* species have the ability to metabolize glucose to produce lactic acid and this genus was found at high levels in CAC (10.29%) and in CAS (16.28%) and may have an active cariogenic role. *Lactobacillus* is found at low levels in endodontic infections with deep caries (Hong et al., 2013; Santos et al., 2011). It has also been suggested by Rôças et al. that altered *Lactobacillus* abundance may be due to the change from cariogenic microbiota to a microbiota that stimulates progression into pulpal tissue causing infection. Shifts in the microbiota composition can be affected by saliva and diet to the outermost pulpal layer, while the inner deeper layer has a different environment in comparison (Rôças et al., 2016). The replacement of *Lactobacillus* with other taxa could be linked to the degree of pain, duration of pain, length of caries destruction, connection with pulp, diet and environmental factors, and this fine detail could reveal reasons for the low levels of *Lactobacillus* in these teeth.

Within CFS there are higher levels of *Capnocytophaga* (10.9%) and *Leptotrichia* (8.1% CFS), and lower levels of *Porphyromonas* and *Neisseria*. There is evidence to suggest that *Capnocytophaga* and *Leptotrichia* are health-associated species (Aas et al., 2008; Jiang et al., 2013; Lif Holgerson et al., 2015; Xu et al., 2014) and *Capnocytophaga* has been found at higher levels in caries-free subjects (Gross et al., 2010; Jiang et al., 2013; Z. Ling et al., 2010). We found lower levels of *Porphyromonas* (9.5% CAS & 6.6% CFS) and *Neisseria* (16.3% CAS & 12.6% CFS) in CFS compared to CAS. Some *Neisseria* species have been shown to play a role in acid production (e.g. *N. gonorrhoeae* and *N. meningitidis*) (Gross et al., 2010), while other *Neisseria* species such as *Neisseria flavescens* have been shown to elicit higher signal of probes when targeted in caries-free children (Crielaard et al., 2011) together with the *Porphyromonas* gram-negative species, *Porphyromonas catoniae*. The association of these species with a caries-free oral status has been further supported by Nyvad et al. (Nyvad et al., 2013). We found a higher abundance of *Porphyromonas catoniae* within the CAS (9.5%). Studies have reported children with high levels of caries harbouring higher levels of *Porphyromonas* in their saliva (Gomar-Vercher et al., 2014), by culture study (Tanner et al., 2011b), and it has been detected in root canals of necrotic deciduous teeth (Fabris et al., 2014) and our findings demonstrate a positive association of *Porphyromonas* with S-ECC.

### 5.3.2. Conclusion

We conclude that there are distinct differences between the caries microbiota and saliva microbiota, with separation of both salivary groups (caries-active and caries-free), showing a clear separation when rare taxa are considered. While the microbiota diversity in the caries dentinal microbiota is lower than both salivary microbiota types, there are clear compositional differences between all groups from phylum to species. The taxa present in the dentinal lesions could be potential instigators that drive migration of infection to the pulp, while the saliva microbiotas in health and disease may be associated with caries-active or caries-free status in these children.

## Chapter 6

### Conclusions

The oral microbiome is a rich and dynamic environment, constantly under change, being the first point of entry for food and liquids into the body, and an open environment to many transient microbes. While the oral cavity is a dynamic ecosystem there are also compositional differences in the oral cavity sub niches such as the subgingival and supragingival gingivae, hard and soft palate, saliva, floor of mouth and between the occlusal fissures of teeth and the smooth surfaces of teeth (Dewhirst et al., 2010). There has been a surge in microbiota research examining microbiota influence on the health and wellbeing of the human body. The influence of birth modality on the infant microbiota is also a research focus for many research groups particularly when these effects can persist throughout life (Biasucci et al., 2008; Bokulich et al., 2016; Curran et al., 2015; Gregory et al., 2015; Huurre et al., 2008). Although there has been increased focus on the effect of birth mode on the gut microbiota, there has not been the same attention into the effect of birth mode on the oral microbiome (Dominguez-Bello et al., 2010; Lif Holgerson et al., 2011). This thesis and ORALMET study aimed to add to the data from the limited number of studies investigation the effect of birth mode on the oral micrbiome (Dominguez-Bello et al., 2010; Lif Holgerson et al., 2011) that were presented at the time of designing the study. In particular, this study aimed to follow the oral microbiota these neonates from birth through to toddler age, which had never been done previously.

The Objectives set out at the beginning of the study were to:

Longitudinal/Cohort study

- 1) Determine if birth mode (SVD v CS) would have an influence on the composition and development of the oral microbiota.
- 2) Describe the changes in the oral microbiota from birth to 3 years of age among breast fed infants.

Cross sectional study

- 3) Compare the oral microbiota (saliva/dentinal microbiota) of children aged less than 5 years with caries (S-ECC) with caries-free children (saliva).

We amplified the V4-V5 region and used high-throughput sequencing of 16S rRNA amplicons to determine microbiota composition of each sample site examined in both studies.

**Longitudinal study: Birth to 1 year of age.**

As mentioned, there have been no longitudinal studies observing the microbial changes of the oral microbiome of a neonate from birth as it grows to an older age. While there have been few studies investigating the influence of birth mode on the oral microbiome (Dominguez-Bello et al., 2010; Lif Holgersson et al., 2011) and studying the microbial composition of the oral microbiome during infancy and childhood (Bokulich et al., 2016; Cephas et al., 2011; Chu et al., 2017; Foxman et al., 2016; Ling et al., 2013), no study to date that we are aware of has followed the same cohort of neonates from birth through infancy into toddlerhood.

The main objective of the ORALMET study were to observe the oral microbial changes of a neonate from birth until 3 years of age, with particular emphasis of the influence of potential microbial transfer of the maternal microbes depending on birth modality (CS or SVD). In total, included in our study were 81 saliva samples were collected from each mother at birth, together with skin (n=37) and vaginal (n=37) samples. At birth 75 neonates were sampled, with follow on of 83 infants sampled at 1 year of age (total number of samples from birth to 1 year of age n=340). We had an initial aim to enrol 100 mothers and their infants (n=50 CS/ n=50 SVD). The recruitment stage was performed in the public maternity department of the CUMH, information leaflets and follow up by telephone was performed for over 200 mothers in their second and third trimester. Given the unpredictability of pregnancy and in particular the timing of birth, this initial phase of recruitment did prove difficult. We achieved a final enrolment of 83 mothers and their infants and when these final study numbers were compared to the previous studies investigating the role of birth mode on the microbiota of the infant (Chu et al., 2017), our final numbers were similar, while compared to other studies our final study number was very high (Dominguez-Bello et al., 2010).

Following the microbial changes in the same neonates from birth through infancy has given us an insight into the dynamics of the oral cavity even at this early stage of life. While the microbiome of the maternal skin and vagina are described, along with the maternal salivary microbiome at birth, we see the impact of birth mode on the oral microbiome of the neonate at a very early stage, but not beyond, where the oral microbiome of the infant continues to develop and change, particularly from 6 months of age. Of interest is that the observation that the timing of change in the oral microbiome of the infant is from 6 months which coincides with the



introduction of solid foods and average eruption time of first deciduous teeth (lower incisors). Other factors which could influence the oral microbiome at this stage and at 1 year of age are the beginning of crawling and introduction of objects and hands into mouth to ease teething symptoms which may also have an influence. While these factors all could have a major influence on the oral microbiome of the infant, these factors are difficult to measure accurately in such a large cohort of infants. We were unable to recall all infants back to the Paediatric clinic at each time point (week 4, 8, 6 months and 1 year) due to distance, feasibility for parents and costs. I was unable to also call out to the homes to complete a full dental examination at each time point of sampling, a and collect detailed diet analysis and oral health analysis, which would have supplemented the study, and is something, which would benefit further work in this field.

This study does however highlight the role birth mode has to play in the oral microbiome of the neonate and that breast feeding does not have a significant role in changing the microbial composition of the oral microbiome. We also demonstrated the diversity differences within the maternal niches (oral/skin/vagina) and how the diversity of the oral microbiome of the infant remains relatively stable at an early age, with compositional changes evident and still developing up to 1 year of age. This study is the largest cohort study of the oral microbiome performed to date, and will provide a solid foundation to drive further work in this field.

### **Longitudinal study: Birth to 2 years of age.**

These findings were a continuation of the ORALMET study, including the data and findings of the oral microbiome of the infant at 2 years of age (n=42). This data was separated from the Year 1 data as this data was separately prepared for publication, while the year 2 data will be submitted along with the year 3 data for publication when all the bioinformatic data for year 3 is analysed. The year 3 data was not included in this thesis due to the timing of collection of samples and the return of sequencing data and bioinformatic analysis; therefore I was unable to include the data as part of this thesis.

The oral microbiota of the infant from 1 year of age continues to mature and develop at 2 years of age. While an adult like stability has been observed with the infant gut microbiota by 2 years of age (Bäckhed et al., 2015; Yatsunen et al., 2012), where the gut microbiota has a high diversity, we did not observe such dynamics at 2 years within the oral microbiota. We observed a complete change in the diversity between samples at 1 week, 1 year to 2 years of age, with complete separation of taxa clustering away from the early stages, and the maternal microbiota. The compositional changes evident at 2 years of age do not show higher diversity, but appears to be similar or slightly lower to an earlier age.

The average abundance of *Streptococcus* in the adult salivary microbiota was found to be around 20% in two previous studies (Cephas et al., 2011; Keijser et al., 2008). We observed that the *Streptococcus* levels decrease from 53.8% to 11.2% from week 1 to 2 years of age. This finding does support a trend towards a more adult like microbiota, but the lower diversity and the compositional differences between the maternal ‘adult’ oral microbiota and infant oral

microbiota at 2 years of age, suggests that the oral microbiome is under continuous change at this age, as previously demonstrated (Crielaard et al., 2011). The addition of the year 3 oral microbiota data will add to these findings and reinforce if there is any evidence of stabilisation beyond 2 years of age. Because there are no longitudinal oral microbiota studies to date from birth, it would be interesting to follow these toddlers from 3 years of age, where the majority of deciduous teeth would be erupted, through to 6 years of age, when the first permanent teeth begin to emerge.

By following these infants from birth through to 6 years of age and observing the oral microbiota changes, it would be constructive to complete dental examinations every 6 months along with detailed diet analysis. This would be important, as mentioned in Chapter 4, where we compared the oral microbiota of those with S-ECC and without caries. We then could document and observe if any microbial changes occur before caries presents. This would give us an insight into any subtle microbial changes that may become apparent within the oral cavity that may increase the caries risk in these children. A future aim following on from this thesis would be to complete this by carrying forward the same cohort group enrolled in the ORALMET study to at least 6 years of age and investigating the oral microbiota, alongside a more detailed dental examination.

Our research findings have given a deeper insight into the diversity and dynamics of the infant oral cavity, both in health and in disease. Both our cross-sectional study of the carious microbiota of children and the longitudinal development of neonates from birth to 2 years of age has given us a platform to work onwards with, with an eventual aim to get a better understanding of what drives this diversity and dynamical changes in young children. A better understanding of

these changes and the action of the microbes within this ecosystem could potentially lead to the development of a clinical application to reduce or prevent the occurrences of dental diseases in children, in particular before they have a chance to develop.

### **Cross Sectional Study.**

In chapter four we described the microbiota of deep dentinal lesions of deciduous teeth of Irish children affected with S-ECC. We also compared the microbiota of the saliva of children with S-ECC to the saliva of children who didn't present with caries at the time of examination and sampling. All children were between the ages of 3-5 years of age. Children with active caries were suffering with chronic dental diseases and undergoing full general anaesthetic due to the scale of dental disease and poor co-operation. While these children were under general anaesthetic, samples were taken (dentine and saliva), that otherwise would have proven difficult in the dental chair. We used advanced sequencing methods to compare the microbiota compositional differences and diversity of taxa between all the sampled sites (saliva/dentine), between the two cohorts of children, for the first time in Irish children.

We identified microbiota compositional differences between both groups, with species associated with the dentinal lesions such as *Streptococcus mutans*, *Prevotella*, *Bifidobacterium* and *Scardovia* species. The diversity of the salivary microbiota was higher than the dentinal microbiota and rare taxa were identified in the active dentinal microbiota in these children with S-ECC that warrant further investigation. While diet analysis was taken into account in this study (FFQ), the influence of other factors such as oral hygiene, plaque levels, measurement of the depth of dentinal lesions and if any pulpal involvement was present was not

investigated. It is difficult to sample these children and collect information of a weekly or daily diet analysis which is a true reflection of the frequency of intake. Because the majority of these children only present in pain at the dentist by which time caries is already active and chronic. Ideally following a cohort of children from birth, before the presence of teeth and into the age of 3-6 years would avoid ambiguous data collection (i.e. diet).

This study does however provide an insight for the first time on the oral microbiota of Irish children, where the rates of dental caries in Irish children are still high. The identification of microbiota associated with the advanced dentinal lesions and microbiota differences in the saliva could be the beginning of future work to highlight this persistent problem in Irish children. Further detailed analysis using both culture and culture independent techniques of these taxa identified in the salivary microbiome and dentinal microbiome would further benefit this cross sectional study, with a particular focus on the taxa within the advancing front of deep dentinal lesions, where the microbial composition can vary considerably to that of occlusal lesions. Together with detailed diet and oral hygiene recording, this additional analysis would advance our understanding of this rapid progressive disease and support the potential development of therapeutics (i.e. with the probiotic potential or with the aim of arresting the advancing lesion), with the overall aim to reduce the destruction of this disease in young children.

## **In Conclusion:**

Hypotheses to be tested:

- There is no difference in the oral microbiome of children
  1. who are delivered by Caesarean section and vaginally: **Confirmed**
  2. from birth to 3 years of age: **Confirmed**
  3. with high caries and those who are caries free: **Confirmed.**

## **Chapter 7**

## Appendices

**Appendix 7.1** Oralmet/Infantmet Questionnaire. Cohort study. Pages 1-3.

**InfantMet Study/OralMet  
Year 1 Questionnaire**

Study Number: IM \_\_\_\_\_

Date: \_\_\_\_\_

Are you still Breast Feeding? Yes No

How long did you **exclusively** breast feed? (Please circle)

2 weeks      1 month      2 months

Less than 4 months      Greater than 4 months

If No when did you discontinue: Date: \_\_\_\_\_

If yes is your infant getting formula with breast milk? \_\_\_\_\_

Name of Formula: \_\_\_\_\_

When did you infant start solids? Date: \_\_\_\_\_

Are there persons smoking in the home? Yes No

Has your child taken any medication in the past year Yes No

If yes please name the medication:

Date taken: \_\_\_\_\_

Has your child taken any antibiotics in the past year? Yes No

If yes please name the antibiotic(s):

Date(s) taken: \_\_\_\_\_

Has your child taken any probiotics in the past year? Yes No

If yes please name the probiotics

Date(s) taken: \_\_\_\_\_

Has your child been diagnosed with colic by a doctor? Yes No

Has your child ever cried for more than 3 hours per  
day for more than 3 days in one week? Yes No

Has your child ever suffered from reflux? Yes No



InfantMet Study/OralMet  
Year 1 Questionnaire

Has your child been diagnosed with a medical condition?    Yes                      No

**Mother:**

Have you taken any medication or antibiotics  
since the birth of your child?                                              Yes                      No

If yes, when?

(please circle)

During breast feeding period                      After breast feeding period

Please name antibiotic:

Date(s) taken: \_\_\_\_\_

Where you (mother) breast fed as a child? (please circle)

Yes                      No                      Don't Know

Do you know if you were born by (please circle)

Natural (vaginal) delivery                      Caesarean section

Other children:              Sons                      Yes / No              Age(s): \_\_\_\_\_

                                    Daughters                      Yes / No              Age(s): \_\_\_\_\_

Are there any other issues affecting you or your child which you think may be  
relevant? \_\_\_\_\_

**Oral Questions:**

Is the water in your home connected to: (please circle)

Public water mains    or    Private well

Have you given your child juice?                      Yes              No

How often does your child drink juice? (please circle)

Daily                      Nightly                      Weekly                      Monthly

When did you last visit your dentist?

InfantMet Study/OralMet  
Year 1 Questionnaire

Date (Approx): \_\_\_\_\_

Filings: (please circle)

None      1-3      4-6      6+

Extractions:      Yes    No

**Appendix 7.2.** Oralmet questionnaire. Cross sectional study. **Pages 1-5.**

## Consent Form

Subject Number:  
 \_OM\_\_\_\_\_

PLEASE USE BLOCK CAPITALS

Child's Full name (initials)  Date Screened

Boy ☐ Girl ☐ Child's Date of Birth:

Child's weight at birth:  Method of delivery:

Address/Area:

Does your child have any allergies or significant Medical History? Yes ☐ No ☐

If yes, Please provide details below: \_\_\_\_\_

Has your child taken antibiotics in the last 6 months? If yes, Please provide details below:  
 \_\_\_\_\_

### CONSENT

I have read and understand the information provided about the Oralmet study. I understand that a dentist will look at my child's teeth.

Parent/legal guardian signature: \_\_\_\_\_ Date: \_\_\_\_\_

Print name: \_\_\_\_\_

Dental record form:

#### CARIES

UPPER											LOWER										
	R					L						R					L				
	E	D	C	B	A	A	B	C	D	E		E	D	C	B	A	A	B	C	D	E
Status											Status										
Condition											Condition										
O											O										
M											M										
B											B										
D											D										
P											P										

TRAUMA

Vitals:

Pulse: \_\_\_\_\_

Weight: \_\_\_\_\_

Height: \_\_\_\_\_

Race: \_\_\_\_\_

**Q1. At what age did you or your child start brushing your child's teeth?**

Tick one box

- My child's teeth are not usually brushed* 1 ☐
- Before 12 months of age* 2 ☐
- Between 12 and 18 months of age* 3 ☐
- Between 19 and 24 months of age* 4 ☐
- After 24 months of age* 5 ☐

**Q2a. How often does your child brush his/her teeth (or have them brushed for him/her)?**

Tick one box

- My child's teeth are not usually brushed* 1 ☐
- Less than once a day (e.g., every second day, once a week)* 2 ☐
- Once a day* 3 ☐
- Twice a day* 4 ☐
- More than twice a day* 5 ☐

**Q2b. Does anyone help your child brush his/her teeth?**

Tick one box

- My child brushes his/her own teeth and does not get any help* 1 ☐
- My child's teeth are not usually brushed* 2 ☐
- Mother/Father* 3 ☐
- Brother/Sister* 4 ☐
- Guardian* 5 ☐
- Nanny/Childminder* 6 ☐

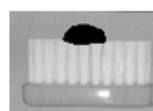
**Q3 When your child's teeth are brushed does he/she use**

- Adult tooth paste* ☐
- Junior/children's toothpaste (low fluoride toothpaste)* ☐
- No toothpaste* ☐
- My child's teeth are not brushed* ☐

**Q4a. These pictures show different amounts of toothpaste on a brush. Which picture shows the amount of toothpaste your child uses?**

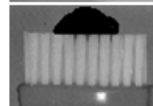
Tick one box

- My child's teeth are not usually brushed* 1 ☐
- My child does not use toothpaste* 2 ☐



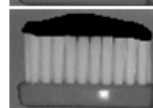
**Picture 1**

*pea-sized amount of toothpaste* 3 ☐



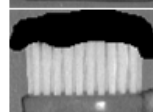
**Picture 2**

*half brush of toothpaste* 4 ☐



**Picture 3**

*full brush of toothpaste* 5 ☐



**Picture 4**

*overflowing brush of toothpaste* 6 ☐

**Q4b. These pictures show children rinsing their teeth after brushing. Which picture shows what your child does?**

Tick one box

*My child's teeth are not usually brushed* 1 ☐



*Picture 1*  
*using the toothbrush* 2 ☐  
*to rinse*



*Picture 2*  
*rinsing directly from* 3 ☐  
*the tap*



*Picture 3*  
*cupping hands to* 4 ☐  
*rinse*



*Picture 4*  
*using glass to rinse* 5 ☐

*My child does not rinse* 6 ☐

*None of these* 7 ☐

**Q5.** How often does your child eat sweet food or sweet drinks (such as biscuits, cakes, sweets, Coca-Cola, Pepsi-Cola, 7-Up, Ribena, fruit drinks, etc.) between normal meals?

Tick one box

*Never* 1 ☐

*Less than once a day: (e.g., once a week,* 2 ☐  
*every second day)*

*Once a day* 3 ☐

*Twice a day* 4 ☐

*Three times a day* 5 ☐

*Four times a day* 6 ☐

*Five times a day* 7 ☐

*Six times or more a day* 8 ☐

*Don't know* 9 ☐

**Q6.** Was your child breast fed as a baby?

*Yes* 1 ☐

*No* 2 ☐

For how long? \_\_\_\_\_

**Q7.** When your son/daughter was a baby did he/she ever have baby or infant formula, or follow-on milk like Progress or Junior Milk (not liquid cow's milk)?

Tick one box

*Yes* 1 ☐

*No/Never* 2 ☐

*Don't know* 3 ☐

**Q8.** How old was your son/daughter when he/she started to drink baby or infant formula, or follow-on milk like Progress or Junior Milk (not liquid cow's milk)?

Tick one box

*Under 1 month* 1 ☐

*1 month – under 2 months* 2 ☐

*2 months – under 4 months* 3 ☐

*4 months – under 6 months* 4 ☐

*6 months – under 9 months* 5 ☐

*9 months – under 1 year* 6 ☐

*1 year – under 1½ years* 7 ☐

*1½ years – under 2 years* 8 ☐

*2 years – under 2½ years* 9 ☐

*2½ years – under 3 years* 10 ☐

*3 years or older* 11 ☐

**Q9. How old was your son/daughter when he/she stopped having baby or infant formula, or follow-on milk like Progress or Junior Milk, even just at bedtime?**

- Tick one box
- Under 1 month* 1 ☐
- 1 month – under 2 months* 2 ☐
- 2 months – under 4 months* 3 ☐
- 4 months – under 6 months* 4 ☐
- 6 months – under 9 months* 5 ☐
- 9 months – under 1 year* 6 ☐
- 1 year – under 1½ years* 7 ☐
- 1½ years – under 2 years* 8 ☐
- 2 years – under 2½ years* 9 ☐
- 2½ years – under 3 years* 10 ☐
- 3 years or older* 11 ☐

**Q10. If your child had a painful baby tooth would you prefer if it was**

- Tick one box
- Filled* 1 ☐
- Taken out* 2 ☐
- Don't know/No opinion* 3 ☐

**Q11. If your child had a painful back tooth and it was not a baby (milk) tooth but a second (permanent) tooth, would you prefer if it was**

- Tick one box
- Filled* 1 ☐
- Taken out* 2 ☐
- Don't know/No opinion* 3 ☐

**Q12. If your child had a painful front tooth and it was not a baby (milk) tooth but a second (permanent) tooth, would you rather it was**

- Tick one box
- Filled* 1 ☐
- Taken out* 2 ☐
- Don't know/No opinion* 3 ☐

**Q13. Do you think it is important to treat decay in baby (primary) teeth?**

- Tick one box
- Yes* 1 ☐
- No* 2 ☐

**Q14. If you answered YES to Question 11 (previous question), is this because**

- Tick one box
- Decayed baby teeth might cause my child pain* 1 ☐
- He/she may have crowding later on if baby teeth are lost early* 2 ☐
- It is important to treat diseased teeth* 3 ☐
- Decayed baby teeth look bad* 4 ☐
- All of the above* 5 ☐
- Other (Please Specify)* 6 ☐

**Q15. Please complete as applicable:**

Enter Age in Years

*My child had his/her first visit to a dentist (including Health Board/ school dentist) at the age of* 1

**Or**

Tick Box

*My child has never been to a dentist* 2 ☐

**Q16. What was the reason for this first visit?**

- Tick one box
- Check up* 1 ☐
- If felt treatment was needed but my child had no pain* 2 ☐
- My child was in pain* 3 ☐
- Was sent an appointment by school dentist* 4 ☐
- Other (e.g., trauma to teeth)* 5 ☐
- My child has never been to the dentist* 6 ☐

Q17. How often does your child go to the dentist?

- Tick one box
- Occasionally* 1 ☐
- Every six months or more often* 2 ☐
- Every 6 – 12 months* 3 ☐
- Every 12 – 24 months* 4 ☐
- Every 2 years/more* 5 ☐
- My child has never been to the dentist* 6 ☐

Q18. In the last 6 months, have you (or your partner) ever had to take time off work to bring your child to a dentist because he/she had toothache?

- Tick one box
- Yes* 1 ☐
- No* 2 ☐
- I (or my partner) have not been working (employed) in the last 6 months* 3 ☐

Q19. In the last 6 months, have you ever had a sleepless night because your child was awake with toothache (excluding teething/cutting teeth)?

- Tick one box
- Yes* 1 ☐
- No* 2 ☐

Q20. In the last 6 months, has your child ever missed school because of toothache?

- Tick one box
- Yes* 1 ☐
- No* 2 ☐

Q21. What type of dentist does your child normally attend?

- Tick one box
- My child has never been to a dentist* 1 ☐
- Health Board (School Dentist)* 2 ☐
- Private Dentist (Republic of Ireland)* 3 ☐
- Private Dentist (Northern Ireland)* 4 ☐
- Dental Hospital* 5 ☐
- Specialist Children's Dentist* 6 ☐

Q22 Please write your child's typical daily food and snack intake:

Breakfast: \_\_\_\_\_

Lunch: \_\_\_\_\_

Dinner: \_\_\_\_\_

Snacks: \_\_\_\_\_

Drinks: \_\_\_\_\_

Q23: Did your child ever take probiotics? \_\_\_\_\_

Q24: Are you served by public water or well water? \_\_\_\_\_

Q25: What is your Occupation? \_\_\_\_\_

Q26: Do you smoke? Or your partner? If yes, How many? \_\_\_\_\_

Q27: If you have any comments you would like to make, please use the space below:

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## Appendix 7.3 Food Frequency Questionnaire (FFQ)

M = month

W = week

D = Day

**ORALMET code: OM**

Meat, fish, poultry - Medium serving (size deck of cards)	Never	<1 / M	1/M	1/W	2/W	5/W	1/D	2/D	4/D	6+/D
Beef (roast / steak)										
Beef: stew										
Beef burger (1 burger)										
Pork (roast / chops / escalopes)										
Lamb (roast / chops / stew)										
Chicken or other poultry e.g. turkey: roast										
Breaded chicken, chicken nuggets, chicken burger										
Bacon / Ham										
Processed meat (Corned beef, Luncheon meats, sausages)										
Savoury pies (e.g. meat, pork, steak & kidney, sausage roll)										
Liver, heart, kidney, paté										
Fish fried (batter/breadcrumbs), baked, grilled, fingers/cakes										
White fish, fresh or frozen (e.g. cod, haddock, plaice, sole)										
Oily fish, fresh or canned (e.g. mackerel, kippers, tuna, salmon, sardines, herring)										
Shellfish (e.g. crab, prawns, mussels)										
Bread and savoury biscuits - One slice or one biscuit	Never	<1 / M	1/M	1/W	2/W	5/W	1/D	2/D	4/D	6+/D
White bread and rolls (including ciabatta & pannini)										
Brown bread and rolls										
Wholemeal bread and rolls										
Wheat-free; Rye bread; spelt bread (specify)										
Cream crackers, cheese biscuits										
Crisp bread, e.g. Ryvita										
Pancakes, muffins, oatcakes										
Scone (white)										
Scone (brown)										



<b>Cereals - One medium sized bowl</b>	<b>Never</b>	<b>&lt;1 / M</b>	<b>1/M</b>	<b>1/W</b>	<b>2/W</b>	<b>5/W</b>	<b>1/D</b>	<b>2/D</b>	<b>4/D</b>	<b>6+/D</b>
Non-ready to eat - Porridge, Readybrek										
High fibre (All Bran/flakes, Weetabix, Shredded wheat)										
Cornflakes, Rice Krispies										
Muesli (e.g. Country Store, Aplen)										
Sugar-coated cereals (e.g. Frosties, Crunchy nut cornflakes)										
<b>Potatoes, rice and pasta - Medium serving = a cupful</b>	<b>Never</b>	<b>&lt;1 / M</b>	<b>1/M</b>	<b>1/W</b>	<b>2/W</b>	<b>5/W</b>	<b>1/D</b>	<b>2/D</b>	<b>4/D</b>	<b>6+/D</b>
Boiled, instant or jacket potatoes										
Mashed potatoes										
Chips / Roast potatoes										
Potato Salad										
White Rice										
Brown Rice										
White/yellow or green pastas (e.g. spaghetti, noodles)										
Wholemeal pasta										
Lasagne (meat based)										
Lasagne (vegetarian)										
Pizza										
<b>Dairy products and fat</b>	<b>Never</b>	<b>&lt;1 / M</b>	<b>1/M</b>	<b>1/W</b>	<b>2/W</b>	<b>5/W</b>	<b>1/D</b>	<b>2/D</b>	<b>4/D</b>	<b>6+/D</b>
Cream (tablespoon)										
Full-fat yoghurt or Greek style yoghurt (125g carton)										
Dairy desserts (125g carton)										
Cheddar cheese (medium serving)										
Low-fat cheddar cheese (medium serving)										
Cottage cheese										
Eggs as boiled, fried, scrambled, poached (one)										
Quiche (medium serving)										
Light salad cream or light mayonnaise (tablespoon)										
Salad cream, mayonnaise (tablespoon)										
French dressing (tablespoon)										
Other salad dressing (tablespoon)										
<b>The following on bread or vegetables (teaspoon):</b>										

Butter										
Lite Butter e.g. Dawn Lite, Connacht Gold										
Sunflower margarine e.g. Flora										
Low-fat margarine (e.g. lowlow)										
Cholesterol Lowering e.g. Flora Pro-Active, DairyGold Heart										
Cream & Veg. Oil spread e.g. Kerrymaid, Dairy Gold										
Olive oil spread e.g. Golden Olive										
<b>Fruit - 1 fruit or a medium serving (a cupful)</b>	<b>Never</b>	<b>&lt;1 / M</b>	<b>1/M</b>	<b>1/W</b>	<b>2/W</b>	<b>5/W</b>	<b>1/D</b>	<b>2/D</b>	<b>4/D</b>	<b>6+/D</b>
Apples										
Pears										
Oranges, satsumas, mandarins										
Grapefruit										
Bananas										
Grapes										
Melon										
Peaches, plums										
Apricots										
Strawberries, raspberries, kiwi fruit										
Blueberries										
Tinned fruit (specify)										
Dried fruit e.g. raisins										
Frozen fruit (specify)										
<b>Vegetables (Fresh, frozen, tinned) - 2 tablespoons</b>	<b>Never</b>	<b>&lt;1 / M</b>	<b>1/M</b>	<b>1/W</b>	<b>2/W</b>	<b>5/W</b>	<b>1/D</b>	<b>2/D</b>	<b>4/D</b>	<b>6+/D</b>
Carrots										
Spinach										
Broccoli, spring greens, kale										
Brussel sprouts										
Cabbage										
Peas										
Green beans, broad beans, runner beans										
Courgettes										
Cauliflower										

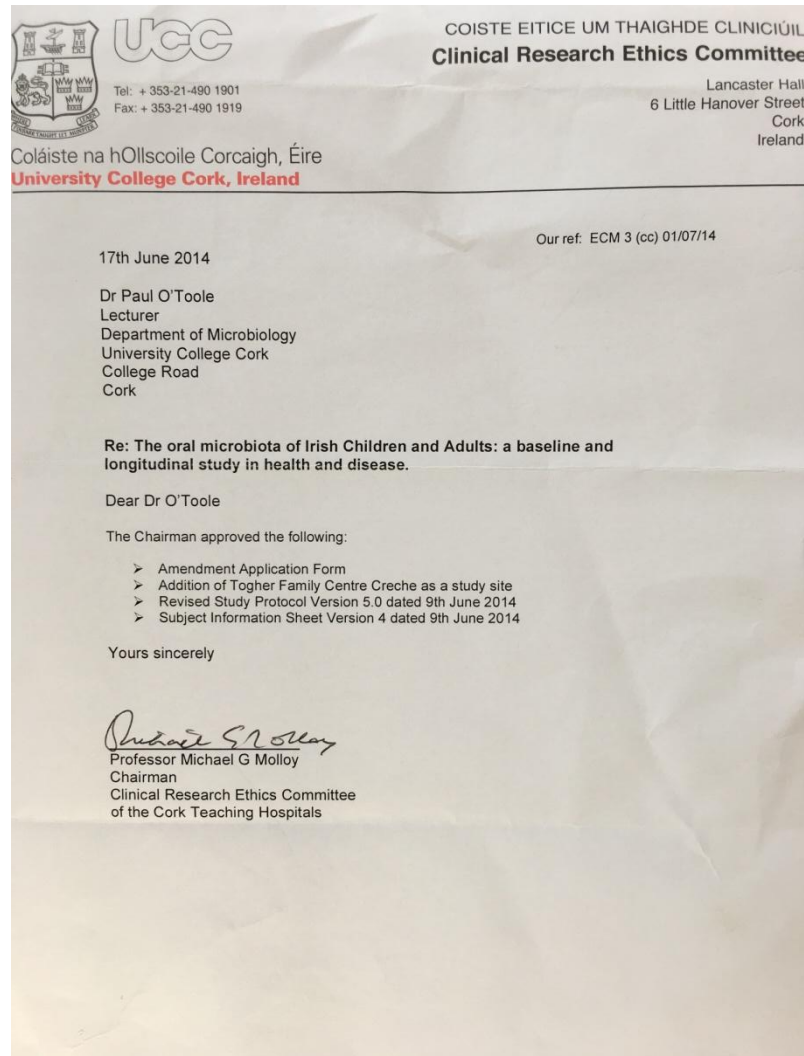
Parsnips, turnips										
Leeks										
Onions										
Garlic										
Mushrooms										
Sweet peppers										
Green salad, lettuce										
Cucumber, celery										
Tomatoes										
Sweetcorn										
Beetroot										
Coleslaw										
Baked beans										
Dried lentils, beans, peas										
Tofu, soya meat, TVP, vegeburger										
<b>Sweets and snacks - medium serving</b>	<b>Never</b>	<b>&lt;1 / M</b>	<b>1/M</b>	<b>1/W</b>	<b>2/W</b>	<b>5/W</b>	<b>1/D</b>	<b>2/D</b>	<b>4/D</b>	<b>6+/D</b>
Chocolate coated sweet biscuits e.g. digestive (one)										
Plain biscuit e.g. marmalade, digestives, rich tea (one)										
Cakes e.g. fruit, sponge										
Buns, pastries e.g. croissants, doughnuts										
Fruit pies, tarts, crumbles										
Sponge puddings										
Milk puddings e.g. rice, custard, trifle										
Ice cream, choc ices, frozen desserts										
Chocolates, singles or squares										
Sweets, toffees, mints										
Sugar added to tea coffee, cereals (teaspoons)										
Sugar substitute e.g. canderel (teaspoon)										
Crisps or other packet snacks										
Peanuts or other nuts										
<b>Soups, sauces and spreads</b>	<b>Never</b>	<b>&lt;1 / M</b>	<b>1/M</b>	<b>1/W</b>	<b>2/W</b>	<b>5/W</b>	<b>1/D</b>	<b>2/D</b>	<b>4/D</b>	<b>6+/D</b>
Vegetable soups: homemade/fresh (1 bowl)										
Vegetable soups: tinned/packet (1 bowl)										

Meat or cream soups: homemade / fresh (1 bowl)										
Meat or cream soups: tinned / packet (1 bowl)										
Sauces e.g. white, cheese, gravy (tablespoon)										
Tomato based sauces e.g. pasta sauces										
Curry-type sauces										
Pickles, chutney (tablespoon)										
Marmite, Bovril (tablespoon)										
Jam, marmalade, honey, syrup (teaspoon)										
Peanut butter (teaspoon)										
Drinks	Never	<1 / M	1/M	1/W	2/W	5/W	1/D	2/D	4/D	6+/D
Tea (cup)										
Herbal tea (cup)										
Whole milk (cup) - cow, goat, soya, rice milk – specify										
Semi-skimmed milk (cup)										
Coffee instant (cup)										
Coffee ground (cup)										
Coffee, decaffeinated (cup)										
Coffee whitener e.g. Coffee-mate (teaspoon)										
Cocoa, Hot Chocolate (cup)										
Horlicks, Ovaltine (cup)										
Wine (glass)										
Beer, Lager or Cider (half pint)										
Low alcohol / alcohol free beer / lager (half pint)										
Port, Sherry, Vermouth, liqueurs (glass)										
Spirits e.g. Gin, Whiskey (Single measure)										
Low calorie or diet soft fizzy drink (glass)										
Fizzy Soft drinks e.g. (Coca Cola - Glass)										
Pure fruit drinks e.g. orange juice (small glass)										
Fruit squash (small glass)										
Functional Foods / Supplements	Never	<1 / M	1/M	1/W	2/W	5/W	1/D	2/D	4/D	6+/D
Probiotic Yoghurts / cheese / milk										
Vitamin supplements (details)										
Mineral supplements (details)										

Ready meals	Never	<1 / M	1/M	1/W	2/W	5/W	1/D	2/D	4/D	6+/D
Ready meal (specify)										
Takeaway (specify)										

#### Appendix 7.4. Ethical approval.

Ethical approval was obtained from the Cork Teaching Hospitals Clinical Research Ethics Committee for the recruitment and sampling of these Cohorts of children. Ethical approval reference: ECM 3 (cc) 01/07/14.



**Appendix 7.5.** Oralmet Consent & Information leaflet.

**SUBJECT INFORMATION SHEET**

**Study Title:** The Oral Microbiota of Irish Children and Adults: a baseline and longitudinal study in health and disease

**Protocol No.:** APC034

**Principal Investigator:** Dr Paul O'Toole

**Co-Investigators:** Professor Helen Whelton, Professor Kinirons, Dr.Eimear Hurley & Dr Catherine Stanton.

**Site of Investigation:** Cork University Hospital (CUH), Cork Dental School and Hospital, University College Cork (UCC), & Teagasc Moorepark Food Research, Fermoy, Co. Cork,

**Why is this study being run?**

Oral hygiene in children is a key part of ensuring healthy teeth in adulthood. A significant number of children in Ireland suffer from pain and sleepless nights with toothache. Because treatment of kids in the dental chair is traumatic, children's teeth that are damaged beyond repair are frequently extracted under anaesthetic in hospital. This can lead then to a higher need for orthodontic treatment and dental-phobia in the treated kids.

Tooth damage due to bacteria is called dental caries. Based on older microbiology techniques, the major bacteria related to caries have been identified

as oral streptococci, with minor parts played by other bacteria. However, recent molecular techniques have shown that some caries can be caused by multiple other bacteria. Importantly, whether or not a person develops caries is influenced by the total pattern of bacteria in the mouth – the *oral microbiota*.

The oral microbiota of Irish babies or children has not been studied using molecular techniques. We will study children (in crèches and primary school) by taking a saliva sample and determining what bacteria are present associated with bad or good teeth. We expect to find differences, and this may help us form a ‘library’ of information, which we can store, and use to prevent children getting these bad, decayed teeth. We will also investigate the oral microbiota of new-born babies from birth to age 3. We will test, as the baby develops and starts eating solid foods and cutting teeth, how the bacteria in the mouth also change. We will cross-reference this information to the data from older children, to build an overlapping picture, from infancy into pre-school kids where the risk of caries is greatest. This will help us identify which bacteria develop to put the child at risk of bad teeth, or promote good teeth.

If you agree to participate in the study you will be asked some general questions and eligibility to participate in the study will be determined.

The visits will take place at the site clinic at Cork University Hospital or Cork Dental School and Hospital.

Two studies will be conducted in parallel. The first study will involve up to 6 visits over 3 years, at different time intervals. 100 neonates and 100 mothers will be involved in the study from birth to 3 years.

The second study will involve one visit. A sample will be collected while the patient is in the Cork University Hospital for a general anaesthetic extraction procedure. 100 healthy males and females, under 6 years of age and adults will be recruited.

## **Study Procedure**

You will be advised of the purpose of the study and the procedures which will be undertaken. You will be given a copy of the Subject Information sheet, which will explain what is required from you and your child. If interested, you will then



be requested to read and sign the Informed Consent form, and receive a signed copy. Your child's suitability to participate in the study will be checked.

In the first study, participation will involve attending the CUH maternity clinic for 6 visits over a period of 3 years, at weeks 1, 4, 8 and at 4, 12, 24 and 36 months. You will be asked some general questions regarding your baby's feeding and oral hygiene and baby's weight will be recorded. A saliva sample will be collected from your child and where plaque is present in older infants, a sample will be collected. You, the Mother, will be asked to provide a saliva and plaque sample, and a clinical nurse will take a vaginal/skin swab at visit one only.

You will be asked to avoid feeding your baby 45 minutes before his/her sample collection and for you to avoid eating for 4 hours before your sample is collected.

The second study will involve one visit. If you or your child is scheduled to undergo a tooth extraction at the General Anaesthetic clinic in Cork University Hospital (CUH), while sedated a saliva and plaque sample will be collected. In addition, a caries sample will be taken immediately from the teeth extracted.

Saliva and plaque sample will also be collected from children who are caries free and have no decayed, missing or filled teeth.

### **What happens if I start the study and change my mind later?**

You do not have to take part in the study, participation is entirely voluntary. Refusal to participate, or discontinuing participation at any time, will involve no penalty, loss of benefits or denial of treatment or services by the Cork Teaching Hospital or the participating doctor.

### **Will I experience any unpleasant side effects?**

No side effects will be felt on taking the sample

**Funding of trial**

There are no cost implications for the Health Services Executive (HSE) or to you. The management of patients and investigative tests will comply with current standards of care. Cost of research tests will be incurred by the Cork Dental School and Hospital and the Alimentary Pharmabiotic Centre, University College Cork.

**Confidentiality**

All the information gathered from this study will be stored on a computer, paper files and will be treated confidentially. You will be identified only by a subject number.

In the event of any publication regarding this study, your identity will not be disclosed.

**What happens if there is anything I do not understand?**

If there is anything you are not sure about, the Doctor will be happy to explain in more detail to yourself or your relatives, guardians (or legal representative if required). The study will be fully explained to you before you decide if you want to take part.

Dr. Paul O'Toole

Department of Microbiology & Alimentary Pharmabiotic Centre, University  
College Cork

Telephone Number: 021 4903997

**CONSENT BY SUBJECT FOR PARTICIPATION IN A HUMAN  
INTERVENTION STUDY**

**Protocol Number:** APC034

**Subject's Name:** \_\_\_\_\_

**Title of protocol:** The Oral Microbiota of Irish Children and Adults: a baseline and longitudinal study in health and disease

**Principal Investigator:** Dr.Paul O'Toole 021-4903997

**Participation in this study is voluntary and you may withdraw at any time for any reason**

The research project and procedure associated with it have been fully explained to me. All experimental procedures have been identified and no guarantees have been given about the possible results. I have had the opportunity to ask questions concerning any and all aspects of the project and any procedures involved. I am aware that participation is voluntary and I may withdraw consent at any time. I am aware that my decision not to participate or to withdraw will not restrict my access to health care services normally available to me. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. I understand that the investigators have such insurance as is required by law in the event of injury resulting from this research.

I, the undersigned, hereby consent to participate as a subject in the above described project conducted at the Cork University Hospital and University College Cork. I have received a copy of this consent form for my records. I understand that if I have any questions concerning this research, I can contact the Doctor listed below. If I have further queries concerning my rights in connection with the research, I can contact the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Lancaster Hall, 6 Little Hanover Street, Cork

After reading the entire consent form, if you have no further questions about giving consent, please sign the where indicated.

Parent's/Guardian's Signature: \_\_\_\_\_

Date: \_\_\_\_\_

dd mon yy

NAME (BLOCK LETTERS): \_\_\_\_\_ Time: \_\_\_\_\_

Witness' Signature: \_\_\_\_\_

Date: \_\_\_\_\_

dd mon yy

NAME (BLOCK LETTERS) \_\_\_\_\_

Investigator's Signature: \_\_\_\_\_

Date: \_\_\_\_\_

dd mon yy

NAME (BLOCK LETTERS) \_\_\_\_\_

## **Appendix 7.6.**

### **Publications & articles under peer review during the course of this thesis.**

A study of primary teeth restored by intracoronal restorations in children participating in an undergraduate teaching programme at Cork University Dental School and Hospital, Ireland.

Authors: E. Hurley, C. Da Mata, C. Stewart, M. Kinirons

Published In March 2015 in ‘The European Journal of Paediatric Dentistry

Decisions on repositioning of intruded permanent incisors; a review and case presentation.

Authors: E. Hurley, C. Stewart. C. Gallagher, M. Kinirons

Accepted for publication September 2017 in ‘The European Journal of Paediatric Dentistry.

Comparison of the salivary and dentinal microbiome of children with severe-early childhood caries to the salivary microbiome of caries-free children.

Authors: Eimear Hurley, Maurice P.J Barrett, Martin Kinirons, Helen Whelton, C. Anthony Ryan, Catherine Stanton, Hugh M.B. Harris and Paul W. O’Toole.

Under peer review at ‘BMC Oral Health’.

The influence of birth mode and breastfeeding duration on the emerging neonatal oral microbiota from birth to 1 year of age, a cohort study.

Authors: Eimear Hurley, Hugh M.B. Harris, David Mullins, Maurice P.J Barrett, Carol Anne O'Shea, Martin Kinirons, Helen Whelton, C. Anthony Ryan, Catherine Stanton, Paul W. O'Toole.

Under peer review at 'Journal of Dental Research'

**Co-Author on the following:**

Maternal microbiota impact on the gut and oral microbiota of naturally delivered, breastfed infants.

Authors: Cian J. Hill, Denise B. Lynch, Eimear Hurley, Eugene M. Dempsey, C. Anthony Ryan, R. Paul Ross, Catherine Stanton and Paul W. O'Toole.

This has been submitted to "The Archives of Disease in Childhood, Fetal and Neonatal Edition" for peer review.

The oral microbiota in colorectal cancer is distinctive and predictive.

This has been accepted for publication to the Journal 'Gut' . September 2017.

Author: Dr. Burkhardt Flemer.

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## *Scaffolding*

Masons, when they start upon a building,  
Are careful to test out the scaffolding;

Make sure that planks won't slip at busy points,  
Secure all ladders, tighten bolted joints.

And yet all this comes down when the job's done  
Showing off walls of sure and solid stone.

So if, my dear, there sometimes seem to be  
Old bridges breaking between you and me

Never fear. We may let the scaffolds fall  
Confident that we have built our wall.

*Seamus Heaney*