Analysis of the oral bacteriome and mycobiome in children with inflammatory bowel disease compared to healthy children by 16S and ITS2 profiling

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

By

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July 2019

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Declaration

I hereby declare that this thesis has not previously been submitted for a degree at this or other university and that is represent my entirely my own work. I agree that this thesis may be lent or copied at the discretion of the librarian, Trinity College Dublin.

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29th April 2019

Summary

The oral cavity is an environment that is rich in microbial life, including bacteria, fungi and viruses. Many hundreds of species of bacteria and fungi have been identified in the oral cavity and these commensal organisms form part of our normal oral microbiota. Oral mucosa is a critical protective interface between external and internal environments and must serve as a barrier to the myriad microbial species present in this warm, moist environment. The oral cavity is the only area of the body in which hard tissues break through the epithelial surface. In many instances, our relationship with these organisms determines our state of oral and perhaps systemic health. For example, poor oral hygiene can lead to transitions in the species composition of the oral microbiota that can lead to tooth decay and in later life, periodontal disease. The periodontal epithelium surrounding the tooth is specialized to form an attachment and seal around each tooth. This unique function imparts special challenges to the tissue and leads to certain vulnerabilities associated with periodontal disease, especially in view of the continual exposure to the bacterial biofilm (dental Plaque) that forms on the tooth surface at the junction of the soft tissue. Thus, this anatomical region is one where there is a significant risk of bacterially and fungal induced infection and inflammation. Maintaining a healthy and diverse oral flora is therefore essential for continued oral health. Analysis of the oral metagenome has lagged somewhat behind studies of the human gut. Little is known about the stability of the oral flora and the effects of some systemic diseases, which can result in oral manifestations and possible changes in the bacteriome and mycobiome community inside the mouth. In the current study alteration in diversity of the oral microbiome and mycobiome in children with IBD were assessed. Tongue and buccal swabs were obtained from newly onset children with CD and UC were recruited (n=38, n=21) in respective, and 36 healthy individuals. DNA was performed for all swabs that were stored at -80°C.

The DNA was then used in PCR, targeting the bacterial 16S gene (V1-V2 region), and ITS2 barcode of fungal DNA. Amplicons were sequenced on the Illumina MiSeq platform. Sequences were analysed with the Mothur and DADA2 pipelines. Additionally, IBD children following therapy were also analysed. Our data demonstrate reduced biodiversity and lower species richness in tongue and buccal microbiota of CD children compared to healthy children. Our findings show a reduction in the levels of several major oral bacterial genera, including *Veillonella*, and *H. parainfluenza*, and increased levels of oral *Enterobacteriaceae* and Actinobacteria in patients with severe IBD. Previous studies have implicated these taxa in the pathogenesis of IBD. In our follow up studies, remission from IBD symptoms was associated with loss of *Enterobacteriaceae* from the oral microbiome. We observed an increase in *C. albicans* and *S. cerevisiae* in the oral mycobiota in IBD. Interestingly, *C. krusei* and *C. dubliniensis* were also observed in the oral cavity in IBD. Overall, the mycobiome changes observed were more related to IBD disease activity compared to microbiome changes. Shifting of the oral microbial community was noted in follow samples from patients after therapy, returning some taxa (*Veillonella*, *Enterobacteriaceae*) to levels close to the normal oral community. The observations in this study indicate that a full understanding of the oral microbiome IBD may be important to understand the aetiology and pathogenesis of the disease and these data may also have practical applications in diagnosis and monitoring disease progression.

Acknowledgements

When I look to this effort, I see many hearts and hands which moulded this work into shape.

To my extended family (brothers and sisters and their families).

A special thanks go also to my brothers and sisters-in law, for their encouragement and support.

With deep gratitude I acknowledge the constant support, encouragement and assistance I received from my supervisor

Prof. Gary Moran.

I owe a deep sense of gratitude to **Prof. David Colman, Prof. Derek Sullivan and Dr. Mary O'Donnell for** their kind help, support and co-operation throughout all my study period.

My heartfelt and gratitude to**, Dr. Paddy Fleming, Dr. Kirsten Fitzgerald, Dr. Seamus Hussey** for support in making this endeavour possible

I gratefully acknowledge the help and assistance given to me by **Dr. Elaine Kenny and Kathleen McGrath** for their guidance and support in sequencing technology.

I wish to place on record my heartfelt and sincere thanks to **Dr. Peter Flanagan, Dr. Peter Kinnevey, Dr. Abdrazak Amer, Dr. Emily Deasy, Dr. Megan Earls, Jessica Fletcher, Elaine Moloney**, **Ajith Selvaraj, Sarah Egan** and to all members of Microbiology research unit at DDUH for their help and valuable suggestions rendered.

I also wish to thank **Isabel Fleischmann** for her patience and help with the bibliography.

MY GRATITUDE TO ALL OF THEM.

KHALID ELMAGHRAWY

Dedication

To…My father's soul.

'' Whose many sacrifices made possible my education''?

To my mother for her encouragement and prayers.

To…My dear wife ***Ghada*** *and my sons* ***Abdelftah, Abdallah,*** *and* ***Amro*** *and to my little daughters* ***Jumana*** *and* ***Tasneem*** *without their constant patience and encouragement I would not have succeeded in my efforts.*

Poster and Oral Presentations conducted during the PhD

Published Abstract (Poster)

1. The oral mucosal microbiome in children with Crohn's disease exhibits reduced biodiversity compared to healthy children, revealed by 16S profiling. *Journal of Oral Microbiology*, 9 (Suppl. 1), 1325254. Poster presented at the European Oral Microbiology Workshop, May 2017, Stockholm, Sweden (Appendix 18)

Poster Presentations

1. The oral microbiome in children with Crohn's disease exhibits reduced biodiversity compared to healthy children, revealed by 16S profiling. TCD Faculty of Health Sciences Research day, September 2016. **Winner of Best Poster**. (Appendix 19)
2. Analysis of the Oral Microbiome and Mycobiome in children with Crohn's disease. IADR Annual Conference, London, July 2018. (Appendix 20).

Oral Presentations

1. The oral microbiome in children with Crohn's disease exhibits reduced biodiversity compared to healthy children, revealed by 16S profiling. National Children's Research Centre Research Day, Our Lady's Children's Hospital Crumlin, December 2016.
2. The impact of Crohn's disease on the Oral Microbiome. Irish Division of IADR, Belfast, November 2017.

List of Abbreviations

Abbreviation	Full Name
AMOVA	Analysis of MOlecular Variance
CD	Crohn's Disease
CDB myco	Crohn's Disease Buccal mycobiota
CDB myco genus	Crohn's Disease Buccal mycobiota at genus level
CDT	Crohn's Disease Tongue
CDT myco	Crohn's Disease Tongue mycobiota
CDT myco genus	Crohn's Disease Tongue mycobiota at genus level
CFU	Colony Forming Unit
CT	Cycle threshold
DC	Dental Caries
DMFT	Decayed/Missing/Filled Teeth index
EEO	Electroendosmosis
GIT	Gastrointestinal tract
HC	Healthy Control
HCB	Healthy Control Buccal
HCB myco	Healthy Control Buccal mycobiota
HCB myco genus	Healthy Control Buccal mycobiota at genus level
HCF	Healthy Control female
HCF	Healthy control female
HCM	Healthy Control male
HCM	Healthy control male
HCT	Healthy Control Tongue
HCT myco	Healthy Control Tongue mycobiota
HCT myco genus	Healthy Control Tongue mycobiota at genus level
HMP	Human Microbiome Project
HOMOVA	HOmogeneity of MOlecular Variance
HSCT	Hematopoietic Stem Cell Transplantation
IBD	Inflammatory bowel disease
IBD myco	Inflammatory bowel disease mycobiota
IBD.FU	Inflammatory bowel disease follow up
IBD.FU myco	Inflammatory bowel disease follow up mycobiota
ISHAM	International Society for Human and Animal Mycology
Jclass	Jaccard index
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
Min	Minutes
NGS	Next Generation sequencing
NMDS	Non-metric Multidimensional Scaling
OTUs	Operational Taxonomic Units
S	Second
Seq	Sequences
Thetayc	Yue and Clayton dissimilarity index

UC	Ulcerative Colitis
UCA	Ulcerative Colitis abscess
UCB	Ulcerative Colitis Buccal
UCB myco	Ulcerative Colitis Buccal mycobiota
UCB myco genus	Ulcerative Colitis Buccal mycobiota at genus level
UCF	Ulcerative Colitis female
UCG	Ulcerative Colitis gingivitis
UCM	Ulcerative Colitis male
UCMR	Ulcerative Colitis traumatic ulcer
UCR	Ulcerative Colitis ulcer
UCS	Ulcerative Colitis dental caries
UCT	Ulcerative Colitis Tongue
UCT myco	Ulcerative Colitis Tongue mycobiota
UCT myco genus	Ulcerative Colitis Tongue mycobiota at genus level
IFX	Infliximab

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Chapter 1

General Introduction

Chapter 1. General Introduction.

1.1 Human microbiota

The human microbiota consists of 10-100 trillion symbiotic microbial cells harboured by each person (Ursell *et al.* 2012). Recently, progress in DNA, RNA and protein analysis, in association with increased computing technology has led to improvement in the analysis of this microbial community. However, the rapid evolution of analysis of microbial communities has led to some misunderstanding of the vocabulary used to explain different aspects of these communities and their environments. For example, the terms microbiome, microbiota, and metagenomics are often confused (Marchesi and Ravel 2015). The term microbiota was first defined by Lederberg and McCray in 2001 and defines the microbial taxa associated with humans, whereas the term microbiome refers to the catalogue of these microbes and their genes. Moreover, the term metagenomics refers to the technique of shotgun sequence characterisation of total genomic DNA, even though the term is often mistakenly used for studies of markers genes such as 16S RNA gene (Ursell *et al.* 2012), and 18S rRNA gene (Marchesi and Ravel 2015). These days, advancements in laboratory technologies for DNA and RNA analysis and sequencing have improved our understanding of human microbial communities. In addition to that, the rapid development in these advanced laboratory methods has led to the discovery of new microbes and their natural communities, and overcomes the drawbacks of conventional culture methods.

Humans are colonized by many microorganisms (bacteria, archaea, fungi, viruses, and protozoa) (Cenit *et al.* 2014). The human microbiome project (HMP) characterized the microbial communities of several niches of the human body including the gut, nasal

cavity, oral cavity, skin, and urogenital system, which was funded by the National Institute of Health (Dave *et al.* 2012) (Figure 1.1).

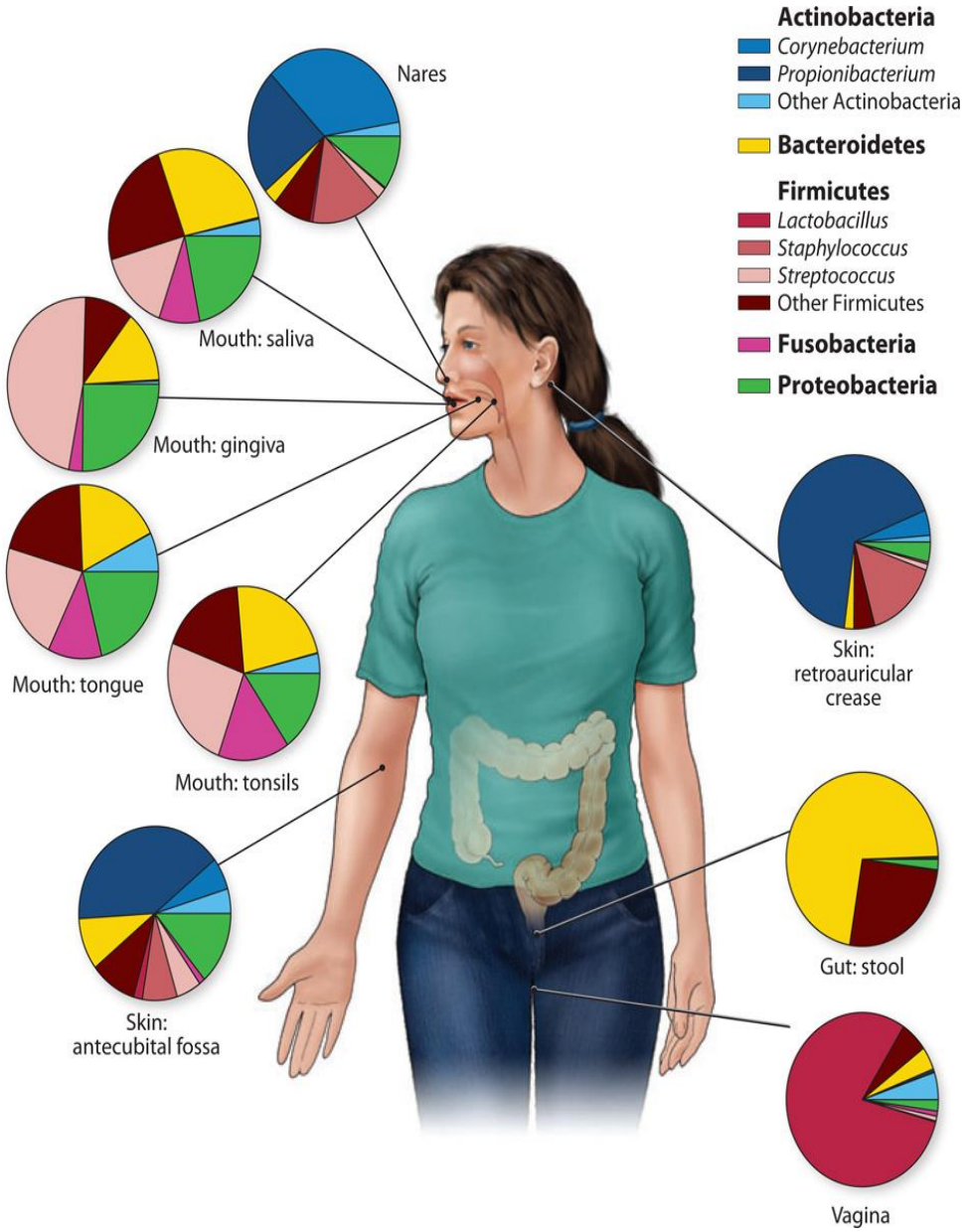


Figure 1.1 Diversity of the human microbiota across the various niches of the body (Grice and Segre 2012).

1.1.1 Bacterial Taxonomy.

The human microbiome is dominated by approximately 12 main bacterial groupings termed phyla. The five dominant phyla are shown in Figure 1.1, namely Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria and Fusobacteria. Distinct organisms within these groups are termed species. Similar species can be grouped to form genera (e.g. *Streptococcus* spp.). The Actinobacteria and Firmicutes account for the majority of Gram positive species in the human microbiome, whereas the Bacteroidetes, Fusobacteria and Proteobacteria are exclusively Gram negative.

1.1.2 Human microbiota in health.

In the human body, microbial cells (the human microbiota) are thought to be at least as numerous as host cells (Dave *et al.* 2012, Cenit *et al.* 2014). The microbiome composition and function differs from person to person depending on various factors, including the location, age, sex, race and diet of the host. The commensal microbiome first colonizes shortly after birth and there is a correlation between the first new-born infant gut microbiota and the vaginal and skin microbiota of the mother (Wang *et al.* 2017). Furthermore, the mode of delivery (natural versus caesarean) can impact on shaping the early gut microbiota composition (Cenit *et al.* 2014). The human microbiota, particularly the gut microbiota are considered to be an essential organ of the human body. The human digestive tract microbiome is made up of hundreds of species in each individual and these microbes carry 150 times more genes compared to the human genome (Wang *et al.* 2017). Microbial population densities in this complex bacterial consortium reach their maximum values in the colon with 10^{11} bacteria per gram of content (Dore and Corthier 2010). The gut microbiota play a critical role in human health and disease and several studies including the HMP have demonstrated the role of gut

flora in human health (Jandhyala *et al.* 2015). For example, the commensal microbiome of the intestine has been implicated in nutrient absorption, mucosal barrier fortification, xenobiotic metabolism, angiogenesis, and postnatal intestinal maturation (Hooper *et al.* 2001). Furthermore, the human microbiota stimulates the immunity (innate and adapative) and plays an important role in maturation of the immune system (Berger *et al.* 2015). Additionally, a study by Nehra *et al.* (2016), assessing the correlation between the gut microbiota and obesity reported that the gut microbiota has the ability to regulate body fat deposition, metabolism, and immune function. In a recent study by Singhal *et al.* (2019), the association between Serotonin Transporter (SERT) deficiency and changes in metabolic function of the gut microbiome in mice was investigated. This study showed SERT deficient mice, which exhibit metabolic features similar to type II diabetes, exhibit gut dysbiosis similar to that described in obesity, providing further links between microbiome changes and metabolic disorders.

Most studies of the gut microbiota have focused on the bacterial component. However, analysis of the fungal microbiome has lagged behind the gut bacteriome, largely due to the fact that the gut mycobiota comprise a numerically small percentage of the gut microbiota (Kumamoto 2016). Recent studies have highlighted the significance of the gut mycobiota in health and development of human disease. Analysis of the fungal community of the intestinal tract was carried out by Heisel *et al.* (2015) who concluded that the diversity of the infant faecal mycobiota was similar to that of the bacteriome community. Several important findings on the gut mycobiome include reports that the gut mycobiome is less stable than the bacterial microbiome (Hallen-Adams and Suhr 2017) and that fungi influence the metabolome of the microbial community (Limon *et al.* 2017). A recent study of the gut mycobiome in heathy subjects showed that the adult human fungal microbiota exhibits lower diversity compared to gut the bacteriome, with

Saccharomyces, *Malassezia*, and *Candida* representing the main fungal genera (Nash *et al.* 2017). Another recent study by Auchtung *et al.* (2018) attempted to differentiate between transiently present fungal species and those that colonize the gastrointestinal tract (GIT). This study detected a very low abundance of fungi in the GIT, and many of these fungi were shown to be transiently present from the diet (*Saccharomyces*) or the mouth (*Candida*) and did not grow in gut-like conditions in vitro, suggesting that the mycobiome may not persist in the gut of individuals with a healthy immune system. However, fungal colonization may be a symptom of disease, for example in patients with acute leukaemia, HSCT recipients and solid organ transplant recipients (Low and Rotstein 2011).

1.1.3 Human microbiota in disease.

Even though microorganisms in host associated ecosystems adapt to minor changes in the environment, huge changes could challenge the whole microbial community, leading to substantial consequences for the host. Perturbation in the microbiota may lead to dysbiosis which can be defined as "changes to the structure of a microbial community that are detrimental to its host" (Miles 2015). Furthermore, changes in the structure of the microbial community can impair important functions, including its ability to resist foreign microorganisms. The human gut microbiota constitutes a complex ecosystem, impacting on human health and well-being. This may impact on the prevalence of several diseases, including allergies, inflammatory bowel diseases (IBD) and also could effect metabolic and degenerative disorders (Dore and Corthier 2010). Changes in microbial species have been closely linked to several diseases of the GIT including cancers, IBD and atopic disease and changes in the gut microbiome may lead to an increased susceptiblilty to such diseases within the gut. Furthermore, changes in the gut microbiota

have also been associated with other extra-intestinal diseases, namely rheumatoid arthrits (RA), type 2 diabetes, obesity, cardiovascular disease and even psychiatric and neurodegenerative diseases such as autism, depression, Alzheimer's Disease, and Parkinson's Disease (Blum 2017, Cenit *et al.* 2014) (Figure 1.2). However, the evidence for a mechanistic link between these diseases and microbiome changes is weak. In the case of diseases of the GIT, the aetiology of many common diseases is still not well-defined in the literature, but recent evidence suggests that environmental and genetic factors could be responsible for perturbation of the homeostasis between the mucosal immune system and the gut microbiota resulting in inflammation (Kałużna-Czaplińska *et al.* 2017). In the present study, we aim to investigate the oral microbial (bacteriome and mycobiome) signatures in treatment naïve children with IBD at baseline and following therapy compared to healthy individuals. In the following section, we discuss in brief the role of the gut microbiota in inflammatory bowel disease (IBD), as well as the role of the oral microbiota in IBD.

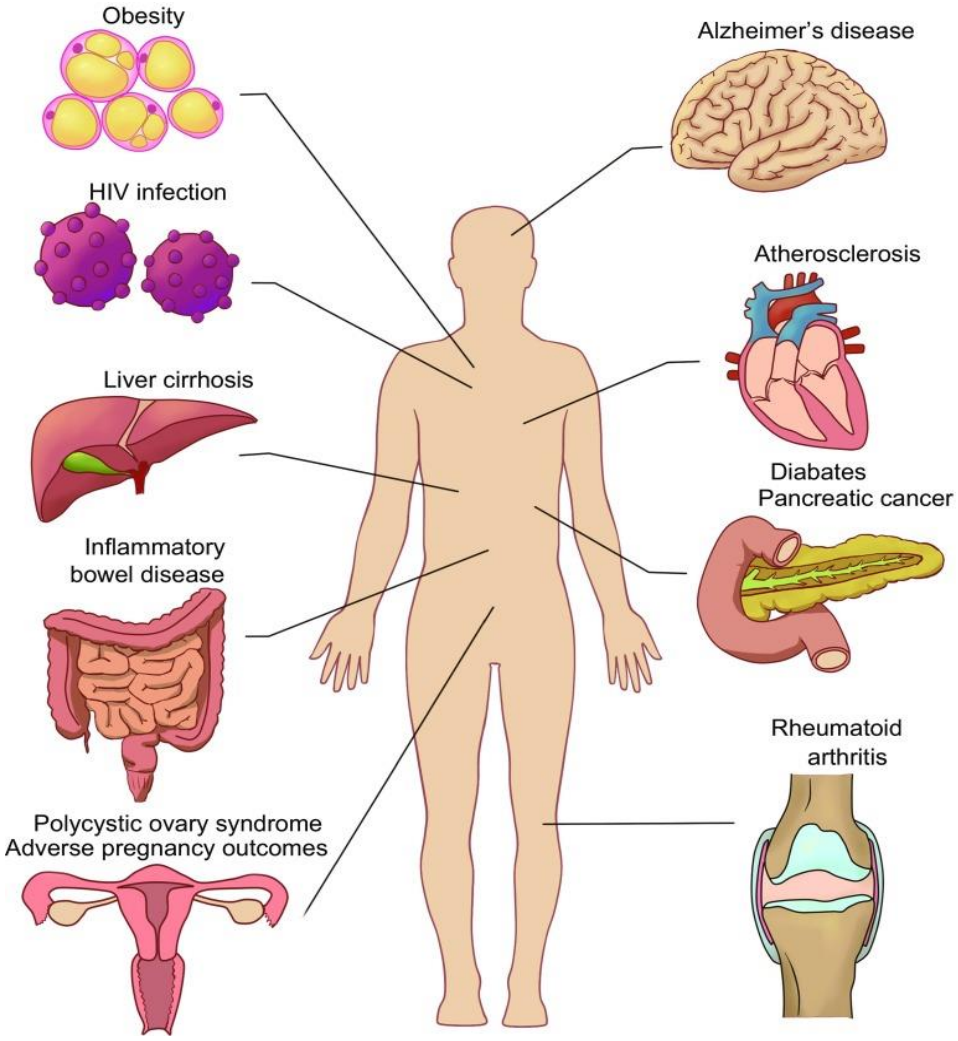


Figure 1.2 Examples of systemic disease associated with dysbiosis in the human microbiota (Gao *et al*. 2018).

1.1.4 Epidemiology and Pathogenesis of IBD.

The most common gastrointestinal disease associated with microbial changes is inflammatory bowel disease (IBD), including several inflammatory conditions of the small intestine and colon. The major types of IBD are Crohn's disease (CD) and ulcerative colitis (UC). The inflammation in CD can affect any part of the gastrointestinal tract (GIT) and affects all layers, while the inflammation in UC is limited to the colon

and only affects the mucosa and superficial submucosa, with deep layers unaffected. The pathogenesis of IBD is still hotly debated, but the disease involves a strong inflammatory response that may be triggered by viral infection or the hosts' own microbiota. Additionally, CD is more common in those who have family members with the disease, indicating that there may be a hereditary component. There is no official database which gives accurate figures, but according to the Irish Society for Colitis and Crohn's disease (www.iscc.ie), it is thought that at least 40,000 people are living with IBD in Ireland. Males and females are affected equally and patients can be diagnosed at any age, including babies and children. According to the ISCC, paediatric cases of IBD have increased three-fold in Ireland since 2003 and in general, most cases are diagnosed between the ages of 15 and 30 (https://iscc.ie/worldibdday/).

1.1.5 Systemic manifestation Crohn's disease.

The variability in location and the transmural nature of Crohn's disease leads to a wide variety of presentations in CD patients. Patients usually present with abdominal pain and diarrhoea. In severe cases, patients may present with systemic symptoms, including fatigue, anorexia, and weight loss (Cheifetz 2013). Crohn's and Colitis UK have listed the main systemic symptoms of CD, including diarrhoea, cramping pain, tiredness and fatigue, feeling generally unwell, loss of appetite and loss of weight and anaemia. Some patients, particularly CD patients may develop strictures and fistula. However, CD symptoms are often variable and not all patients present with diarrhoea or abdominal pain (Feuerstein and Cheifetz 2017).

1.1.6 Classification of Crohn's disease.

Inflammatory bowel disease, including CD and UC have been classified according to different categories, namely age at diagnosis, sex, family history, location of disease, response to treatment (Disease behaviour) (Louis *et al.* 2001). In Rome in 1991, the International Working Party on Crohn's disease proposed a classification based on anatomical distribution, operative history, and clinical behaviour (inflammatory, fistulising, or stenotic disease), however this classification was not appropriate for routine clinical application. Reanalysis of this classification was carried out at the World Congress of Gastroenterology in Vienna in 1998. This later classification was further modified at the Montreal World Congress of Gastroenterology in 2005. The two classifications were compared to each other in an article by Satsangi *et al.* (2006) and are presented in (Appendix 1). Recently, to better characterize IBD, the Paris Classification scheme was introduced and validated by an international group of pediatric IBD experts (Levine *et al.* 2011). The Paris Classification is a new evidence-based consensus recommendation for paediatricians and is a modification of the Montreal criteria (Eszter Muller *et al.* 2014) (Appendix 2). Ileocolonic involvement is the most characteristic disease location in Crohn's disease (CD) based on applying the Paris Classification. In the current study, the disease phenotype of the participants attending Our Lady's Children's Hospital, Crumlin (OLCHC) was recorded using the Paris classification scheme (Appendix 3).

1.1.7 Diagnosis and treatment of Crohn's disease.

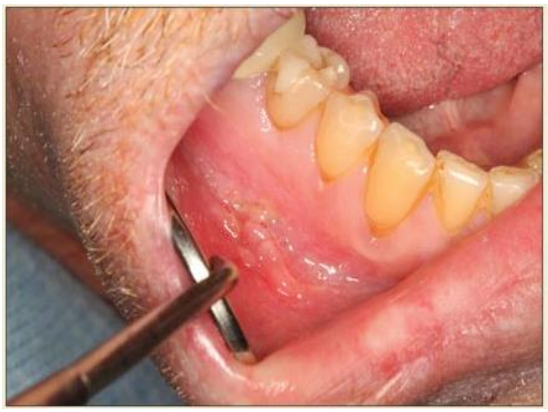
Crohn's disease is a chronic inflammatory disease categorised by relapsing behaviour, occurring at any age, but more often in second and third decade and can affect all segments of gastrointestinal tract (GIT) (Yoon *et al.* 2015). A detailed account of diagnosis and treatment is beyond the scope of this Chapter. However, here we present an outline of diagnostic tools and therapeutic regimens. The diagnostic procedures and therapeutic decisions depend on the individual patient. Several diagnostic tools are used in CD diagnosis, including patient's history and examination and supported by laboratory, serologic, radiologic, endoscopic (colonoscopy, sigmoidoscopy and capsule endoscopy), and histologic findings (Laass *et al.* 2014). Furthermore, ultrasound (Dillman *et al.* 2017), Computed Tomography (CT) Scanning (Dillman *et al.* 2015), and Magnetic Resonance Imaging (MRI) (Yoon *et al.* 2015). Early diagnosis of CD is important to avoid an unfavourable course in CD patients and to allow early treatment (Moon *et al.* 2015). The therapeutic strategy in CD management depends on several factors, namely disease severity, location, behaviour and previous or planned surgery (Laass *et al.* 2014). The treatment approach should be customized to the individual CD patient and focused on inducing and maintaining remission, preventing complications, minimizing toxicity, and improving quality of life (Wei *et al.* 2017). In the present study, the disease severity was categorized for each participant at baseline and following therapy (Appendix 1.4). During the treatment period the studied participants received various therapies depending on patient's status. For example, in the current study antibiotic therapy (flucloxacillin, metronidazole), supplements (iron, vitamin D, multi-vitamins, SandoK [potassium]), anti-inflammatories (Infliximab, Sulfasalazine, Salofalk, Asacol, Becotide, Lansaprazole) and intensive nutritional supplements (e.g. Scandishake drinks) were prescribed depending on the patient's specific presentation (Appendix 5).

1.1.8 Oral Crohn's disease (OCD).

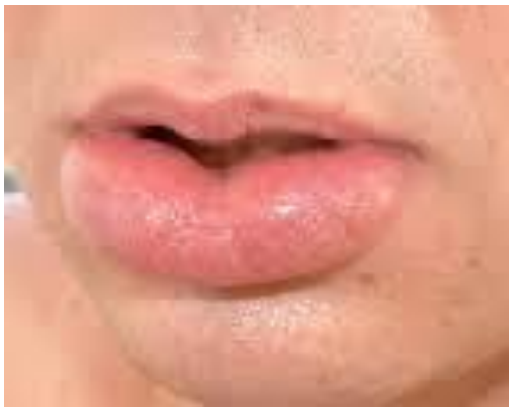
Crohn's disease (CD) has been described in several previous studies as an inflammatory and likely immunologically mediated disease of unknown aetiology (Stavropoulos *et al.* 2004). Inflammation most frequently affects the distal ileum and colon but may occur in any part of the gastrointestinal tract including the mouth (Hussey *et al.* 2011). Crohn's is an inflammatory bowel disease characterized by granulomatous inflammation (Padmavathi *et al.* 2014, Harikishan *et al.* 2012, Woo 2015). The term oral Crohn's disease (OCD) is used to describe patients with intestinal CD who exhibit involvement of the oral cavity with a wide variety of disease-specific oral lesions (Rowland *et al.* 2010).

1.1.8.1 Oral manifestation of Crohn's disease.

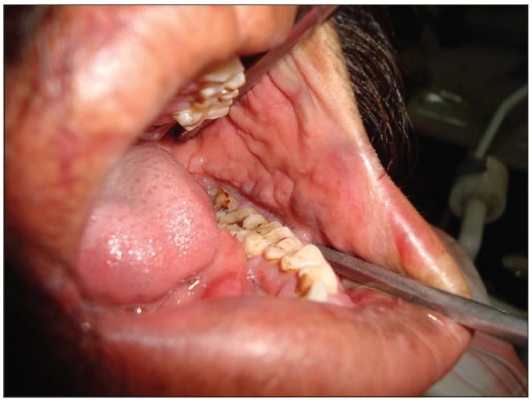
Many oral lesions have been described in CD patients, including swelling of the lips, buccal mucosal swelling or "cobble-stoning", mucogingivitis, deep linear ulceration and mucosal tags (Figure 1.6). In addition to that, submental lymphadenopathy, perioral erythema with scaling, recurrent buccal abscesses, and angular cheilitis are often observed in patients with CD (Rowland *et al.* 2010, Harikishan *et al.* 2012). The oral manifestations of inflammatory bowel diseases (IBD) are diverse and based on their relationship with CD activity can be classified as specific (e.g. mucosal swelling) and non-specific oral lesions (e.g. angular chelitis). The authors of this study identified specific manifestations that were more common in CD patients relative to other types of IBD, such as Indurated tag-like lesions, Cobble-stoning, and Mucogingivitis (Lankarani *et al.*, 2013).



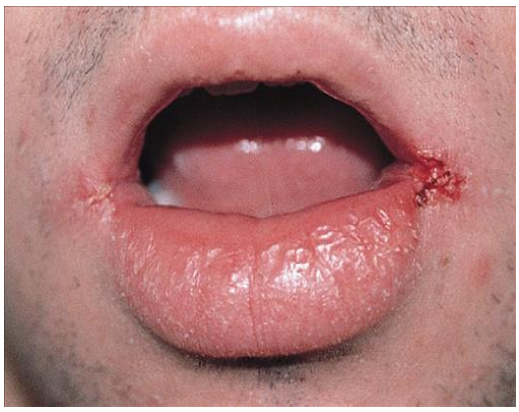
Deep linear ulceration and mucosal tags (Anderson *et al.* 2015)



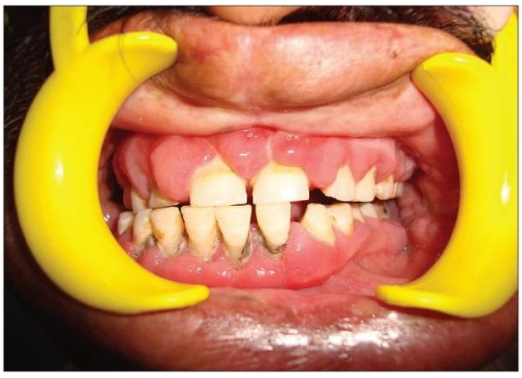
Lip Swelling (Triantafillidis *et al.* 2008)



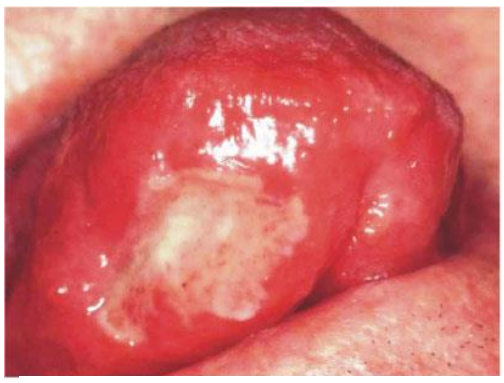
Epithelial “Cobblestone” (Padmavathi *et al.* 2014)



Angular cheilitis (Dupuy *et al.* 1999)



Enlargement of gingiva (Padmavathi *et al.* 2014)



Oral ulceration of tongue (Leao *et al.* 2007)

Figure 1.3 Intra and Extra oral photographs showing oral lesions in Crohn’s disease.

Similar oral manifestations have been recorded in different patient cohorts throughout the world (Rowland *et al.* 2010, Lourenco *et al.* 2010, Hussey *et al.* 2011, Docktor *et al.* 2012, Lankarani *et al.* 2013, Tang *et al.* 2014, Laranjeira *et al.* 2015, Woo 2015, Crippa *et al.* 2016). Although patients with CD can exhibit oral manifestations, paediatric CD has no specific clinical manifestations (Tang *et al.* 2014). Some studies have suggested that oral manifestations are a good cutaneous marker of IBD (Lourenco *et al.* 2010), and are useful diagnostic markers (Katsanos *et al.* 2015).

1.1.9 Microbiome dysbiosis and pathogenesis of IBD.

Recent studies suggest that IBD is the result of an altered immune response to the gut microbiota (Lucas Lopez *et al.* 2017). Alterations to the microbiota have been identified in the gut as well as oral microbiota of patients with IBD, including CD and UC patients (Eckburg *et al.* 2005, Manichanh *et al.* 2006, Gevers *et al.* 2014, Mottawea *et al.* 2016, Eun *et al.* 2016, Lucas Lopez *et al.* 2017, Zhou and Zhi 2016, Docktor *et al.* 2012, Said *et al.* 2014, Schirmer *et al.* 2018). The composition and distribution of the human gut microbiota may be affected by a diversity of factors, including for example, host genetic factors, the immune response and environmental factors. Most studies have focused on the gut bacteriome as the GIT is dominated by bacteria (Sam *et al.* 2017) compared to other human microbiota members. However, other inhabitants and their roles in association with the inflammatory bowel disease are also considered to be important, including the fungal microbiota (mycobiome). Additionally, the bacteriome and mycobiome interactions were characterized in CD patients (Hoarau *et al.* 2016), who suggest that the two gut inhabitants (bacteria and fungi) play an important role in inflammatory bowel disease pathogenesis.

1.1.10 Change in the gut microbiome (bacteria) in Crohn's disease (CD) patients.

Several previous studies have assessed the changes in gut bacteria of CD patients. A study by Seksik *et al.* (2003) reported that *Enterobacteria* are significantly enriched in the faecal flora of patients with CD compared to healthy controls. In another study of the biodiversity of the faecal microbiota in Crohn's disease, using a microarray based strategy, a reduction in normal anaerobic bacterial diversity in CD patients, particularly among the Firmicutes phylum was reported (Manichanh *et al.* 2006). Another study showed that five bacterial species were characteristic of dysbiosis in CD patients, including *Dialister invisus*, *Clostridium* cluster XIVa, *Faecalibacterium prausnitzii*, *Bifidobacterium adolescentis* and *Ruminococcus ganavus* (Joossens *et al.* 2011). In a large paediatric CD cohort study (n=447) by Gevers *et al.* (2014), 16S rRNA sequencing showed that *Escherichia coli*, *Fusobacterium nucleatum*, *Haemophilus parainfluenzae* (*Pasteurellaceae*), *Veillonella parvula*, *Eikenella corrodens* (*Neisseriaceae*), and *Gemella moribillum* were more abundant in the gut of CD patients, whereas *Bacteroides* spp., *Bifidobacterium* spp., *Ruminococcus* spp., *Clostridium* spp., and *Faecalibacterium* spp. exhibited reduced abundance (Figure 1.3).

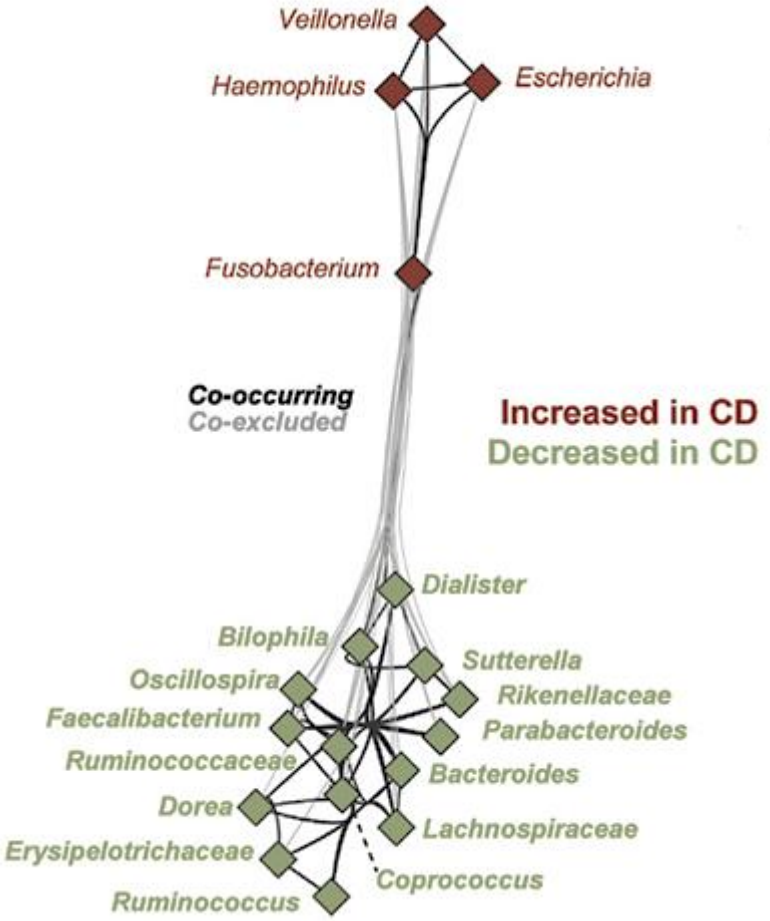


Figure 1.4 Gut microbiota in paediatric IBD (Gevers *et al*. 2014). Colour corresponds to behaviour in disease, with green for those taxa decreased in CD and red for those increased in CD.

In addition to that, they indicated that antibiotic use amplifies the microbial dysbiosis associated with CD. Moreover, they suggested that in early stages of the disease, assessing the rectal mucosa-associated microbiome may have potential for convenient and early diagnosis of CD. Analysis of intestinal microbiota in Korean Crohn's disease patients by Eun *et al.* (2016), sampling stool and mucosal tissue showed increased abundance of Proteobacteria in both faecal and mucosal tissues of CD patients. In addition, Fusobacteria were increased in the mucosal tissue, whereas faecal samples in CD patients were enriched in Actinobacteria. This study also reported increase in the

abundance of some bacterial taxa, including *Enterobacteriaceae, Pseudomonadaceae, Streptococcaceae,* and *Erysipelotrichaceae*. Additionally this later study demonstrated the impact of disease activity, disease location, disease behaviour, and therapeutic medication such as anti-TNF agent on the intestinal microbial community in patients with CD. A study by Shaw *et al.* (2016) measuring calprotectin, disease severity and microbiome dysbiosis in IBD patients showed that increased inflammation and disease severity were associated with the greatest levels of dysbiosis. The CD enriched taxa included the *Enterobacteriaceae, Pasteurellaceae, Fusobacteriaceae, Neisseriaceae, Veillonellaceae,* and *Gemellaceae*. Similar dysbiosis in the intestinal microbiota of IBD children were found by Mottawea *et al.* (2016) using V4-V6 16S sequence analysis. They noted that this microbiome exhibited a reduction in butyrate producers and increased numbers of H_2S producers. In addition, proteomic analysis of the mucosal tissue revealed reduced capacity of mitochondria from IBD patients to detoxify H_2S, suggesting a mechanistic link for microbiome induced inflammation. Wang *et al.* (2018) also used a 16S sequencing approach to study the composition and function of the faecal microbiota in CD patients and healthy controls. This study also identified reduced biodiversity in the gut microbiota in CD patients and also showed a reduction in bacterial taxa responsible for production of short chain fatty acids, including *Anaerostipes, Blautia, Clostridium, Coprococcus, Faecalibacterium, Lachnospira, Odoribacter, Roseburia, Ruminococcus,* and *Sutterella*. Olbjorn *et al.* (2019) also characterised the faecal microbiota in treatment-naïve paediatric IBD patients and non-IBD symptomatic patients and found similar dysbiosis in both groups. Faecal microbiota profiles similarly differentiated IBD and non-IBD symptomatic children from healthy children, with reduced abundances of *Eubacterium* and *Bifidobacterium* species in IBD and non-IBD symptomatic patients compared to healthy children. Examination of the faecal microbiota in adult patients with

Crohn's disease also showed that these patients had lower species richness and diversity, however no changes in microbial richness and diversity were noted over time, even in those patients who exhibited disease remission (Galazzo *et al.* 2019).

Recently, studies of the microbiome in UC patients have provided similar results. Schirmer *et al.* (2018) investigated the gut microbiota in relation to disease severity in UC patients upon diagnosis and following therapy. The results showed a number of bacterial genera were increased in severe UC, including *V. dispar, Aggregatibacter segnis, Campylobacter, Lachnospiraceae. V. parvula, H. parainfluenzae,* and *Megasphaera*. Some studies have investigated IBD affected tissue compared to non-affected tissue in the same patient. The comparison of the microbial communities of adjacent normal mucosa and aphthous ulcers in patients with Crohn's disease were carried out by O'Brien *et al.* (2018). No bacterial imbalances were detected, with the alpha diversity and microbial composition similar in aphthous ulcer and adjacent mucosa in newly diagnosed patients. In more recent time, a study by Li *et al.* (2019), assessing the association between inflammatory bowel disease (IBD) phenotype and ileal disease, showed significant differences in microbiota between macroscopically disease affected and unaffected regions of resected ileum in CD patients. In addition the presence of *Clostridia difficile* infection and NOD2 genotype had significant effects on the ileum-associated microbiota.

Overall, the gut microbiota plays an important role in the development of IBD through the development of inflammation in the gut (Zhang *et al.* 2015). In summary, IBD appears to be associated with reduced biodiversity and reduced levels of normal gut microbes with increases in the levels of *Enterobacteriaceae* and species normally associated with the oral cavity (*Veillonella, Haemophilus, Eikenella*).

1.1.11 Change in the gut mycobiome (fungi) in Crohn's disease (CD) patients.

Fungi are one of the inhabitants of the gut microbiota (Nash *et al.* 2017). Fungi are often overlooked because of their low abundance in the lower GIT of monogastric animals, accounting for only 0.1% of the total microorganisms (Li *et al.* 2018). However, fungi account for approximately 13% of the gut microbial volume with representatives from 140 different fungal genera (Schei *et al.* 2017). The role of the human mycobiome in GIT health and disease status has been largely unexplored (Hager and Ghannoum 2017). Fungi have also been detected in the GIT of other animals, for instance, mice, rates, and pigs (Nash *et al.* 2017). The wide diversity of fungi detected in the human GIT could potentially provide or influence metabolic functions (Auchtung *et al.* 2018). Some investigators have suggested that fungi, including common members of the oral mycobiota such as *C. albicans*, are only transiently found in the gut, as their presence can vary with oral hygiene and diet (Auchtung *et al*. 2018). Recently several studies have investigated the fungal community in association with IBD. For example, characterization of the fungal microbiota in the intestines and faeces in patients with CD by Li *et al.* (2014) showed variation in the structure and diversity of the mucosa-associated gut mycobiome in inflamed and non-inflamed mucosa, suggesting that the mycobiota of inflamed mucosa is distinct from noninflamed mucosa. Additionally, they identified greater differences in the mycobiome community and composition between CD patients and healthy subjects. The predominant fungal composition in the inflamed mucosa was of *Candida* spp., *Gibberella moniliformis, Alternaria brassicicola,* and *Cryptococcus neoformans*, whereas the fecal mycobiota of CD patients was enriched in *C. albicans, Aspergillus clavatus,* and *C. neoformans*. Another study by Mukhopadhya *et al.* (2015), studying the fungal microbiome in children with IBD reported distinct differences in the mycobiome, with higher levels of Basidiomycetes in IBD children. A

study by Sokol *et al.* (2017), characterizing the faecal mycobiome in IBD patients using ITS2 sequencing also noted an increase in the Basidiomycota to Ascomycota abundance ratio in IBD. Although *Candida* was the most abundant fungal genus in the population it was not associated with disease phenotype, however an increase in *S. cerevisiae* was observed in healthy subjects and those IBD patients in remission compared to IBD patients and those with IBD flare. In a study by Chehoud *et al.* (2015), children with IBD had significantly lower faecal mycobiome diversity compared to healthy children.

Recently a study by Limon *et al.* (2019), characterizing the mycobiome associated with intestinal mucosa in CD and healthy subjects, ITS1 sequencing data revealed that CD samples are associated with a significant loss of Ascomycota and an increase in Basidiomycota (specifically *Malassezzia* spp.). Several fungal genera were found in association with CD, including *Malassezia, Cladosporium, Aureobasidium,* and *Fusarium*. CD patients with ileocolonic disease were enriched in *Malassezia* and *Cladosporium*, while Fusarium was mainly decreased in patients with colonic disease. Interestingly, the presence of *Malassezia* spp. showed strong linkage to a CARD9 allele ($CARD9^{S12N}$) associated with CD. CARD9 encodes a signalling adaptor required for antifungal defence and is part of the C-type lectin/CARD9 pathway. Limon *et al*. (2019) went on to demonstrate that *M. restrica* could induce colitis in susceptible mice and that dendritic cells with the $CARD9^{S12N}$ polymorphism produced higher levels of TNF-a and IL-8 in response to *M. restricta* than cells with the wild-type CARD9 allele. These data produce the first evidence that a member of the normal mycobiome may play a role in the pathogenesis of CD in at least a subset of patients.

1.1.12 Changes in the oral microbiota in Crohn's disease CD patients.

The first analysis of the oral microbiome in IBD was carried out using DNA microarrays. In the later study, the analysis of oral samples from the tongue and buccal regions in CD patients indicated that overall microbiome biodiversity of CD patients was significantly reduced compared to healthy controls. Additionally, the tongue samples in CD showed reduced probe activity in two phyla (Fusobacteria and Firmicutes) and additionally CD patients with oral manifestations had a higher level of (ASCA) anti-Saccharomyces cerevisiae antibody (Docktor *et al.* 2012). Another study analysed the salivary microbiota of healthy patients and IBD groups (CD and UC), showing a significant difference in the salivary microbiota composition between healthy groups and IBD patients. Shifts in the oral microbiota composition of CD patients were observed, with higher levels of *Prevotella* and *Veillonella* and reduced *Streptococcus* and *Haemophilus* (Said *et al.* 2014). Furthermore, a recent study by Xun *et al.* (2018) considered the dysbiosis and ecotype of salivary microbiota in IBD patients. The results from this study reported that the CD patients were enriched with *Streptococcus* and *Veillonella* and depleted *Prevotella, Neisseria* and *Haemophilus* genera. The results from these previous studies indicate distinct changes in the oral microbiome of patients with CD, suggesting that these alterations in the oral microbiome differ depending on sampling method, sequencing method, in addition to other factors (e.g. treatment) which might lead to discrepancy between the studies. Most studies concur that the genera *Prevotella, Haemophilus* and *Veillonella* are somehow affected in the oral cavity in IBD, which interestingly matches the genera shown to be enriched in the gut microbiota in CD patients (Gevers *et al.* 2014). The link between changes in the oral microbiome and gut microbiota will be discussed further in later chapters.

The oral mycobiome is not well characterized in IBD patients compared to the oral microbiome. To our knowledge, this is the first study analysing the association of the oral mycobiome with inflammatory bowel disease.

1.2. The oral microbiota.

The oral cavity, or mouth contains an abundance of microorganisms, including viruses, protozoa, fungi, archaea, and bacteria. These are known as the oral microflora, oral microbiota, or more recently as the oral microbiome. We define the human oral microbiome as all the microorganisms that are found on or in the human oral cavity and its contiguous extensions stopping at the distal oesophagus (Dewhirst *et al.* 2010). Approximately 600 prevalent bacterial species have been identified in human oral cavity (Chen *et al.* 2010) and it represents the second most complex community in the body. Up to one third of oral bacterial species are still uncultured (Wade 2013). The mouth contains many different surfaces for colonisation, such as keratinized and non-keratinized epithelial tissues and the hard surfaces of the tooth and each surface appears to harbor a unique microflora (Scannapieco 2013). The oral cavity also contains antimicrobial fluids, including saliva and gingival crevicular fluid to keep the microbial population in check. The predominant bacterial species in the healthy oral cavity were described by Aas *et al.* (2005), showing some species are site specific at one or multiple sites, including buccal, vestibule, tongue dorsum, tongue lateral, hard palate, soft palate, tonsils, tooth surface, and subgingival surface (Figure 1.4). More recent studies have taken the benefit of molecular approaches to characterize both cultivable and non-cultivable species of the oral microbiome. These laboratory methods include PCR amplification and sequencing of the bacterial 16S rRNA gene. The results from these observations revealed that the

majority of oral bacterial species have never been cultivated and identified, at the same time the use of these advanced techniques has led to the discover of 1,000 or more phylotypes (Scannapieco 2013). In addition to the oral microbiome (bacteria), another important component of the oral microbiota is the oral mycobiome.

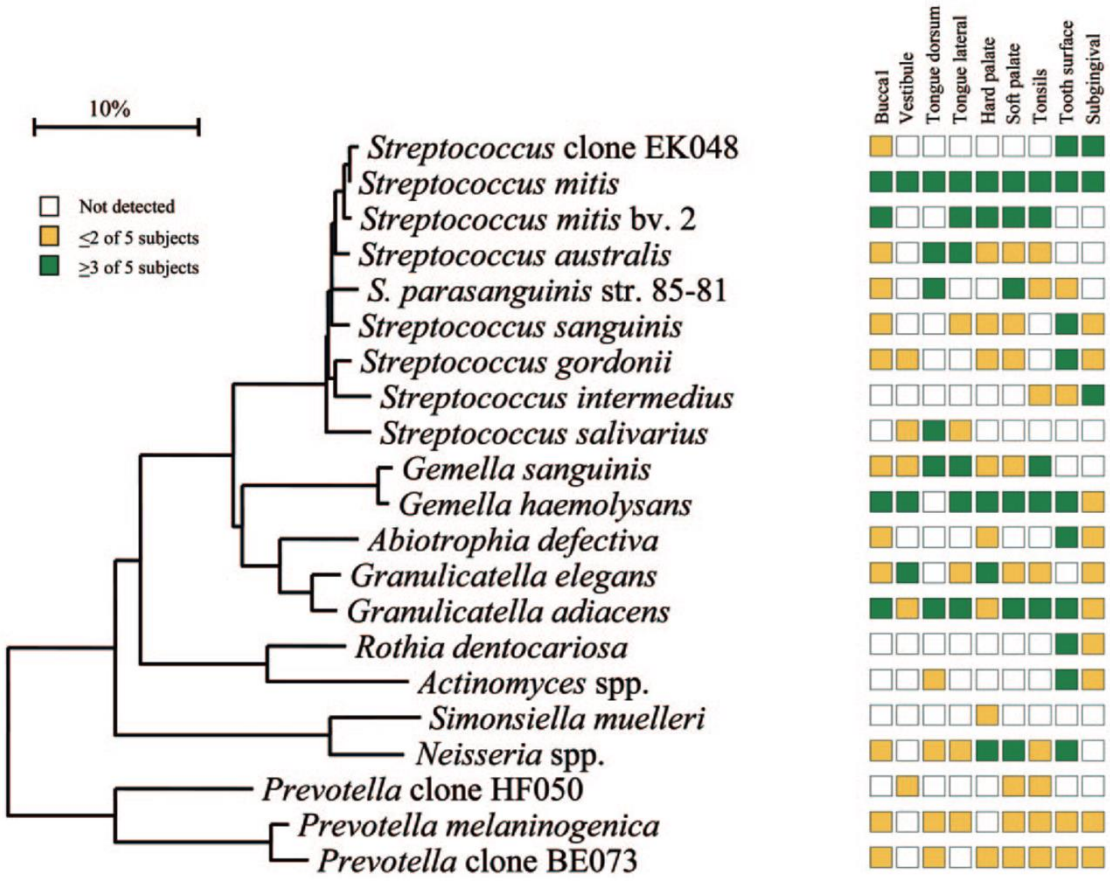


Figure 1.5 Site specificity of predominant bacterial species in the oral cavity (Aas *et al.* 2005)

Characterization of the human mycobiome community, including the oral mycobiome has been slowly evolving compared to oral bacteriome (Bandara *et al.* 2018). The first time the basal oral mycobiome of healthy subjects was described in 2010 by Ghannoum *et al.* (2010), who reported that the oral cavity contains 74 culturable and 11 non-

culturable fungal genera. *Candida* species (*albicans, parapsilosis, tropicalis,* and *metapsilosis*) were the most dominant, isolated from 75% of the studied population. Further studies have identified the fungal genus *Malassezia* as present in the oral cavity (Dupuy *et al.* 2014).

The human oral microbiome is strongly associated with important oral diseases such as tooth decay (dental caries) and gum disease (periodontitis) (Chen *et al.* 2010). However, in recent studies the roles of oral mycobiome were investigated in association with common oral diseases, including dental caries (Al-Ahmad *et al.* 2016, Fechney *et al.* 2019), root canal infection (Persoon *et al.* 2017), and periodontitis (Peters *et al.* 2017), emphasizing the correlation between the oral mycobiome and oral diseases. Furthermore, Changes to the oral microbiome have also been associated with denture stomatitis and in these patients is influenced by the number of teeth and the presence of *C. albicans* (O'Donnell *et al.* 2015). The oral microbiome may also be source of respiratory pathogens (O'Donnell *et al.* 2016).

The oral microbiome has also been implicated as a contribitory risk factor to human health in general, including diseases as cancer, diabettes mellitus, cardiovascular diseases, bacteremia and preterm and low birth weight in infants (He *et al.* 2015). For example, Streptococci are associated with infectious endocarditis and *Fusobacterium* spp. have been linked with progression of cancers throughout the GIT. More recently, the keystone pathogen in periodontal disease (*Porphyromonas gingivalis*) has been linked with rheumatoid arthritis and Alzheimer's disease (Dominy *et al.* 2019). Alterations in the oral microbiota have been noted in response to some systemic diseases, particularly those diseases where patients present with oral manifestations. The oral findings of systemic diseases may even play an important role as an early disease indicator

(Laranjeira *et al.* 2015). Notable oral manifestations of systemic disease. For example, HIV including oral candidiasis, oral hairy leukoplakia, and recurrent aphtous-like ulceration (Askinyte *et al.* 2015, Heron and Elahi 2017), diabetes mellitus including dry mouth, dental caries, periodontal disease, gingivitis, oral candidiasis, burning mouth syndrome, oral lichen planus, geographic tongue and grooved tongue (Kudiyirickal and Pappachan 2015), and Autoimmune diseases including Behcet's disease, Sjögren's syndrome and Crohn's disease, Lupus Erythematosus and Rheumatoid Arthritis (Mays *et al.* 2012). Analysis of the oral metagenome has lagged somewhat behind studies of the human gut. Little is known about the stability of the oral flora and the effects of some systemic diseases, which can result in oral manifestations and possible changes in the bacterial community inside the mouth. In this section, we discuss the oral microbiota (bacteriome and mycobiome) in association with commonly oral diseases (dental caries and periodontitis), and inflammatory bowel disease (oral Crohn's disease).

1.2.1 Oral bacteriome and mycobiome in association with oral diseases.

Microorganisims from the oral cavity are responsible for several infectious diseases. For example caries, periodontitis, root canal infections and alveolar osteitis (Figure 1.5). Caries and periodontitis are the two most common oral diseases, which are caused by bacterial infections (Duran-Pinedo and Frias-Lopez, 2015).

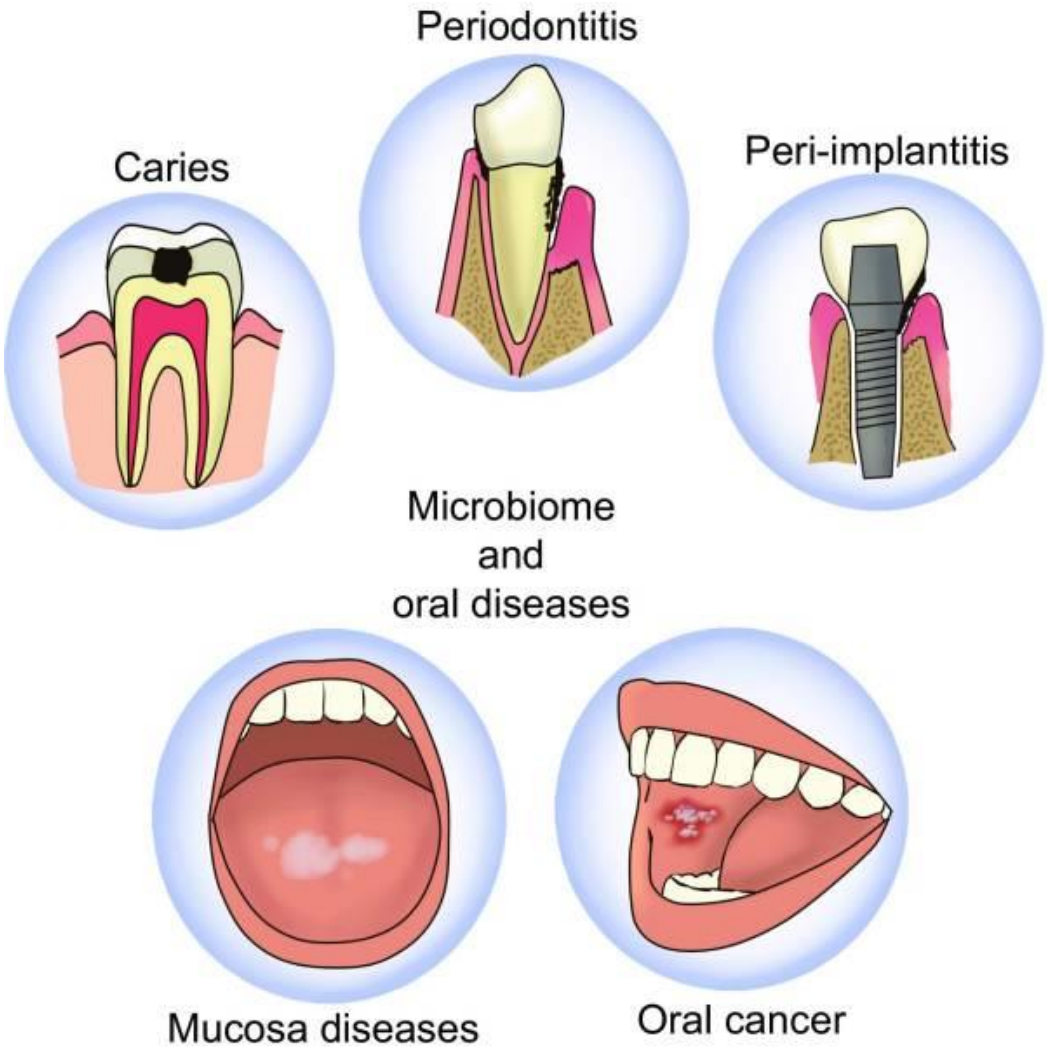


Figure 1.6 Oral diseases associated with dysbiosis in the oral microbiota (Gao *et al*. 2018).

1.2.1.1 Dental caries.

Dental caries has been considered as one of the most common chronic infectious diseases in the world (Aas *et al.* 2008). Furthermore, others consider that it is the most prevalent human disease, affecting 80-90% of the world's population (Simon-Soro and Mira 2015). Most theories for the etiology of dental caries specify the important roles of oral microorganisms in the pathogenesis of dental caries. However, dental caries is a multifactorial disease caused by many factors, including oral bacteria in dental plaque,

presence of fermentable carbohydrates and available tooth surface, in addition to other contributing factors, which are salivary flow rate and salivary buffer capacity (Mathur and Dhillon 2018). Culture-based studies have shown different species to be associated with dental caries, but were largely limited to *Streptococcus mutans* and *Streptococcus sobrinus* (Aas *et al.* 2008). These organisms have been shown to produce copious glucan and fructan fibres using a variety of glycosyl transferase enzymes. These biofims have high acidogenic potential in the presence of dietary carbohydrates and mutans Streptococci can thrive in these acid conditions. During the 1960s and 1970s, there was major progress in the microbiology of dental caries which identified a large group of cariogenic bacteria, largely belonging to the genus *Streptococcus*, including *Streptococcus mutans, Streptococcus sobrinus, Streptococcus cricetus, Streptococcus rattus, Streptococcus downeii,* and *Streptococcus macacae,* that were associated with dental caries. In addition to that lactobacilli were also found in dental plaque which may contribute to the etiology of dental caries by converting dietary fermentable carbohydrates to lactic acid. However, the more recent laboratory methods of studying bacterial species, including molecular methods, have overcome some of the limitations of the previous culture-based methods resulting in identification of oral bacterial species never before cultivated (Belda-Ferre *et al.* 2012, Scannapieco 2013). Using direct pyrosequencing, dental caries are not only dominated by *Streptococcus mutans,* but there are a complex community formed by tens of bacterial species (Belda-Ferre *et al.* 2012). These findings have been confirmed using stringent anaerobic techniques, which identified *Scardovia wiggsiae, Veillonella parvula, Streptococcus cristatus*, and *Actinomyces gerensceriae* in carious lesions (Scannapieco 2013). Population based studies confirm the presence of higher levels of *S. mutans* and *S. sobrinus* in children with active caries (Hurley *et al.* 2019). Interstingly, Johansson *et al.* (2016) showed that

levels of these organisms were highest in communities with limited access to dental care programs. Recent studies have also showed an association between the oral mycobiome and dental caries. In a study by Al-Ahmad *et al.* (2016), assessing the prevalence of *Candida albicans* and *Candida dubliniensis* in caries free and caries active children by culture, revealed for the first time a high prevalence of *C. dubliniensis* in children with caries, suggesting an association of this fungal species with dental caries. Another study by Fechney *et al.* (2019), investigating the mycobiome in dental plaque from children with and without caries did not identify *C. dublineinsis* but showed the presence of three enriched species in children with dental caries, including *C. albicans, Naganishia diffluens,* and *R. mucilaginosa*.

1.2.1.2. Periodontal Disease.

Dental plaque is a complex microbial community, and according to current estimates, dental plaque may contain more than 700 different bacterial species (Jiang *et al.* 2011). The plaque microbiota has an essential role in the etiology of gingivitis and periodontitis by inducing damaging inflammatory responses that account for most of the disease symptoms. A study by Kistler *et al.* (2013) has shown that, in absence of oral hygyeine, the change from healthy periodontium to gingivitis is associated with a change in the bacterial community structure of plaque and an increase in bacterial community diversity. The later study identified new taxa associated with healthty gingiva and gingivitis, confirming the association of a number of apparent periodontal pathogens with gingivitis, for instance *F. nucleatum* subsp. *polymorphum* (Kistler *et al*. 2013). It is now well known that extension of this plaque biofilm into the gingival crevice results in inflammation of the periodontal tissues resulting in periodontal disease (Ge *et al.* 2013). The percentage of the adult population worldwide suffering from periodontal diseases has been estimated

at almost 90%. Analysis of the plaque microbiome in periodontal diseases shows there are differences in the abundance of Operational Taxonomic Units (OTUs) between the shallow and deep periodontal pockets, which confirm the earlier DNA-checkerboard studies. According to the study of Xiuchun Ge in 2013, there were 200 OTUs statistically different between the two types of pockets. Out of the two hundreds OTUs identified, 148 were shown to be significantly more abundant in deep pockets compared to shallow ones including *P. gingivalis, P. endodontalis, F. nucleatum, P. nigrescens, Treponema denticola, Treponema medium, Tannerella forsythia* and *Parvimonas micra*. They also found significantly greater abundance of 52 other species-level OTUs in shallow pockets, including *Streptococcus oralis, Streptococcus sanguinis, Streptococcus gordonii, Rothia dentiocariosa, Veillonella dispar, Actinomyces naeslundii* and *Actinomyces sp*. Recently, the oral mycobiome of individuals with and without periodontal disease was described and compared by Peters *et al.* (2017). ITS sequencing revealed higher abundance of *Candida* in subjects with periodontal disease, with no significant differences in overall oral mycobiome diversity and composition between participants with and without periodontal disease.

1.3 Modern analytical tools for studying the oral microbiome and mycobiome.

Characterization of the human oral microbiome community is limited by the inability to culture the majority of microbes that inhabit the oral cavity (Liu *et al.* 2012). The difficulties in studying the oral microbiome using conventional culture-based techniques have led to new methods (molecular techniques) to allow profiling of the microbiome and metagenomes. The DNA sequence-based approaches to microbiome research are now common but have become increasingly diverse (Kuczynski *et al.* 2011). DNA-based microbiome studies fall into two types. The first technique involves PCR amplicon based gene targeting (for example, 16S rDNA profiling). The second method uses an entire metagenomics approach (shotgun metagenomics). These studies have used numerous sequencing methods, namely capillary (Sanger) sequencing (e.g. the ABI 3730xl DNA analyser), pyrosequencing (e.g. the Roche 454 Genome Sequencer GS) and Illumina's clonal arrays (e.g. the MiSeq and HiSeq2000). Each of these technologies has different chemistries and characteristics read length, depth, sequence accuracy, usability and cost (Kuczynski *et al.* 2011). Many of the current technologies produce short read lengths (~250 bp for Illumina). However, the cost and quality of long-read technologies (e.g. PacBio and Nanopore) is improving and may soon replace Illumina sequencing for amplicon based studies. In the current study, for oral microbiome analysis we amplified the 16S rDNA (Figure 1.7). The 16S rDNA gene has become the gene of choice as the ribosome is present in all living organisms, it contains hyper variable and conserved regions which can be used to design broad spectrum primers and classify organisms at family or genus levels (Kuczynski *et al.* 2011, Zaura 2012). Another reason is the availability of several large databases of reference sequences and taxonomies (Kuczynski *et al.* 2011). In the current study the F27 and R338 primers were used to amplify the V1-

V2 regions of the16S rDNA gene (Figure 1.7) (Kuczynski *et al.* 2011). Diaz *et al*. (2014) have shown that the V1-V2 region of the 16S rRNA allows species level identification of oral bacteria with a similar accuracy to the full length 16S gene.

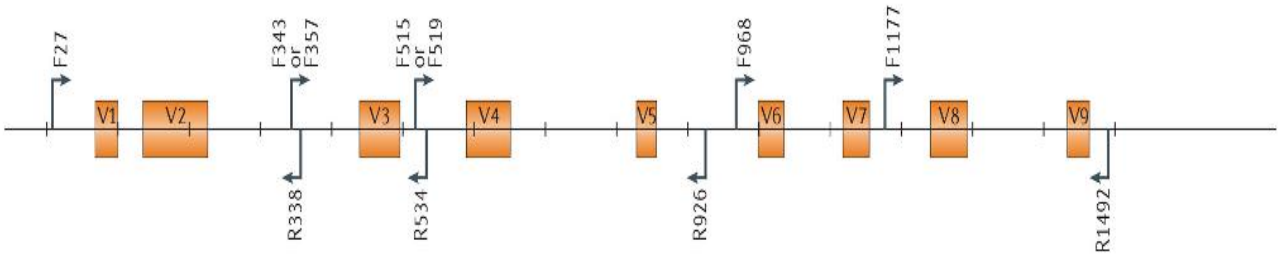


Figure 1.7 The variable region of 16S rDNA and the primer pairs (Kuczynski *et al*. 2011).

For the oral mycobiome analysis, the ITS2 region of the fungal rDNA locus was amplified (Figure 1.8). The Fungal ITS workshop Group expressed a preference for ITS2 over ITS1 as ITS2 has less variation in length, more sequence information available to inform sequence alignment, less PCR bias and has better sequence representation in databases than ITS1 (Heisel *et al.* 2015). In the present study, the Illumina MiSeq platform was used to sequence the 16S rDNA for bacterial analysis and the ITS2 spacer for mycobiome analysis.

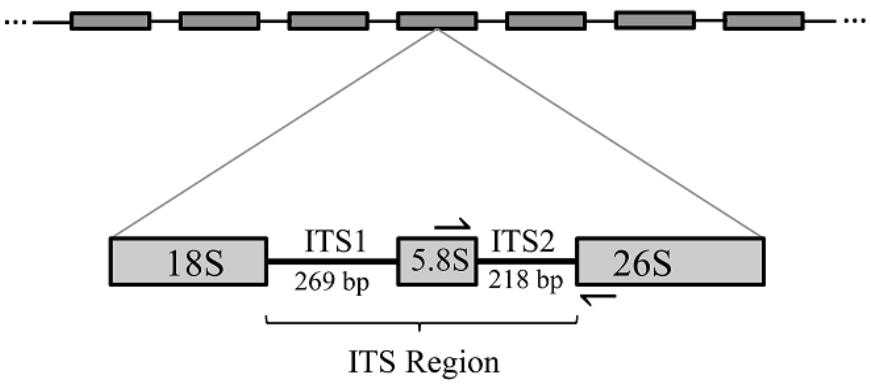


Figure 1.8 Structure of the ITS region of the nuclear ribosomal RNA genes (Zhang *et al*. 2015).

Another important variable in microbiome studies is DNA extraction. In the current study we use a well validated DNA extraction kit (Masterpure DNA Extraction kit, Epicentre Biosciences) that utilises proteinase K and detergent based lysis methods. We supplemented this protocol with an additional incubation with a highly active lysozyme preparation (Ready-Lyse Lysozyme, Epicentre Biosciences) to digest the cell wall of difficult to lyse Gram- positive bacteria. In addition, in order to lyse fungal cells, we included a bead-beating step with 0.1 mm glass beads which has been shown to be necessary for detection of certain fungal species (Diaz *et al*. 2014).

1.4 Aims and Objectives of the study.

This study aimed to analyse the oral mucosal microbiome and mycobiome in paediatric IBD patients. The mucosa was selected rather than the saliva as this is the region where tissue and immune cells directly interact with the microbiome.

It is our hypothesis that the oral microbiome is dysbiotic in IBD (both CD and UC) and that these data may have diagnostic potential.

The initial objectives were to:

1. Characterise the oral mucosal microbiome in treatment naïve paediatric IBD patients using next-generation sequencing to determine if oral dysbiosis occurred in these patients.
2. Characterise the oral mucosal mycobiome in healthy children and children with IBD
3. Determine if rebiosis of the oral cavity occurred following treatment.

These data were collected to determine if the oral microbiome could inform on the pathogenesis of IBD or act as a possible diagnostic tool to rapidly diagnose IBD or assist in disease classification or assessing treatment success.

Chapter 2

Materials and Methods

Chapter 2 Materials and Methods

2.1 Sample collection.

In the current study, 105 children and adults were recruited during the period from July 2014 to October 2015. Samples were collected at Our Lady's Children's Hospital, Crumlin (OLCHC) and Dublin Dental University Hospital (DDUH), Dublin, Ireland. A total of 38 children were diagnosed as suffering from Crohn's disease (CD), and 21 children with ulcerative colitis (UC), whereas a total of 32 children who were attending DDUH, and 4 children were attending OLCHC were grouped as a healthy controls (HC), in addition to 10 healthy adult individuals (Table 2-1). The consent forms were signed by the children's parents (Appendix 6) with the approval of the Ethics (Medical Research) Committee at OLCHC (Appendix 7). The children who were recruited in this study underwent a full mouth examination and hard tissue and soft tissue status was recorded by a dentist (Appendix 8). Two swabs were collected from each child (buccal mucosa and dorsum of the tongue) using Catch-all sample collection swabs (Epicentre). Ethical Approval to collect oral swab specimens from children attending the paediatric clinic at DDUH was granted by the SJH / AMNCH Research Ethics Committee, Dublin (Appendix 9).

The total number of swabs collected from healthy and diseased children was 210 swabs. After the samples were taken, the swabs were immediately transferred to the laboratory at Our Lady's Children's Hospital Crumlin for storage at -80 °C for up to 6 months. Swabs were sent to the microbiology research unit at DDUH for further laboratory procedures. The age of the study population ranged from 5 to 16 years old (Table 2-2). All subjects were examined intra orally, to determine any abnormalities in hard and soft tissues of the oral cavity. For all children the average age was 11.44 years. In both disease

and control groups the majority of children were male. The number of males and females in the Crohn's disease group (CD) were males 82%, females 18%, respectively, whereas in the control group males were 57% compared to 43% of females (Figure 2.1).

Table 2-1 Classification of participants by disease status.

CD	UC	HC	Total
38	21	46	105

Table 2-2 Age distribution of children.

Study group	**Children 5-12**	**Adolescent 13-17**	**Adult 18+**	**Total**
CD	17	21	-	38
UC	12	9	-	21
HC	19	17	10	46

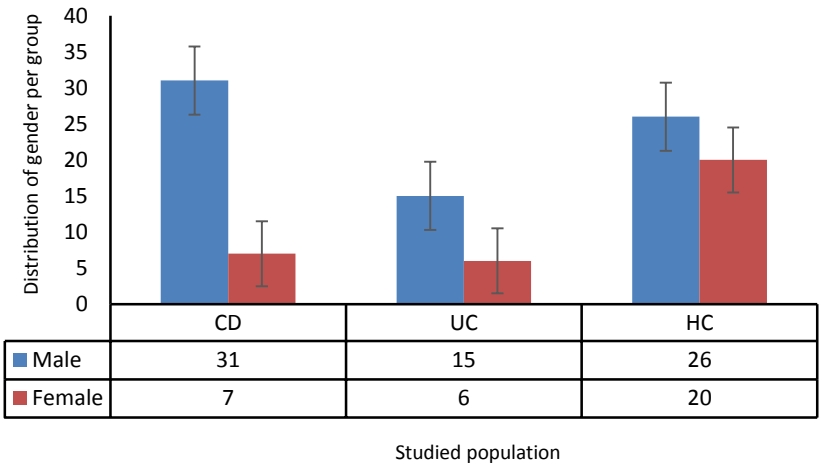


Figure 2.1 Gender distribution in the three studied groups.

2.2 Microbial genomic DNA extraction.

DNA extraction was performed using the Master Pure purification Kit (Epicentre) following the manufacturer's instructions with the addition of a Ready-Lyse Lysozyme incubation step (Epicentre), and a bead beating disruption step (Appendix 10). This protocol, including the additional enzymatic and bead-beating steps, was developed in-house based on recommendations for efficient extraction of DNA from Gram-positive bacteria and fungi, as discussed in section 1.3. The oral swab samples were suspended in 300 µl Tris-EDTA-Salt buffer (TES; 0.1M Tris, pH 8.0, 0.01M EDTA, 0.1M NaCl) in a screw capped tube. Ready-Lyse Lysozyme was diluted to 250 U/µl in TES buffer and the proteinase K was diluted to 1.00 U/µl in the 2x "T and C Lysis solution". A 2 µl volume (500 Units) of the Ready-Lyse Lysozyme solution was added to each sample and incubated at room temperature for 15 minutes, and then each sample was supplemented with 150 µl "T and C Lysis solution" containing 150 Units of proteinase K and incubated for 15 min at 65°C. Next, 250 µl glass beads were added to each tube and disrupted in the bead beater (Mini-BeadBeater 24, Cell Disrupter, BioSpec Products, Inc. Bartlesville, OK. USA) for 30s. The liquid was removed to a 1.5 ml Eppendorf tube, and 1 µl of RNase A was added to the sample and mixed and incubated at 37 °C for 30 min. Next, 175 µl of MPC protein precipitation reagent was added to each sample. The samples were spun for 10 min at 4°C at top speed in a microcentrifuge, following that the supernatant was transferred to a fresh Eppendorf tube leaving the pellet behind. A 500 µl volume of isopropanol was added and mixed by inverting. After centrifugation at top speed at 4°C for 10 min, the liquid was carefully removed without disturbing the DNA pellet. Lastly the samples were dried and suspended in 35 µl TE buffer (Tris-EDTA buffer solution, pH 8.0). To make sure that DNA extraction was successful the concentration of DNA in each sample was measured using the NanoDrop UV Spectrometer followed by agarose

gel electrophoresis, using Agarose Type I, low EEO with a concentration of 1%, and Tris-Borate, EDTA (TBE) consisting of (0.45 M Trizma base, 0.45 M boric acid and 0.01 M EDTA, pH 8.0). The electrophoresis buffer (TBE) was prepared at 10 x concentration, then diluted to 0.5% by adding (500 ml of 10x TBE buffer to 950 ml of distilled water). A mixture of 4 µl of loading dye, 2 µl of Sigma water, and 2 µ⌊ of template DNA was prepared and 6µl of this mixture was injected into 1.5 mm wells. Electrophoresis was performed with 100V, 85mA, 10W for 45 min.

2.3. PCR Analysis for bacterial samples.

ITS2 and 16S rDNA gene regions were amplified for characterization of fungi and bacteria in the oral cavity in IBD children and healthy individuals.

2.3.1 Amplification of 16S gene fragments.

To assess DNA integrity, the whole 16s rRNA gene DNA was amplified using universal bacterial primers 27F and 1492R to assess the quality of DNA in the samples. For Illumina sequencing, PCR was performed using the primers 27F-YM (5´-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGTCAGTCTGTCAGA GTTTGATYMTGGCTCAG -3´), and 338R-R (5´-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTATGGTAATTCATGCTG CCTCCCGTAGRAGT -3´), which target the V1-V2 hypervariability region. The primers were selected based on primers designed by Prof. William Wade (Personal Communication). The 27F and 338R primers were selected as they amplify from a broad range of taxa and produce sequences that can discriminate between closely related

streptococci in the V1 and V2 regions of the 16S rRNA gene. The 27F-YM primer components include Y (C or T) and M (A or C) for best matching. A degenerate base 'R' (A or G) was added close to the 3´ end of the 338R primer to reduce potential bias against certain oral taxa due to a mismatch to 338R at this position. The primers also contain the MiSeq overhang adapter sequences for indexing with the Nextera indexing kit, a 10-nt primer pad and a 2 base pair linker sequence. The primer pads are added to raise the T_m of the sequencing primers to between 60-65°C, following Illumina's recommendations. The reactions were carried out on 10 ng template DNA in 25 µl final volumes. The amplification mix contained microbial genomic DNA (5 ng/µl in 10 mM Tris pH 8.5; 2.5 µl per sample), amplicon PCR reverse primer (1µM; 5µl per sample), amplicon PCR Forward Primer (1µM; 5 µl per sample), and 2x KAPA HiFi Hot Start Ready Mix (12.5 µl per sample). After an initial denaturation at 95°C for 3 min, 25 cycles were performed consisting of denaturation at 95°C for 30s, annealing at 55°C for 30s, and progressive extension at 72°C for 30s. Following the 25 cycles there was a final extension at 72°C for 5 min. The cycle then it holds the reaction at 4°C.

2.3.2 Amplification of ITS2 gene fragment.

The ITS2 region from DNA sample extracts was amplified using the primers ITS2F: 5´-TCGTCGGCAGCGTCAGATGTGTAAGAGACAGATGCCTGTTTGAGCGTC and ITS2R:5´GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTTACCTGATTTGAGGTC. The PCR conditions were carried with total reaction volume of 25 µl, containing, 12.5 µl Phusion High-Fidelity PCR Master Mix (Thermo Scientific) per sample, amplicon PCR reverse primer (1µM; 5µl per sample), amplicon PCR Forward Primer (1µM; 5 µl per sample), and microbial genomic DNA (5 ng/µl in 10 mM Tris pH

8.5; 2.5 µl per sample). The thermocycling conditions consisted of an initiation step at 95°C for 5 min, followed by 30 cycles of denaturation at 98°C for 30 sec, annealing at 56°C for 30 sec, elongation at 72°C for 45°C, and final extension at 70°C for 5 min. All PCR products from ITS2 and 16s rDNA amplification were examined by electrophoresis, using 1% agarose gel.

2.3.3 Purification of PCR product.

Amplicons (16S V1-V2 or ITS2) were purified with AMPure XP beads. Prior to purification the AMPure XP beads were brought to room temperature. PCR products (20 µl per sample) were transferred to a 96-well PCR plate. The amplicon PCR plate was centrifuged at 1000 x *g* at 20°C for 1 min. After vortexing the AMPure XP beads for 30s, 20 µl of AMPure XP beads was added to each well of the amplicon PCR plate. Using a multichannel pipette the entire volume of each well was gently mixed up and down 10 times, followed by incubation at room temperature for 5 min. The 96 well plate was placed on a magnetic stand for 2 min. While the plate was on the magnetic stand, the supernatant was removed using a multichannel pipette. Following that, the beads were washed by adding 200 µl freshly prepared 80% ethanol to each sample well and following incubation for 30s, the supernatant was removed. A second wash using the same procedure was performed. After that, the beads were allowed to air dry for 10 min. After the beads has been dried, the amplicon PCR plate was removed from the magnetic stand and 52.5 µl of 10 mm Tris pH 8.5 was added to each well, and mixed up and down for 10 times. Then plate was incubated at room temperature for 2 min, followed by replacement of the plate on the magnetic stand for 2 minutes or until the supernatant has cleared. Finally, 50 µl of the supernatant was transferred from the Amplicon PCR plate to a new 96 well PCR plate.

2.3.4 Index PCR.

In this step the Nextera XT Index Kit was used to add unique identifying index primers to each set of amplicons. The kit consisted of the Nextera XT Index 1 primers (N701 to N712) and Nextera XT Index 2 primers (S501 to S508). The other items used in this step include 2x KAPA HiFi HotStart Ready Mix, PCR Grade water, 96-well 0.2 ml PCR plates and micro seal film. To begin with, using a multichannel pipette 5 µl of PCR amplicons from 2.3.2 was transferred to a new 96-well plate. The Index 1 and 2 primers were arranged according to the Dual Indexing Strategy (Figure 2.2). A 5 µl volume from each primer tube (1 to 12) in the horizontal row was added to each well in the corresponding column from A to H. The same procedure was used for the primers in the vertical column (1 to 8) by adding 5 µl to each well in the corresponding row from well 1 to 12. In order to make the total volume 50 µl, we added 25 µl of 2x KAPA HiFi Hot Start Ready Mix (V1-V2 region of 16S rDNA); 25 µl of Phusion High-Fidelity PCR Master Mix (ITS2 region), and 10 µl of PCR Grade water to each well of the PCR plate. After this step, the contents of each well were gently pipetted up and down for 10 times, and the PCR plate was covered with a micro seal film and centrifuged at 1000 x *g* at 20°C for 1 min. Finally, the following program was performed for the Index PCR of V1-V2 region of 16S rDNA. After an initial denaturation at 95°C for 3 min, 8 cycles were performed consisting of denaturation at 95°C for 30s, annealing at 55°C for 30s, and progressive extension at 72°C for 30s. Following the 8 cycles there was a final extension at 72°C for 5 min. The cycle then it holds the reaction at 4°C. However, the Index PCR for ITS2 region, the initial denaturation at 95°C for 5 min, with 8 cycles of denaturation at 98°C for 30 sec, annealing at 56°C for 30 sec, elongation at 72°C for 45°C, and final extension at 70°C for 5 min.

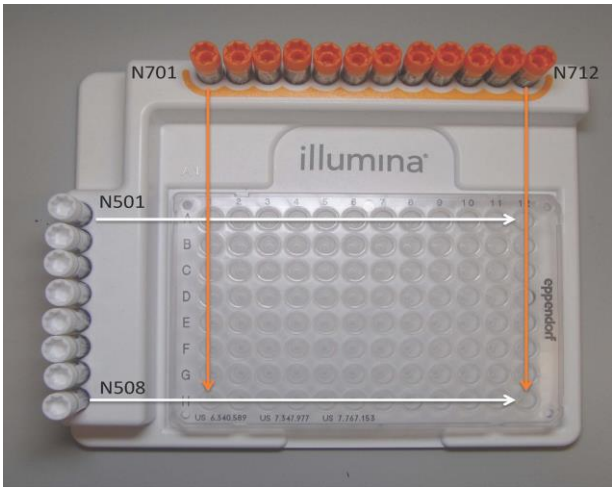


Figure 2.2 The combined dual Index primer plate, which is used in Nextera XT Index kit (Trombetta *et al*. 2014).

2.3.5 Second Purification of PCR product.

This step was carried out using the same procedures as 2.3.3, except that 56 µl of the AMPure XP beads and 27.5 µl of 10 mM Tris pH 8.5 were used in this step, and 25 µl of the supernatant were transferred from the Index PCR plate to a new 96 well PCR plate for library Quantification.

2.3.6 Quantification and library preparation.

The Qubit® ds DNA HS Assay kit 500 assay was used for quantifying the library. A master mix was made by adding 199 µl of ds DNA HS buffer to 1 µl of ds DNA HS Reagent 200x per sample. To calibrate the Qubit® 1.0 Fluorometer, 190 µl of master mix was added to 10 µl of ds DNA HS No1 and No2 standards in thin-wall clear 0.5 ml PCR tubes before each assay. After the calibration has been completed, samples were assessed in 0.5 ml PCR tubes containing 99 µl of master mix (199 µl of ds DNA HS buffer to 1 µl

of ds DNA HS Reagent 200x) and 1 µl of the sample. After that, the concentrations of the samples were calculated using the equation:

$$\text{Concentration of the sample} = \text{QF value} \times \left(\frac{200}{x}\right).$$

Where: QF value = the value given by the Qubit® Fluorometer.

x = the number of microliters of sample you added to the assay tube

At this point the DNA concentration was calculated in nM, based on the size of DNA amplicons as determined by an Agilent Technologies 2100 Bioanalyzer trace, using the equation:

$$\frac{(\text{Concentration in ng/µl})}{(660\ \text{g/mol} \times \text{average library size})} \times 10^6 = \text{concentration in nM}$$

Following that, the concentrated library in nM was diluted using 10 mM Tris pH 8.5 to 4 nM. The final library was pooled onto 1.5 ml Eppendorf tube containing 5 µl from each sample.

2.3.7 Illumina sequencing of the ITS2 and 16S rRNA gene.

The libraries were denatured with NaOH (0.2 N NaOH) and diluted with hybridization buffer (HT1). The next steps for sequencing include:

2.3.7.1 Denature DNA.

0.2 N NaOH was prepared by diluting 200 µl of NaOH 1M in 800µl of water. The bioanalyzer analysis shows that the concentration of pooled final DNA library is 10 nM, in order to bring it into 4 nM, the pooled final DNA library was diluted into the half concentration, 5 µl of 4 nM pooled library were added to 5 µl of 0.2 N NaOH, followed

by incubation at room temperature for 5 min. After that, 990 µl of pre-chilled HT1 was added to 10 µl of denatured DNA, resulting in a 20 pM denatured library in 1 mM NaOH.

2.3.7.2 Dilution of denatured DNA.

Illumina recommends targeting 800-1000K/mm^2 raw cluster densities using MiSeq V3 reagents. To achieve this we routinely used 8 pM loading concentration (240 µl of 20 pM denatured library diluted with 360 µl of pre-chilled HT1 buffer).

2.3.7.3 Denature and dilution of PhiX control.

To denature and dilute the 10 nM PhiX library to the same loading concentration as the amplicon library (8 pM), the following steps were carried out. To denature the PhiX, a 2 µl volume of 10 nM PhiX library were added to 3µl of 10 mM Tris, pH 8.5, diluting the PhiX library to 4 nM. The next step denatured and diluted the PhiX library from 2 nM to 4 nM. Using a Microcentrifuge tube, a 5 µl volume of 4 nM PhiX library was added to 5µl of 0.2 N NaOH. The mixture was vortexed and incubated at room temperature for 5 min to denature the PhiX library into single strands. Following that, 990 µl of pre-chilled HT1 was added to 10 µl of the denatured PhiX library, resulting in a 20 nM PhiX library. The final step was to dilute the denatured 20 nM PhiX library to the same loading concentration as the amplicon library, by adding 240 µl of the denatured 20 nM PhiX library to 360 µl of pre-chilled HT1, the mixture was inverted several times and placed on ice.

2.3.7.4 Combine amplicon library and PhiX control.

The final library mixture must contain 5% PhiX. To achieve this 30 µl of 8 pM denatured PhiX library was added to 570 µl of 8 pM denatured amplicon library. The combined mixture was placed on ice while preparing it for loading onto the MiSeq V3 reagent cartridge.

2.3.7.5 MiSeq workflow.

After denaturation and dilution of the libraries was completed, the pre-filled reagent cartridge was prepared for use, and the library mix was loaded onto the reagent cartridge in the designated reservoir. The flow cell was washed and thoroughly dried and loaded. Next, the PR2 bottle was loaded, and the waste bottle was emptied and the reagent cartridge was loaded. Finally, the run parameters and pre-run check were reviewed and the run was started.

2.4 Processing of sequence Data analysis.

The 16S rRNA and ITS2 amplicon sequences were downloaded from the Illumina BaseSpace data cloud, data sequences were processed and analysed using the Mothur software (Schloss *et al.*, 2009). For ITS2 analysis, we used the DADA2 package.

2.4.1 Processing of 16s rDNA sequence in Mothur.

In the first step contigs were made from the forward and reverse reads. Contigs less than 300 bp and more than 400 bp were removed. Next, identification of the unique sequences was performed, to determine how many unique sequences were present in the data. Following this the sequences were aligned to the V1-V2 region of the 16S gene, using the 16S database from the Human Oral Microbiome Database (HOMD; Version 14.5). Next sequences outside the V1-V2 region were trimmed away (bases 20-714 bp of the alignment), resulting in a decrease in total and unique numbers of sequences. A pre-clustering step was performed to merge sequences less than 2 bases different, leading to a reduction in the number of unique sequences. Further processing was carried out to

remove sequences that were present in only 1 or 2 copies in the entire data set. Finally, chimeras (artefacts generated by mispriming during PCR procedures) were removed from sequences.

2.4.2 Analysis of Operational Taxonomy Units (OTUs) in Mothur.

In this step, sequences were first clustered at the order level to generate separate goups (to reduce memory usage) before sequences were clustered at 98% nucleotide sequence identity. At this stage, the number of OTUs in each sample was identified and the taxonomy of OTUs was determined in Mothur. For some statistical analysis, data sets were normalized by subsampling, where a sample of the data is selected for analysis. The subsample taken corresponds to the number of sequences present in the smallest data set. In this current study the smallest group has 5550 sequences, any samples with a number smaller than this were eliminated.

2.4.3 Processing of ITS2 sequence analysis.

ITS2 sequencing data were analysed using DADA2 pipeline. Following this a Mothur compatible file was generated.

2.4.3.1. DADA2 ITS2 sequence analysis.

DADA2 pipeline sequence processing include several steps. This workflow was based on the tutorial obtained from DADA2 home page (https://benjjneb.github.io/dada2/tutorial.html). The DADA2 pipeline workflow included the following steps:

1. Inspection of read quality profile. The quality profiles of the forward and reverse reads were visually inspected by examining plots of the quality scores for each nucleotide base. Reads were trimmed to remove bases with quality scores <30. The forward reads were truncated at position 240, while reverse reads at position 190.
2. Estimation of the sequencing error rates. The DADA2 algorithm was then used to estimate the sequencing error rates. DADA2 used a machine learning approach to generate a model of the sequencing error rates in the data set.
4. A dereplication step combines all identical sequencing reads to generate a set of unique sequences, with a corresponding "abundance" equal to the number of reads with that unique sequence.
5. In the sample inference step, the sequencing error model generated in step 2 was then applied to the data set, resulting in the correction of spurious sequences and estimation of the true number of sequence variants in the samples.
6. Merge paired reads. Paired forward and reverse reads were than merged to construct full length sequences.
7. Construct sequence table. A table of all samples and sequences was constructed.
8. Remove chimeras. Finally chimeras were removed from the merged sequences (this corresponded to <4% of total sequences in this dataset).

2.4.3.2. Mothur ITS2 sequence analysis.

Following processing in DADA2, a Mothur compatible file was generated. These analyses include several procedures:

1. Removal of non-fungal sequences by filtering out any sequences with <80% sequence homology to the ITS2 region.
2. Classification of the remaining ITS2 sequences to species level in Mothur using the classify.seqs command.
3. Classification of the most abundant fungal sequences by BLAST analysis using the ISHAM (International Society for Human and Animal Mycology) Barcoding Database.
4. Normalization of data sets by subsampling to 5000 sequences.

2.5 Statistical analysis.

Various entities and measurements were used to assess the statistical relationships of the samples, described below.

2.5.1. Alpha diversity measurements.

Alpha diversity measurements measure species richness and biodiversity within a microbial environment. In the current study, two alpha measurements were used to measure enrichment of species in the tongue and buccal samples, which include the rarefaction curve and the Inverse Simpson index.

2.5.1.1 Rarefaction curve.

In ecology, rarefaction is a technique to assess species richness from the results of the sampling. This technique allows the calculation of species richness for a given number of individual samples, based on the generation of a so-called Rarefaction curve, which is a plot of the number of OTUs identified with increased sampling of the sequence data.

2.5.1.2 Inverse Simpson index.

The Inverse Simpson index was used to measure microbial diversity of tongue and buccal samples. In ecology, it is often used to quantify the biodiversity of a habitat. It takes into account the number of species present, as well as the abundance of each species.

The Simpson index D is calculated as:

$$D = \Sigma (n / N)^2$$

n = the total number of organisms of a particular species.

N = the total number of organisms of all species.

Inverse Simpson index = 1/D.

The value of this index starts with 1 as the lowest possible figure. This figure would represent a community containing only one species. The higher the value, the greater the diversity. The maximum value is the number of species (or other category being used) in the sample. For example, if there are five species in the sample, then the maximum value is 5.

2.5.1.3. Shannon index and Chao 1 index.

This Shannon index is commonly used to characterize species diversity in the microbial community. It accounts for both abundance and evenness of the species present. Chao1 is a species richness estimator based on abundance. (Chiu and Chao 2016).

2.5.2. Beta diversity measurements.

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

2.5.2.1. Jaccard index.

The Jaccard (jclass) calculator returns the traditional Jaccard index describing the similarity in membership between two communities.

Jaccard Index = (the number in both sets) / (the number in either set) * 100.

2.5.2.2 Bray-Curtis index.

The Bray-Curtis calculator returns the Bray-Curtis index describing the dissimilarity between the structures of two communities.

$$BCij= 1- 2Cij/Si+Sj$$

Where Cij is the sum of the OTUs in common between both sites. Si and Sj are the total number of OTUs counted at both sites.

2.5.2.3. Yue and Clayton index.

The Yue and Clayton index (Thetayc) describes the dissimilarity between the structures of two communities and measures membership and abundance.

2.5.2.4. Phylogenetic Tree.

A phylogenetic Tree or evolutionary Tree is a branching diagram showing the inferred evolutionary relationships among the samples based upon similarities and differences in the distance matrix. Statistical differences in phylogenetic relationships were determined using unweighted Unifrac and Parsimony tests (Schloss 2008).

2.5.3 LEfSe (Linear discriminant analysis effect size) analysis.

LEfSe is a tool developed by the Huttenhower group to find biomarkers between 2 or more groups using relative abundances. LEfSe analyses the features of the dataset, in this study OTUs, and uses a Wilcoxon matched pairs test to identify significant differences (Segata *et al.* 2011). LEfSe then uses Linear Discriminant Analysis (LDA) to describe the affect sizes of the differences detected among groups of microbial communities. Features are then ranked based on the LDA score in terms of their contribution to the observed difference in community structure.

2.6 Real-time quantitative PCR.

ITS2 gene amplification was used to estimate fungal density in each samples. The oligonucleotide primers used were the ITS2 forward primer ITS1_F_seq (5´-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTTGGTCATTCGAGGAATAA), and the reverse primer ITS2_seq (5´-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTTGCGTTCTTCATCGATGG), (Heisel *et al.* 2015). ITS2 levels were determined by real time quantitative PCR using the ABI7500 Fast Real-Time PCR system (Applied Biosystems) and carried out in 96 well plates. Fast SYBR Green Master Mix was used in a final reaction volume 15 µl. For each sample 50 µl was prepared, containing 25 µl of Fast SYBR Green Master Mix, 2.5 µl of Forward primer, 2.5 µl of Reverse primer, and 20 µl of water. By adding 1 µl of template to the 50 µl Mixture, 3 x 15 µl aliquots (technical replicates) were placed in a 96 well PCR plate. The real time cycling conditions were as follow: 95°C for 20 s, followed by 40 cycles of 95°C for 1 sand annealing and extension at 60°C for 30 s. A standard curve was generated using *C. albicans* SC5314 DNA, ranging in concentration

from 10^7 to ~1 CFU/ml. Amplification data (i.e. CT values) were used to generate a standard curve of *C. albicans* CFUs versus CT value in GraphPad Prism (8.0) and this was used to estimate relative fungal density, expressed as CFU/ml, in clinical samples.

Chapter 3

Analysis of the oral microbiome in treatment naïve children with IBD

Chapter 3 Analysis of the oral microbiome in treatment naïve children with IBD

3.1 Introduction.

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder affecting the gastrointestinal tract (GIT) and can be of unknown aetiology (Xun *et al.* 2018), or likely due to an aberrant immune response to the microbiota (Docktor *et al.* 2012). IBD, including Crohn's disease (CD) and ulcerative colitis (UC), although commonly affecting the colon and terminal ileum (Baumgart *et al.* 2007), may affect the entire GIT from the mouth to the anus (Lucas Lopez *et al.* 2017). Additionally, many previous studies have reported oral manifestations in CD patients, including minor aphthous ulcers, mucogingivitis, angular chelitis, swelling of the lips, mucosal tags, cobble-stoning of the mucosa, deep linear ulceration and orofacial granulomatosis (Katz *et al.* 2003, Ojha *et al.* 2007, Rowland *et al.* 2010, Hussey *et al.* 2011, Docktor *et al.* 2012, Lankarani *et al.* 2013, Muhvic-Urek *et al.* 2016, Tan *et al.*, 2018). Although, there are extensive studies investigating the involvement of the gut microbiota in IBD patients, there is a lack of information regarding the involvement of the oral microbiota in patients with IBD (Gao *et al.* 2018, Xun *et al.* 2018). The oral cavity is a large reservoir of bacteria of more than seven hundred species (Said *et al.* 2014). There is a correlation between the microbial composition of the oral cavity and oral health (Yamashita and Takeshita 2011) and moreover the oral microbiota play a significant role in general health status (Gao *et al.* 2018). Approximately 60% to 80% of the gut microbiota cannot be grown by culture based techniques (Dave *et al.* 2012), whereas about 35% of oral microbiota are uncultivable, resulting in difficulties in investigating the oral microbiome using culture-based techniques (Docktor *et al.* 2012). In 1977, Woese and Fox were the first to show

that DNA sequencing could be used to classify bacteria by molecular characterization of the 16S rRNA gene. In the current study, molecular analysis of the V1-V2 region of the 16S rRNA gene by Illumina sequencing on the MiSeq was used to characterise buccal and tongue communities. The Mothur software tool was used to analyses our data and various statistical measurements were applied, including Alpha and Beta diversity measurements. Previous investigations of the oral microbiome in CD patients have focused on the salivary microbiota (Said *et al.* 2014, Xun *et al.* 2018), whereas a study by Docktor *et al.* (2012) sampled tongue and buccal mucosal sites in CD patients who had received therapeutic medications. In the present study, tongue and buccal sites were sampled from treatment naïve children from the Republic of Ireland with IBD, ranging in age from 5 to 16 years old. In this part of the study, we aim to characterize the composition of the oral microbiota in treatment naïve children with IBD compared to heathy subjects and to identify non-invasive biomarkers to detect IBD.

3.2 Data processing of V1-V2 region the 16S gene.

In this study, analysis included 600 cycle data (2 x 300 bp) from an initial MiSeq run and 500 cycle data (2 x 250 bp) from a second run. In order to generate 250 bp reads, data from the first run was pre-processed in DADA2 to trim the terminal 50 bp. This procedure was done for both tongue and buccal samples individually. Mothur was used to generate contigs from the forward and reverse reads and contigs less than 300 bp and more than 400 bp were removed, resulting in the generation of 21,718,478 sequences in the tongue analysis and 12,116,761 sequences in the buccal analysis (Table 3-1 and Table 3-2).

Table 3-1 Tongue data after contig assembly and trimming procedures and summarizes the number of sequences.

	Alignment Start	Alignment End	Number of Bases	Ambigious Bases	Polymer tracts	Number of Sequences
Minimum:	1	35	35	0	2	1
2.5%-tile:	1	359	359	0	4	542962
25%-tile:	1	373	373	0	5	5429620
Median:	1	377	377	0	5	10859240
75%-tile:	1	380	380	0	5	16288859
97.5%-tile:	1	402	402	16	6	21175517
Maximum:	1	502	502	95	250	21718478
Mean:	1	378	378	1	4	

Total number of sequences: 21,718,478.

Table 3-2 Buccal data after contig assembly and trimming procedures and summarizes number of sequences.

	Alignment Start	Alignment End	Number of Bases	Ambigious Bases	Polymer tracts	Number of Sequences
Minimum:	1	35	35	0	1	1
2.5%-tile:	1	358	358	0	4	302920
25%-tile:	1	373	373	0	5	3029191
Median:	1	377	377	0	5	6058381
75%-tile:	1	379	379	0	5	9087571
97.5%-tile:	1	408	408	15	7	11813842
Maximum:	1	502	502	75	250	12116761
Mean:	1	377	377	1	5	

Total number of sequences: 12,116,761

Identification of unique sequences was performed to determine how many unique contigs were present in the tongue and buccal sample data. For tongue samples, this yielded 2,396,527 sequences whereas buccal samples yielded 1,632,313 unique sequences. Unique sequences were aligned to the V1-V2 region of the 16S gene, using the 16S

reference sequence database from the Human Oral Microbiome Database (HOMD), version 14.5. Sequences that aligned outside this region were trimmed (the 20-714 bp region). Pre-clustering was performed to merge sequences 1 or 2 bases different, leading to reduction in the number of unique sequences and further processing was carried out to remove sequences that were present in only 1 or 2 copies in the entire data set. Chimeras (artefacts generated during PCR procedures) were removed from sequences using UCHIME (R.C. Edgar *et al.* 2014), leading in a reduction in the number of total sequences and unique sequence number (Table 3-3 and Table 3-4).

Operational Taxonomy Units (OTUs) were defined at 98% sequence similarity (0.02 distance). This cut off was based on advice from Professor. William Wade, King's College London (Personal Communication). Our own empirical analysis comparing 0.02 and 0.03 distances indicated that a greater number of Streptococcal taxa could be discriminated at the 0.02 distance. Sequences were also classified at phyla level and genus level for phylotype analysis. For some analysis data sets were normalized by subsampling. In the current study the smallest number of sequences in any sample was 5550 and a subsample of this size was generated from each sample.

Following sequencing data processing, statistical analysis were carried out using the following statistical methods.

- Alpha diversity measurements (rarefaction curve, Inverse Simpson index).
- Beta diversity measurements (Jaccard index [Jclass], Bray-Curtis dissimilarity index, and the Yue and Clayton dissimilarity index [Thetayc]).
- A phylogenetic Tree.
- Linear discriminant analysis (LEfSe).

Table 3-3 Total number of sequences and the number of unique sequences in tongue samples after the final step of data pre-processing.

	Alignment Start	Alignment End	Number of Bases	Ambiguous Bases	Polymer tracts	Number of Sequences
Minimum:	1	564	322	0	3	1
2.5%-tile:	1	564	349	0	4	364194
25%-tile:	1	564	361	0	5	3641932
Median:	1	564	364	0	5	7283864
75%-tile:	1	564	367	0	5	10925795
97.5%-tile:	1	564	378	0	6	14203533
Maximum:	1	564	408	0	8	14567726
Mean:	1	564	364	0	4	

Number of unique sequences: 37,089

Total number of sequences: 14,567,726

Table 3-4 Total number of sequences and the number of unique sequences in buccal samples after the final step of data pre-processing.

	Alignment Start	Alignment End	Number of Bases	Ambiguous Bases	Polymer tracts	Number of Sequences
Minimum:	1	563	323	0	3	1
2.5%-tile:	1	563	345	0	4	192048
25%-tile:	1	563	360	0	5	1920472
Median:	1	563	364	0	5	3840944
75%-tile:	1	563	366	0	5	5761416
97.5%-tile:	1	563	378	0	5	7489840
Maximum:	1	563	398	0	8	7681887
Mean:	1	563	363	0	4	

Number of unique sequences: 23,498

Total number of sequences: 7,681,887

3.3 Results.

To explore the oral microbial features of Crohn's disease in children, 16S rDNA gene sequencing was performed. Tongue and buccal swabs were collected from 38 children with Crohn's disease (treatment naïve patients), 21 children with Ulcerative Colitis (treatment naïve) and 46 healthy subjects. The subjects' demographic and clinical characteristics are summarized in Chapter 2, section 2.1.

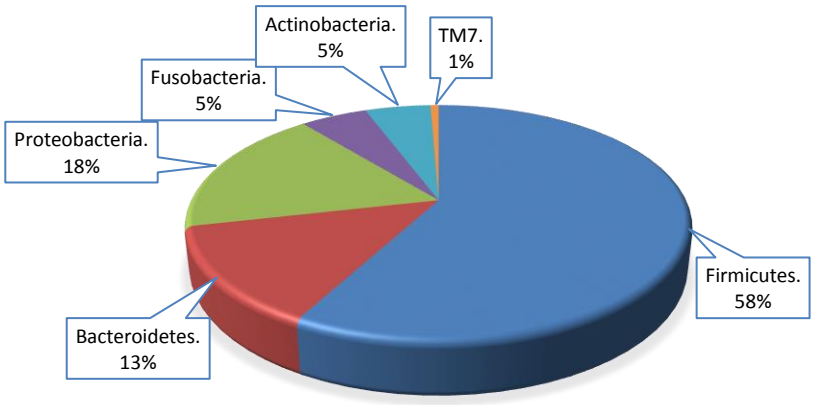
3.3.1 Buccal site analysis.

Data from buccal samples were analysed at different levels of classification (phyla, genus, OTU) using the Mothur software package. Samples were classified as healthy control buccal (HCB), ulcerative colitis buccal (UCB) and Crohn's disease buccal (CDB).

3.3.1.1 Phyla level analysis.

At this level, analysis of buccal samples revealed that Firmicutes was the dominant phylum (58%), followed by Proteobacteria (18%), Bacteroidetes (13%), Actinobacteria (16%), Fusobacteria (5%) and TM7 (1%) (Figure 3.1a). Comparison of the three buccal study groups (CDB, UCB, and HCB) showed that Firmicutes were the most abundant phyla in all groups (58%, 55% and 60% respectively; Figure 3.1b). Analysis with Metastats was carried out to identify differentially abundant buccal phyla between the three study groups. This analysis revealed a significant increase in the levels of the phyla Actinobacteria in CDB and UCB groups compared to healthy children (P=0.015 and P=0.043, respectively).

(a)



(b)

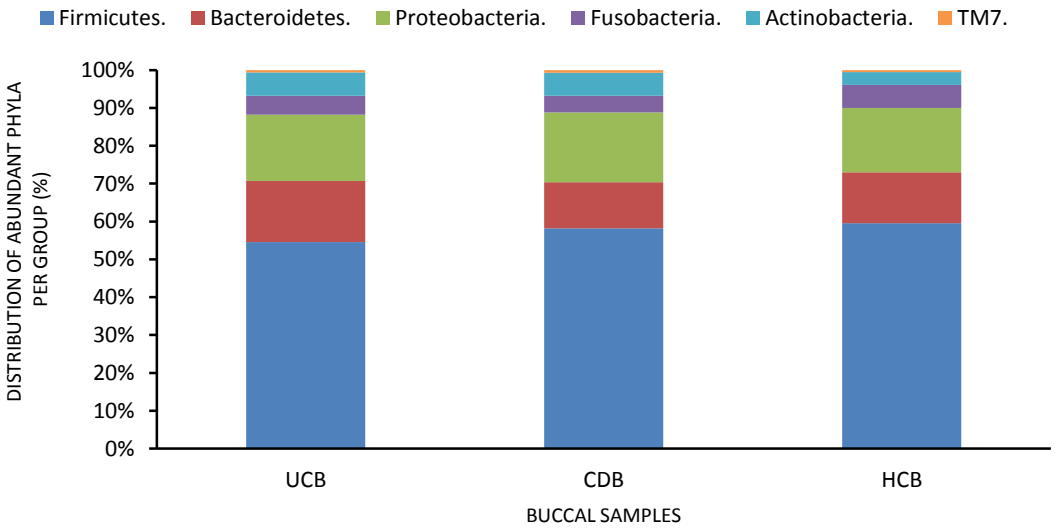


Figure 3.1 (a) Distribution of the microbiota in buccal samples following classification at the phyla level. (b) Comparison of phyla distribution in the three study groups (CDB, UCB, and HCB).

3.3.1.2 Genus level analysis.

Classification of the buccal sequences at genus level identified 174 genera in our analysis. After normalisation of the data by subsampling the number of genera was reduced to 158. When we compared the 5 most abundant species in all three buccal groups, we found that *Streptococcus* spp. was the most dominant genus accounting for 31-35% of reads in each group, followed by the *Pasteurellaceae, Neisseria* spp., *Gemella* spp. and *Prevotella* spp. (Figure 3.2).

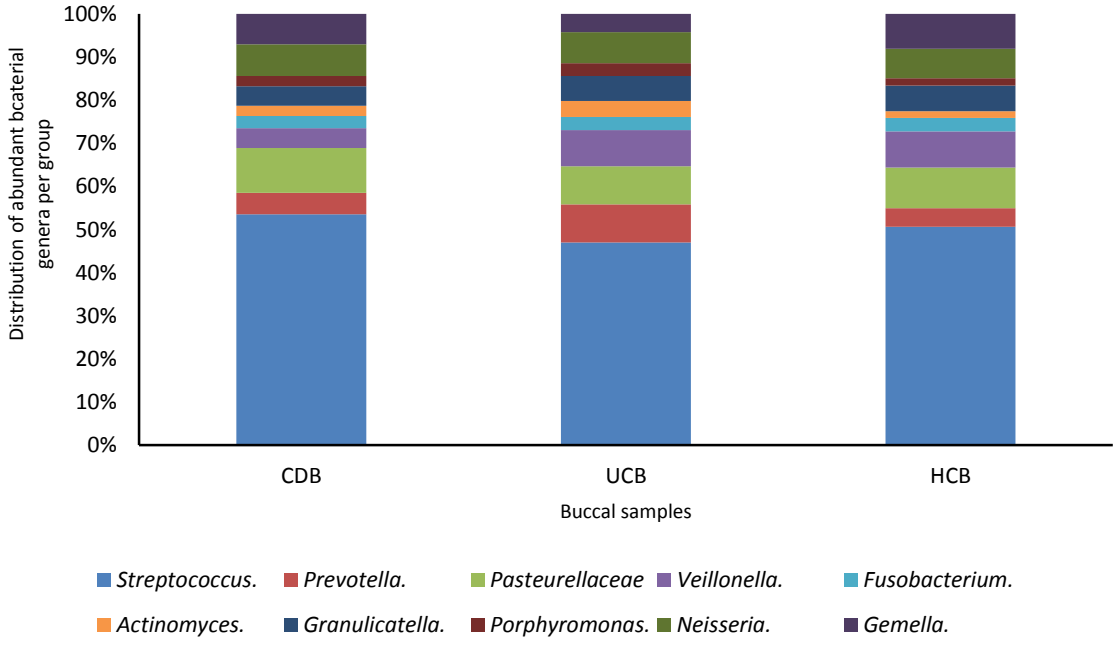


Figure 3.2 The most 10 abundant buccal species in the three study groups samples.

3.3.1.3 OTU level analysis.

For further analysis, sequences with 2% or less nucleotide sequence differences were classified as OTUs. The total number of OTUs identified in all buccal samples was 1,422. Following normalisation by subsampling the number was reduced to 1092 OTUs. 20 OTUs accounted for more than 90% of sequence reads in buccal samples (Figure 3.3).

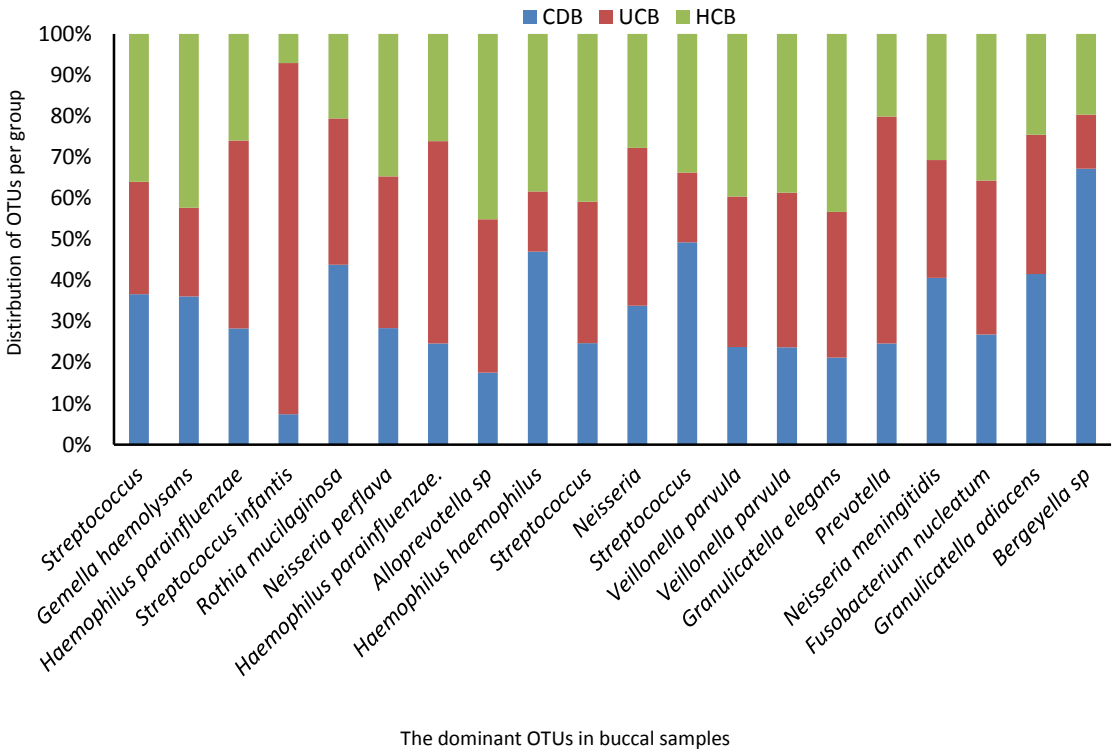


Figure 3.3 The distribution of the dominant OTUs in buccal samples of all studied groups.

3.3.1.4 Alpha diversity measurements.

Species richness within the buccal microbial environment was measured, using different alpha diversity measurements.

3.3.1.4.1 Inverse Simpson Index.

The inverse Simpson and Shannon indices of biodiversity were calculated for each buccal sample. The average inverse Simpson index values for each group show that UCB samples exhibited greater biodiversity compared to CDB and HCB (Table 3-5). HCB samples also had greater inverse Simpson index values relative to CDB samples. The average Shannon index values indicate that CDB samples generally had reduced levels of biodiversity relative to the UCB and HCB study groups (Table 3-5 and Figure 3.4).

Table 3-5 The minimum and maximum numbers of OTUs in buccal samples of all study groups and Shannon and Inverse Simpson values.

SITE	Study groups	Average OTUs	Inverse Simpson	Shannon	Minimum OTU number	Maximum OTU number
Buccal	CDB	111.44	8.92	2.70	17.11	194.89
Buccal	UCB	118.42	11.18	2.89	43.48	190.32
Buccal	HCB	131.58	9.85	2.92	60.06	243.85

3.3.1.4.2 Rarefaction curve.

Using the rarefaction curve, species richness was analysed in buccal samples. Data from buccal samples show that healthy control subjects (HCB) were richer in species compared to the IBD group of children (CDB and UCB). On the other hand, children with UC had greater species richness compared to Crohn's disease children (Figure 3.4).

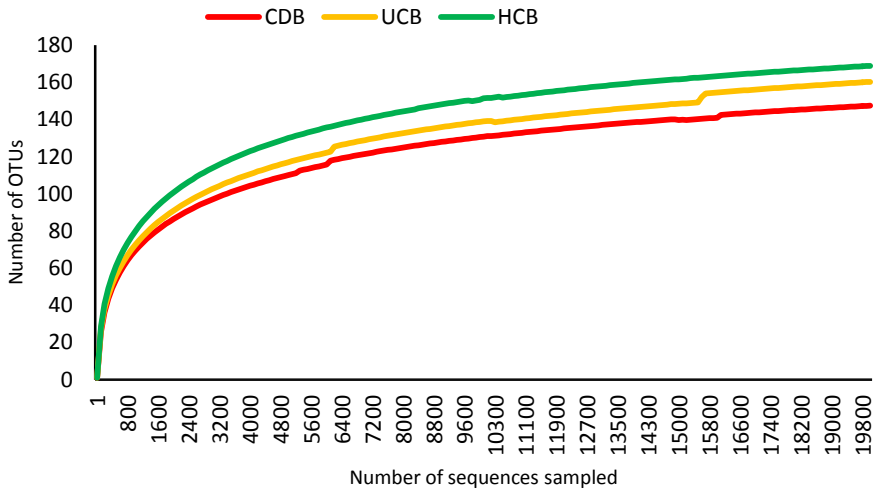


Figure 3.4 Rarefaction curve showing microbial species richness of CDB, UCB and HCB study groups.

3.3.1.5 Beta diversity measurements.

Several methods were used to compare microbial community structure between the different studied groups.

3.3.1.5.1 Phylogenetic tree analysis.

Phylogenetic methods were used to determine the relationships between samples. The Yue and Clayton (Thetayc), the Jaccard (Jclass) and the Bray-Curtis calculators were each used to generate distance matrices showing the relatedness of all samples. Phylogenetic trees were generated in Mothur using these calculated distance values to determine the branch lengths separating different microbial communities. The Parsimony statistical test was used to determine if samples from different studied groups separated and clustered at different branches in the tree. Analysis of trees generated with the

Jaccard index show that communities from the Crohn's disease group (CDB) and the Ulcerative Colitis (UCB) group were significantly different to control HCB samples (Table 3-6). Analysis of trees generated with the Thetayc and Bray-Curtis calculators did not yield any statistically significant differences.

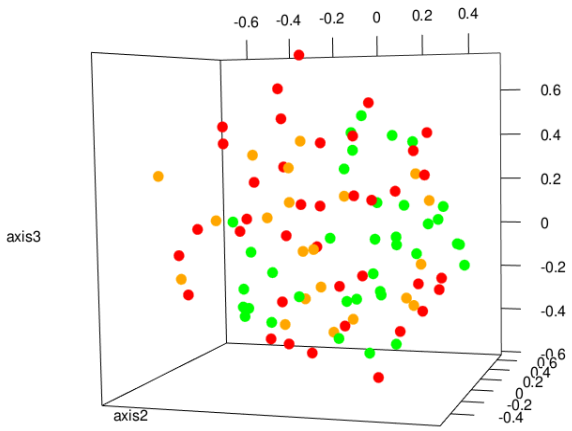
Table 3-6 Results of the Parsimony test examining the phylogenetic relationships in community structure calculated using the Thetayc, Bray-Curtis, and Jaccard calculators.

Groups Tested	Thetayc (P Value)	Bray-Curtis (P Value)	Jaccard (P Value)
Colitis Vs Crohn's	0.898	0.699	0.444
Colitis Vs Healthy	0.765	0.538	0.002**
Crohn's Vs Healthy	0.573	0.082	<0.001**

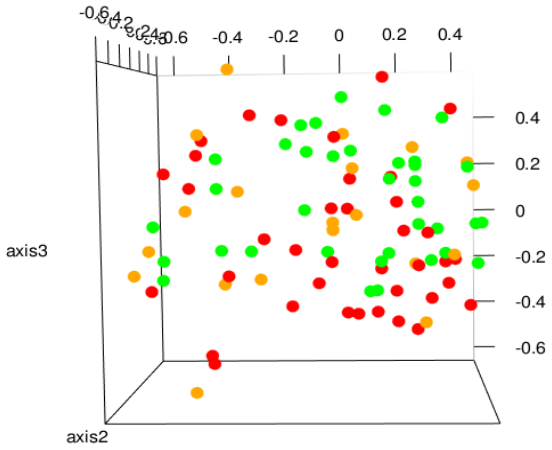
3.3.1.5.2 Non-metric Multidimensional Scaling (NMDS).

NMDS was used to graphically represent the distance between different buccal samples. Distances were calculated using Thetayc, Jclass, and Bray-Curtis calculators. Analysis of Molecular Variance (AMOVA) was carried out to determine if the separation between the study groups in the NMDS plot was significant (Figure 3.5). Using the Jclass distances, AMOVA identified a significant difference in community structure between buccal samples of IBD children (CDB and UCB) and healthy control children (HCB) ($P<0.003$). However, differences in community structure between Crohn's (CDB) and healthy (HCB) were more significant compared to the difference between ulcerative colitis (UCB) and healthy (HCB) ($P<0.001$ and P 0.034, respectively). Analysis of the Thetayc distance matrix and Bray-Curtis disimilarity coefficient matrix showed that communities from buccal samples of Crohn's disease (CDB), ulcerative Colitis (UCB) and healthy children (HCB) were not significantly different (Bray-Curtis $P=0.073$, and Thetayc $P=0.271$).

(a)



(b)



(c)

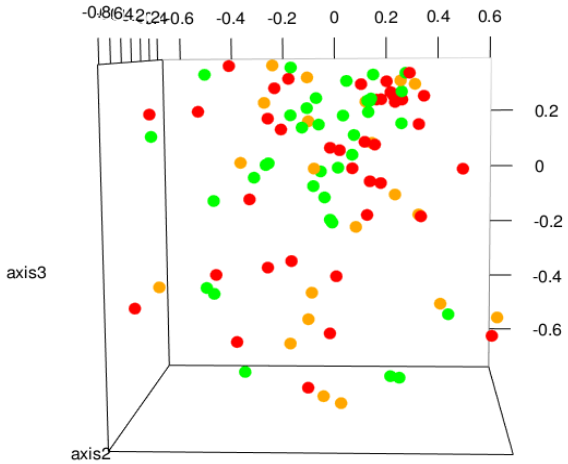


Figure 3.5 NMDS plots of buccal samples from HCB (green), UCB (orange) and CDB (red) study groups generated with distance matrices generated with (a) Thetayc (b) Bray-Curtis and (c) Jclass calculators.

3.3.1.6 LEfSe analysis of buccal samples.

To identify species and OTUs enriched in each group, LEfSe analysis was carried out on buccal samples from the three study groups (CDB, UCB, and HCB). Analysis between IBD children and healthy subjects (HCB) indicates that IBD patients were enriched in Actinobacteria, including *Actinomyces* spp. and *Bergeyella* spp., whereas several streptococcal taxa (*Streptococcus sp.* OTU0010 and OTU0032), *Gemella haemolysans* (OTU0002), *Granulicatella elegans* (OTU0015), and *Leptotrichia spp.* showed greater abundance in the HCB group (Figure 3.6).

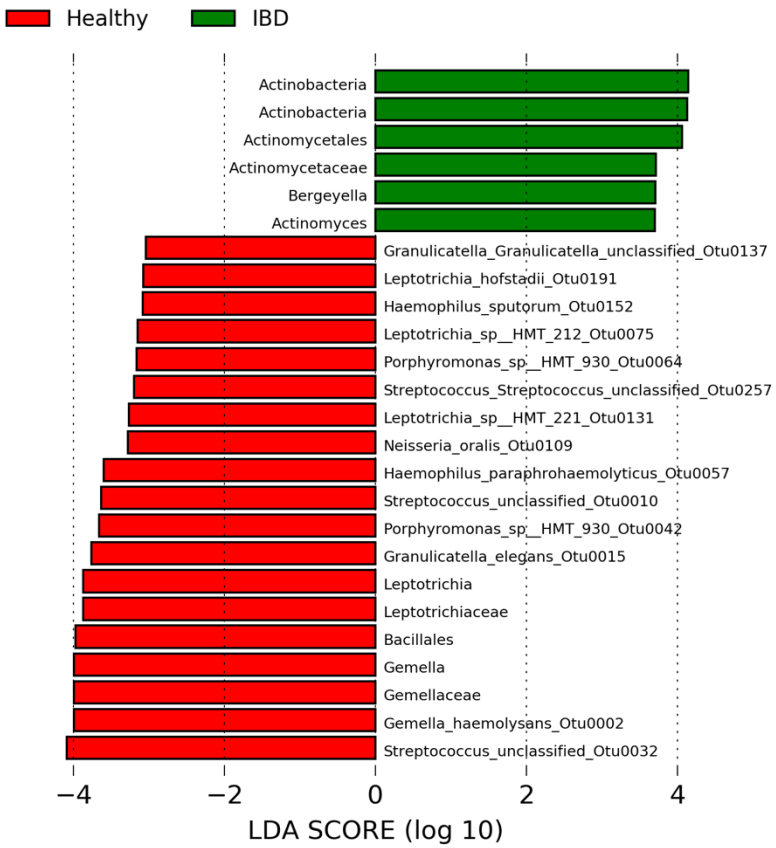


Figure 3.6 Results of LEfSe analysis showing significant enrichments (P <0.05) in buccal IBD samples (CDB UCB) and healthy buccal (HCB) samples, ranked in significance based on the linear discriminatory analysis (LDA) score.

The phylogenetic distribution of the differences between inflammatory bowel disease (IBD) and healthy children (HCB) was also analysed, showing the enrichment in the phylum Actinobacteria in IBD children (Figure 3.7).

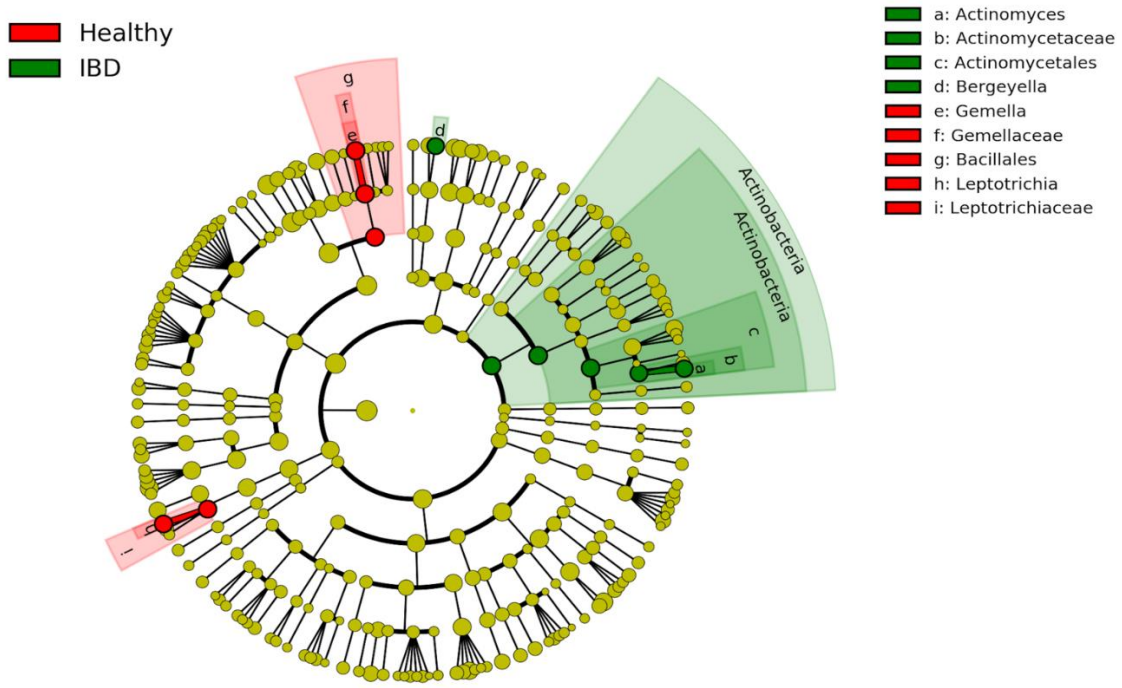


Figure 3.7 Phylogenetic distribution of OTUs enriched in buccal samples from IBD patients (UCB and CDB) and healthy (HCB) patients.

When the IBD groups (CDB and UCB) are analysed individually the results show that CDB were enriched in *Bergeyella* spp. and Actinobacteria (Figure 3.8). *Veillonella* spp. and *Prevotella* spp. were enriched in the UCB group. In healthy subjects, streptoccoci (*Streptococcus sp.* OTU0010 and OTU0032), *Gemella haemolysans* (OTU0002), *Granulicatella elegans* (OTU0015), and *Leptotrichia* spp. were again shown to have greater abundance relative to the IBD groups (Figure 3.8).

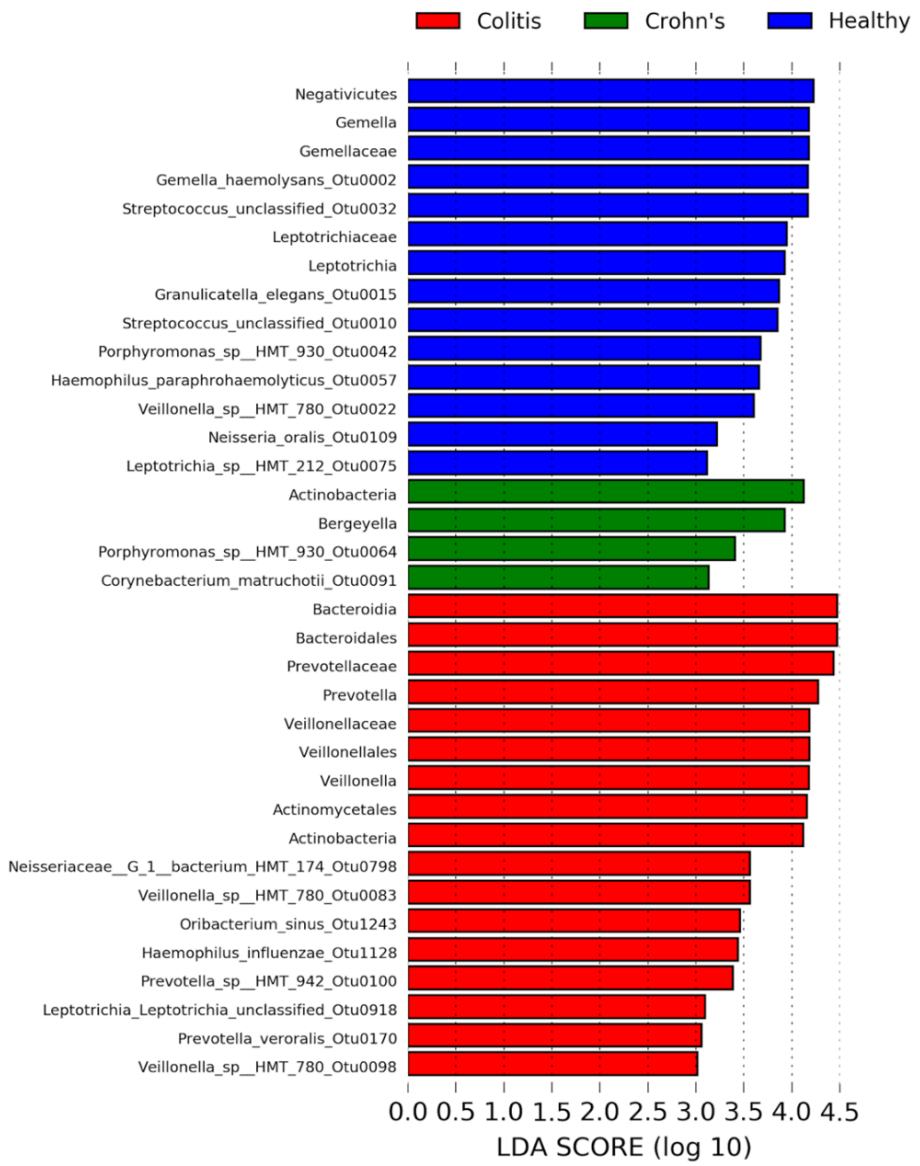


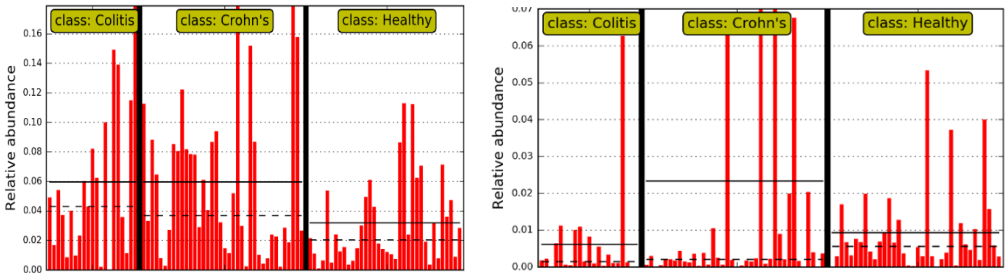
Figure 3.8 Results of LEfSe analysis showing significant enrichments (P<0.05) in buccal samples from CDB, UCB, HCB samples, ranked in significance based on the linear discriminatory analysis (LDA) score

3.3.1.7 Abundance of bacterial species in buccal samples.

Plots showing the abundance of significantly enriched OTUs in each group were generated using the LEfSe package (Hutlab Galaxy server). Each plot shows the abundance of the OTU in each sample analyzed in the CDB, UCB and HCB groups. The

greatest enrichment in buccal samples from children with IBD (both CDB and UCB) were the Actinobacteria (Figure 3.9). LEfSe analysis (Figure 3.8) also indicated an enrichment in *Bergeyella* spp. in CDB samples, however analysis of the plot in Figure 3.9 shows that this enrichments is not uniform. *Veillonella* spp. and *Prevotella* spp. showed reduced abundance in CDB samples, but had a higher abundance in both UCB and HCB samples.

Actinobacteria *Bergeyella* spp.



Veillonella spp. *Prevotella* spp.

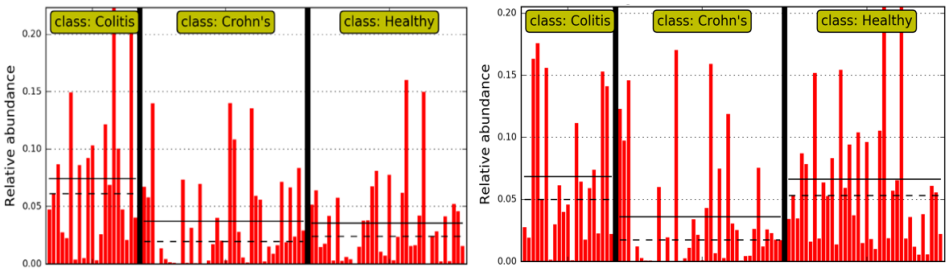
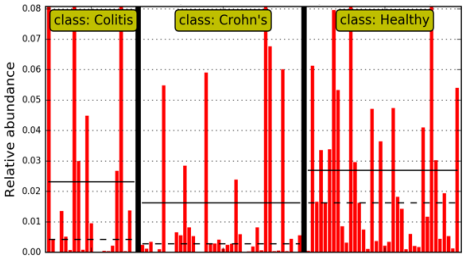


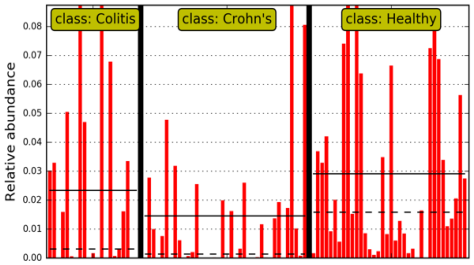
Figure 3.9 Plots showing the abundances of species identified as enriched in buccal samples from IBD patients. Mean values are represented by the solid line, median values by the dotted line.

In buccal samples from the healthy group of children (HCB), the plots confirmed the increased abundance of several OTUs, including *Gemella haemolysans* (OTU0002), *Streptococcus* spp. (OTU0010), *Granulicatella elegans* (OTU0015) and *Leptotrichia* spp. (Figure 3.10).

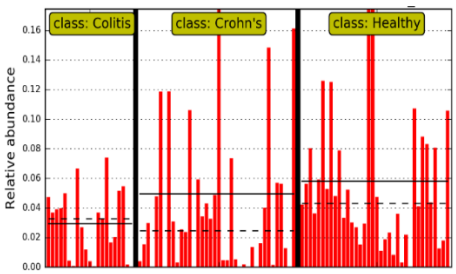
Streptococcus sp. OTU0010



Gemella haemolysans OTU0002



Leptotrichia spp.



Gran. elegans OTU0015

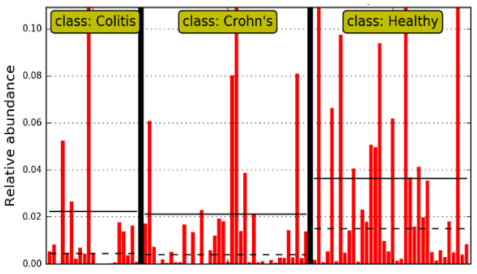


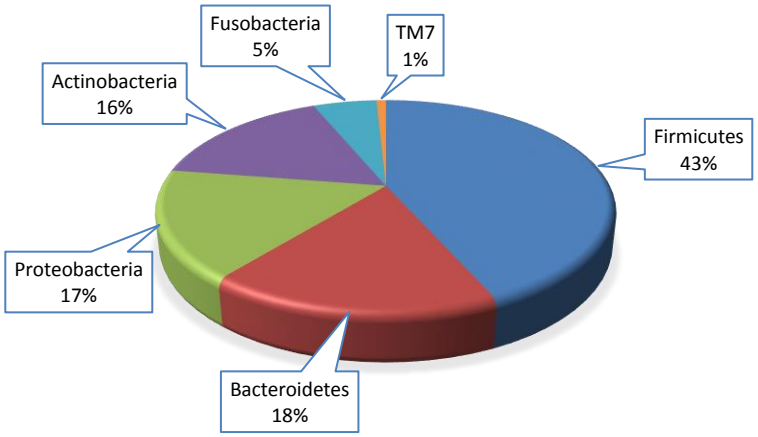
Figure 3.10 Plots showing the abundances of species identified as enriched in buccal samples from healthy patients (HCB). Mean values are represented by the solid line, median values by the dotted line.

3.3.2 Tongue site analysis.

3.3.2.1 Phyla level.

The tongue analysis shows that phyla Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, and TM7 accounted for 99% of sequences. Firmicutes were less abundant on the tongue compared to the buccal mucosa, but still accounted for the highest percentage of reads (43%) (Figure 3.11a). At phyla level, the three study groups including Crohn's disease tongue (CDT), Ulcerative colitis tongue (UCT) and healthy control tongue (HCT) were compared to each other. CDT and UCT children were similar with regards to phyla distribution, with Firmicutes accounting for ~50% of sequences, followed in abundance by Bacteroidetes, Actinobacteria and Proteobacteria (each between 14-17%) with less than 10% Fusobacteria and TM7 (Figure 1.1b). HCT samples showed a different phyla distribution compared to the IBD study groups, with Firmicutes less dominant, representing 36% of sequences. Bacteroidetes, Proteobacteria and Fusobacteria were found at higher levels in the healthy samples (HCT) compared to the IBD samples, whereas levels of Actinobacteria were similar in all study groups (Figure 3.11b). Metastats was used to identify differentially abundant phyla between the tongue groups, which identified significant differences between CDT and HCT children in the phylum Firmicutes (P=0.0009), Bacteroidetes (P=0.009), Proteobacteria (P=0.015), Spirochaetes (P=0.02), and Fusobacteria (P=0.04).

(a)



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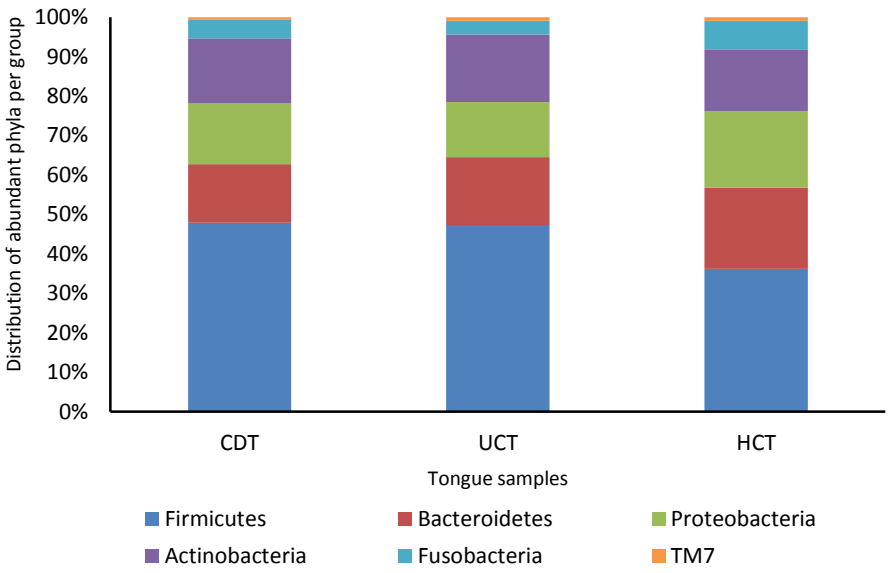


Figure 3.11 (a) Distribution of the microbiota in tongue samples following classification at the phyla level. (b) Distribution of the 6 most the most abundant phyla in tongue samples of all study groups.

3.3.2.2 Genus level.

Mothur identified 133 bacterial genera in our analysis. Following subsampling, the number of genera was reduced to 120. Figure 3.12 was generated to show the abundance of bacterial species in the three studied groups (CDT, UCT, and HCT). These data show that *Streptococcus* spp., *Prevotella* spp., *Rothia* spp., and *Neisseria* spp. were the most abundant genera all study groups accounting for at least 50% of the organisms present in each group.

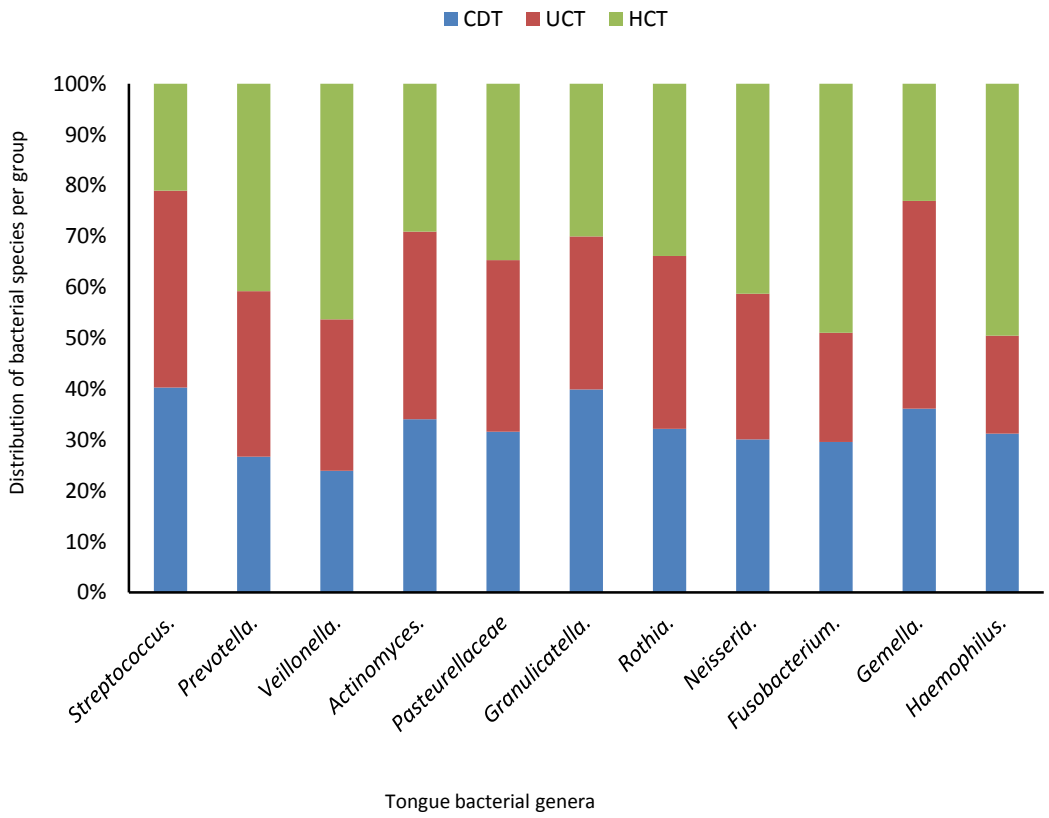


Figure 3.12 Distribution of the 11 most abundant genera in tongue samples of all study groups.

3.3.2.3 OTU level analysis.

For the OTU level analysis, sequences with 2% or less nucleotide sequence differences were classified as OTUs, resulting in the identification of 1617 OTUs. Following normalisation by subsampling, 1108 OTUs remained in the dataset. In the IBD group of children (CDT and UCT) *Streptococcus* sp. (OTU0002) presented at high levels compared to the HCT group. However, in the healthy control group (HCT), *Neisseria* sp. (OTU0003), *Prevotella* sp. (OTU0004), and *Veillonella* sp. (OTU0005) were present at higher levels relative to the CDT and UCT groups (Figure 3.13).

3.3.2.4 Alpha diversity measurements.

These analyses were carried out to measure species richness within the tongue microbial environment.

3.3.2.4.1 Inverse Simpson Index.

The inverse Simpson and Shannon indices of biodiversity were calculated for each tongue sample. These data show that CDT and UCT samples had similar levels of biodiversity, while the HCT group exhibited greater biodiversity compared to the IBD group (Table 3-7). Using Inverse Simpson and Shannon tests, Crohn's disease children (CDT) exhibited reduced biodiversity compared to Ulcerative Colitis (UCT) children and healthy subjects (HCT).

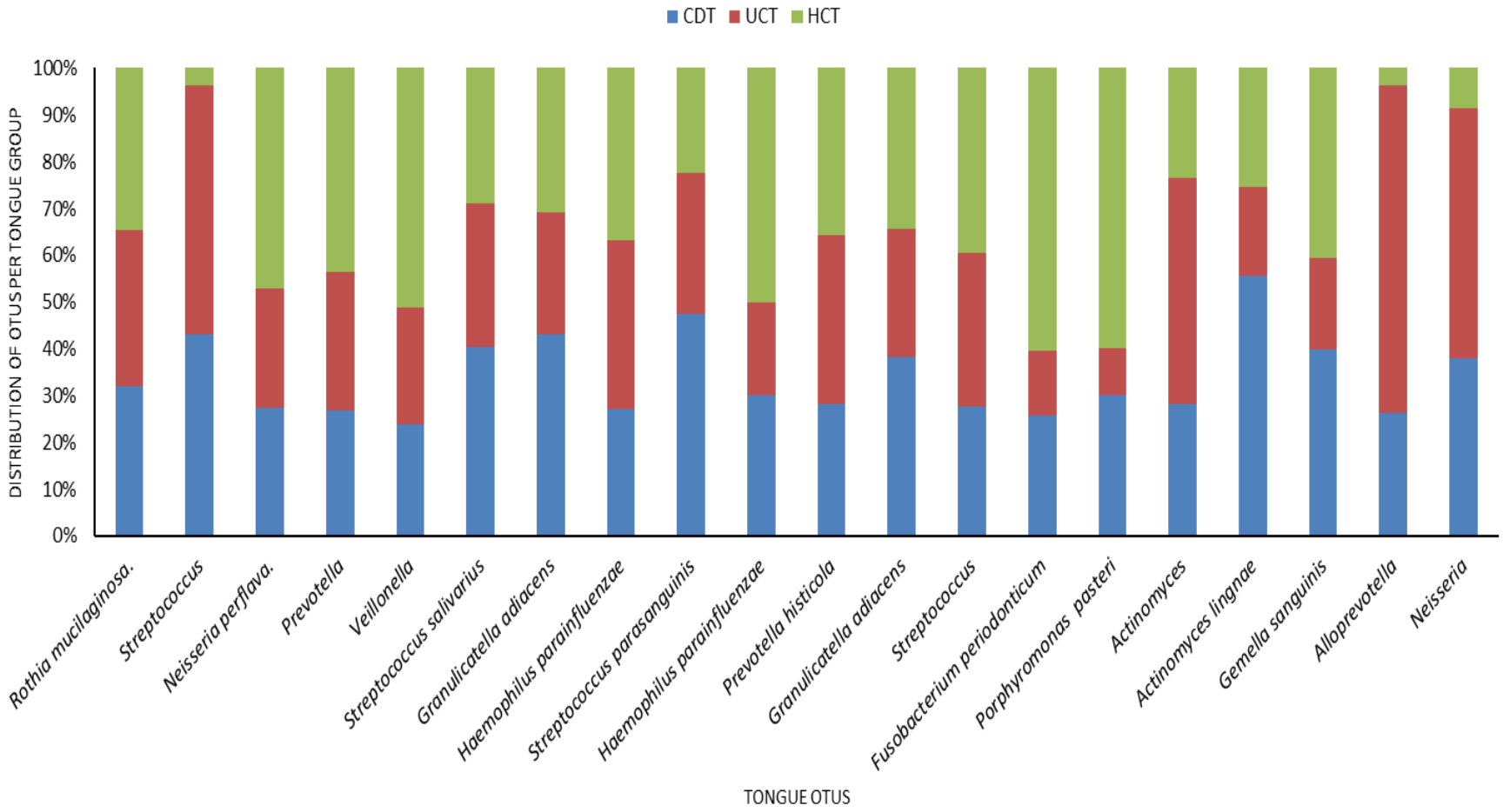


Figure 3.13 The distribution of the most abundant 20 OTUs in tongue samples of CDT, HCT, and HCT groups

Table 3-7 Shannon and Inverse Simpson index values and OTU distributions in the tongue sample study groups.

Site	Study groups	Average OTUs	Inverse Simpson	Shannon Index	Minimum OTU Number	Maximum OTU number
Tongue	CDT	109.3464	12.14	2.92	23.866	179.207
Tongue	UCT	120.0293	12.55	2.96	31.879	258.06
Tongue	HCT	126.8299	13.87	3.22	77.932	214.486

3.3.2.4.2 Rarefaction curve

The rarefaction curve was used to determine species richness in each study group. Analysis of the tongue samples show that HCT samples had greater species richness compared to the IBD group of children (CDT and UCT). On the other hand, children with UC had greater species richness compared to Crohn's disease children (Figure 3.14).

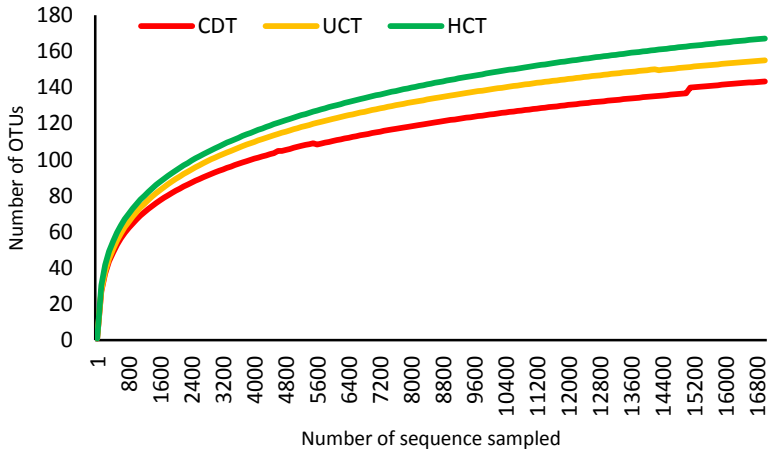


Figure 3.14 Rarefaction curve showing microbial species richness of CDT, UCT and HCT study groups.

3.3.2.5 Beta diversity (community structure) measurements.

Diversity in microbial community structure between different study groups was measured using several methods including phylogenetic analysis and non-metric multidimensional scaling (NMDS).

3.3.2.5.1 Phylogenetic analysis.

Phylogenetic methods were again used to determine the relationships between sample groups using the Thetayc, Jclass and the Bray-Curtis calculators. The Parsimony statistical test was used to determine if samples from different study groups separated and clustered at different branches in the tree. Thetayc, Jclass, and Bray-Curtis calculators each showed that communities from tongue samples of CD patients and healthy subjects (HCT) were statistically significantly different. Ulcerative Colitis (UCT) and healthy controls (HCT) samples also showed statistically significant differences in community structure. Differences between Crohn's disease group (CDT) samples and control HCT samples exhibited the greatest significance ($P < 0.001$). Statistically, no difference was found between CDT and UCT groups (Table 3-8).

Table 3-8 Parsimony test examining the phylogenetic relationships in community structure estimated using the three calculators.

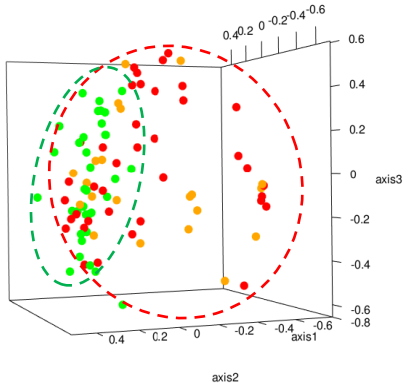
Groups	**Parsimony P value**		
	Thetayc	Jaccard	Bray-Curtis
Colitis vs Crohn's	0.13	0.71	0.27
Colitis vs Healthy	0.002**	0.022*	0.019*
Crohn's vs Healthy	0.005**	<0.001**	0.05

3.3.2.5.2 Non-metric Multidimensional Scaling (NMDS).

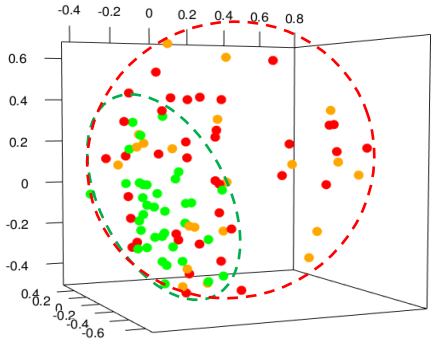
NMDS was used to graphically represent the distance between the samples. Distances were calculated using Thetayc, Jclass, and Bray-Curtis calculators. Analysis of Molecular Variance (AMOVA) was carried out to determine if the separation in the NMDS plot is significant (Figure 3.15). Using the Theta distance metric, the Bray-Curtis dissimilarity coefficient, and Jclass metric, AMOVA analysis of tongue samples shows a significant difference in community structure between tongue samples of IBD children and healthy control children (HCT) ($P < 0.001$). Both Crohn's (CDT) and ulcerative colitis (UCT) sample groups exhibited significant differences from healthy (HCT) samples ($P < 0.001$). On the other hand, no significant difference was found between the tongue samples of Crohn's disease (CDT) and Ulcerative Colitis children (UCT) (Jclass $P=0.337$; Bray-Curtis $P=0.639$ and Thetayc $P=0.836$).

Homogeneity of molecular variance (HOMOVA) was carried out to compare the variation within the study groups, using Bray-Curtis and Theta measurements. The NMDS plots in Figure 3.16 suggest that communities from HCT samples are more homogenous due to their clustering. HOMOVA analysis of both calculators shows a greater degree of variation in samples from IBD children (CDT and UCT), compared to HCT samples ($P<0.001$).

(a)



(b)



(c)

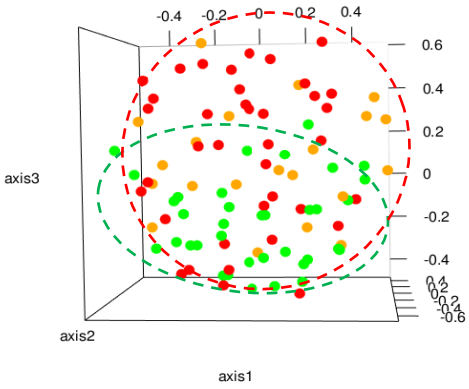


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Figure 3.15 NMDS plots of tongue samples from HCT (green), UCT (orange) and CDT (red) study groups generated with distance matrices generated with (a) Theta (b) Bray-Curtis and (c) Jclass calculators. Red and green circles are used to show healthy and IBD groups and are for visualisation purposes only.

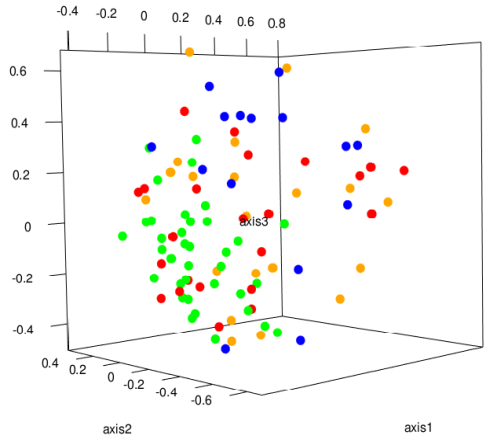
3.3.2.6 Analysis of disease location and severity.

Using the three different matrices, including Thetayc, Jclass, and Bray-Curtis calculators the relationship between oral community structure and the location of the disease symptoms was investigated. Location was classified according to the Paris scheme as L3 (ileocolonic), L4 (upper GI tract) or a mixed phenotype of both L3L4. Although children diagnosed with L4 disease were significantly different from healthy children, no difference was found with the remaining IBD children (CDT, UCT) with a non-L4 diagnosis (Table 3-9). Next, the correlation between L3L4 disease and the three study groups (CDT, UCT, and HCT) was analysed. AMOVA showed a significant difference between children with an L3L4 diagnosis and other non-L3L4 CDT children and healthy control children (Table 3-9 and Figure 3.16).

Table 3-9 P values generated by AMOVA following comparison of tongue microbiomes in children with different disease classifications according to the Paris scheme.

Measurements	***Thetayc***	***Bray-Curtis***	***Jclass***
L4 VS HCT	P<0.0002*	P<0.001*	P<0.001*
L4 VS CDT	P 0.078	P 0.14	P 0.36
L4 VS UCT	P 0.449	P 0.158	P 0.08
L3L4 VS HCT	P <0.0002**	P <0.0001**	P <0.0002**
L3L4 VS CDT	P 0.013*	P 0.047*	P 0.047*
L3L4 VS UCT	P 0.182	P 0.2	P 0.02*

(a)



(b)

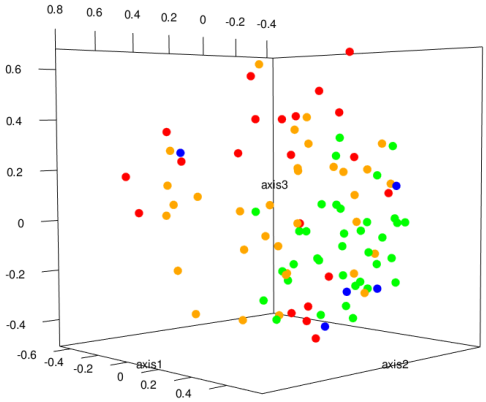


Figure 3.16 NMDS plots showing (a) separation of L3L4 CDT samples (Blue) from non-L3L4 CDT samples (red), UCT samples (orange) and HCT samples (green) and (b) separation of HCT samples (green) from IBD samples classified as inactive (blue), mild-moderate (orange) and severe (red). Distances were calculated using the Bray-Curtis dissimilarity value.

AMOVA analysis was also carried out to assess impact of disease severity on community structure. The severity of the IBD diagnosis was divided into three groups, including inactive, mild-moderate, and severe. AMOVA showed that inactive IBD patients were not significantly different to healthy controls, whereas Mild-moderate and Severe cases of IBD had highly significant differences in community structure compared to healthy control patients (Table 3-10, Figure 3.16).

Table 3-10 P-value results from the correlation between disease severity and the studied groups, using Thetayc and Bray-Curtis calculators.

Disease Groups	***Bray-Curtis***	***Thetayc***
HCT Vs Inactive IBD	P =0.17	P =0.2
HCT Vs mild-moderate IBD	P <0.001**	P <0.001**
HCT Vs severe IBD	P <0.001**	P <0.001**
Inactive IBD Vs mild-moderate IBD	P =0.281	P =0.197
Inactive IBD Vs severe IBD	P =0.151	P =0.126
Mild-moderate IBD Vs Severe IBD	P =0.455	P =0.664

3.3.2.7 LEfSe analysis of tongue OTUs.

Analysis of community structure in section 3.3.1.5 indicated differences in community membership in the three study groups (CDT, UCT, and HCT). To identify the species and OTUs accounting for this, LEfSe analysis was carried out. Patients with IBD were enriched in *Streptococcus* spp., *Neisseria* sp. (OTU0020), and *Gemella* sp. (OTU0021).

On the other hand, *Veillonella* sp. (OTU0005), *Neisseria perflava* (OTU0003), *Prevotella* sp. (OTU0004), *Fusobacterium periodonticum* (OTU0014), *Haemophilus parainfluenzae* (OTU0010) and *Porphyromonas pasteri* (OTU0015) were more abundant in the HCT group (Figure 3.17). Looking at the studied groups individually (CDT, UCT, and HCT), the results show that CDT were enriched in *Streptococcus* sp., including a specific enrichment for *Streptococcus oralis subsp. dentisani clade 058* (OTU0043), and *Actinomyces lingnae* (OTU0017) (Figure 3.18). In the UCT group, four species were enriched, including the dominant streptococcal OTU *Streptococcus* sp. OTU0002 (*S. mitis*), *Gemella* sp. (OTU0021), *Neisseria* sp. (OTU0020), and *Streptococcus* sp. (OTU0023). In healthy subjects, several highly abundant OTUs were enriched including *Veillonella* sp. (OTU0005), *Neisseria perflava* (OTU0003), *Prevotella* sp. (OTU0004), *Fusobacterium periodonticum* (OTU0014), *Haemophilus parainfluenzae* (OTU0010) and *Porphyromonas pasteri* (OTU0015). Furthermore, the data shows enrichment in *Leptotrichia* sp. (OTU0026) and *Leptotrichia* sp (OTU0034).

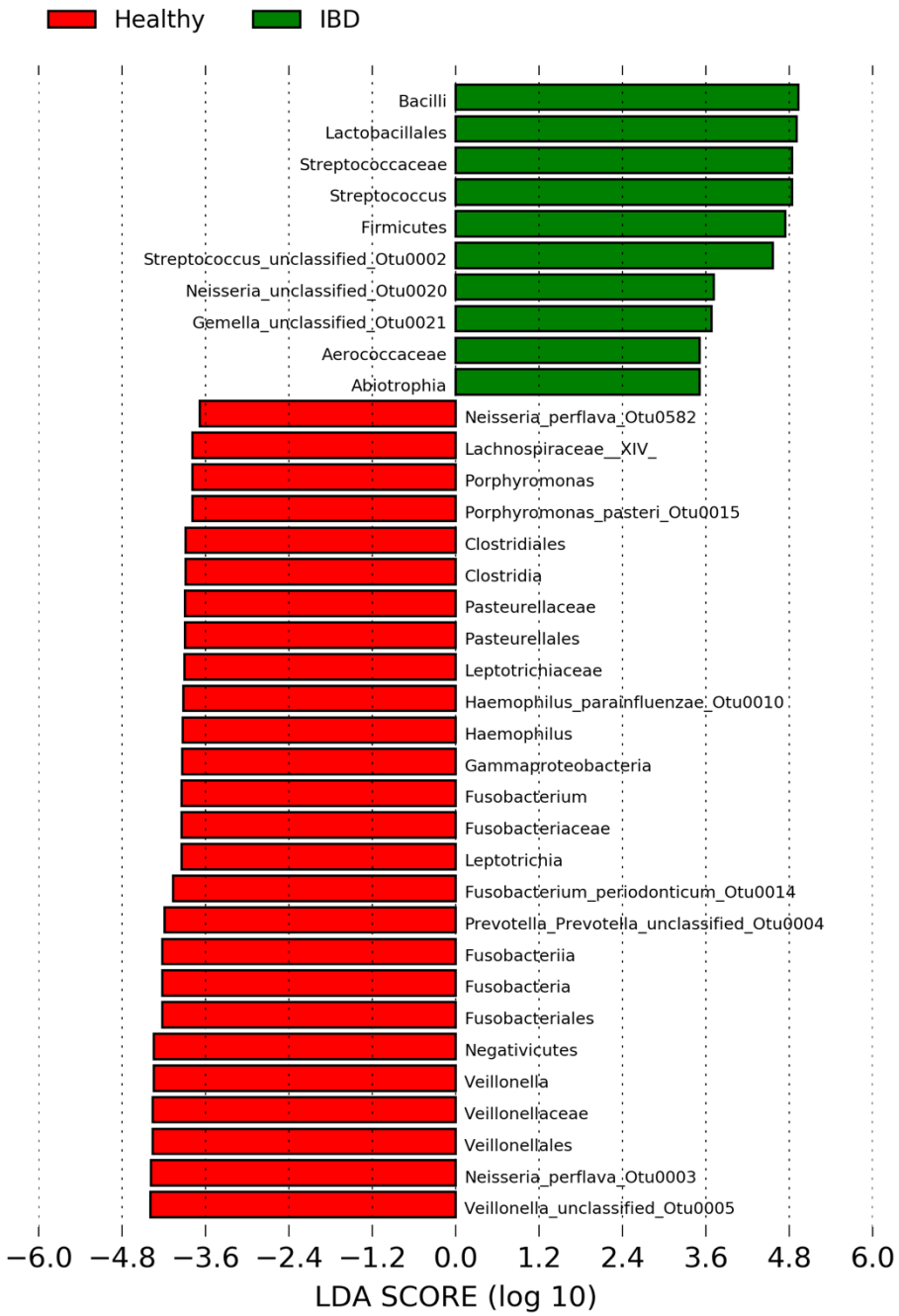


Figure 3.17 Results of LEfSe analysis showing significant enrichments (P <0.05) in HCT and IBD samples, ranked in significance based on the linear discriminatory analysis (LDA) score.

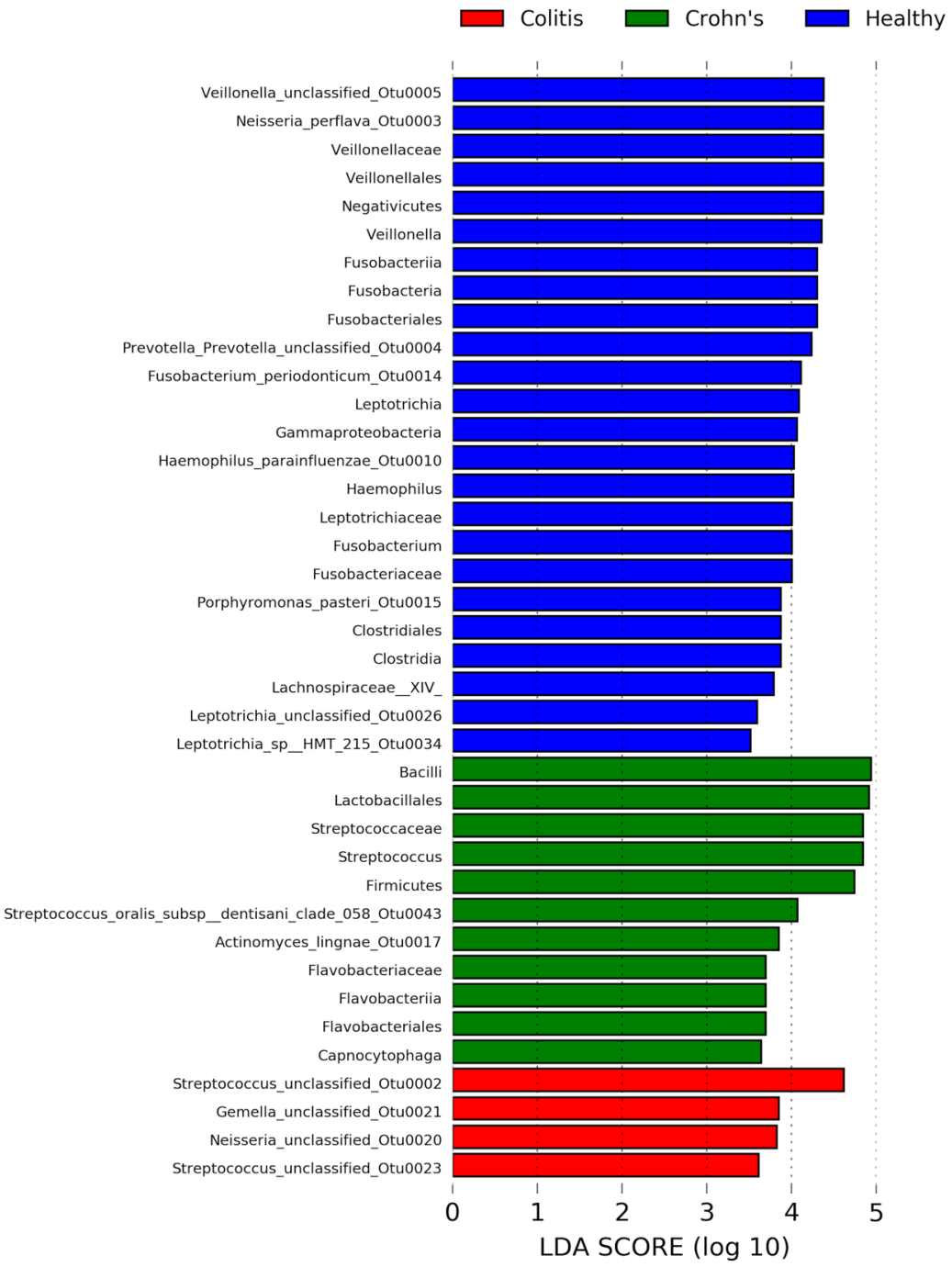


Figure 3.18 Results of LEfSe analysis showing significant enrichments (P <0.05) in Healthy, Crohn's disease and Ulcerative colitis samples, ranked in significance based on the linear discriminatory analysis (LDA) score.

A phylogenetic distribution showing the differences between Crohn's disease children (CDT) and healthy children (HCT) was generated, showing the most differentially abundant bacterial taxa in CDT and HCT study groups, as shown in Figure 3.19.

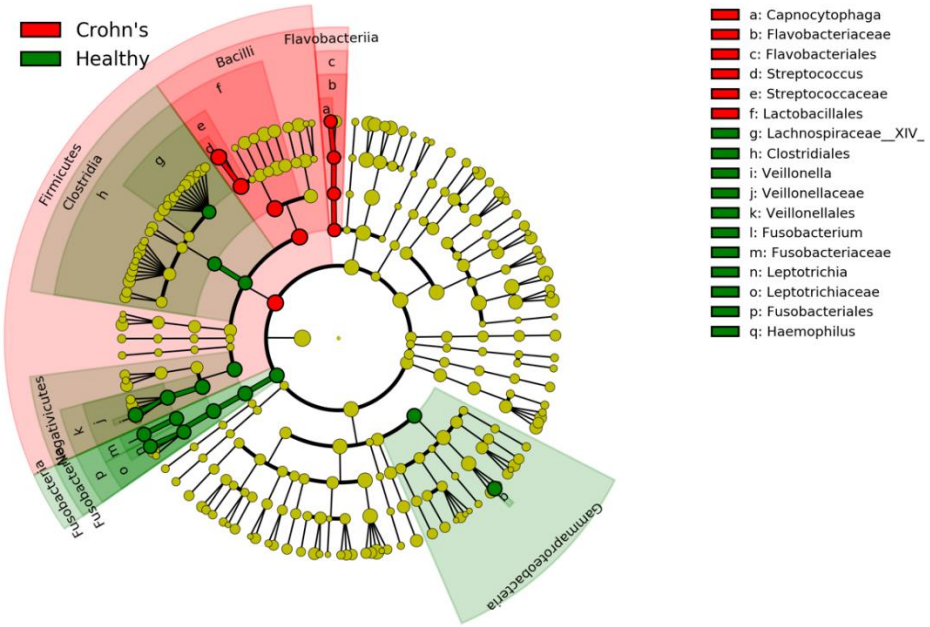
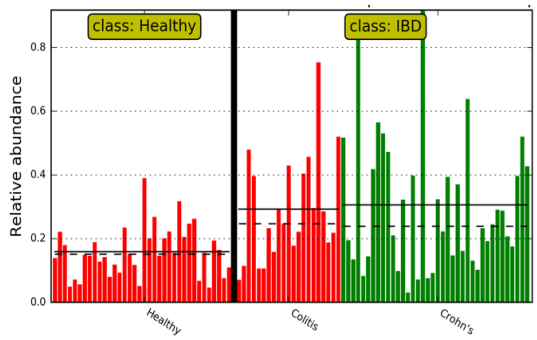


Figure 3.19 Phylogenetic distribution of OTUs with significantly different abundances in CDT and HCT groups.

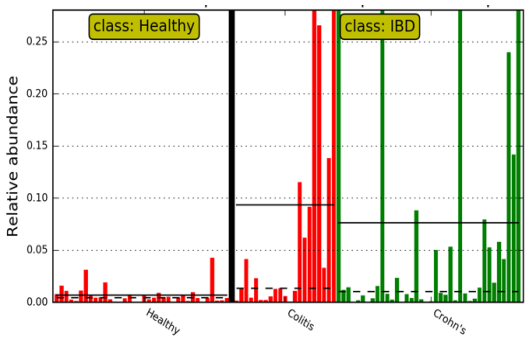
3.3.2.8 Abundant bacterial species in tongue samples.

Plots showing abundances of significantly enriched OTUs in each group were generated using the LEfSe package (Hutlab Galaxy server). The greatest enrichments in tongue samples of IBD children included *Streptococcus spp*., including *Streptococcus sp*. OTU0002 and *Gemella sp.* OTU0021, and *Niesseria* OTU0020 (Figure 3.20).

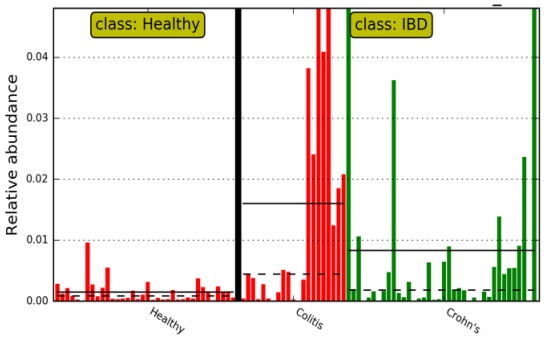
Streptococcus spp.



Streptococcus spp. OTU002



Gemella sp. OTU021



Neisseria sp. OTU020

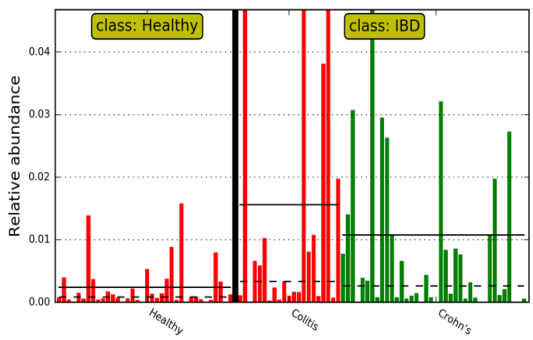
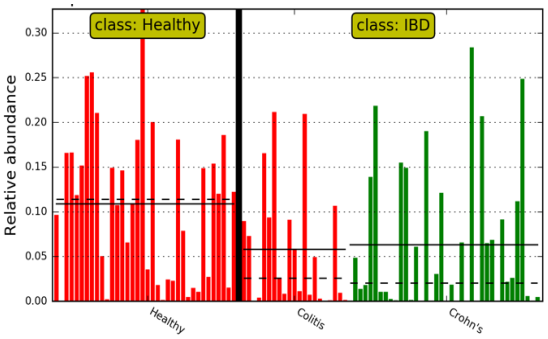


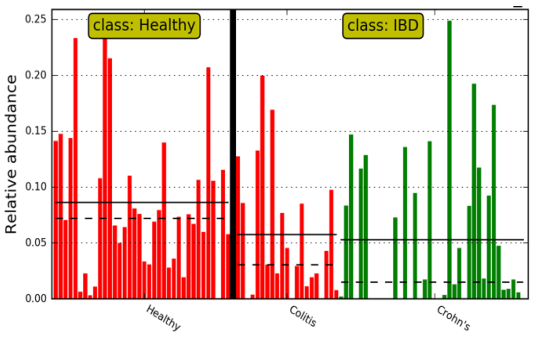
Figure 3.20 Plots showing the abundances of species identified as enriched in tongue samples from IBD patients. Mean values are represented by the solid line, median values by the dotted line.

In the healthy group of children (HCT), the tongue samples showed increased abundance of several highly abundant OTUs, representing major components of the normal oral flora, including the major OTUs of *Neisseria* spp., *Prevotella* spp, *Veillonella* spp. and *Fusobacterium* spp. (Figure 3.21).

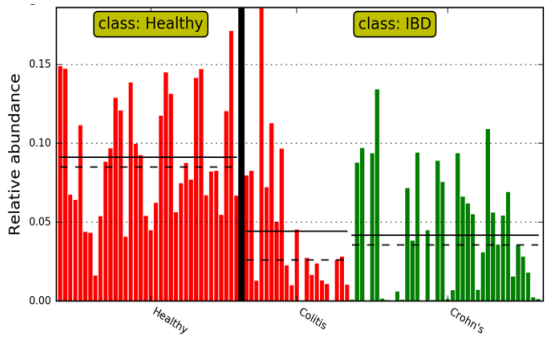
Neisseria perflava OTU003



Prevotella sp. OTU004



Veillonella sp. OTU005



F. periodonticum OTU014

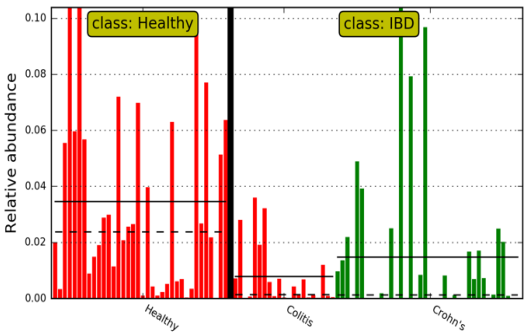


Figure 3.21 Plots showing the abundances of species identified as enriched in tongue samples from healthy patients. Mean values are represented by the solid line, median values by the dotted line.

Comparison of CDT and UCT samples (Figure 3.12) identified a number of taxa that could be potentially used to distinguish Crohn's and ulcerative colitis samples. Inspection of the individual plots indicated that *Gemella* sp. OTU20 was generally more abundant in UCT samples (Figure 1.21). Compared to UCT samples, CDT samples generally had higher levels of *Capnocytophaga* spp. and *Actinomyces lignae* OTU0017 (Figure 3.22).

Capnocytophaga spp. *Actinomyces lignae* OTU017

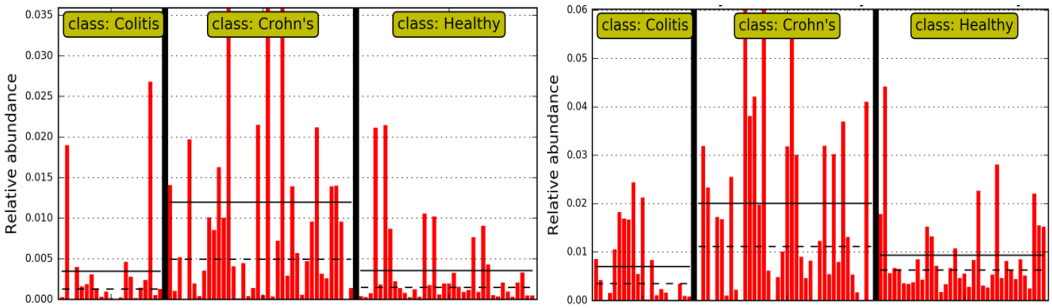
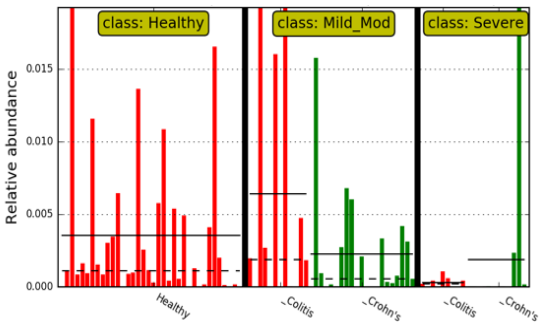


Figure 3.22 Plots showing the abundances of species identified as enriched in tongue samples from CDT patients. Mean values are represented by the solid line, median values by the dotted line.

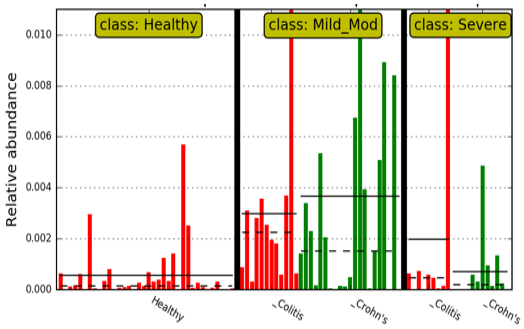
3.3.2.9 Species abundance and disease severity.

Next we carried out LEfSe analysis on the samples from IBD patients categorised as healthy, mild-moderate disease and severe disease based on disease severity scores attributed to the patients at the time of sampling. This analysis identified OTUs that decreased in abundance depending on the severity of IBD. Reduced abundance of several taxa in tongue samples was observed when mild-moderate and severe forms of CD were compared (Figure 3.23). This included taxa shown to be affected in earlier analysis including *Veillonella*, *Neisseria* and *Fusobacterium* species. These changes were not as apparent in UC patients. Unusually, *Streptococcus* OTU0050 was found at higher abundance in patients with mild-moderate disease compared to both healthy and severe IBD patients. This analysis also identified an association between the presence of *Enterobacteriaceae* in tongue samples and severe IBD. Six of the 21 children (29%) with severe IBD harboured low level Enterobacterial carriage, compared to 15% of children with mild-moderate symptoms and 8% of healthy children.

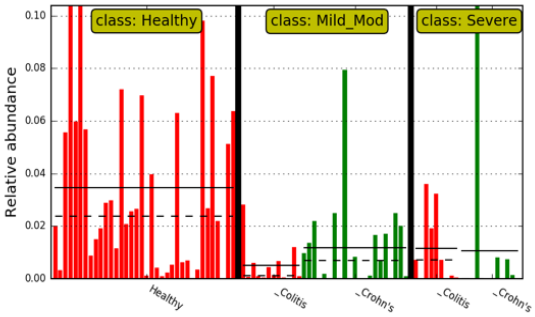
Streptococcus spp. OTU0048



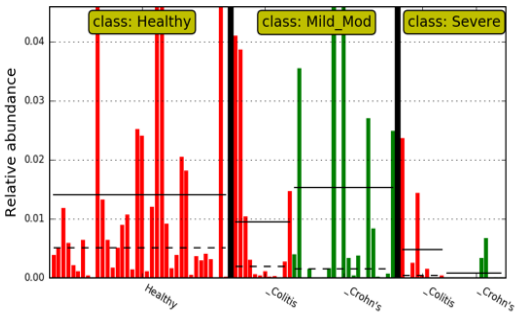
Streptococcus spp. OTU0050



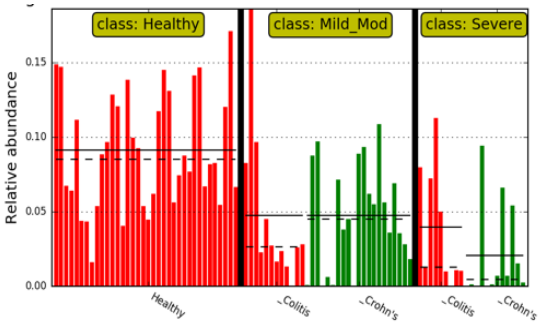
F. periodonticum OTU0014



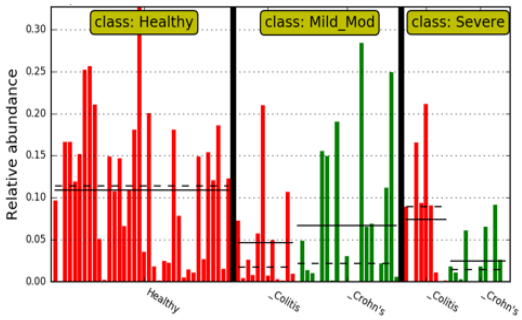
Leptotrichia spp. OTU0024



Veillonella sp. OTU0005



Neisseria perflava OTU0003



Enterobacteriales OTU0218

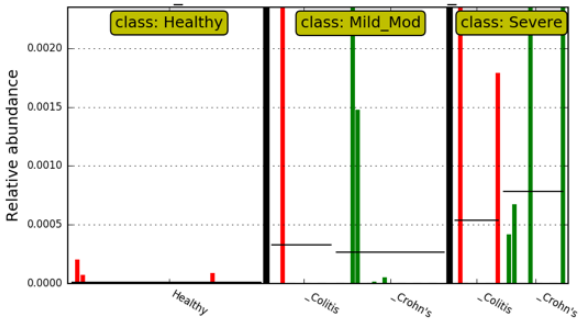


Figure 3.23 Plots showing the abundances of species identified as differentially enriched in IBD patients with mild-moderate and severe IBD. Mean values are represented by the solid line, median values by the dotted line.

3.3.2.10 Impact of age, gender and oral health on the tongue microbiome.

We used AMOVA to determine if age, gender, DMFT or the presence of caries made a significant impact on community structure in our analysis. Using the Bray-Curtis, Thetayc and Jclass metrics we found that gender had no significant impact on community structure. Of the 95 subjects, we recorded DMFT values for 72 patients. Increasing DMFT score did not impact on community structure. Dental caries recorded in 27 children and gingivitis (5 children) did not impact the tongue microbiome. In terms of age, adolescents did not differ from children. Using the Thetayc and Bray-Curtis metric, children under 10 years did not differ from children aged over 10 years. However AMOVA using the Jclass metric of community membership could distinguish children under 10 from those aged over 10 (P 0.015). LEfSe analysis showed that children under 10 harboured higher levels of *F. periodonticum* OTU0014 and children over 10 harboured higher levels of S. *salivarius* OTU0006, *Gemella sanguinis* OTU0018 and *Neisseria sp.* OTU0020. *F. periodonticum* was previously shown to be enriched in healthy children in this study. As the average age of the healthy cohort was 11 years (median 10.5), 1 year lower than the average age of the IBD cohort (12 years; median 13 years), this finding might be influenced by the age difference in the two cohorts.

3.4 Discussion.

This study conducted analysis of the oral microbiome in the first NGS study in treatment-naïve children with IBD, sampling tongue (dorsum) and buccal mucosal surfaces of the oral cavity. Several previous studies assessed the dysbiosis of the gut microbiome in IBD patients based on stool and tissues samples from the intestine (Dirk G *et al*. 2014, Manichanh *et al.* 2006, Baumgart *et al.* 2007, Schirmer *et al*. 2018). However, there have been few studies investigating the diversity of the oral microbiome in children suffering from IBD. The current study evaluates if oral microbial dysbiosis occurs in treatment naïve Crohn's disease (CD) and ulcerative colitis children (UC) compared to healthy subjects (HC). Our cohort study demonstrates a marked difference in microbiome structure and content between the buccal and tongue samples in all the three study groups.

The microbial richness and biodiversity of the oral microbiota was assessed in tongue and buccal samples of IBD patients. Using different methods of calculation in alpha diversity measurements, we examined species richness and biodiversity using the Shannon and Inverse Simpson indices. Overall, the tongue was observed to have greater biodiversity compared to the buccal environment, based on Shannon and inverse Simpson measurements of buccal (Table 3-5) and tongue (Table 3-7). We observed that overall buccal and tongue samples of CD patients had lower bacterial species richness (measured by rarefaction of OTUs) compared to HC and UC groups (Figures 3.8 and 3.22). The biodiversity of samples (measured with the Shannon and Inverse Simpson indices) was also generally less in samples from IBD children. Samples from CD children, both tongue and buccal had reduced biodiversity as assessed by both metrics. Unexpectedly, UC tongue samples had reduced biodiversity whereas buccal samples had similar or greater biodiversity compared to samples from HC subjects. In our findings CD samples showed a significant decrease in overall biodiversity compared to HC samples and this is

consistent with the finding of Docktor *et al.* (2012). In accordance with the study of Docktor *et al.* (2012), tongue and buccal samples from UC patients were not statistically different from HC. On the other hand, no differences in salivary microbiota richness among healthy, CD and UC patients were observed by Said *et al*. (2014). However, in this later study the salivary microbiota of IBD patients was examined rather than mucosal surfaces (Said *et al*. 2014). Also patients were older (22-70 years) and most patients (81%) were under treatment with anti-inflammatory drugs (57%) anti TNF-α (4%) or other immunosuppressive drugs (19%). In another oral study, (Xun *et al.*, 2018) examined salivary samples from IBD patients aged greater than 18 years who had started to receive standardized medication. The results showed that Firmicutes and Bacteriodetes were increased in CD and UC patients compared to HC subjects, with depletion of *Prevotella, Neisseria* and *Haemophilus* and enrichment with *Veillonella* noted in CD patients. Although there are some similarities with our findings, (e.g. depletion of *Haemophilus, Neisseria* and *Prevotella*), the differences between our study and the previous studies could be explained by the fact that patients in our study are treatment naive. It is possible that the anti-inflammatory treatments used in the previous studies reduced the symptoms of IBD and allowed rebalancing of the healthy microbiota. Moreover, a study by Singh and Manning (2016) reported that age and sex can impact on the abundance of specific taxa, suggesting that the differences in patients age between the current study (7-16 years) and all previous studies could be significant.

Beta diversity measurements indicated that the microbiomes of healthy children and children with IBD had different community structures. Community structure was not significantly affected by gender, caries, DMFT and only affected by age using the Jaccard index of community membership. Phylogenetic approaches and NMDS based analysis clearly show that tongue communities from CD patients and UC patients are significantly

different to communities from the tongues of healthy children. However, analysis of buccal communities showed less significant differences. The reasons for this are not clear, but may reflect differences in local immune responses in buccal and lingual mucosa.

In general, the communities from IBD patients exhibited greater variation. This could be seen from their distribution in NMDS plots and was confirmed by HOMOVA analysis which showed greater variation in IBD communities versus healthy communities. From the NMDS plots it can be seen that some IBD patients have communities which were indistinguishable from healthy children. We examined these data to determine if disease classification could clarify this distribution. Using the Paris classification scheme, L4 or L4a diagnosis (i.e. upper GI tract disease) did not separate the CD patients in NMDS plots. However, patients with a complex diagnosis of L3L4, where there is extensive disease throughout the GI tract, separated significantly from non-L3L4 patients and healthy children. Disease severity was also assessed, using Thetayc and Bray-Curtis calculators. Disease severity has been shown to influence species diversity of gut microbiota in CD children (Gevers *et al.* 2014). Our findings showed children classed as inactive were indistinguishable from healthy children but mild-moderate and severe classification were significantly different. Based on these analysis it appears that the extent and severity of IBD influences the tongue community structure.

Previous studies by Eun *et al.* (2016), demonstrated that intestinal microbial composition in patients with CD was effected by variable factors including disease location and behaviour, clinical disease activities and disease treatment. Another study reported that the dissimilarity between IBD stool and rectal microbiota increases with disease severity (Schirmer *et al.* 2018). Our findings could suggest that the factors responsible for impacting the gut microbiota could also affect the oral microbiota of IBD children,

indicating that children who are diagnosed with L3L4 disease or more severe symptoms have greater alterations in oral bacterial communities compared with other disease types. To confirm this further investigations should be done, including analysis of a larger number of patients with variable disease classification. There are many previous studies describing the diversity of gut the microbiota. These studies described the predominant phyla in the healthy human gut microbiome, including the Firmicutes and Bacteroidetes phyla (Eckburg *et al.* 2005, Manichanh *et al.* 2006, D'Argenio and Salvatore 2015, Eun *et al.* 2016). A recent article by Lucas Lopez *et al.* (2017) reported similar results with healthy subjects. Imbalance in gut microbial structures was associated with intestinal symptoms including bloating, abdominal pain, and diarrhoea (Zhang *et al.* 2015). Looking at inflammatory bowel disease (IBD), several studies illustrated changes in this microbiome structure with IBD, including a decrease in the abundance of Firmicutes and Bacteroidetes in the gut microbiota of IBD patients compared to healthy subjects (Cenit *et al.* 2014, Lucas Lopez *et al.* 2017). Another previous study comparing faecal and mucosal tissues of CD patients and HC subjects has reported that relative to HC subjects the abundance of Proteobacteria was increased in both faecal and mucosal tissues. Fusobacteria were increased in mucosal tissues and Actinobacteria were increased in the faecal samples of the CD patients (Eun *et al.* 2016). In our study, the dorsum of tongue and buccal mucosa were sampled from the three study groups (CD, UC, and HC), and 12 phyla were detected at both sites. Tongue samples from IBD patients showed increased levels of Firmicutes but decreased levels of Fusobacteria, Proteobacteria and Bacteroidetes. At buccal sites, phylum distribution was more even, but the Actinobacteria were significantly increased in UC and CD children relative to healthy children. Other previous studies were carried out to examine the oral microbiota in the saliva of IBD patients (Said *et al.* 2014, Xun *et al.* 2018). The first of these studies by Said *et al.* (2014)

showed that Proteobacteria was the only phylum significantly reduced in the salivary microbiota of CD patients. The other study by Xun *et al.* (2018) reported variations in the levels of these phyla in CD (n=13) and UC (n=54) patients, with UC patients exhibiting reduced Bacteroidetes and increased Actinobacteria and CD patients showing reduced Bacteroidetes and Proteobacteria. The phyla level differences in these studies appear to be less severe compared to our study. This may be due to the use of saliva, which represents a sample of all shedding surfaces of the mouth. Our study, which shows differences between tongue and buccal sites, indicates that specific sites may have specific dysbiosis that could be missed by sampling saliva. Additionally, as mentioned previousy, patients in these studies tended to be older and receiving medication. Further investigation are required to expand a better understanding of these differences.

In the present study a total of 133 and 174 bacterial genera were identified in tongue and buccal sites, representing 1617 and 1422 OTUs at each site respectively. A total of 107 and 115 genera were identified in the salivary microbiota by Said *et al.* (2014) and Xun *et al.* (2018), respectively. Our data shows the 10 most abundant genera detected in tongue samples were similar to the buccal genera, although their relative abundances were different at each site. At the OTU level, *Rothia mucilaginosa* OTU0001 was the most abundant OTU in tongue samples and *S. mitis* OTU0001 was most abundant in buccal samples. In order to determine which species and/or OTUs were responsibe for the changes in phyla distribution and community structure, we used the LEfSe test to compare communities from healthy children and children with IBD. In healthy children, this analysis revealed that an extensive number of species and OTUs were found at higher levels in healthy samples. This included several major OTUs of high prevalence in the oral cavity including *Neisseria perflava* OTU0003, *Prevotella* sp., OTU0004 and *Veillonella* sp. OTU005. In children with IBD, loss of these taxa in tongue samples

appears to be associated with increased levels of streptococci, including the major streptococcal taxon, *S. mitis* (OTU0001). OTUs of *Gemella* sp. (OTU0021) and *Neisseria* sp. (OTU0020) were also at greater levels. It was difficult to identify enrichments specific to CD or UC patients, however CD patients tended to harbour higher levels of *Capnocytophaga* sp. and *Actinomyces lignae* OTU0017. The reduced levels of so many taxa in the tongue samples of CD patients supports the overall findings that these patients exhibit reduced species richness and biodiversity compared to healthy children.

Analysis of the buccal data show that dysbiosis is site specific. IBD children generally exhibited greater levels of Actinobacteria, and UC patients exhbited higher levels of *Prevotella* sp. CD patients exhibited some unique changes including reduced levels of *Viellonella* spp., similar to that observed in tongue samples. Overall, analysis of community structure and content indicates that levels of dysbiosis are more severe in tongue samples compared to buccal samples. Recent studies of the gut microbiota in IBD have shown these patients exhibit reduced levels of *Bacteroidales*, *Clostridiales* and *Bifidobacteriaceae* (Gevers *et al*. 2014). In addition to that, many of the enrichments identified in the gut flora of these patients appear to be taxa that have originated from the oral cavity. This includes members of the *Pasteurellaceae* (*H. parinfluenza*), *Neisseriaceae* (*Eikenella corrodens*), *Fusobacteriaceae* (*F. nucleatum*), *Veillonellaceae (V. parvula, V. dispar)* and *Gemellacea* (*G. moribillum*). It is noticable from this list that these organsims are similar to the taxa we have identified in the current study as reduced in abundance in the oral cavties of IBD patients. This raises the possibility that the changes in the distribution of these taxa in the oral and gut environments may be somehow linked and may play a role in the pathogenesis of IBD. Recent studies have shown microorganisms from the saliva of children with CD are highly inflammatory when introduced to the GI tract of susceptible mice. Atarashi *et al.* (2017) showed that

the oral microbiome of children with IBD could induce inflammation in a murine model of IBD and the most inflammatory organism from these oral samples was the enterobacteria *Klebsiella pneumonia*. Our data shows that patients with a severe IBD diagnosis are more likely to harbour *Enterobacteriaceae* in the oral cavity (OTU0218), indicating the the oral cavity may be a reservoir for these bacteria.

3.5 Conclusion.

In conclusion, most studies that have investigated dysbiosis in the gut microbiota of IBD patients indicate alterations in the bacterial community of the gut flora. These include shifts in the microbial structure from the normal human gut microbiota, particularly changes at phyla level. On the other hand, few studies have assessed the diversity of the oral microbiota among those group of patients suffering from IBD. Previous studies of the oral microbiome in IBD involved patients already enrolled to receive therapeutic medications for IBD (i.e. not treatment naïve). Furthermore, the previous studies examined for changes in the salivary microbiota in IBD patients (Said *et al.* 2014, Xun *et al.* 2018), while the only previous study to examine the tongue and buccal site utilised DNA microarrays (Docktor *et al.* 2012). In the present study the IBD children were treatment-naïve and the dorsum of tongue and the buccal mucosa were sampled before and after receiving their treatment. These differences between our study and previous observations could explain the dissimilarities between our finding and the results from previous studies. Overall, the diversity of the oral microbiome of CD children tended to decrease in both tongue and buccal samples compared to HC group. In comparison to healthy control children, Crohn's disease patients had the lowest OTUs numbers (i.e. reduced species richness). In general, healthy buccal and tongue samples had greater species richness compared to CDT and CDB samples. Our findings demonstrated the significant relation between L3L4 disease type and severity IBD children. We theorize that alterations in oral microbial composition are impacted by disease severity and location in the GI tract. Furthermore, the relationship between alterations of the gut and oral microbiota could be a useful diagnostic marker and indicator of disease severity. To evaluate these hypotheses further, a larger cohort may be required and dual oral and gut sampling to determine if oral and gut microbiome changes correlate in the same patient.

Chapter 4

Healthy oral mucosal mycobiome

Chapter 4 Healthy oral mucosal mycobiome

4.1 Introduction.

There are many studies on oral bacterial communities in health and disease. However, few studies have specifically looked at the mycobime of the oral cavity in healthy individuals. The oral mycobiome community in healthy subjects was first characterised by next-generation sequencing in 2010 (Ghannoum *et al*. 2010). Subsequent studies have investigated the oral mycobiome in association with other diseases (oral and systemic), most notably the oral mycobiome in HIV patients (Mukherjee *et al.* 2014, Mukherjee *et al.* 2018). The correlation between oral disease and oral fungi has also been investigated, for instance characterization of oral mycobiome in relation to periodontitis (Peters *et al.* 2017). A recent study comparing the oral mycobiome in children with and without dental caries (Fechney *et al.* 2019). Further studies have investigated the fungal populations on the tongue of institutionalized elderly individuals Li *et al.* (2012) and the oral mycobiome of healthy elderly Japanese subjects (Zakaria *et al.* 2017). Dupuy *et al*. (2014) redefined the composition of the healthy mycobiome revealing the presence of *Malasezzia* spp. as a significant component of the oral mycobiome.

The methodology and techniques of oral fungal characterization often differ from one study to another. For amplicon based studies, the ITS region is the most commonly amplified region of fungal DNA, including the ITS1 and ITS2 regions, in addition to the 18S and 5.8S rDNA regions (Figure 4.1), with several methods of sequencing and numerous sampling methods (Table 4-1). However, the components of the core mycobiome proposed by previous studies are often dissimilar between studies, raising many questions about these different approaches. To obtain a comprehensive profile for the oral mycobiome in children in the present study, we investigated the significance of

the amplified region (ITS1 or ITS2) and the anatomical regions sampled (buccal or tongue) in identifying the core mycobiome of healthy individuals.

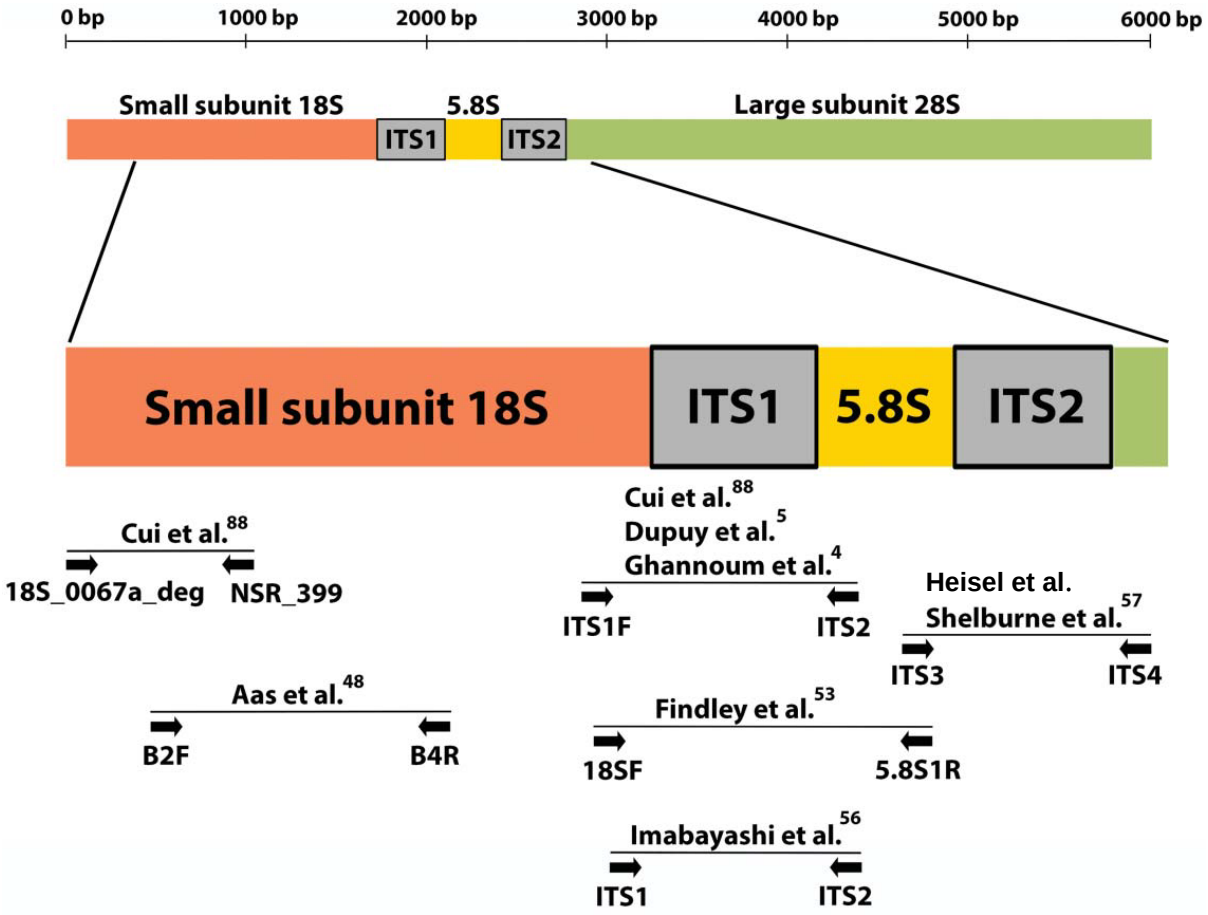


Figure 4.1. Map of the ITS rDNA region and primer sets used in oral mycobiome studies (Diaz *et al*. 2016).

We utilized Next Generation sequencing (NGS) of the primary fungal DNA barcode ITS2, which was found to be a more accurate and sensitive method for studying the mycobiome (stool samples) (Nash *et al.* 2017). In addition to that, to determine whether

ITS2 enables identification of the majority of the mycobiome taxa, the ITS1 region was also amplified from selected samples.

Table 4-1 Previous studies of oral mycobiome characterization in health individuals, showing study population number, sampling method, amplified region of fungal DNA, and sequencing method.

Study	**Study population**	**Sampling method**	**Amplified region**	**Sequencing method**
Ghannoum *et al.* (2010)	20	Oral rinse	ITS1 region	Multitag Pyrosequencing (MTPS)
Li *et al.* (2012)	291	Tongue dorsum	ITS regions	PCR-based molecular techniques
Dupuy *et al.* (2014)	6	Saliva	ITS1, 18S	Pyrosequencing
Zakaria *et al.* (2017)	410	Saliva	ITS1-5.8S-rDNA-ITS2 region	PCR-based molecular techniques
Fechney *et al.* (2019)	17	Dental plaque	ITS2 region	Illumina MiSeq

4.2 Data processing of ITS1and ITS2 region of HC subjects.

In the present study, the ITS2 barcode region was amplified from tongue and buccal samples of healthy individuals and sequenced on the MiSeq using the 500 cycle kit (2 x 250 bp reads). Furthermore, the ITS1 barcode was also amplified from 11 randomly selected samples from the healthy subjects. These procedures were carried out for both tongue and buccal samples individually. DADA2 was used as our main sequence processing tool. Additionally, the Mothur pipeline was used for initial sequence classification following processing in DADA2.

4.2.1. DADA2 pipeline processing workflow for ITS2 sequencing data.

DADA2 pipeline was used to analyse ITS2 sequences from healthy samples. The workflow used here was obtained from the DADA2 pipeline tutorial 1.8 (https://benjjneb.github.io/dada2/tutorial.html). In these analyses, the quality profiles of the forward and reverse reads were visually inspected by examining plots of the quality scores for each nucleotide base. Reads were trimmed to remove bases with quality scores <30. The forward reads were truncated at position 240, while reverse reads at position 190. After this, the sequencing error rates were estimated, and a model of the sequencing error rates in the data set was generated. A set of unique sequences was then generated by combining all identical sequencing reads (Dereplication). The sequencing error model was then applied to the data set, resulting in the correction of spurious sequences and estimation of the true number of sequence variants in the samples, and paired forward and reverse reads were than merged to construct full length sequences. A table of all samples and sequences was constructed, resulting in 3,011 total sequences in healthy

samples. Finally, chimeras were removed from the merged sequences. We identified 598 chimeras out of 3011 unique sequences variants in tongue and buccal samples (<4% total sequences).

4.2.2. DADA2 pipeline processing workflow for ITS1sequencing data.

DADA2 processing of ITS1 sequencing data was carried out in an identical fashion to ITS2 processing, resulting in construction of a sequence table with 2,422 total sequences. A total of 483 chimeras were identified out of 2,422 unique sequence variants in 11 healthy samples (<4% total sequences).

4.2.3. Sequence classification in Mothur pipeline.

Unique sequences and their abundances were exported to Mothur. The non-fungal sequences were removed by filtering out any sequences with <80% sequence similarity to the fungal ITS1 and ITS2 region. The remaining ITS sequences were classified to species level in Mothur using classify.seqs, resulting in 300 fungal species. Further classification of the 30 most abundant fungal sequences was carried out by BLAST analysis using the ISHAM (International Society for Human and Animal Mycology) Barcoding Database. For statistical analysis, data sets were normalized by subsampling to 5000 sequences. Following sequencing data processing, analysis of alpha and beta diversity was carried out as described in chapter 2, section 2.5.

4.3 Results.

Tongue and buccal swabs were obtained from healthy individuals after informed consent was provided by the children's parents. Data from 37 healthy subjects were analysed, the three age groups, including Children (n=20), Adolescent (n=8), and Adult (n=9). The average age of the participants was 14.45 years. Additionally, ITS1 regions were amplified to characterize the oral fungal microbiome in healthy subjects.

4.3.1 Tongue and buccal mycobiota in healthy individuals.

The mycobiome of tongue and buccal samples were analysed and investigated at different levels, including unique sequence and genus level.

4.3.1.1 Analysis of unique sequences in the tongue and buccal mycobiome.

Unique sequences first were analysed without any clustering into OTUs or species phylotypes. In total, 603 unique sequences were identified. The identities of the 30 most abundant sequences were confirmed by BLAST searches of the ISHAM barcoding database, which resulted in classification to the species level. In the case of sequence 21, two identical matches were found, namely *Candida xylopsoci* and *Pichia krudiavezii* (the teleomorph of *C. krusei*). As *C. xylopsoci* is not a human commensal, we have referred to this sequence as *C. krusei* in subsequent sections. The five most dominant fungi were ascomycetous yeasts including, *Candida dubliniensis* (seq1), *Candida albicans* (seq2; seq5; seq32), *Didymella gardenia* (seq3), *Saccharomyces cerevisiae* (seq4; seq12; seq 30; seq35) and *Candida zeylanoides* (seq6) Figure 4.2 (a, b). It can be seen that *C. albicans* and *S. cerevisiae* are represented by multiple sequence types, indicating the

presence of different clades or possibly subspecies in the population. We compared the mycobiome profiles of the participants based on age, grouping them into three age groups including children (5-12), Adolescent (13-17) and Adult (18+). Our data showed the healthy children, adolescents and adults had different mycobiome profiles. Children were highly enriched with *C. dubliniensis* compared to the adult group. On the other hand, *C. albicans* (seq2), *Didymella gardenia* (seq3), *Epicoccum nigrum* (seq8 and. seq11), *Candida krusei* (seq21) and *Alternaria infectoria* (seq61) were more dominant in profiles from adult healthy individuals in both tongue samples (Figure 4.2a), and buccal samples (Figure 4.2b).

4.3.1.2 Alpha diversity measurements.

These measurements were carried out to measure species richness within healthy tongue and buccal mucosa mycobiome environment, including the inverse Simpson index, Chao index and rarefaction curve analysis.

4.3.1.2.1 Inverse Simpson Index.

The Inverse Simpson and Chao1 indices were used to calculate healthy mycobiome biodiversity and richness respectively (Table 4-2). The Inverse Simpson index data showed that the buccal mycobiome in healthy individuals (HCB) exhibited reduced biodiversity compared to the tongue mycobiome (HCT). However, the results from the Chao1 measure of species richness indicated that HCB mycobiota exhibited greater richness compared to HCT group.

The differences between age groups of healthy individuals in mycobiome biodiversity and species richness were also compared. Our data showed that the mycobiome biodiversity indicated by the inverse Simpson index was greater in the adult group compared to the child and adolescent groups. On the other hand, richness (Chao1) was similar in children and adult fungal communities (Table 4-3). When we compared alpha diversity by gender, the males exhibited marginally greater mycobiome richness, however biodiversity was similar in males and females (Table 4-4).

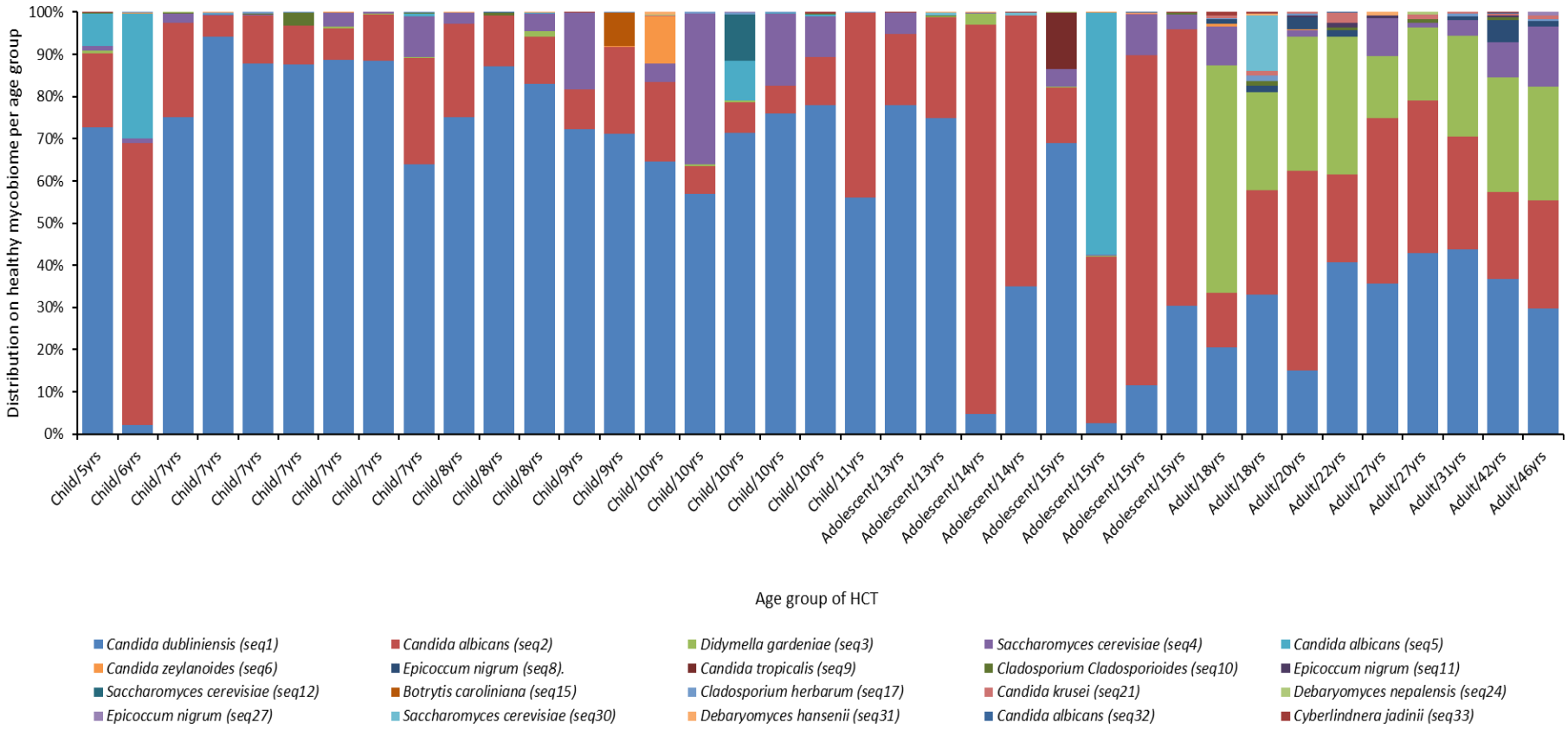


Figure 4.2a Distribution of the 20 most abundant fungal sequences identified by ITS2 sequencing by age groups of healthy controls tongue (HCT).

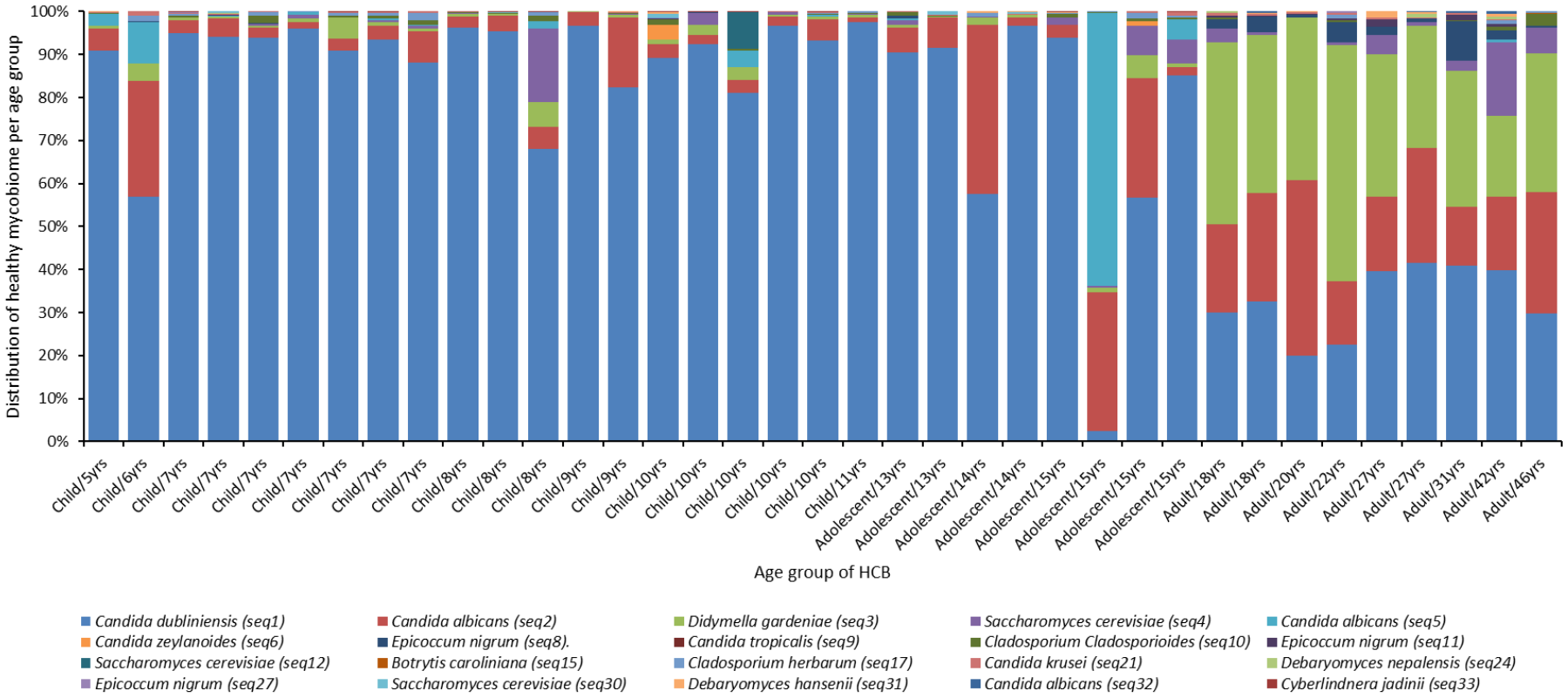


Figure 4.3b Distribution of the 20 most abundant fungal sequences identified by ITS2 sequencing by age groups of healthy controls buccal (HCB)

Table 4-2 The minimum and maximum numbers of fungal sequences (seq) identified in healthy tongue and buccal samples and corresponding Inverse Simpson and Chao1 statistics.

Site	Average seq no.	Chao1	Inverse Simpson	Minimum Seq number	Maximum Seq number
Tongue	14.63	16.99	2.10	5.63	44.73
Buccal	16.82	18.04	1.88	6.52	32.70

Table 4-3 The minimum and maximum numbers of fungal sequences (seq) in the three age group categories and corresponding Inverse Simpson and Chao1 statistics.

Age Group	Average seq no.	Chao1	Inverse Simpson	Minimum Seq number	Maximum Seq number
Child	15.48	18.08	1.49	6.52	30.76
Adolescent	14.32	16.29	1.72	5.63	32.70
Adult	17.55	17.73	3.43	8.77	44.73

Table 4-4 The minimum and maximum numbers of fungal sequences (seq) in Male and Female healthy individuals, and inverse Simpson and Chao1 statistics.

Gender	Average seq no.	Chao1	Inverse Simpson	Minimum Seq number	Maximum Seq number
Female	14.50	15.65	1.96	5.63	30.76
Male	16.59	16.59	2.04	6	44.73

4.3.1.2.2 Rarefaction curve.

The rarefaction curve was used to determine mycobiome species richness in healthy samples. Tongue and buccal sample data showed that buccal samples exhibited greater species richness compared to tongue samples (Figure 4.3).

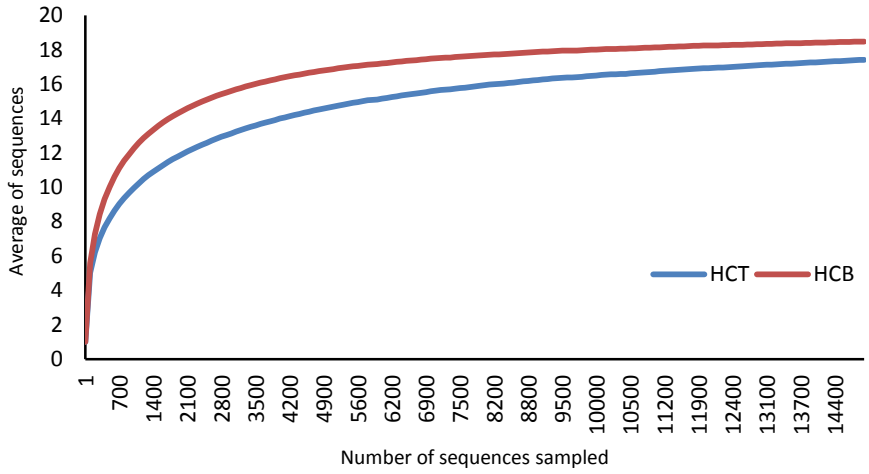


Figure 4.4 Rarefaction curve showing mycobiome species richness of healthy tongue samples (Blue, HCT) and buccal samples (HCB, red).

The species richness of the mycobiome was also compared in the three age groups. Greater species richness was observed in the in adult healthy group, whereas the adolescent group had the lowest species richness (Figure 4.4). We also compared the species richness between male and female participants and found that males exhibited greater species richness compared to females (Figure 4.5).

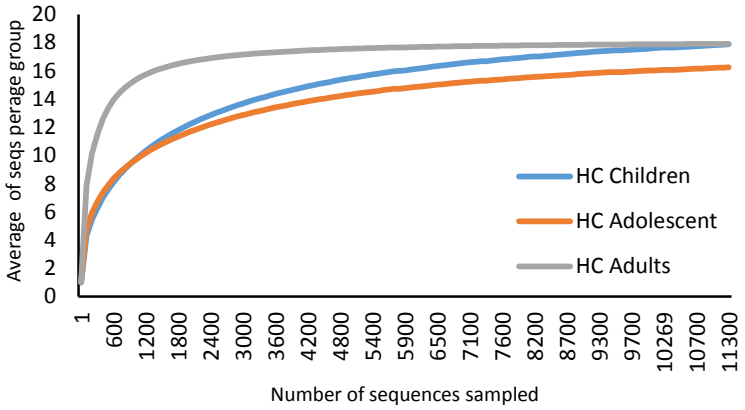


Figure 4.5 Rarefaction curve showing mycobiome species richness in children, adolescents and adults.

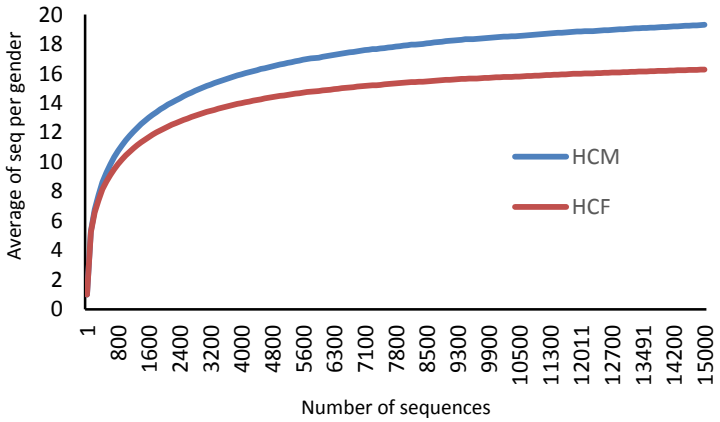


Figure 4.6 Rarefaction curve showing mycobiome species richness of male (HCM), and female (HCF) groups.

4.3.1.3 Beta diversity measurements.

Diversity in healthy mycobiome community structure was measured using Non-metric Multidimensional Scaling (NMDS).

4.3.1.3.1 Non-metric Multidimensional Scaling (NMDS).

NMDS was used to graphically represent the distance between the tongue and buccal samples of healthy controls. Distances were calculated using the Yue and Clayton (Thetayc), Jaccard (Jclass) and Bray-Curtis calculators. Analysis of Molecular Variance (AMOVA) was carried out to determine if the samples separation in the NMDS plot is significant (Figure 4.6 a,b,c).

Using the three measurements (Thetayc, Jaccard, and Bray-Curtis), AMOVA analysis of showed a significant difference in mycobiome community structure between tongue and buccal samples of healthy subjects ($P < 0.001$), (Table 4-5).

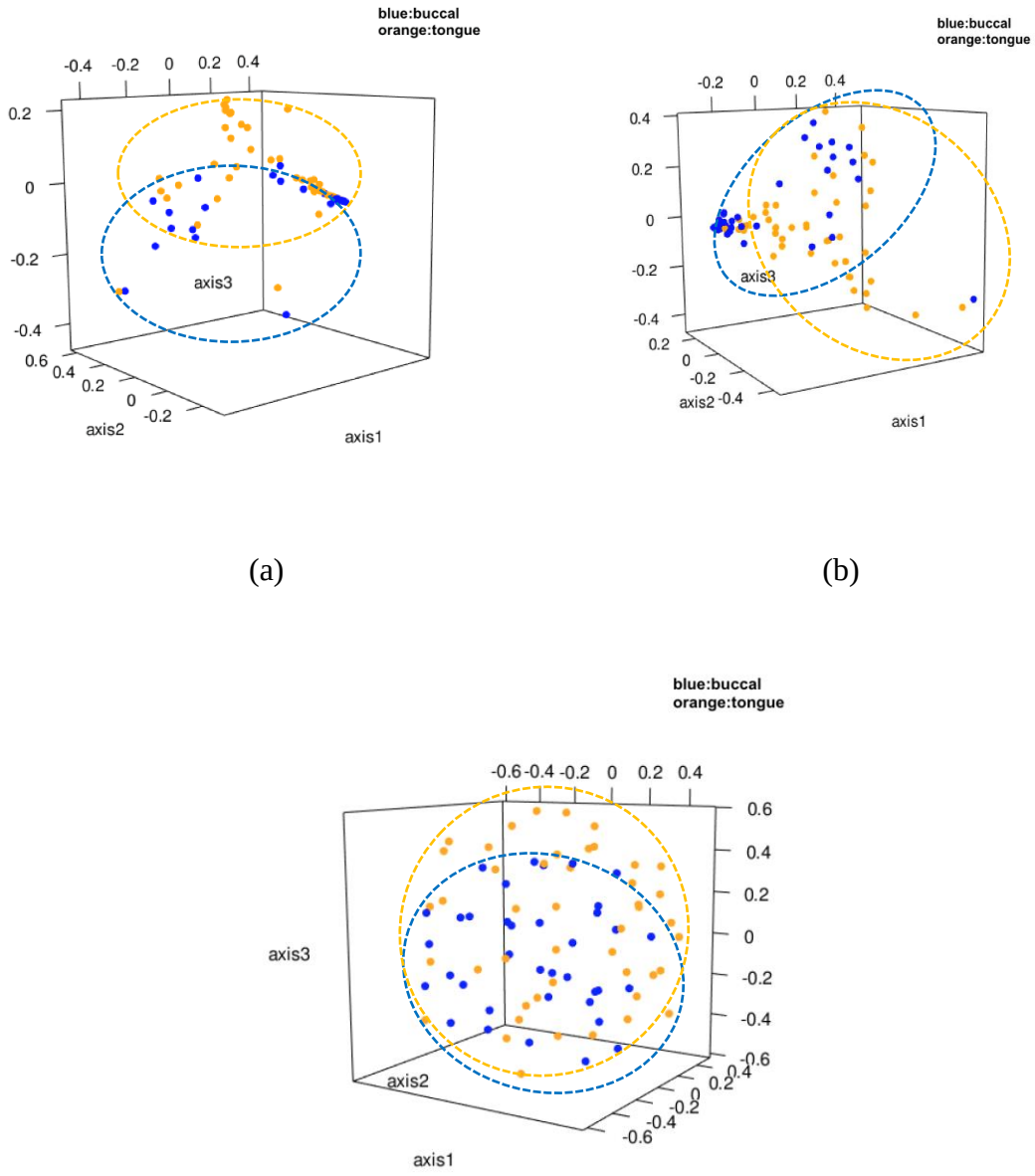


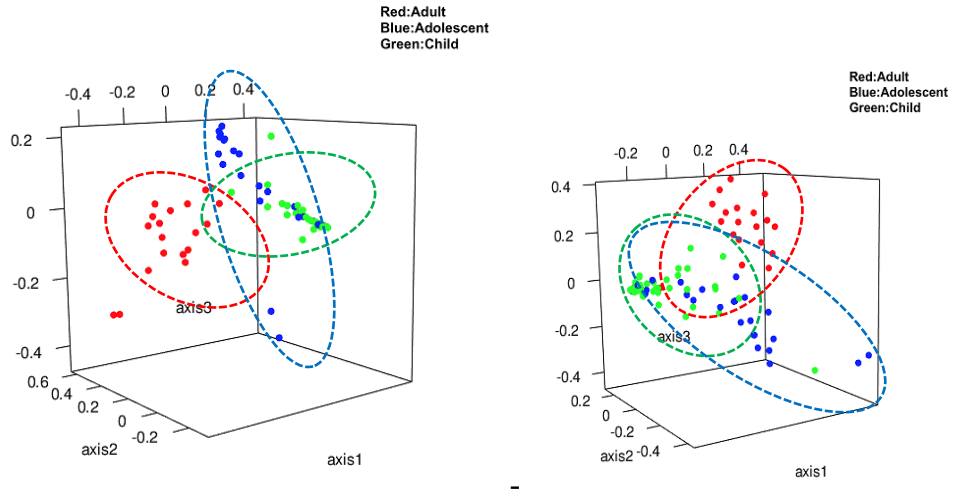
Figure 4.7 NMDS plots of tongue and buccal samples generated with the Thetayc (a), Bray-Curtis (b) and Jclass calculators (c), showing tongue (blue), and buccal (orange) samples. Blue and orange circles are used to show healthy and IBD groups and are for visualisation purposes only.

4.3.1.4 Analysis of age group and mycobiome structure.

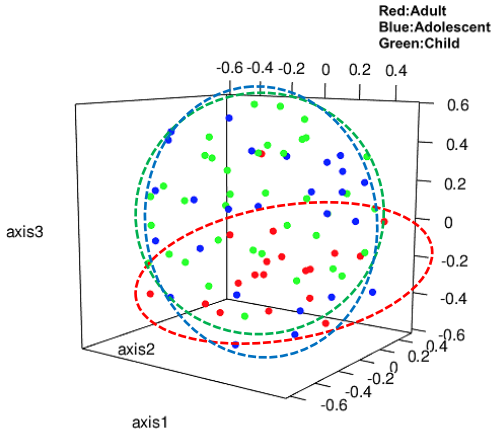
Analysis of Molecular Variance (AMOVA) was carried out to determine if age group (Child. Adolescent, Adults) had a significant effect on community structure (Figure 4.7 a,b,c). Using the three measurements (Thetayc, Jaccard, and Bray-Curtis calculators), AMOVA analysis of age group showed significant differences in mycobiome community structure between children, adolescent and adults ($P < 0.001$). Only the Jaccard based AMOVA test comparing children and adolescents failed to identify a significant difference (Table 4-5).

4.3.1.5 Analysis of gender and mycobiome structure.

Separation of mycobiome communities based on gender in the NMDS plot was determined using AMOVA (Figure 4.8 a,b,c). In all of the three measurements (Thetayc, Jaccard, and Bray-Curtis), AMOVA analysis of male and female groups of healthy individuals showed no difference in mycobiome community structure between male and female groups of healthy subjects. (Table 4-5).



(a) (b)



(c)

Figure 4.8 NMDS plots of mycobiome communities generated with the Thetayc (a), Bray-Curtis (b), and Jclass calculators (c), showing children (green), adolescents (blue), and adults (red) communities. Red, blue and green circles are used to show healthy and IBD groups and are for visualisation purposes only.

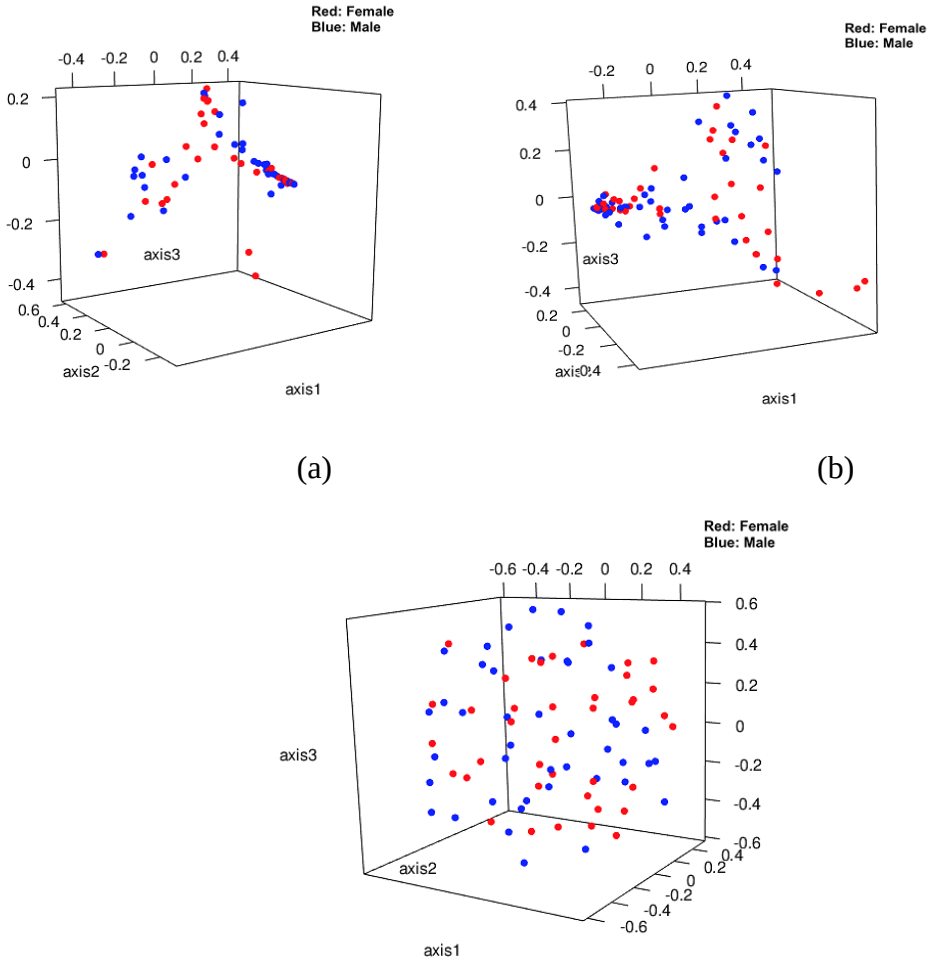


Figure 4.9 NMDS plots of mycobiome communities generated with the Thetayc (a), Bray-Curtis (b), and Jclass calculators (c), showing male (blue) female (red) communities.

Table 4-5 P-value results from AMOVA tests comparing mycobiome communities in tongue and buccal samples, age groups and gender of subjects, using Thetayc, Jclass, and Bray-Curtis calculators.

Comparison	**Thetayc AMOVA P value**	**Bray-Curtis AMOVA P value**	**Jclass AMOVA P value**
Buccal vs tongue	P <0.001**	P <0.001**	P <0.001**
Adolescent vs Adult	P <0.001**	P <0.001**	P <0.001**
Adolescent vs Child	P <0.001**	P <0.001**	P =0.363
Adult vs Child	P <0.001**	P <0.001**	P <0.001**
Male vs Female	P =0.162	P =0.233	P =0.334

4.3.1.6 LEfSe analysis of tongue and buccal healthy mycobiota.

To identify species which may be significantly enriched in different niches, LEfSe analysis was carried out comparing the buccal and tongue samples of healthy subjects. Tongue site was enriched in *C. albicans* (seq2). On the other hand, buccal samples were enriched in different with members of the Davidiellaceae and the Pleosporales, specifically Seq3 (*Didymella gardeniae*) and Seq10 (*Cladospirium cladisporioides*) (Figure 4.9). The phylogenetic distribution of the differences between tongue and buccal niches were examined, showing that the enriched species in buccal site cluster in the Dothideomycetes, whereas the tongue site was enriched in Saccharomycetes (Figure 4.10).

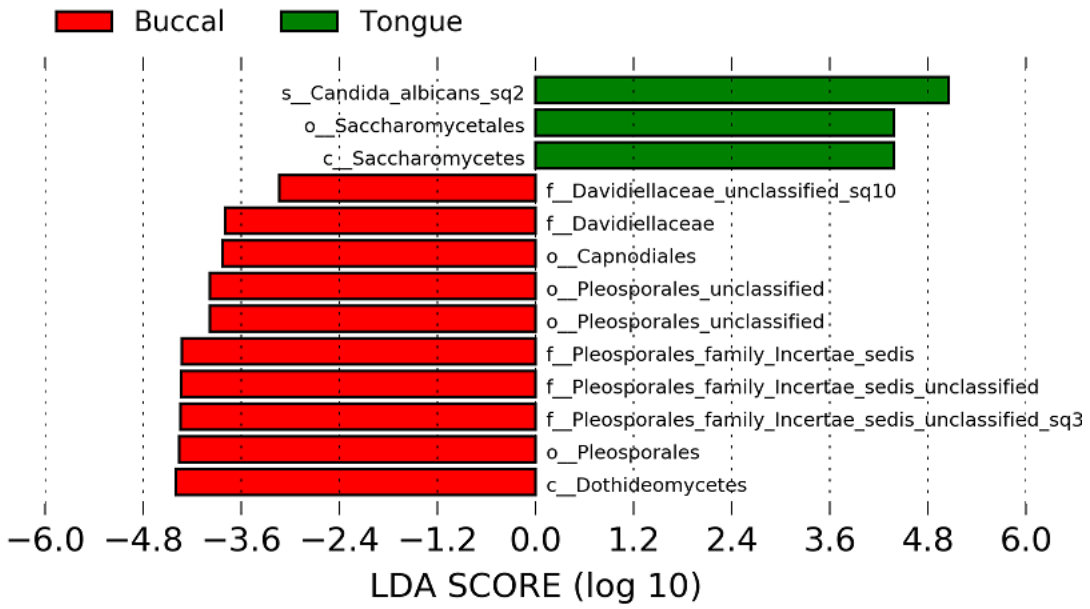


Figure 4.10 LEfSe plot showing the differential enrichment of fungal species in tongue and buccal samples from healthy control group

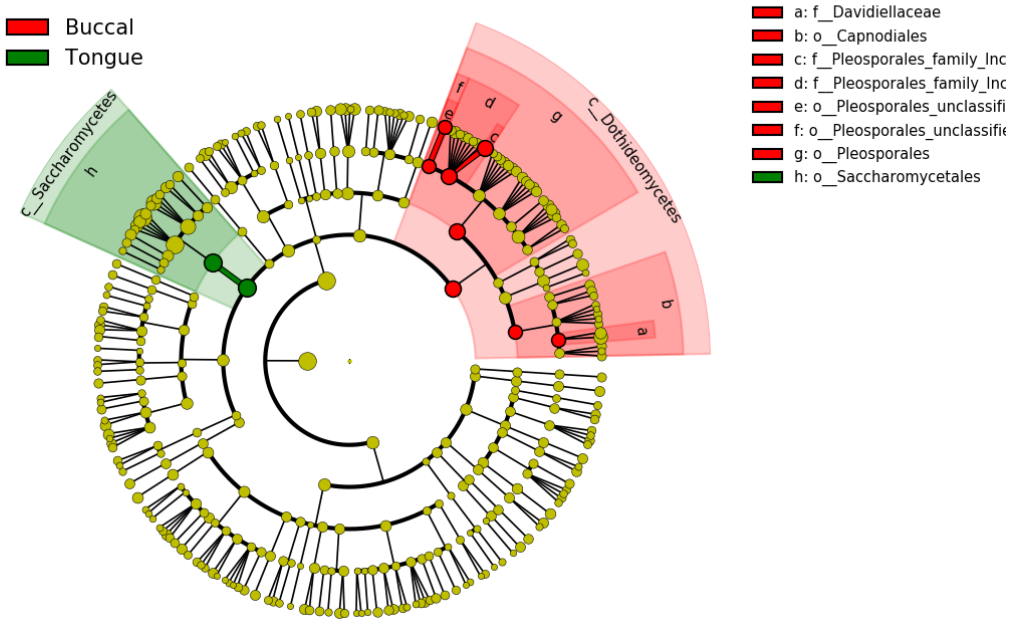


Figure 4.11 Phylogenetic distribution of fungal species enriched in tongue and buccal samples.

4.3.1.7 LEfSe analysis of age groups.

Comparison of the three age groups (child, adolescent and adult) showed that the mycobiota of children was enriched with *C. dubliniensis* (seq1), whereas adults showed enrichment with *Didymella gardenia* (seq3), *Epicoccum nigrum* (seq8, seq11) and *Candida krusei* (Seq21) (Figure 4.11). Adults were also found to harbour higher levels of Basidiomycota. Adolescents appeared to harbour higher levels of *C. albicans* (seq2) and overall higher levels of the genus *Candida*. The phylogenetic distribution of the differences between the age groups of healthy subjects was examined, showing that the enriched species in adults cluster in the Dothideomycetes, whereas children were enriched in Saccharomycetes (Figure 4.12).

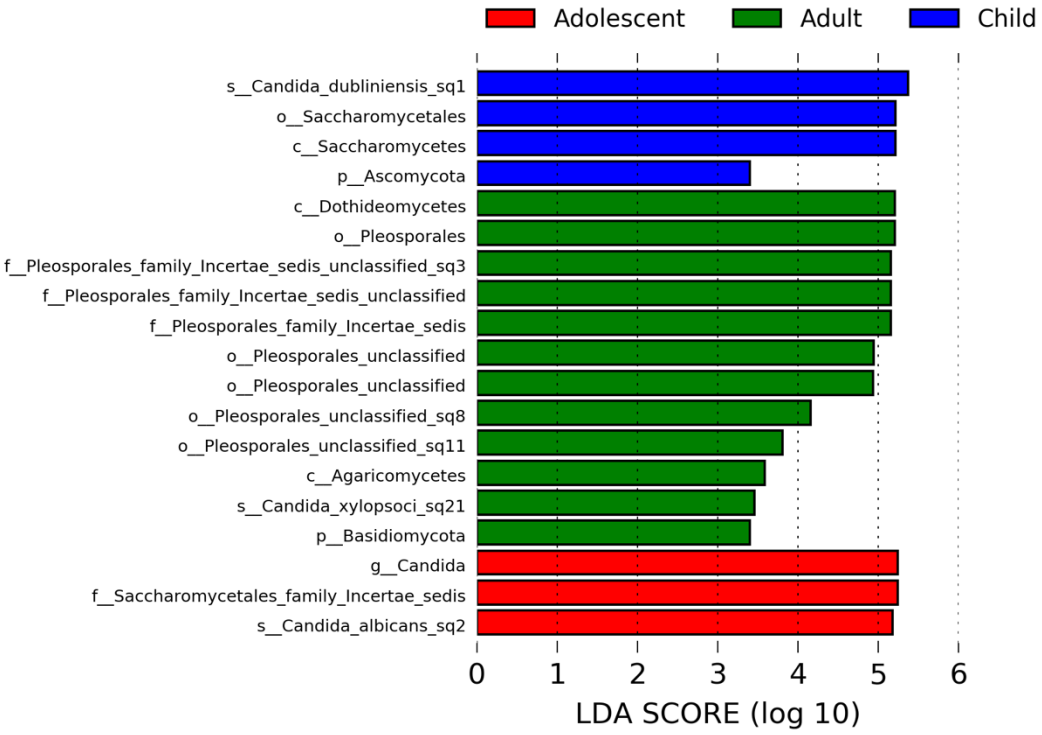


Figure 4.12 LEfSe plot showing the differential enrichment of fungal species in the three age groups of the healthy controls

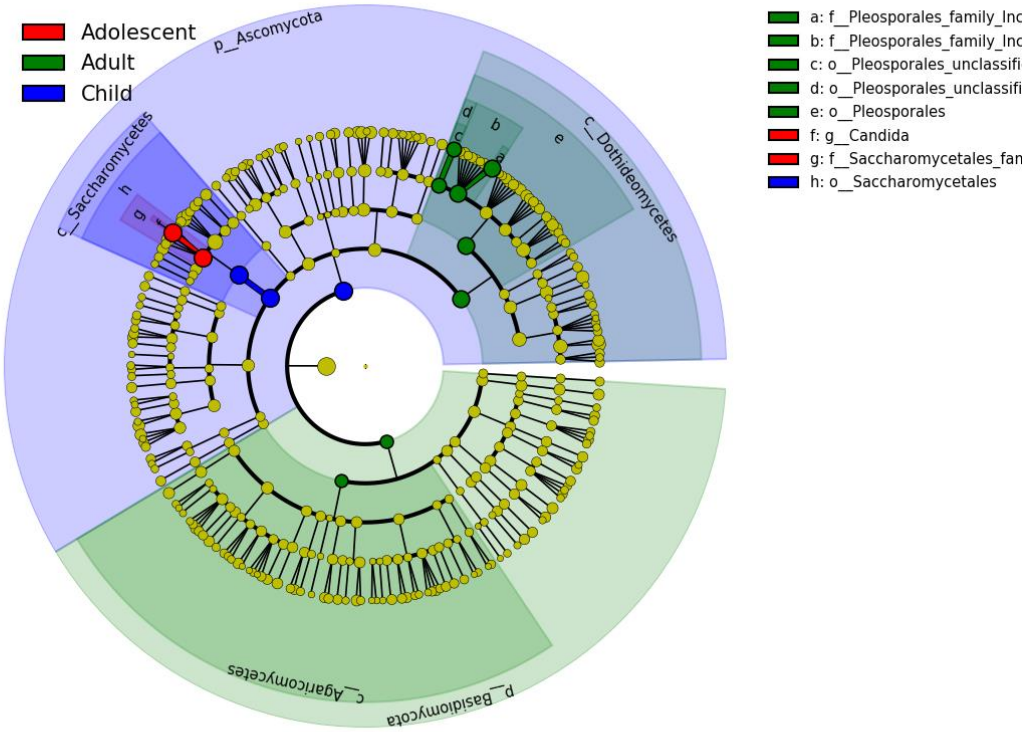
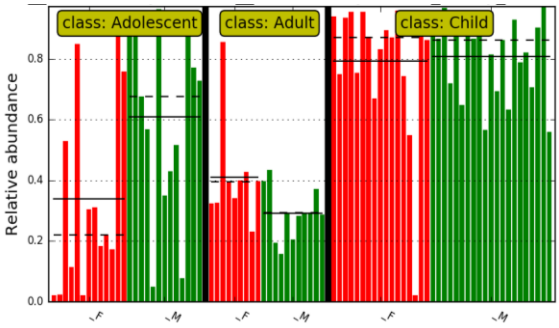


Figure 4.13 Phylogenetic distribution of fungal species enriched in the three age groups of HC subjects.

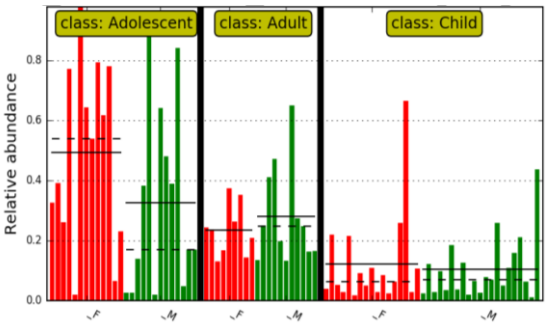
4.3.1.8 Abundant mycobiome taxon in different age groups.

Graphs showing the abundance of significantly enriched fungal species in the mycobiota of the subjects (child, adolescent and adult) were generated using the LEfSe package (Hutlab Galaxy server) (Figure 4.13).

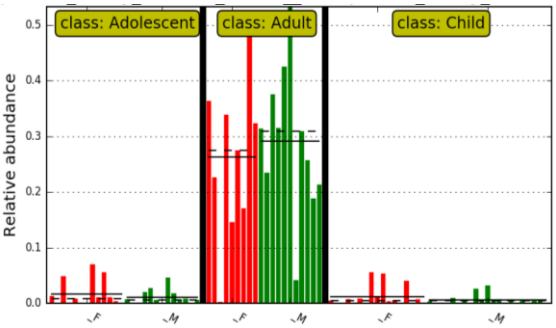
Candida dubliniensis (seq1)



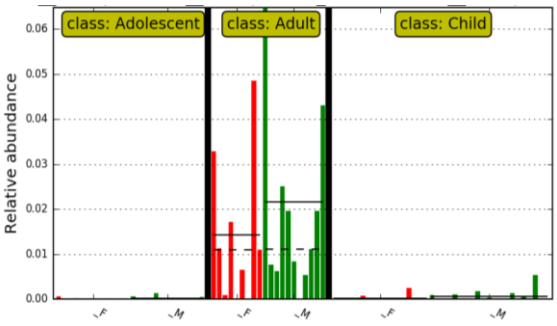
Candida albicans (Seq2)



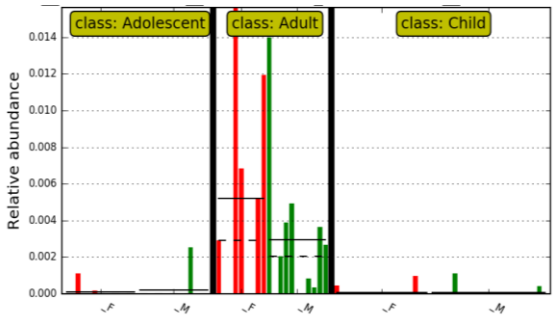
Didymella gardenia (seq3).



Epicoccum nigrum (seq8)



Epicoccum nigrum (seq8)



Candida krusei (Seq21)

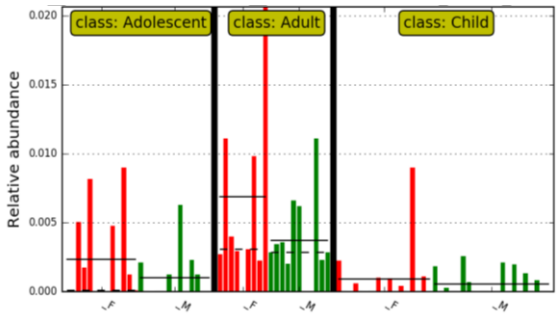


Figure 4.14 Plots showing the abundance of fungal taxa identified in LEfSe analysis of healthy tongue and buccal samples. Mean values are represented by the solid line, median values by the dotted line.

4.3.2 Characterization of tongue and buccal mycobiota in healthy individuals by sequence analysis of the ITS1 region.

The ITS1 region from the DNA samples of 11 healthy individuals was amplified, using specific primers for this region. The forward primer ITS1F CTTGGTGATTTAGAGGAAGTAA, and reverse primer ITS2R GCTTGCGTTCTTCATCGATGC described by Ghannoum *et al*. (2010) were employed. The reactions were carried out in an identical fashion to ITS2 region amplifications as described in chapter 2, section 2.3.2. The resulting data were also processed using DADA2. Analysis of the data from ITS1 sequences showed that healthy control samples were highly enriched in *C. dubliniensis* (seq1), whereas *C. albicans* (seq5) was less abundant. In addition, other mycobiome species were found at high abundance, especially in samples from adolescents including *Rhodotorula mucilaginosa* (seq2), *Fusarium oxysporum* (seq3), *Alternaria alternate* (seq4), *Malassezia restrica* (seq6, seq10, seq17), *Malassezia globosa* (seq8), and *Cladosporium herbarum* (seq12) (Figure 4.14). Interestingly, data from ITS2 sequencing contained lower levels of the Basidiomycetous yeasts *Malassezia* and *Rhodotorula*, which were undistinguished in ITS2 sequencing of the same healthy group.

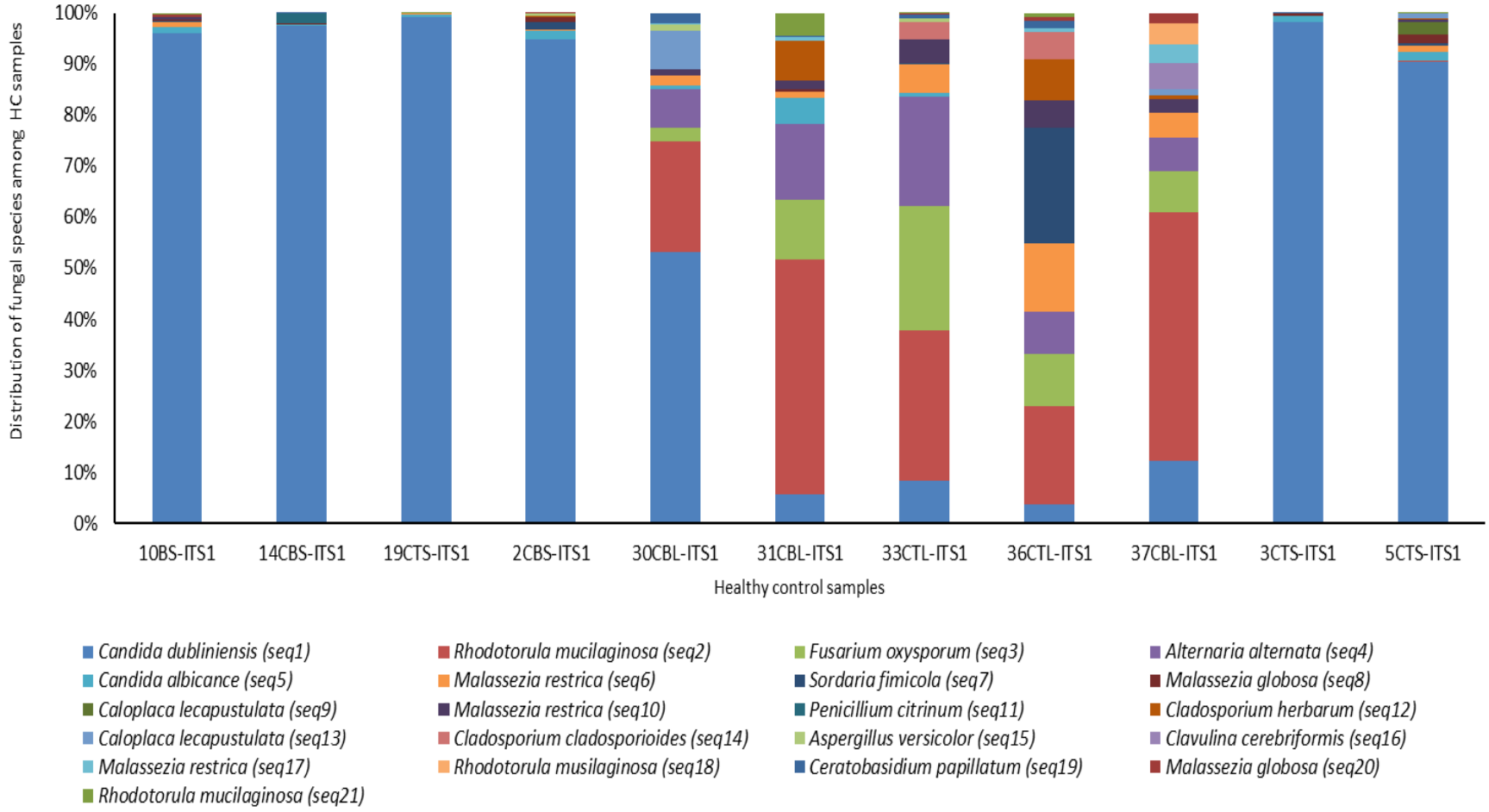


Figure 4.15 Distribution of the most abundant fungal sequences identified by ITS1 sequencing of some samples from healthy controls (HCT and HCB).

4.4 Discussion.

In the current cohort study of tongue and buccal samples, we identified 603 unique ITS2 sequences in the oral cavity of healthy individuals, corresponding a total of 300 species in all study participants (healthy subjects). The oral mycobiome is still poorly characterised in healthy individuals (Bandara, Panduwawala and Samaranayake, 2018). In this section of the present study, we focused on investigation of the oral mycobiome in healthy children, adolescent and adult individuals. Interestingly, *C. albicans* was not the only dominant oral mycobiome species in healthy individuals. Other fungal species were identified in our samples, namely *C. dubliniensis*, *Didymella gardenia*, *S. cerevisiae*, *C. zeylanoides*, *C. tropicalis*, *C. krusei*, *Epicoccum nigrum*, *Cladosporium* spp., *Botrytis caroliniana* and *Debaryomyces* spp amongst others. There are four previous studies evaluating the oral mycobiome in healthy individuals, including Ghannoum *et al.* (2010), Dupuy *et al.* (2014), Li *et al.* (2012), and Zakaria *et al.* (2017). Our findings were somewhat dissimilar to the oral mycobiome described in the first two studies. In the first study by Ghannoum *et al*. (2010), oral rinse samples were obtained from 20 healthy individuals, the ITS1 region was amplified and pyrosequencing was conducted. Ghannoum and colleagues, who for the first time described the basal oral mycobiome of healthy subjects identified 13 components in the basal healthy mycobiome, namely *Candida*, *Cladosporium*, *Aureobasidium*, *Saccharomycetales*, *Dothioraceae*, *Teratosphaeria*, *Alternaria*, *Aspergillus*, *Fusarium*, *Saccharomyces*, *Eurotium*, *Glomus*, and *Cryptococcus.* In the current study, only 2 fungal taxa, *Eurotium* and *Glomus*, were not detected using ITS2. In a second study by Dupuy *et al*. (2014), the oral mycobiome was surveyed using high throughput sequencing (pyrosequencing) of internal transcribed spacer 1 (ITS1) amplicons from saliva. This study detected 7 additional taxa not identified by Ghannoum *et al*. (2010), including, *Malassezia*, *Epicoccum*, *Irpex*,

Cytospora/Vaisa, Phoma, Lenzites, and Sporobolomyces. Six components from this latter study (*Aspergillus, Cryptococcus, Malassezia, Irpex, Cytospora,* and *Lenzites*) were absent from the highly abundant fungi identified in the ITS2 component of the current study (although were present at low abundance). In contrast, using ITS2 sequencing we observed another 8 species of fungi (*Candida dubliniensis*, *Candida zeylanoides*, *Botrytis caroliniana*, *Candida krusei*, *Debaryomyces nepalensis*, *Debaryomyces hansenii*, *Cyberlindnerajadinii,* and *Ganoderma destructans*) in both tongue and buccal samples, which were not recognized in the two previous studies. In the current study we also applied ITS1 primers to a subsection of the samples. This analysis identified a significant amount of *Malassezia* spp. and *Rhodotorula* spp. in the samples. This analysis indicates that the main reason for discrepancies between our ITS2 based survey and previously published data is due to primer choice. Although, *Malassezia* spp. and *Rhodotorula* spp. could be identified in the ITS2 survey, the abundance of these taxa was extremely low (outside the 20 most abundant taxa). The two pioneering observations of the oral mycobiome using ITS1 pyrosequencing by Ghannoum *et al*. (2010) and Dupuy *et al*. (2014) produced findings that were somewhat inconsistent (Bandara *et al*. 2018), and factors such as DNA extraction methods may be important for isolating DNA from some fungi such as *Malassezia* spp. The current study employed a bead beating DNA extraction protocol which allowed identification of *Malassezia* spp. using the ITS1 primer set, but not using the ITS2 amplification protocol. The absence of both *Malassezia* spp. and *Rhodotorula* spp., which are basidiomycetes suggests a lack of specificity of our ITS2 primer set for this phylum. Notably, two members of *Malassezia spp* (*M. restrica, M. globosa*) were identified in our healthy individuals from ITS1 sequences data, which was in line with the findings from Dupuy *et al*. (2014), one that was not recognized by Ghannoum *et al*. (2010). Additionally, *M. restrica* was also detected in the saliva of

elderly individuals (Zakaria *et al.* 2017). *Malassezia* species, an important commensals and previously known to be pathogenic of human skin Dupuy *et al*. (2014) and Bandara (2018). In 2014 *Malassezia* species was discovered by Dupuy and colleagues as predominant commensals in saliva of healthy individuals. In the present study, *Malassezia* species was not detected from ITS2 sequence data of tongue and buccal samples. When we amplified ITS1 region, *Malassezia* species was identified in our samples. To our knowledge, this is the first study discover two members of *Malassezia spp* namely *M. restrica* and *M. globosa* in tongue dorsum and buccal mucosa of the healthy mouth.

The other two studies by Li *et al.* (2012) and Zakaria *et al.* (2017), ITS1- 2.8SrDNA-ITS2 were amplified and quantified by quantitative Real-time PCR. They detected *C. albicans, C. dubliniensis* and *C. tropicalis*, which was consentient with our findings. However, two fungal species from these studies (*Candida globerata* and *Malassezia* spp.) were not detected in the present study. The most unexpected finding from our study was the presence of *C. dubliniensis* at high abundance, particularly in children less than 13 years old. This species was not identified by Ghannoum *et al*. (2010) and Dupuy *et al*. (2014) as a component of the healthy mycobiome in adults. Mukherjee *et al*. (2014) identified *C. dubliniensis* in 17% of HIV-infected patients by ITS1 pyrosequencing, which confirms previous culture based studies (Sullivan *et al.* 1995). Interestingly, we noted a significant reduction in the levels of *C. dubliniensis* with increasing age in adolescents and adult individuals. Adults exhibited a higher abundance of *Didymella gardenia* and *Epicoccum nigrum*. *C. dubliniensis* is one of the pathogenic mycobiome species first detected in oral specimens from HIV-infected and AIDS patients with recurrent oral candidiasis attending the Dublin Dental University Hospital in 1995 (Sullivan *et al.* 1995). However, *C. dubliniensis* is rarely detected in oral mycobiome of

healthy individuals by direct culture and it is responsible for only 2% of cases of candidemia (Sullivan *et al.* 2004). However, this species has been detected as an oral carriage organism and has been involved in oral candidosis in healthy subjects (Ponton *et al.* 2000). Additionally, *C. dubliniensis* was detected in the tongue mycobiome of 59.3% of institutionalized elderly adults Li *et al.* (2012) and in the oral cavity of 38% of community-dwelling healthy individuals (Zakaria *et al.* 2017). Using culture based techniques, Jabra-Rizk *et al*. (2001) cultured *C. dubliniensis* from 13% (4/30) of healthy children examined, which was a similar rate compared to *C. albicans*. Al-Ahmad *et al.* (2016), also using direct culture could detect *C. dubliniensis* in 25% of children with dental caries. These data support the notion that *C. dubliniensis* is a component of the healthy mycobiome and that is comprises a significant element of the mycobiome in healthy children. It is difficult to determine the significance of this finding and it may be related to the less developed immunity found in younger children. Follow-up studies using qRT-PCR and/or culture based detection should be carried out to confirm this finding.

Fungal ITS sequences are variable, and the degree of the ITS intra-species variability is different among the mycobiota, with ITS1 more variable than ITS2 (Diaz *et al.* 2017). Others revealed that ITS2 is a more accurate and sensitive method for studying the mycobiome (stool samples) (Nash *et al.* 2017). We compared our ITS1 sequence data to the ITS1 data from the two previous ITS1 studies by Ghannoum *et al*. (2010) and Dupuy *et al*. (2014). Interestingly, 6 fungal taxa were shared between the present study and the other two studies by Ghannoum *et al*. (2010) and Dupuy *et al*. (2014), including *Candida, Saccharomyces Alternaria, Asperguillus, Fusarium* and *Cladosporium*.

4.5 Conclusion.

Our findings revealed the overall composition of oral mycobiome of healthy individuals, and demonstrated differences between the tongue and buccal fungal communities. Furthermore difference in the oral mycobiome species between healthy children and adult healthy subjects. The dominant oral fungal species were *C. dubliniensis, C. albicans* and *Didymella gardeniae*. All three fungal species were prevalent among 37 healthy individuals. Interestingly, each one of the three abundant fungal species was found predominant in one age group. *C. dubliniensis* was highly predominant in children, whereas adolescent individuals were enrichment in *C. albicans* species. *Didymella gardeniae* (also knowns as *Phoma gardenia*) was prevalent in healthy adult subjects, along with *C. albicans*. When we amplified the ITS1 and ITS2 region from tongue and buccal samples, our data revealed discrepancies between the results from ITS1 and ITS2 sequencing, suggesting that the amplified region of fungal DNA could impact on resolution of the oral mycobiome membership. Further studies are required, investigating the impact of sampling method, amplified region of fungal DNA and sequencing techniques involving a large population of subjects.

Chapter 5

Analysis of oral mycobiome in treatment naïve children with IBD

Chapter 5 Analysis of oral mycobiome in treatment naïve children with IBD

5.1 Introduction.

Associations between the mycobiome and systemic diseases have been well documented, including graft versus host disease, Hirschsprung-associated enterocolitis, colorectal cancer, hepatitis B virus infection and inflammatory bowel disease (Iliev *et al.* 2012, Nash *et al.* 2017, Hager and Ghannoum 2017). In recent studies, using advanced sequencing techniques to measure mycobiome composition in the intestines of Crohn's disease (CD) patients, it was observed that there was a relationship between microbial and mycobial community structure and biodiversity and that mycobiome dysbiosis was seen in IBD patients (Miyoshi *et al.* 2018). The mycobiome component of the gut mycobiota has been studied extensively in IBD and is known to be dysbiotic (Li *et al.* 2014, Chehoud *et al.* 2015, Hoarau *et al.* 2016, Liguori *et al.* 2016, El Mouzan *et al.* 2017, Sokol *et al.* 2017, El Mouzan *et al.* 2018). Investigations of dysbiosis of the mycobiome in IBD have examined human faeces and different regions of the gut, including colonic mucosa and ileal mucosa. On the other hand, the oral microbiome, particularly the oral mycobiome in IBD patients has not been explored, therefore, there is a lack of information regarding the mycobiome in patients with IBD. Thus, in this section of the present study, we attempted to define the characteristics of the oral mycobiome in newly diagnosed treatment-naïve children with IBD. A cohort study was performed whereby tongue and buccal samples were obtained from paediatric IBD patients aged 5 to 16 years with Crohn's disease (CD; n=38) and Ulcerative Colitis (UC; n=21).

5.2 Data processing of the ITS2 region.

In the current study, the ITS2 region was amplified using primers ITS2F and ITS2R (Section 2.3.2) from samples recovered from paediatric IBD patients and sequenced on the MiSeq using the 500 cycle kit (2 x 250 bp reads). This procedure was carried out for both tongue and buccal samples individually. DADA2 was used as our main sequence processing tool. Additionally, the Mothur pipeline was used for initial sequence classification following processing in DADA2.

5.2.1. DADA2 pipeline processing workflow.

Analysis of ITS2 sequences using DADA2 was carried out as described in section 4.2.1. Analysis of the sequences resulted in 3,387 total sequences in tongue samples and 2,422 total sequences in buccal samples. Finally chimeras were removed from the merged sequences. We identified 909 chimeras out of 3387 unique sequences variants in tongue samples (<4% total sequences). Buccal samples yielded 483 chimeras out of 2,422 input unique sequences (<4% total sequences).

5.2.2. Sequence classification in Mothur pipeline.

Unique sequences and their abundances were exported to Mothur. Contaminating non-fungal sequences were removed by filtering out any sequences with <80% sequence similarity to the fungal ITS2 region. Remaining sequences were classified to species and genus level in Mothur using classify.seqs and the ITS2 sequence database from UNITE. Further classification of the most abundant fungal sequences was carried out by BLAST analysis using the ISHAM (International Society for Human and Animal Mycology) Barcoding Database. Following this, data sets were normalized by subsampling to 5000 sequences for statistical analysis. Following sequencing data processing, analysis of alpha and beta diversity was carried out as described in Chapter 2, section 2.5.

5.3 Results.

For oral mycobiome analysis in children with IBD, the internally transcribed spacer 2 region (ITS2 region) between the 18S and 28S rRNA gene was used. The current study analysed tongue and buccal samples from newly diagnosed treatment-naive children with CD (n=38) and children with UC (n=21). Healthy subjects, including children and adults were also recruited (n=46). Participant demographics are summarized in the Materials and Methods.

5.3.1 Tongue mycobiota.

The mycobiome of tongue samples were analysed and investigated at different levels, including species level and sub-species level using the unique sequences from DADA2.

5.3.1.1 Analysis of unique sequences in the tongue mycobiome.

Unique sequences first were analysed without any clustering into OTUs or species. In total, 691 unique sequences were identified. Twenty of the most dominant fungi were Ascomycetes yeasts including *Candida dubliniensis* (seq1), *Candida albicans* (seq2, seq6, seq10, seq12 and seq16), *Saccharomyces cerevisiae* (seq3, seq5 and seq 20), *Candida krusei* (seq.8), *Candida zeylanoides* (seq9), and *Candida tropicalis* (seq.13). Ascomycetes of the class Dothidiomycetes were the next most abundant including *Didymella gardeniae* (seq4), *Cladosporium cladosporiodes* (seq7), *Cladosporium herbarum* (seq14) and *Epicoccum nigrum* (seq18). The most abundant basidiomycetes was ranked 24th most abundant and was classified as *Cryptococcus laurentii* (seq35), (Figure 5.1).

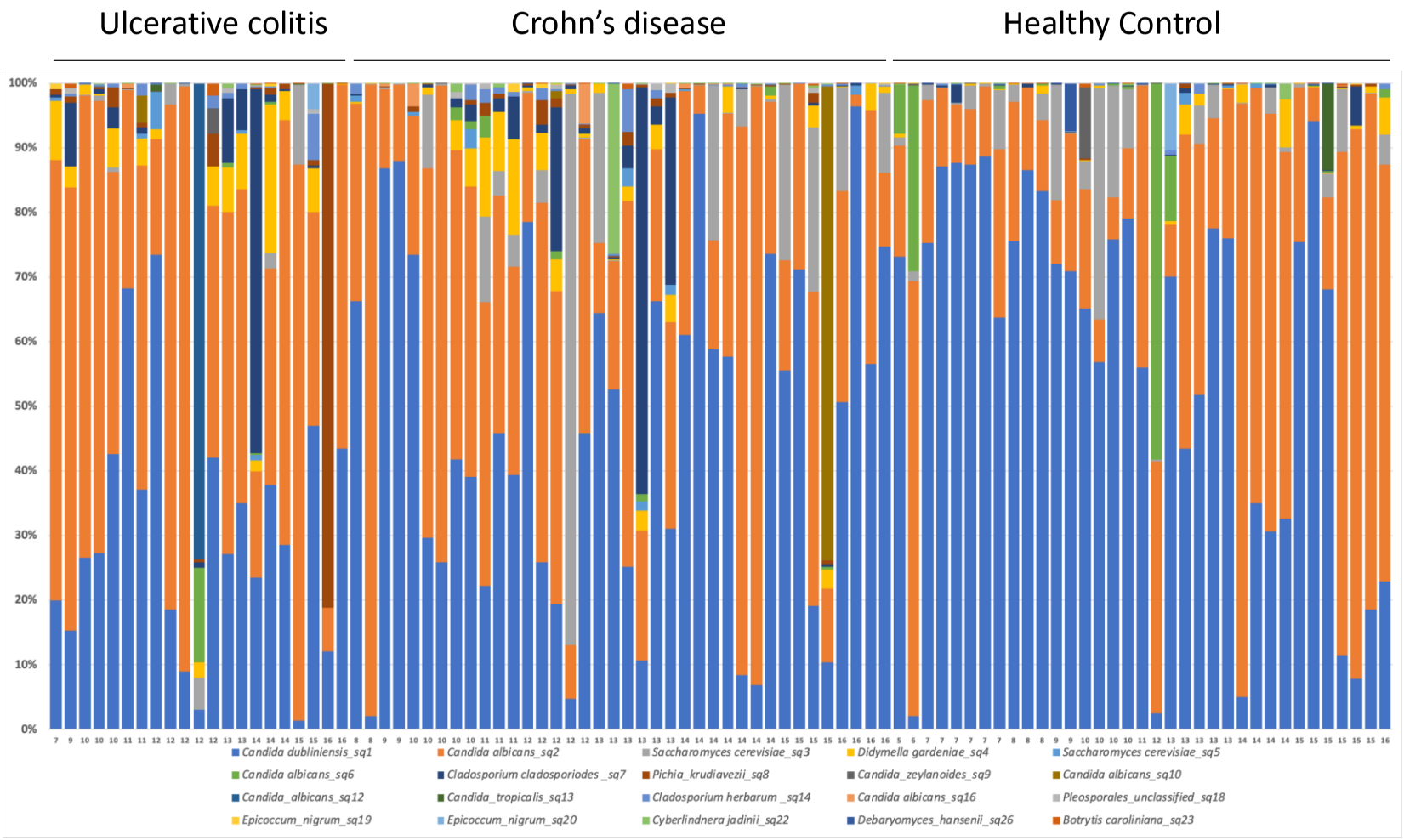


Figure 5.1 Distribution of the 20 most abundant fungal sequences identified by ITS2 sequencing in children with IBD and healthy controls. The ages of each participant are indicated on the bottom axis.

5.3.1.2 Species level tongue mycobiome.

Tongue data were analysed at species level, demonstrating the abundance of sequences clustered at species level. *Candida albicans* (Otu001) and *Candida dubliniensis* (Otu003) were the dominant species. The other most abundant species included *Saccharomyces* spp., *Davidella* spp. and *Cladosporium* spp. (Figure 5.2).

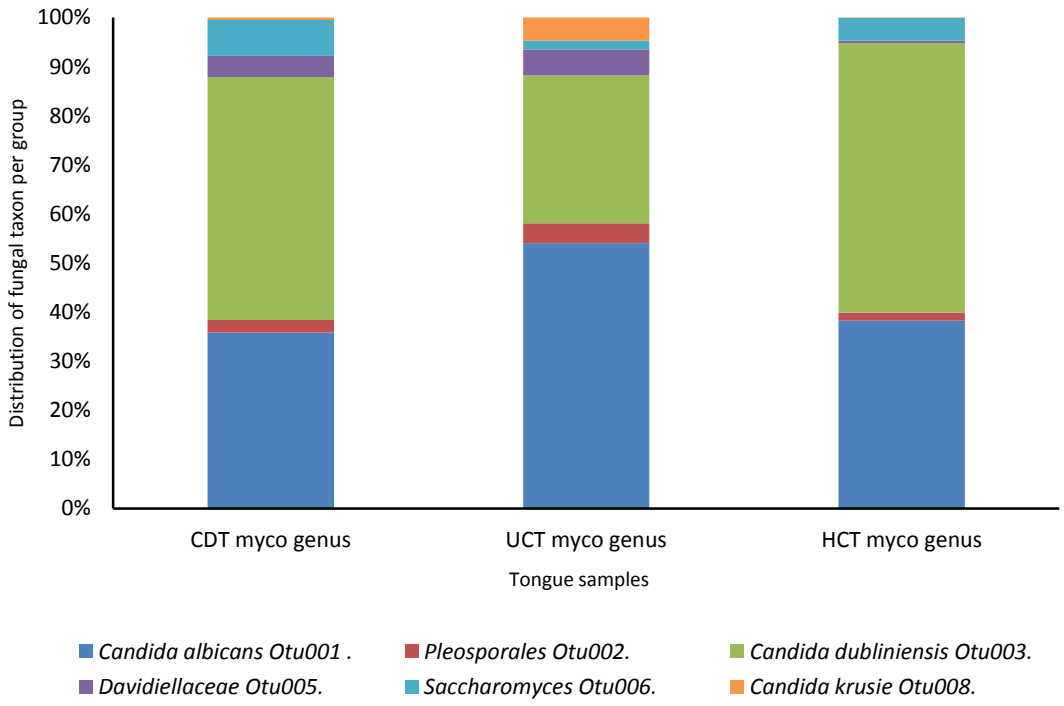


Figure 5.2 The abundance of different fungal taxon in tongue samples and their distribution in each study group.

5.3.1.3 Alpha diversity measurements.

These measurements were carried out to measure species richness within the tongue mycobiome environment, and included the inverse Simpson index, Chao1 index and rarefaction curve analysis.

5.3.1.3.1 Inverse Simpson Index.

The inverse Simpson and Chao1 indices were used to calculate mycobiome biodiversity and richness of tongue samples, respectively, as shown in (Table 5-1). These data showed that the tongue mycobiome in Crohn's disease (CDT), and ulcerative colitis (UCT) groups had similar levels of biodiversity measured with the inverse Simpson index, whereas healthy controls exhibited reduce biodiversity compared to IBD children. However, the results from the Chao test indicated that CDT mycobiota exhibited greater richness compared to other two groups (UCT and HCT).

Table 5-1 The minimum and maximum numbers of fungal sequences (seq) in the study groups and inverse Simpson and Chao1 statistics.

SITE	Study Groups	Average seq number	Chao1	Inverse Simpson	Minimum Seq number	Maximum Seq number
Tongue	CD	15.31	17.02	2.24	4.18	36.77
Tongue	UC	14.72	15.96	2.31	4.64	25.10
Tongue	HC	13.55	16.50	1.74	5.61	23.83

5.3.1.3.2 Rarefaction curve.

The rarefaction curve was used to determine fungal species richness in study samples. Tongue sample data showed that the CDT group of children with Crohn's disease exhibited greater species richness compared to UCT children and HCT subjects. In general, IBD children had greater species richness compared to healthy individuals (Figure 5.3).

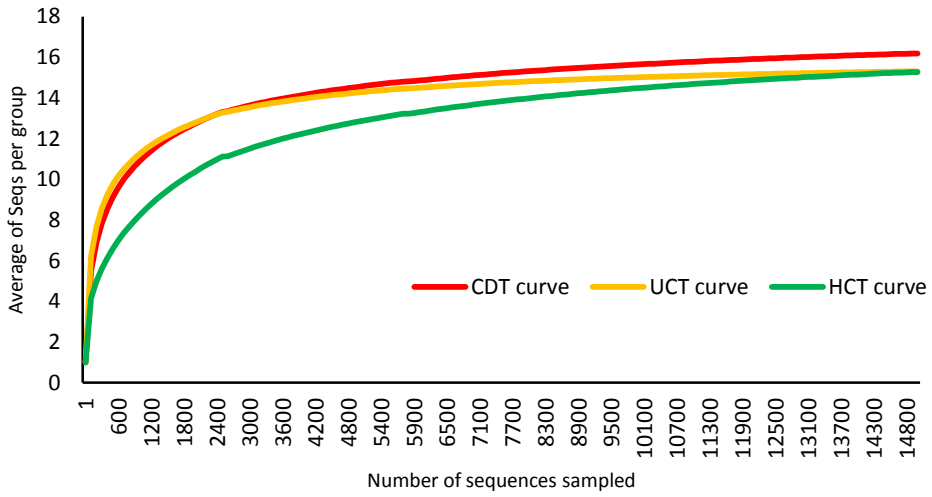


Figure 5.3 Rarefaction curve showing mycobiome species richness of CDT, UCT and HCT study groups.

5.3.1.4 Beta diversity measurements.

Diversity in mycobiome community structure between different samples was measured using Non-metric Multidimensional Scaling (NMDS).

5.3.1.4.1 Non-metric Multidimensional Scaling (NMDS).

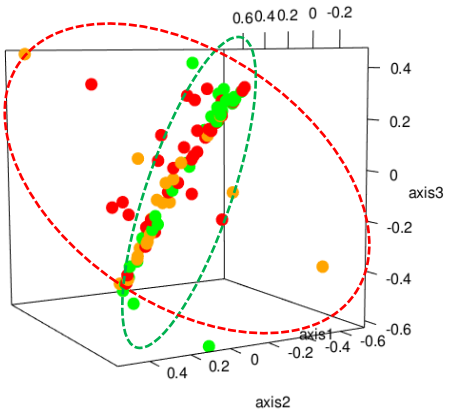
NMDS was used to graphically represent the distance between the tongue samples. Distances were calculated using the Yue and Clayton (Thetayc), Jaccard (Jclass) and Bray-Curtis calculators. Analysis of Molecular Variance (AMOVA) was carried out to determine if the sample separation in the NMDS plot is significant (Figure 5.4 a to c).

Using the Thetayc distance calculator, AMOVA analysis of tongue samples showed no significant difference in mycobiome community structure between tongue samples of IBD children (CDT and UCT) (P=0.062). When the tongue mycobiota of Crohn's disease (CDT) and Ulcerative Colitis children (UCT) were compared separately to healthy subjects, the result showed that there was a significant difference between the mycobiota of UCT and HCT children (P=0.007) but not the CDT samples (P=0.037). Statistically significant differences were noted in the relationship between the tongue mycobiota of CDT and UCT patients (P=0.003). Using the Bray-Curtis similarity coefficient, AMOVA analysis of the tongue mycobiota showed significant differences between IBD children and healthy individuals (P=0.036). In the separate comparison of CDT and UCT with HCT, the data showed a significant difference between UCT and HCT (P=0.002), but no significant difference between CDT and HCT (P=0.191). Using the Jclass calculator, when we compared IBD children and the HCT group, AMOVA of tongue mycobiota showed a significant different between IBD and HCT (P=<0.001). In addition, when CDT and UCT were compared separately to healthy subjects (HCT), both groups were found to be significantly different from HCT samples (P=<0.001 and P=0.006, respcectively). However, two IBD groups (CDT and UCT) were not significantly different (P=<0.474), (Table 5-2).

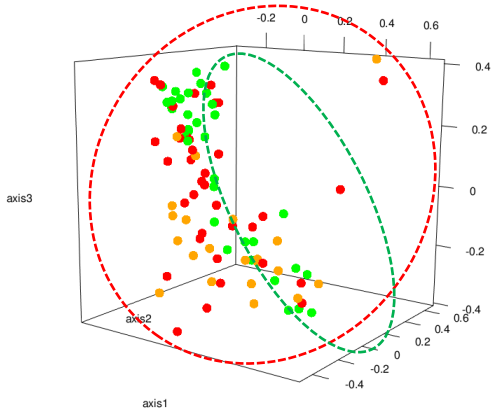
Table 5-2 P-value results from AMOVA tests comparing mycobiome communities in tongue samples (CDT, UCT, and HCT), using Thetayc, Jclass, and Bray-Curtis calculators.

Tongue samples	**Thetayc** **P-value**	**Bray-Curtis** **P-value**	**Jclass** **P-value**
IBD vs Healthy	0.062	0.036*	<0.001**
Colitis vs Crohn's	0.003**	0.001**	0.474
Colitis vs Healthy	0.007**	0.002**	0.006**
Crohn's vs Healthy	0.037*	0.191	<0.001**

(a)



(b)



(c)

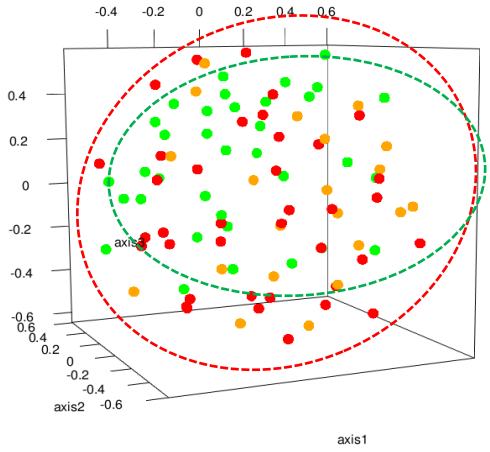


Figure 5.4 NMDS plots of tongue samples generated with the Thetayc (a), Bray-Curtis (b) and Jclass calculators (c) showing CDT (red), UCT (orange) and HCT (green) samples. Red and green circles are used to show healthy and IBD groups and are for visualisation purposes only.

5.3.1.5 Impact of disease location on community structure.

According to the Paris classification of IBD, disease location is one of the three important parameters in disease classification, which can be listed as L1 to L4 (Appendix 1 and Appendix 2). In Chapter 3, the microbiome community structure in children Crohn's disease with extensive disease throughout the GI tract, classified as L3L4, exhibited the greatest differences in community structure. Using three different distance matrices, including Thetayc, Jclass, and Bray-Curtis calculators, the relation between L3L4 disease phenotype and the mycobiome community structure in the three study groups were assessed.

In our study, using the three calculators, no significant differences were found between the children classified as L3L4 and the remaining CDT samples (Table 5-3). The L3L4 samples were also not significantly different from UCT samples. However, using the Jclass calculator, AMOVA identified a significant difference between L3L4 type disease and the HCT group (P=0.001).

Table 5-3 P-value results from AMOVA analysis of the L3L4 disease type in CD and the study groups, using Thetayc, Jclass, and Bray-Curtis calculators.

Measurements	***All groups***	**L3L4 vs HCT**	**L3L4 vs CDT**	**L3L4 vs UCT**
Thetayc	P=0.018*	P=0.357	P=0.735	P=0.079
Bray-Curtis	P=0.01*	P=0.299	P=0.765	P=0.059
Jclass	P=<0.001**	P=0.001**	P=0.281	P=0.136

5.3.1.6 Impact of disease severity on community structure.

We next examined whether the tongue mycobiome was affected by the severity of disease, including inactive, mild, moderate, and severe. Using the three previous distance measurements (Thetayc, Jclass, and Bray-Curtis) the relationship between heathy subjects and IBD children classified by disease severity was examined. The Thetayc and Bray-Curtis calcuators showed no differences between healthy (HCT) and IBD children classified as inactive, mild, moderate, and severe types, respectively (P=0.375; P=0.295). On the other hand, the Jaccard measurement reported a significant differences between HCT and the four disease phenomena (P=0.012). Specifically, a significant difference identified between HCT samples and IBD samples exhibiting both moderate disease (P<0.001) and severe disease (P=0.032), but not mild or inactive disease. (Table 5-4).

Table 5-4 AMOVA P value results from the correlation between disease severity and community structure in the study groups, measured using the Jclass calculator

Disease Groups	**AMOVA P (Jclass)**
HCT vs inactive IBD	P=0.359
HCT vs mild IBD	P=0.099
HCT vs moderate IBD.	P<0.001**
HCT vs severe IBD	P=0.032*
Inactive IBD vs mild IBD	P=0.572
Inactive IBD vs moderate IBD	P=0.428
Inactive IBD vs severe IBD	P=0.568
Mild IBD vs moderate IBD	P=0.927
Mild IBD vs severe IBD	P=0.799
Moderate IBD vs severe IBD	P=0.056

5.3.1.7 LEfSe analysis of tongue mycobiota OTUs.

To identify species which may be significantly enriched in each study group, LEfSe analysis was carried out comparing the three study groups (CDT, UCT, and HCT). Children with inflammatory bowel disease (IBD) were enriched in different fungal taxon, *Didymella gardeniae, Cladosporium cladosporiodes* and *C. krusei*. On the other hand *Candida dubliniensis* exhibited greater abundance in samples from HCT samples (Figure 5.5). The phylogenetic distribution of the differences between IBD healthy children (HCT) were examined, showing that the enriched species in IBD children cluster in the Dothideomycetes (Figure 5.6).

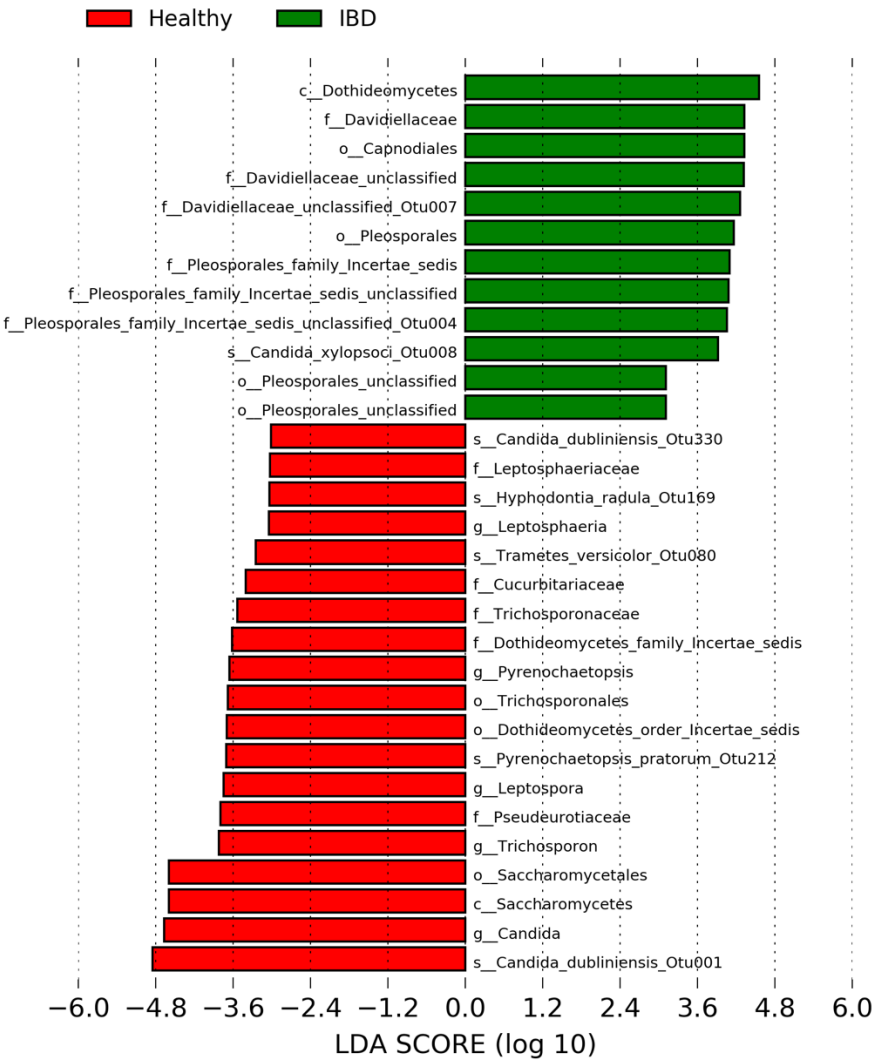


Figure 5.5 . LEfSe plot showing the differential enrichment of fungal species in tongue samples from IBD children and the healthy control group

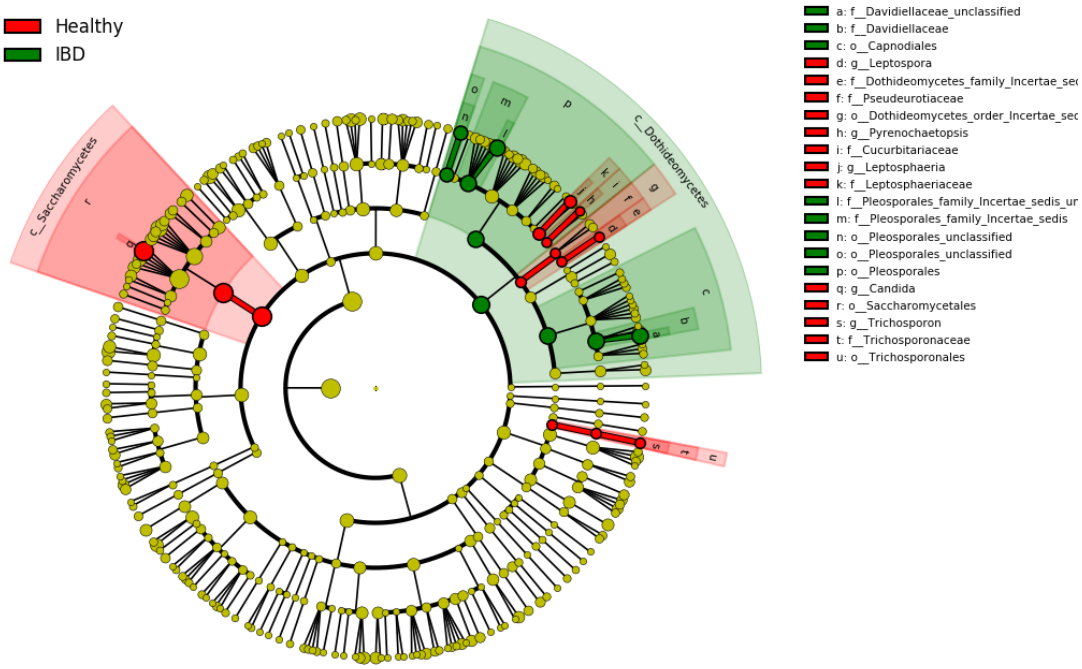


Figure 5.6 Phylogenetic distribution of fungal species enriched in tongue samples in IBD and HCT groups.

Comparison of the tongue mycobiota in CDT and UCT samples to healthy subjects (HCT), showed that the mycobiota of CDT samples were enriched with *S. cerevisiae* and the UCT samples showed enrichment in *Didymella gardenia*, *Cladosporium cladosporiodes*, and *C. krusei* (Figure 5.7).

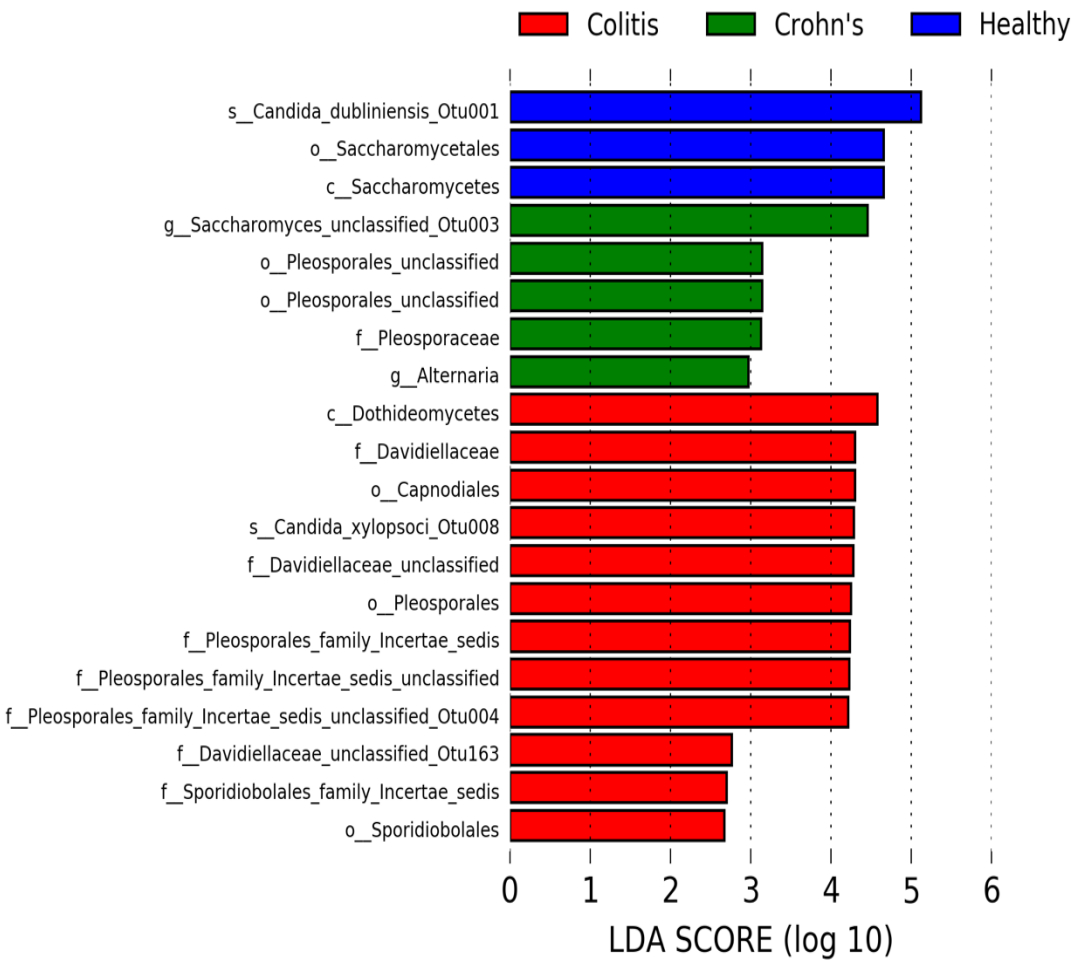


Figure 5.7 LEfSe plot showing the differential enrichment of fungal species in tongue samples from children with ulcerative colitis, Crohn's disease and the healthy control group.

5.3.1.8 Abundant mycobiome taxon in tongue samples.

Graphs showing the abundance of significantly enriched fungal species in tongue mycobiota in each group were generated using the LEfSe package (Hutlab Galaxy server). Each plot shows abundance data for statistically significantly enriched taxa in HCT, CDT and UCT samples (Figure 5.8).

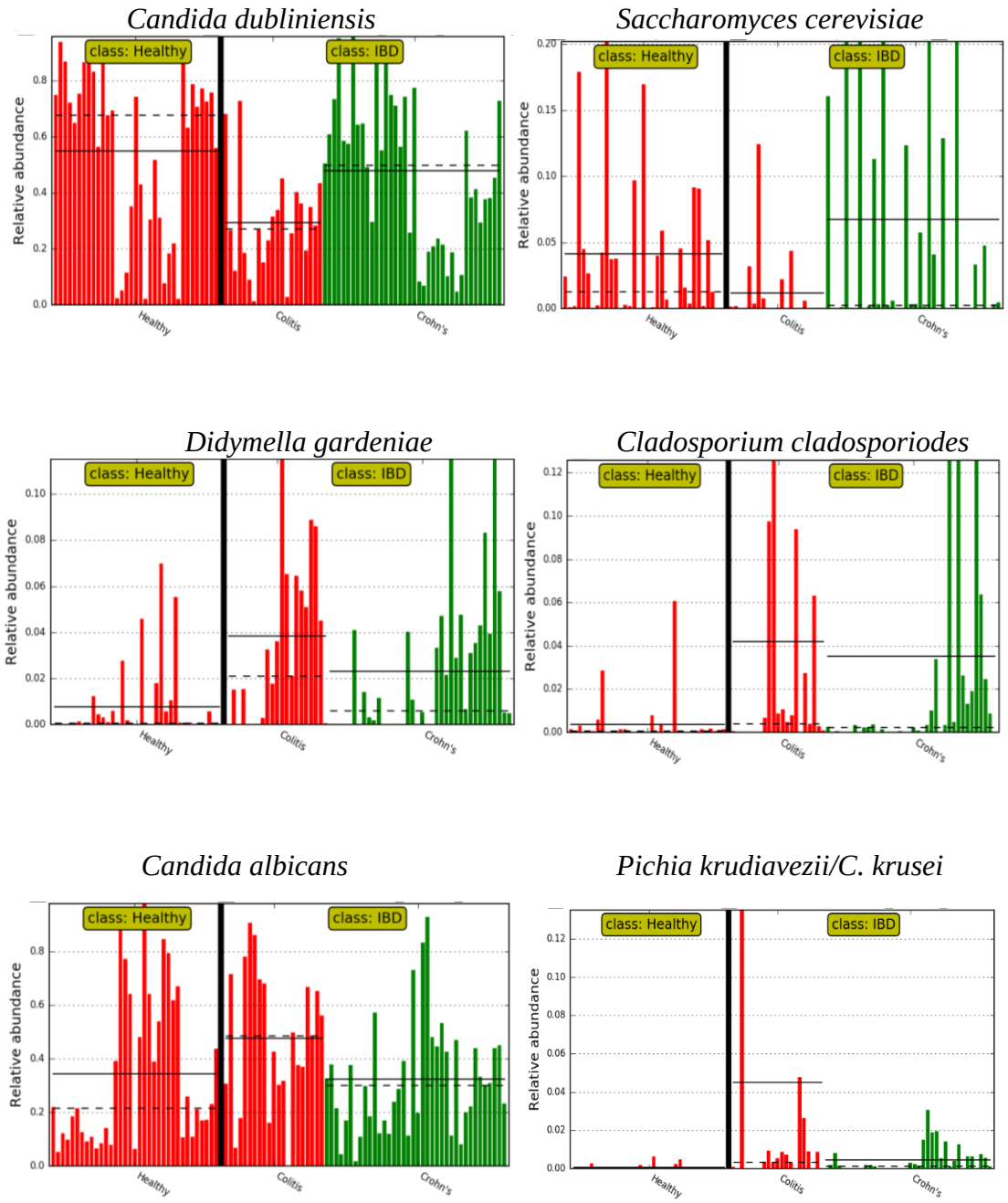


Figure 5.8 LEfSe plot showing the differential enrichment of fungal species in tongue samples from children with ulcerative colitis, Crohn's disease and the healthy control group. Mean values are represented by the solid line, median values by the dotted line.

5.3.2 Buccal mycobiota.

The buccal mycobiota of Crohn's disease (CDB), ulcerative colitis (UCB) and healthy children (HCB) were analysed and investigated at different levels.

5.3.2.1 Analysis of unique sequences in the buccal mycobiome.

The fungal ITS2 sequences from buccal samples were dominated by six yeasts including, *Candida dubliniensis* (seq1), *Candida albicans* (seq2 and seq5), *Didymella gardeniae* (seq.3), *Saccharomyces cerevisiae* (seq4), *Cladosporium cladosporioides* (seq6), and *C. krusei* (seq7). In contrast to the tongue mycobiome, the 20 most abundant fungal sequences did not include *C. zelanoides* or *C. tropicalis* and included two sequences representing *Alternaria infectoria.* Only two species of basidiomycete were detected among the 50 most abundant sequences, namely *Schizophyllum commune* (seq56), and *Resinicium bicolor* (seq65). (Figure 5.9).

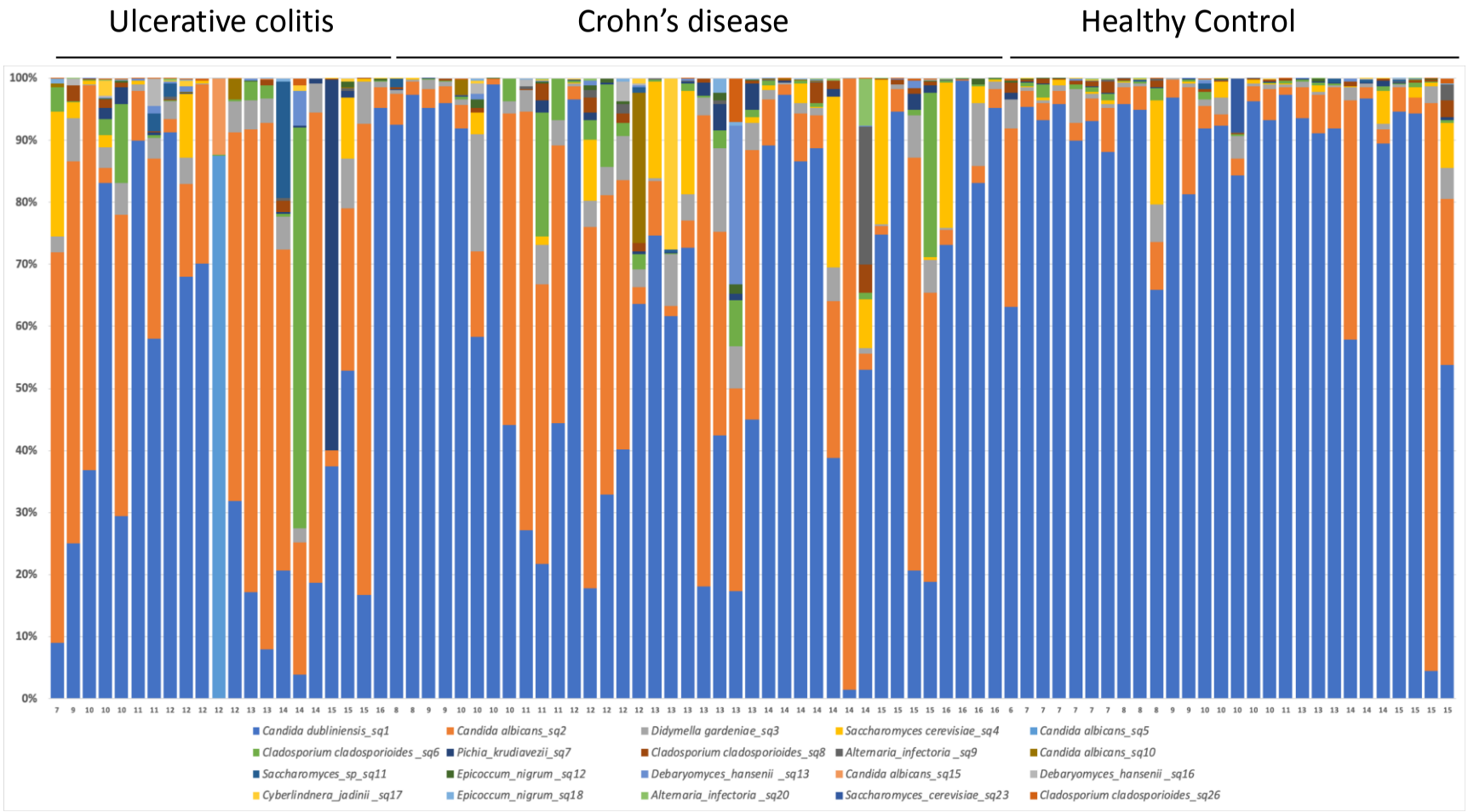


Figure 5.9 Distribution of the 20 most abundant fungal sequences identified by ITS2 sequencing in children with IBD and healthy controls. The ages of each participant are indicated on the bottom axis.

5.3.2.2 Analysis of the buccal mycobiome at species level resolution.

Buccal data were analysed at species level, to identify the most abundant fungal species, including *Candida albicans*, *Candida dubliniensis*, *Saccharomyces cerevisisae*, *Didymella gardeniae* and *Cladosporium cladosporiodes*. (Figure 5.10).

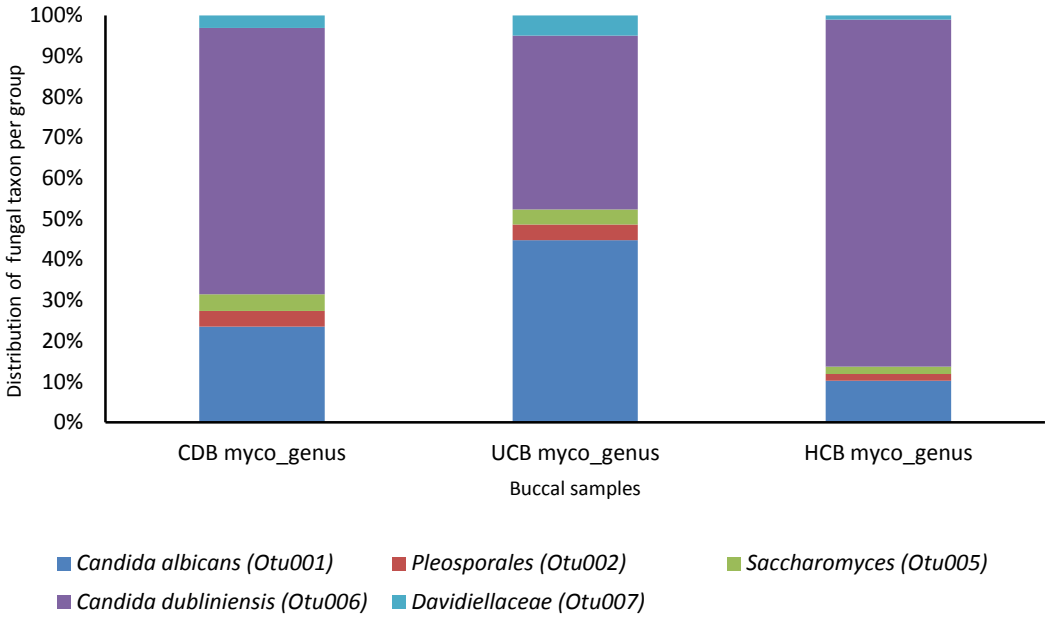


Figure 5.10 The six most abundant fungal taxon in buccal samples.

5.3.2.3 Alpha diversity measurements of buccal mycobiota.

Alpha diversity measurements, including the inverse Simpson and Chao indices and the rarefaction curve were used to measure species richness within the buccal mycobiome environment.

5.3.2.3.1 Inverse Simpson Index.

The inverse Simpson and Chao1 index was used to estimate mycobiome biodiversity in buccal samples (Table 5-5). The results from inverse Simpson index indicated that CDB

mycobiota exhibited greater biodiversity compared to UCB and HCB groups (Table 5-5). Estimation of species richness with the Chao1 test showed that CDB mycobiota exhibited reduced richness compared with UCB, and HCB.

Table 5-5 The minimum and maximum numbers of fungal sequences per sample in the study groups and average inverse Simpson and Chao1 values.

SITE	Study Groups	Average Seqs	Chao1	Inverse Simpson	Min Seq number	Max Seq number
Buccal	CDB	14.48	15.98	2.07	2.48	38.41
Buccal	UCB	15.14	16.31	1.99	4.89	38.41
Buccal	HCB	14.77	17.19	1.35	5.92	28.65

5.3.2.3.2 Rarefaction curve.

Using a rarefaction curve, fungal species richness in buccal samples was determined. The analysis showed that species richness was similar in all three sample types. Healthy control samples exhibited a greater number of species at large sample depths (>5000 sequences) (Figure 5.11).

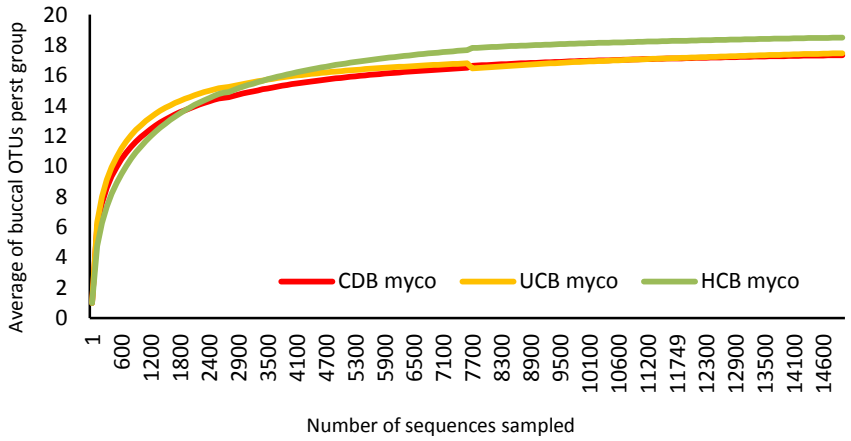


Figure 5.11 Showing rarefaction curve analysis of mycobiome species richness in CDB, UCB and HCB study groups.

5.3.2.4 Beta diversity measurements of buccal mycobiota.

Non-metric Multidimensional Scaling (NMDS) was used to measure the diversity in mycobiome community structure between different buccal environments.

5.3.2.4.1 Non-metric Multidimensional Scaling (NMDS).

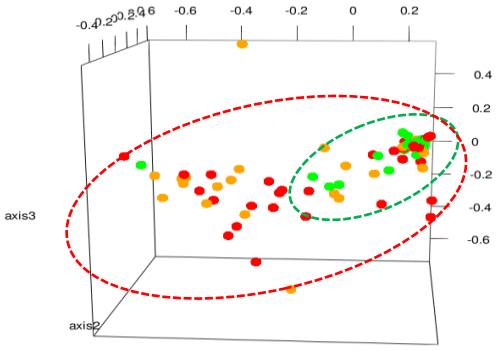
NMDS was used to graphically represent the distance between buccal samples. Distances were calculated using Thetayc, Jclass, and Bray-Curtis calculators. Analysis of Molecular Variance (AMOVA) was carried out to determine if the separation in the NMDS plot is significant (Figure 5.12 a to c). Using the Bray-Curtis dissimilarity coefficient, AMOVA of buccal samples showed a significant difference in mycobiome community structure between buccal samples of IBD and healthy control subjects (P=0.001). When UCB and CDB samples were compared to healthy controls (HCB), both UCB and CDB samples were found to be significantly different from the healthy buccal samples (P<0.001 and P=0.008, respectively). Differences were also found between the buccal mycobiota of

CDB and UCB children (P=0.025). Using, the Thetayc calculator, AMOVA also demonstrated a significant difference between healthy buccal samples and UCB samples (P<0.001), and also between CDB samples the healthy buccal samples (P=0.007). CDB and UCB samples were also significantly different (P 0.023) The Jclass calculator did not identify significant differences between any of the groups. (Table 5-6).

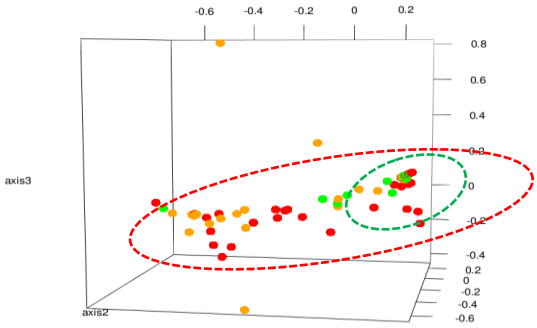
Table 5-6 P-value results from AMOVA tests comparing mycobiome comunities in buccal samples (CDB, UCB, and HCB), using Thetayc, Jclass, and Bray-Curtis calculators.

Buccal samples	**Thetayc P-value**	**Bray-Curtis P-value**
IBD vs Healthy	0.002**	0.001**
Colitis vs Crohn's	0.023*	0.025*
Colitis vs Healthy	<0.001**	<0.001**
Crohn's vs Healthy	0.007**	0.008**

(a)



(b)



(c)

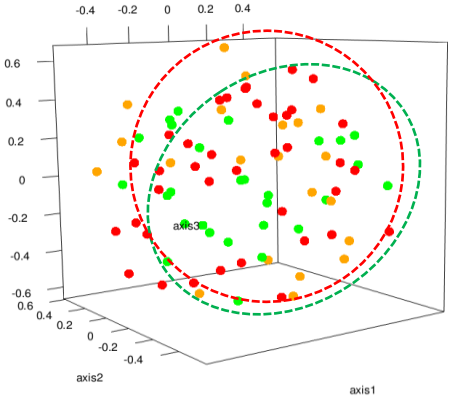


Figure 5.12 NMDS plots of buccal samples generated with the Thetayc (a), Bray-Curtis (b) and Jclass calculators (c) showing CDB (red), UCB (orange) and HCB (green) samples. Red and green circles are used to show healthy and IBD groups and are for visualisation purposes only.

5.3.2.5 Impact of disease location on community structure.

The influence of Crohn's disease classification on the buccal mycobiome was analysed. As described previously, we examined whether extensive CD (L3L4 classification) had a greater impact on the microbiome relative to other classifications. While buccal mycobiome communities from CD patients with an L3L4 diagnosis were significantly different from healthy control (HCB) and ulcerative colitis (UCB) communities ($P<0.05$), there was no significant difference to communities from the non-L3L4 CD patients, using the Yue & Clayton and Bray-Curtis calculators (Table 5-7).

Table 5-7 P-value results from AMOVA tests comparing L3L4 disease type and the study groups, using Thetayc, Jclass, and Bray-Curtis calculators.

Measurements	All groups	L3L4 vs HCB	L3L4 vs CDB	L3L4 vs UCB
Thetayc	P<0.001**	P=0.029*	P=0.373	P=0.026*
Bray-Curtis	P<0.001**	P=0.015*	P=0.505	P=0.036*

5.3.2.6 Impact of disease severity on community structure.

We examined whether disease severity in IBD (inactive, mild, moderate, and severe) impacted on community structure. Analysis of communities with the Thetayc and Bray-Curtis dissimilarity metrics showed that healthy communities were significantly different from mild, moderate and severe cases of IBD (Table 5-8). Inactive cases of IBD were not significantly different from healthy samples or borderline significant (P 0.043 and 0.057).

Table 5-8 Comparison of buccal mycobiome community structures in IBD patients based on disease severity. Communities were compared using the Thetayc, Jclass, and Bray-Curtis calculators and differences in community structure were identified using AMOVA.

Healthy and IBD Groups	**Bray-Curtis P value**	**Thetayc P value**	**Jclass P value**
All groups	P=<0.001**	P=<0.001**	P=0.009**
HCB vs inactive IBD	P=0.043*	P=0.057	P=0.035*
HCB vs mild IBD	P=0.008**	P=0.011*	P=0.462
HCB vs moderate IBD	P=<0.001**	P=<0.001**	P=0.016*
HCB vs severe IBD.	P=0.003**	P=0.003**	P=0.224

5.3.2.7 LEfSe analysis of buccal mycobiota.

To identify species significantly enriched in each study group, LEfSe analysis was performed. Children with IBD were mainly enriched with *Candida albicans* (seq2) and *Didymella gardeniae* (Seq3). In contrast, *Candida dubliniensis* (seq1) was found to be enriched in the healthy group of children. A large number of other low abundance fungi were identified in healthy children but the levels of colonisation were not significant (<0.01% or reads), (Figure 5.13). Analysis of the phylogenetic distribution of the differences between IBD and healthy children (HCB) showed an increase in fungi of the class Dothideomycetes in IBD Saccharomycetes in healthy children (Figure 5.14).

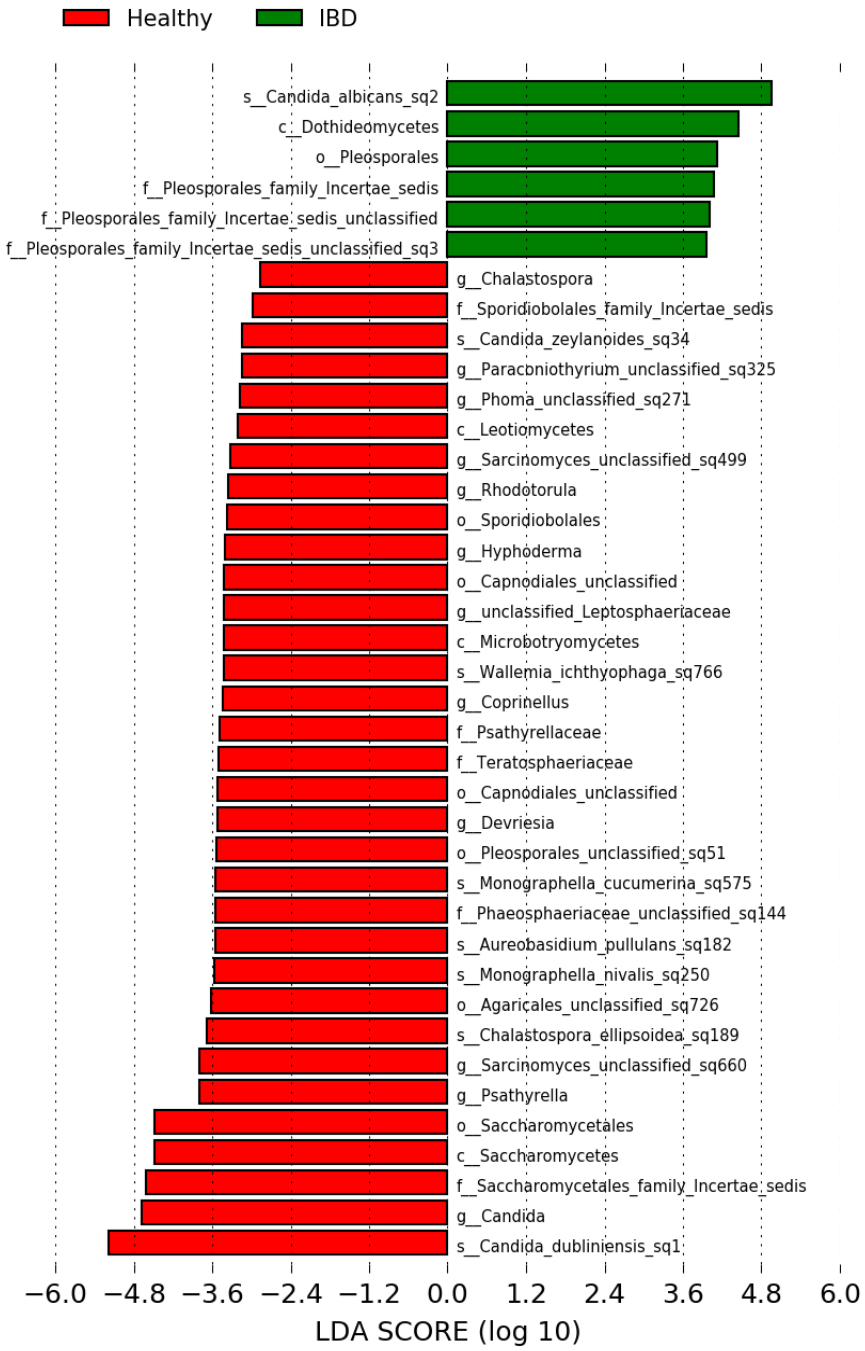


Figure 5.13 LEfSe plot showing the differential enrichment of fungal species in buccal samples from children with ulcerative colitis, Crohn's disease and the healthy control group.

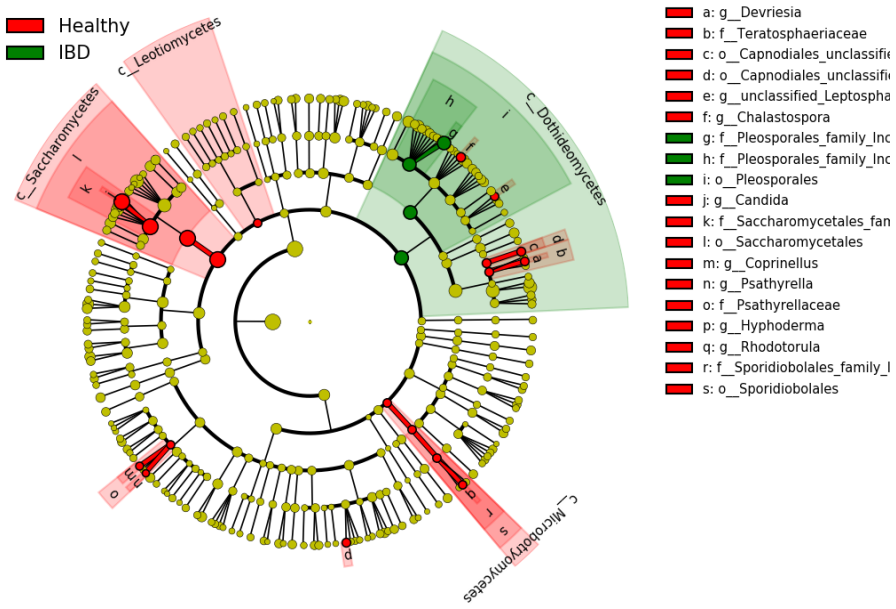


Figure 5.14 The distribution of fungal taxa enriched in buccal samples in IBD and HCB groups.

We next compared the buccal mycobiota of Crohn's disease and ulcerative colitis patients together with samples from healthy children. *Candida dubliniensis* (seq1) was again enriched in the healthy children. *Candida albicans* (seq2 and seq15) was found to be most enriched in the ulcerative colitis patients and *Didymella gardeniae* (Seq3) in Crohn's disease pateints (Figure 5.15).

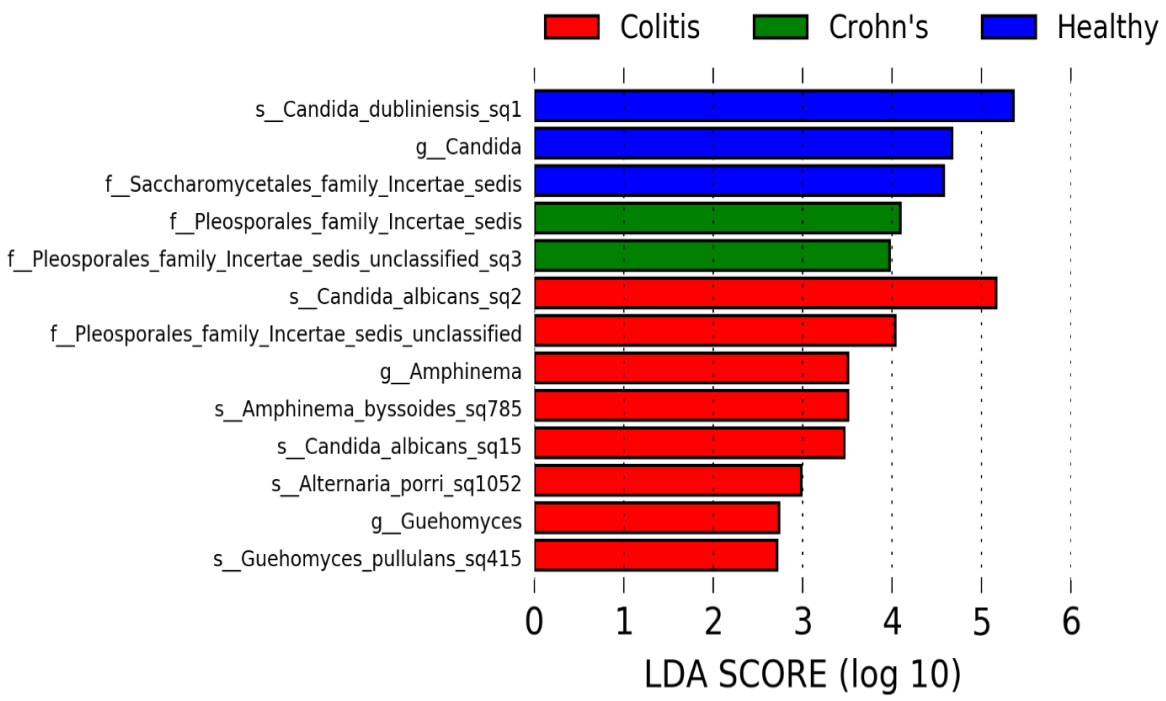
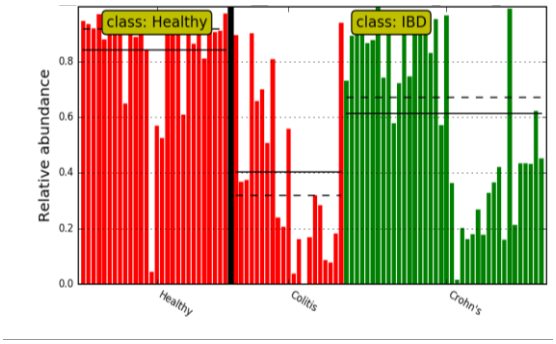


Figure 5.15 LEfSe analysis plot showing enriched taxa in buccal samples from the CDB, UCB and HCB study groups.

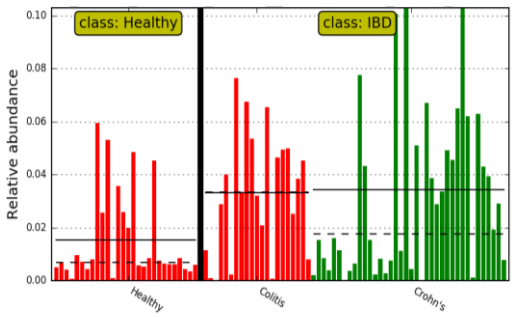
5.3.2.8 Abundant mycobiome taxa in buccal samples.

Graphs showing the abundance of significantly enriched fungal species in the buccal mycobiota in each group were generated using the LEfSe package (Hutlab Galaxy server). Each plot shows statistically significant differences between taxa enriched in HCB, CDB and UCB samples (Figure 5.16).

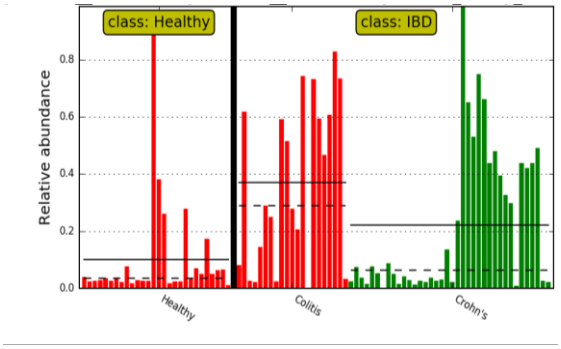
Candida dubliniensis seq1



Didymella gardenia seq3



Candida albicans seq2



Candida albicans seq15

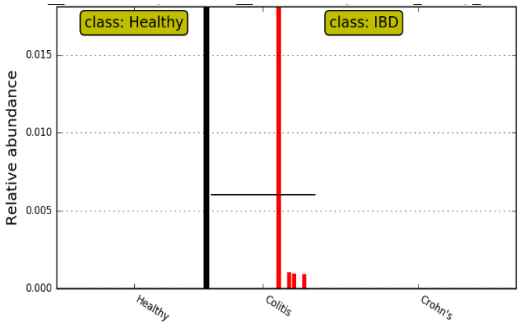


Figure 5.16 Plots showing the abundance of fungal taxa identified in LEfSe analysis of buccal samples from CD and UC children. Mean values are represented by the solid line, median values by the dotted line.

5.3.3 Estimation of fungal load by qRT-PCR.

The fungal load within given tongue and buccal samples was determined using real time quantitative PCR. *C. albicans* SC5314 DNA was used to generate a standard curve. The CT values obtained by real- time PCR were used to calculate the concentration of fungal DNA in a given sample, based on the standard curve established with *C. albicans* SC5314 DNA (Figure 5.17). The mean CT values for each samples were used to interpolate the standard curve and the fungal density present in each samples was expressed as CFU/ml.

Our finding indicated that the fungal DNA concentration was similar in CD children and HC individuals (tongue and buccal samples) (Figure 5.18 a, b).

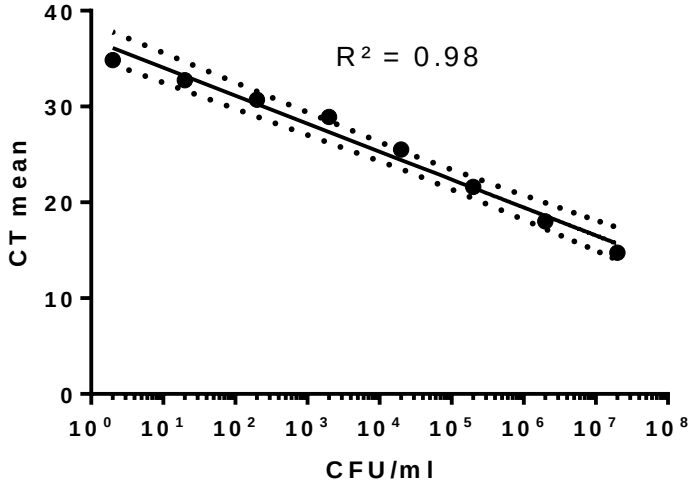
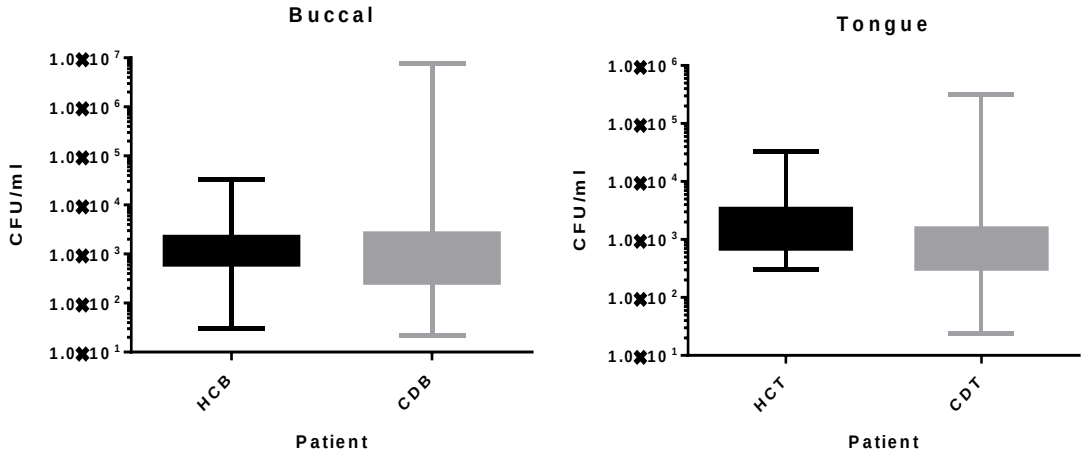


Figure 5.17 The standard curve generated with *C. albicans* SC5314 DNA.



(a) (b)

Figure 5.18. Quantification of fungal DNA as CFU/ml in given samples. (a) Fungal DNA concentration in buccal samples (CDB and HCB). (b) Fungal DNA concentration in tongue samples (CDT and HCT).

5.4 Discussion.

The mycobiome community in the human GI tract represents only a small proportion of the microbiome, accounting for about 0.02% of the gut microbiota compared to bacteriome (99.1%). However, the role of fungi in the pathogenesis of IBD is suggested based on animal and human observations (El Mouzan *et al.* 2017). Previous studies were carried out to characterize the gut mycobiome in IBD patients. However, there is currently a lack of information regarding the oral mycobiome in healthy individuals, and in its relation to oral and systemic diseases (Zakaria *et al.* 2017). Few studies have assessed the oral mycobiome in healthy subjects (Ghannoum *et al.* 2010, Dupuy *et al.* 2014; Zakaria *et al.* 2017). To our knowledge, this study represents the first time the oral mycobiome has been described in treatment naïve children with IBD. In the current study, we investigated if dysbiosis of the oral mycobiome occurs in children with IBD, sampling the dorsum of the tongue and buccal mucosa. A total of 210 samples (tongue and buccal) from IBD patients and controls were analysed. Our results indicated that across all tongue and buccal samples, we observed two main phyla (Ascomycota and Basidiomycota), with the majority of sequence reads in the samples from the phylum Ascomycota. Interestingly, at the phyla level our findings were consistent with some previous studies, assessing the gut mycobiome in IBD (Chehoud *et al.* 2015). In contrast, other previous reports describing the gut fungal community in children with CD found that Basidiomycota were more dominant compared to Ascomycota (Mukhopadhya *et al.* 2015, Liguori *et al.* 2016, Sokol *et al.* 2017, El Mouzan *et al.* 2017). The discrepancy between the previous observations and our study are likely due to differences in the community composition between oral and gut microbiota, or could be explained by differences in the impacts of IBD on gut and oral microbiota. Furthermore, the differences in outcomes could be related to disease severity itself. In addition to that, using different

methodologies to amplify fungal sequences might play a role in these differences. A proper comparison of dysbiosis in the oral and gut mycobiota in IBD patients requires investigation of both sites in the same individuals. In the current study, the sequences of ITS2 regions were subjected to BLAST analysis using the ISHAM Barcoding Database. Oral samples of IBD children were enriched in *Candida albicans, Didymella gardeniae* (family Pleosporales), *Cladosporium cladosporiodes* (family Davidiellaceae), *Saccharomyces cerevisiae* (family Saccharomycetaceae), *C. krusei* (family Saccharomycetales), *Debaryomyces hansenii* (family Saccharomycetales), *Cyberlindnera jadinii* (order Pleosporales), and *Alternaria infectoria* (family Davidiellaceae). The healthy samples were more enriched in *Candida dubliniensis.*

Using LEfSe our data showed *Saccharomyces cerevisiae* was overrepresented in tongue samples of CD children. Although not all CD children were colonised with *S. cerevisiae*, in 9 of the 37 CD children *S. cerevisiae* represented greater than 10% of the mycobiome, compared to 3 of 35 healthy children. Previous studies have detected anti-*S. cerevisiae* antibodies more frequently in patients with CD compared to UC and HC subjects (29-69% of CD patients), (Chehoud *et al.,* 2015), leading to speculation that *S. cerevisiae* may be involved in inducing inflammation. However, some studies have shown that *S. cerevisiae* is more abundant in non-inflamed mucosa compared to inflamed mucosa of CD patients (Li *et al.*, 2014; Liguori *et al.*, 2016; Sokol *et al.*, 2017). The findings that *S. cerevisiae* is overrepresented in intact or non-inflamed tissue of CD patients could be explained by anti-inflammatory effects of *S. cerevisiae* (Sokol *et al.,* 2017). The unexpected abundance of *S. cerevisiae* in the dorsum of tongue in CD children might reflect the shifting fungal microbiota that occurs in the gut mycobiome in CD, particularly in inflamed parts of the gastrointestinal tract (GIT). Hoarau et al. (2016) reported that *Saccharomyces cerevisiae* and *Candida tropicalis* were the most common known fungal

species in stool samples from CD patients. Furthermore, *Saccharomyces cerevisiae* and *Saccharomyces bayanus* were significantly more abundant in stool samples from CD patients (El Mouzan *et al.*, 2018). As many CD patients in the current study have high levels of oral *S. cerevisiae*, it may be possible that the gut *S. cerevisiae* reported by El Mouzan *et al.* (2018) and Hoarau et al. (2016) could have originated in the oral cavity of these patients. However, we did not observe any enrichment in *C. tropicalis* in the oral cavity. The findings of El Mouzan *et al.* (2018) and Hoarau et al. (2016) were in line the results from our study, reporting that the phylum of Ascomycota was the most abundant fungal phyla in CD children. In contrast, other studies have reported that Basidiomycota was the dominant phylum in the gut mycobiome of CD patients (Li *et al.*, 2014; Liguori *et al.*, 2016; El Mouzan *et al.*, 2017; Sokol *et al.*, 2017). However, the abundance of Ascomycota and Basidiomycota could be related to the disease phenotype in IBD (Sokol *et al.*, 2017). Moreover, the differences in the results from gut mycobiota studies could be explained by type of samples collected from the recruited individuals (i.e. stool or mucosal). The biodiversity and richness of the oral mycobiome was assessed in tongue and buccal samples of IBD patients. Overall, the tongue was observed to have greater biodiversity compared to the buccal environment, based on the inverse Simpson index, whereas the Chao1 metric showed similar levels of species richness overall in tongue and buccal samples. The tongue groups showed the greatest differences in biodiversity, with CDT samples and UCT samples exhibiting greater species richness (Figure 5.3) and biodiversity compared to HCT groups. Buccal groups did not exhibit as great a difference in alpha diversity metrics, suggesting differences in the fungal community composition between oral regions. The increased oral mycobiome biodiversity in IBD is at odds with some previous studies demonstrating reduced biodiversity in the gut mycobiome in CD patients (Chehoud *et al.* 2015, Sokol *et al.* 2017). In contrast, other characterizations of

gut fungal microbiota in IBD patients showed no difference in alpha biodiversity between CD patients and normal individuals (Liguori *et al.* 2016, El Mouzan *et al.* 2017). However, the study by Li *et al.* (2014) reported that adults with CD had increased biodiversity of the gut mycobiome compared to the healthy subjects. The difference in outcomes from previous observations of the gut fungal microbiota in CD patients could be explained by several factors, including samples collected from either faeces or mucosa (ileal or colon), patient age, treatment stage of the CD patients and methods of fungal characterization (pyrosequencing, Illumina sequencing). These results indicate that the complexity of gut fungal community in IBD patients, making the difficulty in comparing dysbiosis in oral and gut mycobiota.

LEfSe analysis identified several trends in buccal and tongue samples from IBD patients that explain these changes in community structure. In general, the IBD patients examined here exhibit reduced levels of *C. dubliniensis*, the dominant oral mucosal yeast in children. The reduced levels of *C. dubliniensis* in IBD patients may be due to loss of oral bacteria that promote *C. dubliniensis* in the oral cavity (Figure 5.10) or increased antifungal immunity in the oral cavity. Reduced *C. dubliniensis* appears to be associated with increased levels of *C. albicans*, *Didymella gardeniae*, *Cladosporium cladosporiodes*, *Saccharomyces cerevisiae* and *C. krusei,* which likely accounts for the increased biodiversity in the IBD patients.

According to the Paris classification of IBD, considering the disease location as one of the three predominant parameters in disease classification, the L3L4 disease type is located throughout the GIT, including the oral cavity and represents a severe manifestation. In the current study the relationship between L3L4 disease type and the oral mycobiome of the three study groups were assessed. AMOVA analysis of data from tongue and buccal samples showed that Crohn's disease children with L3L4 disease did

not have communities significantly different from non-L3L4 patients. However, healthy subjects exhibited significant differences in community structure from L3L4 CD patients in both tongue samples (Jclass calculator) and buccal samples (Thetayc, and Bray-Curtis calculators), (Table 5-7). We also hypothesised that the oral mycobiome might be influenced by IBD severity. In this study, disease severity was grouped into four categories including inactive, mild, moderate, and severe. Analysis of tongue communities using AMOVA did not identify any difference in community structure using the Thetayc and Bray-Curtis calculators. However the Jaccard measurement reported a significant difference in community membership between HCT samples and moderate IBD (P=<0.001) and HCT versus severe IBD (P=0.032). Analysis of the buccal mycobiome data showed a significant difference between HCB samples and IBD samples in each of the four categories of disease severity. The Thetayc calculator indicated that inactive disease was not significantly different from healthy controls. However increasing disease severity (from mild to moderate and severe) was associated with increased significance in terms of changes in community structure assessed using AMOVA (P=0.011 to P<0.003). These data suggest that the mycobiome changes observed are related to IBD disease activity. The level of fungal DNA was calculated using real-time PCR. Our data revealed the similarity in fungal DNA density between CD children and HC subjects, indicating that IBD is not associated with significantly higher or lower carriage levels of fungi (Heisel *et al.* 2015).

5.5 Conclusion.

In the current study, we focused on defining the community structures of the oral fungal microbiota in children with Crohn's disease, particularly those adhering to tongue and buccal mucosa of the oral cavity. Our findings illustrate that the fungal microbiota of the tongue and buccal environments were dominated by the phylum Ascomycetes. Samples from IBD patients showed greater biodiversity and in the case of tongue samples exhibited greater fungal species richness compared to HCT subjects. These changes may be related to the reduced bacterial biodiversity in the oral cavity which may present new niches for oral fungi to colonise. The changes observed in fungal diversity and relative abundance in the oral cavity may have a relevant impact on CD aetiology, however this requires further investigation. This study provides preliminary data on the possible correlation between the changes in oral fungal community and early identification of CD. Further observations are required to better understand the alteration of oral mycobiota in CD children and to turn this information into new diagnostic approaches to identify Crohn's disease.

Chapter 6

Analysis of oral microbiome and mycobiome in IBD children following therapy

Chapter 6 Analysis of oral microbiome and mycobiome in IBD children following therapy

6.1 Introduction.

The oral microbiome and mycobiome communities are important factors in maintaining health and preventing disease. The majority of previous studies have focused on characterization of gut microbiome and mycobiota in health and disease. Some previous studies have characterized the oral microbiota in association with oral diseases (dental caries, periodontitis, and oral cancer). For example, a study by Ge *et al.* (2013) who investigated the oral microbiome in shallow and deep periodontal pockets (periodontitis) and Chalmers *et al.* (2015) who investigated oral microbiome changes associated with dental caries. The oral mycobiome was also assessed in relation to periodontitis by Peters *et al.* (2017) and a recent study by Fechney *et al.* (2019), studied the oral mycobiome in children with and without dental caries. Analysis of oral bacteria and fungi in relation to systemic diseases (inflammatory bowel disease, irritable bowel disease, obesity, HIV, systemic cancer) have been carried out in several previous studies, for instance analysis of the oral mycobiome in HIV patients Mukherjee *et al.* (2014) studied the relationships between the oral microbiota and pancreatic cancer (Michaud and Izard 2014). Recently, many studies have focused on chronic conditions which affect the gastrointestinal tract (IBD), which are likely due to an aberrant immune response to the microbiota and other factors (Docktor *et al.* 2012). IBD, including both CD and UC, has become one of the most studied human conditions linked to the gut microbiota (Kostic *et al.* 2014). In this Chapter, we compare the oral microbiome and mycobiome in children with Crohn's disease at two stages: baseline diagnosis and following therapy. To date, this is the first study to characterise the oral microbiota (bacteria and fungi) in treatment naïve children

with IBD and at a follow up stage, giving a dynamic view of the oral microbiota in CD patients. Oral bacterial and fungal microbiota were analysed at baseline, and following treatment regimens for IBD. In previous studies, differences in gut microbiota have been noted at baseline and over time following therapy (Schirmer *et al.* 2018). Management of inflammatory bowel disease (IBD) depends on characterising disease aggressiveness and monitoring response to treatment (Sartor and Wu 2017). In the present study, the recruited children were sampled at baseline, then after 2 years further samples were collected from the same patients. During the treatment period the study population received various therapies, depending on patient's status. This may have included antibiotic therapy (flucloxacillin), antimicrobial therapy (metronidazole), supplements (iron, vitamin D, multi-vitamins, SandoK [potassium]), anti-inflammatories (Infliximab, Sulfasalazine, Salofalk, Asacol, Becotide, Lansaprazole) and intensive nutritional supplements (e.g. scandishake drinks) (Appendix 5). The treatment of Crohn's disease is rapidly changing. A review by Cheifetz (2013) found that immunomodulators and biologics are now the preferred treatment options for Crohn's disease. A recent study showed the effectiveness and safety of the TNF-alpha blocker Infliximab in CD and UC (Gheorghe *et al*. 2019). On the other hand, the efficacy and safety of antibiotics for maintenance of remission in CD are unclear and need further investigations (Townsend *et al.* 2019). Changes in bacterial and fungal profiles were observed following therapy, suggesting that the alteration in microbiota occurred as a response to the therapeutic agents and reduced disease severity.

6.2 Results.

In order to investigate the alterations in oral bacterial and fungal communities in IBD patients between the time of diagnosis and follow up analysis after treatment, twenty-one patients from the initial study provided a second swab from the dorsum tongue during the period between May to October 2017 (CD=18, UC=3) Table 6-1. These patients had received a variety of treatments based on their clinical presentation and all had exhibited an improvement in their clinical condition based on their severity scores (Appendix 4). To explore the oral microbial and mycobiome features of Crohn's disease in children at baseline and following therapy, 16S rDNA gene and ITS2 barcode sequencing were performed.

Table 6-1 Follow up IBD patients showing enrolment, follow up date and treatment period.

Study Number	sample ID	Diagnosis	Age	Gender	Date of Enrolment	Date of Follow up	Treatment period
356	4TFU	Colitis	10	M	06/03/2015	14/07/2017	2 years
417	38TFU	Colitis	12	F	2015	16/06/2017	2 years
460	41TFU	Colitis	12	M	07/12/2015	02/02/2018	3 years
298	31TFU	Crohn's	8	M	09/10/2015	22/09/2017	2 years
320	12TFU	Crohn's	14	M	17/04/2015	13/10/2017	2 years
347	3TFU	Crohn's	12	M	13/02/2015	10/11/2017	2 years
351	1TFU	Crohn's	13	M	20/02/2015	2017	2 years
357	20TFU	Crohn's	12	M	25/03/2015	05/07/2017	2 years
362	13TFU	Crohn's	10	M	24/03/2015	16/06/2017	2 years
363	5TFU	Crohn's	12	M	26/03/2015	31/08/2017	2 years
373	19TFU	Crohn's	8	M	14/04/2015	21/09/2017	2 years
389	18TFU	Crohn's	13	M	2015	16/06/2017	2 years
411	30TFU	Crohn's	9	F	27/07/2014	17/04/2018	4 years
435	33TFU	Crohn's	15	M	15/09/2015	29/08/2017	2 years
459	40TFU	Crohn's	14	M	04/12/2015	02/02/2018	3 years
667	45TFU	Crohn's	15	M	16/06/2017	20/10/2017	4 months
673	46TFU	Crohn's	12	M	30/06/2017	08/09/2017	3 months
679	47TFU	Crohn's	13	M	14/07/2017	13/10/2017	3 months
717	48TFU	Crohn's	11	F	09/10/2017	2018	3 months
726	50TFU	Crohn's	12	F	06/11/2017	21/12/2017	1 month
764	54TFU	Crohn's	10	M	07/03/2018	15/05/2018	2 months

6.2.1 Microbial tongue follow up.

The tongue samples at baseline and in the follow-up period were analysed. 16S sequencing data was used to analyse microbial diversity and species richness in newly diagnosed IBD patients before treatment (IBD) and in the follow up group (IBD.FU).

6.2.1.1 Follow up analysis at Phyla level.

The 16S sequencing data of IBD and IBD.FU samples revealed 8 dominant phyla, as expected. When we compared the two tongue groups (IBD and IBB.FU), our data showed that the follow up group (IBD.FU) exhibited increased Actinobacteria and reduced levels of Bacteroidetes compared to the same samples before treatment (IBD; Figure 6.1). Metastats analysis was carried out to identify differentially abundant tongue phyla between IBD and IBD.FU groups. This analysis revealed no significant difference in the levels of the 8 most dominant phyla.

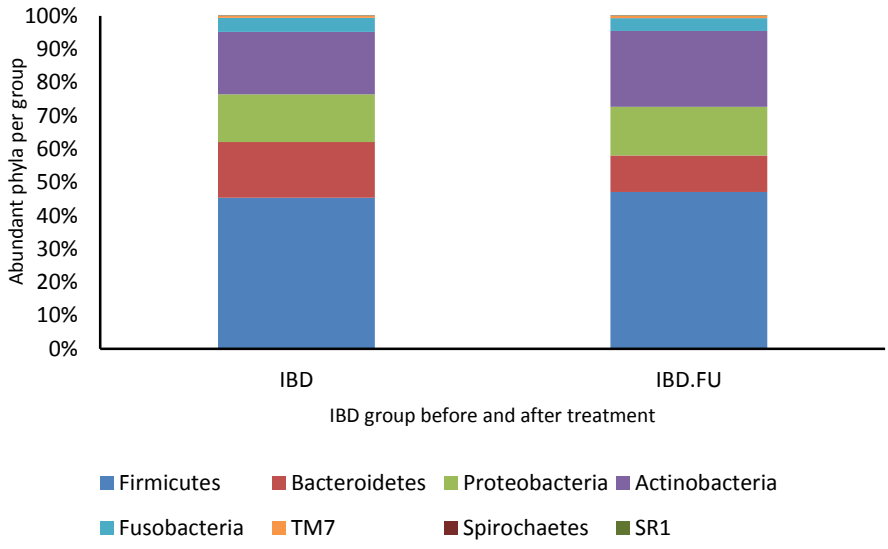


Figure 6.1 Comparison of phyla distribution in the two IBD groups (IBD and IBD.FU).

6.2.1.2 Genus level analysis.

After normalisation of the data by subsampling, our data showed *Streptococcus, Prevotella, Veillonella, Actinomyces, Rothia, Granulicatella* and *Neisseria* were the dominant bacterial genera in both IBD groups (IBD and IBD.FU), while other genera, including *Porphyromonas, Fusobacterium, Leptotrichia,* and *Gemella* were less abundant (Figure 6.2). When we compared the two IBD groups, our findings indicated that following therapy, the follow up samples had greater levels of *Streptococcus, Veillonella, Rothia, Neisseria, Porphyromonas* and *Haemophilus* compared to IBD group. However, these differences were not statistically significant (Metastats P >0.05). However, Metastats did identify a significant reduction in the level of Enterobacteriaceae (P=0.012), *Bergeyella* spp. (P=0.003) and an unclassified Bacteriodales species (OTU016; P <0.0001) following treatment.

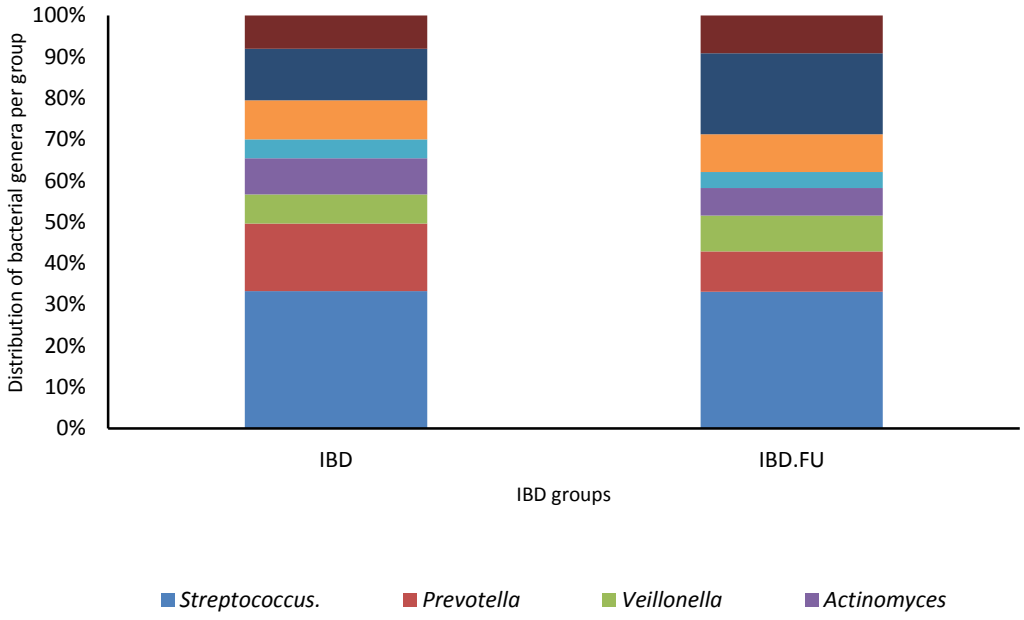


Figure 6.2 The abundant tongue fungal species in the follow up and treatment naïve IBD samples.

6.2.1.3 OTU level analysis.

Sequences with 2% or less nucleotide sequence differences were classified as OTUs. The total number of OTUs identified in all tongue samples was 1,617. Following normalisation by subsampling the number was reduced to 1188 OTUs. 20 OTUs accounted for more than 90% of sequence reads in tongue samples (Figure 6.3).

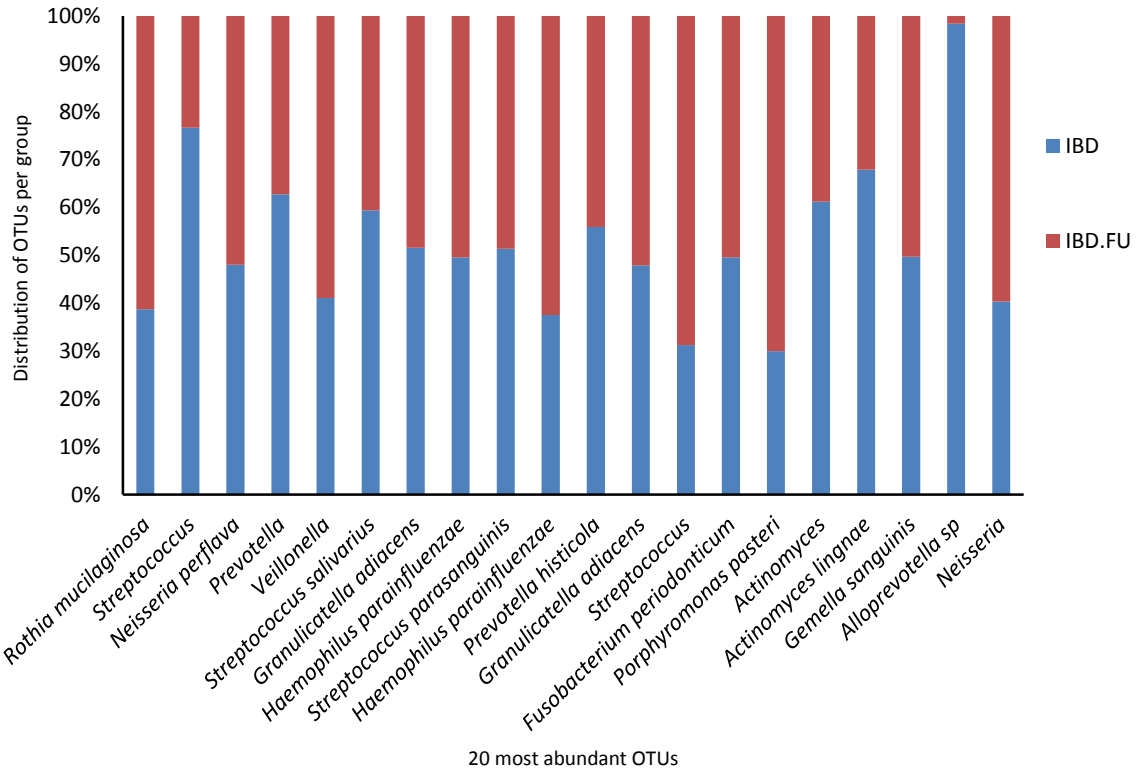


Figure 6.3 The distribution of the 20 most dominant OTUs between tongue samples of both IBD groups (IBD and IBD.FU).

6.2.1.4 Alpha diversity measurements.

Species richness within the follow up microbial environment was measured, using the Inverse Simpson and Shannon alpha diversity measurements.

6.2.1.4.1 Inverse Simpson Index.

The inverse Simpson and Shannon indices of biodiversity were calculated for follow up samples. The average inverse Simpson index and Shannon index values for each group show that IBD.FU and IBD samples exhibited similar biodiversity, while the number of OTUs were different between the two groups (Table 6-2).

Table 6-2 The minimum and maximum numbers of OTUs in tongue samples of all IBD and IBD.FU groups and Shannon and Inverse Simpson values.

SITE	Study groups	Average OTUs	Inverse Simpson	Shannon	Minimum OTU number	Maximum OTU number
Tongue	IBD.FU	119.07	11.93	2.99	73.57	173.23
Tongue	IBD	105.95	11.64	2.90	35.55	162.607

6.2.1.4.2 Rarefaction curve.

Species richness was analysed in follow up samples. Data from tongue samples showed that the follow up group (IBD.FU) had greater bacterial species richness compared to treatment naïve children (IBD). On the other hand, healthy children had greater species richness compared to all IBD children (Figure 6.4).

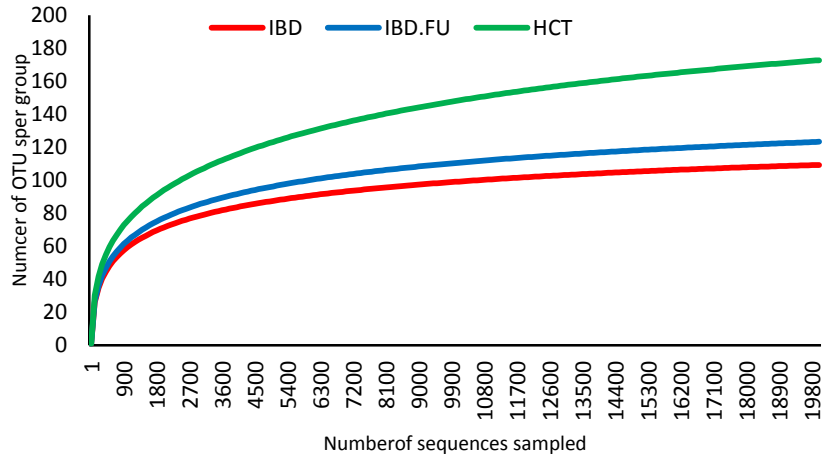


Figure 6.4 Rarefaction curve shows microbial species richness of IBD and IBD.FU groups and healthy subjects.

6.2.1.5 Beta diversity measurements.

Beta-diversity measurements were used to compare microbial community structure between IBD children before and after treatment.

6.2.1.5.1 Phylogenetic analysis of follow up samples.

Phylogenetic methods were used to determine the relationships between IBD and IBD.FU samples. Each of the three calculators (The Yue and Clayton [Thetayc], the Jaccard [Jclass] and the Bray-Curtis) were used to generate distance matrices, showing the relatedness of all samples. Phylogenetic trees were generated in Mothur using these calculated distance values to determine the branch lengths separating different microbial communities. The Parsimony statistical test was used to determine if samples from different IBD groups separated and clustered at different branches in the tree. Analysis of trees generated with Thetayc and Bray-Curtis calculators show that communities from

the treatment naïve children with IBD (IBD) were significantly different in samples from same IBD children after treatment (IBD.FU). Analysis of trees generated with the Jaccard index did not yield any statistically significant differences (Table 6-3).

Table 6-3 The results Parsimony tests examining the phylogenetic relationships in community structure calculated using the Yue and Clayton (Thetayc), the Jaccard (Jclass) and the Bray-Curtis calculators.

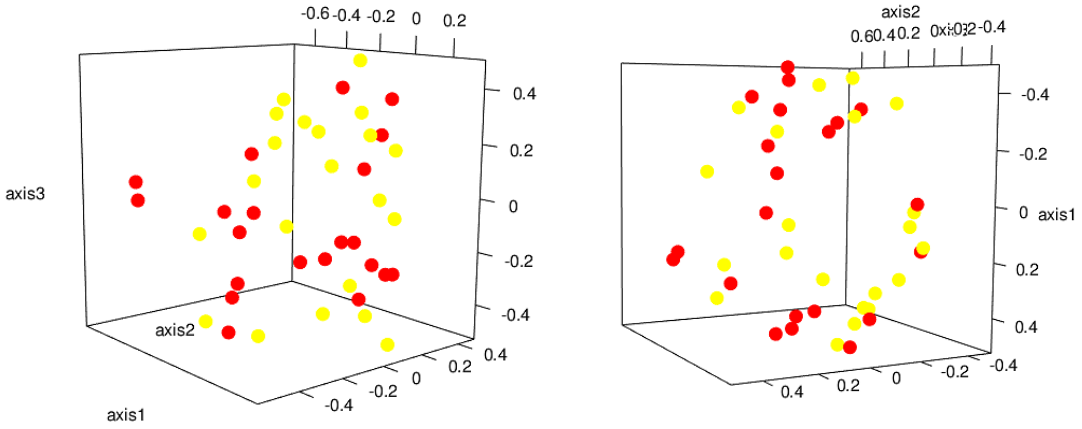
Groups	P value Bray-Curtis	P value Thetayc	P value Jaccard
Healthy vs IBD	0.003**	<0.001**	<0.001**
Healthy vs IBD.FU	0.024*	0.008**	0.002**
IBD vs IBD.FU	0.016*	0.022*	0.907

6.2.1.5.2 Non-metric Multidimensional Scaling (NMDS).

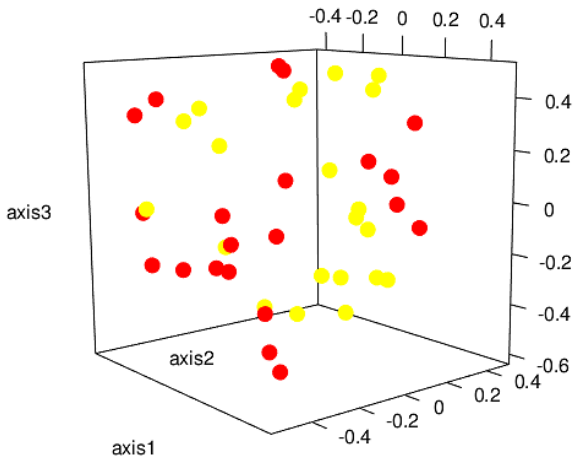
NMDS was used to graphically represent the distance between different tongue samples. Distances were calculated using Thetayc, Jclass, and Bray-Curtis calculators. Analysis of Molecular Variance (AMOVA) was carried out to determine if the separation between the treatment naïve and follow up groups in the NMDS plot (Figure 6.5). Using the Thetayc, the Jaccard and the Bray-Curtis calculators, AMOVA identified a significant difference in community structure between tongue samples of IBD.FU children and healthy control children (HCT), respectively (P=0.0032; P=0.0013; P=0.0002). However, differences in community structure between IBD children before treatment and after treatment (IBD.FU) were not significantly different compared to the difference between healthy subjects and IBD children (Table 6-4).

Table 6-4. Comparison of tongue microbiome community structures in Healthy, IBD and IBD Follow up communities using the Thetayc, Jclass, and Bray-Curtis calculators using AMOVA.

Groups	**Bray-Curtis P value**	**Thetayc P value**	**Jclass P value**
All groups	0.0001**	0.0001**	<0.0001**
Healthy vs IBD	<0.0001**	<0.0001**	<0.0001**
Healthy vs IBD.FU	0.0013**	0.0032**	0.0002**
IBD vs IBD.FU	0.0709	0.0892	0.0746



(a) **(b)**



(c)

Figure 6.5 NMDS plots of tongue samples from IBD (red), and IBD.FU (yellow) IBD groups generated with distance matrices generated with (a) Thetayc (b) Bray-Curtis and (c) Jclass calculators.

HOMOVA analysis was also carried out on the same groups (Healthy, IBD, and IBD Follow up) in order to determine if there were differences in the levels of variability in the communities from the different groups. Using the Thetayc, and the Bray-Curtis calculators, HOMOVA identified significantly greater variation in community structure in tongue samples of IBD children before treatment (IBD) and children with IBD at follow up (IBD.FU) (Table 6-5).

Table 6-5 Comparison of tongue microbiome community structures in Healthy, IBD, and IBD Follow up. Communities were compared using the Thetayc and Bray-Curtis calculators and differences in community structure were identified using HOMOVA.

Groups	Bray-Curtis P value	Thetayc P value
All groups	<0.001**	<0.001**
Healthy vs IBD	<0.001**	<0.001**
Healthy vs IBD. FU	<0.001**	0.002**
IBD vs IBD. FU	0.025*	0.047*

6.2.1.6 LEfSe analysis of follow up samples.

To identify species and OTUs enriched in each group, LEfSe analysis was carried out on tongue samples from the two IBD study groups (IBD and IBD.FU). Analysis between IBD children before treatment (IBD) and after receiving treatment (IBD.FU) indicated that IBD treatment naïve patients were enriched in Actinobacteria (Act) and Proteobacteria (Pro) including *Actinomyces lingnae* (Act), *Actinomyces massiliensis*

(Act), *Actinomyces georgiae* (Act), *Rothia mucilaginosa* (Act), *Haemophilus* spp. (Prot) and the *Enterobacteriales* (Prot). Furthermore, the follow up group (IBD.FU) showed enrichments in the phyla Firmicutes, including *Streptococcus* spp. and *Veillonella* spp. (Figure 6.6).

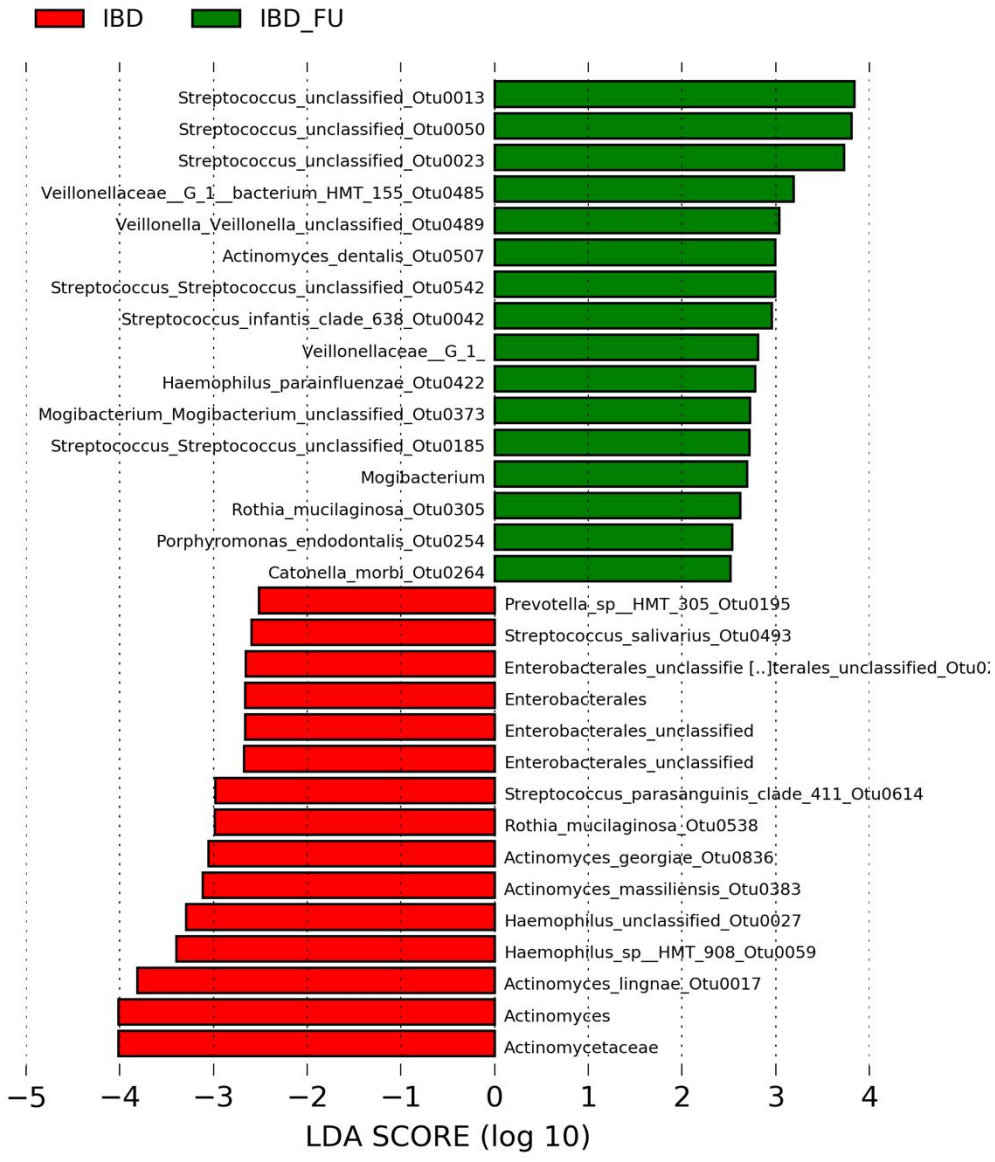


Figure 6.6 Results of LEfSe analysis showing significant enrichments in tongue samples from treatment naïve IBD patients (IBD) and follow up samples (IBD.FU), ranked in significance based on the linear discriminatory analysis (LDA) score.

Next, samples were categorised into two groups based on disease severity at baseline (mild to moderate or severe) and both groups were compared with their respective follow up samples. A number of enrichments were identified in follow-up samples from patients diagnosed with severe disease at baseline, including several streptococcal OTUs (OTU0023 and OTU0050) and a number of other low abundance OTUs. Patients with mild-moderate disease at follow-up exhibited increased levels of *Veillonella* sp. OTU005, an OTU identified as partly responsible for the oral dysbiosis in IBD (Chapter 3). Baseline mild-moderate samples exhibited higher levels of *Actinomyces* spp. (Figure 6.7).

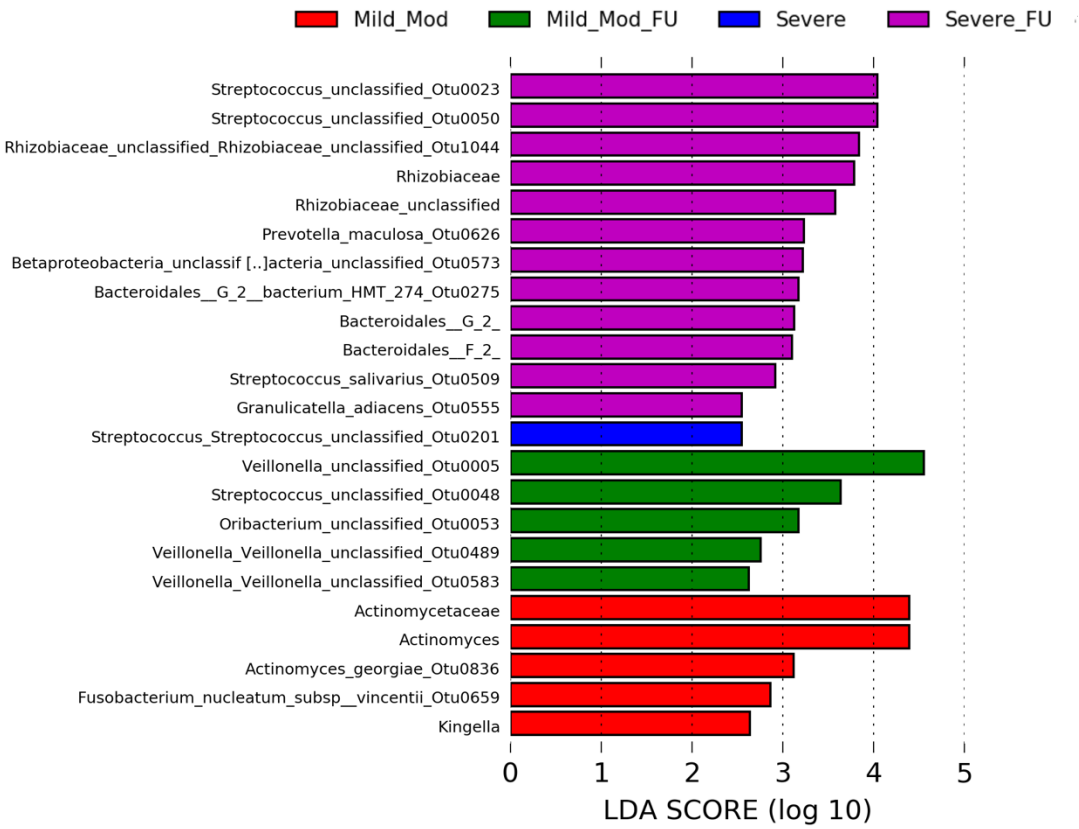


Figure 6.7 Results of LEfSe analysis showing significant enrichments in follow up samples (IBD.FU) based on disease severity, and ranked in significance based on the linear discriminatory analysis (LDA) score.

6.2.1.7 Abundance of bacterial species in follow up samples.

Plots showing the abundance of significantly enriched OTUs in each group were generated using the LEfSe package (Hutlab Galaxy server). Each plot shows the abundance of the OTU in each sample analyzed in IBD and IBD.FU groups. These plots show that *A. lignae* (OTU0017) exhibited reduced abundance following treatment, most noticeably in those with severe IBD. In addition, the *Enterobacteriales* could be identified at low abundance in 7 children at baseline, but only 1 child post treatment. This OTU is represented by 8 sequences with greatest similarity (~99%) to *Serratia marcescens*, *Klebsiella aerogenes* and *Klebsiella pneumonia* (Figure 6.8). *A. lignae* was previously shown to be Crohn's disease enriched (Figure 3.18).

Actinomyces lignae (OTU0017) Enterobacteriales

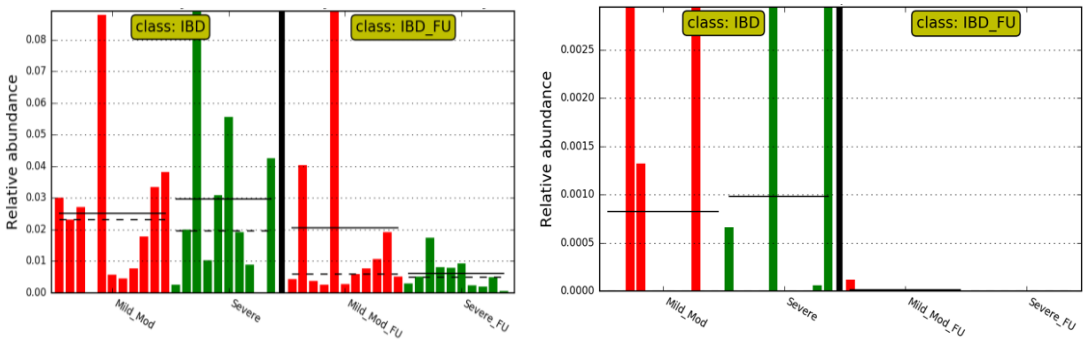
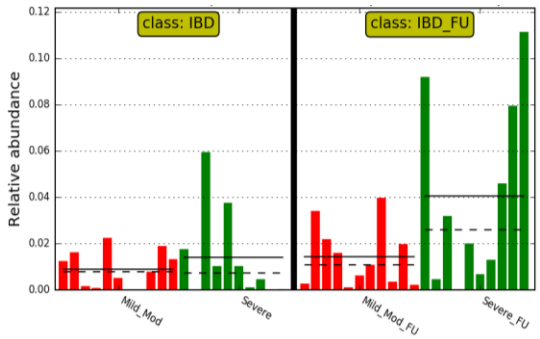


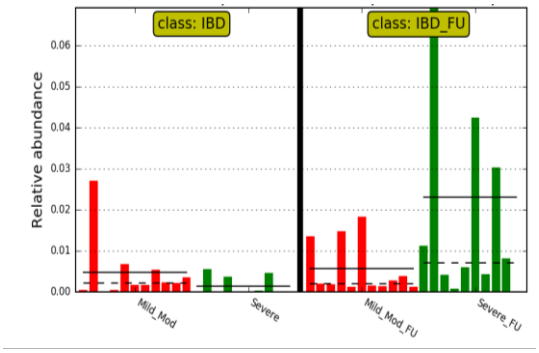
Figure 6.8 Plots showing the abundances of species identified as reduced in tongue samples from IBD.FU patients. Mean values are represented by the solid line, median values by the dotted line.

Next we examined plots of organisms that LEfSe indicated exhibited increased abundance following treatment of IBD. Several streptococcal OTUs were identified in Figure 6.6 and BLAST analysis of the OTU sequences showed that three of these OTUs corresponded to *S. infantis* (Figure 6.9).

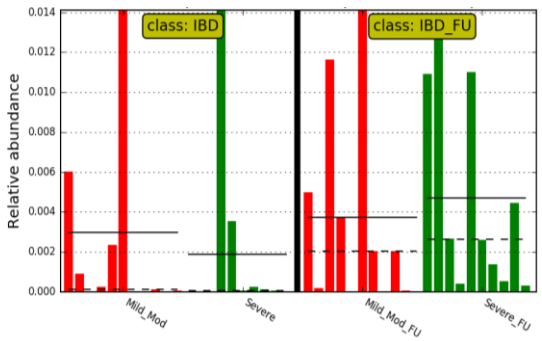
Streptococcus infantis (OTU0013)



Streptococcus infantis (OTU0023)



Streptococcus infantis (OTU0042).



Streptococcus sanguinus (OTU0050).

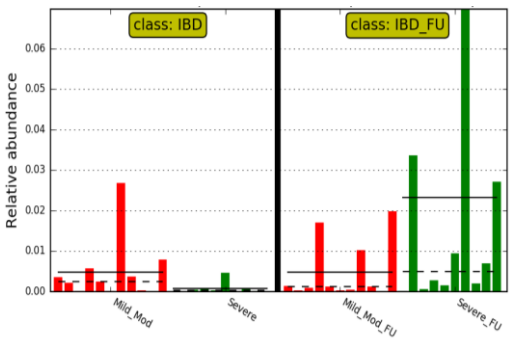
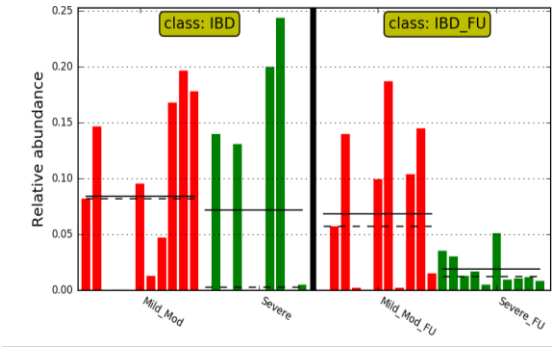


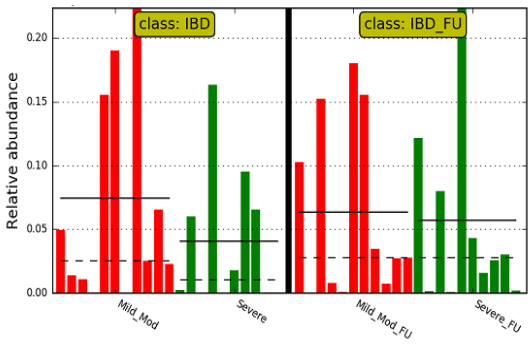
Figure 6.9 Abundance of streptococcal OTUs identified in LEfSe analysis in IBD patients and post treatment follow-up samples (IBD.FU). Mean values are represented by the solid line, median values by the dotted line.

The follow up samples (IBD.FU) from patients exhibiting mild to moderate disease were also enriched in *Veillonella* sp. (OTU0005), one of the organisms shown to be depleted in IBD (Figure 3.17). We next examined plots for some of the other IBD depleted OTUs identified in (Chapter 3). Of these, the two major *H. parainfluenza* OTUs (OTU0008 and OTU0010) when taken together showed increased abundance of *H. parainfluenza* in follow up samples. On the other hand *Pr. melaninogenica* OTU0003, *N. perflava* OTU0004 and *Fusobacterium* sp. OTU0014 did not exhibit obviously increased abundance, although their occurrence was more evenly distributed in the follow up patients (Figure 6.10).

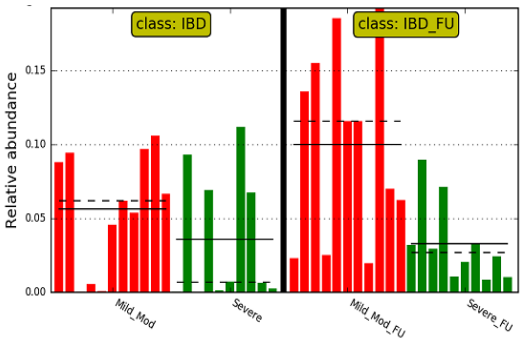
Pr. melaninogenica (OTU0003)



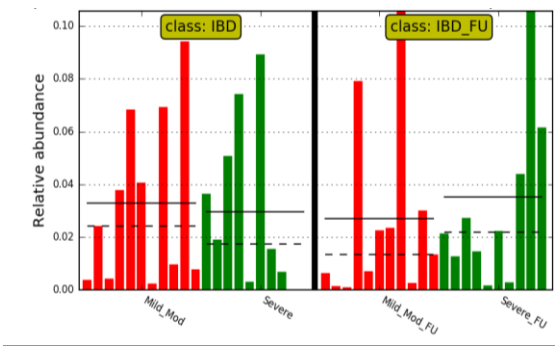
Neisseria perflava (OTU0004)



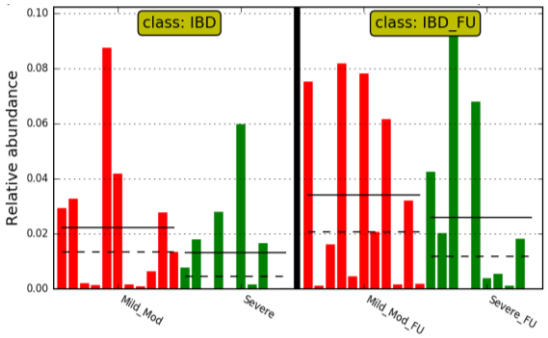
Veillonella sp. (OTU0005)



H. parainfluenza (OTU0008)



H. parainfluenza (OTU0010)



Fusobacterium sp. (OTU0014)

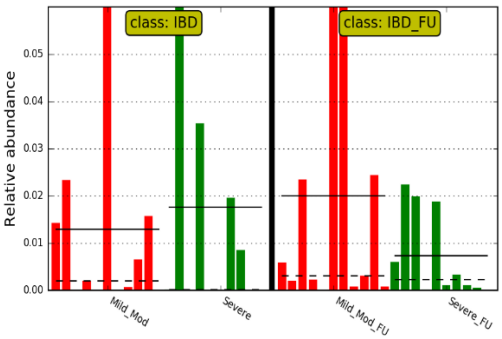


Figure 6.10 Abundance of selected OTUs in IBD patients and post treatment follow-up samples (IBD.FU). Mean values are represented by the solid line, median values by the dotted line.

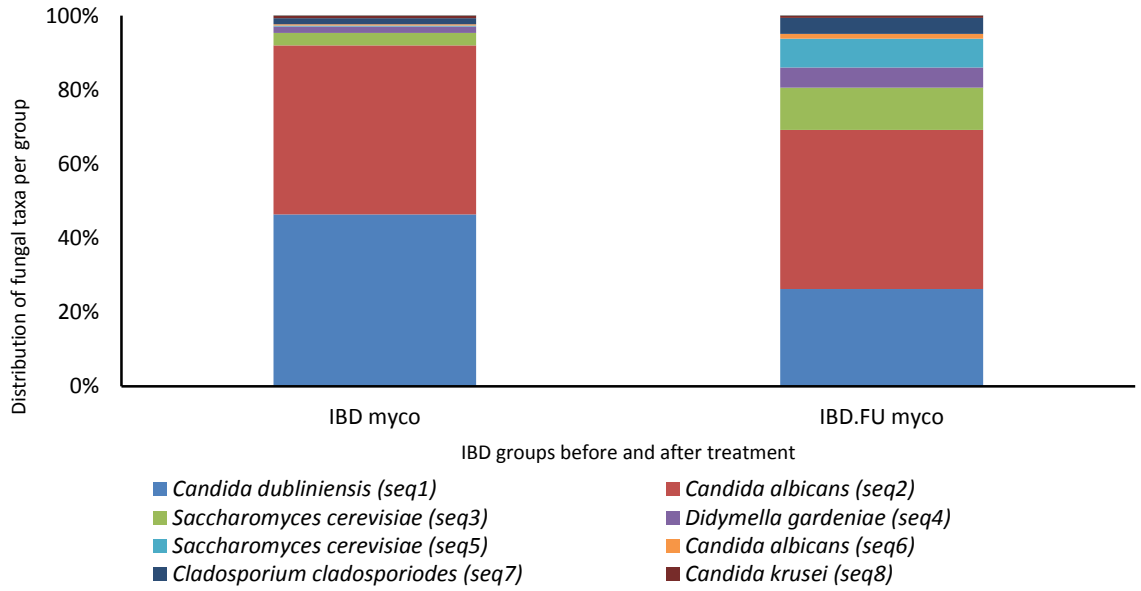
6.2.2 Tongue mycobiome following therapy.

The oral mycobiome of IBD children before and after treatment were analysed. The ITS2 region of fungal rDNA was amplified and sequenced. The correlation between the two IBD groups (IBD and IBD.FU) were assessed at taxonomic different levels.

6.2.2.1 Analysis of mycobiome sequences of follow up samples.

Sequencing data from the amplified ITS2 region revealed the dominant fungal taxon in IBD and IBD.FU tongue samples. The 10 most abundant fungi in both IBD samples were *Candida dubliniensis* (Seq.1), *Candida albicans* (Seq.2, Seq.6, Seq.10, Seq.16), *Saccharomyces cerevisiae* (Seq.3, Seq.5), *Didymella gardeniae* (Seq.4), *Cladosporium cladosporiodes* (Seq.7), *Candida krusei* (Seq.8) and *Candida zeylanoides* (Seq.9) (Figure 6.11a). When we compared the two tongue sample groups (IBD and IBD.FU), the data showed that *Candida dubliniensis* was decreased in the follow up group. However, the follow up group (IBD.FU) exhibited increased levels of several fungal taxa which were shown to be members of healthy mycobiota of the oral cavity, including *Saccharomyces cerevisiae, Didymella gardenia, Cladosporium cladosporiodes* and *Candida albicans* (Seq.6) (Figure 6.11b).

(a)



(b)

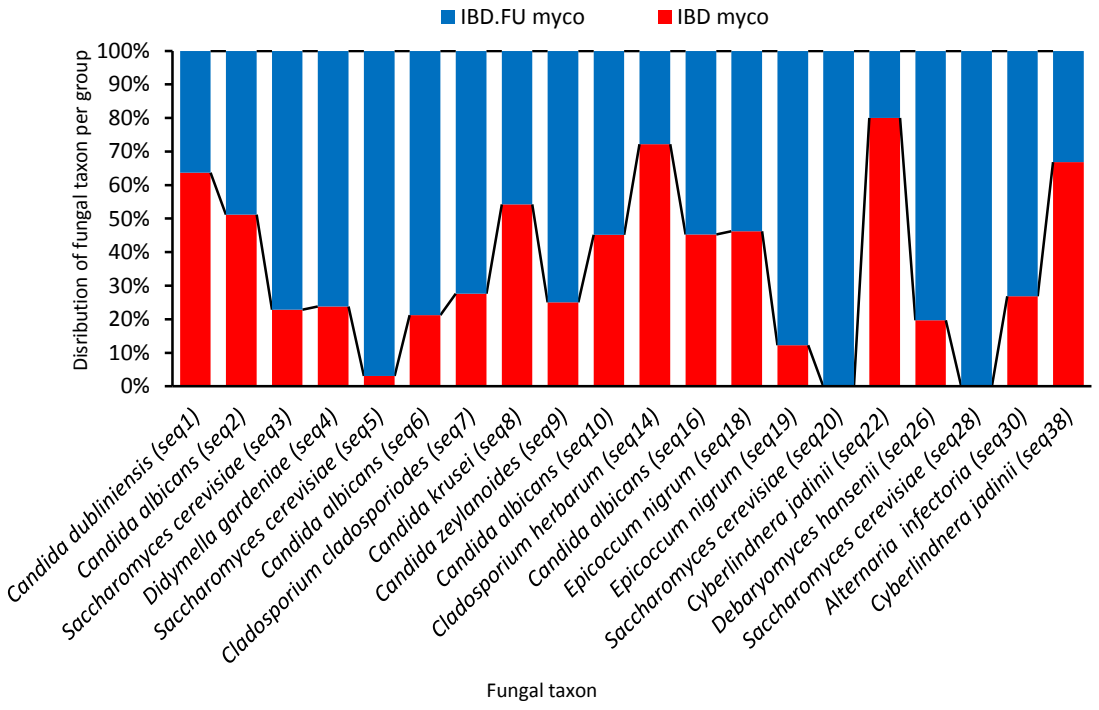


Figure 6.11 (a) Distribution of the fungal microbiota in follow up samples at sequence level. (b) Comparison of mycobiome distribution in the two IBD groups (IBD and IBD.FU).

6.2.2.2 Alpha diversity measurements.

Fungal species richness within the IBD follow up microbial environment was measured, using alpha diversity measurements, including Inverse Simpson and Chao1 values.

6.2.2.2.1 Inverse Simpson Index.

The inverse Simpson and Chao1 values of biodiversity were calculated for treatment naïve children with IBD and the IBD follow up samples. The average of inverse Simpson index and values for each group show that IBD and IBD.FU samples exhibited similar biodiversity. The species richness measured using the Chao1 diversity index revealed a greater fungal species richness in IBD.FU compared to children before follow up (IBD), while the number of OTUs were different between the two groups. All IBD children (including all IBD patients with no follow up samples) exhibited similar fungal biodiversity and species richness compared to IBF.FU. When we compared IBD.FU group with children and adult healthy subjects, Chao1 measurement of richness showed greater fungal richness with healthy individuals compared to IBD.FU children. Furthermore, similar fungal biodiversity were seen between HC group and IBD.FU children (Table 6-6).

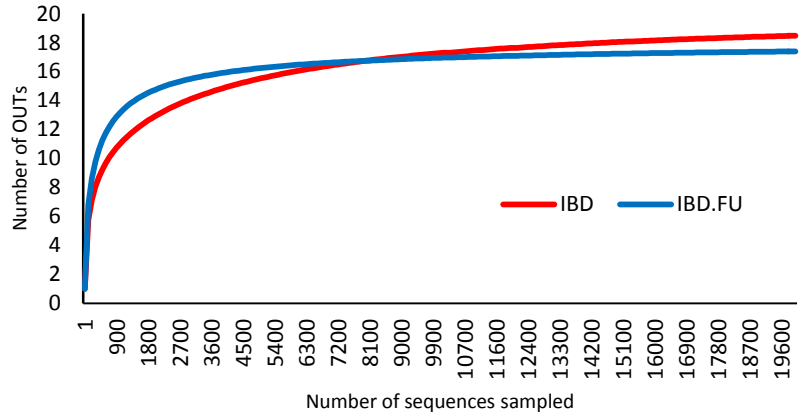
Table 6-6 The minimum and maximum numbers of fungal OTUs in tongue samples of all IBD and IBD.FU groups and Chao1 and Inverse Simpson values.

SITE	Study groups	Average OTUs	Inverse Simpson	Chao1	Minimum OTU number	Maximum OTU number
Tongue	IBD	15.53	2.22	17.55	4.18	36.76
Tongue	IBD.FU	16.23	2.36	16.79	9	28.74
Tongue	Healthy subjects	14.79	2.13	17.13	5.57	43.86

6.2.2.2.2 Rarefaction curve.

Species richness within the fungal community of IBD children before and after treatment was analysed. Data from tongue samples show that the treatment naïve children (IBD) was more enrichment in fungal species compared to follow up group (IBD.FU), Figure 6.12a. On the other hand, healthy children had similar species richness to IBD children before treatment (IBD.FU), Figure 6.12b.

(a)



(b)

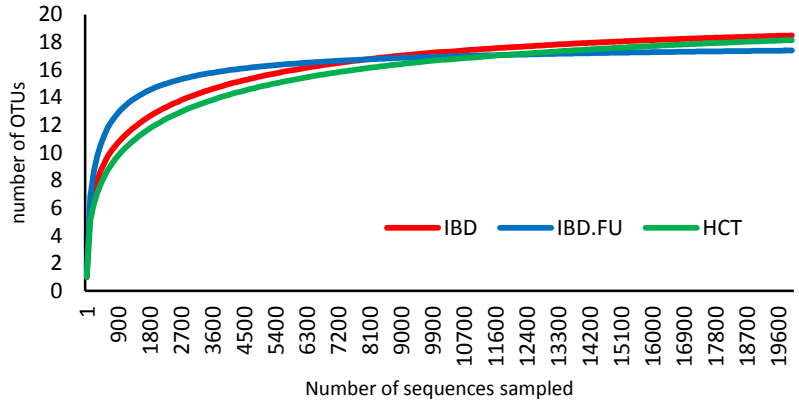


Figure 6.12 (a) Rarefaction curve shows microbial species richness of IBD and IBD.FU groups. (b) Comparing the species richness between groups of IBD and healthy subjects.

6.2.2.3 Beta diversity measurements.

Beta-diversity measurements were used to compare microbial community structure between IBD children before and after treatment.

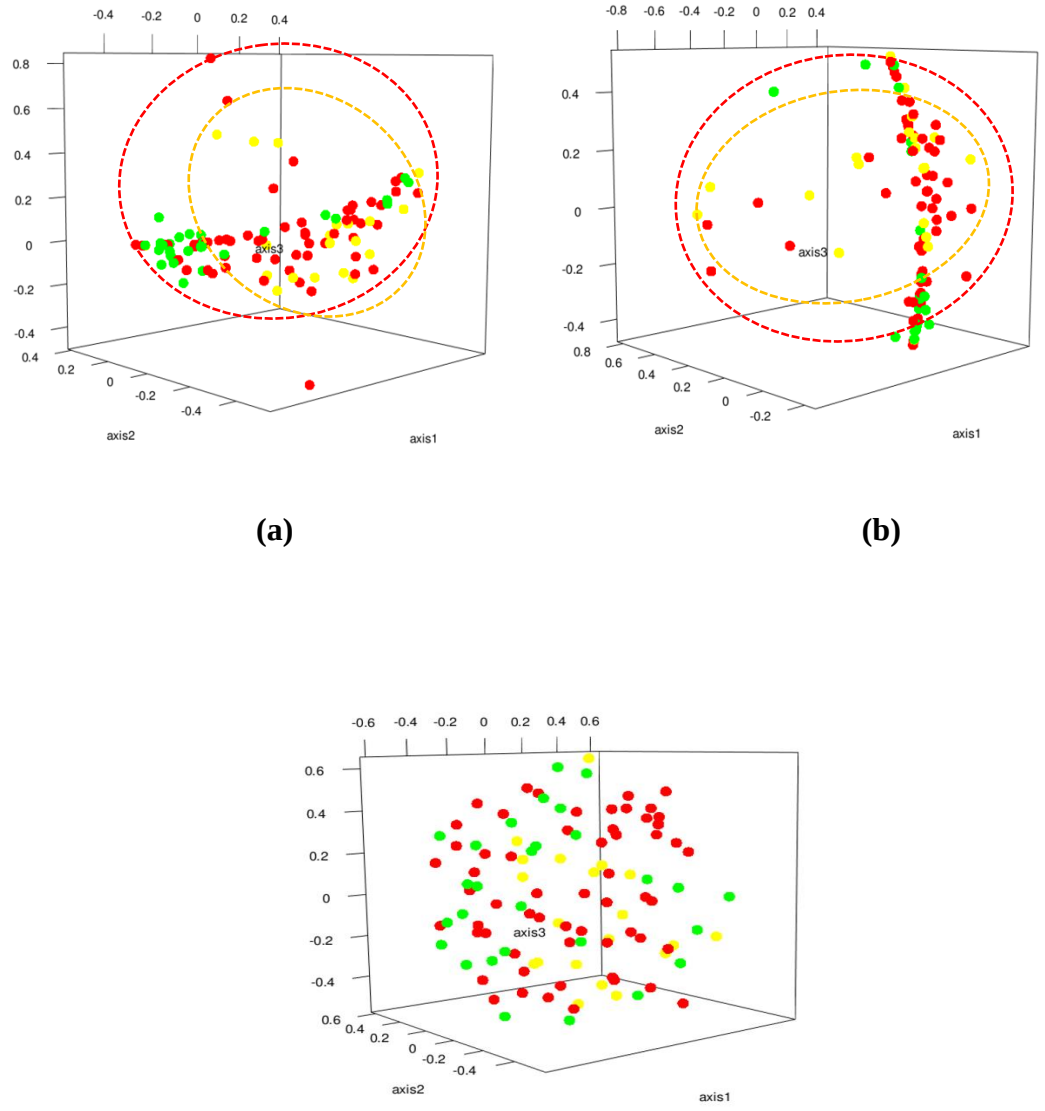
6.2.2.3.1 Non-metric Multidimensional Scaling (NMDS).

NMDS was used to graphically represent the distances between different tongue samples of IBD and IBD.FU groups. Distances were calculated using Thetayc, Jclass, and Bray-Curtis calculators. Analysis of Molecular Variance (AMOVA) was carried out to determine if the separation between the fungal communities of treatment naïve and follow up groups in the NMDS plot was significant (Figure 6.13). Using the Thetayc, the Jaccard and the Bray-Curtis calculators, AMOVA identified a significant difference in fungal community structure between tongue samples of IBD and IBD.FU groups (P=0.02; P=0.019; P=0.03, respectively). Both IBD and IBD.FU populations were significantly different from healthy children ($P < 0.01$).

6.2.2.4 LEfSe analysis of follow up samples.

To identify the fungal species enriched in each group, LEfSe analysis was carried out on tongue samples from the two IBD study groups (IBD and IBD.FU). Analysis between IBD children before treatment (IBD) and after receiving treatment (IBD.FU) indicated that IBD treatment naïve patients were enriched in several fungal taxon of the phyla Ascomycota (A) and Basidiomycota (B), *Candida dubliniensis* (seq1) (A), *Botrytis caroliniana* (seq23) (A), family *Tremellales* (B), *Cryptococcus* spp. (B), *Trechispora* spp. (B) and class *Leotiomycetes* (A). However, the follow up group (IBD.FU) showed

enrichment only with Ascomycota, which included the class *Dothideomycetes* (order pleosporales) represented by *Didymella gardenia*. Additionally *Candida albicans* (seq6), *Aspergillus_fumigatus* (seq143) and *Fusarium* spp. were also identified (Figure 6.14).



(a) **(b)**

(c)

Figure 6.13 NMDS plots of tongue samples from IBD (red), IBD.FU (yellow) and Healthy children (green) with distance matrices generated with (a) Thetayc (b) Bray-Curtis and (c) Jclass calculators. Red and green circles are used to show healthy and IBD groups and are for visualisation purposes only.

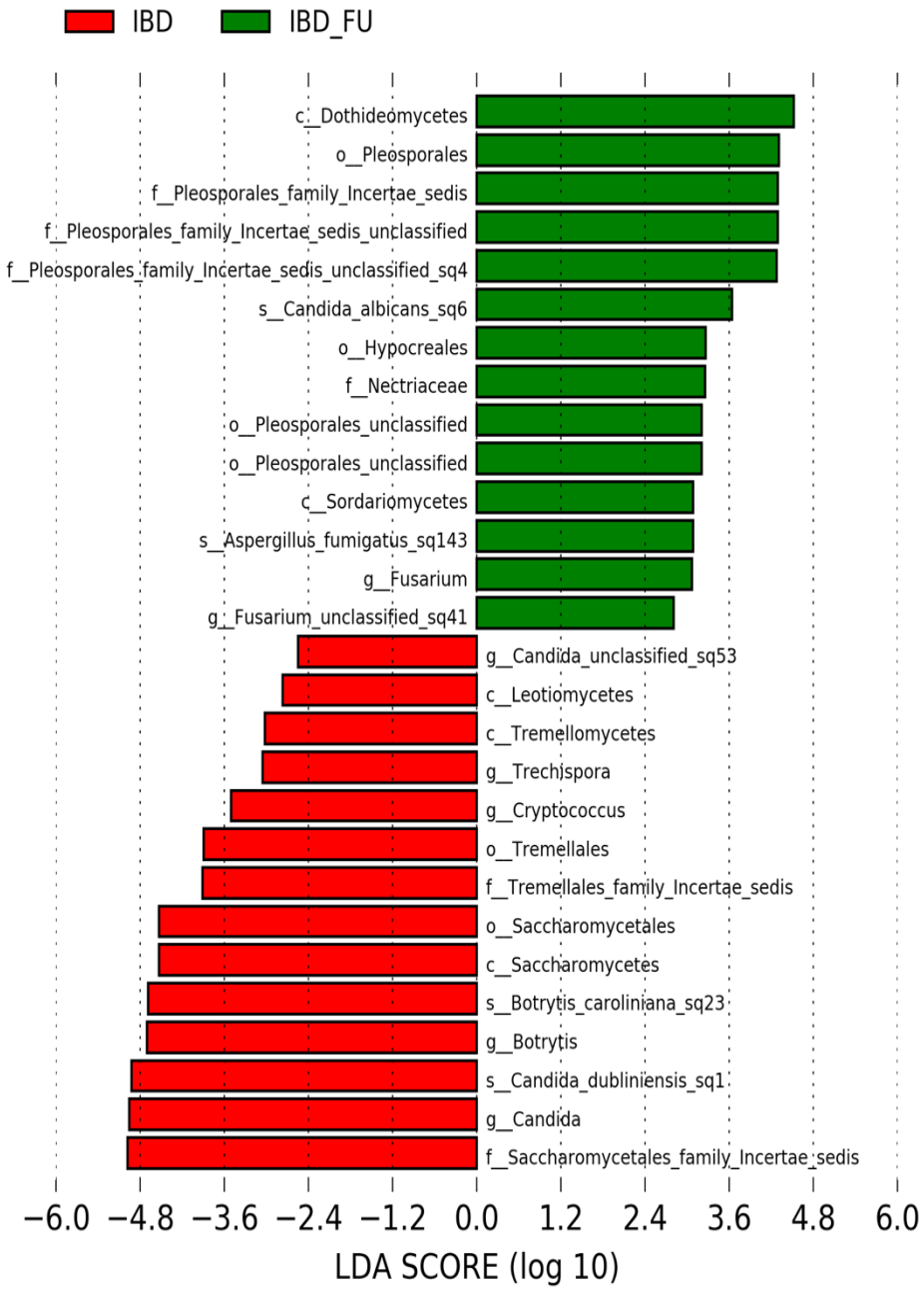
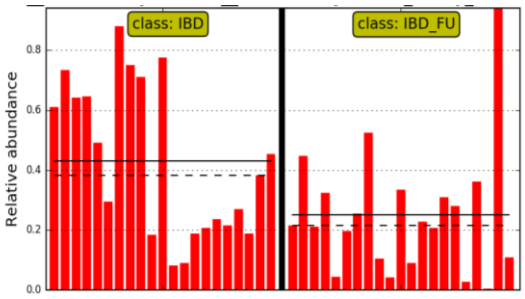


Figure 6.14 Results of LEfSe analysis showing significant enrichments in tongue mycobiota of IBD samples, treatment naïve (IBD) and tongue follow up samples (IBD.FU), ranked in significance based on the linear discriminatory analysis (LDA) score.

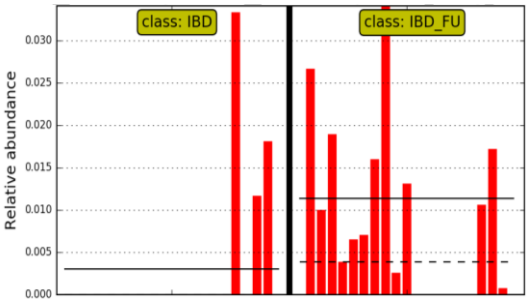
6.2.2.5 Abundance of mycobiome species in follow up samples.

Plots showing the abundance of significantly enriched OTUs in each group were generated using the LEfSe package (Hutlab Galaxy server). Each plot shows the abundance of the OTU in each sample analyzed in the IBD and IBD.FU groups (Figure 6.15). *C. dubliniensis* (Seq1) was reduced in follow up samples which showed higer levels of *C. albicans* (Seq6). *Didymella gardenia* (Seq4) and *Fusarium* spp. also showed higher levels in IBD.FU samples.

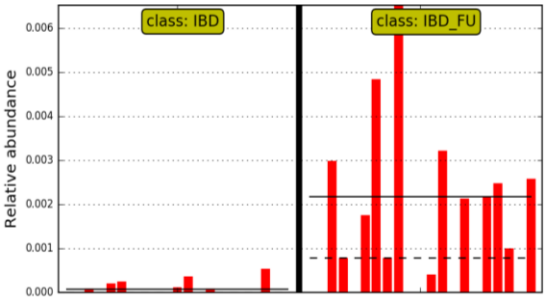
C. dubliniensis (Seq1)



C. albicans (Seq 6)



Fusarium spp.



Didymella gardenia (Seq 4)

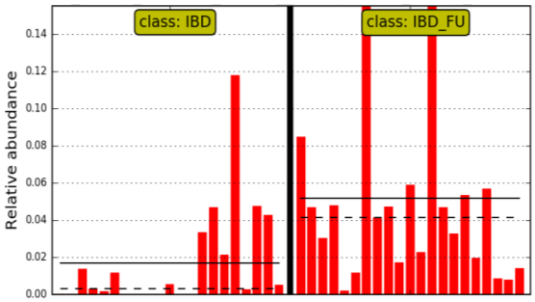


Figure 6.15 Plots showing the abundances of fungal taxon identified as enriched in tongue samples from IBD and IBD.FU patients. Mean values are represented by the solid line, median values by the dotted line.

6.2.3 ITS2 Real Time PCR analysis of fungal load in follow up samples.

The fungal density in the CDT. FU group was assessed using real time quantitative PCR. Our data revealed that the level of fungal DNA concentration in follow up samples was similar to CDT samples at baseline (Figure 6.16). A paired T test found no significant difference in fungal carriage.

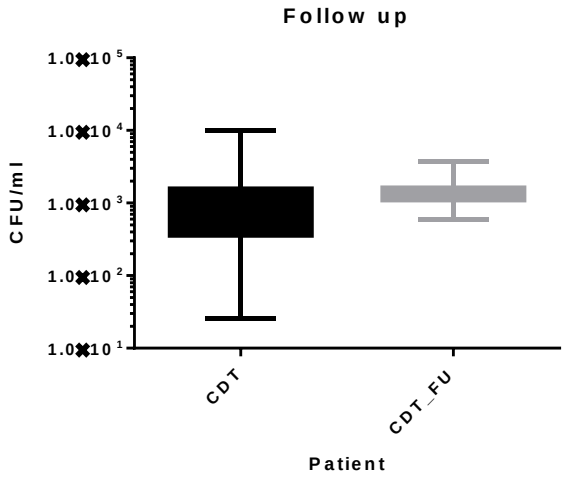


Figure 6.16 Quantification of fungal DNA as CFU/ml in follow up samples

6.3 Discussion.

In the current study, the oral bacteriome and mycobiome of treatment naïve IBD patients was compared before and after treatment. The number of children with follow-up samples was 21 (CD=18, UC=3). Differences in the oral bacterial and fungal profiles of treatment naïve IBD patients pre- and post-treatment have not yet been described and to our knowledge, this is the first investigation of the oral microbiome and mycobiome following treatment for IBD. Our findings demonstrated a marked change in overall biodiversity and species richness between the two communities at different levels of classification.

6.3.1 The oral bacteriome of follow up samples.

We examined the V1-V2 region of 16S rRNA of tongue bacterial DNA from 21 follow up children with IBD. Our data showed increased abundance of the phyla Firmicutes, Proteobacteria, Actinobacteria and TM7 among the follow up samples (IBD.FU) compared to pre-treatment children (IBD). In terms of biodiversity, the two IBD groups showed similar levels of microbial diversity, whereas the IBD.FU samples exhibited greater species richness compared to the treatment naïve IBD group. However, healthy samples had much greater species richness compared to both IBD groups. In terms of community structure, the IBD and IBD.FU samples were not significantly different, although the HOMOVA test indicated that the IBD.FU samples were more homogenous in nature, which might reflect stabilisation of the microbiome. In general, the IBD.FU samples showed less Actinobacteria, including the Crohn's enriched taxon *A. lignae* (OTU0017) and the Enterobacteriales. Oral Enterobacteriaceae have been implicated as potential inflammatory pathobionts and reduced levels of these organisms could be indicative of a return to normal GI health. the Enterobacteriaceae has been identified as

a significant biomarker for disease (Wright *et al.*, 2015). *K. pneumonia* is one of the Enterobacteriaceae family, which can colonize mucosal surfaces in human and causes opportunistic infections (Guo *et al.*, 2012). One recent study by Wang *et al.* (2018), characterizing the composition of the faecal bacterial community in CD patients before and during treatment with IFX revealed depletion of bacterial taxa, including *Enterobacteriaceae, Enterococcaceae, Planococcaceae*, and *Streptococcaceae* in CD patients following treatment with IFX, shifting the gut microbial community post – IFX treatment close to normal a gut community. In the present study, we also show the loss of the Enterobacteriaceae family (*Klebsiella sp*) and *Actinomyces* (*A. lignae*) following treatment. In the current study, IFX therapy was used in the treatment of the CD patients (Appendix 5), and the similarity in the response to IFX therapy between the oral and gut microbiota (depletion of some microbial taxa during treatment) could be a useful biomarker of treatment outcome. A number of streptococcal OTUs and *Veillonella* sp. OTU0005 also showed increased abundance in IBD.FU following treatment. Our findings indicated that *Streptococcus* spp were more abundant in follow up samples from patients with severe IBD and *Veillonella* sp. OTU0005 tended to increase in samples from mild-moderate patients on follow-up. This is in agreement with the results from Schirmer *et al*. (2018), who found that *Veillonella* spp. were indicative of IBD and returned to healthy levels following therapy, suggesting that the alterations in gut microbiota compositions observed following treatment might be mirrored in the oral microbiota in patients with UC and/or CD.

6.3.2 The oral mycobiome of follow up samples.

To compare the oral mycobiome in treatment naïve children with IBD before and after treatment, ITS2 region was amplified from tongue samples. In the current study, we described for the first time the oral mycobiome in Crohn's disease children before and

after treatment using Illumina sequencing methods and showed differences in diversity and species richness in the tongue mycobiota. Notably, *Candida dubliniensis* was reduced in the IBD.FU group compared before therapy. Additionally several other fungal taxa were enriched in follow up samples (which were previously detected as members of healthy oral mycobiota), including *Saccharomyces cerevisiae*, *Didymella gardenia*, and *Candida albicans*, suggesting that a clear shift of the tongue mycobiota occurred following therapy. Although community structure in IBD.FU did not resemble healthy children, which have lower biodiversity, our data suggest an increase in species richness following treatment for IBD. Although biodiversity increases in the follow up samples, we observed a reduction in the Basidiomycota, which was in line with an analysis of gut mucosa in CD patients (Liguori *et al.* 2016). Our data showed enrichment of IBD children with the Basidiomycota phylum compared to IBD patients following therapy, which exhibited an increase in Ascomycota. These results were consistent with the previous observations, showing a high abundance of Basidiomycota in the mucosal and faecal mycobiota in CD (El Mouzan *et al.* 2017), and faecal microbiota of IBD patients (Sokol *et al.* 2017). In present study, the IBD.FU patients had greater biodiversity according to the inverse Simpson value, whereas measurement of richness with Chao 1 revealed less fungal richness in the follow up samples. The rarefaction curve shows greater richness in the IBD.FU samples within the first ~7,000 sequences sampled (indicating the presence of more high abundance fungi, i.e. increased biodiversity). However, the level in richness increases in the IBD samples at higher sampling depths (indicating greater numbers of low abundance fungal taxa).

To date, all previous observations of the mycobiota in IBD have analysed the gut mycobiota using different sampling methods (Li *et al.* 2014, Hold *et al.* 2014, Mukhopadhya *et al.* 2015, Chehoud *et al.* 2015, Liguori *et al.* 2016, El Mouzan *et al.*

2017, Sokol *et al.* 2017, El Mouzan *et al.* 2018). However, none of the previous studies investigate the oral mycobiota in IBD patients at baseline and following therapy, and to our knowledge, this is the first report on the oral fungal microbiota in newly diagnosed treatment naïve children with CD, as well as at the follow up stage (following therapy).

6.4 Conclusion.

In the current study, we present the first report of the oral mycobiome in treatment naïve children with CD in comparison with post-treatment follow-up samples of the same population. We detected differences in the fungal composition between the treatment naïve samples and samples recovered following therapy, and a clear shift of fungal composition from Basidiomycota phylum, which was significantly abundant in IBD patients to Ascomycota phylum which were more dominant in healthy mycobiota (Sokol *et al.* 2017, El Mouzan *et al.* 2017). Alteration of the oral mycobiome following therapy was noted, this was represented by an increase in the abundance of some members of oral mycobiome (Ghannoum *et al.* 2010), including *Candida albicans*, *Didymella gardenia*, *Cladosporium cladosporiodes* and *Fusarium* spp.. These changes could be a result of antibacterial therapy and anti-inflammatory therapy or could represent a return to a healthy diverse mycobiome. Changes in the microbiota also suggest a return to a healthier microbial community post-treatment. We observed a reduction in the pathobionts of the Enterobacteriaceae and increased levels of the oral health associated species *Veillonella* and *Haemophilus*. This study provides preliminary data on the alterations of the oral microbiota in newly diagnosed IBD patients and following therapy. We theorize that oral microbiome alterations following therapy might be a consequence of disease remission and reduced inflammation. Further studies are required to determine whether the mircobiome profile following therapy could be a guide for treatment success and disease management in IBD.

Chapter 7

General Discussion

Chapter 7 General discussion.

7.1 Oral microbiota (Bacteriome and Mycobiome) in CD patients.

The alteration of oral microbiota composition may be critical in the pathogenesis of IBD. From the results discussed previously in chapter 3 and 5 of this work, the tongue and buccal microbiota of CD children exhibted reduced biodiversity and lower species richness compared to healthy children. Subsequent analysis showed differences at the phyla level accounting for change in overall biodiversity in the present study. This study showed that the tongue and buccal mucosa are different environments and the tongue was observed to have greater biodiversity compared to the buccal environment (Figure 3.1 and 3.11). Tongue biodiversity was reduced in CD patients. Previous study by Said *et al*. (2014) observed no differences in salivary microbiota richness between CD, UC patients and healthy controls. Our study indicates that examination of mucosal surfaces, specifically the tongue, may be a more sensitive indicator of dysbiosis than saliva, which represents organisms shed from all oral surfaces. Additionally, in the current study several genera were enriched in patients with IBD, including *Actinobacteria, Bergeyella* spp., *Veillonella* spp. and *Prevotella* spp in buccal samples (Figure 3.9), and the greatest enrichments in tongue samples of IBD children included *Streptococcus* spp., *Gemella* spp., and *Neisseria* (Figure 1.20). Our finding shows a reduction in the levels of several major oral bacterial genera, including *Prevotella, Veillonella*, *H. parinfluenza,* and *F. periodonticum* in the oral microbita of IBD patients. Identification of these genera is surprising, as recent studies of the gut microbiota in IBD patients have shown an increase in *H. parinfluenza* sp, *Veillonellaceae, Neisseriaceae,* and *Fusobacteriaceae* in CD patients (Gevers *et al.* 2017), and *H. parainfluenza and Veillonella* in UC patients (Schirmer *et al.* 2018). Previous studies have suggested that the two bacterial taxa, *H.*

parinfluenza and Veillonella spp. induce dendritic cell maturation, with the mature dendritic cells producing cytokines which may drive inflammation (Larsen *et al.* 2012). The discerpency in the distribution of these taxa in the oral and gut environments may be somehow linked to oral-gut transit and may play a role in the pathogenesis of IBD. It is not clear how this transit mechanism would result in depletion of these taxa from the oral cavity. Although oral-gut transit of these microbes is likely common place, it is unlikely that these children simply swallow all of these organisms leading to depletion in the oral cavity. Perhaps, in susceptible children, stimulation of the immune response in the GI tract by these bacteria could lead to enhanced systemic immunity to these organsims which could result in depletion in the oral cavity. Transit of these organisms likely happens in all children, but perhaps a genetic susceptibility to colonisation with these oral taxa exists in these children and this results in intense inflammation that leads to IBD and depletion of these taxa from the oral cavity (Figure 7.1). These responses could be more effective on the tongue mucosa compared to the buccal mucosa resulting in more severe dysbiosis at this location. Another important taxon implicated in inducing inflammation in IBD patients was reported by Atashari *et al.* (2017), who reported that *Klebsiella pneumonia* from the oral cavity of IBD patients could induce intestinal inflammation in mice (Atarashi *et al.* 2017). In this study the oral microbiome from IBD children was inoculated in mice and resulted in enrichment for *Enterobacteriaceae* in the murine GIT. These taxa (*Klebsiella* spp) have been shown to be resistant to multiple antibiotics and can cause infection (Pamer 2016). Atashari *et al*. (2017) isolated *Klebsiella pneumonia* strain (Kp-2H7) from the human salivary microbiota and reported that Kp-2H7 induced severe inflammation, acting as a gut pathobiont. Additionally, the results observed a link between oral-derived bacateria and inflammatory TH1 cell responses, revealing that Kp2H7 drives TH1 cell induction, with marked accumulation of TH1 cells in the murine

colon. These data clearly show that the oral cavity may be a reservoir for these bacteria, and that trasit from the oral cavity to the gut may play a role in inducing inflammation in IBD patients.

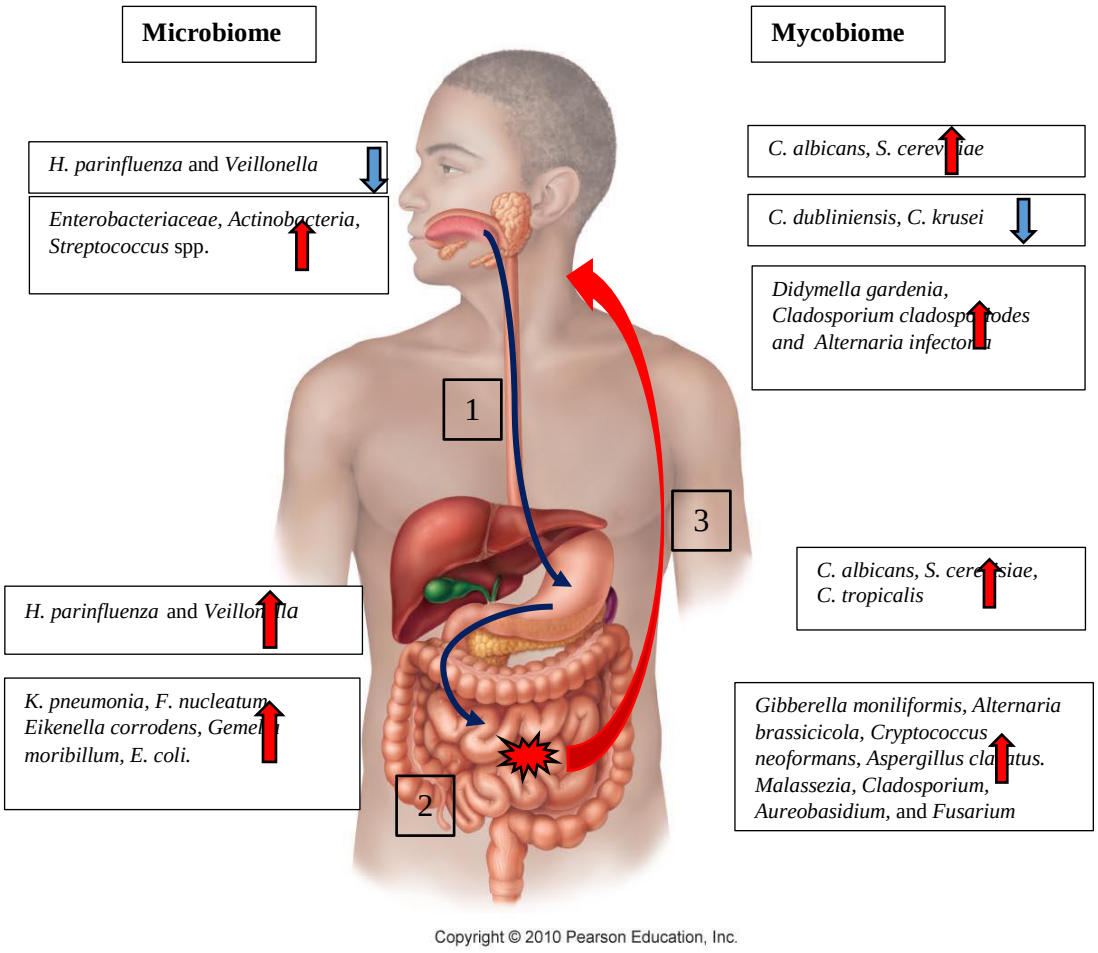


Figure 7.1 Detailing oral-gut transit and the effects on the taxa in oral and gut microbiota in IBD patient. (1) Transit of organsisms from the oral cavity to gut. The presence of *Haemophilus*, *Veillonella* and *Fusobacterium* species in the gut suggests a high rate of transit. (2) Colonization of gut microbiota, and inflammation of the ileum occurs in susceptible IBD patients. (3) The inflammatory response may result in a strong systemic immune response against the oral invaders, manifesting as oral Crohn's and resulting in depletion of these oral taxa. The reduced biodiversity of the microbiota may allow growth of variuos fungi resulting in increased mycobiome biodiversity.

Atarashi *et al.* (2017) suggested that these species could be used a biomarker in IBD diagnosis, as well as in planning of treatment stategies for IBD. Further investigations are required for a better understanding of *Klebsiella* spp. and IBD. Our data support a role for oral *Enterobacteriaceae* in inducing severe inflammation in IBD. In this study, the greatest levels of oral *Enterobacteriaceae* were observed in patients with severe IBD. This OTU was most similar to *Serratia* and *Klebsiella* spp. In our follow up studies, remission from IBD symptoms was associated with loss of *Enterobacteriaceae* from the oral microbiome. If the oral cavity is the source of these inflammatory pathobionts, reduced oral carriage may be crucial for disease remission and perhaps analysis of oral levels of *Enterobacteriaceae* could be used to monitor treatment success.

In addition to the oral microbiome signature in IBD patients, the oral mycobiome community composition was also assessed in this study. In the current study, oral samples of IBD children were enriched in several fungal taxa, namely *Candida albicans, Didymella gardeniae*, *Cladosporium cladosporiodes, Saccharomyces cerevisiae*, *C. krusei*, *Debaryomyces hansenii, Cyberlindnera jadinii*, and *Alternaria infectoria*. We observed increased biodiversity and prevalence of these fungal taxa in the oral mycobiota of IBD patients. Increased *C. albicans* and *S. cerevisiae* were also seen in gut microbiota in patients with IBD (Figure 7.1). These fungal taxa induce distinct dendritic cell responses in murine models, leading to interleukin secretion (Sokol *et al.* 2017). We hypothesise that the gut *S. cerevisiae* reported by El Mouzan et al. (2018) and Hoarau *et al*. (2016), and *C. albicans* by Sokol *et al.* (2017) could have originated in the oral cavity of these patients. Interestingly, *C. krusei* was also observed in the oral cavity in IBD, although this yeast has not been reported in the GIT of IBD patients. In model mice, *C. krusei* is able to disseminate to the CNS and promote local inflammation (Sanches *et al.* 2018). However, the role of *C. krusei* in the GIT is unclear, and requires further

investigations. Increased fungal biodiversity may be a result of the reduced bacterial biodiversity in IBD patients, where more space and nutrients could be available for fungi to colonise. Whether any of these fungi can then travel to the gut and promote inflammation requires further investigation.

7.2 Crohn's disease location and severity.

Using the Paris classification scheme, our data show that patients with a complex diagnosis of L3L4 have a significantly different oral microbiome from non-L3L4 patients and healthy children, suggesting that greater alterations occur in the oral bacterial communities of patients with L3L4 disease types. The oral mycobiome of Crohn's disease children with L3L4 disease did not differ significantly from non-L3L4 patients. These data suggest that the most severe inflammatory reactions are required to disrupt oral homeostasis. This finding was supported by the disease severity score. Our study shows a reduction in abundance of several bacterial taxa in tongue samples in severe cases of IBD. Analysis of tongue mycobiota communities using AMOVA showed a significant difference in community membership of moderate IBD ($P<0.001$) and severe IBD ($P=0.032$) compared to HCT, whereas buccal mycobiome data shows alterations in buccal mycobiota are associated with increased disease severity. Our findings could suggest that the disease location and severity of IBD could influence the oral microbiota of IBD children, leading to alterations in oral bacterial communities. Overall, the mycobiome changes observed were more related to IBD disease activity compared to microbiome.

7.3 Oral microbiota following CD therapy

In this study, shifting of the oral microbial community was noted in follow up patients, returning some taxa to levels close to the normal oral community. Similar results were reported in the gut microbiota in CD patients following IFX therapy (Wang *et al.* 2018). Additionally, the gut microbiome of UC patients showed alterations of microbial composition over time in association with disease severity and following treatment (Schirmer *et al.* 2018). Interestingly, our findings show increased abundance of certain *Streptococcus* spp. OTUs and *Veillonella* spp, with depletion of *A. lignae* and Enterobacteriales in IBD children following therapy. *Veillonella* spp. appear to be an important marker organism for dysbiosis in IBD and restoration of healthy levels could be used as a biomarker for remission. The similarity in the response to therapy between the oral and gut microbiota suggest that the alterations in the gut microbiota composition observed following treatment might be mirrored in the oral microbiota in patients with UC and/or CD. Interestingly, several members of healthy oral mycobiota, namely *Saccharomyces cerevisiae*, *Didymella gardenia,* and *Candida albicans* were increased in IBD patients following therapy, indicating a clear shift of the tongue mycobiota occurred following therapy. The healthy oral mycobiome appears to be associated with the Ascomycota and increased levels of these taxa indicate alterations of the oral mycobiota in remission associated with oral health.

7.4 Oral mycobiota in healthy individuals.

Our ITS2 sequences data of tongue and buccal samples of healthy individuals report similar oral healthy mycobiome core, which was listed by Ghannoum and colleagues (2010), namely *Candida, Cladosporium, Aureobasidium, Saccharomycetales, Dothioraceae, Teratosphaeria, Alternaria, Aspergillus, Fusarium, Saccharomyces, Eurotium, Glomus,* and *Cryptococcus.* However, two fungal taxa, *Eurotium* and *Glomus* were not detected in our data using ITS2. Additionally, our study detected another 8 genera of fungi, including *Candida dubliniensis*, *Candida zeylanoides*, *Botrytis caroliniana*, *Candida krusei*, *Debaryomyces nepalensis*, *Debaryomyces hansenii*, *Cyberlindnera jadinii,* and *Ganoderma destructans* in both tongue and buccal samples, which were not recognized in the previous studies (Ghannoum *et al.* 2010, Dupuy *et al.* 2014). In the present study ITS1 primers were applied to a subsection of the samples, resulting in identification a high amount of *Malassezia* spp. and *Rhodotorula* spp compared to the low amount which was detected using ITS2 primers. These data explain the discrepancy between the current and previous studies, confirming that the amplified region of ITS spacer has impact on species identification and a lack of specificity of ITS2 primer to the Basidomycetes phylum. Unexpectedly, our data shows high abundance of *C. dubliniensis,* suggesting that *C. dubliniensis* is a component of the healthy mucosal mycobiome and that it comprises a significant element of the mycobiome in healthy children, which was consistent with previous culture based observations by (Li *et al.* 2012, Zakaria *et al.* 2017, Jabra-Rizk *et al*. 2001,Al-Ahmad *et al*. 2016), characterizing the healthy oral mycobiome.

7.5 The future of oral microbiota in Crohn's disease patients.

Current advances in next generation sequencing (NGS) allow better understanding and identification of oral and gut community composition in health as well as in association with other diseases. The oral microbiota in Crohn's disease is not well characterized, in particular the oral mycobiome (oral fungal microbiome) compared the gut microbiota. Several lines of evidence emphasize that the microbiome has an impact on either onset or perpetuation of Crohn's disease (O'Brien *et al.* 2018), and numerous children diagnosed with IBD will be exposed to several episodes of flare and remission, requiring multiple periods of medication (Zhu *et al.* 2018). To date the current study is the first study to characterize the oral bacteriome and mycobiome in treatment naïve children with IBD and following treatment. Our findings correspond to sequence analysis of the V1-V2 region of the 16S rDNA gene and the ITS2 region of fungal DNA from 59 children with IBD, showing that there are some correlations between the results of the oral and gut microbiome analysis in IBD patients. However, a full understanding of the relationship between oral and gut dysbiosis in patients with IBD required further analysis.

The hypothesis outlined in Figure 7.1 supports the notion that oral pathobionts that transfer to the gut from the oral cavity may be partly responsible for the inflammatory reactions in the gut of IBD patients. This raises the possibility that control of the oral microbiome may be a way to control the progression of IBD. This could be achieved by improved oral hygiene to reduce bacterial load in the oral cavity or by using an oral probiotic that may replace or inhibit oral pathobionts such as *K. pneumonia* or *Veillonella* spp.

The current study shows the potential of the oral cavity as a source of biomarkers for the diagnosis and categorisation of IBD patients. Future investigations could involve additional 'omic' technologies for biomarker discovery (Horgan and Kenny, 2011). Whole genome sequencing or shotgun metagenomics may be capable of identifying specific pathobionts unique to IBD patients that may be involved in the pathogenesis of CD or UC. Functional profiling of the dysbiotic oral and gut communities by profiling of mRNA (transcriptomics), protein (proteomics) or metabolite (metabolomics) patterns may reveal additional biomarkers and elucidate the functional role of these communities in the pathogenesis of IBD. These tools are fully applicable to IBD and may provide more effective diagnostic markers or potential therapeutic targets for patients with CD and UC (Fiocchi 2014).

For a better understanding of the association between the human oral microbiota and IBD the following studies are required:

1. A large cohort study, comparing the oral and gut microbiota community composition from same children with IBD. Newly recruited patients should be sampled at both sites.

2. Examine the oral microbiota in a large cohort of patients following therapy. In the current cohort of children, only 21 swabs out of 59 IBD children were sampled following therapy (36%).

3. Examine the significance of the *Enterobacteriaceae* in the oral cavity of children with IBD. The levels of *Enterobacteriaceae* could be used as a marker of disease severity and could be used to identify disease remission. This may be achieved by qRT-PCR. Shot-gun metgenomic sequencing may also give a more accurate identification of these organisms to the species level.

4. Characterize the oral and gut fungal community in Crohn's patients by Illumina sequencing. A combination of ITS1 and ITS2 sequencing may be required to identify all taxa. Recent studies have shown that *Candida tropicalis* and *Malasezzia* spp. are enriched in the gut flora of Crohn's patients.

The observations in this study indicate that a full understanding of the oral microbiome IBD may be important to understand the aetiology and pathogenesis of the disease and these data may also have practical applications in diagnosis and monitoring disease progression.

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Appendices

Appendix 1. Comparison of Vienna and Montreal Classifications for Crohn's disease (Satsangi *et al.*, 2006).

Table 1 Vienna and Montreal classification for Crohn's disease

	Vienna	Montreal
Age at diagnosis	A1 below 40 y A2 above 40 y	A1 below 16 y A2 between 17 and 40 y A3 above 40 y
Location	L1 ileal L2 colonic L3 ileocolonic L4 upper	L1 ileal L2 colonic L3 ileocolonic L4 isolated upper disease*
Behaviour	B1 non-stricturing, non-penetrating B2 stricturing B3 penetrating	B1 non-stricturing, non-penetrating B2 stricturing B3 penetrating p perianal disease modifier†

*L4 is a modifier that can be added to L1–L3 when concomitant upper gastrointestinal disease is present.
†"p" is added to B1–B3 when concomitant perianal disease is present.

Appendix 2. Comparison of Montreal and Paris Classifications for Crohn's disease (Eszter Muller *et al.*, 2014).

	Montreal Classification	Paris Classification
Age at diagnosis	A1: below 17 years A2: 17–40 years A3: above 40 years	A1a: 0–<10years A1b: 10–<17 years A2: 17–40 years A3: >40 years
Location	L1: terminal ileal/ limited cecal disease L2: colonic L3: ileocolonic L4*: isolated upper disease	L1: distal 1/3 ileum / limited cecal disease L2: colonic L3: ileocolonic L4a: upper disease proximal to ligament of Treitz* L4b: upper disease distal to ligament of Treitz and proximal to distal 1/3 ileum*
Behaviour	B1: nonstricturing nonpenetrating B2: stricturing B3: penetrating p: perianal disease modifier	B1: nonstricturing nonpenetrating B2: stricturing B3: penetrating B2B3: both penetrating and stricturing disease, either at the same or different times p: perianal disease modifier
Growth	—	G0: no evidence of growth delay G1: growth delay

*In both the Montreal and Paris Classification systems L4 and L4a/L4b may coexist with L1, L2, and L3, respectively.

Appendix 3. Disease classification of the studied population.

sample ID	Diagnosis	Age	Gender	Classification
21T&B	Colitis	11	F	PancolitisS1
4 T&B	Colitis	10	M	E2S0
2 T&B	Colitis	16	M	PancolitisS1
8 T&B	Colitis	16	M	E2S1
29 T&B	Colitis	10	M	PancolitisS0
35 T&B	Colitis	12	M	PancolitisS0
38 T&B	Colitis	12	F	E2S0
43 T&B	Colitis	15	F	E3S1
41 T&B	Colitis	12	M	L2B1
59 T&B	Colitis	9	M	E1, S0
60 T&B	Colitis	14	M	E4, S0
61 T&B	Colitis	11	M	E4, S0
62 T&B	Colitis	14	F	E2, S1 -> E4, S1 (rescope)
63 T&B	Colitis	16	M	IBD-U (rectal sparing)
64 T&B	Colitis	12	F	IBD-U, E2, S0
65 T&B	Colitis	13	M	Atypical, S0
66 T&B	Colitis	12	M	E4, S0
67 T&B	Colitis	10	M	Atypical UC, S1 (macroscopic rectal and sigmoidal sparing)
68 T&B	Colitis	7	M	IBD-U (rectal sparing)
69 T&B	Colitis	13	M	E4, S0
70 T&B	Colitis	14	F	E1, S0
31 T&B	Crohn's	8	M	L3B1P
12 T&B	Crohn's	14	M	L3B1
3 T&B	Crohn's	12	M	L3L4bB1
11 T&B	Crohn's	16	M	L3B1
6 T&B	Crohn's	14	M	L3L4aB1
1 T&B	Crohn's	13	M	L3L4aB1
20 T&B	Crohn's	12	M	L3L4aL4bB1
13 T&B	Crohn's	10	M	L3B1
5 T&B	Crohn's	12	M	L3L4aB3P
14 T&B	Crohn's	14	F	L2L4aB1P
19 T&B	Crohn's	8	M	L3L4aB1
18 T&B	Crohn's	13	M	L2L4aB1
16 T&B	Crohn's	14	M	L2,L4a,B1
17 T&B	Crohn's	16	M	L2B1
15 T&B	Crohn's	14	M	L3B1
27 T&B	Crohn's	9	F	L2B1
36 T&B	Crohn's	16	M	L3,L4a,L4b,B1
28 T&B	Crohn's	15	M	L3,L4a,B1
34 T&B	Crohn's	16	M	L3L4aL4bB1

30 T&B	Crohn's	9	F	L3L4aL4bB1
33 T&B	Crohn's	15	M	L3B2
42 T&B	Crohn's	14	M	L3L4bB1
37 T&B	Crohn's	10	F	PB3
40 T&B	Crohn's		M	L3L4aPB1
45 T&B	Crohn's	15	M	L4a, L2, P, B1, G0
46 T&B	Crohn's	12	M	L4a, L4b, L3, B1, G0
47 T&B	Crohn's	13	M	L4b, L1, L5, P, B1, G1
48 T&B	Crohn's	11	F	L2, B1, G0, P
49 T&B	Crohn's	15	M	Penile
50 T&B	Crohn's	12	F	L4a, L2, B1, G0
51 T&B	Crohn's	12	M	No MRE
52 T&B	Crohn's	13	M	L4a, L3, B1, G0
53 T&B	Crohn's	13	M	L3 - No MRE
54 T&B	Crohn's	10	M	L2, P, B1, G0 for now, no MRE yet.
55 T&B	Crohn's	11	M	L3, P, G0 - No MRE
56 T&B	Crohn's	13	M	L4a, L3, P, B1 - query B2 stricture possible but not confirmed as per MRE report.
57 T&B	Crohn's	11	M	L2, B1, G?
58 T&B	Crohn's	10	F	L4a, L4b, L2, B1, G0

Appendix 4. Disease severity of the studied population.

study number	Severity at Baseline (PCDAI [=95]/PUCAI[=85])	Severity at Follow up	sample ID	Diagnosis
345	65/85	n/a	21T&B	Colitis
356	25/85	0/85	4 T&B	Colitis
364	65/85	Transitioned	2 T&B	Colitis
366	75/85	Transitioned	8 T&B	Colitis
408	20/85	Due Nov 2017	29 T&B	Colitis
416	55/85	Due Dec 2017	35 T&B	Colitis
417	75/85	0	38 T&B	Colitis
458	75/85	Transitioned	43 T&B	Colitis
460	52.5/95	43133	41 T&B	Colitis
705	40/85	n/a	59 T&B	Colitis
727	60/85	n/a	60 T&B	Colitis
746	35/85	Due December 2019	61 T&B	Colitis
749	80/85	Due December 2018	62 T&B	Colitis
754	50/85	Due January 2019	63 T&B	Colitis
756	50/85	Due Feb 2019	64 T&B	Colitis
773	60/85	Due March 2019	65 T&B	Colitis
779	60/85	Due March 2019	66 T&B	Colitis
781	75/85	Due April 2019	67 T&B	Colitis
784	50/85	Due April 2019	68 T&B	Colitis
785	50/85	Due April 2019	69 T&B	Colitis
792	30/85	Due May 2019	70 T&B	Colitis
298	57.5/95	0/95	31 T&B	Crohn's
320	12.5/95	0/95	12 T&B	Crohn's
347	45/95	0/95	3 T&B	Crohn's
348	40/95	Transitioned	11 T&B	Crohn's
349	Oct-95	Due Feb 18	6 T&B	Crohn's
351	22.5/95	Due Jan 18	1 T&B	Crohn's
357	37.5/95	0/95	20 T&B	Crohn's
362	40/95	0/95	13 T&B	Crohn's
363	47.5/95	0/95	5 T&B	Crohn's
370	57.5/95	15/95	14 T&B	Crohn's
373	45/95	0/95	19 T&B	Crohn's
389	20/95	May-95	18 T&B	Crohn's
390	7.5/95	Due Dec 2017	16 T&B	Crohn's
393	57.5/95	Transitioned	17 T&B	Crohn's

394	Oct-95	UK	15 T&B	Crohn's
395	40/95	Relocated	27 T&B	Crohn's
402	40/95	Transitioned	36 T&B	Crohn's
407	32.5/95	Transitioned	28 T&B	Crohn's
410	25/95	Transitioned	34 T&B	Crohn's
411	47.5/95	Due Jan 2018	30 T&B	Crohn's
435	UK	0	33 T&B	Crohn's
444	32.5/95	Transitioned	42 T&B	Crohn's
445	Oct-95	n/a	37 T&B	Crohn's
459	50/95	43119	40 T&B	Crohn's
667	50/95	n/a	45 T&B	Crohn's
673	30/95	n/a	46 T&B	Crohn's
679	30/95	n/a	47 T&B	Crohn's
717	60/95	n/a	48 T&B	Crohn's
720	n/a	n/a	49 T&B	Crohn's
726	22.5/90 (no ESR available)	n/a	50 T&B	Crohn's
728	32.5/95	n/a	51 T&B	Crohn's
751	n/a (no ESR)	Due January 2019	52 T&B	Crohn's
759	45/95	17.5/95	53 T&B	Crohn's
764	n/a (no ESR, no height)	Due March 2019	54 T&B	Crohn's
765	50/95	Due March 2019	55 T&B	Crohn's
772	27.5/95	Due April 2019	56 T&B	Crohn's
778	37.5/95	Due April 2019	57 T&B	Crohn's
795	20/95	Due May 2019	58 T&B	Crohn's

Appendix 5. Medical therapies received by the studied population.

study number	Treatments since diagnosis	Treatment at Follow up	sample ID	Diagnosis
345	PO Pred, Sulfasalazine, Vit D, Galfer	n/a	21 T&B	Colitis
356	Salofalk, Vit D	Salofalk, Vit D	4 T&B	Colitis
364	Asacol, flucloxacillin,Becotide, Lansaprazole, Infliximab, Sando K supplements, scandishake drinks, lyrica, multivitamin	Transitioned	2 T&B	Colitis
366	Salofalk, PO Pred, IV methylpred, Infliximab, fortisip, pentasa, asacol, omeprazole, Metronidazole, Galfer, Calcium, Multivitamin, IV Iron, Probiotic	Transitioned	8 T&B	Colitis
408	Pentasa, Omeprazole, Galfer,	Due Nov 2017	29 T&B	Colitis
416	IV Methylpred, Sulfasalazine, PO Pred, Lansaprazole	Due Dec 2017	35 T&B	Colitis
417	Sulfasalazine, rectal Tx, Vit D,	0	38 T&B	Colitis
458	Sulfasalazine, PO Pred, Vit D, Galfer	Transitioned	43 T&B	Colitis
460	Metronidazole, Infliximab, Aciclover, Galfer, Vit D	43133	41 T&B	Colitis
705	Mesalazine (Pentasa)	n/a	59 T&B	Colitis
727	Mesalazine (asacol), Prednisalone, omeprazole,	n/a	60 T&B	Colitis
746	Sulfasalazine, Oral pred, Rectal pred, 6mp, Galfer, Vitamin D, Fortisip/Juice as supplement,	Due December 2019	61 T&B	Colitis
749	Asacol PO, Mesalzine PR, IV Metronidazole, IV Penicillin (Augmentin), Oral Pred, IV Hidrocortisone, IV Methylpred, Omeprazole, Vitamin D, Iron, Infliximab, Salofalk PO, Rectal Steroid (Colifoam),	Due December 2018	62 T&B	Colitis
754	Sulofalk, Oral Pred, Omeprazole, Vitamin D, Sulofalk Suppository, Predsol enema, Amoxicillin, Clarythromicin, 6-mp, Iron,	Due January 2019	63 T&B	Colitis
756	EEN only as supplement, Infliximab, IV iron, Sulfasalazine PO, Mesalazine PO, Sulofalk PR, Colifoam PR, Omeprazole, Vitamin D, Movicol, Lactulose, Buscopan, Dulcolax	Due Feb 2019	64 T&B	Colitis

773	Pentasa PO, Omeprazole, Azathioprin, Vitamin D, Ursofalk, (Asthma inhalers becotie/salbutamol)	Due March 2019	65 T&B	Colitis
779	Pentasa PO, Mesalazine PR, Vitamin D, Iron, Biocult supplement	Due March 2019	66 T&B	Colitis
781	Sulfasalazine	Due April 2019	67 T&B	Colitis
784	Sulfasalazine, Rectal steroids (Colifoam), Vitamin D, Galfer,	Due April 2019	68 T&B	Colitis
785	Asacol PO, Oral Pred, Cod Liver oil, Vitamin D, Galfer, Ensure as supplement, Tetracycline (acne), Infliximab,	Due April 2019	69 T&B	Colitis
792	Mesalazine PR, Vitamin D, Movicol	Due May 2019	70 T&B	Colitis
298	EEN, IV Methylpred, Oral pred, Vit D, Lansoprazole, Ciprofloxin, Infliximab, Methotrexate	Methotrexate, Infliximab	31 T&B	Crohn's
320	EEN, 6-MP, Losec, Galfer, Vit D	6-MP, Vit D, Galfer	12 T&B	Crohn's
347	EEN, PO Pred, Vit D6-MP, Galfer,	6-MP, Vit D,	3 T&B	Crohn's
348	EEN, 6-MP, Vit D, Spatone	Transitioned	11 T&B	Crohn's
349	EEN, Lansaprazole, Imuran, Methotrexate, Folic acid, Glafer	Due Feb 18	6 T&B	Crohn's
351	Pentasa, 6-MP, Vit D, Omeprazole	Due Jan 18	1 T&B	Crohn's
357	EEN, 6-MP, Omeprazole, Vit D, Budesonide,	6-MP, Vit D	20 T&B	Crohn's
362	Lansaprazole, Infliximab, EEN, Vit D, Adalimumab, 6-MP	Adalimumab, 6-MP, Vit D	13 T&B	Crohn's
363	Lansaprazole, Infliximab, Vit D, Ferrum+potassium phospahte, EEN	Vit D	5 T&B	Crohn's
370	Metronidazole, Lansaprazole, Infliximab, Vit D, Scheriproct topical	Infliximab	14 T&B	Crohn's
373	EEN, 6-MP, Vit D, Fresubin, Infliximab	6-MP, Infliximab	19 T&B	Crohn's
389	EEN, 6-MP, Vit D, Infliximab	6-MP, Infliximab	18 T&B	Crohn's
390	EEN, Vit D, Budesonide, Omeprazole,	Due Dec 2017	16 T&B	Crohn's
393	Pentasa, Amoxicillin, IV methylpred, Galfer, Pred, Infliximab,	Transitioned	17 T&B	Crohn's
394	Lansaprozole, EEN, Vit D, 6-MP,	UK	15 T&B	Crohn's
395	EEN	Relocated	27 T&B	Crohn's
402	Omerprazole, EEN, Lansaprasole, MTx, Vit D, Infliximab	Transitioned	36 T&B	Crohn's
407	EEN, 6-MP, Omeprazole, Pred, Budesonide	Transitioned	28 T&B	Crohn's
410	EEN, Lansaprazole, 6-MP, Vit D	Transitioned	34 T&B	Crohn's

411	Omeprazole, 6-MP, Vit D, Infliximab, Azathiopurine	Due Jan 2018	30 T&B	Crohn's
435	6-MP, EEN, Vit D, Asacol	Asacol, 6-MP	33 T&B	Crohn's
444	IV Mmethylpred, PO Pred, Omeprazole, EEN, IV iron, Sofar, mTx, Multivitamin, Vit D, Infliximab	Transitioned	42 T&B	Crohn's
445	P/A Abscess Drained	n/a	37 T&B	Crohn's
459	Lansaprazole, EEN, 6-MP, Omeprazole	43119	40 T&B	Crohn's
667	EEN, vitamin D, lansoprazole	n/a	45 T&B	Crohn's
673	EEN, lansoprazole	n/a	46 T&B	Crohn's
679	EEN	n/a	47 T&B	Crohn's
717	EEN, metronidazole,	n/a	48 T&B	Crohn's
720	PPI	n/a	49 T&B	Crohn's
726	EEN	n/a	50 T&B	Crohn's
728	mesalazine (salofalk), omeprazole, 6-mercaptopurine,	n/a	51 T&B	Crohn's
751	Lansoprazole, EEN, Asacol,	Due January 2019	52 T&B	Crohn's
759	Mesalazine PO, EEN, 6mp, Vitamin D, Galfer	6mp, Vitamin D, Galfer	53 T&B	Crohn's
764	EEN, Metronidazole, 6mp, Galfer, Fortijuice and Scandishake supplement,	Due March 2019	54 T&B	Crohn's
765	EEN, Azathioprin, Vitamin D, Budesonide, Liquid paraffin PRN,	Due March 2019	55 T&B	Crohn's
772	EEN, Ciprofloxin, Omeprazole, Infliximab,	Due April 2019	56 T&B	Crohn's
778	Metronidazole, EEN, Infliximab, Vitamin D,	Due April 2019	57 T&B	Crohn's
795	EEN, Azathioprin, Galfer, Vitamin D, Duphalac	Due May 2019	58 T&B	Crohn's
734	Omeprazole,EEN (Fortijuse/Sip, Ensure), Infliximab, Vitamin D, Methotrexate PO	Due Nov 2018		Crohn's
798	EEN, Vitamin D, IV Iron, Infliximab	Due May 2019		Crohn's
799	Sulfasalazine, Galfer	Due May 2019		Crohn's
803	EEN, Vit D, Lansoprazole, Lactulose, Infliximab	Due July 2019		Crohn's
806	Lansoprazole, EEN, Vitamin D, 6mp	Due June 2019		Crohn's
816	Lansoprazole, EEN, Methotrexate SC	Due July 2019		Crohn's
819	EEN failed, oral pred	Due July 2019		Crohn's
821	Metronidazole,	Due September 2019		Crohn's

Appendix 6. Patient consent form.

PATIENT CONSENT FORM

Analysis of Oral Microbiome in Healthy Children and Adolescents

Principal Investigators:

Dr Gary Moran, Dr Paddy Fleming and Dr Kirsten Fitzgerald

This study and this consent form have been explained to me. My dentist has answered all my questions to my satisfaction. I believe I understand what will happen if I agree to be part of this study.

I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. I have received a copy of this agreement.

PARTICIPANT'S NAME……..

PARTICIPANT'S SIGNATURE: ……

Date: ……./……./……..

Where the participant is under 18 years old, the form must be signed by a person legally competent to give consent.

NAME OF CONSENTER, PARENT or
GUARDIAN……...

SIGNATURE…….

RELATION TO PARTICIPANT……

Statement of investigator's responsibility: I have explained the nature, purpose, procedures, benefits, risks of, or alternatives to, this research study. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

Physician's signature:……..

Date: ……/……/……

(Keep the original of this form in the participant's medical record, give one copy to the participant, keep one copy in the investigator's records)

Appendix 7. Ethics (Medical research) committee office.

ETHICS (MEDICAL RESEARCH)
COMMITTEE OFFICE

Tel: + 353 (01) 409 6307/6243
Fax: + 353 (01) 455 8873
Email: ethics.committee@olchc.ie
Website: www.olchc.ie

Dr Seamus Hussey
Consultant Paediatric Gastroenterologist
Our Lady's Children's Hospital
Crumlin
Dublin 12
20th April 2011

REC Reference: GEN/193/11

Re Determinants and Outcomes for Children and Adolescents
With Inflammatory Bowel Disease (DOCHAS)
Chief Investigators: Professor Billy Bourke, Dr. Seamus Hussey, Dr. Ben Hope

Dear Dr Hussey

Further to our previous correspondence dated 16th February 2011.

At their meeting which took place on 19th April 2011, the Ethics (Medical Research) Sub-Committee reviewed and approved the following amended documentation which was requested by them:

 Age-Related Assent/Consent Forms and Information Leaflets i.e. under 8 years of age; 8 – 12 years; 12 – 14 years; 14 – 16 years, Parent/Guardian.

 The Assent/Consent Forms and Information Leaflets which were amended to include a "Withdrawal Clause" and advice that the extra samples of tissue will be used for research and that patients will not get results from the analysis of the samples.

Yours sincerely
Claire Rice
Secretary
Ethics (Medical Research) Committee
Email: ethics.committee@olhsc.ie
CC: Professor Billy Bourke.

Appendix 8. Patient's leaflets form for swab collection and oral examination.

Oral Microbiome Swab Return Form

Affix Patient information Sticker here

Working Medical Diagnosis

……..

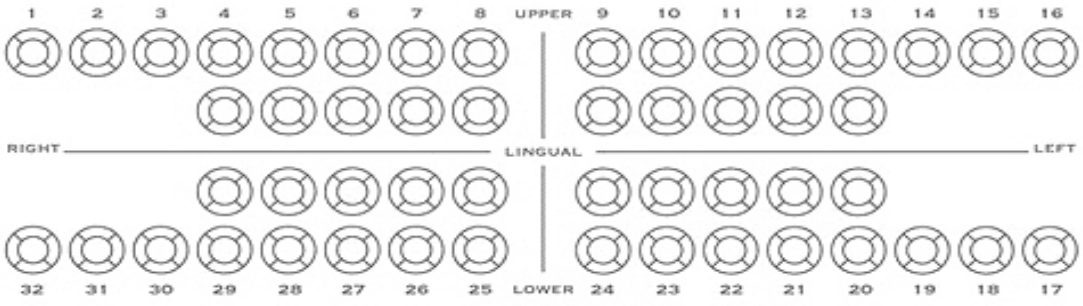
Soft Tissue Examination

_____

_____

_____

Hard Tissue Examination:



Signature:_____ Date:_____

Appendix 9. SJH/ AMNCH research ethics committee.

SJH / AMNCH RESEARCH ETHICS COMMITTEE.

Confidential Research Protocol, Pilot 2010

Please place an "X" or ✓ after the appropriate response in the boxed areas. NA is an abbreviation for Not Applicable.

1. **Title of research project**: Analysis of the oral microbiome in a control population of healthy children & adolescents

2. **Name of Chief Investigator** – who should ordinarily be a hospital consultant:
Prof. Gary Moran, Associate Prof.in Microbiology

DECLARATION BY SUPERVISOR

I confirm that the information provided in this protocol is correct. I also undertake to provide an annual report on the anniversary of Research Ethics Committee approval with details of the number of subjects who have been recruited, the number who have completed the study and details of any adverse effects.

Signed: [signature] Date: 08/07/2015
(Chief Investigator)

Please PRINT name of signatory here
..... PROF. GARY MORAN

Research Ethics Committee opinion:

Approved subject to :

Approved without conditions.

Signed: (Chair)

Date:

Appendix 10. DNA extraction protocol.

DNA extraction from Oral Swab specimens for microbiome analysis

Materials:

MasterPure DNA purification Kit (Epicentre).

Ready-Lyse Lysozyme Solution (Epicentre).

Methods:

1) Oral swab specimens (Catch-All™ Sample Collection Swab) should be collected and stored at -80˚C

2) Dilute the Ready-Lyse Lysozyme to 250 U/µl in TES buffer (1 µl + 19 µl).

3) Thaw the swabs and resuspend the collected material in 300 µl TES buffer in a screw capped tube.

4) Add 2 µl of ReadyLyse Lysozyme. Incubate at room temperature for 15 mins with occasional mixing

5) While the Lysozyme digestion is incubating, dilute 1 µl of Proteinase K in 150 µl 2x T and C Lysis solution for each swab sample.

6) Add 150 µl of the Proteinase K/T and C mixture to each tube and incubate for 15 min at 65˚C

7) Add 250 µl of glass beads and disrupt in the bead beater for 30s

8) Remove the liquid from the tube above to a 1.5 ml Ependorf. Cool the samples to 37˚C and add 1 µl of RNase A to the sample and mix and incubate at 37˚C for 30 min.

8) Place the samples on ice. Add 175 µl of MPC protein precipitation reagent and vortex for 10s.

9) Spin for 10 min at 4˚C at top speed and transfer the supernatant to a fresh tube leaving the pellet behind.

10) Add 500 µl of isopropanol and mix by inverting.

11) Pellet the DNA by centrifugation at top speed at 4˚C for 10 min.

12) Carefully remove the liquid without disturbing the DNA pellet.

13) Dry and resuspend in 35 µl TE buffer.

Appendix 11. ITS2 Sequence identities.

sequences	Mothur Classifucation	ISHAM blast result
sq1_size_5883316_	s__Candida_dubliniensis_unclassified/ seq 1.	Candida_dubliniensis/ seq 1.
sq2_size_1508625_	s__Candida_albicans_unclassified/seq 2	Candida_albicans/seq 2
sq3_size_619725_	f__Pleosporales_family_Incertae_sedis_unclassified/seq 3.	Peyronellaea glomerata/seq 3.
sq4_size_250833_	g__Saccharomyces_unclassified/seq 4	Saccharomyces cerevisiae/seq 4
sq5_size_172843_	s__Candida_albicans_unclassified/seq 5	Candida_albicans/seq 5
sq6_size_141909_	f__Davidiellaceae_unclassified/seq 6	Cladosporium cladosporioides/seq 6
sq7_size_110389_	s__Candida_xylopsoci_unclassified/seq 7	Pichia kudriavzevii /seq 7
sq8_size_41711_	f__Davidiellaceae_unclassified/seq 8	Cladosporium cladosporioides/seq 8
sq9_size_41459_	s__Xenobotryosphaeria_calamagrostidis_unclassified/seq 9	Xenobotryosphaeria calamagrostidis/seq 9
sq10_size_35799_	s__Candida_albicans_unclassified/seq 10	Candida_albicans/seq 10
sq11_size_34673_	g__Saccharomyces_unclassified/seq 11	Saccharomyces cerevisiae/seq 11
sq12_size_34390_	o__Pleosporales_unclassified/seq 12	Microsphaeropsis olivacea/seq 12
sq13_size_25997_	s__Debaryomyces_hansenii_unclassified/seq 13	Debaryomyces_hansenii/seq 13
sq15_size_24689_	s__Candida_albicans_unclassified/seq 15	Candida_albicans/seq 15
sq16_size_18423_	s__Debaryomyces_prosopidis_unclassified/seq 16	Debaryomyces_hansenii/seq 16
sq17_size_17984_	s__Cyberlindnera_jadinii_unclassified/seq 17	Cyberlindnera_jadinii/seq 17
sq18_size_17010_	o__Pleosporales_unclassified/seq 18	Paraphoma fimeti/seq 18
sq20_size_13959_	s__Alternaria_metachromatica_unclassified/seq 20	Alternaria triticina/seq 20
sq25_size_8899_	g__Saccharomyces_unclassified/seq 25	Saccharomyces cerevisiae/seq 25
sq27_size_7891_	f__Davidiellaceae_unclassified/seq 27	Cladosporium cladosporioides/seq 27
sq28_size_7213_	s__Fusarium_sp_59_4_unclassified/seq 28	Fusarium poae/seq 28
sq33_size_5942_	s__Monographella_nivalis_unclassified/seq 33	Microdochium nivale /seq 33
sq34_size_5880_	s__Candida_zeylanoides_unclassified/seq 34	Candida_zeylanoides/seq 34
sq37_size_5311_	s__Hanseniaspora_uvarum_unclassified/seq 37	Hanseniaspora_uvarum/seq 37
sq39_size_5238_	s__Aureobasidium_pullulans_unclassified/seq 39	Aureobasidium_pullulans/seq 39

Appendix 12. ITS1 Sequence identities.

sequences	ISHAM blast result	Mothur identity
sq1_size_10732114_	Candida_dubliniensis Seq.1	s__Candida_dubliniensis_unclassified Seq.1.
sq2_size_7174794_	Candida_albicans Seq.2	s__Candida_albicans_unclassified Seq.2.
sq3_size_1116069_	Saccharmyces cerevisiae Seq.3	g__Saccharomyces_unclassified Seq.3.
sq4_size_755624_	Didymella gardeniae Seq.4	f__Pleosporales_family_Incertae_sedis_unclassified Seq.4.
sq5_size_303784_	Saccharmyces cerevisiae Seq.5	g__Saccharomyces_unclassified Seq.5.
sq6_size_292485_	Candida_albicans Seq.6	s__Candida_albicans_unclassified Seq.6.
sq7_size_251484_	Cladosporium Cladosporiodes Seq.7	f__Davidiellaceae_unclassified Seq.7.
sq8_size_99991_	Candida xylopsoci Seq.8	s__Candida_xylopsoci_unclassified Seq.8.
sq9_size_80460_	Candida_zeylanoides Seq.9	s__Candida_zeylanoides_unclassified Seq.9.
sq10_size_68464_	Candida_albicans Seq.10	s__Candida_albicans_unclassified Seq.10.
sq12_size_60065_	Candida_albicans Seq.12	s__Candida_albicans_unclassified Seq.12.
sq13_size_54203_	Candida_tropicalis Seq.13	s__Candida_tropicalis_unclassified Seq.13.
sq14_size_52031_	Cladosporium Herbarum Seq.14	f__Davidiellaceae_unclassified Seq.14.
sq16_size_41115_	Candida_albicans Seq.16	s__Candida_albicans_unclassified Seq.16.
sq18_size_39459_	Epicoccum nigrum Seq.18	o__Pleosporales_unclassified Seq.18.
sq19_size_37045_	Epicoccum nigrum Seq.19	o__Pleosporales_unclassified Seq.19.
sq20_size_34897_	Saccharmyces cerevisiae Seq.20	g__Saccharomyces_unclassified Seq.20.
sq22_size_33991_	Cyberlindnera_jadinii Seq.22	s__Cyberlindnera_jadinii_unclassified Seq22.
sq23_size_27634_	Colletotrichum truncatum Seq.23	s__Botrytis_caroliniana_unclassified Seq.23.
sq26_size_20092_	Debaryomyces hansenii Seq.26	g__Debaryomyces_unclassified Seq.26.
sq28_size_17061_	Saccharmyces cerevisiae Seq.28	g__Saccharomyces_unclassified Seq.28.
sq30_size_14440_	Alternaria infectoria Seq.30	o__Pleosporales_unclassified Seq.30.
sq31_size_14340_	Debaryomyces nepalensis Seq.31	s__Debaryomyces_prosopidis_unclassified Seq.31.
sq35_size_10089_	Cryptococcus anemochreius Seq.35	s__Cryptococcus_laurentii_unclassified Seq.35.
sq36_size_10032_	Candida_albicans Seq.36	s__Candida_albicans_unclassified Seq.36.

Appendix 13. Top 100 Tongue sample OTUs.

Tongue OTU	Taxonomy
Otu0001	Actinobacteria;Rothia;mucilaginosa.
Otu0002	Firmicutes;Streptococcus;Streptococcus_unclassified.
Otu0003	Proteobacteria;Neisseria;perflava.
Otu0004	Bacteroidetes;Prevotella;Prevotella_unclassified.
Otu0005	Firmicutes;Veillonella;Veillonella_unclassified.
Otu0006	Firmicutes;Streptococcus;salivarius.
Otu0007	Firmicutes;Granulicatella;adiacens.
Otu0008	Proteobacteria;Haemophilus;parainfluenzae.
Otu0009	Firmicutes;Streptococcus;parasanguinis_clade_411.
Otu0010	Proteobacteria;Haemophilus;parainfluenzae.
Otu0011	Bacteroidetes;Prevotella;histicola.
Otu0012	Firmicutes;Granulicatella;adiacens.
Otu0013	Firmicutes;Streptococcus;Streptococcus_unclassified.
Otu0014	Fusobacteria;Fusobacterium;periodonticum.
Otu0015	Bacteroidetes;Porphyromonas;pasteri.
Otu0016	Actinobacteria;Actinomyces;Actinomyces_unclassified.
Otu0017	Actinobacteria;Actinomyces;lingnae_[Not_Validly_Published].
Otu0018	Firmicutes;Gemella;sanguinis.
Otu0019	Bacteroidetes;Alloprevotella;sp._HMT_473.
Otu0020	Proteobacteria;Neisseria;Neisseria_unclassified.
Otu0021	Firmicutes;Gemella;Gemella_unclassified.
Otu0022	Bacteroidetes;Prevotella;pallens.
Otu0023	Firmicutes;Streptococcus;Streptococcus_unclassified.
Otu0024	Bacteroidetes;Prevotella;nanceiensis.
Otu0025	Firmicutes;Streptococcus;sp._HMT_066.
Otu0026	Fusobacteria;Leptotrichia;Leptotrichia_unclassified.
Otu0027	Proteobacteria;Haemophilus;Haemophilus_unclassified.
Otu0028	Actinobacteria;Actinomyces;sp._HMT_172.
Otu0029	Firmicutes;Streptococcus;Streptococcus_unclassified.
Otu0030	Firmicutes;Streptococcus;sp._HMT_074.
Otu0031	Firmicutes;Solobacterium;moorei.
Otu0032	Actinobacteria;Actinomyces;graevenitzii.
Otu0033	Fusobacteria;Fusobacterium;Fusobacterium_unclassified.
Otu0034	Fusobacteria;Leptotrichia;sp._HMT_215.
Otu0035	Actinobacteria;Atopobium;parvulum.
Otu0036	Saccharibacteria_(TM7);Saccharibacteria_(TM7)_[G-1];bacterium_HMT_352.
Otu0037	Firmicutes;Streptococcus(100);Streptococcus_unclassified(100).
Otu0038	Actinobacteria;Actinomyces(100);sp._HMT_180.
Otu0039	Firmicutes;Streptococcus(100);sp._HMT_066.
Otu0040	Firmicutes;Granulicatella;elegans.
Otu0041	Bacteroidetes;Alloprevotella;sp._HMT_308.
Otu0042	Firmicutes;Streptococcus;infantis_clade_638.

Otu0043	Firmicutes;Streptococcus;oralis_subsp._dentisani_clade_058.
Otu0044	Firmicutes;Streptococcus;parasanguinis_clade_411.
Otu0045	Firmicutes;Peptostreptococcus;stomatis.
Otu0046	Firmicutes;Veillonella;parvula.
Otu0047	Fusobacteria;Leptotrichia;sp._HMT_221.
Otu0048	Firmicutes;Streptococcus;Streptococcus_unclassified.
Otu0049	Bacteroidetes;Prevotella;salivae.
Otu0050	Firmicutes;Streptococcus;Streptococcus_unclassified.
Otu0051	Synergistetes;Pyramidobacter;piscolens.
Otu0052	Firmicutes;Streptococcus;australis.
Otu0053	Firmicutes;Clostridia;Oribacterium;Oribacterium_unclassified.
Otu0054	Firmicutes;Clostridia;Lachnoanaerobaculum;orale.
Otu0055	Firmicutes;Streptococcus;oralis_subsp._dentisani_clade_058.
Otu0056	Firmicutes;Streptococcus;gordonii.
Otu0057	Bacteroidetes;Porphyromonas;pasteri.
Otu0058	Bacteroidetes;Porphyromonas;sp._HMT_930.
Otu0059	Proteobacteria;Haemophilus;sp._HMT_908.
Otu0060	Proteobacteria;Campylobacter;concisus.
Otu0061	Firmicutes;Streptococcus;intermedius.
Otu0062	Bacteroidetes;Porphyromonas;pasteri.
Otu0063	Saccharibacteria_(TM7);Saccharibacteria_(TM7)_[G-6];bacterium_HMT_870.
Otu0064	Firmicutes;Streptococcus;sp._HMT_066.
Otu0065	Firmicutes;Clostridia;Lachnoanaerobaculum;Lachnoanaerobaculum_unclassified.
Otu0066	Firmicutes;Megasphaera;micronuciformis.
Otu0067	Firmicutes;Peptostreptococcaceae_[XI][G-1];sulci.
Otu0068	Firmicutes;Veillonella;sp._HMT_780.
Otu0069	Actinobacteria;Corynebacterium;Corynebacterium_unclassified.
Otu0070	Proteobacteria;Enterobacteriaceae;Enterobacteriaceae_unclassified.
Otu0071	Bacteroidetes;Porphyromonas;gingivalis.
Otu0072	Firmicutes;Streptococcus;australis.
Otu0073	Firmicutes;Abiotrophia;defectiva.
Otu0074	BacterBacteroidetes;Porphyromonas;Porphyromonas_unclassified.
Otu0075	Bacteroidetes;Prevotella;sp._HMT_313.
Otu0076	Bacteroidetes;Porphyromonas;pasteri.
Otu0077	Proteobacteria;Haemophilus;Haemophilus_unclassified.
Otu0078	Actinobacteria;Actinomyces;sp._HMT_180.
Otu0079	Proteobacteria;Neisseria;meningitidis.
Otu0080	Firmicutes;Stomatobaculum;Stomatobaculum_unclassified.
Otu0081	Firmicutes;Streptococcus;Streptococcus_unclassified.
Otu0082	Firmicutes;Streptococcus;Streptococcus_unclassified.
Otu0083	Proteobacteria;Lautropia;mirabilis.
Otu0084	Firmicutes;Streptococcus;sp._HMT_057.
Otu0085	Actinobacteria;Rothia;aeria.
Otu0086	Fusobacteria;Leptotrichiaceae_unclassified.
Otu0087	Firmicutes;Veillonella;Veillonella_unclassified.
Otu0088	Firmicutes;Streptococcus;Streptococcus_unclassified.

Otu0089	Bacteroidetes;Capnocytophaga;leadbetteri.
Otu0090	Firmicutes;Abiotrophia;defectiva.
Otu0091	Bacteroidetes;Bergeyella;sp._HMT_931.
Otu0092	Bacteroidetes;Prevotella;oulorum.
Otu0093	Firmicutes;Oribacterium;Oribacterium_unclassified.
Otu0094	Bacteroidetes;Bergeyella;sp._HMT_322.
Otu0095	Bacteroidetes;Prevotella;sp._HMT_306.
Otu0096	Bacteroidetes;Capnocytophaga;sputigena.
Otu0097	Actinobacteria;Actinomyces;Actinomyces_unclassified.
Otu0098	Proteobacteria;Aggregatibacter;Aggregatibacter_unclassified.
Otu0099	Firmicutes;Veillonella;sp._HMT_780.
Otu0100	Bacteroidetes;Porphyromonas;sp._HMT_930.

Appendix 14. Top 100 Buccal OTUs.

Buccal OTU	Taxonomy
Otu0001	Streptococcus(100);Streptococcus_unclassified(89).
Otu0002	Gemella(100);haemolysans(51);
Otu0003	Haemophilus(100);parainfluenzae(100).
Otu0004	Streptococcus(100);infantis_clade_431(77).
Otu0005	Rothia(100);mucilaginosa(100).
Otu0006	Neisseria(100);perflava(100).
Otu0007	Haemophilus(100);parainfluenzae(100).
Otu0008	Alloprevotella(100);sp._HMT_473(100).
Otu0009	Haemophilus(100);Haemophilus_unclassified(62).
Otu0010	Streptococcus(100);Streptococcus_unclassified(86).
Otu0011	Neisseria(100);Neisseria_unclassified(73).
Otu0012	Streptococcus(100);Streptococcus_unclassified(100).
Otu0013	Veillonella(100);parvula(100).
Otu0014	Veillonella(100);parvula(51).
Otu0015	Granulicatella(100);elegans(100).
Otu0016	Prevotella(100);Prevotella_unclassified(100).
Otu0017	Neisseria(100);meningitidis(100).
Otu0018	Fusobacterium(100);nucleatum_subsp._polymorphum(51).
Otu0019	Granulicatella(100);adiacens(100).
Otu0020	Bergeyella(100);sp._HMT_206(100).
Otu0021	Leptotrichia(100);wadei(60).
Otu0022	Veillonella(100);sp._HMT_780(100).
Otu0023	Porphyromonas(100);pasteri(99).
Otu0024	Streptococcus(100);salivarius(97).
Otu0025	Fusobacterium(100);periodonticum(100).
Otu0026	Haemophilus(100);sp._HMT_908(86).
Otu0027	Streptococcus(100);gordonii(100).
Otu0028	Streptococcus(100);parasanguinis_clade_411(94).
Otu0029	Streptococcus(100);oralis_subsp._dentisani_clade_058(99).
Otu0030	Pyramidobacter(100);piscolens(100).
Otu0031	Granulicatella(100);adiacens(100).
Otu0032	Streptococcus(100);Streptococcus_unclassified(100).
Otu0033	Abiotrophia(100);defectiva(100).
Otu0034	Prevotella(100);nanceiensis(100).
Otu0035	Prevotella(100);histicola(100).
Otu0036	Porphyromonas(100);pasteri(60).
Otu0037	Streptococcus(100);intermedius(99).
Otu0038	Porphyromonas(100);pasteri(96).
Otu0039	Streptococcus_unclassified(86).
Otu0040	Lautropia(100);mirabilis(100).
Otu0041	Actinomyces(100);lingnae_[Not_Validly_Published](100).
Otu0042	Porphyromonas(100);sp._HMT_930(100).

Otu0043	Streptococcus(100);Streptococcus_unclassified(98).
Otu0044	Porphyromonas(100);gingivalis(100).
Otu0045	Actinomyces(100);Actinomyces_unclassified(98).
Otu0046	Streptococcus(100);australis(73).
Otu0047	Streptococcus(100);Streptococcus_unclassified(100).
Otu0048	Streptococcus(100);Streptococcus_unclassified(100).
Otu0049	Capnocytophaga(100);leadbetteri(100).
Otu0050	Rothia(100);aeria(100).
Otu0051	Abiotrophia(100);defectiva(100).
Otu0052	Granulicatella(100);elegans(100).
Otu0053	Streptococcus(100);Streptococcus_unclassified(75).
Otu0054	Leptotrichia(100);sp._HMT_225(100).
Otu0055	Capnocytophaga(100);sputigena(100).
Otu0056	Alloprevotella(100);sp._HMT_473(100).
Otu0057	Haemophilus(100);paraphrohaemolyticus(99).
Otu0058	Actinomyces(100);sp._HMT_180(61).
Otu0059	Actinomyces(100);Actinomyces_unclassified(78).
Otu0060	Haemophilus(100);Haemophilus_unclassified(100).
Otu0061	Gemella(100);sanguinis(100).
Otu0062	Prevotella(100);sp._HMT_317(52).
Otu0063	Rothia(100);Rothia_unclassified(95).
Otu0064	Porphyromonas(100);sp._HMT_930(100).
Otu0065	Porphyromonas(100);pasteri(96).
Otu0066	Lachnoanaerobaculum(100);Lachnoanaerobaculum_unclassified(83).
Otu0067	Streptococcus(100);Streptococcus_unclassified(100).
Otu0068	Prevotella(100);pallens(100).
Otu0069	Fusobacterium(100);nucleatum_subsp._animalis(100).
Otu0070	Prevotella(100);salivae(100).
Otu0071	Campylobacter(100);gracilis(100).
Otu0072	Staphylococcus(100);aureus(100).
Otu0073	Actinomyces(100);graevenitzii(100).
Otu0074	Gemella(100);morbillorum(100).
Otu0075	Leptotrichia(100);sp._HMT_212(100).
Otu0076	Saccharibacteria_(TM7)_[G-1](100);bacterium_HMT_346(100).
Otu0077	Enterobacteriaceae_unclassified(99);Enterobacteriaceae_unclassified(99).
Otu0078	Haemophilus(100);parahaemolyticus(60).
Otu0079	Aggregatibacter(100);Aggregatibacter_unclassified(64).
Otu0080	Streptococcus(100);mutans(100).
Otu0081	Streptococcus(100);Streptococcus_unclassified(100).
Otu0082	Prevotella(100);oulorum(100).
Otu0083	Veillonella(100);sp._HMT_780(100).
Otu0084	Leptotrichia(100);sp._HMT_215(100).
Otu0085	Saccharibacteria_(TM7)_[G-6](100);bacterium_HMT_870(100).
Otu0086	Alloprevotella(100);sp._HMT_308(99).
Otu0087	Leptotrichia(100);shahii(100).
Otu0088	Prevotella(100);oris(100).

Otu0089	Campylobacter(100);concisus(100).
Otu0090	Leptotrichia(100);Leptotrichia_unclassified(63).
Otu0091	Corynebacterium(100);matruchotii(100).
Otu0092	Streptococcus(100);sp._HMT_066(78).
Otu0093	Capnocytophaga(100);sputigena(100).
Otu0094	Escherichia(100);coli(100).
Otu0095	Leptotrichia(100);hongkongensis(100);
Otu0096	Porphyromonas(100);pasteri(52).
Otu0097	Streptococcus(100);oralis_subsp._dentisani_clade_058(100).
Otu0098	Veillonella(100);sp._HMT_780(100).
Otu0099	Leptotrichia(100);Leptotrichia_unclassified(93).
Otu0100	Prevotella(100);sp._HMT_942(100).

Appendix 16. Tongue sample mycobiome showing Mothur classifications and ISHAM BLAST identification.

Sequences	ISHAM BLAST	Mothur
sq1_size_10732114_	Candida_dubliniensis Seq.1	s__Candida_dubliniensis_unclassified Seq.1.
sq2_size_7174794_	Candida_albicans Seq.2	s__Candida_albicans_unclassified Seq.2.
sq3_size_1116069_	Saccharmyces cerevisiae Seq.3	g__Saccharomyces_unclassified Seq.3.
sq4_size_755624_	Didymella gardeniae Seq.4	f__Pleosporales_family_Incertae_sedis_unclassified Seq.4.
sq5_size_303784_	Saccharmyces cerevisiae Seq.5	g__Saccharomyces_unclassified Seq.5.
sq6_size_292485_	Candida_albicans Seq.6	s__Candida_albicans_unclassified Seq.6.
sq7_size_251484_	Cladosporium Cladosporiodes Seq.7	f__Davidiellaceae_unclassified Seq.7.
sq8_size_99991_	Candida xylopsoci Seq.8	s__Candida_xylopsoci_unclassified Seq.8.
sq9_size_80460_	Candida_zeylanoides Seq.9	s__Candida_zeylanoides_unclassified Seq.9.
sq10_size_68464_	Candida_albicans Seq.10	s__Candida_albicans_unclassified Seq.10.
sq12_size_60065_	Candida_albicans Seq.12	s__Candida_albicans_unclassified Seq.12.
sq13_size_54203_	Candida_tropicalis Seq.13	s__Candida_tropicalis_unclassified Seq.13.
sq14_size_52031_	Cladosporium Herbarum Seq.14	f__Davidiellaceae_unclassified Seq.14.
sq16_size_41115_	Candida_albicans Seq.16	s__Candida_albicans_unclassified Seq.16.
sq18_size_39459_	Epicoccum nigrum Seq.18	o__Pleosporales_unclassified Seq.18.
sq19_size_37045_	Epicoccum nigrum Seq.19	o__Pleosporales_unclassified Seq.19.
sq20_size_34897_	Saccharmyces cerevisiae Seq.20	g__Saccharomyces_unclassified Seq.20.
sq22_size_33991_	Cyberlindnera_jadinii Seq.22	s__Cyberlindnera_jadinii_unclassified Seq22.
sq23_size_27634_	Colletotrichum truncatum Seq.23	s__Botrytis_caroliniana_unclassified Seq.23.
sq26_size_20092_	Debaryomyces hansenii Seq.26	g__Debaryomyces_unclassified Seq.26.
sq28_size_17061_	Saccharmyces cerevisiae Seq.28	g__Saccharomyces_unclassified Seq.28.
sq30_size_14440_	Alternaria infectoria Seq.30	o__Pleosporales_unclassified Seq.30.
sq31_size_14340_	Debaryomyces nepalensis Seq.31	s__Debaryomyces_prosopidis_unclassified Seq.31.
sq35_size_10089_	Cryptococcus anemochreius Seq.35	s__Cryptococcus_laurentii_unclassified Seq.35.
sq36_size_10032_	Candida_albicans Seq.36	s__Candida_albicans_unclassified Seq.36.

Appendix 17. Buccal sample mycobiome showing Mothur classifications and ISHAM BLAST identification.

Sequences	Muthor	ISHAM blast
sq1_size_5883316_	s__Candida_dubliniensis_unclassified/ seq 1.	Candida_dubliniensis/ seq 1.
sq2_size_1508625_	s__Candida_albicans_unclassified/seq 2	Candida_albicans/seq 2
sq3_size_619725_	f__Pleosporales_family_Incertae_sedis_unclassified/seq 3.	Peyronellaea glomerata/seq 3.
sq4_size_250833_	g__Saccharomyces_unclassified/seq 4	Saccharomyces cerevisiae/seq 4
sq5_size_172843_	s__Candida_albicans_unclassified/seq 5	Candida_albicans/seq 5
sq6_size_141909_	f__Davidiellaceae_unclassified/seq 6	Cladosporium cladosporioides/seq 6
sq7_size_110389_	s__Candida_xylopsoci_unclassified/seq 7	Pichia kudriavzevii /seq 7
sq8_size_41711_	f__Davidiellaceae_unclassified/seq 8	Cladosporium cladosporioides/seq 8
sq9_size_41459_	s__Xenobotryosphaeria_calamagrostidis_unclassified/seq 9	Xenobotryosphaeria calamagrostidis/seq 9
sq10_size_35799_	s__Candida_albicans_unclassified/seq 10	Candida_albicans/seq 10
sq11_size_34673_	g__Saccharomyces_unclassified/seq 11	Saccharomyces cerevisiae/seq 11
sq12_size_34390_	o__Pleosporales_unclassified/seq 12	Microsphaeropsis olivacea/seq 12
sq13_size_25997_	s__Debaryomyces_hansenii_unclassified/seq 13	Debaryomyces_hansenii/seq 13
sq15_size_24689_	s__Candida_albicans_unclassified/seq 15	Candida_albicans/seq 15
sq16_size_18423_	s__Debaryomyces_prosopidis_unclassified/seq 16	Debaryomyces_hansenii/seq 16
sq17_size_17984_	s__Cyberlindnera_jadinii_unclassified/seq 17	Cyberlindnera_jadinii/seq 17
sq18_size_17010_	o__Pleosporales_unclassified/seq 18	Paraphoma fimeti/seq 18
sq20_size_13959_	s__Alternaria_metachromatica_unclassified/seq 20	Alternaria triticina/seq 20
sq25_size_8899_	g__Saccharomyces_unclassified/seq 25	Saccharomyces cerevisiae/seq 25
sq27_size_7891_	f__Davidiellaceae_unclassified/seq 27	Cladosporium cladosporioides/seq 27
sq28_size_7213_	s__Fusarium_sp_59_4_unclassified/seq 28	Fusarium poae/seq 28
sq33_size_5942_	s__Monographella_nivalis_unclassified/seq 33	Microdochium nivale /seq 33
sq34_size_5880_	s__Candida_zeylanoides_unclassified/seq 34	Candida_zeylanoides/seq 34
sq37_size_5311_	s__Hanseniaspora_uvarum_unclassified/seq 37	Hanseniaspora_uvarum/seq 37
sq39_size_5238_	s__Aureobasidium_pullulans_unclassified/seq 39	Aureobasidium_pullulans/seq 39

Appendix 18. European Oral Microbiology Workshop poster.

JOURNAL OF ORAL MICROBIOLOGY, 2017
SUPPLEMENT, 1325254
https://doi.org/10.1080/20002297.2017.1325254

Taylor & Francis
Taylor & Francis Group

EOMW Stockholm 2017 – 12th European Oral Microbiology Workshop

The oral mucosal microbiome in children with Crohn's disease exhibits reduced biodiversity compared to healthy children, revealed by 16s profiling

Khalid Elmaghrawy

Trinity College, University of Dublin, Dublin, Ireland

ABSTRACT

The oral microbiome was examined in a cohort of treatment naïve children diagnosed with Crohn's disease (n=27, CD) or ulcerative colitis (n=6, UC). A cohort of 28 children were grouped as a healthy control (HC) group. Bacterial DNA was extracted from tongue and buccal swabs and the V1-V2 region of the 16s gene was amplified and sequenced using the MiSeq. Sequences were analysed with the Mothur pipeline.

Reduced biodiversity of the tongue was indicated by differences in the inverse Simpson's index for both sites (CD tongue=9.39; HC=12.87). Analysis of species richness by rarefaction showed a significant reduction in species richness in CD tongue samples compared to HC tongue samples. Analysis of community structure and membership using AMOVA showed that the populations on HC and CD tongues were significantly different ($P < 0.001$). LEfSe analysis identified 20 OTUs that were significantly enriched on the tongues of healthy children including *H. parainfluenzae, N. flavescens, F. periodonticum, Streptococcus sp., Porphyromonas sp., Actinomyces sp*. Children with Crohn's have an altered microflora that may contribute to their oral health problems. These data could potentially be used to diagnose a patients overall gastrointestinal health.

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Appendix 19. TCD Faculty of Health Sciences Research day. Winner of Best Poster.

Appendix 19

The oral microbiome in children with Crohn's disease exhibits reduced biodiversity compared to healthy children, revealed by 16s profiling

Khalid Elmaghrawy, Paddy Fleming, Kirsten Fitzgerald, Tara Raftery, Billy Bourke, Anne-Marie Broderick, Seamus Hussey & Gary Moran.
School of Dental Science TCD and Our Lady's Children's Hospital Crumlin. Email: gpmoran@dental.tcd.ie

Introduction

Crohn's disease is an inflammatory bowel disease that manifests as a result of genetic and environmental factors that contribute to pathogenesis (1). Dysbiosis of the gut microbiome has been identified as a key contributor to the pathogenesis of the disease. In addition to gut manifestations, Crohn's sufferers often exhibit oral ulceration (2). Few studies have examined the oral microbiome in this cohort and the current study uses Illumina sequencing of treatment naïve children to identify oral dysbiosis.

Aims

To determine if oral microbial dysbiosis occurs in Crohn's
To identify non-invasive diagnostic markers
To improve oral health of children with Crohn's

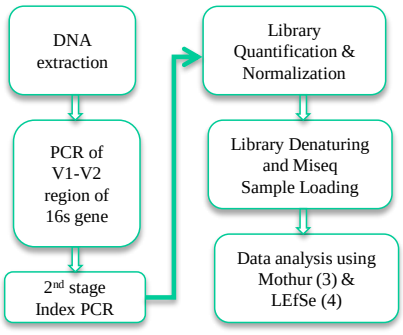
Methods

Ethical approval was granted by the Joint Hospitals Research Ethics Committee (JREC). A total of 62 children were included in the study. Children's ages ranged from 6-16 yrs old. Crohn's and IBD patients were treatment naïve. Control children were those attending the Dublin Dental Hospital for routine check ups. Swabs were recovered form the tongue and buccal cavity using Epicentre Catch-all swabs. DNA was extracted using the Master-Pure DNA extraction kit. The V1V2 region of the 16s gene was amplified from each sample and this was sequenced using the Illumina MiSeq. Data analysis was carried out using the Mothur pipeline (3) with further analysis carried out using LEfSe (4)

Table 1. Categorisation of diseased and healthy children

Crohn's	IBD	Ulcerative Colitis	Healthy Control	Total
23	10	5	24	62

Figure 1. Outline of the analysis pipeline:



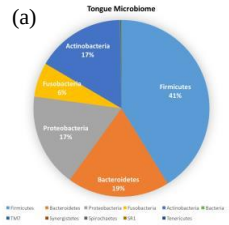
References

1. Gevers D, Kugathasan S et al. 2014. The Treatment-Naive Microbiome in New-Onset Crohn's Disease. Cell Host and Microbe 15:382–392.
2. Hussey S, Fleming P et al. 2011. Disease outcome for children who present with oral manifestations of Crohn's disease. European Archives of Paediatric Dentistry 12:167–169.
3. Schloss PD, Westcott SL et al. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537–7541. 1.
4. Segata N, Izard J et al. 2011. Metagenomic biomarker discovery and explanation. Genome Biol 12:R60.

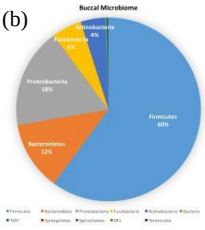
Acknowledgements
This work was supported by the Board of Dublin Dental University Hospital and the National Children's Research Centre. KE is supported by a Scholarship from the Libyan Government. The authors wish to thank Kathleen McGrath and Elaine Kenny at TrinSeq for patience and help with the MiSeq.

Overview of the Buccal and Tongue Microbiomes

(a)



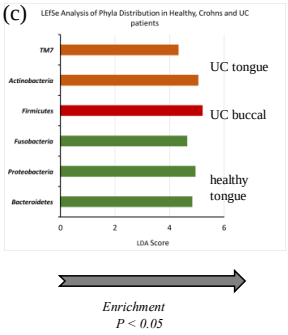
(b)



Sequence reads from tongue and buccal swabs were assigned to the major bacterial phyla as shown in (a) and (b).

The distribution of these Phyla in healthy patients, UC patients, and Crohn's patients was analysed using LEfSe. LEfSe uses a Kruskal-Wallis (KW) sum-rank test to detect features with significant differential abundance. Biological significance is subsequently investigated using the (unpaired) Wilcoxon rank-sum test. As a last step, LEfSe uses Linear Discriminant Analysis (LDA) to estimate the effect size of each differentially abundant feature.
The tongues of healthy children were enriched for Bacteroidetes (Gram –ve), Proteobacteria (Gram –ve) and Fusobacteria (Gram –ve fusiforms).

(c)

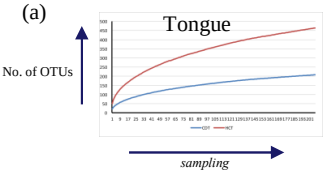


Enrichment
P < 0.05

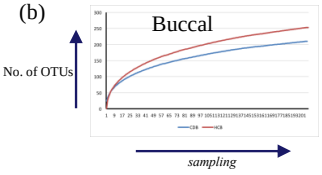
Species level classification

Unique sequences were classified to the species level or OTU (Operating Taxonomic Unit). We compared species richness in Crohn's and healthy patient's buccal and tongue samples by rarefaction (random sampling of increasing numbers of sequence reads). Healthy microbiomes exhibited increased species richness, and this was most pronounced on the tongue (a).

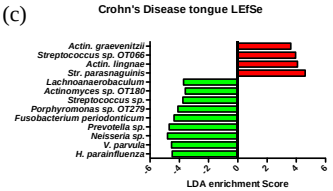
(a)



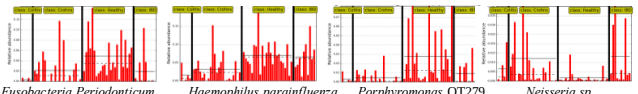
(b)



(c)



(c) Analysis of OTU levels on the tongues of children with Crohn's revealed significant enrichment for *Streptococcus sp*. OT066 and *Actinomyces* sp. However, there was a significant drop in levels of several major oral taxa including *V. parvula, H. parainfluenza and F. periodonticum.*



Fusobacteria Periodonticum *Haemophilus parainfluenza* *Porphyromonas* OT279 *Neisseria sp.*

Conclusions

- Children with Crohn's disease exhibit reduced oral biodiversity
- This dysbiosis is most pronounced on the tongue
- Healthy children have higher levels of several abundant oral OTUs
- These data may be used to develop non-invasive diagnostic tests to characterise a child's gastrointestinal health.

Appendix 20. IADR Annual Conference 2018 poster.

Analysis of the oral microbiome and mycobiome in children with Crohn's disease

Khalid Elmaghrawy, Paddy Fleming, Kirsten Fitzgerald, Seamus Hussey & Gary Moran.
School of Dental Science TCD and Our Lady's Children's Hospital Crumlin. Email: gpmoran@dental.tcd.ie

Introduction

Crohn's disease is an inflammatory bowel disease that manifests as a result of genetic and environmental factors that contribute to pathogenesis. Dysbiosis of the gut microbiome has been identified as a key contributor to the pathogenesis of the disease. In addition to gut manifestations, Crohn's sufferers often exhibit oral ulceration. Few studies have examined the oral microbiome in this cohort and the current study uses Illumina sequencing of treatment naïve children to identify oral dysbiosis.

Aims

To determine if oral microbial dysbiosis occurs in Crohn's
To identify non-invasive diagnostic markers
To improve oral health of children with Crohn's

Methods

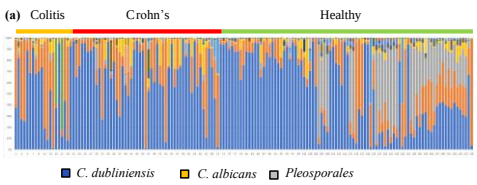
Ethical approval was granted by the Joint Hospitals Research Ethics Committee (JREC). A total of 61 children were included in the study. Children's ages ranged from 6-16 yrs old. Crohn's and ulcerative colitis patients were treatment naïve. Control children were those attending the Dublin Dental Hospital for routine check ups. Swabs were recovered form the tongue and buccal cavity using Epicentre Catch-all swabs. DNA was extracted using the Master-Pure DNA extraction kit. The V1V2 region of the 16s gene was amplified from each sample and this was sequenced using the Illumina MiSeq. ITS2 region of the fungal rDNA was amplified using specific primer (ITS2F and ITS2R). Data analysis was carried out using the Mothur pipeline with further analysis carried out using LEfSe.

Table 1. Categorisation of diseased and healthy children

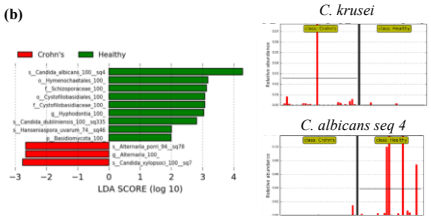
Crohn's	Ulcerative Colitis	Healthy Control	Total
38	21	36	95

Overview of the Mycobiome

Sequence reads from tongue and buccal swabs were assigned to the major fungal phyla using the UNITE ITS2 database. *Candida dubliniensis* was the most abundant fungal species in our analysis, followed by *C. albicans* (a).

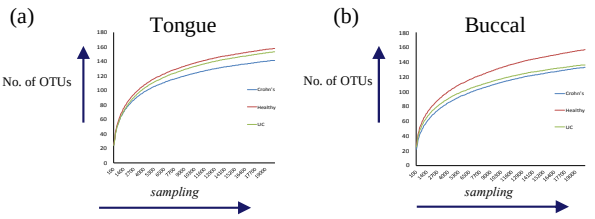


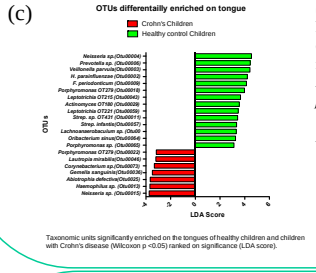
LEfSe identified that *C. albicans* sequence type 4 was more abundant in healthy children, whereas *C. xylopsoci* (*C. krusei*) was more prevalent in Children with Crohn's, although at relatively low abundance (b)



Bacterial classification

Unique sequences were classified to the species level or OTU (Operating Taxonomic Unit). We compared species richness in Crohn's and healthy patient's buccal and tongue samples by rarefaction (random sampling of increasing numbers of sequence reads). Healthy microbiomes exhibited increased species richness (a and b).

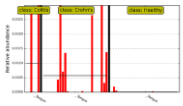




Taxonomic units significantly enriched on the tongues of healthy children and children with Crohn's disease (Wilcoxon p <0.05) ranked on significance (LDA score).

(c) Analysis of OTU levels on the tongues of healthy children compared to those with Crohn's disease revealed significant enrichment for several major oral taxa including *V. parvula, Neisseria sp., Prevotella sp, H. parainfluenza and F. periodonticum.*
Several Crohn's patients exhibited enrichment for *Klebsiella pneumonia* (d).

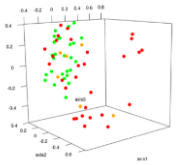
(d)



Text

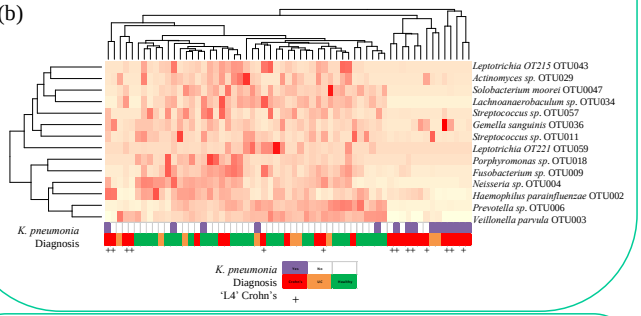
L4 Diagnosis is associated with bacterial dysbiosis

(a)



(a) Analysis of community structure using NMDS to visualise Bray-Curtis dissimilarity values revealed that Crohn's patients exhibited diverse community structures (red=Crohn's, green=control, orange=UC).
(b) A heatmap was generated using Vegan to examine the relative abundance of the enriched taxa identified using LEfSe. Crohn's patients exhibited reduced levels of the selected taxa and this was most apparent in those with an 'L4' classification, indicative of upper GI tract involvement.

(b)



Conclusions

- Children with Crohn's disease exhibit reduced bacterial biodiversity but increased mycobiome diversity
- This dysbiosis is most pronounced on the tongue
- The 'L4' diagnsosis is associated with the most severe dysbiosis
- These data may be used to develop non-invasive diagnostic tests to characterise a child's gastrointestinal health.

Acknowledgements

This work was supported by the Board of Dublin Dental University Hospital and the National Children's Research Centre. KE is supported by a Scholarship from the Libyan Government. The authors wish to thank Kathleen McGrath and Elaine Kenny at TrinSeq for patience and help with the MiSeq.