How do changes in the environment alter the oral microbiome of adolescents

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Declaration

I hereby certify that the work embodied in this thesis is the result of my own investigations, except where otherwise stated in the acknowledgments; the contents of this thesis are entirely my work. This has not been submitted, in part or full, for a degree of this or any other university or examination board.

Violeta Kakiora

23/07/2021

Acknowledgements

I am extremely grateful to Professor Andrew Smith, Professor Paul Ashley and Professor Davit Spratt for their support, help and guidance that allowed me to successfully complete this project. I would also like to thank, Dr Erni Marlina for helping me to familiriase with the laboratory work I had to carry out, Mr Tony Brooks from UCL genomics for processing the samples and Dr Silke Rath, for her enormous assistance, from carrying out the bioinformatics analysis, analysing and interpreting the findings and results, she was always very willing and happy to support me despite her busy schedule. A big thank you to Haileybury's school staff, especially to Miss Gwynneth Norris and Mr Arthur Kattavenos, for their excellent teamwork throughout this study, from agreeing to collaborate on this project, participant's recruitment, samples collection arrangements and warm accommodation during our visits to the school campus.

Abstract

Introduction

The oral cavity is one of the most complex and diverse microbial habitats across the human body. The oral microbiome is a causative factor in conditions such as dental caries and periodontal disease but can have a significant role in the pathogenesis of nonoral diseases. The composition of the human oral microbiome is affected by multiple factors, including the environment and host genetics. Previous studies have demonstrated that environmental effects have dominated over genetic effects, with shared household having a significant effect on the microbial composition. Furthermore, the establishment of the salivary microbiome early in life affects its long-term composition.

Aims and objectives

The aim of this study is to analyse the oral microbiome composition of adolescents and investigate the diversity of the bacterial communities in depth of time. Additionally, to determine whether the oral bacterial community is stable in adolescents and how the shared school environment may influence the oral microbiome composition of boarder students.

Methods and materials

Saliva samples were collected from 17 participants at nine time points within one academic year. Participants of this study are students of a boarding school. The age range was from 11 to 16 years. Students were divided in two groups according to their boarding status: boarders and non-boarders. A total of 135 saliva samples were collected and analysed. Microbial composition of saliva was assessed by sequencing all the variable regions (V1-V9) of the 16S rRNA gene.

Results

Five major phyla predominated in both groups; *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, *Fusobacteria*, forming 99.37% of the total phyla observed. Taxonomic analysis at genus level revealed that *Rothia* was the most

abundant genera, followed by *Streptococcus*, *Neisseria, Haemophilus* and *Prevotella*. The most abundant species identified within all the participants and all sampling points is *Rothia mucilaginosa, Streptococcus oralis* and *Haemophilus parainfluenzae*. Boarders had significantly higher alpha diversity compared to the group of non-boarder group. The beta diversity of the boarder group revealed no clear trend on the similarity of the oral microbiome, which could indicate that boarders share a more similar oral microbiome composition. However, it was noted that having spent more days at school, the oral microbiome composition of boarder students tends to become more similar.

Conclusion

This study concluded that the oral microbiome of adolescents does not remain stable within the studied period of one academic year and that shared environment may have an important role in the shaping of the oral microbiome of adolescents. However, there is a need for further studies involving larger population, equal number of participants in each group and longer sampling time, in order to determine more accurately the effect of shared environment in the composition of the oral microbiome of adolescents.

Impact of statement

Comprehensive investigation of the composition of the oral microbiome of adolescents is of great importance for understanding not only the normal bacterial ecosystem of the oral cavity at this age, but also to make any possible associations with potential causes of these normal communities' disturbances, in order to understand disease mechanisms and develop prevention and treatment strategies. The oral microbial ecosystem of adolescents has been shown to be different and more complex compared to the adult oral microbiome.

Recent studies have demonstrated that the influence of a shared living environment is potentially of greater value than the shared genetics, as it was traditionally thought, in the formation of the oral microbiome composition (Shaw et al., 2017a). How the environmental component affects the composition of the oral microbiome of children, is a research field that is not well investigated.

To our knowledge this is the first study in which the adolescents' oral microbiome was analysed over a period of ten months, involving a cohort of boarder and non-boarder students. This cohort gave the opportunity to investigate whether the component of the "shared household environment" was of any influence on the composition of the oral microbiome.

Saliva samples were collected and all the variable regions of the 16S rRNA gene were sequenced. A rich dataset was obtained referring not only to bacteria, but to fungi as well.

The results show that a shared environment is leading to significantly higher alpha diversity. The beta diversity of the boarder group revealed no clear trend on the similarity of the oral microbiome, which could indicate that participants on that group could share a more similar oral microbiome composition. However, it was noted that having spent more days at school, the oral microbiome composition of boarder students tends to become more similar.

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List of Abbreviations

AD	Alzheimer's disease
ADT	Aerodigestive tract
AEC	Esophageal Adenocarcinoma
AGEs	Advanced Glycation End Products
AIDS	Acquired immunodeficiency syndrome
AMBN	Ameloblastin gene
AMELX	Amelogenin gene
AMR	Antimicrobial resistance
ARG	Antimicrobial resistant gene
ART	Anti-retroviral treatment
ASV	Amplicon sequence variant
BE	Barrett esophagus
BLAST	Basic local alignment searching tool
BMI	Body mass index
bp	Based pair
BSL	Biosafety level
CDH	Children's Dental Health
CNS	Central nervous system
CRP	C-reactive protein
CVD	Cardiovascular disease
dmft	Decayed missing filled teeth (primary dentition)
DSPP	Dentine sialophosphoprotein
EBV	Epstein-Barr virus
EC	Electronic cigarette
ESV	Exact sequence variant
GI	Gastroesophageal
GOS	Galacto-oligosaccharided
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HGT	Horizontal gene transfer

HIV	Human immunodeficiency virus
HOMD	Human Oral Microbiome Database
HOMIM	Human Oral Microbiome Identification Microarray
HOT	Human Oral Taxon
HPV	Human papilloma virus
ICDAS	International Caries Detection and Assessment System
IFNs	Interferons
KEGG	Kyoto Encyclopedia of Genes and Genomes
LPS	Lipopolysaccharides
MAFFT	Multiple Alignment Fast Fourier Transform
NGS	Next generation sequencing
nMDS	Non-metric Multi-dimensional
OLP	Oral lichen planus
OPG	Osteoprotegerin
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PRIMER	Plymouth Routines In Multivariate Ecological Research
RANKL	Receptor activator of nuclear factor kappa beta
RDP	Ribosomal database project
REC	Research Ethics Committee
SCFA	Short-chain fatty acid
T1DM	Type 1 diabetes mellitus
T2D	Type 2 diabetes
V regions	Hypervariable regions
VLBW	Very low birth weight
WHO	World Health Organisation
ZOTU	Zero-radius operational taxonomic unit

1 Literature Review and Introduction

1.1 Microorganisms

1.1.1 Definition

Microorganisms are very small living structures, having a mass of less than 10^{-5} g and a length of less than 500μm. Microorganisms are very old as they are thought to inhabit the Earth for more than 3.5 million years and are an indispensable component of all ecosystems. There is no environment that has been studied until now, regardless the temperature, the pH and the pressure, that did not host microorganisms. Consequently, they can be found in water, in the air, in the soil and in the human body (Bishop and Bishop, 2014).

1.1.2 Classification

Traditionally, microorganisms were classified into two groups, the Prokarya and the Eukarya, based on their morphology, the environment they were isolated from, the means by which they were generating energy, their nutrient requirements and their mode of replication. After the contribution of molecular biology and the collection of sequence data, this classification has now changed. The current three domains are: the bacteria and the Archaea, which are prokaryotes, and the Eukarya, which are eukaryotes (Baker et al., 2017) (Figure 1-1).

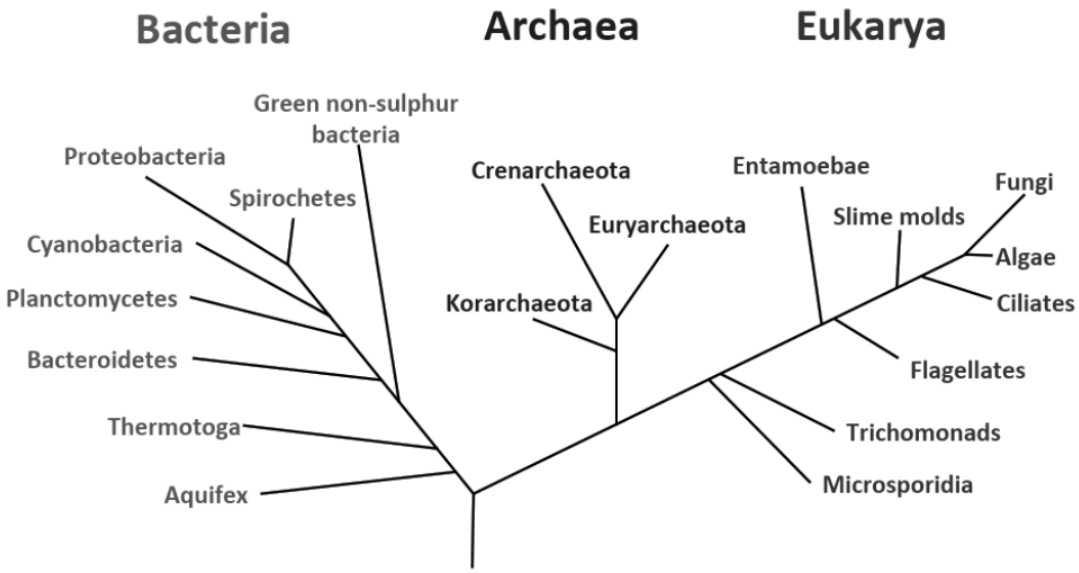


Figure 1-1: Classification of microorganisms.

Prokaryotes, share a number of similarities in their structure, such as the absence of a membrane surrounding the genome, the lack of introns in the encoded genes, the nonappearance of intracellular organelles and the ribosomal subunits of 30S and 50S (Bishop and Bishop, 2014). This review is focusing mainly on bacteria, which is the most common inhabitant of the human body. There are different classification bases for bacteria according to cell structure, cellular metabolism and different cell components. Phenotypic classification is based on the morphological differences of bacteria. Bacteria are classified into five groups according to their basic shapes: spherical (cocci), rod (bacilli), spiral (spirilla), comma (vibrios) and corkscrew (spirochaetes). Phylogenetic classification, on the other hand, is based on the relatedness among various groups of microorganisms. There are four main steps in order to phylogenetically analyse bacteria: to select a suitable phylogenetic marker, to obtain the molecular sequences, to align the multiple sequences and finally to create a phylogenetic tree. The rank-based classification of bacteria, also known as taxonomy, includes in descending order, the phylum, class, order, family, genus and species (Figure 1-2) (Janda, 2019).

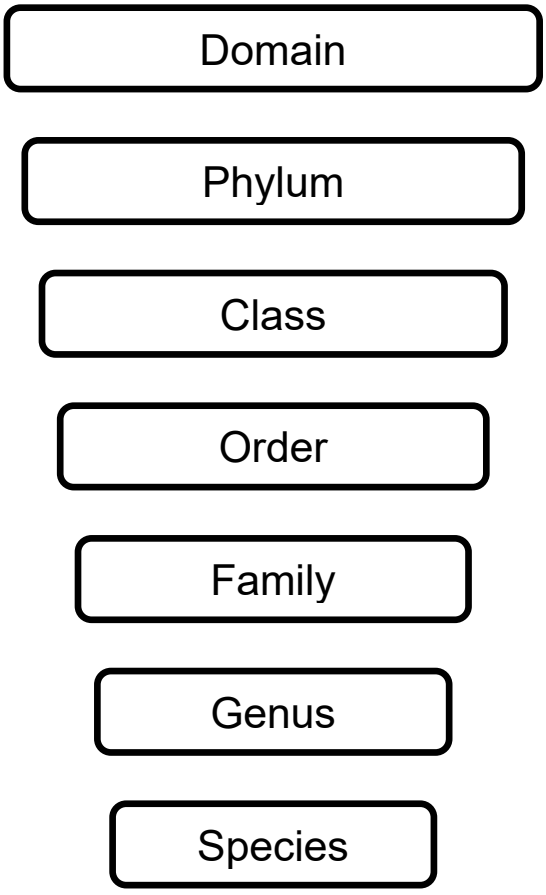


Figure 1-2: Taxonomic ranks or levels of bacteria in descending order.

1.2 Oral Microbiome

1.2.1 Definition

The oral microbiome is defined as the collection of the different microorganisms that are found in the oral cavity (Shaw et al., 2017a). It is one of the most diverse communities found in the human body, with over 600 to 1000 different taxa species at an average density of 1.4×10^8 organisms per milliliter (Dewhirst et al., 2010b) (Gomez and Nelson, 2017). The oral cavity is a unique environment that consists of several different microenvironments, that are hosting highly divert microbial populations. Consequently, distinct microbial composition can be found in the periodontal sulcus, the hard palate, the tongue, the buccal mucosa and the saliva (Krishnan et al., 2017).

1.2.2 Acquisition and Development

The establishment of bacteria in the oral cavity is a dynamic process that starts at birth, when the sterile mouth of the neonate first gets exposed to the environment.

The effect the mode of delivery has on the composition of microbiome in different body sites of the newborn, is broadly being studied. Children born with natural, vaginal birth have a more diverse gut microbiome composition, in comparison with caesarian mode (c-section), that have higher *Clostridium difficile* and delayed acquisition of *Bifidobacterial* and *Escherichia coli* (Lif Holgerson et al., 2011). Evidence has also pointed positive connection between the placenta environment and the establishment of the newborn oral cavity (Han et al., 2009). A study looking at the oral microbiota of three-month-old infants, 38 of which were delivered by c-section and 25 by natural vaginal birth, using the Human Oral Microbiome Identification Microarray (HOMIM), found significant differences in the type and the number of bacteria colonising the mouth (vaginal 79 species, c-section 54) (Lif Holgerson et al., 2011). Another similar, but more recent study, looked at the oral bacteria in two groups of newborns, one group of vaginal birth and the other group of c-section birth. The authors found significant differences in the oral microbial composition of the two groups. The vaginal

group had increased *Lactobacillus*, *Prevotella* and *Gardnerella*, while the c-section group elevated numbers of *Petrimonas*, *Bacteroides, Pseudomonas, Staphylococcus* and *Bifidobacterium*. Furthermore, the c-section group was found more likely to develop asthma, obesity, diabetes, eczema, allergic rhinitis in the future (Li et al., 2018). The same research team, investigated whether very low birth weight (VLBW) infants that were delivered by natural or c-section, had different oral microbiome composition and concluded that the delivery mode was affecting the oral microbiota of VLBW infants (Li et al., 2020).

A different study was made aiming to ascertain the bacterial taxonomic composition and possible metabolic function of the neonatal and early infant microbiota across five different body sites (antecubital fossa, retro- auricular creased, keratinised gingiva, anterior nares and stool) and to evaluate the effect of the mode of delivery. They concluded that the neonatal microbial community structure at the time of delivery did not demonstrate strong body site specificity until the 6th week after birth. The authors also found that the mode of delivery was only affecting the composition of the microbiome in the different sites only short-term after birth (Chu et al., 2017).

Following birth, the initial, transient microbial colonisation occurs via the passive transmission of microorganisms from milk, food and saliva, mainly from the mother (Cephas et al., 2011). It has been suggested that some oral bacteria reach infants mouth through breastfeeding (Ruiz et al., 2019). The establishment of the oral microbiome is rapid within the few first years of life. The diversity of the oral microbiome is increasing from 0-3 years, reaching a peak after the eruption of the primary teeth (Song et al., 2013).

Epithelial mucosal surfaces in oral cavities of dentulous infants are mainly colonised by aerobic and facultatively anaerobic species, such as *Streptococcus mitis, S. salivarius* and *S. oralis*. This first colonisation community is also known as pioneer community. Further maturation of the oral microbiome gradually results in the colonisation of more bacterial species, such as, Gram-negative anaerobes (*Prevotella melaninogenica, Fusobacterium nucleatum, Veillonella*). The eruption of the primary dentition seems to be an important milestone in the development of oral microbiome. Kononen et al. showed in their study that the event of teeth eruption is associated with

significant increase of both the richness and the diversity of the oral microbiome (Könönen et al., 1992). This period coincides with the initial acquisition of *S. mutans,* which is a potential cariogenic bacterium, known as “Window of infectivity” (18 to 26 months of life) (Caufield et al., 1993).

Following, the age from 6 to 12 years is the stage of mixed dentition. Permanent teeth start to erupt, and primary teeth begin to exfoliate. This change is affecting the supragingival, subgingival oral microbiome composition (Mason et al., 2018) and the susceptibility to gingivitis (Matsson, 1993).

Moving from childhood to adolescence, hormonal fluctuations seem to affect the oral microbiome composition. Studies have shown that during puberty changes in the oral microbiota are mainly associated with the increased gingival bleeding, due to increased vascular permeability which results in alteration of the oral plaque architecture (Kaan et al., 2021). Recent evidence is showing that the oral microbiome of adolescents is more diverse compared to the one of adults. A recent study by Burcham et al investigated the differences between the oral microbiome of young people with an average age of 10.12 years and adults with an average age of 34.15. The authors concluded that there was an observed trend of higher median richness and evenness in youth samples (Burcham et al., 2020).

The maturation process of oral microbiome continues into adulthood, and it is subjected to continuous perturbation even after it is established (Sampaio-Maia and Monteiro-Silva, 2014). The oral microbiome has been broadly characterised compared to other microbiomes, but less than the gut microbiome. The Human Oral Microbiome Database (HOMD) is a directory where the full-length 16S rRNA gene sequences of more than 600 prokaryote species that are present in the human oral cavity is recorded, as well as genome sequences where possible (www.homd.org). This “microbiome library” stores all the information on microorganisms identified in the human aerodigestive tract (ADT), organised in genus, species, status (named, unnamed but cultivated, uncultivated phylotype) and body site level. Today, it is believed that only an approximate of 30% of taxa remains uncultivated (Verma et al., 2018). The different oral species and taxa were identified and named based on the 16S rRNA and a unique Human Oral Taxon (HOT) number, a taxon ID, was given to

each of them (Chen et al., 2010). The oral microbiome is dominated by six major phyla, which are mainly forming the core oral microbiome: *Firmicutes* (36.7%), *Bacteroidetes* (17.3%), *Proteobacteria* (17.1%), *Actinobacteria* (11.6%), *Spirochaetes* (7.9%) and *Fusobacteria* (5.2%) containing 96% of the taxa (Dewhirst et al., 2010a). At genus level the most abundant bacteria genera are: *Streptococcus, Veillonella, Selenomonas, Gemella, Fusobacterium, Prevotella, Lactobacillus, Neisseria, Dialister, Actinomyces, Capnocytophaga, Granulicatella* and eleven more bacteria taxa with mean abundance of 1.5% or less as shown in Figure 1-3(Costalonga and Herzberg, 2014).

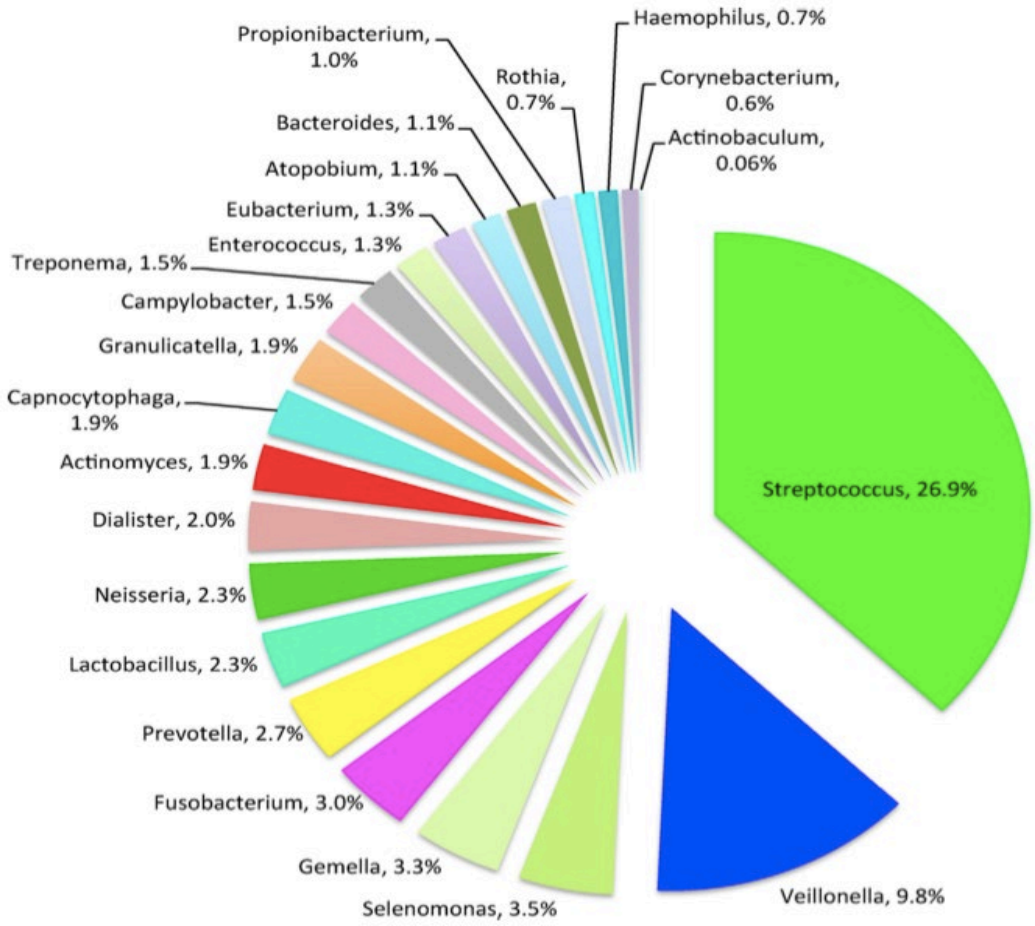


Figure 1-3: The core oral microbiome at genus level. Courtesy of Costalonga and Herzberg, 2014.

The oral microbiome develops and matures into adulthood and is affected by various internal and external conditions, as well as daily physiochemical fluctuations. Internal

factors that influence the composition of oral microbiome are genes and the immune system. External factors are defined as diet, lifestyle, oral hygiene and the environment. It is now believed that people who share the same home environment tend to share more similar species of oral bacteria (Shaw et al., 2017a) (Lax et al., 2014). The changes and interaction between all these different aspects make the composition of oral microbiome a dynamic, constantly developing process. Studies have shown that adult individuals develop a stable oral microbiome “fingerprint” over a time period of a few months up to a year, even after significant changes of events of oral hygiene, like flossing (Utter et al., 2016). However, there is only little knowledge on how stable the oral microbiome of adolescents is over time and how this may be influenced by different factors.

1.2.3 Factors affecting the oral microbiome composition

1.2.3.1 Diet

The primary substrate for oral bacterial growth is not the food that the host ingests directly (Shaw et al., 2017b). The main nutrition source for the oral microbes are endogenous nutrients provided by saliva, tissue excludes, crevicular fluids, degenerating host cells and other bacterial metabolites (Wade, 2013). Kato et al in their recent study tried to associate diet and human oral microbiome by 16s rRNA metagenomic sequencing. It was found that saturated fatty acids were related with high prevalence of *Betaproteobacteria* and *Fusobacteria*. Vitamin C was also found to be related to *Fusobacteria*, but also to *Leptotrichiacea* and *Lachnospiraceae* families. The glycemic load was found to be related with high prevalence of *Lactobacillaceae*. Dietary carbohydrates have long been recognised as having a crucial impact on microbial ecology, as dietary sugars supply the oral microorganisms with readily available structures for, which rely on carbohydrates for energy sources. However, the authors suggest that further investigation is needed to have a more accurate relation between nutrition and the oral microbiome (Kato et al., 2017). Additionally to diet, the composition of tap water was also related to important changes in the abundnace of several bacterial genera. This points to an important role of drinking water in shaping the oral microbiome (Willis et al., 2018).

1.2.3.2 Smoking

The WHO suggests that tobacco usage is one of the biggest public health threats of all time, leading to the death of more than eight million people annually. The lethal consequences of smoking are widely known and therefore smoking has been the subject of many studies relating to human health. Cigarette smoke has been proven to contain more than 5,000 chemicals, 98 of which are listed as hazardous smoke components (Talhout et al., 2011). As smokers are periodically exposed to these toxicants, they are more likely to develop chronic obstructive pulmonary disease, cardiovascular disease and cancer (Stampfli and Anderson, 2009). In mouth related conditions, smokers have been proven to face higher prevalence of not only oral cancer but also periodontitis, as smoking significantly alters the microbial ecology of the smokers' mouth. A big study compared the oral microbiome of 1,204 mericans in three groups current, former and never smokers. In current smokers' group higher levels of *Firmicutes*, *Actinobacteria* and less of *Proteobacteria* were observed. The study concluded that a possible mechanism through which smoking is altering the oral microbial ecology is the influence of oral oxygen availability, while simultaneously having consequences for microbial degradation of xenobiotics. An important finding was also that the overall oral microbiome composition of people who used to smoke did not have a difference from people who never smoked. This may be a suggestion that smoking-related changes in the oral microbiome composition are transient (Wu et al., 2016). In another study microbiota of eight oral sites and the nasal swab of never and current smokers were compared. The authors concluded that smoking significantly affects only the microbiome in the buccal mucosa (Yu et al., 2017). Potentially the alteration of the microbial ecology by the toxic smoke components could also be achieved through antibiotic effects or other mechanisms (Macgregor, 1989).

During the last years electronic cigarettes (ECs) are widely used instead of tobacco cigarettes and are particularly popular amongst adolescents. ECs principally enclose glycol, diacetyl, vegetable glycerin and nicotine (You et al., 2015) and commercial ECs have been reported to contain only very low levels of toxic blends (Kosmider et al., 2016).

There has only been one study done so far to compare the oral and gut bacterial communities of EC vapor and tobacco smokers. The authors extracted DNA out of 30 participants gut and saliva samples. Following 16s rRNA gene sequencing, they came up with the conclusion that EC users do not have significantly different oral or gut microbiota compared to the tobacco smokers (Stewart et al., 2018). A recent systematic review by Yang et al., investigated the oral health impacts of EC usage, in terms of mouth, throat, periodontal, oral microbiome composition effects as well as accidental or traumatic injuries as a result of EC device explosion. The review was not conclusive in regards to EC usage and altered oral microbiome composition compared to non-smokers or cigarette smokers, as some studies suggested higher prevalence of C*andida albicans, Rothia, Haemophilus, Fusobacteria* and *Prevotella* and others found no effect of EC usage on both microbiome diversity and taxonomic abundance (Yang et al., 2020).

1.2.3.3 Stress

Amongst other environmental factors, psychosocial stress can also affect the composition of oral microbiome. A study has tested the hypothesis that stress may affect the microbial colonisation process (adhesion and co-adhesion). The results of this study revealed that a saliva pellicle formed by saliva that is secreted during an acute stress period, promotes the adherence of oral *Streptococci* and *Helicobacter pylori*. Different kinds of stressors and bacteria, had different effects on the adherence (Bosch et al., 2003). Recently, a study was conducted to assess the effect of mental health disorders in the shaping of oral microbiome in adolescents. The study included a cohort of 66 individuals, from 11 to 18 years of age. The authors concluded that the microbial diversity did not differ between participants with low and high self-reported depression and anxiety symptoms. However, the oral microbiome composition was different on the high-symptoms group, comprising of higher levels of *Spirochaetaceae, Actinomyces*, *Treponema, Fusobacterium* and *Leptotrichia spp*. (Simpson et al., 2020).

1.2.3.4 Genetics and Environment

How genetics can affect the oral microbiome can vary from direct alteration on the salivary composition or the immune phenotype to indirectly through gene-diet interactions (Bonder et al., 2016). However, genetics usually interact with the environment. So far, there is not a certain correlation between genetics and oral microbiome, with the role of the environment starting to be more dominant.

There are a number of published studies trying to correlate the genetic information and the environment with the oral microbiome. A study was made to compare the salivary microbiome of twins. The authors concluded that the oral microbiome was not significantly different in monozygotic and dizygotic twins. Also, the similarities started to decrease when the twins stopped sharing the same house, suggesting the importance of shared household rather than genetics (Stahringer et al., 2012).

Shaw et al. investigated the role of the environment and host genetics in shaping the human oral microbiome. All samples were taken from the ultraorthodox Ashkenazi Jewish community, giving the opportunity to compare salivary microbiome within a large number of individuals living in separate locations but sharing a similar diet, lifestyle and genetic background. The most abundant genera were found to be *Streptococcus, Rothia, Neisseira* and *Prevotella*. The results concluded that the host genetic similarity is weakly correlated with salivary microbiome similarity. Furthermore, it was shown that the shared household is the dominant factor affecting salivary microbiome composition and that individuals tend to share the same oral microbiome even if they do not share the same household anymore, indicating a persistent effect of household. Even though the oral microbiome is not completely fixed and can change over time, the establishment of the oral microbiome early in life may lead to the persistence of a similar composition over several years (Shaw et al., 2017a). A similar study was done, aiming to investigate the salivary bacterial DNA profiles of two different families using 16S rRNA gene amplicon sequencing. The study indicated that the most abundant phyla were *Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria* and *Actinobacteria* and that the similarity within the oral microbiome of parent and child was weaken over time, supporting the importance of the shared household rather than the genetic background (Sundström et al., 2019).

1.2.3.5 Immune system

The first line of immunity in the oral cavity, as in all other barrier surfaces such as skin and other mucosa surfaces, is the innate immune system. The main cells participating in this stage are myeloid and lymphoid cells, such as neutrophils, macrophages and dendritic cells. Natural killer (NK) cells are also part of the innate immune system. A rapid inflammatory response is initiated in response to tissue damage, infection or genotoxic stress, via pattern recognition receptors (Gasteiger et al., 2017). Initially, neutrophils and monocytes are recruited. Following, macrophages and dendritic cells are acting as mediators to initiate a delayed activation of the adaptive immune system response. T cells and B cells are part of the adaptive immune system (Figure 1-3). Individuals with compromised immune system response, face a higher risk of bacterial infection and a significant proportion of these infections develop in the oral cavity. Immune deficiency and compromised innate system response can lead to numerous oral manifestations such as ulceration, periodontitis and oral candidiasis (Peacock et al., 2017).

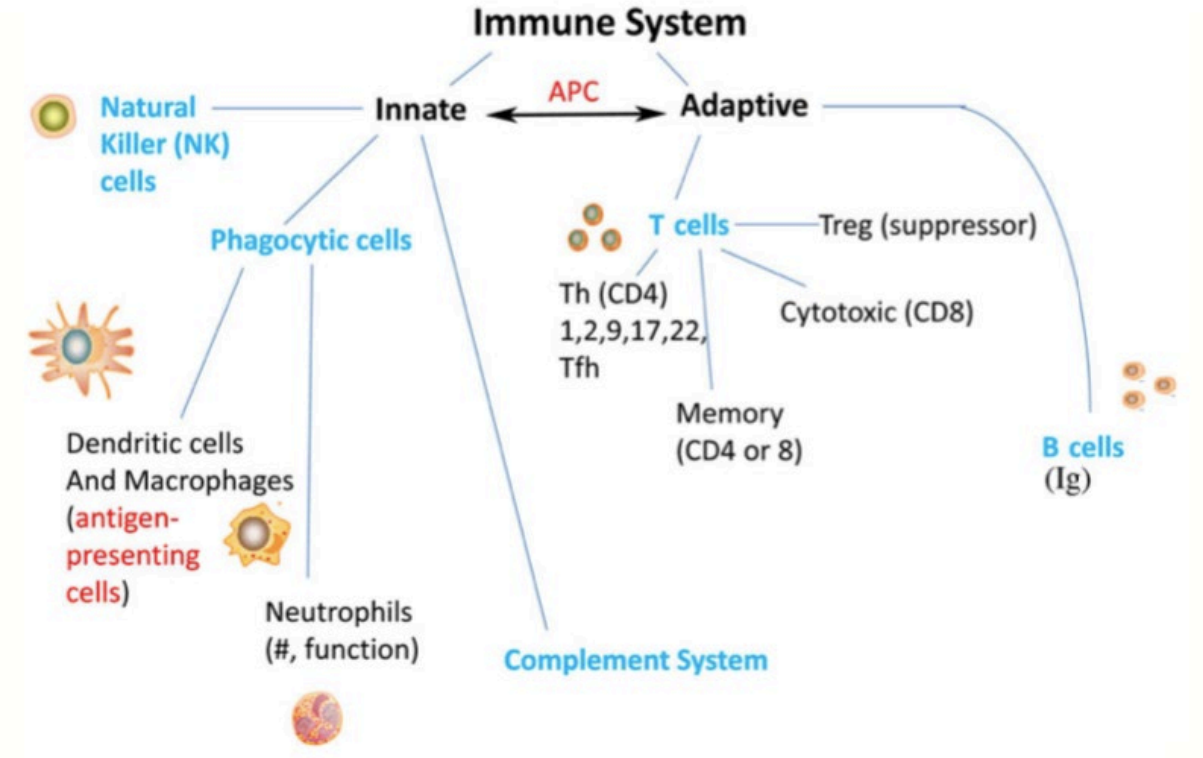


Figure 1-4: Overview of the innate and adaptive immune systems. Courtesy of Peacock et al., 2017.

1.2.3.6 Saliva

Primary saliva is secreted by the major (parotid, submandibular, sublingual) and minor salivary glands. After its secretion, primary saliva is modified and ends up as whole saliva, which is a complex mixture of many different molecules. The role of saliva in the defense system of the tooth surfaces and the oral cavity is generally crucial to oral health. Saliva is forming an acquired pellicle, which is a thin layer of different salivary proteins which covers the tooth surfaces. This pellicle has an important role in the homeostasis of the crystal growth in the tooth surface, a physio-chemical defense, bacterial adhesion and can also act as an elimination surface for transient pathogenic microbes. Saliva supports the immune defense of the oral mucosal surfaces, by its antimicrobial properties and elimination of microbes. Furthermore, saliva is essential for the healing of the mucosa (lesions, wounds, ulcers). There are numerous peptides, proteins and enzymes with defensive properties in the saliva, which are multifunctional and provide an efficient oral defensive network. Some examples of these molecules are the following: defensins, histatins, lysozyme, immunoglobins, amylase, cystatins, peroxidases, statherin, lactoperoxidase. The regional concentration of these molecules is regulated and is controlled by immune or inflammatory reactions of the oral mucosa (Fabian et al., 2012).

Saliva microbiota is a very good site to study human oral microbiome since the sampling is non-invasive, fast, distinguished from other oral microbiomes and easy to build up an individual bacterial profile (Segata et al., 2012).

1.2.3.7 Antimicrobials

The general term antimicrobials is referring to all the chemical substances and drugs that can eliminate or reduce the growth of microbes. Depending on the type of microorganisms there are different antimicrobials to act against them, such as antibacterials/ antibiotics, antiviral, antifungal and antiparasitic (Holmes et al., 2016). There are natural antimicrobials deriving from the diet, such as plant-based essential oils (Prakash et al., 2018, Donsi and Ferrari, 2016) and flavonoids (e.g., quercetin and apigenin) (Wu et al., 2008). Additionally, anthropogenically added preservatives act as antimicrobials (e.g., sodium benzoate). Last but not least, products used for

personal hygiene like chlorhexidine and triclosan have antimicrobial properties (Brading and Marsh, 2003).

1.2.3.7.1 Antibiotics

The discovery of antibiotics is one the most important milestones in medicine. Antibiotics have given the chance of not only treating but also preventing a series of bacterial infections, which were incurable and even fatal before.

Alexander Fleming was the one who accidentally discovered the first antibiotic back in 1928 at St Mary's Hospital Medical School in London. One of his plates with growing culture of Staphylococcus was left open and was contaminated by the fungus Penicillin notatum. Fleming noticed that within the Staphylococcus culture where the fungus grew bacteria-free zones were developed. Penicillin is the antibiotic chemical produced by Penicillium notatum.

Apart from Penicillins there are nine more antibiotic classes. Each class consists of different drugs with similar chemical and pharmacological properties. The remaing classes are: Tetracyclines, Cephalosporins, Quinolones, Lincomycins, Macrolides, Glycopeptides, Aminoglycosides and Carbapenems.

Antibiotics are extensively prescribed in modern medicine. Sepsis or bacteremia, skin and soft tissue infections, central nervous system infections like meningitis, respiratory infections like pneumonia and urinary tract infections are only some of the indications of antibiotics prescription (Timmons et al., 2018).

Antibiotics are also widely used in dentistry, either prophylactically or therapeutically. A dentist can prescribe antibiotics as a part of prophylactic regime in a primary (prevention of initial infection) or secondary level (prevention of infection in a distant site). Additionally, antibiotics can be prescribed when treating odontogenic or

nonodontogenic infections, primary (as first- line treatment for infection) or adjunctive (in conjunction with a surgical intervention) (Stein et al., 2018).

1.2.3.7.1.1 Antimicrobial Resistance

Unfortunately, the misuse and overuse of antimicrobials have led to one of the most worrying public health threats globally, the antimicrobial resistance (AMR). As AMR it is as “the capability of bacteria to multiply in the presence of drug concentrations that are mostly inhibitors of the same species or equal to the maximum achievable concentration during the therapeutic use” (Patini et al., 2020). The WHO is setting off the alarm bells as AMR is threatening the effective prevention and treatment of infections, making them hard to be treated, increasing the risk of their spread, causing severe illness and death, by making the antimicrobial medications ineffective. AMR can occur due to chromosomal mutation in specific genes, like in Mycobacterium tuberculosis, where rifampicin resistance repeatedly arises through predictable mutations in the rpoB gene (Ford et al., 2013). Another way through which AMR can develop, is over the evolution of novel genes that arise resistance to specific antibiotics, such as the mobilised colistin resistance to mcr-1 gene. These genes, can be transferred, in bacteria, on mobile genetic elements (Liu et al., 2016). The more complex a microbial community is, the higher the antimicrobial tolerance. In multi-species biofilms a number of resistant mechanisms can develop, such as physical barriers, mutual cross- species protection and the development of tolerance phenotypes (Hathroubi et al., 2017).

1.2.3.7.1.2 Antimicrobial Resistance and Oral Microbiome

The horizontal gene transfer (HGT) seems to have an active contribution to the promotion of AMR as antimicrobial resistant genes (ARG) are transferred between different unrelated pathogens (Lerminiaux and Cameron, 2019). The fact that the oral microbiome is structured in biofilms, which consist of different species, favors HGT events. Since the oral microbiome is constantly exposed to different antimicrobial agents, genes with increased tolerance to these compounds are developed. Conjugation with mobile genetic elements, such as plasmids and transposons, transduction by bacteriophages and natural transformation by extracellular DNA,

allows the ARGs to be transmitted. Hence, oral microbiome is a rich source for ARGs (Shaw et al., 2017b).

1.2.3.7.2 Probiotics

The World Health Organisation defines probiotics as “living organisms which when are administered in adequate amounts confer a health benefit for the host”. The main bacteria with probiotic characteristics are a series of lactobacilli and bifidobacterial species.

It is now believed that the beneficial role of probiotics is versatile. First of all, probiotics can actively help in the maintenance of a healthy gut flora. As dietary fibers are fermenting, bacteria with probiotic properties have the ability to adhere to the epithelium of colon and to create colonies, surviving in the presence of bile acids and without harming the host. The presence of probiotics is enhancing the conversion of dietary fibers to short-chain fatty acids (SCFAs), such as butyrate, propionate and acetate, which are crucial for the normal function of the GI tract. Further favorable acts of probiotics are: the management of lactose intolerance, the prevention of colon cancer, the decrease of cholesterol, the decrease of blood pressure, the increase of immune function, the prevention of infection, the decline of antibiotic associated diarrhea and the decrease of inflammation (Smith and Jones, 2012).

Probiotics have also been suggested to have a potential positive role in the oral microbiome. Presumable ways of how probiotics can act in oral cavity are: shift in the bacterial biofilm composition, modulation of the immune response and metabolic effects (Rastogi et al., 2011). A systematic review by Gruner et al. was made to explore whether probiotics are beneficial, compared to placebo controls, for prevention and treatment of caries and periodontal disease. Overall, 50 studies were included, and the authors examined different aspects of these two oral diseases. Summarising their findings, probiotics were giving significantly higher chance of reduced *Streptococcus Mutans* and *Lactobacilli*, reducing the bleeding on probing, reducing the gingival index and the depth of pockets in probing. On the contrary, no significant difference was identified in the numbers of periodontal pathogens, in the plaque index and the incidence and experience of caries was also not significantly decreased. The authors

concluded that there is not sufficient evidence to support the use of probiotics for the management of caries, in contrast with managing gingivitis or periodontitis where the indications were supportive (Gruner et al., 2016).

1.2.3.7.3 Prebiotics

A dietary prebiotic is defined as “a selectively fermented ingredient that results in specific changed in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit upon host health”. Prebiotics do not have just protective outcomes on the GI system but also in the CNS, immune system and cardiovascular system. They are naturally found in low levels in the foods and their main source is in prebiotic supplements. Prebiotics also have a positive symbiotic activity with the probiotics. Main prebiotics are: the fructans, like inulin and fructo-oligosaccharides/ oligofructose, galacto-oligosaccharides (GOS) and starch and glycose-derived oligosaccharides (Davani-Davari et al., 2019).

1.3 Oral microbiome and disease

The daily disturbance and removal of the oral biofilms, that gradually builds up in the mouth, is crucial for the prevention of the establishment and progression of oral and also systemic diseases (Marsh, 2018).

1.3.1 Oral diseases and the oral microbiome

1.3.1.1 Caries

The term caries or tooth decay is describing the process and the clinical manifestation of the disturbance of the tooth structure coherence (Kidd and Fejerskov, 2004).

According to WHO caries is a major public health problem, where it appears in the list of top widespread noncommunicable disease worldwide. The Global Burden of Disease Study in 2017 estimated that 2.3 billion people suffer from caries of permanent teeth and more than 530 million children suffer from caries of primary teeth (Gbd, 2018).

The Children's Dental Health (CDH) Survey is a study that was conducted in 2013, that measured the dental caries incidence within children of different age groups (5, 8, 12 and 15 years of age) in England, Wales and Northern Ireland (PHE, 2013). Findings demonstrated that 31% of the 5-year-olds, 46% of the eight-year-olds, 34% of the 12-year-olds and 46% of 15-year-olds were affected by dental caries. The mean number of teeth affected at each age group was 0.9, 1.4, 0.8 and 1.4, respectively. Each of the affected children had a mean of 3 teeth (5 and 8 years old), 2.5 teeth (12 years old) and 3.1 teeth (15 years old) affected.

Dental caries is a multifactorial disease with three main components: diet, oral microbiome and susceptibility.

Studies have shown that if of the overall sugar intake is less than 10% of the total daily energy, there is a possibility of the reduction of caries. It is also important to consider not only the amount of the sugar intake, but also the frequency and the timing of the

consumption. The less harmful consumption pattern suggested against the development of caries if the sugar intake is restricted to mealtimes, the decreased consumption time and the preference for low viscosity sugars. The longer bacteria are in contact with the sugars increases the risk of caries development (Moynihan and Kelly, 2014).

The oral microbiome has a major role in the development of caries. Certain bacterial species, like *Streptococcus mutans*, *Lactobacilli* and *Actinomyces* have a central role in the development of dental caries as these bacteria have the ability to adhere to the enamel salivary pellicle and to other plaque bacteria. Additionally, they are both strong acid producers by metabolising fermentable carbohydrates, increasing the risk for caries incidence (Forssten et al., 2010). Other examples of potentially cariogenic bacteria are; *Bifidobacterium dentium, Rothia dentocariosa, Streptococcus cristatus, Streptococcus salivarious, Streptococcus sobrinus and Streptococcus wiggsiae (*Struzycka*,* 2014*)*.

A number of different suggestions have been made regarding how the oral microbiome promotes the development of dental caries. The “Specific plaque hypothesis” proposed that a certain type of bacteria, the *Streptococcus mutans* along with *Lactobacilli*, are responsible for the development of caries. The initial acquisition of *Streptococcus mutans* occurs during a clearly marked period of the child’s biological development known as “Window of infectivity”, with an average age of MS acquisition occurring approximately during the first 26 months of life (Dasanayake et al., 1993).

However, recent studies have suggested that in the process of caries development more bacteria, rather than *Streptococcus mutans* and *Lactobacilli*, of the oral microbiome are actually involved. This theory is supported by the “Ecological plaque hypothesis” (Takahashi and Nyvad, 2011). The ecological hypothesis, as shown in Figure 1-5, is described below. Lactic acid is produced after the metabolism of sugar (Takahashi, 2005). The presence of the lactic acid in the oral environment causes a drop of the pH. A pH lower than the critical 5.5 is leading to the release of ions, such as: calcium, phosphate and hydroxide. However, if the pH increases again or the oral microbiome is disturbed, remineralisation of enamel can be noted (González-Cabezas, 2010). There is a dynamic process of mineral gain and loss, which defines

the re- and de- mineralisation, equally. If the demineralisation process dominates, the tooth structure is derogated, and a cavity is created (Peters, 2010) (Pitts et al., 2017).

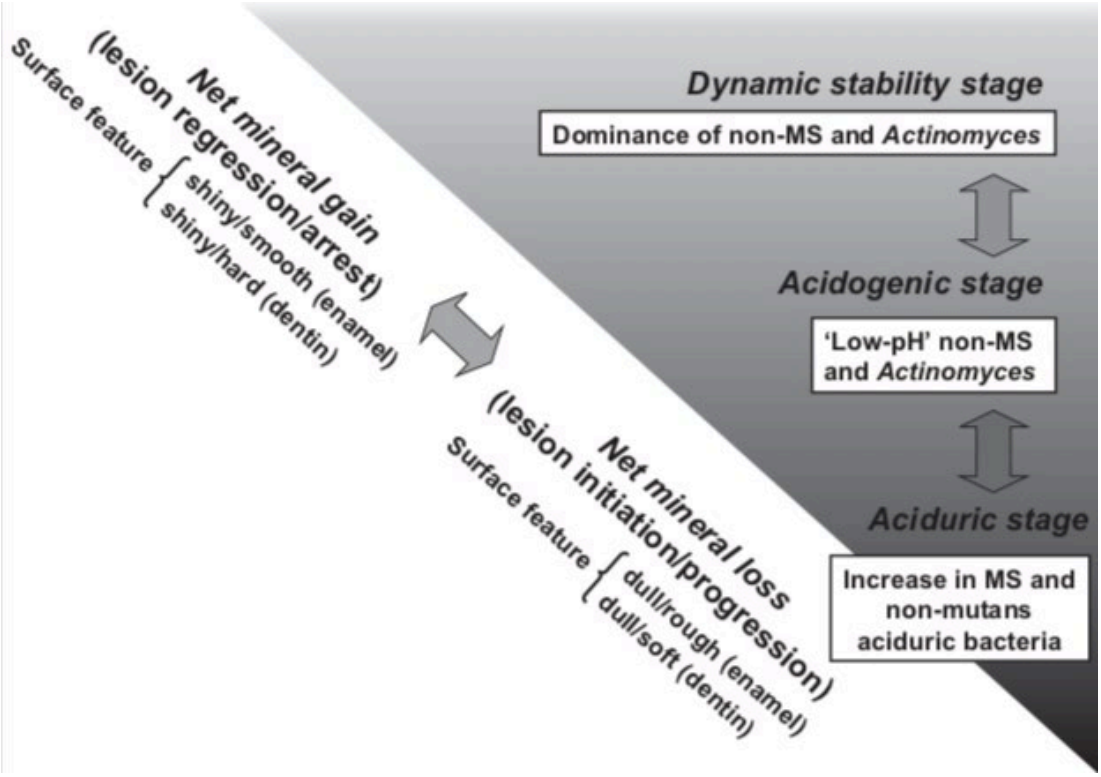


Figure 1-5: Ecological Plaque Hypothesis. Courtesy of Takahashi and Nyvad,2011.

As a multifactorial disease, caries, emerge from a mosaic of environmental and genetic factors. A series of twin studies have been conducted aiming to link heredity and dental caries. Caries experience showed greater similarity within monozygotic twins, less in dizygotic twins and even less in no related pairs, with environmental factors having a greater influence then genetic contribution (Mansbridge, 1959). Goodman et al reported significant heritability for oral microorganisms, including *Streptococci*, salivary flow rate, salivary pH and salivary amylase activity (Goodman et al., 1959). Research performed in mice had shown that genetic factors can affect the salivary flow rate and the hardness of enamel and subsequently make the teeth more or less vulnerable to caries (Endo et al., 2014).

A few different organic matrix molecules are interacting to form the bulk of enamel and dentine. Amelogenin, enamelin, ameloblastin, tuftelin and dentine sialophosphoprotein are some examples. Their production is controlled by different genes. The amelogenin gene (AMELX) resides on the p arm of the X chromosome and its locus is Xp22.31-p22.1. This gene is responsible for the formation of scaffold for enamel crystallites and the control of their growth. The ameloblastin gene (AMBN) is located in chromosome 4 and is a key adhesion molecule for enamel formation that binds and maintains the different phenotypes of ameloblasts. The dentine sialophosphoprotein gene (DSPP) encodes two proteins: the dentine phosphoprotein and the dentine sialoprotein which are involved in the mineralisation process of dentine. Tuftelin has an active part in the first stages of mineralisation. Kalikrein 4 and metalloproteinase 20 are responsible for the order placement of the organic matrix of enamel making it harder, less porous and unstained. Mutation defects in these genes are resulting in the abnormal production of proteins, both qualitive and quantitative, and in the defective mineralisation of the developing teeth. Thus, they are responsible for different diseases, like dentinogenesis imperfecta and amelogenesis imperfecta. Furthermore, they influence the bacterial adherence and the durability of enamel, making these teeth more susceptible to dental caries (Opal et al., 2015).

1.3.1.2 Periodontitis

Periodontal diseases involve the clinical manifestation of pathologic inflammatory conditions occurring in the gingiva, alveolar bone and periodontal ligament around teeth. These inflammatory processes are the response to bacterial accumulation on the surfaces of the teeth, dental prosthesis or oral mucosa (dental plaque) and the formation of biofilm (Loesche and Grossman, 2001). The most common periodontal diseases are gingivitis and periodontitis. Gingivitis is defined as the reversible inflammation of the gingiva in the absence of periodontal breakdown. Gingivitis can eventually progress to periodontitis. Periodontitis is characterised by the irreversible breakdown of the connective tissue attachment and alveolar bone resorption (Armitage, 1999). Untreated gingivitis may eventually progress to periodontitis, while effective long-term, control of gingivitis can prevent progressive attachment loss (Trombelli et al., 2018). Untreated periodontitis will gradually result in the apical

migration of the junctional epithelium, pocket formation and eventually lead to tooth mobility, limited mastication ability and finally to tooth loss (Oliver et al., 1998). Furthermore, the aetiopathogenesis and control of several systemic diseases and conditions such as cardiovascular disease, diabetes, pulmonary diseases and obesity have been associated with untreated periodontitis (Liu et al., 2012).

Dental caries and the wide range of periodontal diseases are the two dominant oral torments worldwide (Petersen, 2003). A plethora of epidemiological studies has been conducted in several different countries in order to record the prevalence and the severity of periodontal disease (Petersen and Ogawa, 2005). A recent study evaluating the population in the United States of America concluded that nearly two-thirds of the dentate adults over 65 years are affected by periodontitis (Eke et al., 2016).

Bacterial-derived factors stimulate the inflammatory response in the gingivae. For many years the research was focused on specific pathogens that initiate the inflammatory process, such as *Porphyromonas gingivalis*, *Porhyromonas intermedia and Aggregatibacter actinomycetemcomitans (Socransky et al.,* 1977). Newer studies have proposed the ecologic plaque hypothesis suggesting that non-specific plaque accumulation may lead to inflammation (Marsh, 1994). When homeostasis between microbial communities and their environment is perturbed, dysbiosis dominates and disease is introduced.

P. gingivalis is a gram-negative anaerobic bacterium and is a member of the red complex bacteria (Socransky et al., 1998). It has been associated with deep pocket depths and bleeding on probing and its virulence stems primarily from the secretion of proteases that are involved in tissue destruction and are called gingipains. These enzymes, such as Kgp and RgpA, have the ability to evade the host immune system by inactivating the complement system, cytokines and antimicrobial peptides and degrading immunoglobulins. Additionally, gingipains restrict the antibacterial activities of neutrophils and increase the vascular permeability.

In periodontal disease, the cellular inflammatory infiltrate of T cells, B cells, macrophages, and neutrophils within gingival connective tissue is increased, with a

simultaneous increase in the secretion of inflammatory mediators. These inflammatory cells also interact with osteoblasts, periodontal ligament, and gingival fibroblasts. Receptor activator of nuclear factor kappa beta (RANKL)-mediated osteoclastogenesis plays a major role in inflammatory bone resorption, and its expression is increased in periodontitis. In inflammatory pathological states, activated T cells may mediate bone resorption through excessive production of RANKL and activated T and B cells are major RANKL sources in diseased periodontal tissue. Gingival fibroblasts are heterogenic in that they produce osteoprotegerin (OPG) in response to lipopolysaccharides (LPS) and Interleukin-1 family (IL-1), suggesting a protective role to suppress osteoclast formation. However, they may also produce Interleukin-6 family (IL-6) and interferons (IFNs) and thus enhance the inflammatory process. Some periodontopathogenic bacteria such as *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* have mechanisms to induce RANKL in osteoblasts and gingival fibroblasts. When stimulated, osteoblasts and periodontal ligament fibroblasts express RANKL. RANKL is upregulated whereas OPG is downregulated in periodontitis compared to periodontal health, resulting in an increased RANKL/ OPG ratio and subsequent bone loss (Cochran, 2008, McCauley and Nohutcu, 2002).

1.3.1.3 Oral lichen planus

Lichen planus (LP) is a common, chronic and inflammatory disease which affects skin, oral and genital mucosa, scalp and nails (Lodi et al., 2005).

Oral lichen planus (OLP) affects 0.1-4% of the population, with middle aged females between 30- 60 years being more likely to have the disease (Lodi et al., 2005). The clinical manifestations can vary and be persistent for long time periods or years, with switching calm and exacerbation cycles (Ismail et al., 2007). Some common clinical manifestations of OLP are white striations, papules, plaques, mucosal atrophy, erosion and blisters. The affected oral sites can also be various, with buccal mucosa, tongue, gingival and lips being the dominant (Eisen, 1999). According to the clinical expression of the disease, there are two phenotypes of OLP: the reticular and the erosive type. The reticular type is asymptomatic with the main feature of Wickham

stripe, whereas, the erosive is symptomatic with high malignant potential (Zakrzewska, 2001).

The exact aetiology of OLP is not yet fully clear as there are some practical difficulties in the studies which make its understanding quiet complex. Not only does the OLP appearance usually overlap with other mucosal conditions but also there is potential coexistence of non- OLP inflammatory conditions in the same individual. A few potential mechanisms have been suggested to play an important role in OLP pathogenesis. Local and systemic inducers of cell- mediated hypersensitivity, stress, autoimmune response to epithelial antigens versus dysregulated response to external antigens and viral infections, are some of the proposed triggers (Kurago, 2016).

Several viruses have been linked with OLP, such as human immunodeficiency virus (HIV) (Ficarra et al., 1993), cytomegalovirus (HCMV) (Sun et al., 1996), papilloma virus (HPV) (Sand et al., 2000), Epstein- Barr (EBV) (Sand et al., 2002) and Hepatitis C virus (HVC) (Konidena and Pavani, 2011). Furthermore, it is suggested that Candida infection is present in 37-50% of patients with OLP (Lundstrom et al., 1984). All these can indirectly affect the synthesis of the oral microbiome and the immune system and the evolution of the disease. The role of the bacteria in the advancement of OLP was studied by comparing the identity of oral microbiome between OLP patients and healthy controls. The authors suggested that OLP patients had significantly higher levels of *Porphyromonas* and *Solobacterioum* and lower *Haemophilus*, *Corynebacterium,* Cellulosimcrobium and *Campylobacter* in their saliva (Wang et al., 2016).

1.3.2 Systemic disease and the oral microbiome

1.3.2.1 Cardiovascular disease

Cardiovascular disease (CVD) continues to be one of the main global causes of death. CVD is associated with many risk factors, many of which indicate a non- healthy lifestyle. Main factors increasing the possibility of CVD are: high blood pressure, high cholesterol levels, overweight and obesity, physical inactivity, poor diet, smoking and type 2 diabetes (Cotti et al., 2011).

There is enough information that oral infection is directly associated with the development of CVD. The accused dental infections are mainly the periodontal disease and the periapical periodontitis. Although these oral conditions have important differences in their pathogenesis and etiology, they share the same microbiota (Gram-negative anaerobic) (Noiri et al., 2001), (Barkhordar et al., 1999) and they are both able to increase the systemic levels of cytokine. Three separate theories have been proposed to explain how an oral infection could potentially lead to CVD. First of all, transient bacteremia can cause metastatic spread of the infection. Secondly, metastatic injury by circulating oral microbial toxins. And lastly, metastatic inflammation arising from an immune response to oral microorganisms (Thoden van Velzen et al., 1984). However, oral infection is not a causality of CVD, as one can exist without the other (Hofmann, 2011).

Dentists as health professionals are obliged to have an active role in promoting an overall healthy lifestyle aiming to eliminate CVD. This can be achieved through different health promoting schemes (Touger-Decker, 2010).

1.3.2.2 HIV

The Human Immunodeficiency Virus (HIV) continues to be a major global public health threat. HIV primarily affects the immune system and seriously impairs the patients' defensive system, making them more vulnerable to infection and a few types of cancer. HIV destroys and weakens the normal function of immune cells and affected individuals become immunosuppressant. The most advanced stage of HIV infection is Acquired Immunodeficiency Syndrome (AIDS), which can take from 2 to 15 years to clinically manifest. AIDS, according to WHO, is defined by the development of certain types of cancer, infections or other severe clinical manifestations.

HIV infection has been associated with raised predominance of oral mucosal infections and dysregulation of oral microbiome, including the overgrowth of *Candida albicans* and the development of candidiasis. Candidiasis results from the loss in neutrophil recruitment to the oral tissue through a depletion in the number of mucosal-associated Th17 lympocytes. Oral manifestations have been reported in up to 50% of

HIV- infected individuals and up to 80% of those who have progressed to AIDS. The commonest clinical appearance of oral candidiasis is of easily removable, curdlike white plaques, underneath which lies raw or bleeding mucosa (pseudomembranous candidiasis). Alternative oral manifestations include: an erythematous form, characterised by patchy reddening of the mucosa and depapillation of the dorsal surface of the tongue (hyperplastic candidiasis), with white plaques that cannot be rubbed away, and angular cheilitis with hurtful fissuring of the commissures. Compromised oral immunity in HIV infection may predispose patients to periodontal disease, potentially increasing the risk of cardiovascular disease. The precise effects HIV infection has on oral microbiome are complicated by potential effects of the anti-retroviral treatment (ART). A study comparing HIV-positive individuals to healthy controls found only small differences in the composition of the salivary microbiome, although certain taxa including *Haemophilus parainfluenzae* were significantly correlated with HIV-positive individuals (Schiodt et al., 1990)

1.3.2.3 Obesity

The WHO defines obesity as the abnormal or excessive fat accumulation that is of public health concern. The most common way to measure obesity is the body mass index (BMI), which is calculated by dividing an individual's weight (in kilograms) by the square of his/ her height (in meters). A BMI of equal or more than 30 is considered obesity, whereas BMI equal and more than 25 overweight.

Obesity can cause systemic inflammation and therefore is considered to be the risk factor for many life- threatening chronic diseases, such as metabolic disease (type 2 diabetes) (Kahn et al., 2006), cancer and CVD (Singer et al., 2014). The adipocytes (lipocytes and fat cells) have the ability to secrete proinflammatory cytokines, such as IL-6, TNF-a and c-reactive protein (CRP), in the systemic circulation (Galic et al., 2010).

Research has been done trying to find a possible association between dental caries and obesity, as the two conditions share quite a similar aetiology (Spiegel and Palmer, 2012). As described in the relevant chapter of this review in "Caries Aetiology" diet, and more specifically the frequent consumption of extrinsic sugars, is included as a

key factor for the development of the disease. Similarly, a caloric intake consisting mainly of sugar intake is likely to lead to obesity (Gibson, 1997). However, research to date cannot identify a clear relationship between the above conditions, with some reporting a positive and some others a negative relation (Kantovitz et al., 2006). Peng et al. suggested that this controversary results could be an effect of poor designing and the lack of using more variable indices than BMI which only measures general adiposity and cannot distinguish fat mass, muscle mass, bone mass or location of the fat. Therefore, they designed a study using different indices to describe the obesity status (general, central, peripheral) of 300 5-year-old in Hong Kong, China. Their results showed positive association between obesity and caries in general weight to height proportion (W/H) and weight to waist circumference (W/C). When W/H increases one unit the caries incident, measured in dmft (decayed, missing, filled primary teeth) increases by 41% and equally when W/C is one unit higher, caries incident is 72% higher (Peng et al., 2014).

Given that obesity has serious health implications, dentists are obliged to participate to the battle against obesity and even more specifically children obesity, by taking advantage of the frequent contact they have with parents and young patients (Vann et al., 2005).

1.3.2.4 Diabetes

Type 1 diabetes mellitus (T1DM), also known as insulin- dependent diabetes mellitus, is an autoimmune chronic disease, which is commonly diagnosed within children, teenagers and young adults (Atkinson et al., 2014). The condition is characterised by insufficient production of insulin by Beta cells of pancreas, which is leading to abnormal blood glucose levels. 5% of all diabetic patients suffer from T1DM. T1DM is not preventable nor treatable, with the existing treatments, however different types of exogenous insulin (rapid-, short-, intermediate-, and long- acting insulin) can be administrated to patients in order to achieve blood glucose control (Li et al., 2017). Available treatment has improved significantly the level of patient's life.

On the other hand, Type 2 diabetes (T2D), also known as insulin-resistant diabetes, is the most common type and is characterised by normal insulin secretion which is

however not recognised by the target cells in the different organs of the body. In order balance to be achieved in the blood glycose levels, more insulin is secreted in pancreas (DeFronzo, 2004). T2D is strongly associated with lifestyle and unhealthy way of living, more specifically lack of body exercise and unhealthy diet.

Oral health and oral diseases have also been linked with diabetes. Periodontal disease was identified to have the strongest association with this systemic condition. On one hand, periodontal disease is a risk factor for the development of diabetes, on the other hand it may affect the metabolic control of a diabetic patient (Liccardo et al., 2019). It is broadly acknowledged that diabetes results from persistent elevated stress and chronic inflammation. The excessive formation and accumulation of Advanced Glycation End Products (AGEs) is an important cause of this inflammation and the most common cause of diabetic complications (Vlassara and Uribarri, 2014). AGEs have the ability to bind with neutrophils and trigger inflammatory response reinforced by cytokines. Neutrophils are also stimulated by LPS of Gram-negative bacteria, which are present in the biofilm in periodontal patients. Consequently, the whole inflammatory process is reinforced resulting in increased destruction of periodontal tissues and harshness of diabetes (Rønningen and Enersen, 2012).

1.3.2.5 Barrett's esophagus and Esophageal adenocarcinoma

Barrett's esophagus (BE) is an endoscopically visible metaplasia of the columnar epithelium in the esophagus with histological detection of a specialised intestinal metaplasia (Labenz, 2016). BE is recognised to be the precursor lesion for the development of Esophageal Adenocarcinoma (AEC) (Rustgi and El-Serag, 2014). Patients with BE face 10- 40 fold higher risk to develop AEC, however the progression rate of BE to neoplasm is particularly low (0.1- 0.3% annually). Furthermore, there are identified risk factors for BE to evolve to AEC, such as gastroesophageal reflux disease, obesity, high dietary fat and smoking. The five-year survival rate for patients with AEC is generally poor (almost 17%) (Snider et al., 2016).

A case-control study was conducted in order to identify any potential oral microbiome differences in patients with BE and control patients. It was concluded that EB patients had significantly higher levels of *Firmicutes* and lower levels of *Proteobacteria*

compared to healthy controls. The authors suggested that these findings could potentially be used in favor of diagnosis of EB patients using oral microbiome analyses (Snider et al., 2018).

1.3.2.6 Alzheimer's disease

Alzheimer's disease (AD) is a condition named after Dr. Alois Alzheimer who first described it in 1907. AD is a pervasive neurodegenerative disorder and Alzheimer's disease patients could face a wide range of clinical symptoms from mild loss of memory to serious cognitive impairment, with decreased global function and subsequent behavioral disturbance (Richards and Hendrie, 1999). The pathophysiology of AD is mainly associated with the extracellular deposition of β-amyloid (Aβ) plaques (Imbimbo et al., 2005).

Recently *Porhyromonas gingivalis*, which is known to be the cornerstone in the development of chronic periodontitis (Darveau et al., 2012), was identified in the brain of Alzheimer's disease patients. Furthermore, toxic proteases produced by these bacteria called gingipains were found. Dominy et al in their study found that gingipain immunoreactivity in AD brain was significantly bigger than in brains of non- AD patients and that DNA of *P. gingivalis* was identified in the brains and cerebrospinal fluid of AD individuals (Dominy et al., 2019). These findings acknowledge oral bacteria as a potential risk factor for the development of brain disease.

1.3.2.7 Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease of unknown aetiology. It is characterised by symmetric, erosive synovitis that mainly affects the joints. RA affects 1% of the adult population worldwide (Hochberg, 1981). Most patients with RA experience a chronic fluctuating course of disease that, despite therapy, may result in progressive joint destruction, deformity, disability, and in some cased premature death (Newsome, 2002).

Human studies have demonstrated that RA is associated with specific alterations in oral and gut microbiome composition when comparing healthy individuals and patients

with RA. The oral microbiome composition of patients with RA was found to be significantly enriched with *Lactobacillus salivarius* and *Haemophilus spp* was depleted. These findings suggest that there might be potential ways of using the oral microbiome composition for prognosis and diagnosis of RA (Zhang et al., 2015). Furthermore, *P. gingivalis* was recently associated with the aetiopathogenesis of RA as demonstrated by a mice study. Researchers showed that the presence of *P. gingivalis* in mice models either triggered or worsened the symptoms of arthritis (Perricone et al., 2019), (Frid et al., 2020).

1.4 Studying the oral microbiome

1.4.1 Methods to analyse the composition of oral microbiome

1.4.1.1 The culture-based method

Traditionally the bacterial species within a sample could be identified by in vitro culturing. Culturing is based on the growth of specific species using suitable selective or non-selective media. This method allowed the isolation of a diverse range of bacteria through the years; however, it has some important limitations in the analysis of the oral microbiome. It is an expensive, sensitive and of high skill- requirements technique. But most importantly it is a method of narrow spectrum in the isolation of bacterial species that are inhibited in the oral cavity, with an estimate of 50- 60% oral phyla being uncultivable (Vartoukian et al., 2010, Kolenbrander, 2000).

1.4.1.2 Molecular techniques

In order to bridge the above- mentioned limitations, a number of different molecular techniques have been advanced and are used in the research of the oral microbiome. These approaches are now based on the DNA sequencing. The molecular techniques could be divided in two types, the first involves the entire metagenomics approach, also known as shotgun metagenomics or simply metagenomics and the second the polymerase chain reaction (PCR) amplicon-based targeting.

1.4.1.2.1 Metagenomics

The shotgun metagenomics describe the untargeted sequencing of all the microbial genomes that are present within a sample. This method reveals the full profile of taxonomic composition and is advantageous in providing rich data sets on the functional potential of microbial communities (Quince et al., 2017). Metagenomics studies, however, are poor in determining the identity of the microorganisms. The metagenomic analysis of raw sequences could be briefly described as follows. Optionally, the raw sequences are massed into contiguous sequences. Following, a functional annotation database, such as Kyoto Encyclopedia of Genes and Genomes (KEGG), is used to assess the functional potential of the sequences. The results are used to identify any metabolic pathways and are compared to already known metagenomic studies. Lastly, these data go through multivariate analyses and visualisations (Kuczynski et al., 2011).

1.4.1.2.2 Amplicon- based targeting

On the other hand, PCR amplicon- based techniques are able to profile selected organisms or single marker genes and cannot identify the entire genomic context. In these studies, the ribosomal small subunit (16S rDNA) gene is, most commonly, the gene of choice as the ribosome is present in all living organisms and it contains hypervariable (V regions) and conserved regions which can be used to identify and classify different bacteria (Woo et al., 2008). The 16S rRNA marker gene is comprised by approximately 1542 base pairs and contains nine hypervariable regions as shown in Figure 1-6.

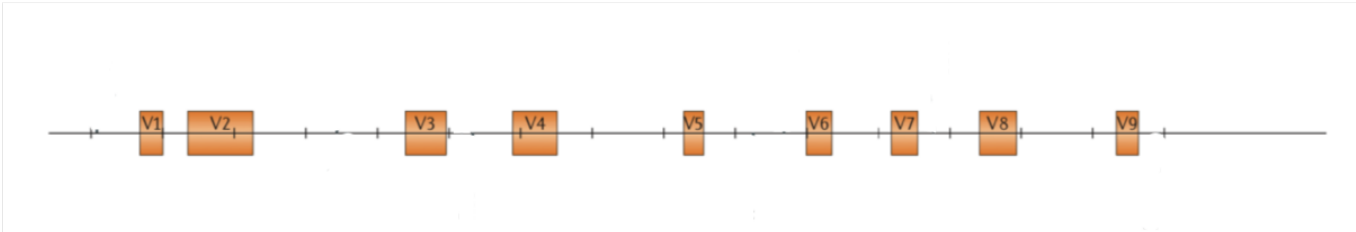


Figure 1-6: The variable regions of the 16S rDNA. Adapted from Kuczynksi et al. 2011.

Designated primers are used to target the amplification of specific V regions or multiple consecutive regions (Kozich et al., 2013) (Kumar et al., 2014).

All things considered, although identifying oral microbes using a marker gene is a forceful technique, it can be also inaccurate. Oral microbes can have highly similar 16S rRNA gene sequence, but different genomic composition. They can also have identical 16S rRNA gene sequences, but very different gene supplements due to mobile DNA (Tansirichaiya et al., 2016). Another limitation is related to the choice of the primer that is used or the PCR amplification. Primers discrepancy can result in distinct PCR amplification from diverse bacterial families, leading to biased conclusions (Morales and Holben, 2009).

1.4.2 Processing biological samples and DNA

1.4.2.1 Sample collection

Saliva is of great diagnostic value and collecting saliva samples is a simple, predictable and non-invasive procedure that has a great value not only in the study of oral microbiome, but also in endocrinology (hormones can be detected in saliva such as estradiol, progesterone, testosterone, cortisol), sports medicine and doping control, forensic science and immunology. There are different methods of collecting saliva samples. The less complex is the passive drooling and active spitting into a container. It can be argued that the detriment of this technique is the lack of standardisation in the salivary flow rate. To increase the amount of flow rate per minute, when needed, different stimuli can be used, such as paraffin wax, chewing gum, citric acid or lemon drops. The ideal stimuli should not absorb, modify or interfere in any way with the saliva components (Gröschl, 2017).

At present there is no standardised saliva sample collection protocol. Lim et al conducted a study aiming to determine, amongst others, the influence that different saliva sample collection techniques have in the oral microbiome analysis. They concluded that there was no significant difference in the oral microbiome composition of volunteers that were either directly spitting, drooling or oral rinsing while sampling (Lim et al., 2017). Henson and Wong suggest as an optimal whole saliva collection protocol the following: Inform participants regarding the time of collection, better aiming for 8-10 am when possible and advise them not to eat, drink, perform any oral

hygiene procedures for at least one hour before the sample collection. Following, provide participants distilled drinking water and ask them to rinse their mouth for one minute. After five minutes, participants are asked to spit into a 50mL sterile tube an approximate volume of 5mL. Always remind participants not to cough up mucus resulting in unwanted phlegm collection. Specimens are stored in ice and should be transferred to the lab immediately (Henson and Wong, 2010). Regardless the saliva collection protocol that is used in each study, it is very important to maintain consistent collecting procedures and conditions, while sampling both different participants and same participants within distinctive collection points. By optimising the collection, processing and storage of the saliva samples, optimal measurement of salivary analytes is succeeded.

1.4.2.2 DNA extraction

Whole saliva was found to provide increased bacterial and genome information. Storage for 36 months in -20 ^{o}C decreases the quality of the samples by 5 or even 10 times depending on the extraction method (Durdiaková et al., 2012).

It is often not realistic, especially in large studies, to perform the extraction of the DNA on the same day of sampling. In this case, saliva samples must be stored prior to processing. With regards to the ideal storage conditions, a study was conducted in Singapore in order to test the purity and quality of the extracted DNA of the same samples in five different storage conditions. The authors assessed the purity of DNA by the measurements of the spectrophotometer at 260nm and more specifically the ratio of $OD_{260/280}$ (good purity ratio: 1.8-2.0) and the PCR product band volume for each of the five storage circumstances. They concluded that DNA extraction within a week from the day of the sampling, in temperature of either -70 ^{o}C or 4 ^{o}C, caused only minimal, not statistically significant deterioration of the purity and quality of the extracted DNA. Additionally, DNA can successfully be extracted within a month with a decrease of 17-30% in PCR products (Ng et al., 2004).

1.4.2.3 Sequencing methodologies

Over the last fifty years many researchers have developed different techniques and technologies, aiming to determine the exact order of nucleic acids in polynucleotide chains, in other words, to sequence DNA and RNA molecules (Heather and Chain, 2016). It required a lot of effort, from 1953 when Watson and Crick recognised DNA as a three-dimensional structure, to 1965 when Robert Holley and his colleagues managed to produce the first nucleic sequence of alanine tRNA from *Saccharomyces cerevisiae* and to 1972 when Walter Fier, using Sanger's two-dimensional fractionation method, produced the first complete protein-coding gene sequence of the coat protein of bacteriophage MS2.

In the last couple of years, the sequencing industry has been dominated by Illumina to the point of nearly monopoly and the company can be considered the greatest contributor of next generation sequencing. Illumina have adopted a sequencing-by-synthesis approach, utilising fluorescently labelled reversible-terminator nucleotides, on clonally amplified DNA templates immobilised to an acrylamide coating on the surface of a glass flow cell. The Illumina Genome Analyser and the HiSeq have set the standard for high throughput massively parallel sequencing (Quail et al., 2012). The MiSeq is Illumina's most consolidated next generation sequencing tool. It was introduced by the company in 2011. This particular instrument uses a reversible-terminator sequencing-by-synthesis technology to provide end-to-end sequencing solutions. The MiSeq is able to perform, in just a single run, onboard cluster generation, amplification, genomic DNA sequencing, and data analysis, including base calling, alignment and variant calling. Furthermore, is one of the smallest benchtop sequencers. It performs both single and paired-end runs with adjustable read lengths from 1 × 36 bp (base pairs) to 2 × 300 bp. A single run can produce output data of up to 15 Gb in 4 hours of runtime and can output up to 25 M single reads and 50 M paired-end reads. Hence, MiSeq provides an ideal platform for rapid turnaround time. MiSeq is also a cost-effective tool for various analyses focused on targeted gene sequencing, metagenomics, and gene expression studies. For these reasons, MiSeq has become one of the most widely used next generation sequencing platforms (Ravi et al., 2018).

Other broadly used sequencing platforms are: The Ion Torrent Personal Genome Machine (PGM) by Thermo Fisher Scientific, the single molecule real time (SMRT) sequencer by Pacific Biosciences (PacBio) and the MinION platform by Oxford Nanopore Technologies (ONT). A brief comparison of these sequencing platforms in terms of mode of action, average read length measured by bp, advantages and disadvantages is demonstrated in Table 1-1 (Petersen et al., 2019).

Table 1-1: Comparison of sequencing platforms. Courtesy of Petersen et al., 2019.

Sequencing platform	Chemistry	Avg read length (bp)	Advantage(s)	Disadvantage(s)[a]
Illumina	Sequencing by synthesis; fluorescently labeled deoxynucleoside triphosphates	≤300	High accuracy	Short reads, high capital cost, long TAT
Thermo Fisher Ion Torrent	Sequencing by synthesis; detection of hydrogen ions	≤400	High accuracy	Short reads, high capital cost, long TAT
Pacific Biosciences	Sequencing by synthesis: SMRTbell replication	≥500	Long reads	High capital cost, variable accuracy, long TAT
Oxford Nanopore	Measures the changes in current as biological molecules pass through the nanopore	≥500	Long reads, low capital cost, short TAT	Low accuracy

1.4.2.4 Bioinformatics analysis tools

The raw data that arise following PCR and DNA sequencing must then be analysed aiming to accurate taxonomic assignment. To minimise the artefacts, the raw sequences are filtered through quality testing, through which low quality sequences are removed.

The arising clean sequences are then clustered into operational taxonomic units (OTUs) and they represent sequence reads that have a similarity above a certain cutoff (Kuczynski et al., 2011). Historically, a willful cutoff of 97% 16S rRNA gene sequence similarity level is accepted (Wayne, 1988). Below that level, sequences are considered to be different units. There are three different methods to assign sequences into OTUs. The first is closed, where seed sequences are used from a reference database. Closed reference OTUs are fast to create but are subject to reference bias. The second is called de novo, and the sequences are clustered without any reference database. In this method reference bias is minimised, however the method is expensive and can change with changed samples. The third, the open, is a combination of the previous two, where the sequences similar to reference being firstly closely clustered and the remaining, more novel sequences being de novo clustered

(Edgar, 2017). There are different bioinformatic pipelines for the analysis of amplicon sequencing data available, that can be used for OTU-level flows like MOTHUR (Schloss et al., 2009), QIIME-uclust (Bolyen et al., 2019) and USEARCH-UPARSE. A study to compare these pipelines, concluded that MOTHUR and USEARCH-UPARSE produce not significantly different number of OTUs. Authors also suggest that users of QIIME-uclust should switch to an alternative pipeline, because of the extensive spurious OTUs composed (Prodan et al., 2020).

The standard method of OTU-clustering is gradually replaced by a new alternative approach based on amplicon sequence variants (ASVs), also known as exact sequence variants (ESVs) and zero-radius OTUs (ZOTUs). Here, following the first quality check, a further quality process takes place. This process is also known as denoising and involves the computational correction of errors to a single nucleotide basis and removal of the chimeric sequences from the raw data. Chimeric sequences are artefacts that appear following the PCR process and represent the formation of a product that is a combination of two or more parent sequences (Bishop and Bishop, 2014). ASV-based analysis is advantageous as less false sequences are included in the microbiome analyses and since ASVs are consistent biological entities, independent of reference database, they provide reproducibility and comparability across different studies (Callahan et al., 2016). Broadly used bioinformatic pipelines for ASV-level flows are: Qiime2-Deblur, DADA2 (Callahan et al., 2016) and USEARCH-UNOISE. ASV-based analysis was used in this research project and will be described in further details in the relevant material and methods chapter.

Following the identification of OTUs or ASVs, these should be assigned to specific taxa. The most broadly used taxonomic classifier tool is the Ribosomal Database Project (RDP) algorithm. RDP classifies, with high level of accuracy, taxa based on the co-occurrence of 8-mers in a query sequence and a reference database (Cole and Tiedje, 2014). Other taxonomic classifiers are, the Greengenes and Silva databases.

2 Aims and objectives

2.1 Aims of the study

The aim of this study is to analyse, in terms of bacterial relative abundance at phylum, genus and species level, the oral microbiome composition of adolescents in depth of time. Furthermore, to investigate the diversity of the bacterial communities, with regard to richness, evenness, similarity and presence or absence of bacteria taxa. Additionally, to determine which is the effect of the shared school environment if any, on the oral microbiome of boarder students.

2.2 Objectives

Oral health questionnaires were collected and a basic clinical examination was performed in order to gather and analyse not only demographic information but also to investigate the oral health status of the participants. Saliva samples were collected from students of Haileybury boarding school at different time points. This allowed us to understand how the oral microbiome changes over time. Students were divided in two groups according to their boarding status: boarders and non-boarders. Allocating participants in these two groups, gave us the chance to compare the oral microbiome of adolescents with an unchanged household environment with ones which sustained significant changes in their living environment. The microbial composition analysis of saliva was assessed by sequencing all the variable regions (V1-V9) of the 16S rRNA gene, a technique which gave us the opportunity to obtain rich set of sequencing data in order to more accurately identify bacterial taxa.

3 Materials and methods

3.1 Sponsorship

This research project is being funded by a research grant awarded by Biomedical Research Centre (BRC) University College Hospital.

3.2 Ethics statements

This human study was approved by the University College London (UCL) Research Ethics Committee (REC) (Project identification: 7567/001). Initial ethical approval was obtained and was valid until the 31st of August 2020 (Appendix 1. Ethical Approval). Following submission of the Annual Continuing Review Approval Form requesting extension of the ethical approval, REC approved increased validation to 31st of August 2021 (Appendix 2. Ethics Extension Approval). An information sheet outlining the study aims and objectives was given to participants, version for young adults (Appendix 3. Participation Information Sheet For Young Adults), and their parents, version of parent/ guardian information sheet (Appendix 4. Parent/ Guardian Information Sheet). Written informed consent was obtained by all parents of students who volunteered to participate in this study (Appendix 5. Consent Form For Adolescents In Research Studies).

3.3 Cohort

In this study, all saliva samples were collected from students of Hailebury school. Haileybury is an independent co-educational school, located in Hertfordshire, in southern England. Haileybury offers boarding and day provision for students aged from 11 to 18 years (https://www.haileybury.com).

Participants were asked to complete an oral health questionnaire (Appendix 6. Oral Health Questionnaire), including basic demographic, general and oral health questions and their boarding status. Participants were, then, allocated in two groups. The first group, the control group, was the non-boarders. Participants in that group

lived at home and travelled in and out of school on a daily basis, suggesting that their household remained unchanged. The second group, the test group, was the boarders. Boarder students have left home and lived with their classmates on site at Haileybury during the term time and their household had significantly changed. The students needed to be new pupils of the school, in order to participate in the study. Participants were either in year 7, 9 or 11. Each participant was allocated to a unique identification code, in the form of Hx, which they carried through the study.

3.4 Clinical screening

A basic clinical examination was performed when the participants entered the study. A clinical screening protocol was developed (Appendix 7. Screening Protocol) and a data collection form was created (Appendix 8. Clinical Data Collection Form). The oral examination included the inspection of the oral mucosa, caries experience and plaque assessment. Caries and restorations experience were recorded by DFT index, using the modified ICDAS criteria. Additionally, unrestorable decay was noted. Caries scored as follows:

0 No evidence of caries.
A Initial caries.
3 Localised enamel breakdown due to caries with no visible dentine.
4 Underlying dark shadow from dentine.
5 Distinct cavity with visible dentine.
6 Extensive distinct cavity with visible dentine.

The plaque was assessed using the Silness-Löe Index. This was based on the recording both soft debris and mineralised deposits on four surfaces (buccal, lingual, mesial and distal) of six teeth, the upper right first permanent molar (16), the upper right second permanent incisor (12), the upper left first permanent premolar (24), the lower right first permanent molar (36), the lower left second permanent incisor (32) and the lower right first permanent premolar (44). Each surface was given a score from 0-3. The scores from the four areas of the tooth were added and divided by four in order to give the plaque index for the tooth with the following scores and criteria:

0 No plaque.
1 A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may be seen in situ only after application of disclosing solution or by using the probe on the tooth surface.
2 Moderate accumulation of soft deposits within the gingival pocket, or the tooth and gingival margin which can be seen with the naked eye.
3 Abundance of soft matter within the gingival pocket and/ or on the tooth and gingival margin.

The oral mucosa was checked in a systematic way, following the order of upper right, upper left, lower left, lower right, making sure that all of the soft tissues, including the lips and cheeks were adequately examined. The Pulp, Ulceration, Fistula and Abscess index (PUFA) was used. P was noted when there was open pulp expose in the permanent dentition, U if obvious ulceration was present, F in case of clinical evidence of fistula in permanent dentition and A if abscess was detected. The scores and criteria for PUFA index are the following:

0 No lesions evident.
1 A single lesion present.
2 Two or more lesions present.

Furthermore, significant findings as the presence or absence of any orthodontic appliances or other dentures or dental anomalies of enamel or dentine were recorded.

3.5 Sampling

As shown in Figure 3-1, first step of the next generation sequencing methodology, is the saliva sample collection. Saliva sampling was planned to be performed in the beginning, middle and end of each of the three terms of the school year 2018-2019 (Table 3-1), with a total of nine saliva sample collection points. Sampling at beginning of autumn term, September 2019, was also included. Sampling cycles were identified as Sy (S1 to S10).

Table 3-1: Term dates for school year 2018-2019.

Terms (2018-2019)	Starting	Ending	Half term starting	Half term ending
Autumn	03/09/2018	14/12/2018	22/10/2018	02/11/2018
Spring	07/01/2019	12/04/2019	16/02/2019	22/02/2019
Summer	23/04/2019	28/06/2019	25/05/2019	02/06/2019
Autumn	04/09/2019			

In every sampling point, the participants were provided with a 14 ml sterile tube prepared with 2 ml of saliva buffer. Saliva buffer reagents are shown in Table 3-2. Each tube had a printed sticker label, in the form of HxSy, allocated to the individual participant. Students were asked to spit an approximate of 3ml of saliva inside the tubes provided. In case of absence, or unavailability of the participant during the sample collection, the school staff attempted to perform the collection at another time as close as possible to the original collection date. If this was not possible samples were not collected.

Table 3-2: Saliva buffer mixtures. Courtesy of Erni Marlina PhD Thesis,2020.

Reagent	**Final volume**	**Final concentration**
1 M Tris pH 8.0	50 ml	50 mM
0.5 M EDTA pH 8.0	100 ml	50 mM
Sucrose	17.2 gr	50 mM
3 M NaCl	33.33 ml	100 mM
10% SDS	100 ml	1%
dH2O	Top up to 1 L	

Following saliva sample collection, specimens were transferred in ice to the laboratory, where they were kept in -20 ^{o}C until proceeding to the next step, the DNA extraction.

3.6 DNA extraction

Total DNA was extracted using a bacterial genomic DNA purification kit, the PurElute^{TM} Bacterial Genomic Kit (EdgeBio, Gaithersburg, USA). The kit contains: 25ml of Spheroplast Buffer, 6ml of Lysis 1, 6ml of Lysis 2, 6ml of Advamax^{TM} 2 Beads and 6ml of Extraction Buffer. Components of the kit are demonstrated in Figure 3-2.

As per the manufacturers' instructions, the Sheroplast Buffer was the only component that was stored in -20°C and the rest in 4 °C. The following protocol was followed for every saliva sample, each containing 2 ml of saliva buffer and 2-5ml saliva.

2 ml of bacterial culture with an OD_{600} value was centrifuged for 5 minutes, at 10,000 g, at 4 °C, to obtain pellet. Then the supernatant was removed, and the same centrifuging process was repeated once again. I added 400 µl of Spheroplast buffer and vortexed at the highest speed until the pellet was re-suspended. Following that step, the samples were incubated for 10 minutes at 37 °C. Next, 100 ul of Lysis 1 and 100 ul Lysis 2 were added to each sample. Samples were vortexed for 10 seconds at low speed before centrifuged at for 3 minutes, 18,000 g, at 4 °C. Then, 100 µl of Advamax 2 beads added were added. The next step was to gently invert the sampling tube 10 times. Another centrifuging cycle, followed. Next the supernatant was transferred to a clean 2 ml tube, clearly labeled. An equal volume (800 µl) of Isopropanol was added and centrifuged at 18,000 g for 2minutes. The supernatant was then decanted before DNA washing by 750 µl of 70% ethanol before centrifugation at 18,000 g for 2 minutes. The DNA samples were then air dried for approximately 40 minutes, until no ethanol was left, before being re-suspended in 100 µl of dH2O. The concentration of the total DNA was measured with a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). The tubes containing the extracted DNA were kept in -80 °C, until the next step.

Figure 3-1: The five components of the PurElute™ Bacterial Genomic Kit by EdgeBio.

3.7 PCR

The extracted DNA samples were handed to the UCL Genomics to proceed with PCR and sequencing. Each sample was in 1,5 ml Eppendorf tube, clearly labelled with the sample details. Samples were accompanied with a spreadsheet detailing each samples' elution volume, DNA concentration ng/uL, 280/ 260 and 230/ 260 ratios, as measured from the NanoDrop Spectophotometer (Appendix 9. DNA concentration measured with NanoDrop Spectophotometer).

The Swift Amplicon 16S+ITS Panel by Swift Biosciences was used. This kit enables the preparation of high quality targeted next-generation sequencing (NGS) libraries. Each sequencing run can include up to 96 samples. The kit provides a single primer pool covering all the variable regions of the 16S rRNA gene (V1-V9) and the Fungi ITS 1 and ITS 2 genes. This kit was selected, because of the opportunity it provides, to gain maximum information of the complex bacterial communities by sequencing the entire V regions of the 16S rRNA gene (Table 3-3). The fungal finding of the tested samples is a subject of another thesis in progress (Tengku Ab Malek, DDent project, unpublished thesis in preparation). The detailed protocol that was followed to prepare sequencing libraries can be found in the following link https://swiftbiosci.com/swift-amplicon-16s-its-panel/. Briefly, the samples were re-quantified with the Qubit DNA HS assay (https://www.thermofisher.com/order/catalog/product/Q32851#/Q32851). Any samples outside of the range of the kit were re-quality controlled (QC'd) with the Qubit DNA BR assay (https://www.thermofisher.com/order/catalog/product/Q32850). Library preparation included two main steps, one pre-PCR and one post-PCR. The first, the multiple PCR step endured 70 minutes. A master mix with the components, was created. Components were placed on ice for at least 10 minutes prior to pipetting, letting them to reach 4 (from -20 ^{o}C). Components, apart from enzymes, were vortexed and centrifuged. Following the multiplex PCR was performed as shown in Table 3-4. The second step endured 20 minutes. A second master mix was created. Size selection and clean-up step 1, was followed by the index step and finally the size selection and clean-up step 2. Once the library was prepared, library quantification was performed. An equal volume of each sample (5μL) was pooled and was QC'd via qPCR (QuantaBiosparQ Universal Library Quant Kit run on the QuantaBio Q qPCR

machine). The DNA libraries were sequenced using the Illumina MiSeq with a v2 500 cycle run (2x250bp run). There was no deviation from any manufacturer instructions.

Table 3-3: Key characteristics and performance of Swift Amplicon 16S + ITS Panel (https://swiftbiosci.com/swift-amplicon-16s-its-panel/).

Specification	Feature	16S + ITS Panel
Input	Input DNA required	10pg-50ng
Workflow	Time required Multiplexing on MiSeq v2 Standard	2 hours Up to 96
Design	Number of amplicons Average amplicon size Genes covered	5 16S rRNA +2 Fungal 475 bp Bacterial 16S + Fungal ITS

Table 3-4: Multiplex PCR conditions

(https://www.thermofisher.com/order/catalog/product/Q32850).

PCR STEP	CYCLING CONDITIONS
Multiplex PCR Thermocycler Program	Lid heating ON 30 sec 98 °C 10 sec 98 °C 5 min 63 °C — 4 cycles 1 min 65 °C 10 sec 98 °C 1 min 64 °C — 22 cycles 1 min 65 °C Hold 4 °C
Indexing Thermocycler Program	Lid heating OFF 20 min 37 °C

The raw sequencing data were uploaded in the form of FASTQ files in Base Space.

3.8 Sequence processing and analysis

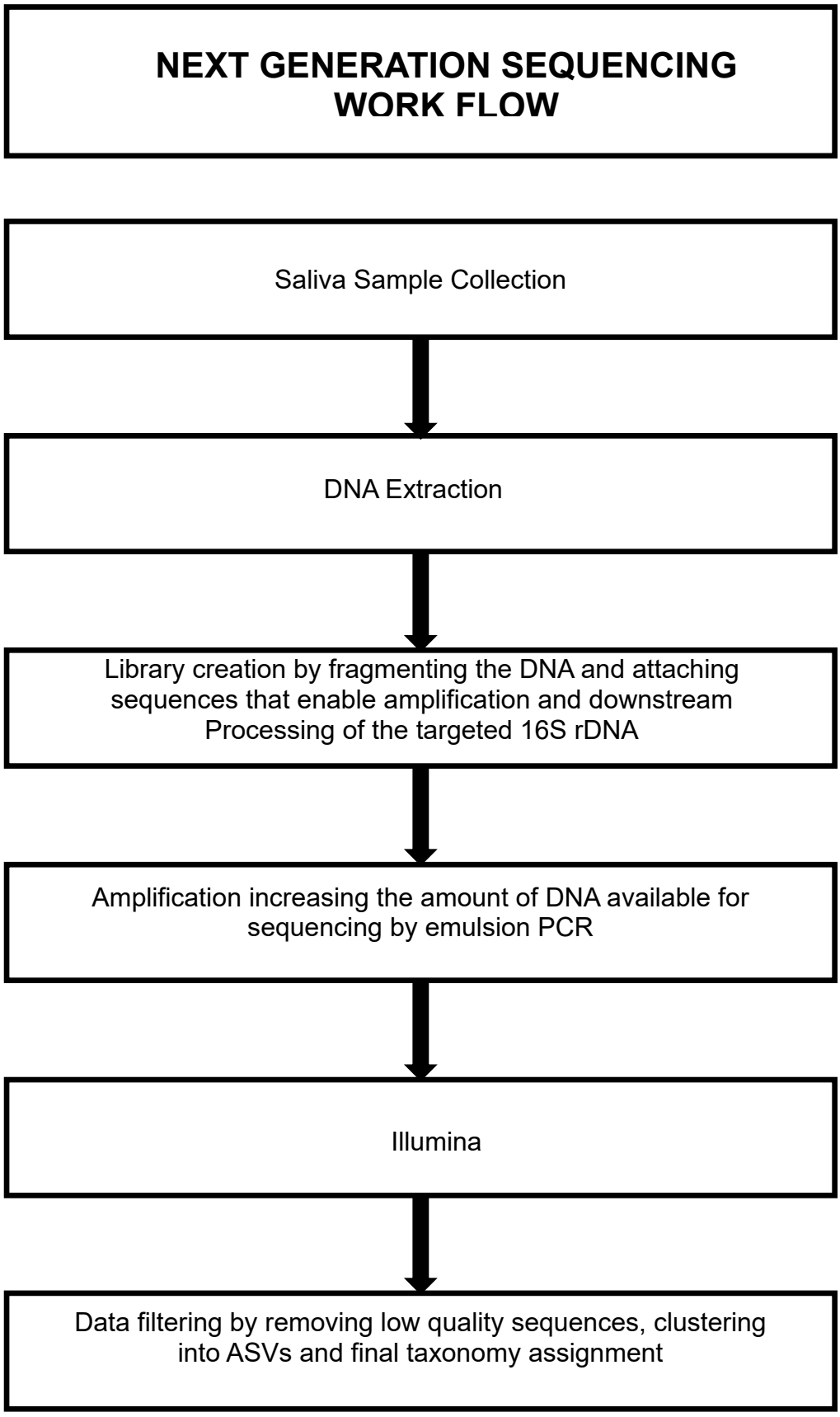


Figure 3-2: Methodology flowchart.

Bioinformatics analysis was performed using the Swift 16S SNAP-APP for 16S, an open source pipeline (https://github.com/swiftbiosciences/16S-SNAPP-py3), for analysing 16S rRNA gene sequencing data consisting of amplicons from multiple variable regions. The Swift 16S SNAP-APP associates sequence reads from all nine (V1-V9) variable regions of the 16S rRNA gene to their presumable template sequences. The pipeline that was followed is briefly described in Figure 3-3. The pipeline starts with primer trimming using the package Cutadapt. Trimming was then followed by quality filtering, denoising, paired- end merging and chimera removal using parts of the DADA2 pipeline. During these initial steps, short and low-quality reads were filtered out and amplicon sequence variants (ASVs) were identified. ASVs were later split into single reads and duplicate reads were dereplicated using the package VSEARCH. That way a dataset of unique sequences set was formed. The basic local alignment searching tool (BLAST) was used to search and map the reads to reference nucleotide sequences derived from the 16S rRNA gene database of the ribosomal database project (RDP). The aligned read pairs were allocated to selected templates via Python3 scripts. From template-aligned read pairs consensus sequences were computed. These sequences, together with the sequence features of individual non-aligned read pairs, were classified using the RDP classifier at species level in order to generate an abundance lineage table. Template sequences were used to construct reference trees using the programs Multiple Alignment using Fast Fourier Transform (MAFFT) and FASTTREE.

First steps of 16S SNAPP processing of the raw data produced a spreadsheet with primer-trimmed, filtered, forward and reversed denoising, merged sequences and the final number of non-chimera reads (full spreadsheet report of DADA2 denoising can be found in Appendix 10). A threshold of minimum 10.000 non-chimera reads was set. Samples with lower than 10.000 non-chimera reads were omitted (samples H4S6 and H7S6). ASV count, feature, taxonomy and lineage tables were generated, as well as a phylogenic tree.

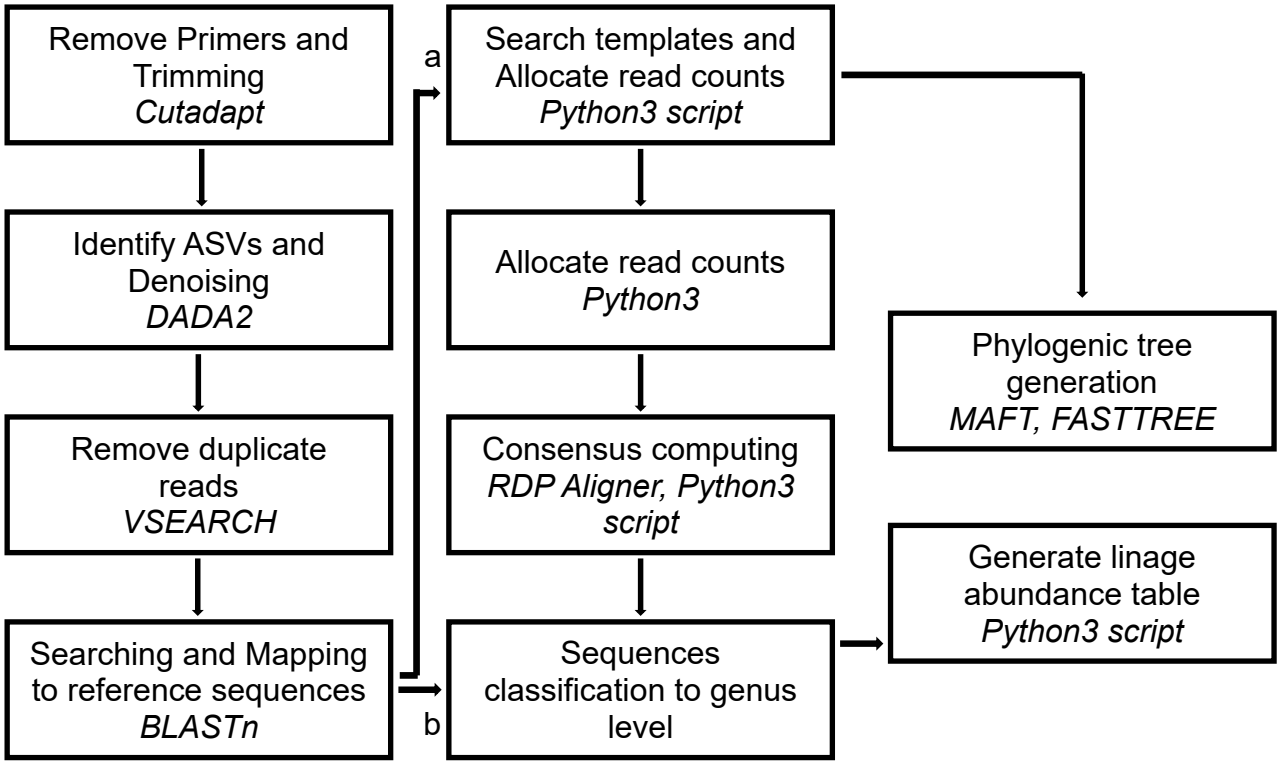


Figure 3-3: Overview of the Swift 16S SNAP-APP Pipeline.

3.9 Statistical analysis

Different entities and measurements were used to assess the statistical relationships between the samples, as described in detail below.

3.9.1 Clinical examination and oral health questionnaires

Data gathered from the clinical examination forms and oral health questionnaires were computerised and organised in an Excel spreadsheet, in order to be appropriately analysed, and generate tables and figures.

3.9.2 Taxonomic analysis

Taxonomic analysis at phylum, genus and species level was performed. ASV count, feature, taxonomy and lineage tables that were generated, were manipulated in spreadsheets. The relative abundance was calculated by the total number of individual phyla/ genera/ species divided by the total number of phyla/ genera/ species population multiplied by one hundred. Subsequently figures and tables were developed, using Microsoft Excel version 16.16.27.

3.9.3 Alpha diversity measurements

Alpha diversity measures the species richness and biodiversity within a microbial environment. In other words, how many different species could be detected in a microbial community within one sample and how are the microbes balanced to each other (evenness), whether different species have equal abundance or some species dominate others (Poos et al., 2009).

3.9.3.1 Rarefaction

In reality there is a difference between the actual distribution of microbes within a community and the observed based on the sequencing. This may result to miss interpretation of the microbial distribution. This problem arises from what we call low

sampling depth, which is practically the low total frequency of observed features. Rarefaction is fixing this problem, enabling researchers to accurately present and compare microbial communities, as diversity metrics are sensitive to total frequency. In rarefaction, frequencies are subsampled without replacement until all samples have the same total samples with fewer sequences than the even sampling depth and are been filtered out of the feature table. This is a technique to assess species richness from the results of the sampling. This particular approach, allows the calculation of species richness for a given of individual samples, based on the generation of a so-called Rarefaction curve, which is a plot the number of OTUs or ASVs identified with increased sampling of sequence data (Weiss et al., 2017).

3.9.3.2 Chao1, Shannon, Simpson's Indices

Chao1 index assumes that the number of organisms identified for a taxon has a Poisson distribution and corrects for variance. It is estimating the diversity from abundance data and is useful for data skewed towards rare abundances.

The Shannon index (non-phylogenetic) is commonly used to characterise species diversity in the microbial community. It accounts for both the effective number of species detected, their abundance and evenness of the species present.

The Simpson's index is a measure which accounts for both the number of species observed within a population and the relative abundance of each of these species (Xia et al., 2018).

The non-parametric statistical test of the Wilcoxon rank sum test was used to investigate whether two independent cohorts consist of samples that were selected from populations having the same alpha diversity distribution (Xia et al., 2018). The null hypothesis is that the probability that a randomly selected value from one population is less than a randomly selected value from a second population, is equal to the probability of being grater. P-values less than 0.01 suggest that the null hypothesis can be rejected, confirming that the samples of two studied populations that were randomly selected have different alpha diversity distributions.

3.9.4 Beta diversity measurements

Beta diversity measurements compare the diversity in microbial communities between different environments (Legendre et al., 2005). This analysis requires a distance matrix, which is made by comparing the similarity of every sample to each other using a dissimilarity estimator. Two different methods to estimate dissimilarity were used, described below.

The Bray-Curtis calculator returns the Bray-Curtis index by describing the dissimilarities between the structures of two communities and is based on the abundance or read count data (Ricotta and Podani, 2017).

The Jaccard calculator is based on the presence or absence of species and does not include any abundance information. It is demonstrating the difference in microbial composition between two samples (Ricotta et al., 2019).

Each sample is a point and the distance between the points represents the similarity of those samples. The closer the points are in the plot, the more similar the bacterial communities are. This was observed using non-metric Multi-Dimensional Scaling (nMDS) with two dimensions (Kenkel and Orloci, 1986). For the nMDS plots the PRIMER (Plymouth Routines in Multivariate Ecological Research) -e (version 7) was used.

4 Results

4.1 Samples

Saliva collection was performed at eight different time points from September 2018 to June 2019 (Figure 4-1). S8 is not included as no saliva samples were obtained in May 2019. Time spent at school during the three school terms, is defined by the blue areas and the school holidays by the orange. Boarder students left the school premises and presumably travelled home during every holiday period. The maximum days continuously spent at school prior sampling are 39 days when S3 was collected.

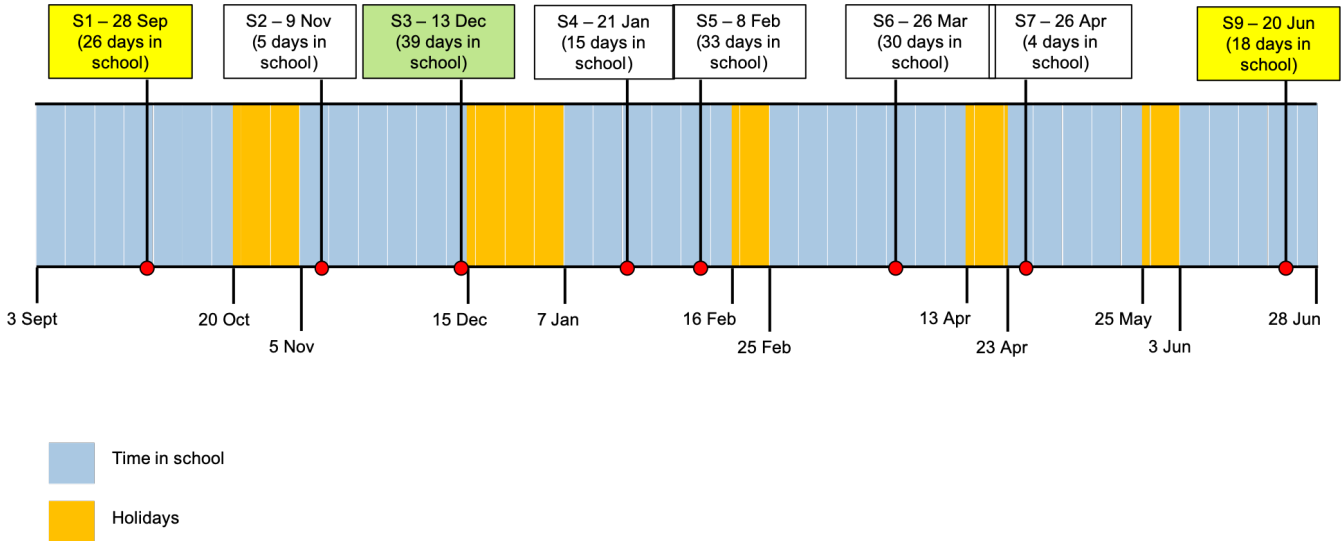


Figure 4-1: Sampling timeline for the school year. Period covers September 2018 to July 2019.

In September 2018, when the study started, 17 students were recruited, 4 non-boarders and 13 boarders, 4 females and 13 males, with an average age of 12.23 years (9 participants were 11 years old, 7 were thirteen and 1 was 16). Each participant was allocated to a unique identification code, which they will carry through the study, in the form of Hx (H1 to H18). Participant H10, was not included in the study as no saliva samples were collected from this party.

Seven saliva samples were not successfully collected. Because each Swift Amplicon 16S+ITS Panel could run up to 96 samples (two kits were available that had to be shared within two projects running at our laboratory during this time) a final of 135 samples were handed for sequencing, as shown in Table 4-1. Cells in grey demonstrate saliva samples that were not able to be collected, cells without a check represent samples that were not included in the sequencing process. Five samples from S10 (September 2019) were included.

Table 4-1: Saliva samples collected from each participant.

Participant	Group	Gender	School Year	S1	S2	S3	S4	S5	S6	S7	S9	S10
H1	NB	F	7	✓	✓	✓	✓	✓	✓	✓	✓	✓
H2	B	F	7	✓	✓	✓	✓	✓	✓	✓	✓	✓
H3	B	M	7	✓	✓	✓	✓	✓	✓	✓	✓	✓
H4	NB	M	7	✓	✓	✓	✓	✓	✓	✓	✓	✓
H5	NB	M	7	✓	✓	✓	✓	✓	✓	✓	✓	✓
H6	B	M	7	✓	✓	✓	✓	✓	✓	✓	✓	
H7	B	F	12	✓	✓	✓	✓	✓	✓		✓	
H8	B	M	7	✓	✓	✓	✓	✓	✓	✓	✓	
H9	B	M	7	✓	✓	✓	✓	✓	✓	✓	✓	
H11	B	M	9	✓	✓	✓	✓	✓	✓	✓	✓	
H12	B	M	9	✓	✓	✓	✓	✓	✓	✓	✓	
H13	B	F	9	✓	✓	✓	✓		✓		✓	
H14	B	M	9	✓	✓	✓	✓	✓	✓	✓	✓	
H15	B	M	9	✓	✓	✓	✓	✓	✓	✓	✓	
H16	NB	M	7	✓	✓	✓	✓	✓	✓	✓	✓	
H17	B	M	9	✓	✓	✓	✓	✓		✓	✓	
H18	B	M	9	✓	✓	✓	✓	✓		✓	✓	

4.2 Oral health questionnaire findings

All the questionnaires were completed in September 2018. Participants were allocated in two groups according to their boarding status. Four students were non-boarders

(H1, H4, H5, H16) and thirteen students formed the group of boarders (H2, H3, H6, H7, H8, H9, H11, H12, H13, H14, H15, H17, H18). The non-boarder group was comprised of one female and three males. In the boarder group three participants were girls and ten are boys. In total the cohort was composed of four females and thirteen males. Participants had a mean age of 12.2 years, with all the non-boarders being at the age of 11 years and the boarders had a mean age of 12.6 years (Table 4-2).

Table 4-2: Sample distribution.

GROUP	**Nr of children, (%)**	**Gender**	**Age in years, mean**
Boarders	13 (76%)	Female= 3 Male= 10	12.6
Non-Boarders	4 (24%)	Female= 1 Male= 3	11

The vast majority of participants, twelve in total, were from the United Kingdom, two sibling participants were coming from Croatia (H6, H12), one from India (H7), one from Malaysia (H15) and one from Germany (H13) (Figure 4-2).

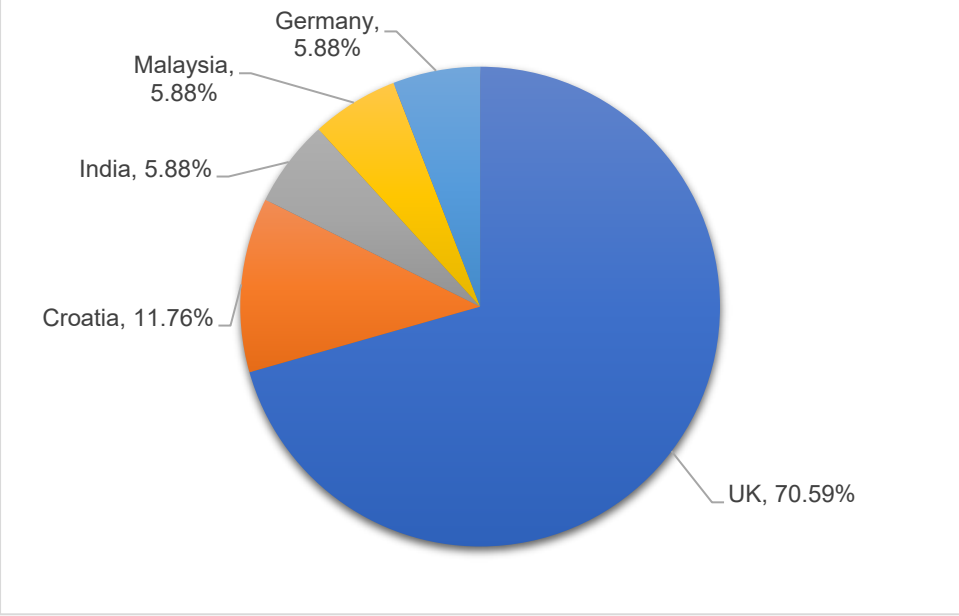


Figure 4-2: Sample distribution frequency according to country of origin

H8 and H17 were also siblings. Fourteen participants owned pets (82.35%), as shown in Table 4-3.

Table 4-3: Sample distribution frequency of owned pets.

PET	Nr of children, (%)
Cat	7, (46.67%)
Dog	5, (33.33%)
Hamster	2, (13.33%)
Fish	1, (6.67%)

Only two participants (H6, H14) were not involved in sports activities, and they were the only ones that did not have a mouthguard. All the participants stated that they brush their teeth twice daily, six of them (35.29%) were using mouthwash (H5, H7, H9, H13, H14, H16) and four students (23.52%) were having fixed orthodontic appliances (H6, H12, H13, H18). No participant had a false tooth or using a denture to replace a missing tooth. H5 and H16 were consuming probiotic drinks (11.76%). Medically, H5 had asthma and H3, H5 and H6 had eczema.

4.3 Clinical examination findings

Caries experience, that was measured with the ICDAS score, showed that three teeth had initial caries, corresponding to score A. All the early caries were detected in first permanent molar teeth, two upper and one lower, in three different participants (H1, H8, H18). Localised enamel breakdown due to caries with no visible dentine, corresponding to score 3, affected two first molar teeth of the same individual, H2. The same participant was the only one with an existing restoration of their upper right first permanent molar. No teeth with either underlying dark shadow from dentine (score 4) or distinct (score 5) or extensive (score 6) cavity with visible dentine were detected (Table 4-4).

Table 4-4: Caries experience measured with the ICDAS score.

ICDAS Score	Nr of affected teeth
A	3
3	2
4	0
5	0
6	0

The average plaque score of all participants was 0.125. Seven participants scored 0 (H1, H3, H11, H13, H14, H16, H17), three had a plaque score of 0.1 with one out of six teeth having plaque adhering to the free gingival margin and adjacent area of the tooth (H8, H12, H18), H15 had a plaque score of 0.3, three a score of 0.6 (H5, H6, H7), H4's score was 0.8, H9 scored 1 and H2 had the highest plaque score of 1.4.

The PUFA index revealed one single ulceration lesion present in the oral mucosa of participant H15.

4.4 Computational analysis of raw data

Raw sequences from 134 samples were processed via DADA2. One sample, H5S1, produced no reads during sequencing thus was not included in the findings. Following primmer trimming 17,522,895 sequence reads were obtained in total. Computing filtering, forward (FWD) denoising, reverse (RV) denoising and merging resulted on a total final of 13,879,849 non-chimera reads. The average of merged non-chimera reads was 83%. Overall 21% of the initial sequence reads were filtered and removed. A threshold of a minimum of 10,000 non-chimera reads was set in order to omit low quality samples. This excluded H4S6 (9,091 reads) and H7S6 (11,764 reads). Full DADA2 report can be found in Appendix 10.

The asv_table included 129,585 ASVs (from asv_1 to asv_129585) and 64,547 different features were identified in the feature table. The feature table consists of all consensus sequences and ASVs that could not be mapped to any reference sequence. These features were then clustered in a taxonomy table and finally

presented on a lineage table. Bacteria were identified to the most descending level possible, as demonstrated in Figure 1-2.

4.5 Taxonomic Analysis

4.5.1.1 Phylum level

Taxonomic analysis at phylum level revealed that 99.54% of observed bacteria were classified and 0.46% remained unclassified. Five major phyla predominated in both groups; *Firmicutes* (29.63%), *Actinobacteria* (25.62%), *Proteobacteria* (23.71%), *Bacteroidetes* (16.11%), *Fusobacteria* (4.29%), forming 99.37% of the total classified phyla observed. Nine phyla (*Campilobacterota, Candidatus Saccharibacteria, Cyanobacteria/ Chloroplast, Deferribacteres, Spirochaetes, SR1, Synergistetes, Tenericutes* and *Verrucomicrobia*) were found in lower proportions comprising the rest of 0.17% of the observed classified phyla.

4.5.1.2 Genus level analysis

Genus level taxonomic analysis allowed classification of 132 different genera (95.44%) and 4.56% of bacterial taxa remained unclassified at genus level. *Rothia* (21.96%) is the most abundant genera, followed by *Streptococcus* (21.61%), *Neisseria* (11.62%), *Haemophilus* (10.6%) and *Prevotella* (10.25%). Seven further genera had relative abundance of more than 0.91% and the rest of the classified genera formed 4.06% of the total relative abundance, as shown in Figure 4-3.

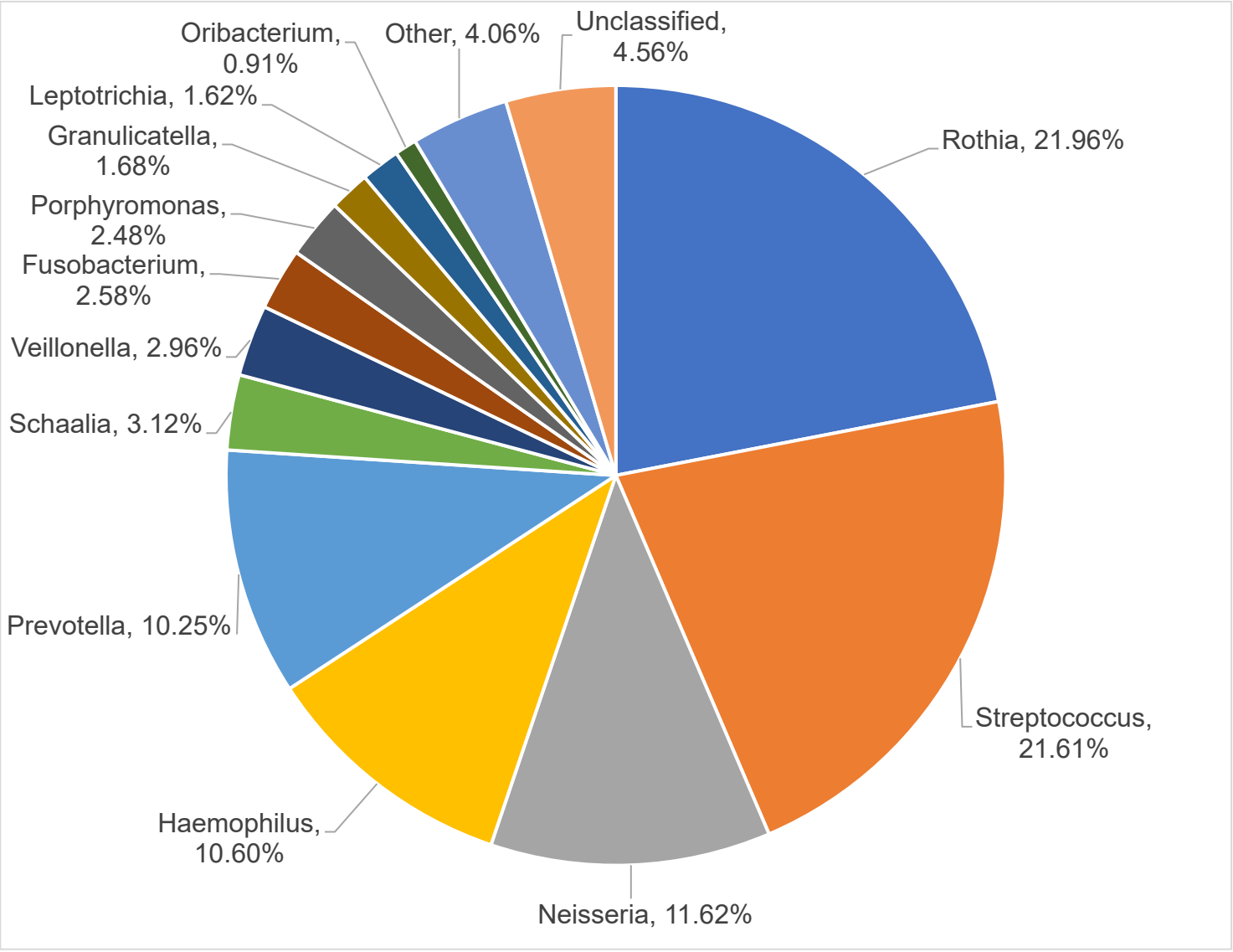


Figure 4-3: Relative abundance at genus level of all samples and sampling points including classified and unclassified genera.

4.5.1.3 Species level analysis

4.5.1.3.1 Most abundant species within the entire cohort

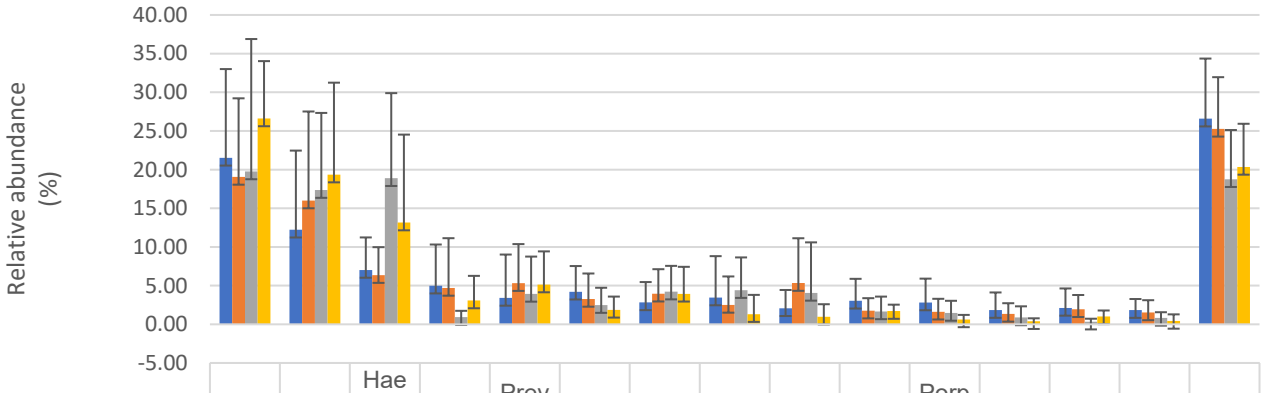
Descending the taxonomic analysis to species level, the most abundant species identified within all the participants and all sampling points was *Rothia mucilaginosa* (20.43%), followed by *Streptococcus oralis* (16.46%) and *Haemophilus parainfluenzae* (9.38%). The most abundant species, including unclassified taxa at species level are shown in Table 4-5.

Table 4-5: Relative abundance at species level of all samples and sampling points expressed in percentages.

Species	Mean abundance (%)
Rothia mucilaginosa	20.43
Streptococcus oralis	16.46
Haemophilus parainfluenzae	9.38
uncl.Neisseria	4.35
Prevotella melaninogenica	3.95
Neisseria mucosa	3.45
Schaalia odontolytica	3.08
Neisseria perflava	3.05
uncl.Prevotella	2.95
uncl.Streptococcus	2.67
Porphyromonas pasteri	2.04
uncl.Prevotellaceae	1.86
uncl.Fusobacterium	1.40
uncl.Bacteroidales	1.03
Other	23.90

4.5.1.3.2 Comparison between boarders and non-boarders at S1 and S9

The most abundant species identified in the two studied groups, boarders and non-boarders, were compared between S1 and S9, as shown in Figure 4-4. Sampling points S1 and S9 were selected for comparison as they are supposed to represent the original oral microbiome composition and how this was shaped ten months later. In the boarder group, there was on average an increasing abundance of *Streptococcus oralis* (by 3.78%) and unclassified *Prevotella* (by 3.26%) from S1 to S9, with the rest of most abundant species showing similar abundances in both groups. In the non-boarder group, there was a noticeable increase in the mean abundance of *Rothia mucilaginosa* (by 6.85%). On the other hand, *Haemophilus parainfluenzae*, *Neisseria perflava* and unclassified *Prevotella* showed a decreasing trend from S1 to S9.



	Rothia_mucilaginosa	Streptococcus_oralis	Haemophilus_parainfluenzae	uncl. Neisseria	Prevotella_melaninogenica	Neisseria_mucosa	Schaalia_odontolytica	Neisseria_perflava	uncl. Prevotella	uncl. Streptococcus	Porphyromonas_pasteri	uncl. Prevotellaceae	uncl. Fusobacterium	uncl. Bacteroidales	other
BoarderS1	21.52	12.23	7.02	4.99	3.41	4.21	2.83	3.46	2.07	3.04	2.82	1.84	2.12	1.84	26.59
BoarderS9	19.05	16.00	6.36	4.70	5.32	3.28	3.96	2.51	5.33	1.77	1.63	1.35	1.94	1.53	25.27
Non-BoarderS1	19.76	17.36	18.89	0.93	3.93	2.48	4.23	4.42	4.06	1.67	1.47	0.88	0.34	0.83	18.76
Non-BoarderS9	26.61	19.36	13.15	3.08	5.14	1.87	3.94	1.31	0.98	1.71	0.63	0.40	1.02	0.44	20.35

Figure 4-4: Relative abundance at species level at S1 and S9 in boarder and non-boarder groups. Data expressed by percentages and standard deviation.

More detailed figures demonstrating the mean percentages of the most abundant species for each participant at each sampling point can be found in Appendix 12. (Species relative abundance for each participant at each sampling point)

4.5.1.3.3 Comparison of bacterial composition between two sibling pairs (H6,12 and H8,17)

Among the participants of the boarder group two pairs were siblings, H6 and H12 as well as H8 and H17, respectively. A comparison of the composition of the oral microbiome of the siblings was made, in order to assess whether each of these two pairs demonstrated similar changes of bacterial species over the sampling period of ten months, as shown in Figure 4-5. No strong associations were able to be made for either of the two pairs.

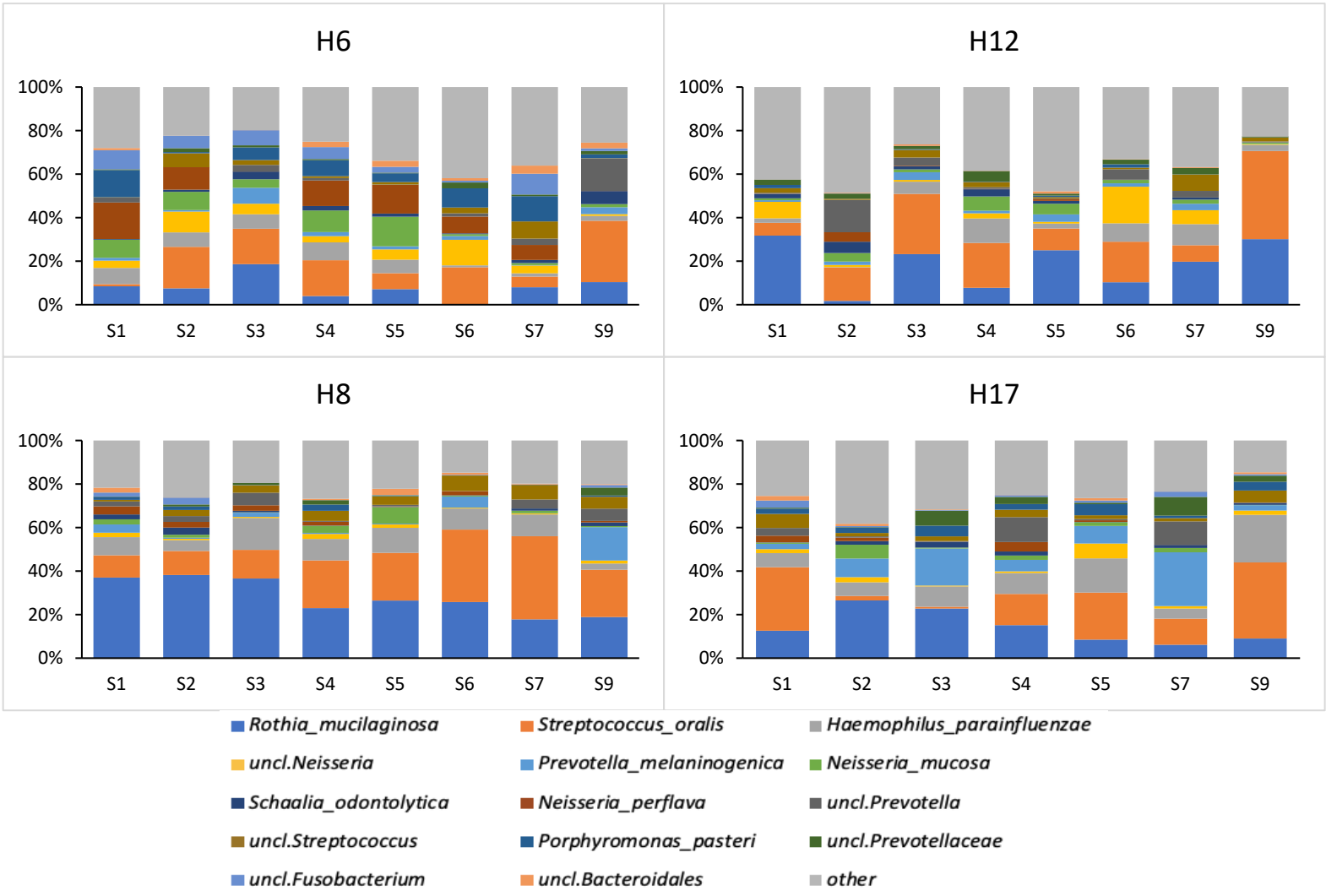


Figure 4-5: Comparison of bacterial composition at species level between two sibling pairs at all sampling points. A. Siblings H6 and H12. B. Siblings H8 and H17.

4.6 Alpha diversity

4.6.1 Rarefaction

Using the rarefaction curve, based on the abundance lineage table, species richness per sample was analysed. H2S7 shows the greatest species richness (223 species), followed by H6S9 (218 species) and H7S9 (215 species). On the other hand, H16S7, H4S and H8S6 proved to be the least rich samples, with 123, 130, 132 species observed, respectively (Figure 4-6).

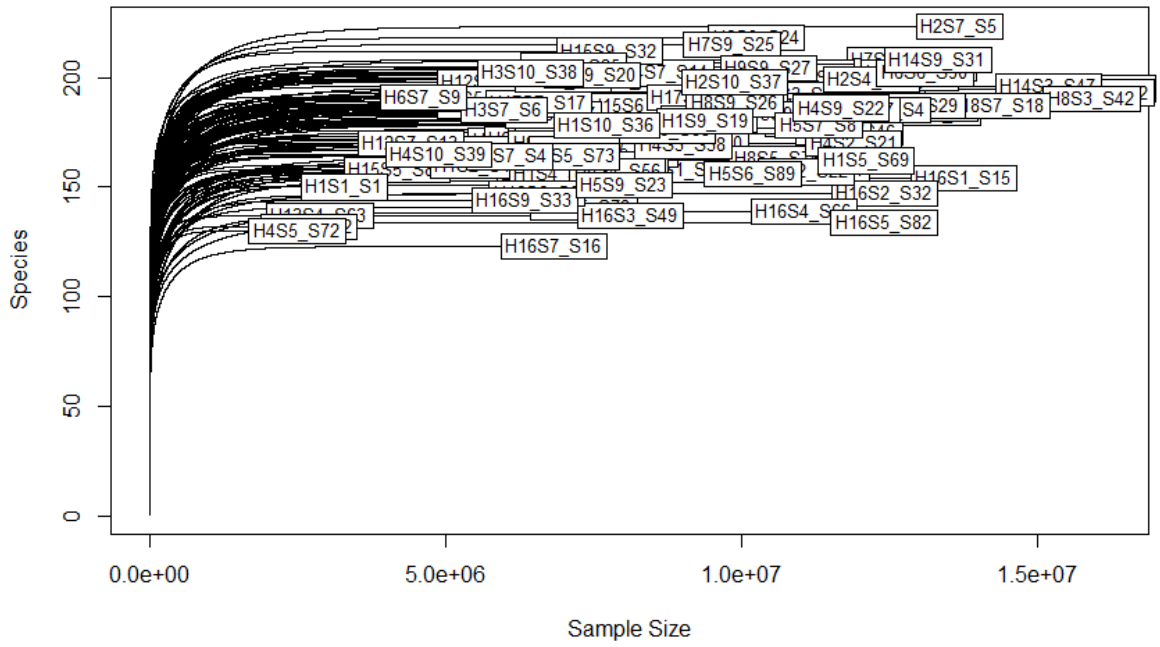
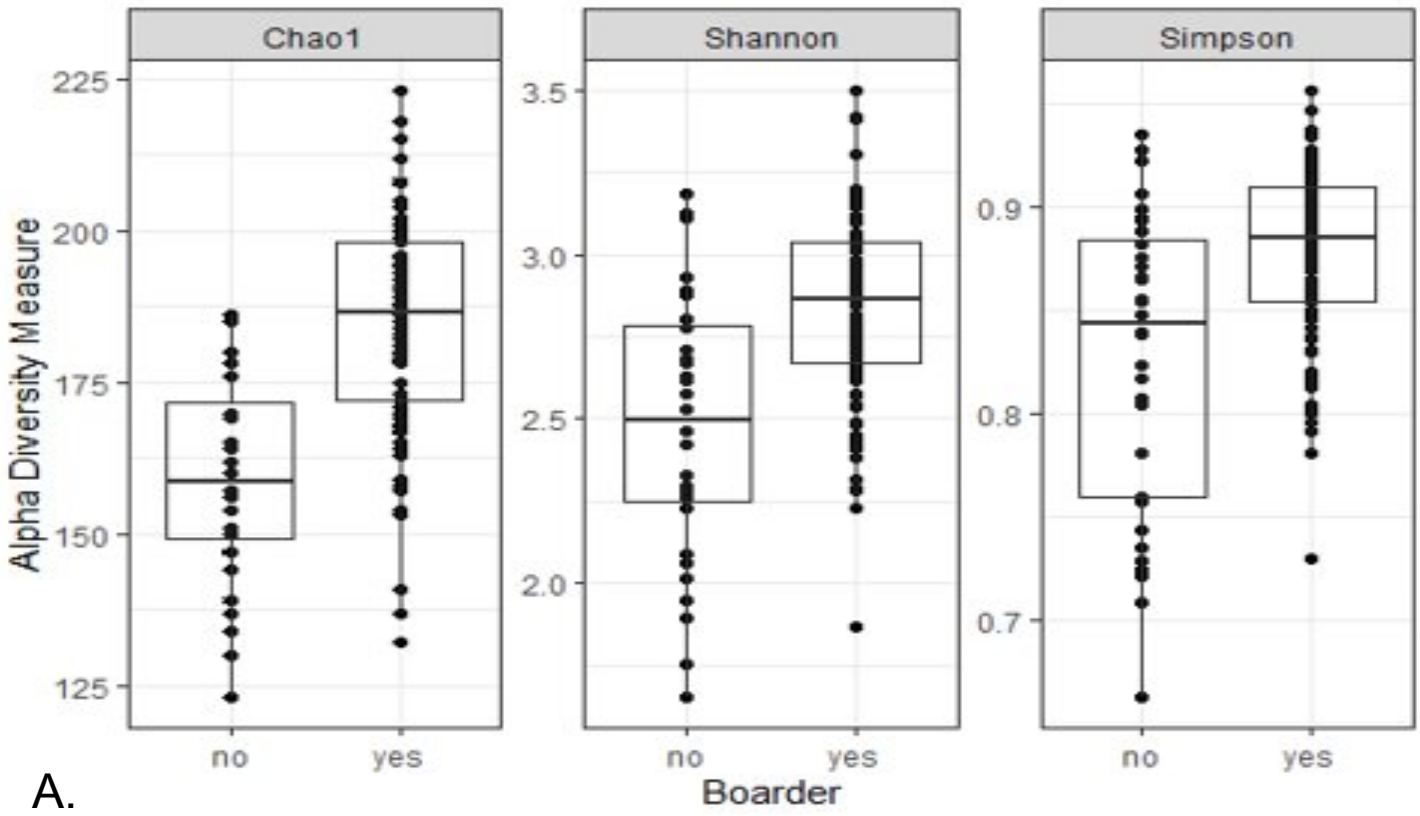


Figure 4-6: Rarefaction curve showing microbial species richness of all studied samples

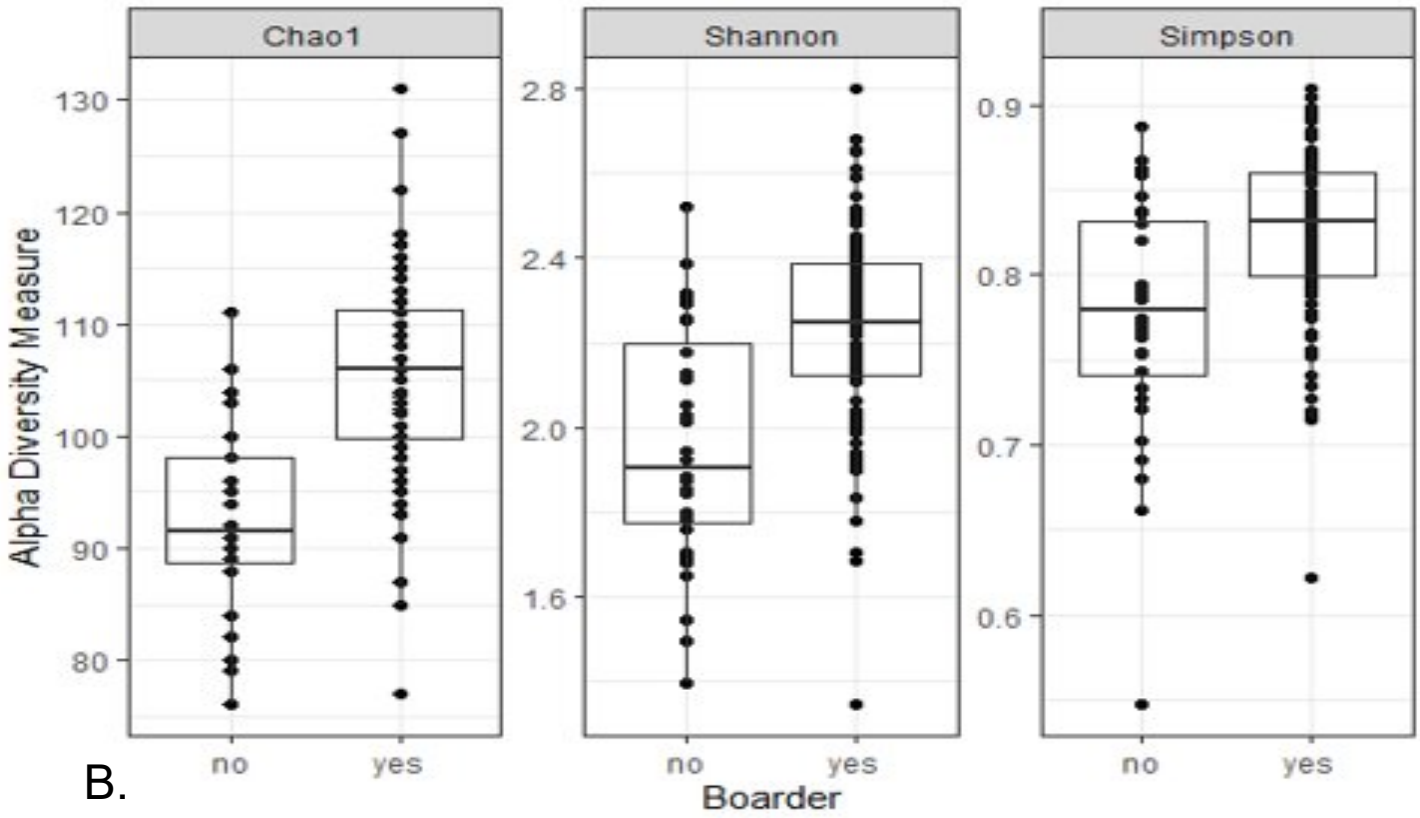
4.6.2 Alpha diversity between boarders and non-boarders

The Chao1, Shannon and Simpson's indices of biodiversity were calculated for each of the two groups of the study, boarders (yes) and non-borders (no). Alpha diversity was calculated based on raw lineage table at species level, lineage table at genus level and feature table (Figure 4-7). The pairwise comparison was performed using the Wilcoxon rank sum test with continuity correction. The p value was adjusted using the Holm method. There was statistically significant ($p < 0.01$) difference in the alpha diversity within the two groups, with higher diversity in boarder group. It was found that the group of boarders had significantly higher alpha diversity compared to the group of non-boarders.



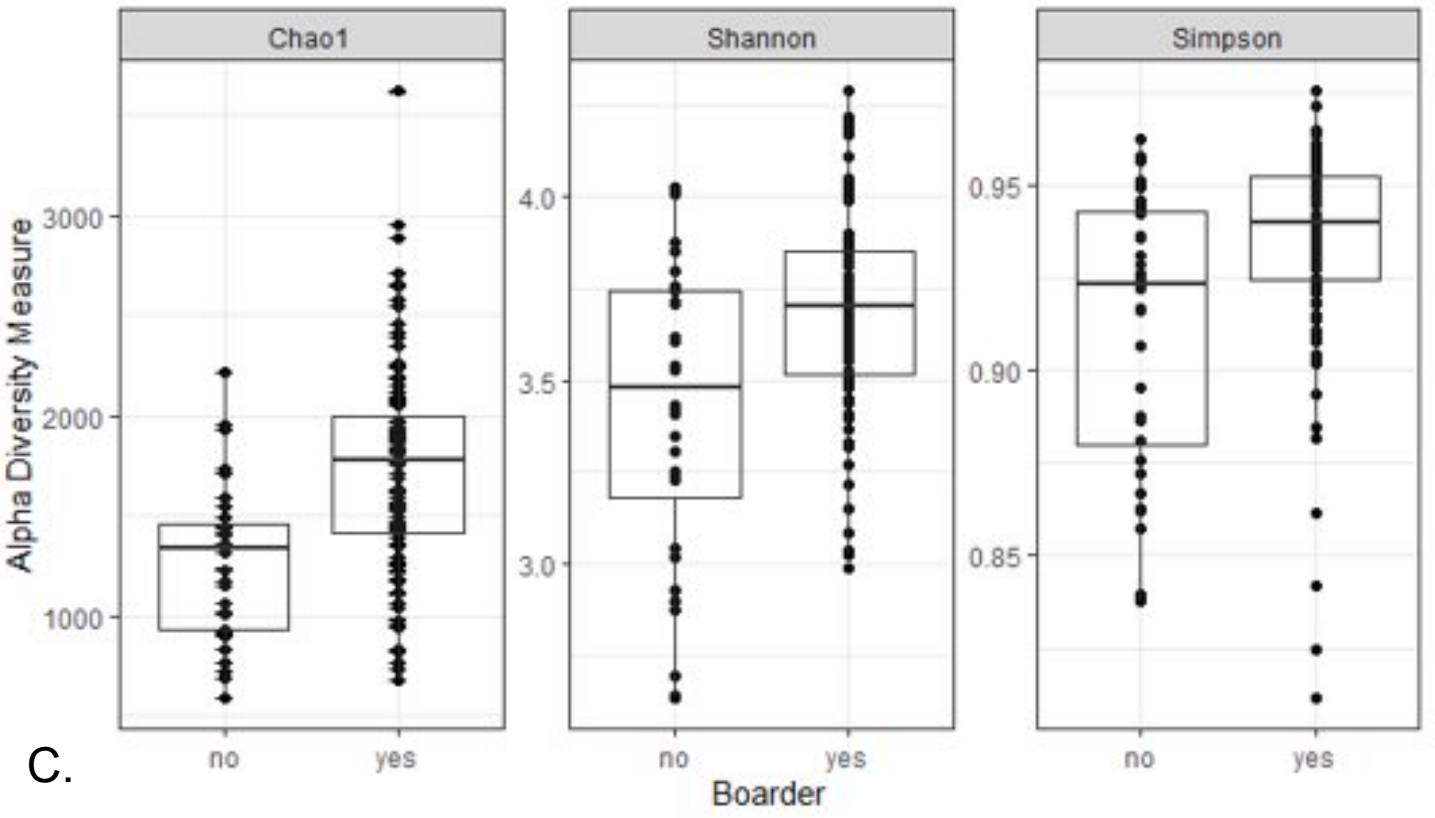
A.

p= 1.5^{-9} p= 1.3^{-5} p= 2.9^{-4}



B.

p= 1.1^{-9} p= 2.9^{-6} p= 2.4^{-4}



C.

p= 4.1^{-6} p= 1.1^{-3} p= 1.5^{-3}

Figure 4-7: Alpha Diversity between boarders (yes) and non-boarders (no) are depicted as box plots. A. Based on the raw lineage table at species level, B. Based on the raw lineage table at genus level, C. Based on the raw feature table. P-values is less than 0.01 suggesting that the null hypothesis is rejected, confirming that the samples of two studied populations that were randomly selected have different alpha diversity distributions.

4.6.3 Alpha diversity at S1 and S9

When comparing the alpha diversity in all samples during the first saliva collection point, on September 28, 2018 (S1) and the last, on June 20, 2019 (S9), there was no significant change of the oral microbiome diversity from the one point to the other ($p>0.01$). However, statistically significant difference ($p<0.01$) was observed in the Chao1 index within only the boarder group. Boarders seem to have a trend of increasing oral microbiome alpha diversity at S9 compared to S1. Comparing the difference in alpha diversity in the two groups from S1 to S9, Chao1 index for the

boarders showed significant diversity. Non-boarders, on the other hand, seemed to have no difference in the alpha diversity when comparing S1 to S9.

4.7 Beta diversity Non-Metric Multidimensional Scaling plots

Non-Metric Multidimensional Scaling (nMDS) plots were used to graphically represent the distance between the samples. Each sample is a point and the distance between the points represents the similarity of those samples. The closer the points are in the plot, the more similar the bacterial communities are.

4.7.1 Bray-Curtis similarity between boarders and non-boarders

In Figure 4-8 the beta diversity of the two studied, boarder (yes) and non-boarder (no), groups is demonstrated at all sampling points. Non-boarder participants were expected to not have a particular similarity in the diversity of their oral microbiome, whereas the opposite was expected for the boarders. It could be argued that there is a slightly higher concentration of boarder samples in the central area of the graph, however, there is no clear trend on the similarity of the boarder group that could indicate that participants on that group could share a more similar oral microbiome composition.

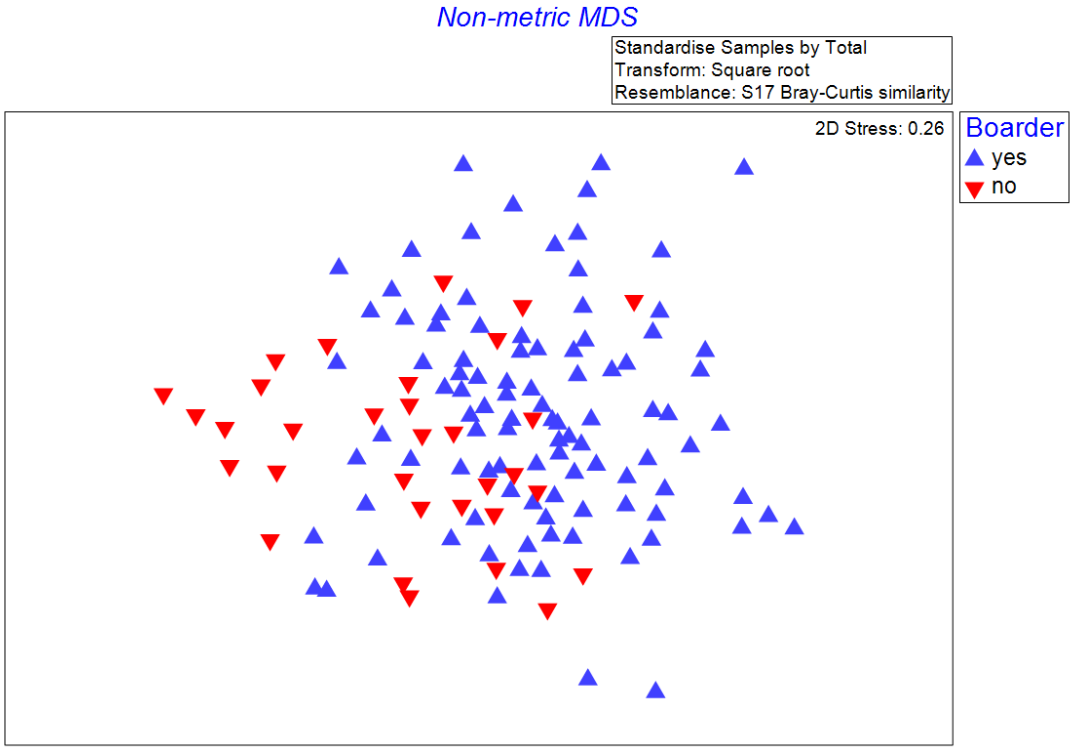


Figure 4-8: Beta Diversity (Bray-Curtis similarity) based on species level between boarders (B) / non- boarders (NB). The blue arrows, representing the boarder participants, are marginally concentrated in the central area of the graph. The red arrows, representing the non- boarder participants are widenly distributed in the graph.

4.7.2 Bray-Curtis similarity at S1, S3 and S9 between boarders and non-boarders

The graphs in Figure 4-9 show: in the blue marked area the majority of the samples at S1, excluding H6S1 and H14S1 and in the red marked area the majority of the samples at S9, excluding H7S9, in the boarder group. The marked area at S9 gets slightly wider. This indicates that the oral microbiome tends to become more dissimilar from S1 to S9, since the marked areas move apart. The oral microbiome of boarder participants did not seem to get more similar over the time period of ten months that was examined.

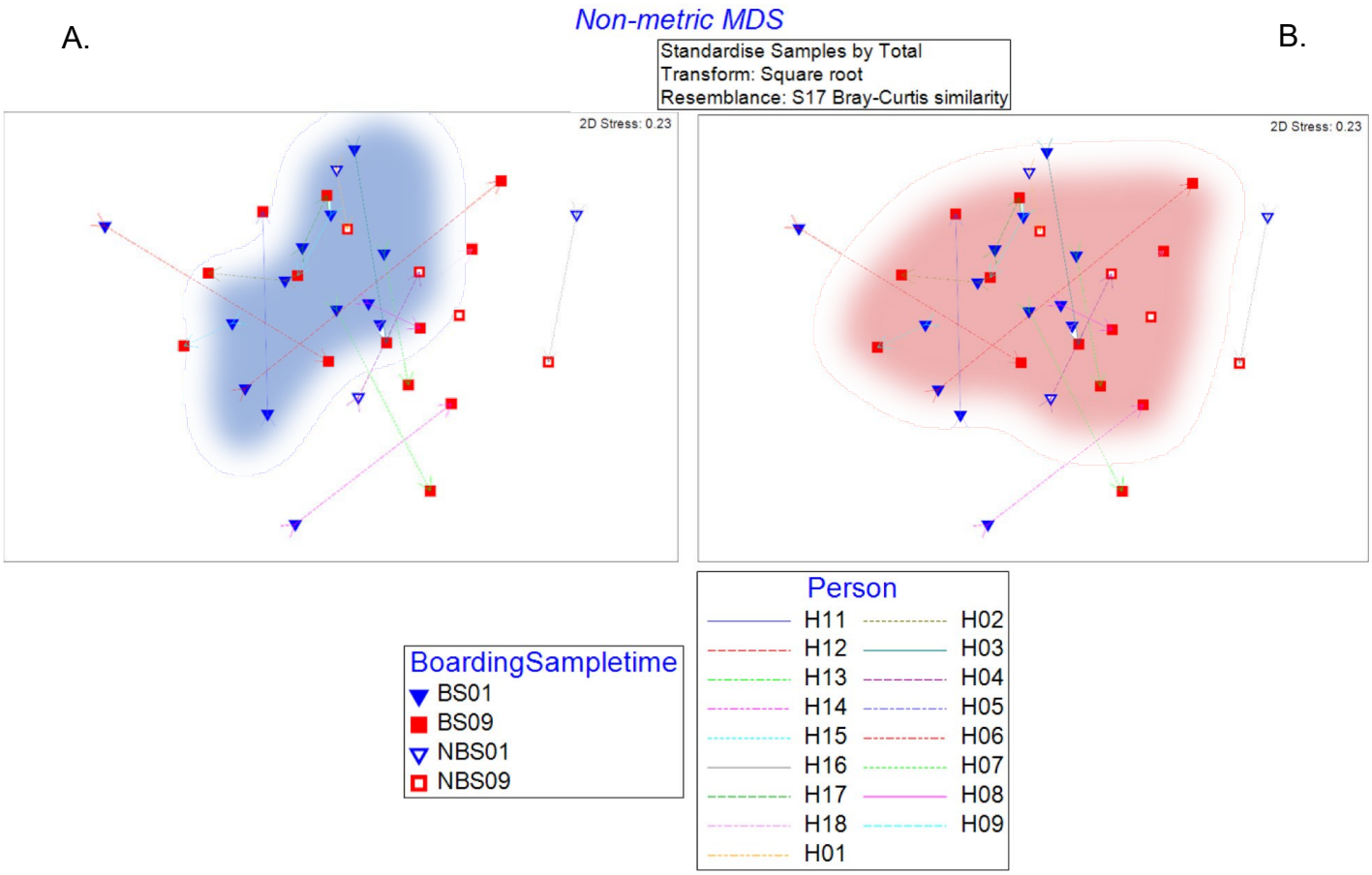


Figure 4-9: Beta Diversity (Bray-Curtis similarity) based on species level at S1 and S9 between boarders (B) / non- boarders (NB). A. Marked blue area involves the majority the BS01 samples. B. Marked red area involves the majority of BS09 samples. The marked area at B. gets slightly wider compared to A. This indicates that the oral microbiome tends to become more dissimilar from S1 to S9.

Boarders were travelling back to their homes at school holidays both at mid-terms and at the end of each term. The maximum days spent at school before sampling were 39 days, at S3. This is supported by Figure 4-10 where the majority of samples at S3, excluding H7S3, are included in the marked orange area. Samples of boarders at S3 form a tighter cluster than samples at S1 and S9, which are widely spread on the nMDS plot, thus, showing a similar oral bacterial community composition at S3.

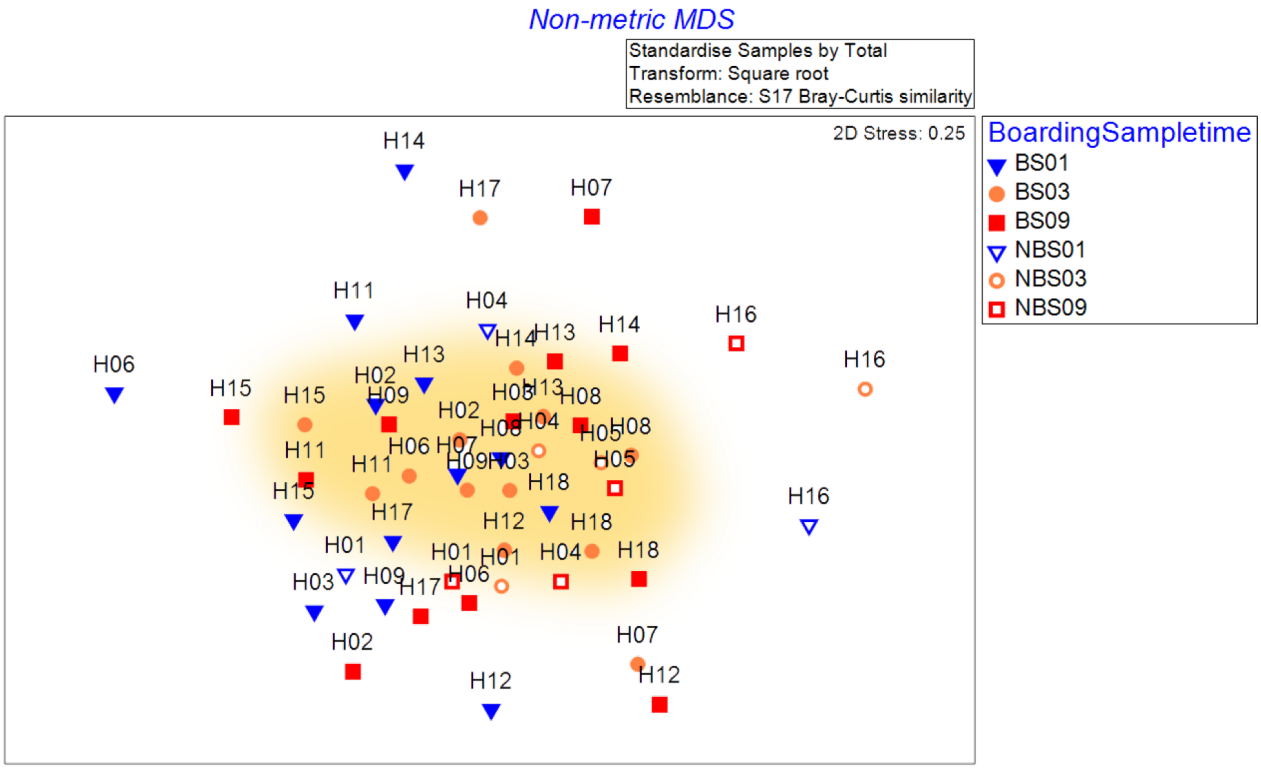


Figure 4-10: Beta Diversity (Bray-Curtis similarity) based on species level at S1, S3 and S9 between boarders (B) / non- boarders (NB). Marked orange area involves the majority of BS03 samples. Samples of boarders at S3 form a tighter cluster than samples at S1 (Figure 4-9,A) and S9 (Figure 4-9,B), which are widely spread on the nMDS plot, showing a more similar oral bacterial community composition at S3.

4.7.3 Jaccard calculator at S1 and S9 between boarders and non-boarders

In order to determine if the microbial composition changed either between boarders and non-boarders or S1 and S9 a Jaccard index was calculated. The Jaccard calculator is based on the presence or absence of species and does not include any abundance information. If therefore, reveals differences in microbial composition between samples.

The Figure 4-11 demonstrates that presence and absence of species from S1 to S9 for both boarders and non-boarders shifts to one direction. This means that both groups have similar gain or loss of bacterial species. Species that were not identified at S1, such as *Peptostreptococcaceae incertae sedis* (from 0% at S1 to 0.00017% at S9)*, Clostridium sensu stricto* (from 0% to 1.26685 E-05) and *Turicimonas muris* (from 0% at S1 to 7.92867 E-05 at S9) were evident at S9, but in very low mean abundances. Furthermore, all boarders started from similar points (marked darker blue area) and moved to again similar points (marked dark red area), excluding H2S9. On the other hand, non-boarders highlighted with yellow, although they had a similar trend they ended up in significantly further apart on the plot.

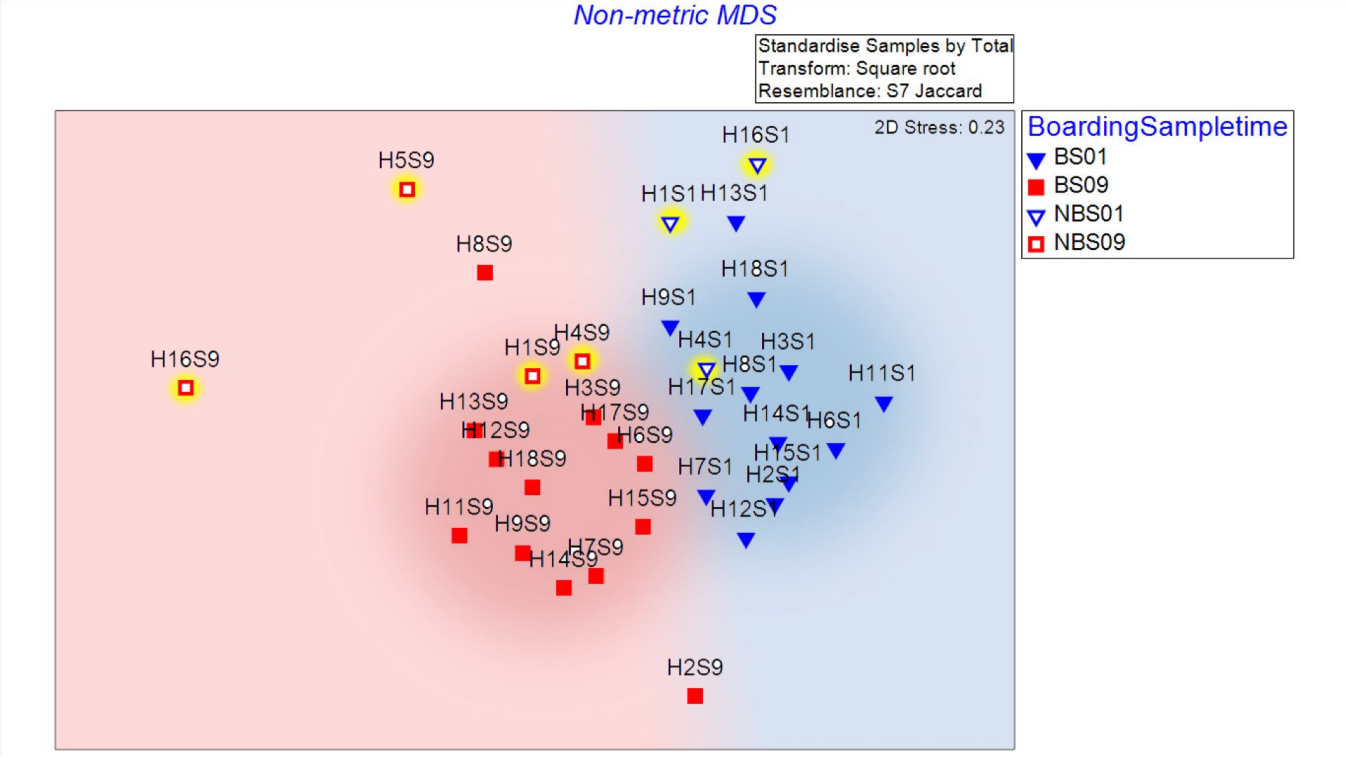


Figure 4-11: Beta diversity (Jaccard calculator) based on species level at S1 and S9 between boarders (B) / non- boarders (NB). Marked blue area involves all samples at S1. Dark blue area involves all the BS01 samples. Marked red area involves all the samples at S9. Dark red area involves the majority of BS09 samples. NB samples at S1 and S9 are highlighted with yellow. This graph demonstrates that presence and absence of species from S1 to S9 for both boarders and non-boarders shifts to one direction. Species that were not identified at S1 were evident at S9. All boarders started from similar points (marked darker blue area) and moved to again similar points (marked dark red area). Non-boarders highlighted with yellow, although they had a similar trend they ended up in significantly further apart on the plot.

4.7.4 Comparison of bacterial diversity between two sibling pairs (H6,12 and H8,17)

As already described, H6 is a sibling of H12 and H8 of H17. It was therefore of interest to investigate the bacterial diversity between these siblings over the course of the school year. Beta diversity (Bray-Curtis similarity) was calculated to compare only these two pairs of siblings at all sampling points, as shown in Figure 4-12. S1 for H6 and H12 are represented by distant points on the graph, suggesting dissimilarity in the oral microbiome composition. The same pattern is followed through time from S2 to S9, with composition at S3 showing the closest similarity. On the other hand, H8 and H17, present with a closely related oral microbiome diversity at the beginning of sampling, at S1 and at the end, at S9. The composition in middle time points, however, shows greater dissimilarity compared to S1 and S9. As, due to the small number of sibling pairs (n=2), there is no clear trend noted in the similarity of beta diversity of genetically related participants of this study.

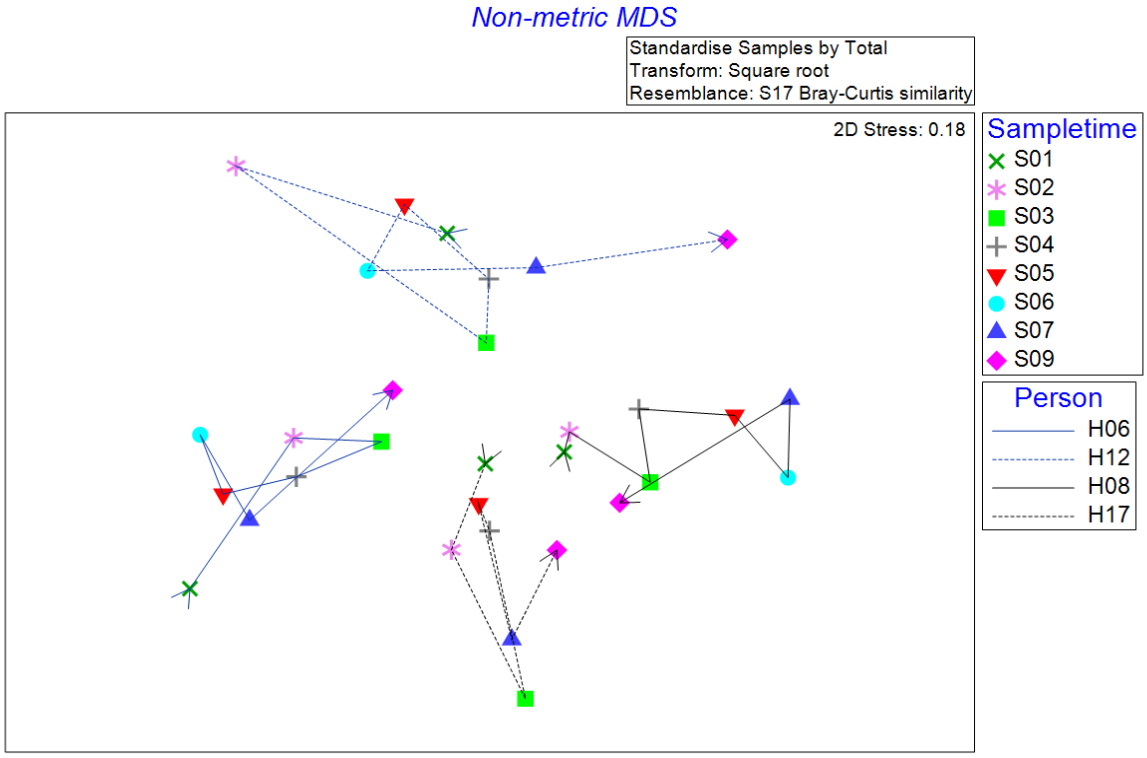


Figure 4-12: Beta Diversity (Bray-Curtis similarity) based on species level at all sampling points between two pairs of siblings (H6, H12 and H8, H17). S1 for H6 and H12 are represented by distant points on the graph, suggesting dissimilarity in the oral microbiome composition. The same pattern is followed through time from S2 to S9, with composition at S3 showing the closest similarity. H8 and H17, present with a closely related oral microbiome diversity at the beginning of sampling, at S1 and at the end, at S9. The composition in middle time points shows greater dissimilarity compared to S1 and S9.

5 Discussion

Comprehensive investigation of the composition of the oral microbiome of adolescents is of great importance for understanding not only the normal bacterial ecosystem of the oral cavity at this age, but also to make any possible associations with potential causes of disturbance to these normal communities. Gaining a greater understanding of how the microbiome forms and adapts during development will improve our understanding of disease mechanisms and develop prevention and treatment strategies. The oral microbial ecosystem of adolescents has been shown to be different and more diverse compared to the adult oral microbiome (Burcham et al., 2020).

The composition of the oral microbiome of children and adolescents has mainly been studied in relation to dental caries and periodontal health (Johansson et al., 2016), but not involving any shared environment elements. Shared genetics was traditionally thought to have a major role in the shaping of oral microbiome (Tierney et al., 2019). There are a number of published studies trying to correlate the genetic information and the environment with the oral microbiome. A study was made to compare the salivary microbiome of twins. The authors concluded that the oral microbiome was not significantly different in monozygotic and dizygotic twins. Also, the similarities started to decrease when the twins stopped sharing the same house, suggesting the importance of shared household rather than genetics (Stahringer et al., 2012), (Freire et al., 2020). However, evidence raised from recent studies has highlighted the influence of a shared living environment in the formation of the oral microbiome composition. This is now believed, to potentially have greater value than the shared genetics (Shaw et al., 2017a) (Song et al., 2013). A recently published study demonstrated the results of investigating the oral microbiome of mothers, both biological and adoptive and their children. Children were aged from three months old to 12 years old. The authors concluded that the mechanisms that are forming the oral microbiome are common among humans and not affected by the genetic relationship but from the contact between individuals and their shared environment (Mukherjee et

al., 2021). However, how the shared environmental component affects the composition of the oral microbiome of children, is still a research field that is not well investigated.

To our knowledge this is the first study in which the adolescents' oral microbiome was analysed over a period of ten months, involving a cohort of boarder and non-boarder students. This cohort gave the opportunity to investigate whether the component of the "shared household environment" was of any influence on the composition of the oral microbiome.

Oral health

The findings from our study demonstrate that the caries experience noted in the studied population was different, and lower than the general population, of the same age, as measured by the (CDH) Survey (PHE, 2013). Since the cohort of our study has a mean of 12.23 years of age, comparisons can be made with the age group of 12 years from the CHD survey. Findings of CHD survey reported that 34% of the 12-year-old children are affected by dental caries, with a mean number of teeth affected at 0,8 and a mean number of teeth affected in children with caries at 2.5. Furthermore, unrestored carious permanent teeth were 19%. In the present study, only one child had a single restoration in a permanent tooth, representing 5.8% of the cohort. None of the participants had caries extending to dentine. Although, it is obvious that our relatively small sample size (n=17) cannot conclude to any statistically significant differences, our findings could be a strong indicator supporting another aspect of the CHD survey, focusing on the socioeconomic aspect of dental caries. CHD survey demonstrated that children coming from lower income families, measured by their right to access free school meals, are more likely to suffer oral disease. The socioeconomic impact in the development of dental caries has been thoroughly studied in many countries around the world, including Sweden (André Kramer et al., 2018), the United Stated of America (Caplan and Weintraub, 1993), Abu Dhabi (Elamin et al., 2018) and Greece (Gizani et al., 2009), all concluding that economic deprivation is associated with higher caries incidence. Furthermore, children coming from low-income families are more likely not to be brought to their dental checkup appointments and to brush their teeth less than twice daily. The only participant (H2) who had localised enamel

breakdown due to caries with no visible dentine affecting two first molar teeth and had an existing restoration of their upper right first permanent molar, had a very low mean abundance of *Streptococcus mutans* (0.0005%) even lower compared to the mean abundance of the rest of the cohort (0.00186%). These findings, support the multibacterial aetiology of caries and the “Ecological plaque hypothesis” (Takahashi and Nyvad, 2011) rather than the “Specific plaque hypothesis” (Emilson and Krasse, 1985). This is also consistent to the findings of a recent study which suggests that there is no association with cariogenic bacteria and the incidence of dental caries (Inquimbert et al., 2019). In regard to the oral hygiene status of the cohort of our study, this is again superior compared to general population of the same age group in the United Kingdom. Participants of our study had an average plaque score of 0.125, consistent with gingival health. On the contrary, two thirds of the 12 year old British children suffer from gingival inflammation (White et al., 2006).

Most abudant phyla

Observed prevalence at phylum level showed some inconsistency compared with previous studies. Comparing the most abundant phyla of the sample of this study with the most abundant phyla as established by the core oral microbiome (Dewhirst et al., 2010b), both agree that Firmicutes is the most abundant phylum. The core oral microbiome suggests that the second most abundant phyla is Bacteroidetes (17.3%), but in this study this phylum was found to be the fourth commonest (16.11%). A larger proportion of Actinobacteria (25.62%) was detected in the cohort of this study compared to the core oral microbiome (11.6%). Proteobacteria was the third most abundant phylum in both. Fusobacteria was the least common amongst the most abundant phyla. Spirochaetes was found to be the fifth commonest phylum in the core oral microbiome (7.9%), however in this study this phylum counted only for 0.0017% of the total composition.

Most abudant genera

The most dominant genus detected in the adult oral microbiome is *Streptococcus* (26.9%), followed by *Veillonella* (9.8%). The rest genera comprise less than 5.0% of the total abundance (Costalonga and Herzberg, 2014). In our study, with a cohort comprising of adolescents, the vast majority of observed genera belong to *Rothia*

(21.96%), followed by *Streptococcus* (21.61%) and *Neisseria* (11.62%). These findings support that the oral microbiome at the studied age is not yet fixed and continues to undergo significant alteration until adulthood (Kaan et al., 2021). *Prevotella* and *Veillonela* have been associated with periodontal disease and Neisseria with periodontal health (Yamashita and Takeshita, 2017). *Neisseria* was found to have relatively high mean abundance and *Veillonela* low, compared to adult individuals, confirming a pattern of a healthier periodontium in our cohort. Interestingly, *Prevotella* was found in higher abundance compared to adults (10.1% compared to 2.7%).

Alpha diversity

Studying the alpha diversity of the two groups, it was found that the group of boarders had significantly higher alpha diversity compared to the group of non-boarders. Additionally, there was statistically significant higher diversity of the boarders at the end of the sampling period compared to the beginning. This did not apply in the non-boarder group. Boarders having a more diverse oral microbiome composition, could be a result of frequent changes of their environment, changing between school environment, shared with other co-boarder students, during terms and home environment during holiday periods. Another possible explanation for this, could be that the boarder group is comprising of an international mixture of Croatian, Briton, Indian, Malaysian and German students. Studies have demonstrated that different ethnic group variations are associated with the presence of specific bacterial taxa (Gupta et al., 2017). Thus, shared school environment could potentially result in the mutual exchange of ethnicity-specific taxa, forming a more diverse oral microbiome in the boarder group. However, this could also be a result of more participants being in the boarder than the non-boarder group (n=13 and n=4).

Beta diversity

Investigation of the beta diversity revealed that, on the contrary, of what was expected, the oral microbiome of boarders is not more similar than the non-boarders and also tends to became more dissimilar at the end of the ten-month sampling period compared to the beginning. These findings imply that there must be other contributing factors that shape the oral microbiome composition more than the shared

environment. These other factors could include diet habits, stress, BMI, puberty and hormonal changes, smoking and the saliva composition. It could also be argued that the time of the shared school environment was not enough to alter the composition of the oral microbiota as boarders were frequently travelling back to their home environment during school holidays. The maximum days spent at school before sampling were 39 days. At that point boarders formed a tighter cluster, and shared a more similar oral microbiome composition. The analysis of presence and absence, revealed that both groups had similar gain or loss of bacterial species. However, only the boarders who were sharing the same school environment ended to closer points in the nMDS plot, unlike the non-boarders who ended up in significantly apart points in the plot, possibly affected individually from their own home environments. Interestingly, the comparison of the bacterial diversity between two sibling pairs showed no clear oral microbiome similarity, even though these participants always shared the same living environment, either their home during holidays or the school campus during terms and were also genetically related. All four individuals forming the two pairs are males, but one sibling of each group is in year 7 at school and the other in year 9. Siblings being in different school years could potentially explain their oral microbiome composition dissimilarities, as they had minimal interaction during school term time. Although, this is a small number of sibling pairs (n=2), these findings could indicate that neither the shared environment (Shaw et al., 2017a) nor the genetic component (Demmitt et al., 2017) are the main factors shaping the oral microbiome composition of adolescents. All things considered, the findings from our study show that the oral microbiome of adolescents does not remain stable within the studied period of one academic year, unlike microbiome of adults which is shown to be relatively stable from several months up to a couple of years (Utter et al., 2016). However, it is not clear, either from our study, or the reported literature exactly how many days are required for the oral microbiome of adolescents to transiently or permanently change.

Study limitations

Methodology

Considering our study design and methodology retrospectively we found some weak points that should be highlighted and improved, in order to provide better quality future

results. First of all, we concluded that the sampling points through the academic year of 2018-2019 were relatively close to one another. Secondly, the exact dates of sampling should have been calculated more precisely, aiming to represent longer period of shared school environment for the border group. To optimise the study design saliva samples collection should have been performed in the beginning (September), middle (February/ March) and end of the school year (June).

In this study the pH of the salivary samples was not determined, even though differences in the pH could contribute to different oral microbiome composition.

Regarding the cohort of the study, first of all, the number of participants is relatively small and secondly, the two groups were not equally distributed. Boarders are dominating. On one hand, this is normal as the majority of the students at Haileybury are boarders. On the other hand, this numeric discrepancy could skew the results of the study when comparisons were made between the two grouped populations. Therefore, interpreting the results was particularly challenging. Differences and trends observed for different aspects, as in detailed outlined in the Results chapter, could not confidently be attributed to specific factors, such as demographic characteristics (age, gender etc.) or boarding status. For instance, the higher alpha diversity of the boarders compared to the non-boarders, could potentially be related to the discrepancy of the number of participants within the two groups and not to the fact that boarders have a constant change in their living environment. A richer cohort, comprising of equally assigned group members, would provide more statistically significant results and more confident conclusions. Similarly, demographic associations would be stronger and would allow more comparisons, meaning that the information obtained from the oral health questionnaire would be of greater research value. Oral health questionnaires could also be enriched to include the participants' diet habits, if for example any of the participants were following a vegan or vegetarian diet or even culture based eating habbits. Additionally, information on height and weight and subsequently the BMI of participants would be of great value. The questionnaires could also provide useful information regarding participant's habbits, such as smoking, nail biting and additional facts concerning personal hygiene, like frequency of hand washing.

Using the Swift Amplicon 16S+ITS Panel by Swift Biosciences to analyse our samples, gave us the opportunity not only to sequence all the hypervariable regions of the 16S rRNA gene and obtain a rich dataset to investigate, but also to obtain information on the mycobiome of the same population at the same time. However, the Swift 16S SNAP-APP bioinformatics analysis pipeline that was used, is not broadly used and different issues were faced during the bioinformatics analysis. We found the pipeline to be relatively slow when running many samples at the same time, without the option of continuing unfinished processes at a later time. The workflow was trained to assign sequences at a genus level and a test version had be used in order to allow species level classification and analysis.

Impact of COVID-19

All things consider, it is very important to highlight the significant impact that the outburst of COVID-19 had on this study. In September 2019, during the beginning of the second year of the study, 9 more students were recruited, from which 2 are non-boarders and 7 boarders, 4 females and 5 males, with an average age of 13.8 years. However, on January 2020 the WHO acknowledged the COVID-19 as a Public Health Emergency of International Concern and on 11 March 2020 COVID-19 was declared a pandemic. University College London (UCL) classified saliva as category 3 biosafety hazard (Cat 3), which means that it must be handled in a biosafety level 3 facility (Cat 3 facility) (Table 5-1). Biosafety level 3 (BSL-3) is applicable to clinical, diagnostic, teaching, research, or production facilities where work is performed with agents that may cause serious or potentially lethal disease through inhalation, to the personnel, and may contaminate the environment. This practically meant that we were not able to collect saliva or use it from any person unless they had a negative qPCR test, as we had no access to any Cat 3 facilities to store and process the saliva samples. Furthermore, boarders had to return to their families as Haileybury was closed. Consequently, the sampling had to be interrupted and no further saliva samples were collected after December 2019. Unfortunately, this had multiple effects on this study. Firstly, saliva samples that were already collected could not be processed, either at DNA extraction level or PCR and sequencing, or even both and subsequently are not included in the results of this study. This included samples 39 (H5S10 to H18S10, H1S11 to H18S11, H19S1 to H27S1, H19S2 to H27S2). Secondly, the new cohort of

participants could also not be included. This resulted in a significant smaller number of participants than initially planned, 17 instead of 26 that were actually recruited. But most importantly, the follow up period of the study reduced from two years to just one year. This disturbance, affected not only the aims of this study but also the quality and significance of the results.

Table 5-1: Containment level according to sample type

Sample Type	Containment Level of Respiratory Samples
Confirmed or presumptive COVID-19 positive samples	Level 3
Samples from people of unknown status	Level 3
Convalescent or recovered COVID-19 patient samples (without negative test results)	Level 2 (If >42 days after onsent of symptoms AND if asymptomatic)
Confirmed COVID-19 negative samples	Level 2
Inactivated samples	Level 2

6 Conclusion and Future work

In this study, 135 saliva samples of 17 participants were collected at eight different time points from September 2018 to June 2019 and analysed. The cohort comprised of 4 non-boarders and 13 boarders, 4 females and 13 males, with an average age of 12.23 years. 12 participants were Britons, 2 sibling participants were coming from Croatia, one from India, one from Malaysia and one from Germany. 14 participants owned pets and 15 were involved in sports activities and all of them were using a mouthguard. All the participants stated that they brush their teeth twice daily, 35.29% were using mouthwash and 23.52% were having fixed orthodontic appliances. No participant had a false tooth or using a denture to replace a missing tooth. 11.76% were consuming probiotic drinks. Medically, one participant had asthma and three had eczema. Clinical examination revealed that three teeth had initial caries all detected in first permanent molar teeth, two upper and one lower, in three different participants. Localised enamel breakdown due to caries with no visible dentine affected two first molar teeth of the same individual. The same participant was the only one with an existing restoration of their upper right first permanent molar. No teeth with either underlying dark shadow from dentine or distinct or extensive cavity with visible dentine were detected. The average plaque score of all participants was 0.125. The PUFA index revealed one single ulceration lesion present in the oral mucosa.

Five major phyla predominated in both groups; *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, *Fusobacteria*, forming 99.37% of the total phyla observed. Taxonomic analysis at genus level revealed that *Rothia* was the most abundant genera, followed by *Streptococcus*, *Neisseria, Haemophilus* and *Prevotella*. The most abundant species identified amongst all the participants and all sampling points are *Rothia mucilaginosa*, followed by *Streptococcus oralis* and *Haemophilus parainfluenzae*. Boarders showed a trend of increasing abundance of *Streptococcus oralis* and unclassified *Prevotella* through sampling time. Non-boarder's oral microbiome composition showed an increase in the mean abundance of *Rothia mucilaginosa* and a decreasing trend of *Haemophilus parainfluenzae*, *Neisseria perflava* and unclassified *Prevotella* from the first to the last sampling point of the ten-month observed period. Boarders had significantly higher alpha diversity compared to

the group of non-boarders. Within the boarder participants there was statistically significant difference in the alpha diversity at the last sampling point compared to the first, this was not valid for the non-boarders. The beta diversity of the boarder group revealed no clear trend on the similarity of the oral microbiome that could indicate that participants on that group could share a more similar oral microbiome composition. However, it was noted that following spending more days at school the oral microbiome composition of boarder students tends to become more similar. The comparison of bacterial diversity between two sibling pairs showed no clear similarity of oral microbiome diversity of the genetically related participants of this study.

This is an ongoing project and the presentation of the results for the mycobiome findings, of the same samples presented in this study, is a work in progress. Furthermore, our research team has already formulated an action plan for the next academic year. As mentioned, it is planned to limit sampling times to three per year, beginning middle and end. Strengthening the cohort with new recruits, targeting non-boarders and possibly female participants, has been prioritised, in order to enhance the dataset available. A second school is aimed to be included in the study. This will be a state school linked with Haileybury. This step will allow us to assess whether the results are reproduceable, but also could produce data that could be used to demonstrate changes in the species linked to environmental effects. Students from different schools could also show similar changes in the diversity of oral microbiome, but these could differ between the two sites. Finally, it would be interesting to perform metagenomic analysis to look for transfer of AMR genes and transposable elements.

7 References

ANDRÉ KRAMER, A. C., PETZOLD, M., HAKEBERG, M. & ÖSTBERG, A. L. 2018. Multiple Socioeconomic Factors and Dental Caries in Swedish Children and Adolescents. *Caries Res,* 52**,** 42-50.

ARMITAGE, G. C. 1999. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol,* 4**,** 1-6.

ATKINSON, M. A., EISENBARTH, G. S. & MICHELS, A. W. 2014. Type 1 diabetes. *The Lancet,* 383**,** 69-82.

BAKER, J. L., BOR, B., AGNELLO, M., SHI, W. & HE, X. 2017. Ecology of the Oral Microbiome: Beyond Bacteria. *Trends Microbiol,* 25**,** 362-374.

BARKHORDAR, R. A., HAYASHI, C. & HUSSAIN, M. Z. 1999. Detection of interleukin-6 in human dental pulp and periapical lesions. *Endod Dent Traumatol,* 15**,** 26-7.

BISHOP, Ö. T. & BISHOP, Ö. T. 2014. *Bioinformatics and data analysis in microbiology / edited by Özlem Taştan Bishop*, Norfolk, England : Caister Academic Press.

BOLYEN, E., RIDEOUT, J. R., DILLON, M. R., BOKULICH, N. A., ABNET, C. C., AL-GHALITH, G. A., ALEXANDER, H., ALM, E. J., ARUMUGAM, M., ASNICAR, F., BAI, Y., BISANZ, J. E., BITTINGER, K., BREJNROD, A., BRISLAWN, C. J., BROWN, C. T., CALLAHAN, B. J., CARABALLO-RODRÍGUEZ, A. M., CHASE, J., COPE, E. K., DA SILVA, R., DIENER, C., DORRESTEIN, P. C., DOUGLAS, G. M., DURALL, D. M., DUVALLET, C., EDWARDSON, C. F., ERNST, M., ESTAKI, M., FOUQUIER, J., GAUGLITZ, J. M., GIBBONS, S. M., GIBSON, D. L., GONZALEZ, A., GORLICK, K., GUO, J., HILLMANN, B., HOLMES, S., HOLSTE, H., HUTTENHOWER, C., HUTTLEY, G. A., JANSSEN, S., JARMUSCH, A. K., JIANG, L., KAEHLER, B. D., KANG, K. B., KEEFE, C. R., KEIM, P., KELLEY, S. T., KNIGHTS, D., KOESTER, I., KOSCIOLEK, T., KREPS, J., LANGILLE, M.

G. I., LEE, J., LEY, R., LIU, Y. X., LOFTFIELD, E., LOZUPONE, C., MAHER, M., MAROTZ, C., MARTIN, B. D., MCDONALD, D., MCIVER, L. J., MELNIK, A. V., METCALF, J. L., MORGAN, S. C., MORTON, J. T., NAIMEY, A. T., NAVAS-MOLINA, J. A., NOTHIAS, L. F., ORCHANIAN, S. B., PEARSON, T., PEOPLES, S. L., PETRAS, D., PREUSS, M. L., PRUESSE, E., RASMUSSEN, L. B., RIVERS, A., ROBESON, M. S., 2ND, ROSENTHAL, P., SEGATA, N., SHAFFER, M., SHIFFER, A., SINHA, R., SONG, S. J., SPEAR, J. R., SWAFFORD, A. D., THOMPSON, L. R., TORRES, P. J., TRINH, P., TRIPATHI, A., TURNBAUGH, P. J., UL-HASAN, S., VAN DER HOOFT, J. J. J., VARGAS, F., VÁZQUEZ-BAEZA, Y., VOGTMANN, E., VON HIPPEL, M., WALTERS, W., et al. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol,* 37**,** 852-857.

BONDER, M. J., KURILSHIKOV, A., TIGCHELAAR, E. F., MUJAGIC, Z., IMHANN, F., VILA, A. V., DEELEN, P., VATANEN, T., SCHIRMER, M., SMEEKENS, S. P., ZHERNAKOVA, D. V., JANKIPERSADSING, S. A., JAEGER, M., OOSTING, M., CENIT, M. C., MASCLEE, A. A., SWERTZ, M. A., LI, Y., KUMAR, V., JOOSTEN, L., HARMSEN, H., WEERSMA, R. K., FRANKE, L., HOFKER, M. H., XAVIER, R. J., JONKERS, D., NETEA, M. G., WIJMENGA, C., FU, J. & ZHERNAKOVA, A. 2016. The effect of host genetics on the gut microbiome. *Nat Genet,* 48**,** 1407-1412.

BOSCH, J. A., TURKENBURG, M., NAZMI, K., VEERMAN, E. C., DE GEUS, E. J. & NIEUW AMERONGEN, A. V. 2003. Stress as a determinant of saliva-mediated adherence and coadherence of oral and nonoral microorganisms. *Psychosom Med,* 65**,** 604-12.

BRADING, M. G. & MARSH, P. D. 2003. The oral environment: the challenge for antimicrobials in oral care products. *Int Dent J,* 53**,** 353-62.

BURCHAM, Z. M., GARNEAU, N. L., COMSTOCK, S. S., TUCKER, R. M., KNIGHT, R. & METCALF, J. L. 2020. Patterns of Oral Microbiota Diversity in Adults and Children: A Crowdsourced Population Study. *Sci Rep,* 10**,** 2133.

CALLAHAN, B. J., MCMURDIE, P. J., ROSEN, M. J., HAN, A. W., JOHNSON, A. J. A. & HOLMES, S. P. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nature methods,* 13**,** 581-583.

CAPLAN, D. J. & WEINTRAUB, J. A. 1993. The oral health burden in the United States: a summary of recent epidemiologic studies. *J Dent Educ,* 57**,** 853-62.

CAUFIELD, P. W., CUTTER, G. R. & DASANAYAKE, A. P. 1993. Initial acquisition of mutans streptococci by infants: evidence for a discrete window of infectivity. *J Dent Res,* 72**,** 37-45.

CEPHAS, K. D., KIM, J., MATHAI, R. A., BARRY, K. A., DOWD, S. E., MELINE, B. S. & SWANSON, K. S. 2011. Comparative Analysis of Salivary Bacterial Microbiome Diversity in Edentulous Infants and Their Mothers or Primary Care Givers Using Pyrosequencing. *PLOS ONE,* 6**,** e23503.

CHEN, T., YU, W.-H., IZARD, J., BARANOVA, O. V., LAKSHMANAN, A. & DEWHIRST, F. E. 2010. The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. *Database,* 2010.

CHU, D. M., MA, J., PRINCE, A. L., ANTONY, K. M., SEFEROVIC, M. D. & AAGAARD, K. M. 2017. Maturation of the infant microbiome community structure and function across multiple body sites and in relation to mode of delivery. *Nature Medicine,* 23**,** 314.

COCHRAN, D. L. 2008. Inflammation and bone loss in periodontal disease. *J Periodontol,* 79**,** 1569-76.

COLE, J. R. & TIEDJE, J. M. 2014. History and impact of RDP: a legacy from Carl Woese to microbiology. *RNA Biol,* 11**,** 239-43.

COSTALONGA, M. & HERZBERG, M. C. 2014. The oral microbiome and the immunobiology of periodontal disease and caries. *Immunol Lett,* 162**,** 22-38.
COTTI, E., DESSÌ, C., PIRAS, A. & MERCURO, G. 2011. Can a chronic dental infection be considered a cause of cardiovascular disease? A review of the literature. *International Journal of Cardiology,* 148**,** 4-10.
DARVEAU, R. P., HAJISHENGALLIS, G. & CURTIS, M. A. 2012. Porphyromonas gingivalis as a potential community activist for disease. *J Dent Res,* 91**,** 816-20.
DASANAYAKE, A. P., CAUFIELD, P. W., CUTTER, G. R. & STILES, H. M. 1993. Transmission of mutans streptococci to infants following short term application of an iodine-NaF solution to mothers' dentition. *Community Dent Oral Epidemiol,* 21**,** 136-42.
DAVANI-DAVARI, D., NEGAHDARIPOUR, M., KARIMZADEH, I., SEIFAN, M., MOHKAM, M., MASOUMI, S. J., BERENJIAN, A. & GHASEMI, Y. 2019. Prebiotics: Definition, Types, Sources, Mechanisms, and Clinical Applications. *Foods,* 8.
DEFRONZO, R. A. 2004. Pathogenesis of type 2 diabetes mellitus. *Med Clin North Am,* 88**,** 787-835, ix.
DEMMITT, B. A., CORLEY, R. P., HUIBREGTSE, B. M., KELLER, M. C., HEWITT, J. K., MCQUEEN, M. B., KNIGHT, R., MCDERMOTT, I. & KRAUTER, K. S. 2017. Genetic influences on the human oral microbiome. *BMC Genomics,* 18**,** 1.
DEWHIRST, F. E., CHEN, T., IZARD, J., PASTER, B. J., TANNER, A. C., YU, W. H., LAKSHMANAN, A. & WADE, W. G. 2010a. The human oral microbiome. *J Bacteriol,* 192**,** 5002-17.
DEWHIRST, F. E., CHEN, T., IZARD, J., PASTER, B. J., TANNER, A. C. R., YU, W.-H., LAKSHMANAN, A. & WADE, W. G. 2010b. The Human Oral Microbiome. *Journal of Bacteriology,* 192**,** 5002-5017.
DOMINY, S. S., LYNCH, C., ERMINI, F., BENEDYK, M., MARCZYK, A., KONRADI, A., NGUYEN, M., HADITSCH,

U., RAHA, D., GRIFFIN, C., HOLSINGER, L. J., ARASTU-KAPUR, S., KABA, S., LEE, A., RYDER, M. I., POTEMPA, B., MYDEL, P., HELLVARD, A., ADAMOWICZ, K., HASTURK, H., WALKER, G. D., REYNOLDS, E. C., FAULL, R. L. M., CURTIS, M. A., DRAGUNOW, M. & POTEMPA, J. 2019. Porphyromonas gingivalis in Alzheimer's disease brains: Evidence for disease causation and treatment with small-molecule inhibitors. *Science Advances,* 5.

DONSI, F. & FERRARI, G. 2016. Essential oil nanoemulsions as antimicrobial agents in food. *J Biotechnol,* 233**,** 106-20.

DURDIAKOVÁ, J., KAMODYOVÁ, N., OSTATNÍKOVÁ, D., VLKOVÁ, B. & CELEC, P. 2012. Comparison of different collection procedures and two methods for DNA isolation from saliva. *Clin Chem Lab Med,* 50**,** 643-7.

EDGAR, R. C. 2017. Accuracy of microbial community diversity estimated by closed- and open-reference OTUs. *PeerJ,* 5**,** e3889.

EISEN, D. 1999. The evaluation of cutaneous, genital, scalp, nail, esophageal, and ocular involvement in patients with oral lichen planus. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod,* 88**,** 431-6.

EKE, P. I., WEI, L., BORGNAKKE, W. S., THORNTON-EVANS, G., ZHANG, X., LU, H., MCGUIRE, L. C. & GENCO, R. J. 2016. Periodontitis prevalence in adults ≥ 65 years of age, in the USA. *Periodontol 2000,* 72**,** 76-95.

ELAMIN, A., GAREMO, M. & GARDNER, A. 2018. Dental caries and their association with socioeconomic characteristics, oral hygiene practices and eating habits among preschool children in Abu Dhabi, United Arab Emirates - the NOPLAS project. *BMC Oral Health,* 18**,** 104.

EMILSON, C. G. & KRASSE, B. 1985. Support for and implications of the specific plaque hypothesis. *Scand J Dent Res,* 93**,** 96-104.

ENDO, C., YAMAMOTO, S. & SHIMIZU, K. 2014. Study on factors that affect caries susceptibility in mice. *Pediatric Dental Journal,* 24**,** 39-45.

FABIAN, T. K., HERMANN, P., BECK, A., FEJERDY, P. & FABIAN, G. 2012. Salivary defense proteins: their network and role in innate and acquired oral immunity. *Int J Mol Sci,* 13**,** 4295-320.

FICARRA, G., FLAITZ, C. M., GAGLIOTI, D., PILUSO, S., MILO, D., ADLER-STORTHZ, K. & EVERSOLE, L. R. 1993. White lichenoid lesions of the buccal mucosa in patients with HIV infection. *Oral Surg Oral Med Oral Pathol,* 76**,** 460-6.

FORD, C. B., SHAH, R. R., MAEDA, M. K., GAGNEUX, S., MURRAY, M. B., COHEN, T., JOHNSTON, J. C., GARDY, J., LIPSITCH, M. & FORTUNE, S. M. 2013. Mycobacterium tuberculosis mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. *Nat Genet,* 45**,** 784-90.

FORSSTEN, S. D., BJORKLUND, M. & OUWEHAND, A. C. 2010. Streptococcus mutans, caries and simulation models. *Nutrients,* 2**,** 290-8.

FREIRE, M., MOUSTAFA, A., HARKINS, D. M., TORRALBA, M. G., ZHANG, Y., LEONG, P., SAFFERY, R., BOCKMANN, M., KUELBS, C., HUGHES, T., CRAIG, J. M. & NELSON, K. E. 2020. Longitudinal Study of Oral Microbiome Variation in Twins. *Sci Rep,* 10**,** 7954.

FRID, P., BARANIYA, D., HALBIG, J., RYPDAL, V., SONGSTAD, N. T., ROSÈN, A., BERSTAD, J. R., FLATØ, B., ALAKWAA, F., GIL, E. G., CETRELLI, L., CHEN, T., AL-HEBSHI, N. N., NORDAL, E. & AL-HARONI, M. 2020. Salivary Oral Microbiome of Children With Juvenile Idiopathic Arthritis: A Norwegian Cross-Sectional Study. *Front Cell Infect Microbiol,* 10**,** 602239.

GALIC, S., OAKHILL, J. S. & STEINBERG, G. R. 2010. Adipose tissue as an endocrine organ. *Mol Cell Endocrinol,* 316**,** 129-39.

GASTEIGER, G., D'OSUALDO, A., SCHUBERT, D. A., WEBER, A., BRUSCIA, E. M. & HARTL, D. 2017. Cellular Innate Immunity: An Old Game with New Players. *J Innate Immun,* 9**,** 111-125.

GBD, D. 2018. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet,* 392**,** 1789-1858.

GIBSON, S. A. 1997. Non-milk extrinsic sugars in the diets of pre-school children: association with intakes of micronutrients, energy, fat and NSP. *Br J Nutr,* 78**,** 367-78.

GIZANI, S., PAPAIOANNOU, W., HAFFAJEE, A. D., KAVVADIA, K., QUIRYNEN, M. & PAPAGIANNOULIS, L. 2009. Distribution of selected cariogenic bacteria in five different intra-oral habitats in young children. *Int J Paediatr Dent,* 19**,** 193-200.

GOMEZ, A. & NELSON, K. E. 2017. The Oral Microbiome of Children: Development, Disease, and Implications Beyond Oral Health. *Microb Ecol,* 73**,** 492-503.

GONZÁLEZ-CABEZAS, C. 2010. The Chemistry of Caries: Remineralization and Demineralization Events with Direct Clinical Relevance. *Dental Clinics of North America,* 54**,** 469-478.

GOODMAN, H. O., LUKE, J. E., ROSEN, S. & HACKEL, E. 1959. Heritability in dental caries, certain oral microflora and salivary components. *Am J Hum Genet,* 11**,** 263-73.

GRÖSCHL, M. 2017. Saliva: a reliable sample matrix in bioanalytics. *Bioanalysis,* 9**,** 655-668.

GRUNER, D., PARIS, S. & SCHWENDICKE, F. 2016. Probiotics for managing caries and periodontitis: Systematic review and meta-analysis. *J Dent,* 48**,** 16-25.

GUPTA, V. K., PAUL, S. & DUTTA, C. 2017. Geography, Ethnicity or Subsistence-Specific Variations in Human Microbiome Composition and Diversity. *Front Microbiol,* 8**,** 1162.

HAN, Y. W., SHEN, T., CHUNG, P., BUHIMSCHI, I. A. & BUHIMSCHI, C. S. 2009. Uncultivated bacteria as etiologic agents of intra-amniotic inflammation leading to preterm birth. *J Clin Microbiol,* 47**,** 38-47.

HATHROUBI, S., MEKNI, M. A., DOMENICO, P., NGUYEN, D. & JACQUES, M. 2017. Biofilms: Microbial Shelters Against Antibiotics. *Microb Drug Resist,* 23**,** 147-156.
HEATHER, J. M. & CHAIN, B. 2016. The sequence of sequencers: The history of sequencing DNA. *Genomics,* 107**,** 1-8.
HENSON, B. S. & WONG, D. T. 2010. Collection, storage, and processing of saliva samples for downstream molecular applications. *Methods Mol Biol,* 666**,** 21-30.
HOCHBERG, M. C. 1981. Adult and juvenile rheumatoid arthritis: current epidemiologic concepts. *Epidemiol Rev,* 3**,** 27-44.
HOFMANN, B. M. 2011. Does oral infection cause cardiovascular disease? Oral and moral challenges. *Community Dent Oral Epidemiol,* 39**,** 385-92.
HOLMES, A., MOORE, L., SUNDSFJORD, A., STEINBAKK, M., REGMI, S., KARKEY, A., GUERIN, P. & PIDDOCK, L. 2016. Understanding the mechanisms and drivers of antimicrobial resistance. *Lancet,* 387**,** 176-187.
IMBIMBO, B. P., LOMBARD, J. & POMARA, N. 2005. Pathophysiology of Alzheimer's Disease. *Neuroimaging Clinics of North America,* 15**,** 727-753.
INQUIMBERT, C., BOURGEOIS, D., BRAVO, M., VIENNOT, S., TRAMINI, P., LLODRA, J. C., MOLINARI, N., DUSSART, C., GIRAUDEAU, N. & CARROUEL, F. 2019. The Oral Bacterial Microbiome of Interdental Surfaces in Adolescents According to Carious Risk. *Microorganisms,* 7.
ISMAIL, S. B., KUMAR, S. K. & ZAIN, R. B. 2007. Oral lichen planus and lichenoid reactions: etiopathogenesis, diagnosis, management and malignant transformation. *J Oral Sci,* 49**,** 89-106.
JANDA, J. M. 2019. Proposed nomenclature or classification changes for bacteria of medical importance: Taxonomic Update 4. *Diagn Microbiol Infect Dis,* 94**,** 205-208.
JOHANSSON, I., WITKOWSKA, E., KAVEH, B., LIF HOLGERSON, P. & TANNER, A. C. 2016. The Microbiome

in Populations with a Low and High Prevalence of Caries. *J Dent Res,* 95**,** 80-6.
KAAN, A. M. M., KAHHAROVA, D. & ZAURA, E. 2021. Acquisition and establishment of the oral microbiota. *Periodontol 2000,* 86**,** 123-141.
KAHN, S. E., HULL, R. L. & UTZSCHNEIDER, K. M. 2006. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature,* 444**,** 840-6.
KANTOVITZ, K. R., PASCON, F. M., RONTANI, R. M. & GAVIAO, M. B. 2006. Obesity and dental caries--A systematic review. *Oral Health Prev Dent,* 4**,** 137-44.
KATO, I., VASQUEZ, A., MOYERBRAILEAN, G., LAND, S., DJURIC, Z., SUN, J., LIN, H. S. & RAM, J. L. 2017. Nutritional Correlates of Human Oral Microbiome. *J Am Coll Nutr,* 36**,** 88-98.
KENKEL, N. C. & ORLOCI, L. 1986. Applying Metric and Nonmetric Multidimensional Scaling to Ecological Studies: Some New Results. *Ecology,* 67**,** 919-928.
KIDD, E. A. & FEJERSKOV, O. 2004. What constitutes dental caries? Histopathology of carious enamel and dentin related to the action of cariogenic biofilms. *J Dent Res,* 83 Spec No C**,** C35-8.
KOLENBRANDER, P. E. 2000. Oral microbial communities: biofilms, interactions, and genetic systems. *Annu Rev Microbiol,* 54**,** 413-37.
KONIDENA, A. & PAVANI, B. V. 2011. Hepatitis C virus infection in patients with oral lichen planus. *Niger J Clin Pract,* 14**,** 228-31.
KÖNÖNEN, E., ASIKAINEN, S. & JOUSIMIES-SOMER, H. 1992. The early colonization of gram-negative anaerobic bacteria in edentulous infants. *Oral Microbiol Immunol,* 7**,** 28-31.
KOSMIDER, L., SOBCZAK, A., PROKOPOWICZ, A., KUREK, J., ZACIERA, M., KNYSAK, J., SMITH, D. & GONIEWICZ, M. L. 2016. Cherry-flavoured electronic cigarettes expose users to the inhalation irritant, benzaldehyde. *Thorax,* 71**,** 376-7.

KOZICH, J. J., WESTCOTT, S. L., BAXTER, N. T., HIGHLANDER, S. K. & SCHLOSS, P. D. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol,* 79**,** 5112-20.

KRISHNAN, K., CHEN, T. & PASTER, B. J. 2017. A practical guide to the oral microbiome and its relation to health and disease. *Oral Dis,* 23**,** 276-286.

KUCZYNSKI, J., LAUBER, C. L., WALTERS, W. A., PARFREY, L. W., CLEMENTE, J. C., GEVERS, D. & KNIGHT, R. 2011. Experimental and analytical tools for studying the human microbiome. *Nat Rev Genet,* 13**,** 47-58.

KUMAR, R., EIPERS, P., LITTLE, R. B., CROWLEY, M., CROSSMAN, D. K., LEFKOWITZ, E. J. & MORROW, C. D. 2014. Getting started with microbiome analysis: sample acquisition to bioinformatics. *Curr Protoc Hum Genet,* 82**,** 18.8.1-29.

KURAGO, Z. B. 2016. Etiology and pathogenesis of oral lichen planus: an overview. *Oral Surg Oral Med Oral Pathol Oral Radiol,* 122**,** 72-80.

LABENZ, J. 2016. [Barrett's esophagus]. *Internist (Berl),* 57**,** 1079-1092.

LAX, S., SMITH, D. P., HAMPTON-MARCELL, J., OWENS, S. M., HANDLEY, K. M., SCOTT, N. M., GIBBONS, S. M., LARSEN, P., SHOGAN, B. D., WEISS, S., METCALF, J. L., URSELL, L. K., VAZQUEZ-BAEZA, Y., VAN TREUREN, W., HASAN, N. A., GIBSON, M. K., COLWELL, R., DANTAS, G., KNIGHT, R. & GILBERT, J. A. 2014. Longitudinal analysis of microbial interaction between humans and the indoor environment. *Science,* 345**,** 1048-52.

LEGENDRE, P., BORCARD, D. & PERES-NETO, P. R. 2005. ANALYZING BETA DIVERSITY: PARTITIONING THE SPATIAL VARIATION OF COMMUNITY COMPOSITION DATA. *Ecological Monographs,* 75**,** 435-450.

LERMINIAUX, N. A. & CAMERON, A. D. S. 2019. Horizontal transfer of antibiotic resistance genes in clinical environments. *Can J Microbiol,* 65**,** 34-44.
LI, H., WANG, J., WU, L., LUO, J., LIANG, X., XIAO, B. & ZHU, Y. 2018. The impacts of delivery mode on infant's oral microflora. *Sci Rep,* 8**,** 11938.
LI, H., ZHANG, Y., XIAO, B., XIAO, S., WU, J. & HUANG, W. 2020. Impacts of delivery mode on very low birth weight infants' oral microbiome. *Pediatrics & Neonatology,* 61**,** 201-209.
LI, W., HUANG, E. & GAO, S. 2017. Type 1 Diabetes Mellitus and Cognitive Impairments: A Systematic Review. *J Alzheimers Dis,* 57**,** 29-36.
LICCARDO, D., CANNAVO, A., SPAGNUOLO, G., FERRARA, N., CITTADINI, A., RENGO, C. & RENGO, G. 2019. Periodontal Disease: A Risk Factor for Diabetes and Cardiovascular Disease. *Int J Mol Sci,* 20.
LIF HOLGERSON, P., HARNEVIK, L., HERNELL, O., TANNER, A. C. R. & JOHANSSON, I. 2011. Mode of birth delivery affects oral microbiota in infants. *Journal of dental research,* 90**,** 1183-1188.
LIM, Y., TOTSIKA, M., MORRISON, M. & PUNYADEERA, C. 2017. The saliva microbiome profiles are minimally affected by collection method or DNA extraction protocols. *Sci Rep,* 7**,** 8523.
LIU, B., FALLER, L. L., KLITGORD, N., MAZUMDAR, V., GHODSI, M., SOMMER, D. D., GIBBONS, T. R., TREANGEN, T. J., CHANG, Y. C., LI, S., STINE, O. C., HASTURK, H., KASIF, S., SEGRÈ, D., POP, M. & AMAR, S. 2012. Deep sequencing of the oral microbiome reveals signatures of periodontal disease. *PLoS One,* 7**,** e37919.
LIU, Y. Y., WANG, Y., WALSH, T. R., YI, L. X., ZHANG, R., SPENCER, J., DOI, Y., TIAN, G., DONG, B., HUANG, X., YU, L. F., GU, D., REN, H., CHEN, X., LV, L., HE, D., ZHOU, H., LIANG, Z., LIU, J. H. & SHEN, J. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China:

a microbiological and molecular biological study. *Lancet Infect Dis,* 16**,** 161-8.
LODI, G., SCULLY, C., CARROZZO, M., GRIFFITHS, M., SUGERMAN, P. B. & THONGPRASOM, K. 2005. Current controversies in oral lichen planus: report of an international consensus meeting. Part 1. Viral infections and etiopathogenesis. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod,* 100**,** 40-51.
LOESCHE, W. J. & GROSSMAN, N. S. 2001. Periodontal Disease as a Specific, albeit Chronic, Infection: Diagnosis and Treatment. *Clinical Microbiology Reviews,* 14**,** 727.
LUNDSTROM, I. M., ANNEROTH, G. B. & HOLMBERG, K. 1984. Candida in patients with oral lichen planus. *Int J Oral Surg,* 13**,** 226-38.
MACGREGOR, I. D. 1989. Effects of smoking on oral ecology. A review of the literature. *Clin Prev Dent,* 11**,** 3-7.
MANSBRIDGE, J. N. 1959. Heredity and dental caries. *J Dent Res,* 38**,** 337-47.
MARSH, P. D. 1994. Microbial ecology of dental plaque and its significance in health and disease. *Adv Dent Res,* 8**,** 263-71.
MARSH, P. D. 2018. In Sickness and in Health-What Does the Oral Microbiome Mean to Us? An Ecological Perspective. *Adv Dent Res,* 29**,** 60-65.
MASON, M. R., CHAMBERS, S., DABDOUB, S. M., THIKKURISSY, S. & KUMAR, P. S. 2018. Characterizing oral microbial communities across dentition states and colonization niches. *Microbiome,* 6**,** 67.
MATSSON, L. 1993. Factors influencing the susceptibility to gingivitis during childhood--a review. *Int J Paediatr Dent,* 3**,** 119-27.
MCCAULEY, L. K. & NOHUTCU, R. M. 2002. Mediators of periodontal osseous destruction and remodeling: principles and implications for diagnosis and therapy. *J Periodontol,* 73**,** 1377-91.
MORALES, S. E. & HOLBEN, W. E. 2009. Empirical testing of 16S rRNA gene PCR primer pairs reveals variance in target

specificity and efficacy not suggested by in silico analysis. *Appl Environ Microbiol,* 75**,** 2677-83.
MOYNIHAN, P. J. & KELLY, S. A. 2014. Effect on caries of restricting sugars intake: systematic review to inform WHO guidelines. *J Dent Res,* 93**,** 8-18.
MUKHERJEE, C., MOYER, C. O., STEINKAMP, H. M., HASHMI, S. B., BEALL, C. J., GUO, X., NI, A., LEYS, E. J. & GRIFFEN, A. L. 2021. Acquisition of oral microbiota is driven by environment, not host genetics. *Microbiome,* 9**,** 54.
NEWSOME, G. 2002. Guidelines for the management of rheumatoid arthritis: 2002 update. *J Am Acad Nurse Pract,* 14**,** 432-7.
NG, D. P., KOH, D., CHOO, S. G., NG, V. & FU, Q. 2004. Effect of storage conditions on the extraction of PCR-quality genomic DNA from saliva. *Clin Chim Acta,* 343**,** 191-4.
NOIRI, Y., LI, L. & EBISU, S. 2001. The localization of periodontal-disease-associated bacteria in human periodontal pockets. *J Dent Res,* 80**,** 1930-4.
OLIVER, R. C., BROWN, L. J. & LOE, H. 1998. Periodontal diseases in the United States population. *J Periodontol,* 69**,** 269-78.
OPAL, S., GARG, S., JAIN, J. & WALIA, I. 2015. Genetic factors affecting dental caries risk. *Aust Dent J,* 60**,** 2-11.
PATINI, R., MANGINO, G., MARTELLACCI, L., QUARANTA, G., MASUCCI, L. & GALLENZI, P. 2020. The Effect of Different Antibiotic Regimens on Bacterial Resistance: A Systematic Review. *Antibiotics (Basel),* 9.
PEACOCK, M. E., ARCE, R. M. & CUTLER, C. W. 2017. Periodontal and other oral manifestations of immunodeficiency diseases. *Oral diseases,* 23**,** 866-888.
PENG, S. M., WONG, H. M., KING, N. M. & MCGRATH, C. 2014. Is dental caries experience associated with adiposity status in preschool children? *Int J Paediatr Dent,* 24**,** 122-30.
PERRICONE, C., CECCARELLI, F., SACCUCCI, M., DI CARLO, G., BOGDANOS, D. P., LUCCHETTI, R., PILLONI, A., VALESINI, G., POLIMENI, A. & CONTI, F.

2019. Porphyromonas gingivalis and rheumatoid arthritis. *Curr Opin Rheumatol,* 31**,** 517-524.
PETERS, M. C. 2010. Strategies for noninvasive demineralized tissue repair. *Dent Clin North Am,* 54**,** 507-25.
PETERSEN, L. M., MARTIN, I. W., MOSCHETTI, W. E., KERSHAW, C. M. & TSONGALIS, G. J. 2019. Third-Generation Sequencing in the Clinical Laboratory: Exploring the Advantages and Challenges of Nanopore Sequencing. *J Clin Microbiol,* 58.
PETERSEN, P. E. 2003. The World Oral Health Report 2003: continuous improvement of oral health in the 21st century--the approach of the WHO Global Oral Health Programme. *Community Dent Oral Epidemiol,* 31 Suppl 1**,** 3-23.
PETERSEN, P. E. & OGAWA, H. 2005. Strengthening the prevention of periodontal disease: the WHO approach. *J Periodontol,* 76**,** 2187-93.
PHE 2013. Children's Dental Health (CDH) Survey.
PITTS, N., ZERO, D., MARSH, P. D., EKSTRAND, K., WEINTRAUB, J., RAMOS-GOMEZ, F., TAGAMI, J., TWETMAN, S., TSAKOS, G. & ISMAIL, A. 2017. Dental caries. *Nature Reviews Disease Primers,* 3**,** 1-16.
POOS, M. S., WALKER, S. C. & JACKSON, D. A. 2009. Functional-diversity indices can be driven by methodological choices and species richness. *Ecology,* 90**,** 341-7.
PRAKASH, A., BASKARAN, R., PARAMASIVAM, N. & VADIVEL, V. 2018. Essential oil based nanoemulsions to improve the microbial quality of minimally processed fruits and vegetables: A review. *Food Res Int,* 111**,** 509-523.
PRODAN, A., TREMAROLI, V., BROLIN, H., ZWINDERMAN, A. H., NIEUWDORP, M. & LEVIN, E. 2020. Comparing bioinformatic pipelines for microbial 16S rRNA amplicon sequencing. *PLoS One,* 15**,** e0227434.
QUAIL, M. A., SMITH, M., COUPLAND, P., OTTO, T. D., HARRIS, S. R., CONNOR, T. R., BERTONI, A., SWERDLOW, H. P. & GU, Y. 2012. A tale of three next generation sequencing platforms: comparison of Ion

Torrent, Pacific Biosciences and Illumina MiSeq sequencers. *BMC Genomics,* 13**,** 341.
QUINCE, C., WALKER, A. W., SIMPSON, J. T., LOMAN, N. J. & SEGATA, N. 2017. Shotgun metagenomics, from sampling to analysis. *Nat Biotechnol,* 35**,** 833-844.
RASTOGI, P., SAINI, H., DIXIT, J. & SINGHAL, R. 2011. Probiotics and oral health. *Natl J Maxillofac Surg,* 2**,** 6-9.
RAVI, R. K., WALTON, K. & KHOSROHEIDARI, M. 2018. MiSeq: A Next Generation Sequencing Platform for Genomic Analysis. *Methods Mol Biol,* 1706**,** 223-232.
RICHARDS, S. S. & HENDRIE, H. C. 1999. Diagnosis, Management, and Treatment of Alzheimer Disease: A Guide for the Internist. *JAMA Internal Medicine,* 159**,** 789-798.
RICOTTA, C., ACOSTA, A. T. R., BACARO, G., CARBONI, M., CHIARUCCI, A., ROCCHINI, D. & PAVOINE, S. 2019. Rarefaction of beta diversity. *Ecological Indicators,* 107**,** 105606.
RICOTTA, C. & PODANI, J. 2017. On some properties of the Bray-Curtis dissimilarity and their ecological meaning. *Ecological Complexity,* 31**,** 201-205.
RØNNINGEN, K. S. & ENERSEN, M. 2012. Diabetes and oral health. *Norsk Epidemiologi,* 22.
RUIZ, L., BACIGALUPE, R., GARCIA-CARRAL, C., BOIX-AMOROS, A., ARGUELLO, H., SILVA, C. B., DE LOS ANGELES CHECA, M., MIRA, A. & RODRIGUEZ, J. M. 2019. Microbiota of human precolostrum and its potential role as a source of bacteria to the infant mouth. *Sci Rep,* 9**,** 8435.
RUSTGI, A. K. & EL-SERAG, H. B. 2014. Esophageal carcinoma. *N Engl J Med,* 371**,** 2499-509.
SAMPAIO-MAIA, B. & MONTEIRO-SILVA, F. 2014. Acquisition and maturation of oral microbiome throughout childhood: An update. *Dental research journal,* 11**,** 291-301.
SAND, L., JALOULI, J., LARSSON, P. A. & HIRSCH, J. M. 2000. Human papilloma viruses in oral lesions. *Anticancer Res,* 20**,** 1183-8.

SAND, L. P., JALOULI, J., LARSSON, P. A. & HIRSCH, J. M. 2002. Prevalence of Epstein-Barr virus in oral squamous cell carcinoma, oral lichen planus, and normal oral mucosa. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod,* 93**,** 586-92.

SCHIODT, M., BAKILANA, P. B., HIZA, J. F., SHAO, J. F., BYGBJERG, I. B., MBAGA, I., VESTERGAARD, B. F., NIELSEN, C. M., LAURITZEN, E., LERCHE, B. & ET AL. 1990. Oral candidiasis and hairy leukoplakia correlate with HIV infection in Tanzania. *Oral Surg Oral Med Oral Pathol,* 69**,** 591-6.

SCHLOSS, P. D., WESTCOTT, S. L., RYABIN, T., HALL, J. R., HARTMANN, M., HOLLISTER, E. B., LESNIEWSKI, R. A., OAKLEY, B. B., PARKS, D. H., ROBINSON, C. J., SAHL, J. W., STRES, B., THALLINGER, G. G., VAN HORN, D. J. & WEBER, C. F. 2009. Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Applied and Environmental Microbiology,* 75**,** 7537.

SEGATA, N., HAAKE, S. K., MANNON, P., LEMON, K. P., WALDRON, L., GEVERS, D., HUTTENHOWER, C. & IZARD, J. 2012. Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat and stool samples. *Genome Biol,* 13**,** R42.

SHAW, L., RIBEIRO, A. L. R., LEVINE, A. P., PONTIKOS, N., BALLOUX, F., SEGAL, A. W., ROBERTS, A. P. & SMITH, A. M. 2017a. The Human Salivary Microbiome Is Shaped by Shared Environment Rather than Genetics: Evidence from a Large Family of Closely Related Individuals. *mBio,* 8**,** e01237-17.

SHAW, L. P., SMITH, A. M. & ROBERTS, A. P. 2017b. The oral microbiome. *Emerging Topics in Life Sciences,* 1**,** 287.

SIMPSON, C. A., ADLER, C., DU PLESSIS, M. R., LANDAU, E. R., DASHPER, S. G., REYNOLDS, E. C., SCHWARTZ, O. S. & SIMMONS, J. G. 2020. Oral microbiome composition, but not diversity, is associated with adolescent anxiety and depression symptoms. *Physiol Behav,* 226**,** 113126.

SINGER, K., ENG, D. S., LUMENG, C. N., GEBREMARIAM, A. & J, M. L. 2014. The relationship between body fat mass percentiles and inflammation in children. *Obesity (Silver Spring),* 22**,** 1332-6.

SMITH, A. & JONES, C. L. 2012. *Probiotics: Sources, Types and Health Benefits,* Hauppauge, UNITED STATES, Nova Science Publishers, Incorporated.

SNIDER, E. J., COMPRES, G., FREEDBERG, D. E., GIDDINS, M. J., KHIABANIAN, H., LIGHTDALE, C. J., NOBEL, Y. R., TOUSSAINT, N. C., UHLEMANN, A.-C. & ABRAMS, J. A. 2018. Barrett's esophagus is associated with a distinct oral microbiome. *Clinical and translational gastroenterology,* 9**,** 135-135.

SNIDER, E. J., FREEDBERG, D. E. & ABRAMS, J. A. 2016. Potential Role of the Microbiome in Barrett's Esophagus and Esophageal Adenocarcinoma. *Digestive Diseases and Sciences,* 61**,** 2217-2225.

SOCRANSKY, S. S., HAFFAJEE, A. D., CUGINI, M. A., SMITH, C. & KENT, R. L., JR. 1998. Microbial complexes in subgingival plaque. *J Clin Periodontol,* 25**,** 134-44.

SOCRANSKY, S. S., MANGANIELLO, A. D., PROPAS, D., ORAM, V. & VAN HOUTE, J. 1977. Bacteriological studies of developing supragingival dental plaque. *J Periodontal Res,* 12**,** 90-106.

SONG, S. J., LAUBER, C., COSTELLO, E. K., LOZUPONE, C. A., HUMPHREY, G., BERG-LYONS, D., CAPORASO, J. G., KNIGHTS, D., CLEMENTE, J. C., NAKIELNY, S., GORDON, J. I., FIERER, N. & KNIGHT, R. 2013. Cohabiting family members share microbiota with one another and with their dogs. *Elife,* 2**,** e00458.

SPIEGEL, K. A. & PALMER, C. A. 2012. Childhood dental caries and childhood obesity. Different problems with overlapping causes. *Am J Dent,* 25**,** 59-64.

STAHRINGER, S. S., CLEMENTE, J. C., CORLEY, R. P., HEWITT, J., KNIGHTS, D., WALTERS, W. A., KNIGHT, R. & KRAUTER, K. S. 2012. Nurture trumps nature in a longitudinal survey of salivary bacterial communities in

twins from early adolescence to early adulthood. *Genome Res,* 22**,** 2146-52.
STAMPFLI, M. R. & ANDERSON, G. P. 2009. How cigarette smoke skews immune responses to promote infection, lung disease and cancer. *Nat Rev Immunol,* 9**,** 377-84.
STEIN, K., FARMER, J., SINGHAL, S., MARRA, F., SUTHERLAND, S. & QUINONEZ, C. 2018. The use and misuse of antibiotics in dentistry: A scoping review. *J Am Dent Assoc,* 149**,** 869-884.e5.
STEWART, C. J., AUCHTUNG, T. A., AJAMI, N. J., VELASQUEZ, K., SMITH, D. P., DE LA GARZA, R., 2ND, SALAS, R. & PETROSINO, J. F. 2018. Effects of tobacco smoke and electronic cigarette vapor exposure on the oral and gut microbiota in humans: a pilot study. *PeerJ,* 6**,** e4693-e4693.
STRUZYCKA, I. 2014. The oral microbiome in dental caries. *Pol J Microbiol,* 63**,** 127-35.
SUN, A., CHANG, J. G., KAO, C. L., LIU, B. Y., WANG, J. T., CHU, C. T., YUAN, J. H. & CHIANG, C. P. 1996. Human cytomegalovirus as a potential etiologic agent in recurrent aphthous ulcers and Behcet's disease. *J Oral Pathol Med,* 25**,** 212-8.
SUNDSTRÖM, K., MISHRA, P., PYYSALO, M., LEHTIMÄKI, T., KARHUNEN, P. & PESSI, T. 2019. Similarity of salivary microbiome in parents and adult children. *PeerJ PrePrints*.
TAKAHASHI, N. 2005. Microbial ecosystem in the oral cavity: Metabolic diversity in an ecological niche and its relationship with oral diseases. *International Congress Series,* 1284**,** 103-112.
TAKAHASHI, N. & NYVAD, B. 2011. The role of bacteria in the caries process: ecological perspectives. *J Dent Res,* 90**,** 294-303.
TALHOUT, R., SCHULZ, T., FLOREK, E., VAN BENTHEM, J., WESTER, P. & OPPERHUIZEN, A. 2011. Hazardous compounds in tobacco smoke. *International journal of environmental research and public health,* 8**,** 613-628.

TANSIRICHAIYA, S., MULLANY, P. & ROBERTS, A. P. 2016. PCR-based detection of composite transposons and translocatable units from oral metagenomic DNA. *FEMS Microbiol Lett,* 363.

THODEN VAN VELZEN, S. K., ABRAHAM-INPIJN, L. & MOORER, W. R. 1984. Plaque and systemic disease: a reappraisal of the focal infection concept. *J Clin Periodontol,* 11**,** 209-20.

TIERNEY, B. T., YANG, Z., LUBER, J. M., BEAUDIN, M., WIBOWO, M. C., BAEK, C., MEHLENBACHER, E., PATEL, C. J. & KOSTIC, A. D. 2019. The Landscape of Genetic Content in the Gut and Oral Human Microbiome. *Cell Host Microbe,* 2*6***,** 283-295.e8.

TIMMONS, V., TOWNSEND, J., MCKENZIE, R., BURDALSKI, C. & ADAMS-SOMMER, V. 2018. An evaluation of provider-chosen antibiotic indications as a targeted antimicrobial stewardship intervention. *Am J Infect Control,* 46**,** 1174-1179.

TOUGER-DECKER, R. 2010. Diet, cardiovascular disease and oral health: Promoting health and reducing risk. *The Journal of the American Dental Association,* 141**,** 167-170.

TROMBELLI, L., FARINA, R., SILVA, C. O. & TATAKIS, D. N. 2018. Plaque-induced gingivitis: Case definition and diagnostic considerations. *J Clin Periodontol,* 45 Suppl 20**,** S44-s67.

UTTER, D. R., MARK WELCH, J. L. & BORISY, G. G. 2016. Individuality, Stability, and Variability of the Plaque Microbiome. *Frontiers in Microbiology,* 7.

VANN, W. F., JR., BOUWENS, T. J., BRAITHWAITE, A. S. & LEE, J. Y. 2005. The childhood obesity epidemic: a role for pediatric dentists? *Pediatr Dent,* 27**,** 271-6.

VARTOUKIAN, S. R., PALMER, R. M. & WADE, W. G. 2010. Strategies for culture of 'unculturable' bacteria. *FEMS Microbiol Lett,* 309**,** 1-7.

VERMA, D., GARG, P. K. & DUBEY, A. K. 2018. Insights into the human oral microbiome. *Arch Microbiol,* 200**,** 525-540.

VLASSARA, H. & URIBARRI, J. 2014. Advanced glycation end products (AGE) and diabetes: cause, effect, or both? *Current diabetes reports,* 14**,** 453-453.
WADE, W. G. 2013. The oral microbiome in health and disease. *Pharmacol Res,* 69**,** 137-43.
WANG, K., LU, W., TU, Q., GE, Y., HE, J., ZHOU, Y., GOU, Y., VAN NOSTRAND, J. D., QIN, Y., LI, J., ZHOU, J., LI, Y., XIAO, L. & ZHOU, X. 2016. Preliminary analysis of salivary microbiome and their potential roles in oral lichen planus. *Sci Rep,* 6**,** 22943.
WAYNE, L. G. 1988. International Committee on Systematic Bacteriology: announcement of the report of the ad hoc Committee on Reconciliation of Approaches to Bacterial Systematics. *Zentralbl Bakteriol Mikrobiol Hyg A,* 268**,** 433-4.
WEISS, S., XU, Z. Z., PEDDADA, S., AMIR, A., BITTINGER, K., GONZALEZ, A., LOZUPONE, C., ZANEVELD, J. R., VÁZQUEZ-BAEZA, Y., BIRMINGHAM, A., HYDE, E. R. & KNIGHT, R. 2017. Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome,* 5**,** 27.
WHITE, D. A., CHADWICK, B. L., NUTTALL, N. M., CHESTNUTT, I. G. & STEELE, J. G. 2006. Oral health habits amongst children in the United Kingdom in 2003. *Br Dent J,* 200**,** 487-91.
WILLIS, J. R., GONZÁLEZ-TORRES, P., PITTIS, A. A., BEJARANO, L. A., COZZUTO, L., ANDREU-SOMAVILLA, N., ALLOZA-TRABADO, M., VALENTÍN, A., KSIEZOPOLSKA, E., COMPANY, C., ONYWERA, H., MONTFORT, M., HERMOSO, A., IRAOLA-GUZMÁN, S., SAUS, E., LABEEUW, A., CAROLIS, C., HECHT, J., PONOMARENKO, J. & GABALDÓN, T. 2018. Citizen science charts two major "stomatotypes" in the oral microbiome of adolescents and reveals links with habits and drinking water composition. *Microbiome,* 6**,** 218.
WOO, P. C., LAU, S. K., TENG, J. L., TSE, H. & YUEN, K. Y. 2008. Then and now: use of 16S rDNA gene sequencing

for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clin Microbiol Infect,* 14**,** 908-34.

WU, D., KONG, Y., HAN, C., CHEN, J., HU, L., JIANG, H. & SHEN, X. 2008. D-Alanine:D-alanine ligase as a new target for the flavonoids quercetin and apigenin. *Int J Antimicrob Agents,* 32**,** 421-6.

WU, J., PETERS, B. A., DOMINIANNI, C., ZHANG, Y., PEI, Z., YANG, L., MA, Y., PURDUE, M. P., JACOBS, E. J., GAPSTUR, S. M., LI, H., ALEKSEYENKO, A. V., HAYES, R. B. & AHN, J. 2016. Cigarette smoking and the oral microbiome in a large study of American adults. *Isme j,* 10**,** 2435-46.

XIA, Y., SUN, J. & CHEN, D.-G. 2018. Community Diversity Measures and Calculations. *In:* XIA, Y., SUN, J. & CHEN, D.-G. (eds.) *Statistical Analysis of Microbiome Data with R.* Singapore: Springer Singapore.

YAMASHITA, Y. & TAKESHITA, T. 2017. The oral microbiome and human health. *J Oral Sci,* 59**,** 201-206.

YANG, I., SANDEEP, S. & RODRIGUEZ, J. 2020. The oral health impact of electronic cigarette use: a systematic review. *Crit Rev Toxicol,* 50**,** 97-127.

YOU, R., LU, W., SHAN, M., BERLIN, J. M., SAMUEL, E. L., MARCANO, D. C., SUN, Z., SIKKEMA, W. K., YUAN, X., SONG, L., HENDRIX, A. Y., TOUR, J. M., CORRY, D. B. & KHERADMAND, F. 2015. Nanoparticulate carbon black in cigarette smoke induces DNA cleavage and Th17-mediated emphysema. *Elife,* 4**,** e09623.

YU, G., PHILLIPS, S., GAIL, M. H., GOEDERT, J. J., HUMPHRYS, M. S., RAVEL, J., REN, Y. & CAPORASO, N. E. 2017. The effect of cigarette smoking on the oral and nasal microbiota. *Microbiome,* 5**,** 3.

ZAKRZEWSKA, J. M. 2001. Re: Mollaoglu N. Oral lichen planus: a review. Br J Oral Maxillofac Surg 2000; 38: 370-377. *Br J Oral Maxillofac Surg,* 39**,** 407.

ZHANG, X., ZHANG, D., JIA, H., FENG, Q., WANG, D., LIANG, D., WU, X., LI, J., TANG, L., LI, Y., LAN, Z., CHEN, B., LI,

Y., ZHONG, H., XIE, H., JIE, Z., CHEN, W., TANG, S., XU, X., WANG, X., CAI, X., LIU, S., XIA, Y., LI, J., QIAO, X., AL-AAMA, J. Y., CHEN, H., WANG, L., WU, Q. J., ZHANG, F., ZHENG, W., LI, Y., ZHANG, M., LUO, G., XUE, W., XIAO, L., LI, J., CHEN, W., XU, X., YIN, Y., YANG, H., WANG, J., KRISTIANSEN, K., LIU, L., LI, T., HUANG, Q., LI, Y. & WANG, J. 2015. The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. *Nat Med,* 21**,** 895-905.

8 Appendices

Appendix 1. Ethical Approval

17th July 2018

Dr Andrew Smith Microbial Diseases Eastman Dental Institute UCL

Dear Dr Smith

Notification of Ethics Approval with Provisos
Project ID/Title: 7567/001: Do changes in environment alter the oral microbiome?

Further to the review of your application at the recent meeting of the UCL REC, I am pleased to confirm in my capacity as Joint Chair of the UCL Research Ethics Committee (REC) that your study has been ethically approved by the UCL REC until **31st August 2020.**

Ethical approval is subject to the following conditions:

Notification of Amendments to the Research

You must seek Chair's approval for proposed amendments (to include extensions to the duration of the project) to the research for which this approval has been given. Each research project is reviewed separately and if there are significant changes to the research protocol you should seek confirmation of continued ethical approval by completing an 'Amendment Approval Request Form' http://ethics.grad.ucl.ac.uk/responsibilities.php

Adverse Event Reporting – Serious and Non-Serious

It is your responsibility to report to the Committee any unanticipated problems or adverse events involving risks to participants or others. The Ethics Committee should be notified of all serious adverse events via the Ethics Committee Administrator (ethics@ucl.ac.uk) immediately the incident occurs. Where the adverse incident is unexpected and serious, the Joint Chairs will decide whether the study should be terminated pending the opinion of an independent expert. For non-serious adverse events the Joint Chairs of the Ethics Committee should again be notified via the Ethics

Committee Administrator within ten days of the incident occurring and provide a full written report that should include any amendments to the participant information sheet and study protocol. The Joint Chairs will confirm that the incident is non-serious and report to the Committee at the next meeting. The final view of the Committee will be communicated to you.

Final Report

At the end of the data collection element of your research we ask that you submit a very brief report (1-2 paragraphs will suffice) which includes in particular issues relating to the ethical implications of the research i.e. issues obtaining consent, participants withdrawing from the research, confidentiality, protection of participants from physical and mental harm etc.

Office of the Vice Provost Research, 2 Taviton Street University College London
Tel:+44(0)2076798717
Email: ethics@ucl.ac.uk

http://ethics.grad.ucl.ac.uk/

In addition, please:

- ensure that you follow all relevant guidance as laid out in UCL's Code of Conduct for Research: http://www.ucl.ac.uk/srs/governance-and-committees/resgov/code-of-conduct-research

- note that you are required to adhere to all research data/records management and storage procedures agreed as part of your application. This will be expected even after completion of the study.

With best wishes for the research. Yours sincerely

Professor Michael Heinrich
Joint Chair, UCL Research Ethics Committee

Cc: Professor Dave Spratt

Appendix 2. Ethics Extension Approval

Ethics ID Number: 7567/001

Dear Violeta,

The REC has approved your attached extension request and the ethics approval of this study has therefore been extended to **31/08/2021**. Please take this email as confirmation of that approval.

IMPORTANT: For projects collecting personal data only

You should inform the Data Protection Team – data-protection@ucl.ac.uk of your proposed amendments to include a request to extend ethics approval for an additional period.

Best wishes,
Lola

Lola Alaska
Research Evaluation Administrator

Appendix 3. Participation Information Sheet For Young Adults

Participant Information Sheet For Young Adults

UCL Research Ethics Committee Approval ID Number: 7567/001

Version 1 25/05/2018

YOU WILL BE GIVEN A COPY OF THIS INFORMATION SHEET

Title of Study: Do changes in environment alter the oral microbiome?

Secondary Title: Do changes in environment that you grow up in change the bacterial community in your mouth?

Department: Microbial Diseases, Eastman Dental Institute

Name and Contact Details of the Researcher(s):

Name and Contact Details of the Principal Researchers:

Dr Andrew Smith (Andrew.m.smith@ucl.ac.uk)

Prof David Spratt (d.spratt@ucl.ac.uk)

Invitation

We'd like you to help us with a research project. You need to understand why we are doing this and what taking part will mean. So please take time to read the following information carefully and discuss it with your parent(s)/guardian and teachers if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this.

What is the project's purpose?

Background

The oral microbiome is the name given to all the microscopic creatures or bugs (bacteria, fungi and viruses) that live in our mouths. Everybody has an oral microbiome and each one is unique in the same way as a fingerprint. A number of these bugs can be very beneficial to our health and scientists and dentists are very interested in understanding how the different types of bugs in our mouths change as we age.

From birth to now, your mouth has gone through a lot of changes. We know that these physical changes (e.g. teeth) will influence and change the types of bugs that live in our mouths. We now think that other factors might affect the types of bacteria we have, and where you live could be one of them.

Aims

The aim of this study is to see if your surroundings (ie where you live) can affect the type of bacteria you have in your mouth

Why is this research important?

Currently we do not understand enough about how the oral bacteria develop and how stable they are in children and young adults. But we do know that the type of bacteria we have can influence everything from caries through to major health problems like diabetes. We have designed this study to help investigate this.

What do we want to do?/ What will happen to me if I take part?

We want to recruit new students entering the school at Year's 7, 9 and 11. We will ask students to complete a general dental health questionnaire in the first appointment and we will also collect nine saliva samples (each one is less than a teaspoon of saliva). The saliva samples will be collected at the beginning, middle and end of each term. This will allow us to work out what bugs are living in your mouth and monitor any changes that may occur over the school year. We will also conduct two dental examinations (one at the beginning of term and a second at the end of term three) in order to determine your overall oral health during the study. The students will be put into one of two groups depending if they are non-boarders (Group 1, a stable environment) or boarders (Group 2, a changed environment). We will then determine the identity of the oral bacteria at each sampling point (for each student) and see if it changes with time. We will then compare the changes observed between Group 1 and Group 2. This will help us understand if the student's environment influences the bacterial community in the mouth.

Why have I been chosen?

You have been asked to take part because you are a new student attending secondary school and are enrolled in either year 7, 9 or 11. In order to take part in the study you will have to get permission from your parent or legal guardian.

Do I have to take part?

The study is entirely voluntary and it is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep. You and

a parent or primary carer will need to read and sign a consent form. You can withdraw at any time without giving a reason and without any penalty or impact on your education. If you decide to withdraw you will be asked what you wish to happen to the information you have provided up that point.

Are there any risks to me?
There no risks that we can see. If the dental examination finds a problem which might need looking at in more detail, we will contact your parents and dentist to follow this up.

What are the possible benefits of taking part?
There won't be any obvious benefits for you. But the information we get from this study might help children in the future.

What if something goes wrong?
If you have any complaints relating to the study or any of the members of the research team you can contact in confidentiality the Principle Investigators Dr Andrew Smith or Professor David Spratt (contact details at the end of this document). However you can also take your complaint to the Chair of the UCL Research Ethics Committee (ethics@ucl.ac.uk), if you are not satisfied with how you have been treated or if the issue is with principle investigators.

Will my taking part in this project be kept confidential?
All the information that we collect about you during the course of the research will be kept strictly confidential.

Limits to confidentiality
Please note that assurances on confidentiality will be strictly adhered to unless evidence of wrongdoing or potential harm is uncovered. In such cases the University may be obliged to contact relevant statutory bodies/agencies.

Please note that confidentiality will be maintained as far as it is possible, unless during our conversation I hear anything which makes me worried that someone might be in danger of harm, I might have to inform relevant agencies of this.

What will happen to the results of the research project?
The results from this study will be presented at clinical, dental and scientific meetings in the UK and abroad. We will also publish the findings in international peer reviewed journals. We will also return to your school the following year and present the findings to the students and teachers. We will explain the findings and participate in a question and answer session. Any data we present will be anonymised

Data Protection Privacy Notice

Notice:

The data controller for this project will be University College London (UCL). The UCL Data Protection Office provides oversight of UCL activities involving the processing of personal data, and can be contacted at data-protection@ucl.ac.uk. UCL's Data Protection Officer is Lee Shailer and he can also be contacted at data-protection@ucl.ac.uk**.**

Your personal data will be processed for the purposes outlined in this notice. The legal basis that would be used to process your personal data will be the provision of your and your parents/legal guardians consent. You can provide your consent for the use of your personal data in this project by completing the consent form that has been provided to you.

Your personal data will be processed so long as it is required for the research project. We will remove all identifiable information from the personal data you provide and will endeavour to minimise the processing of personal data wherever possible.

If you are concerned about how your personal data is being processed, please contact UCL in the first instance at data-protection@ucl.ac.uk. If you remain unsatisfied, you may wish to contact the Information Commissioner's Office (ICO). Contact details, and details of data subject rights, are available on the ICO website at: https://ico.org.uk/for-organisations/data-protection-reform/overview-of-the-gdpr/individuals-rights/

Who is organising and funding the research?

This research project was designed by the principle investigators and is being funded by a research grant awarded by Biomedical Research Centre (**BRC**) University College Hospital.

Contact for further information

Principle Investigators

Dr Andrew Smith
Andrew.m.smith@ucl.ac.uk
020 76796008

Professor David Spratt
d.spratt@ucl.ac.uk
020 34561107

Thank you for reading this information sheet and for considering taking part in this research study. If you take part in the study you will be able to keep a copy of the information sheet and signed consent form for your records.

Appendix 4. Parent/ Guardian Information Sheet

Parent/Guardian Information Sheet

UCL Research Ethics Committee Approval ID Number: 7567/001

Version 1 25/05/2018

YOU WILL BE GIVEN A COPY OF THIS INFORMATION SHEET

Title of Study: Do changes in environment alter the oral microbiome?

Secondary Title: Do changes in environment that you grow up in change the bacterial community in your mouth?

Department: Microbial Diseases, Eastman Dental Institute

Name and Contact Details of the Researcher(s):

Name and Contact Details of the Principal Researchers:

Dr Andrew Smith (Andrew.m.smith@ucl.ac.uk)

Prof David Spratt (d.spratt@ucl.ac.uk)

Invitation

Your Child is being invited to take part in a scientific research project. Before you decided if you would be willing for your child to enrol in the study, it is important for you to understand why the research is being done and what participation will involve. Please take time to read the following information carefully and discuss it with your child. Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you would be happy for your child to take part in the study.

If you are happy for your child to participate in the study could you please sign the consent form and return it to the school. We will then invite your child to a recruitment meeting where we will go over the study answer any questions they may have and then get them to fill in the consent form that you have signed.

What is the project's purpose?

Background

We are very interested in how the community of microbes (bacteria, fungi and viruses) in our mouths changes as we age. When we are born our mouths lack any oral

microbes. In the early period of our growth it becomes colonised by bacteria, which are typically acquired from our parents or primary care giver. As we grow up, our oral bacterial community develops to become a complex and diverse ecosystem with hundreds of species present.

We have recently acquired new evidence showing that where we live and our immediate family has a significant effect on our oral microbes. What is also surprising is that our own genes (DNA) play almost no role. Previously your DNA was thought to play a crucial role in forming and defining oral microbial communities. These new findings therefore provide the possibility of altering an individual's oral microbes through changing the environment they live in. It is now clear that both oral disease and indeed other diseases, such as heart disease and diabetes, are influenced by the types of the bacteria that live in the mouth. If we were able to "re-programme" the microbial community within the mouth we could be able to confer substantial health benefits. However, in adults it is very difficult to alter the oral microbial communities permanently. What we don't know is when, in our development, the oral microbial communities become fixed.

Aims

The aim of this study is to determine if the oral bacterial community is stable in adolescents and how the environment may influence the composition of this bacterial community.

Why is this research important?

Despite being almost completely preventable by good oral hygiene, a third of children in England suffer from tooth decay (caries). It is the most common reason why 5-9 year olds are admitted to hospital and undergo general aesthetic. The NHS spends £30 million per year on hospital based tooth extractions for children. Caries is caused by acid producing bacteria that destroy the tooth's enamel. We believe that while the oral bacterial community is still developing, we can intervene to create a community that does not cause caries, this would be a major leap forward and save all of the pain and suffering associated with tooth decay and treatment. Currently we do not understand enough about how the oral bacteria develop and how stable they are in children and young adults. We have designed this study to help answer these questions.

Proposed study

We believe that there must be a period in human development in which the oral microbial community can be altered permanently and point where it becomes fixed. To accurately find this period we need to follow subjects over time. There are only a few points in human development where this type of study can easily be performed one of which is when people of similar age come together and reside in a new environment. In the first year of secondary school students have a dramatic change in lifestyle in some cases. Students who enrol in boarding school will have a major change in environment and this will enable us to determine how much of an effect environment has on the oral microbial community and how stable it becomes over time.
We will recruit new students entering the school at Year's 7, 9 and 11. We will require the students to complete a general oral health questionnaire during their first appointment and we will also collect nine saliva samples (each one is less than a teaspoon of saliva). The saliva samples will be collected at the beginning, middle and end of each term. The students will be put into one of two groups depending if they are non-boarders (Group 1, a stable environment) or boarders (Group 2, a changed environment). We will then determine the identity of the oral bacteria at each sampling point (for each student) and see if it changes with time. We will then compare the changes observed between Group 1 and Group 2. This will help us understand if the student's environment influences the bacterial community in the mouth.

Why have you chosen my child?
We have identified your child as a potential participant in our study because they are a new student attending secondary school and are enrolled in either year 7, 9 or 11. In order for your child to take part in the study they will need to get permission from their parent or legal guardian, agree to answer the questionnaire and provide nine saliva samples during the coming school year. We will also conduct two oral examinations (one at the beginning of term and a second at the end of term three) in order to determine your child's overall oral health during the study.

Does my child have to take part?
The study is entirely voluntary and it is up to you and your child to decide whether or not to take part. If your child takes part you will be given this information sheet to keep. You and your child will need to read and sign a consent form. They can withdraw at any time without giving a reason and without prejudice on their education. If they decide to withdraw they will be asked what they wish to happen to the data they have provided up that point.

What will happen to your child if they take part?
The study will last for a year and your child will be required to attend nine appointments (beginning, middle and end of each term). All of the appointments will be conducted at school and within term time. The appointments will last approximately 5-10 minutes and they will be required to provide a saliva samples (less than a teaspoon in volume and they will just need to spit into a sterile tube that we provide at the meeting) and answer a lifestyle questionnaire. On the first and last appointments they will also be asked to undergo an oral examination, which should last approximately 5 minutes. In

the first meeting they will be assigned into group 1 (non-boarders) or group 2 (boarders) depending on their residential status at the school.

What are the possible disadvantages and risks of taking part?
The saliva samples that are collected for the study will only be used to isolate bacterial DNA and carries no identified risks to the participants. The oral examination may identify a hitherto unknown condition, which may require additional investigation. In these circumstances the fully qualified paediatric dentist who conducted the examination will send a letter to you as their legal guardian explaining the findings and advising on the best course of action.

What are the possible benefits of taking part?
Whilst there are no immediate benefits for those people participating in the project, it is hoped that this work will lead to a greater understanding of how the oral bacterial community develop and how much of this is influenced by a person's environment. If the oral bacteria are found to be dynamic (not fixed) in adolescents then it could be possible to artificially manipulate the bacterial composition in order to provide beneficial effects on an individual. An indirect benefit will be the greater knowledge of the mouth and the importance of oral microbiome that the participant will gain.

What if something goes wrong?
If you or your child has any complaints relating to the study or any of the members of the research team you can contact in confidentiality the Principle Investigators Dr Andrew Smith or Professor David Spratt (contact details at the end of this document). However you can also take your complaint to the Chair of the UCL Research Ethics Committee (ethics@ucl.ac.uk), if you are not satisfied with how you have been treated or if the issue is with principle investigators.

Will my child taking part in this project be kept confidential?
All the information that we collect about your child during the course of the research will be kept strictly confidential. Upon enrolment your child will be assigned a unique project code which will be linked to their data. This pseudonymised data means that they cannot be identified in any ensuing reports or publications.

Limits to confidentiality

Please note that assurances on confidentiality will be strictly adhered to unless evidence of wrongdoing or potential harm is uncovered. In such cases the University may be obliged to contact relevant statutory bodies/agencies.

Please note that confidentiality will be maintained as far as it is possible, unless during our conversation I hear anything which makes me worried that someone might be in danger of harm, I might have to inform relevant agencies of this.

What will happen to the results of the research project?

The results from this study will be presented at clinical, dental and scientific meetings in the UK and abroad. We will also publish the findings in international peer reviewed journals. We will also return to your school the following year and present the findings to the students and teachers. We will explain the findings and participate in a question and answer session. All data will be presented in a pseudonymised fashion where no individual can be identified.

Data Protection Privacy Notice

Notice:
The data controller for this project will be University College London (UCL). The UCL Data Protection Office provides oversight of UCL activities involving the processing of personal data, and can be contacted at data-protection@ucl.ac.uk. UCL's Data Protection Officer is Lee Shailer and he can also be contacted at data-protection@ucl.ac.uk***.***

Your child's personal data will be processed for the purposes outlined in this notice. The legal basis that would be used to process your child's personal data will be the provision of your child's and your consent. You and your child can provide consent for the use of your child's personal data in this project by completing the consent form that has been provided.

Your child's personal data will be processed so long as it is required for the research project. We will pseudonymise the personal data your child provides and will endeavour to minimise the processing of personal data wherever possible.

If you or your child are concerned about how personal data is being processed, please contact UCL in the first instance at data-protection@ucl.ac.uk. If you remain unsatisfied, you may wish to contact the Information Commissioner's Office (ICO). Contact details, and details of data subject rights, are available on the ICO website at: https://ico.org.uk/for-organisations/data-protection-reform/overview-of-the-gdpr/individuals-rights/

Who is organising and funding the research?

This research project was designed by the principle investigators and is being funded by a research grant awarded by Biomedical Research Centre (**BRC**) University College Hospital.

Contact for further information

Principle Investigators

Dr Andrew Smith
Andrew.m.smith@ucl.ac.uk
020 76796008

Professor David Spratt
d.spratt@ucl.ac.uk
020 34561107

Thank you for reading this information sheet and for considering taking part in this research study. If you take part in the study you will be able to keep a copy of the information sheet and signed consent form for your records.

Appendix 5. Consent Form For Adolescents In Research Studies

Please complete this form after you have read the Information Sheet and/or listened to an explanation about the research.

Title of Study: Do changes in environment alter the oral microbiome?

Department: Microbial Diseases, Eastman Dental Institute

Name and Contact Details of the Researcher(s):

Principle researchers
Professor David Spratt
d.spratt@ucl.ac.uk
Dr Andrew Smith
Andrew.m.smith@ucl.ac.uk

Name and Contact Details of the UCL Data Protection Officer: Sara Green S.green@ucl.ac.uk

This study has been approved by the UCL Research Ethics Committee: Project ID number: 7567/001

Thank you for considering taking part in this research. The person organising the research must explain the project to you and your parent(s)/guardian before you agree to take part. If you or your parent(s)/guardian have any questions arising from the Information Sheet or explanation already given to you, please ask the researcher before you decide whether to join in. You and your parent(s)/guardian will be given a copy of this Consent Form to keep and refer to at any time.

		Tick Box
	I confirm that I have read and understood the Information Sheet for the above study. I have had an opportunity to consider the information and what will be expected of me. I have also had the opportunity to ask questions which have been answered to my satisfaction and would like to take part in the study.	
	I consent to the processing of my personal information associated with my lifestyle and oral health for the purposes explained to me. I understand that such information will be handled in accordance with all applicable data protection legislation.	
	I understand that all personal information will remain confidential and that all efforts will be made to ensure I cannot be identified.	
	I understand that my information may be subject to review by responsible individuals from the University for monitoring and audit purposes.	
	I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason, without my legal rights being affected. I understand that if I decide to withdraw, any personal data I have provided up to that point will be deleted unless I agree otherwise.	
	I understand the potential risks of participating and the support that will be available to me should I become distressed during the course of the research.	
	I understand that no promise or guarantee of benefits have been made to encourage you to participate.	
	I understand that the data will not be made available to any commercial organisations but is solely the responsibility of the researcher(s) undertaking this study.	
	I understand that I will not benefit financially from this study or from any possible outcome it may result in in the future.	
	I understand that the information I have submitted will be published as a report and I wish to receive a copy of it. Yes/No	
	I hereby confirm that I understand the inclusion criteria as detailed in the Information Sheet and explained to me by the researcher.	
	I hereby confirm that: (a) I understand the exclusion criteria as detailed in the Information Sheet and explained to me by the researcher; and (b) I do not fall under the exclusion criteria.	

	I have informed the researcher of any other research in which I am currently involved or have been involved in during the past 12 months.	
	I am aware of who I should contact if I wish to lodge a complaint.	
	I agree to complete the questionnaire associated with the study on oral health (see Information sheet)	
	I agree to provide saliva samples at specified time points throughout the study (see information sheet)	
	I would be happy for all of my data and information generated during the study to be pseudonymised and retained for 20 years in accordance with current University College London records Management policy. I would be happy for the data I provide to be archived at the Department of Microbial Diseases, University College London. I understand that other authenticated researchers will have access to my pseudonymised data.	

I confirm that I understand that by ticking/initialling each box below I am consenting to this element of the study. I understand that it will be assumed that unticked/initialled boxes means that I DO NOT consent to that part of the study. I understand that by not giving consent for any one element that I may be deemed ineligible for the study.

If you would like your contact details to be retained so that you can be contacted in the future by UCL researchers who would like to invite you to participate in follow up studies to this project, or in future studies of a similar nature, please tick the appropriate box below.

	Yes, I would be happy to be contacted in this way	
	No, I would not like to be contacted	

_____	_____	_____
Name of participant	Date	Signature
_____	_____	_____
Name of Parent/Guardian	Date	Signature
_____	_____	_____
Researcher	Date	Signature

Appendix 6. Oral Health Questionnaire

Oral health questionnaire

Do changes in environment alter the oral microbiome?

Principal Investigators: Dr Andrew Smith, Prof Dave Spratt, Dr Paul Ashley

Version 1 (22/05/2018)

UCL Research Ethics Committee Approval ID Number: 7567/001

In order to understand better how the bugs in your mouth can interact with your body, we need to know a little bit more about you. Please try and answer the questions below as best as you can.

The information you give us is confidential.

1. About you

1.1 Unique Identification code……..…..

1.2 Age (years)

……

1.3 Gender (please circle) Male

Female

1.4 What year are you in

……

2. About your home

2.1 Are you a boarder? (please circle) Yes

No

2.2 Which country is home in?

…….....

2.3 How many brothers/sisters do you have?

……

2.4 Do you have any pets at home? (please circle) Yes

No

If the answer was yes to 2.4, what pets do you have?

……..…

……..

3. About your health and fitness

3.1 Have you had antibiotics in the last 3 months? Yes

No

3.2 Do you have asthma? (please circle) Yes

No

3.3 Do you have eczema? (please circle) Yes

No

3.4 Do you drink Yakult or Actimel more than once a week? (please circle) Yes

No

3.5 Are you on any of the school sports teams? (please circle) Yes

No

If the answer was yes to 3.5, which teams?

……..…
……..

3.6 How many times a month do you swim in a swimming pool

……

3.7 Do you ever wear a sports mouthguard? (please circle) Yes

No

4. About your mouth

4.1 How many times a day do you clean your teeth?

……

4.2 How many times a day do you use mouthrinse?

……

4.3 Have you got any fillings? (please circle) Yes No

4.4 Have you had any teeth extracted? (please circle) Yes

No

4.5 Have you ever had dental braces? (please circle) Yes

No

If the answer was yes to 4.5, do you have retainers? (please circle Yes

No

4.6 Do you have dental braces now? (please circle) Yes

No

4.7 Do you wear a denture with a false tooth? (please circle) Yes

No

Appendix 7. Screening Protocol

Do changes in environment alter the oral microbiome?
Screening protocol V2
Paul Ashley 6/11/18

Environment and Cross-infection control
The participant should be seated in a comfortable chair, which has good head support, and which allows the examiner to access and examine the head and mouth.

A suitable external bright light source should be used such as a head torch or lamp.

The instruments should be laid out on a clean tissue out of sight of the participant (if possible) and allowing easy access.
The light source should be set at the highest power setting and dark eye protection glasses placed on the subject.
Each examiner should carry sufficient sets of sterile **disposable** instruments to ensure that there are sterile instruments for every examination. Following the examination these should be disposed. Examiners should wear a clean pair of **latex-free gloves** for the examination of each participant along with a mask and eye protection. Appropriate cross-infection procedures must be followed throughout.

An equipment list is in Appendix 1

Data collection
Where possible questionnaires for additional data should be completed by participants as they wait for the oral examination.
When scoring clinical outcomes, if in doubt score ‘low’.
Examples of data collection forms are in appendix 2.

Examiner training
The training should be structured to provide a clear understanding of the nature and aims of the study or screening exercise together will the assessment/examination procedures and completion of appropriate documentation. Preferably, examiners should be trained and compared to a gold-standard examiner. Furthermore, it will be

important to assess their consistency in measurements (within-individual repeatability).

Caries and restorations (DFT)

Caries and restorations should be scored using modified ICDAS criteria (https://www.iccms-web.com/). Caries will be recorded at tooth level with the most severe score on any surface being recorded. Caries will be regarded as more severe than a restoration if both are recorded on one tooth.

MODIFIED ICDAS

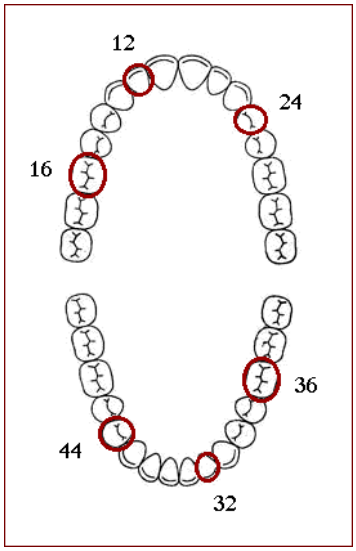
0	No evidence of caries
A	Initial caries
3	Localised enamel breakdown due to caries with no visible dentine
4	Underlying dark shadow from dentine
5	Distinct cavity with visible dentine
6	Extensive distinct cavity with visible dentine

In addition, teeth with unrestorable decay should be noted. This can be defined as:
“Untreated teeth with extensive dentinal decay have obvious loss of tooth structure, with a cavity both deep and wide so that dentine is clearly visible on the walls and at the base. Such a cavity would involve at least half of a tooth surface, and teeth coded in this way are so broken down that it is inconceivable that there is not pulp involvement and so restoration of the tooth would be very involved or impossible.” (Adult Dental Health Survey, 2009)

Plaque

Plaque will be measured using the Silness-Löe Index (https://www.mah.se/CAPP/Methods-and-Indices/Oral-Hygiene-Indices/Silness-Loe-Index/)

The measurement of the state of oral hygiene by Silness-Löe plaque index is based on recording both soft debris and mineralized deposits on the following teeth (Primary teeth can be substituted for permanent teeth. Missing teeth are not substituted).



Each of the four surfaces of the teeth (buccal, lingual, mesial and distal) is given a score from 0-3. The scores from the four areas of the tooth are added and divided by four in order to give the plaque index for the tooth with the following scores and criteria:

The Plaque Index System

Scores	Criteria
0	No plaque
1	A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may be seen in situ only after application of disclosing solution or by using the probe on the tooth surface.
2	Moderate accumulation of soft deposit s within the gingival pocket, or the tooth and gingival margin which can be seen with the naked eye.
3	Abundance of soft matter within the gingival pocket and/or on the tooth and gingival margin.

Swellings/abscesses/infections

Pulp, Ulceration, Fistula and Abscess (PUFA Index)

Description of conditions to be recorded in PUFA

P = open pulp in permanent dentition

U = obvious ulceration

F = fistula in permanent dentition

A= abscess in permanent dentition

Codes and criteria:PUFA

0 = No lesions evident

1 = A single lesion present

2 = 2 or more lesions present

The mouth should be examined in the following order (upper right, upper left, lower left, lower right), ensuring that the lips or cheeks are gently retracted to allow the soft tissues to be examined.

A single code (0, 1 or 2) will be called for each of the four conditions examined.

Other

In addition the presence or absence of any orthodontic appliances or any other dentures will be recorded.

Appendix 8. Clinical Data Collection Form

Clinical Data Collection form Patient ID……

Clinician …….. Date ……

PUFA index (Pulp, Ulceration, Fistula, Abscess)

	Code **0=no lesions, 1=single lesion, 2=2 or more lesions**
Pulp	
Ulcer	
Fistula	
Abscess	

Plaque examination (0,1,2,3)

UR6	UR2	UL4
LR4	LL2	LL6

DFT (ICDAS criteria for caries, code restoration as R)

Tooth	**18**	**17**	**16**	**15**	**14**	**13**	**12**	**11**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**
Tooth	**48**	**47**	**46**	**45**	**44**	**43**	**42**	**41**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**

Tooth	**55**	**54**	**53**	**52**	**51**	**61**	**62**	**63**	**64**	**65**
Tooth	**85**	**84**	**83**	**82**	**81**	**71**	**72**	**73**	**74**	**75**

Appendix 9. DNA concentration measured with NanoDrop Spectophotometer

CODING	260/280	260/230	ng/uL
H1S1	0.94	1.13	35.7
H2S1	2.19	1.45	29.4
H3S1	2.12	1.07	14.8
H4S1	1.81	1.35	138.2
H5S1	1.52	2.01	4.8
H6S1	1.84	1.49	125.0
H7S1	1.65	0.55	18.8
H8S1	1.71	0.86	7.7
H9S1	2.00	1.20	6.2
H11S1	2.14	1.83	4.8
H12S1	1.64	3.51	4.1
H13S1	1.58	0.85	217.9
H14S1	1.97	2.32	35.3
H15S1	1.38	2.33	2.3
H16S1	1.74	0.86	9.2
H17S1	2.01	2.38	25.8
H18S1	2.04	1.56	10.6
H1S2	0.63	1.22	3.5
H2S2	1.75	2.24	6.6
H3S2	2.38	1.78	7.5
H4S2	1.96	2.38	190.1
H5S2	1.80	1.09	16.4
H6S2	1.43	1.66	24.2
H7S2	1.89	1.85	45.3
H8S2	1.53	3.53	6.8
H9S2	1.60	1.04	99.7

H11S2	2.41	1.63	8.2
H12S2	-1.99	9.09	1.0
H13S2	2.18	2.30	6.7
H14S2	2.00	2.39	46.6
H15S2	1.50	0.87	182.6
H16S2	4.75	1.15	2.4
H17S2	1.64	1.04	366.4
H18S2	0.74	1.25	11.8
H1S3	-5.32	2.29	2.2
H2S3	2.08	1.85	14.5
H3S3	2.35	1.06	8.3
H4S3	1.87	2.29	29.3
H5S3	-0.7	-0.73	0.5
H6S3	6.13	3.23	1.8
H7S3	1.62	2.15	15.5
H8S3	1.73	1.38	425.8
H9S3	1.69	1.81	8.8
H11S3	6.66	-1.1	0.8
H12S3	4.49	2.85	1.8
H13S3	-0.91	5.87	0.5
H14S3	1.91	2.21	47.0
H15S3	-4.25	2.72	2.2
H16S3	2.99	1.43	6.7
H17S3	1.93	1.58	19.9
H18S3	4.36	1.76	6.1
H1S4	0.60	1.05	-1.1
H2S4	2.34	1.72	14.1
H3S4	0.29	0.38	-0.7
H4S4	-0.17	-0.33	0.2

H5S4	0.44	0.91	-0.5
H6S4	2.28	0.75	5.1
H7S4	3.36	1.06	2.8
H8S4	3.06	1.69	7.6
H9S4	1.67	1.33	205.5
H11S4	1.23	1.91	6.1
H12S4	1.64	1.40	4.1
H13S4	0.04	0.35	0.0
H14S4	2.02	2.58	370.2
H15S4	2.24	2.39	2.8
H16S4	1.94	2.25	6.6
H17S4	1.70	1.75	8.0
H18S4	3.51	2.21	10.2
H1S5	0.75	0.73	3.1
H2S5	1.85	2.52	16.7
H3S5	1.61	1.27	52.8
H4S5	1.81	1.53	214.9
H5S5	0.56	1.03	2.5
H6S5	0.45	1.64	1.4
H7S5	1.30	2.13	14.9
H8S5	1.36	1.79	14.0
H9S5	1.67	2.39	33.1
H11S5	1.11	1.40	10.3
H12S5	0.32	0.59	0.6
H14S5	1.71	2.40	37.4
H15S5	1.52	1.84	21.6
H16S5	0.67	0.94	2.6
H17S5	1.33	1.51	8.8
H18S5	0.54	1.08	3.7

H1S6	0.76	0.47	-2.1
H2S6	-0.65	-0.27	1.1
H3S6	1.87	1.56	31.2
H4S6	1.85	2.22	236.2
H5S6	1.80	1.40	16.2
H6S6	1.92	2.58	67.7
H7S6	-9.36	0.34	-1.2
H8S6	1.99	1.80	16.4
H9S6	2.02	1.92	82.4
H11S6	1.78	-2.79	4.7
H12S6	3.72	8.39	14.6
H13S6	5.48	0.81	3.6
H14S6	2.07	2.22	120.0
H15S6	1.85	1.15	44.5
H16S6	1.71	1.57	12.0
H1S7	4.09	0.72	2.2
H2S7	1.83	2.01	35.4
H3S7	1.27	0.95	4.3
H4S7	1.79	1.73	1076.8
H5S7	1.87	1.54	30.3
H6S7	1.58	1.01	13.8
H8S7	1.71	1.56	14.2
H9S7	2.04	2.03	99.0
H11S7	2.98	1.13	6.9
H12S7	1.93	0.99	4.8
H14S7	1.80	1.33	21.5
H15S7	1.60	1.06	11.8
H16S7	1.46	1.03	8.8
H17S7	2.08	2.23	47.1

H18S7	2.04	1.55	12.6
H1S9	-0.44	0.12	0.1
H2S9	2.38	1.36	7.1
H3S9	1.81	14.06	9.6
H4S9	2.51	-1.69	2.8
H5S9	-0.05	0.04	-0.1
H6S9	9.43	1.40	3.8
H7S9	2.15	1.22	16.5
H8S9	6.46	18.35	7.6
H9S9	-4.52	0.43	-0.6
H11S9	2.55	1.67	6.9
H12S9	1.86	1.65	11.6
H13S9	2.48	1.04	3.2
H14S9	2.00	1.12	3.5
H15S9	1.72	0.64	4.8
H16S9	0.99	-0.11	0.4
H17S9	2.06	2.03	15.1
H18S9	1.54	1.62	10.9
H1S10	-7.53	0.81	1.9
H2S10	2.34	1.66	8.4
H3S10	-2.22	0.63	1.3
H4S10	1.95	1.19	5.5

Appendix 10. DADA2

Sample	Primer_trimmed	Filtered	Denoised_FWD	Denoised_REV	Merged	Non_chimera
H11S1_S10	178539	172504	172061	171835	171410	141452
H11S2_S27	155693	151348	150968	150748	150384	121026
H11S3_S44	100924	98282	98052	97917	97699	80542
H11S4_S61	132017	128789	128417	128290	127940	100742
H11S5_S78	101985	99823	99624	99536	99352	84481
H11S6_S94	153565	149651	149297	149039	148701	117307
H11S7_S12	96501	93041	92791	92727	92499	82329
H11S9_S28	176428	170602	170253	169958	169636	138167
H12S1_S11	142992	138313	137862	137543	137117	116012
H12S2_S28	105365	102406	102094	101927	101626	87281
H12S3_S45	143780	139892	139539	139391	139054	116724
H12S4_S62	80741	78455	78192	78010	77763	64037
H12S5_S79	76005	74109	73911	73791	73604	64294
H12S6_S95	87894	85332	85116	85034	84831	77038
H12S7_S13	89910	86603	86380	86261	86054	77913
H12S9_S29	164462	158335	158007	157837	157530	134685
H13S1_S12	163968	159266	158934	158703	158401	131834
H13S2_S29	141532	137322	137074	136961	136724	116935
H13S3_S46	155219	151141	150840	150795	150506	125984
H13S4_S63	63058	60942	60813	60747	60623	54780
H13S6_S96	97677	94550	94337	94287	94084	77435
H13S9_S30	150431	145516	145175	144952	144633	113648
H14S1_S13	226409	219353	218720	218500	217911	177735
H14S2_S30	118815	115556	115268	115151	114872	92465
H14S3_S47	232268	225932	225315	225092	224501	163444
H14S4_S64	130978	127182	126800	126641	126281	104381
H14S5_S80	179173	174356	173929	173738	173334	123478
H14S6_S1	78628	75907	75726	75691	75520	61768
H14S7_S14	130101	125989	125481	125364	124910	92901
H14S9_S31	212939	204808	204280	203941	203448	149645
H15S1_S14	176110	170591	170135	169914	169479	139310
H15S2_S31	147937	143551	143218	142984	142664	114545
H15S3_S48	146108	142248	141922	141740	141435	119552
H15S4_S65	162352	157408	157039	156829	156482	127446
H15S5_S81	55641	54189	54094	54036	53946	48225
H15S6_S2	122420	118479	118151	118026	117723	88805
H15S7_S15	89887	86797	86570	86499	86299	79022
H15S9_S32	154404	149274	148883	148707	148349	128641
H16S1_S15	160431	154596	154266	154088	153772	141218
H16S2_S32	154692	149625	149395	149208	148986	130187
H16S3_S49	104214	100111	99889	99707	99497	85491
H16S4_S66	159930	153714	153411	153207	152920	135731
H16S5_S82	151399	146160	145924	145732	145511	128916

H16S6_S3	77922	75015	74802	74728	74532	67914
H16S7_S16	82235	78965	78749	78655	78460	72786
H16S9_S33	77792	74473	74289	74196	74032	67738
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H17S2_S33	90138	87233	87014	86912	86706	74764
H17S3_S50	109863	106146	105858	105724	105447	86397
H17S4_S67	150653	146337	145927	145717	145332	118050
H17S5_S83	102260	99686	99449	99298	99072	81713
H17S7_S17	92557	89191	88980	88781	88591	72521
H17S9_S34	137291	131856	131419	131177	130761	103176
H18S1_S17	107025	102646	102405	102188	101961	84037
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H18S3_S51	77175	74470	74284	74158	73991	64161
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H18S7_S18	214604	204401	203864	203472	202958	148736
H18S9_S35	104046	99902	99565	99347	99031	76680
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H1S4_S52	106469	102734	102532	102352	102166	90869
H1S5_S69	149959	144741	144485	144303	144056	123685
H1S6_S85	116960	113515	113269	113158	112922	95511
H1S7_S4	125579	121111	120818	120728	120453	99284
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H2S10_S37	147778	142452	142126	141957	141659	113425
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H2S5_S70	126492	123277	123043	122892	122674	106650
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H2S7_S5	236655	229660	228933	228806	228118	174269
H2S9_S20	105072	100862	100631	100441	100232	90005
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H3S4_S54	95434	92371	92107	91940	91696	72390
H3S5_S71	126736	122858	122597	122463	122211	105070
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H3S9_S21	120660	115196	114967	114781	114565	99595
H4S1_S4	174754	170037	169653	169477	169116	142990
H4S10_S39	65606	63069	62907	62835	62686	58833

H4S2_S21	164796	158507	158183	157745	157436	122280
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H4S4_S55	124342	119748	119414	119191	118875	95828
H4S5_S72	32839	31601	31499	31428	31330	25693
H4S6_S88	10112	9699	9666	9655	9625	9091
H4S7_S7	154388	148875	148468	148325	147955	126360
H4S9_S22	147607	141260	140915	140742	140413	123352
H5S2_S22	157659	151977	151591	151195	150824	122511
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H5S6_S89	126315	122616	122338	122140	121883	104237
H5S7_S8	148130	143382	143016	142858	142518	121510
H5S9_S23	97902	93422	93252	93004	92847	85084
H6S1_S6	196247	192260	191738	191536	191034	157910
H6S2_S23	91762	88824	88654	88457	88296	77057
H6S3_S40	149963	145441	145083	144847	144510	122093
H6S4_S57	111960	109120	108773	108635	108308	88647
H6S5_S74	119986	117375	117085	116930	116656	99865
H6S6_S90	233893	227619	226524	226144	225100	153170
H6S7_S9	98997	95958	95647	95528	95248	83735
H6S9_S24	174829	167548	166946	166551	165980	122684
H7S1_S7	204583	198648	198051	197764	197195	154643
H7S2_S24	173872	166942	166422	166118	165619	135118
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H7S6_S91	13057	12537	12465	12422	12359	11764
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H8S3_S42	223410	216604	216134	215862	215418	169774
H8S4_S59	96072	92974	92696	92529	92270	73879
H8S5_S76	142321	138681	138439	138323	138095	119408
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H8S7_S10	86141	82750	82502	82400	82172	69652
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H9S4_S60	133561	130156	129835	129645	129341	102008
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H9S7_S11	99798	96354	95938	95787	95407	74821
H9S9_S27	152396	147741	147333	147065	146683	115072

Appendix 11. Poster presented on "Eastman Dental Institute Away Day" (May 2019)

Do changes in environment alter the oral microbiome?

Violeta Kakiora[1], Paul Ashley[1], David Spratt[2], Andrew Smith[2]

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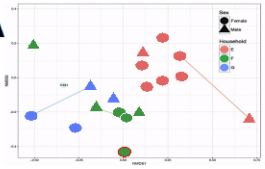
Introduction

We are interested in how the community of microbes in our mouths change as we age. We have recently acquired new evidence demonstrating that where we live and our immediate family has a significant effect on the composition of our oral microbiome and our own genes play almost no role (Figure 1).[1] These new findings provide the possibility of altering an individual's oral microbes through changing the environment they live in.

A



B

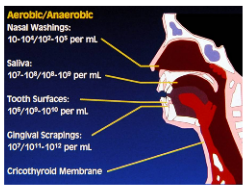
variable	R^2	p
run	0.0358	0.022
household	0.1378	0.011
gender	0.018	0.747
age	0.0359	0.012
residual	0.7725	
TOTAL	1	

Figure 1: Role of household and age on the oral microbiota (**A**) PCA plot with (**B**) PERMANOVA analysis with Adonis. Households clustered with a significant *p=0.011* and was responsible for 13.78% of the variation in the oral microbiome data. Age accounted for ~3.5% of the variability (*p=0.012*).

Background

The oral microbiome is the collection of microorganisms that live in the oral cavity (bacteria, viruses, archaea, fungi).[2] This unique microecosystem harbor over 800 to 1000 different oral bacterial taxa.[3] Both oral disease and other diseases, such as heart disease, diabetes and Alzheimer's disease,[4] are influenced by the types of bacteria that live in the mouth.

A



B

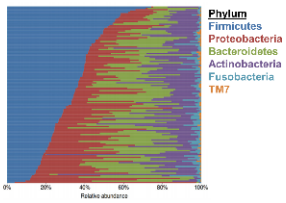


Figure 2: The complexity and variability of the oral microbiome. (**A**) Bacterial numbers in the different environments within the oral/nasal cavity. (**B**) Relative abundance of the six major bacterial phyla found in saliva and sorted by decreasing *Firmicutes* content (n=271).[1]

Aims

The aim of this study is to determine if the oral bacterial community is stable in adolescents and how the environment may influence the composition of this bacterial community.

Study design

New students entering a boarding school at Years 7, 9 and 11 will be recruited over a two-year period. Participants will complete a general health questionnaire, will undergo basic dental clinical examination and saliva samples will be collected (beginning, middle, end of each term). Two groups will be formed: the control group of non- boarders and the test group of boarders.

Analysis of results

Once saliva samples are collected they will be analyzed as follow:

1. Extraction and purification of the DNA in the saliva.
2. Performing DNA sequencing of a specific gene (16s rRNA gene) to identify all the bacteria in each sample.
3. Compare the data generated from the test and control groups to detect if it is different

Data collection from 1st Cycle (n= 17) (Figures 3)

Age	No
11	8
12	1
13	6
14	1
15	0
16	1

Figure 3a: Age

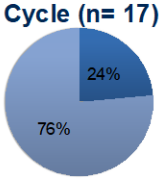


Figure 3b: Gender

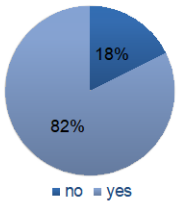


Figure 3c: Pets

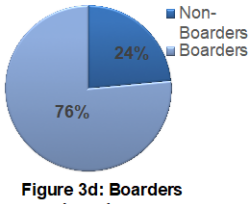


Figure 3d: Boarders non- boarders

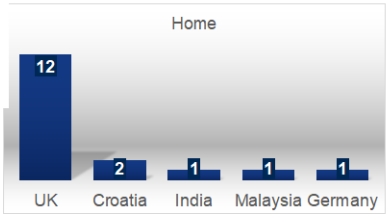


Figure 3e: Home country

ICDAS	Score
A	3
3	2
4	0
5	0

Figure 3g: ICDAS score of permanent dentition

Discussion

Studying adolescents who undergo a dramatic change in lifestyle and a major change in environment, will enable us to determine how much of an effect environment has on the oral microbial community. The longitudinal study will also allow us to look at stability of the oral microbiome in adolescents between the ages of 11-18 years. The possibility of altering an individual's oral microbiome through changing their environment may confer substantial health benefits.

References

1. Shaw L. et al. The human Salivary Microbiome is Shaped by Shared Environment Rather than Genetics: Evidence from a Large Family of Closely Related Individuals. mBio 8:e01237-17
2. Shaw L. et al. The oral microbiome. Emerging Topics in Life Sciences (2017) **1** 287-296
3. Gomez A. et al. The Oral Microbiome of children: Development, Disease and Implications beyond oral health. Microb Ecol (2017) 73:492-503
4. Dominy et al. Porphyromonas gingivalis in Alzheimer's disease brains: Evidence for disease causation and treatment with small- molecule inhibitors. Sci. Adv. 2019; 5 : eaau3333

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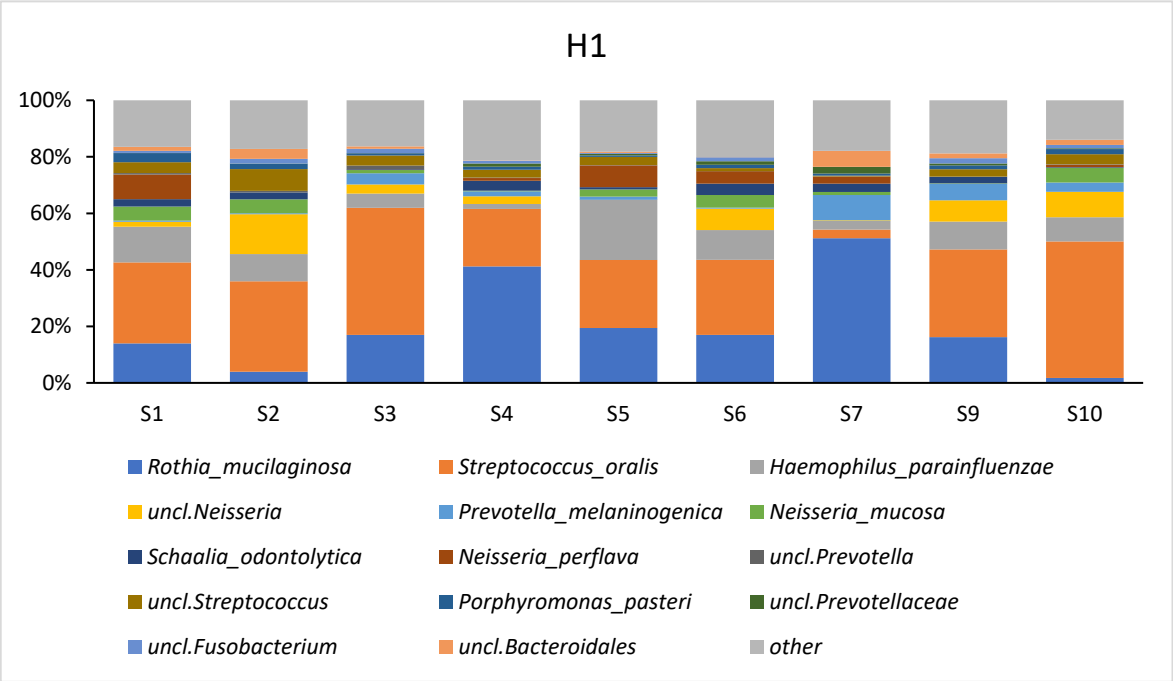
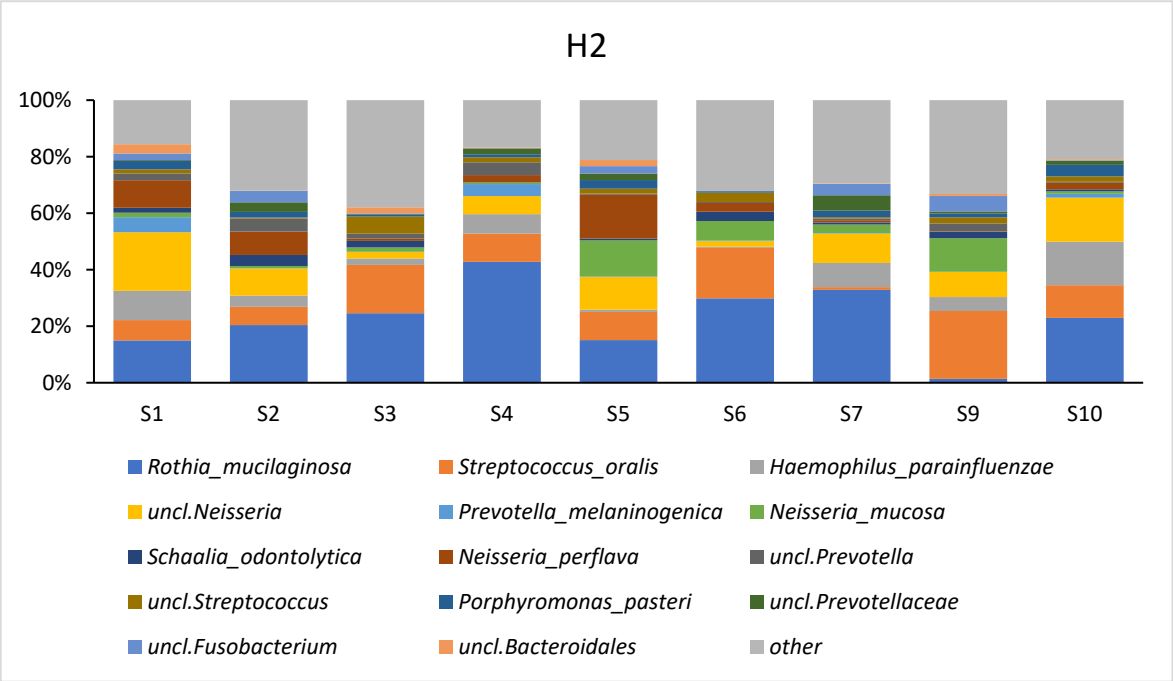
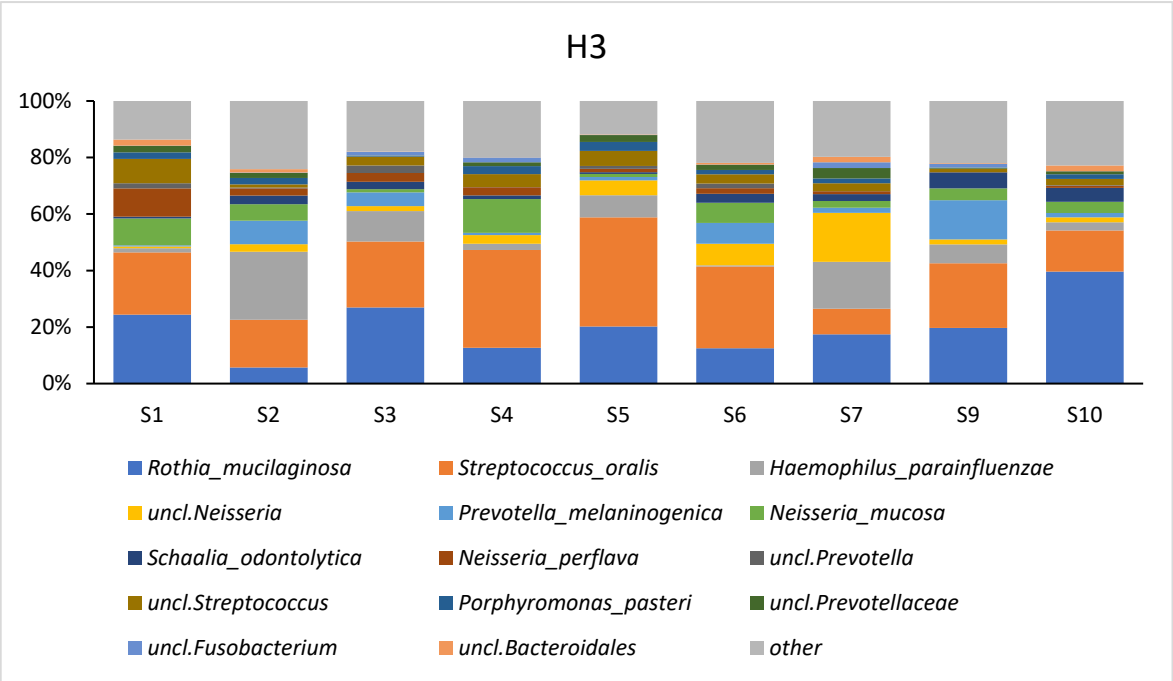
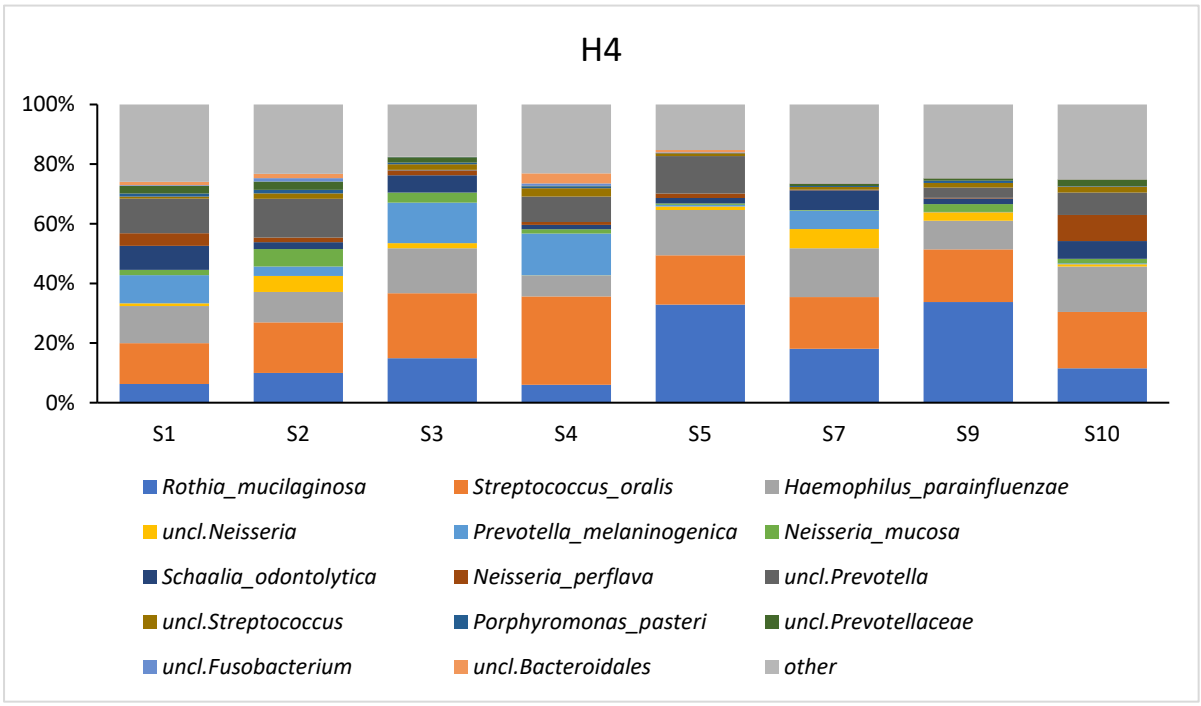
Appendix 12. Species relative abundance for each participant at each sampling point






H3
100%
80%
60%
40%
20%
0%
S1
S2
S3
S4
S5
S6
S7
S9
S10
Rothia_mucilaginosa
Streptococcus_oralis
Haemophilus_parainfluenzae
uncl.Neisseria
Prevotella_melaninogenica
Neisseria_mucosa
Schaalia_odontolytica
Neisseria_perflava
uncl.Prevotella
uncl.Streptococcus
Porphyromonas_pasteri
uncl.Prevotellaceae
uncl.Fusobacterium
uncl.Bacteroidales
other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100%
80%
60%
40%
20%
0%
S1
S2
S3
S4
S5
S7
S9
S10
Rothia_mucilaginosa
Streptococcus_oralis
Haemophilus_parainfluenzae
uncl.Neisseria
Prevotella_melaninogenica
Neisseria_mucosa
Schaalia_odontolytica
Neisseria_perflava
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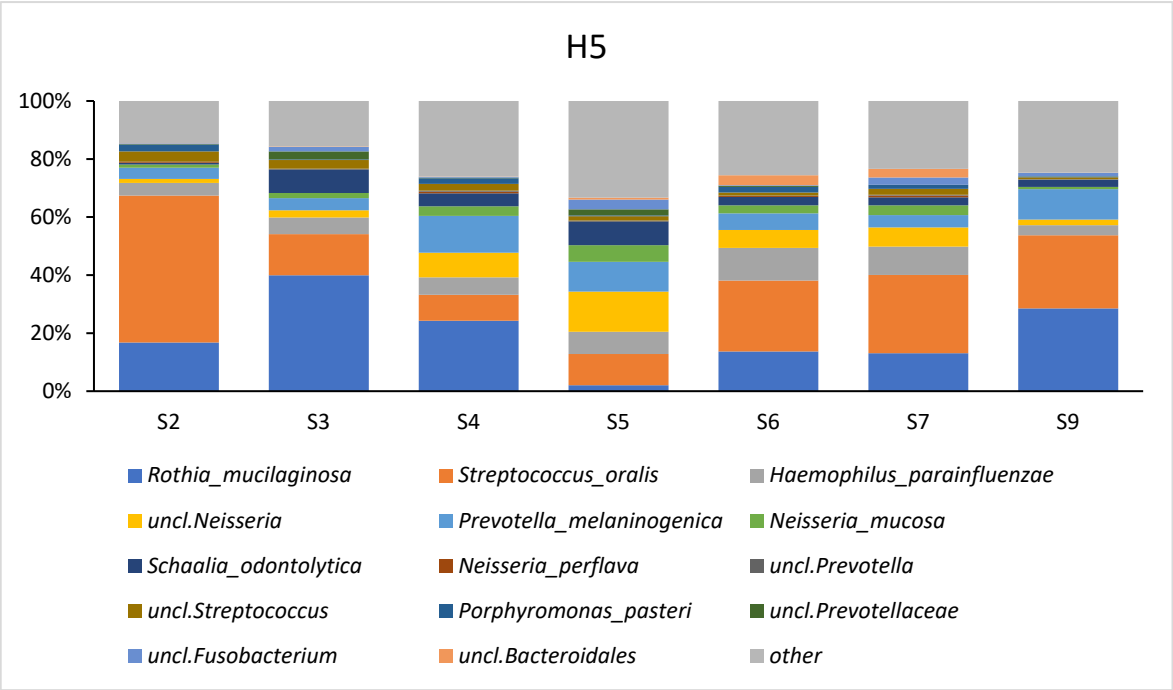
uncl.Prevotellaceae
uncl.Fusobacterium
uncl.Bacteroidales
other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S2
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S4
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S6
S7
S9
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Streptococcus_oralis
Haemophilus_parainfluenzae
uncl.Neisseria
Prevotella_melaninogenica
Neisseria_mucosa
Schaalia_odontolytica
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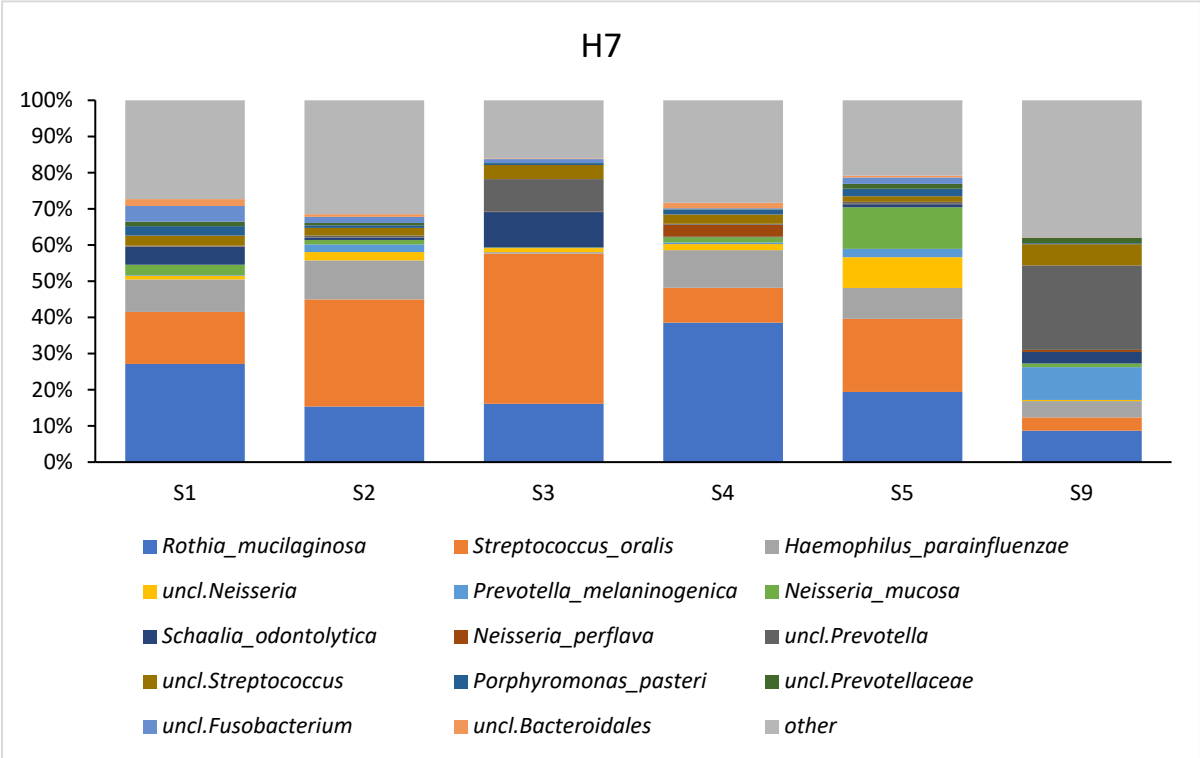
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80%
70%
60%
50%
40%
30%
20%
10%
0%
S1
S2
S3
S4
S5
S9
Rothia_mucilaginosa
Streptococcus_oralis
Haemophilus_parainfluenzae
uncl.Neisseria
Prevotella_melaninogenica
Neisseria_mucosa
Schaalia_odontolytica
Neisseria_perflava
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uncl.Bacteroidales
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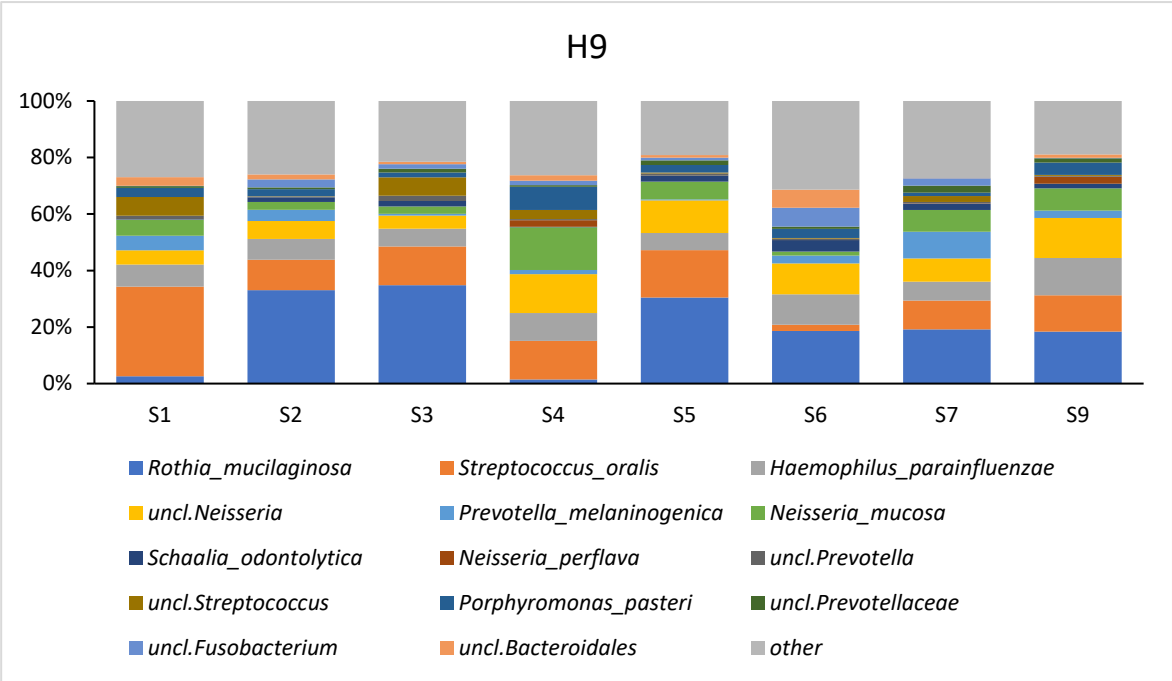
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80%
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40%
20%
0%
S1
S2
S3
S4
S5
S6
S7
S9
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Streptococcus_oralis
Haemophilus_parainfluenzae
uncl.Neisseria
Prevotella_melaninogenica
Neisseria_mucosa
Schaalia_odontolytica
Neisseria_perflava
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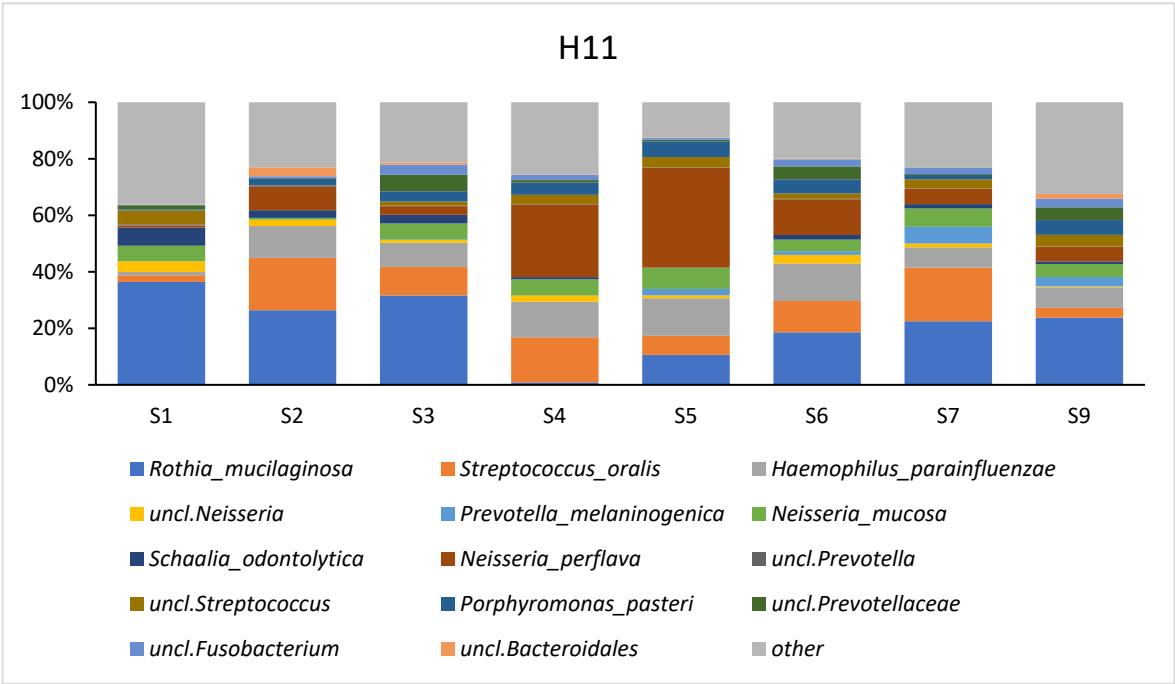
uncl.Bacteroidales
other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S1
S2
S3
S4
S5
S6
S7
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Streptococcus_oralis
Haemophilus_parainfluenzae
uncl.Neisseria
Prevotella_melaninogenica
Neisseria_mucosa
Schaalia_odontolytica
Neisseria_perflava
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uncl.Bacteroidales
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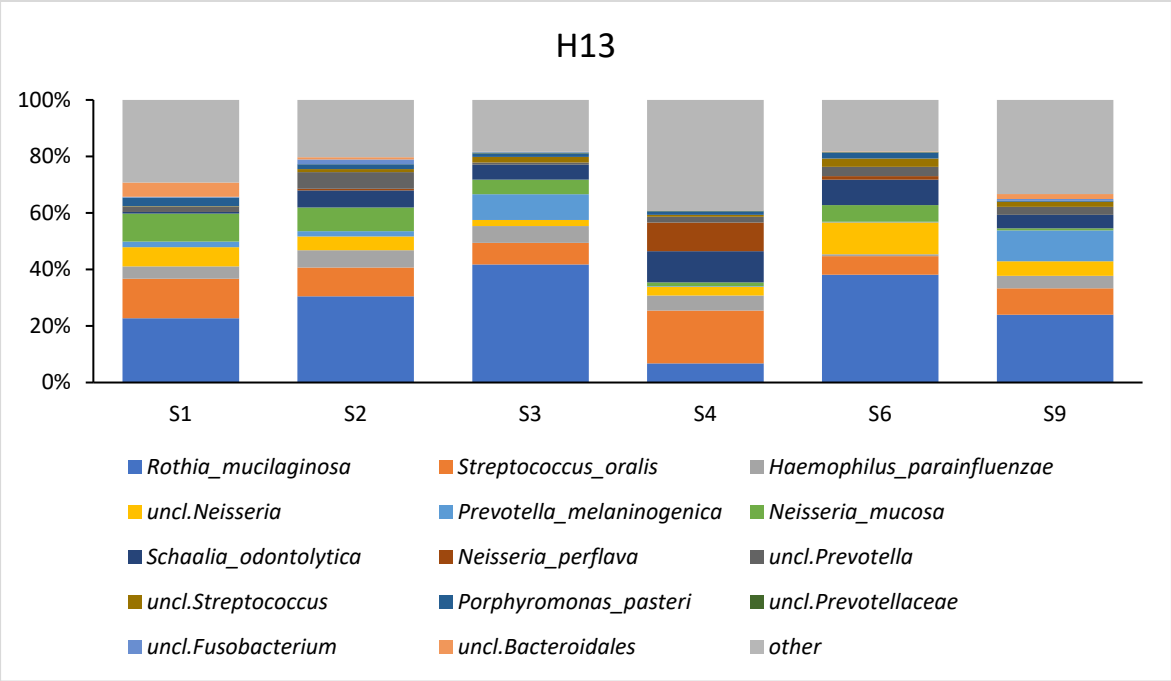
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80%
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40%
20%
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S1
S2
S3
S4
S6
S9
Rothia_mucilaginosa
Streptococcus_oralis
Haemophilus_parainfluenzae
uncl.Neisseria
Prevotella_melaninogenica
Neisseria_mucosa
Schaalia_odontolytica
Neisseria_perflava
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uncl.Streptococcus
Porphyromonas_pasteri
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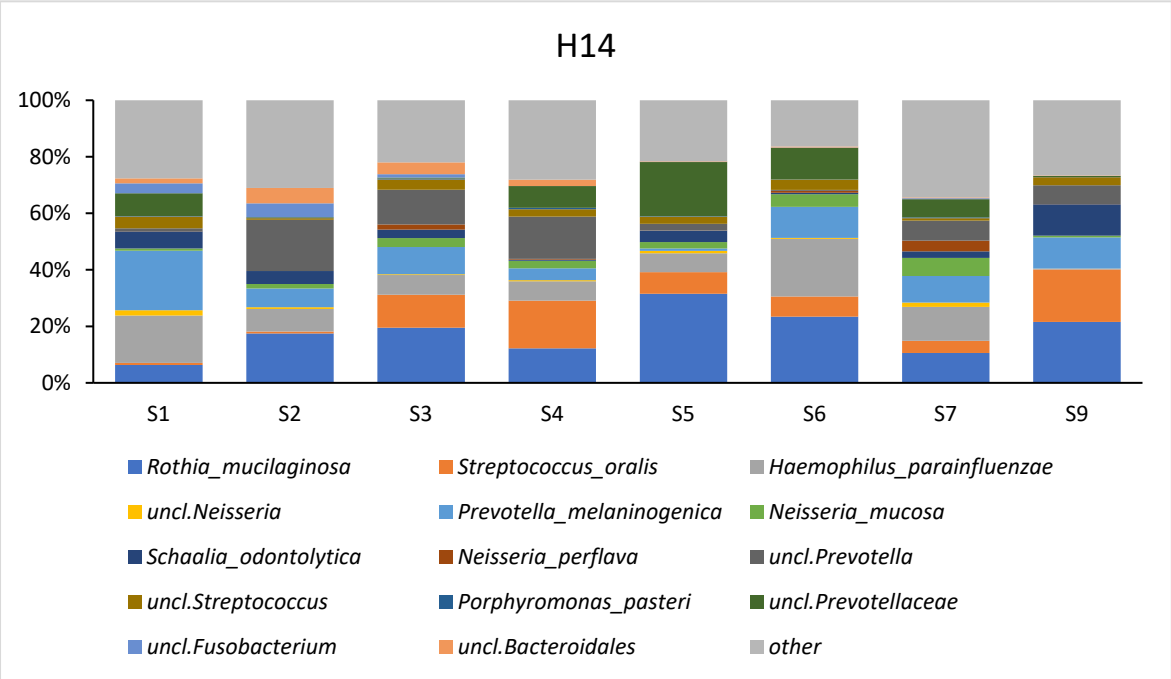
other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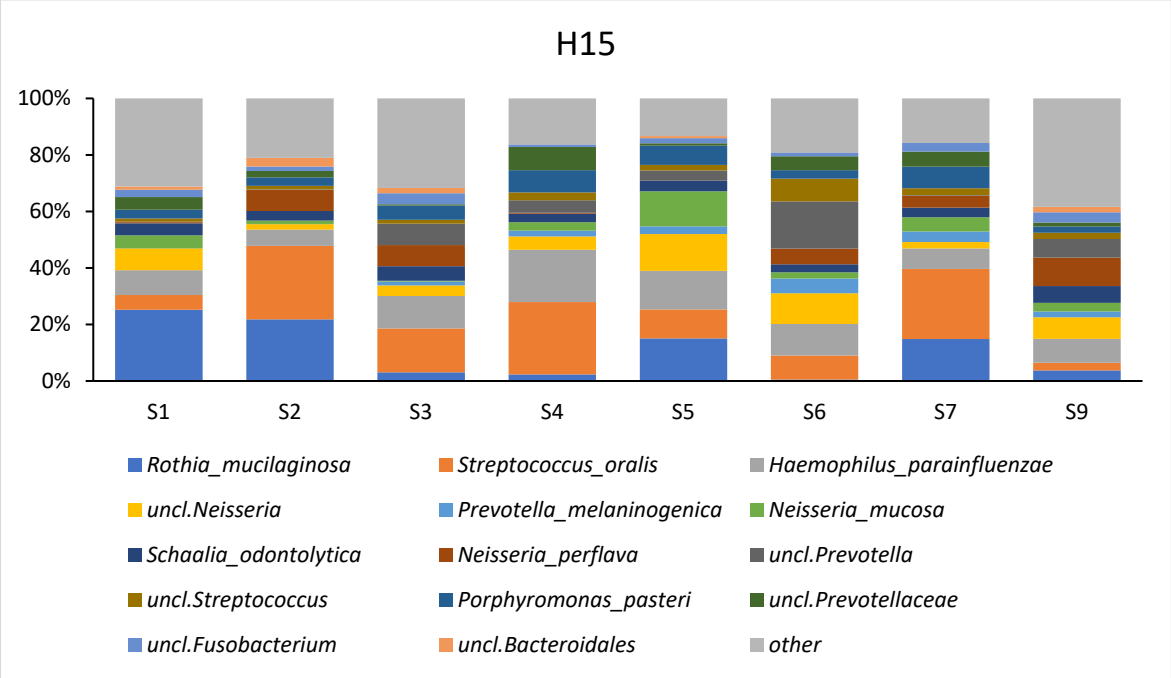
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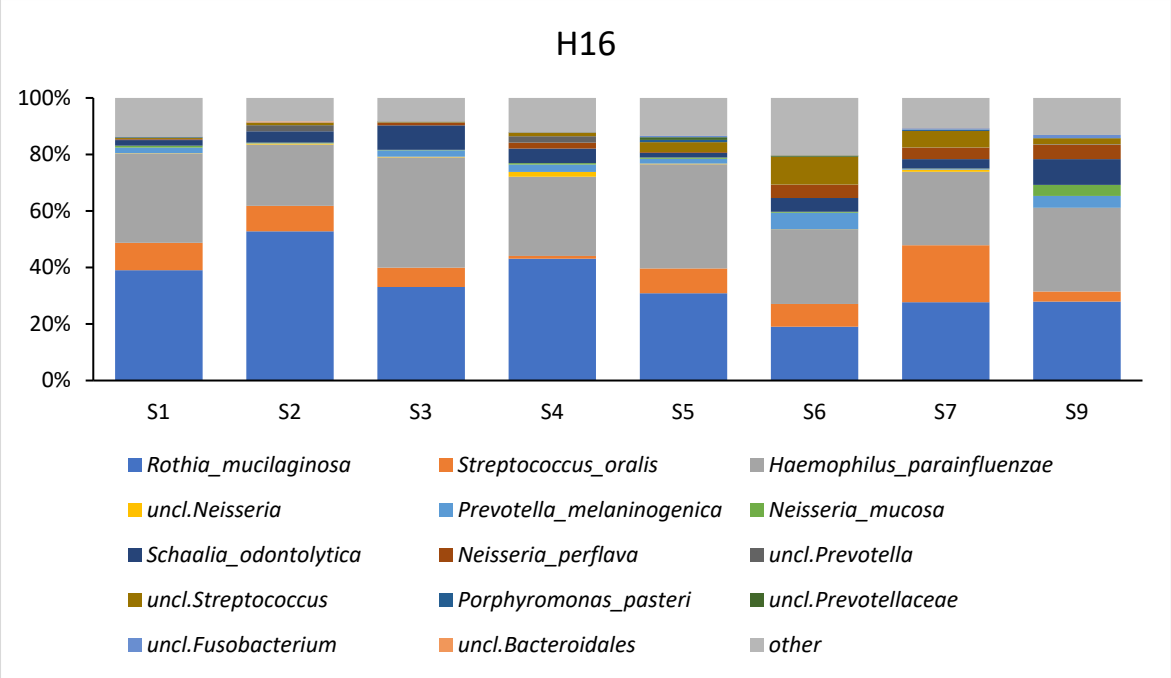
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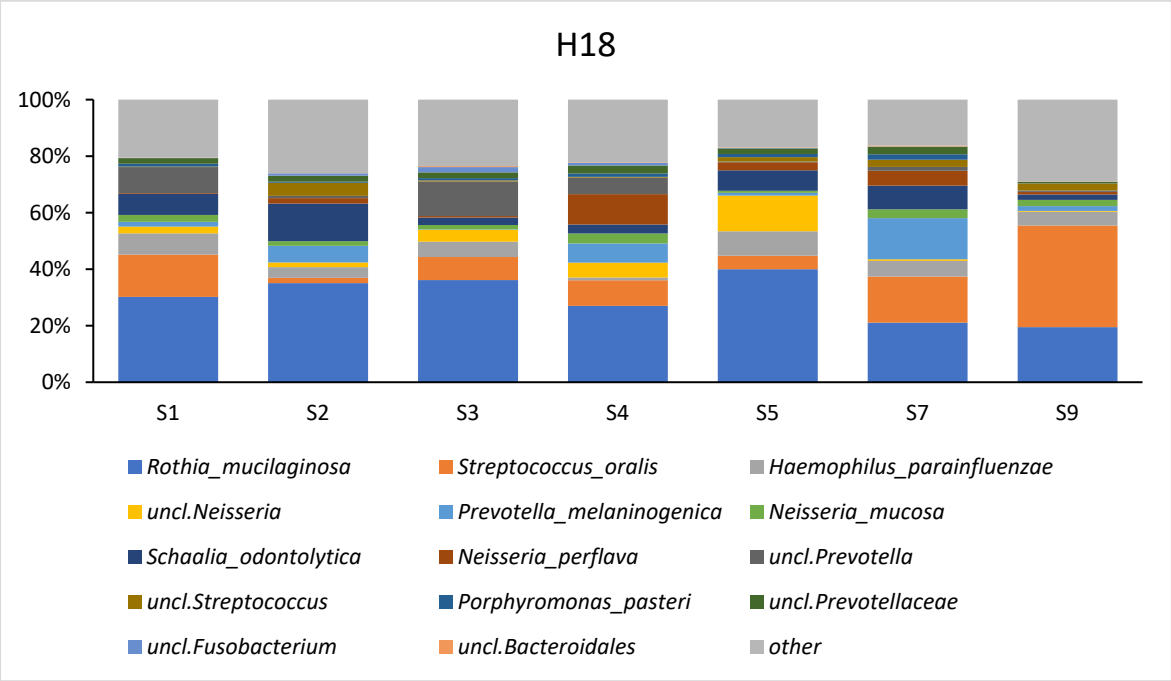
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other