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DECIPHERING THE SALIVARY MICROBIOME IN CROHN'S DISEASE PATIENTS WITH DIFFERENT FACTORS CONTRIBUTING TO DYSBIOSIS

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جامعة الإمارات العربية المتحدة
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College of Medicine and Health Sciences

Department of Pharmacology and Therapeutics

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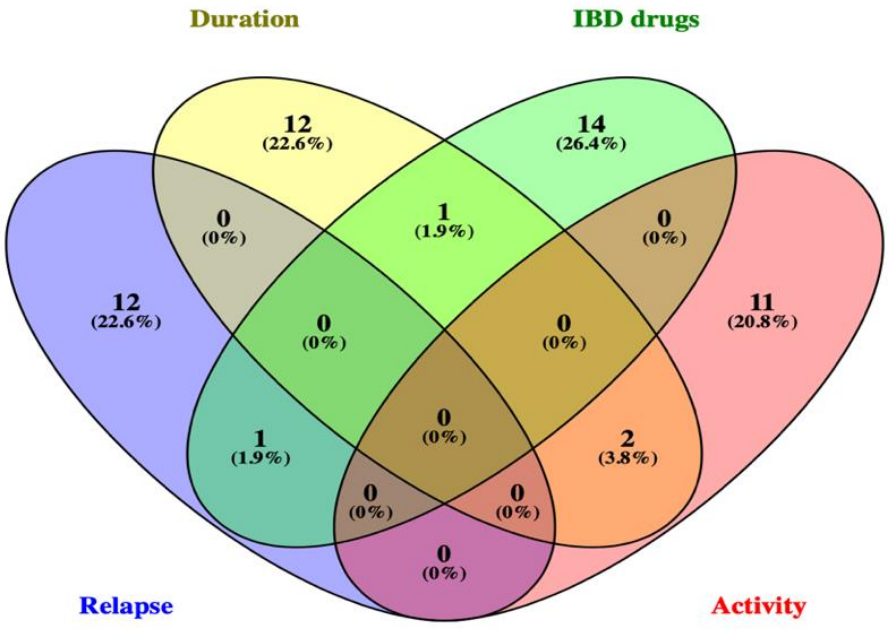

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This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Medical Sciences (Pharmacology and Toxicology)

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Cover: Dysbiosis in Crohn’s Disease is caused by the interaction of several factors.

(Photo: By Hala Elzayat)

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Declaration of Original Work

I, Hala Elzayat, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Deciphering the Salivary Microbiome in Crohn's Disease Patients with Different Factors Contributing to Dysbiosis*", hereby, solemnly declare that this is the original research work done by me under the supervision of Dr. Farah Al-Marzooq in the College of Medicine and Health Sciences at UAEU. This work has not previously formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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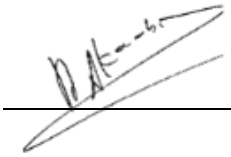
Signature 

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Abstract

Crohn's Disease (CD) is a chronic Inflammatory Bowel Disease (IBD), common in the UAE. Microbiota is necessary to maintain a balanced gut environment which is essential for good health. Dysbiosis can predispose to many diseases including CD. The oral cavity has the second largest and most diverse microbiota after the gut harboring over 700 species of bacteria. This study aims to investigate the alterations in the salivary microbiome in patients with CD compared to Healthy controls (HC). It also aims to compare CD patients for salivary microbiome complexity and diversity according to different factors that can contribute to dysbiosis, including oral health, IBD drug use, disease duration, activity of the disease and relapse of symptoms. Finally, it aims to find any correlation between the inflammatory biomarkers in CD with their levels in saliva, and any possible link to oral dysbiosis.

A total of 80 saliva samples were collected from CD patients and HC (n=40 in each group) seeking healthcare from two hospitals in Abu Dhabi, UAE. Information related to the participants' oral and general health was recorded. DNA was extracted from saliva and sequenced using Oxford nanopore technology for salivary microbiome profiling. Salivary supernatant was used to measure inflammatory biomarkers including C-reactive protein (CRP) and Calprotectin (CAL) by Enzyme-linked immunosorbent assay (ELISA). Data was analyzed using appropriate bioinformatics and biostatistics tools.

Obvious differences in the salivary microbiome of CD were found when compared to HC. Five dominant species were enriched in CD and depleted in HC, namely *Veillonella dispar, Megasphaera stantonii, Prevotella jejuni, Dolosigranulum pigrum* and *Lactobacillus backii*. Oral health is confirmed to have paramount significance in the dysbiosis of the oral microbiota since most significant features are cariogenic such as *Streptococcus mutans* or periopathogenic such as *Fusobacterium periodonticum*. Loss of operational taxa diversity was shown by multiple alpha diversity indices, as well as dissimilarities between CD samples that were interpreted through beta diversity measures. The activity of the disease, duration and the relapse of symptoms also had great impacts on the shift or disruption of the normal balance of the oral

microbiota. Interestingly, treatment with biologicals led to the emergence of a novel species called *Simonseilla muelleri*. When immunomodulatory agents were used in conjunction with biologicals, pathogenic species such as *Salmonella enterica*, *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were recognized. Finally, inflammatory biomarkers were also analyzed confirming an association with significance of being biomarkers for the presence of inflammatory bowel disease and reduction of diversity in the oral microbiome.

In conclusion, we were able to decipher the salivary microbiome of CD patients and prove that the interplay of variable factors contributed to dysbiosis. Each factor seems to have a unique effect on the oral microbiome. Nevertheless, oral health status was found to be of greatest impact. Poor oral health contributes to oral dysbiosis and hence can induce bowel inflammation, especially in the presence of oral periodontal disease such as periodontitis which is obviously an inflammatory condition. Oral health had the greatest impact according to the hypothesis of the ingestion of the tremendous amount of saliva being a reservoir of different microbial species (pathogenic or opportunistic), contributing to dysbiosis in CD patients. In addition, IBD drugs had equivalent influence as the oral health in terms of dysbiosis. Saliva can be used as a tool to detect bacterial dysbiosis and some degree of inflammation, since it is less invasive and more convenient.

Our study is considered unique as this type of in-depth salivary microbiome analyses in CD is established for the first time in the UAE, utilizing a sequencing technique with high resolution enabling the characterization of microbiota down to the species level, in addition to the involvement of multiple factors that added to its uniqueness.

Keywords: Crohn’s disease, Inflammatory bowel disease, Microbiota, Microbiome, Dysbiosis, IBD drugs, Diversity, Inflammatory biomarkers, C-reactive protein (CRP) and Calprotectin (CAL).

Title and Abstract (in Arabic)

فك شفرة الميكروبيوم اللعابي في مرضى كرون المصابين بعوامل مختلفة تساهم في خلل التوازن الميكروبي

الملخص

يعد مرض كرون (CD) مرض التهاب أمعاء مزمن (IBD)، وهو شائع في الإمارات العربية المتحدة. المحتوى الميكروبيومي ضروري للحفاظ على بيئة أمعاء متوازنة وكذلك ضروري لصحة جيدة. يمكن أن يؤهب اختلال التوازن الميكروبي للعديد من الأمراض بما في ذلك داء كرون. يحتوي تجويف الفم على ثاني أكبر مجاميع الميكروبات وأكثرها تنوعًا بعد الأمعاء التي تأوي أكثر من 700 نوع من البكتيريا. تهدف هذه الدراسة إلى التحقق في تغيرات الميكروبيوم اللعابي في المرضى الذين يعانون من داء كرون مقارنة بمجموعه اشخاص اصحاء. وتهدف أيضًا إلى مقارنة مرضى كرون من حيث تعقيد وتنوع الميكروبيوم اللعابي وفقًا لعوامل مختلفة يمكن أن تسهم في اختلال التوازن الميكروبي، بما في ذلك صحة الفم وتعاطي عقاقير مرض التهاب الأمعاء ومدة المرض ونشاط المرض وانتكاس الأعراض. أخيرًا، تهدف إلى العثور على أي ارتباط بين المؤشرات الحيوية الالتهابية في مرض كرون ومستوياتها في اللعاب، وأي ارتباط محتمل بخلل التوازن الميكروبي في الفم. تم جمع 80 عينة لعاب من مجموعة من مرضى داء كرون ومجموعه اشخاص اصحاء (40 في كل مجموعة) الذين يتلقون رعاية صحية من مستشفيين في أبو ظبي، الإمارات العربية المتحدة. تم تسجيل المعلومات المتعلقة بصحة الفم والصحة العامة للمشاركين. تم استخراج الحمض النووي من اللعاب وتسلسله باستخدام تقنية أكسفورد النانوية لتشخيص الميكروبيوم اللعابي. استخدم طاف اللعاب لقياس المؤشرات الحيوية الالتهابية بما في ذلك بروتين سي التفاعلي CRP والكالبروتكتين (CAL) عن طريق مقايسة الممتز المناعي المرتبط بالإنزيم (ELISA). تم تحليل البيانات باستخدام المعلومات الحيوية المناسبة وأدوات الإحصاء الحيوي. تم العثور على اختلافات واضحة في الميكروبيوم اللعابي لمرضى داء كرون بالمقارنة مع اشخاص اصحاء. وجدت خمسة أنواع سائدة في مرض كرون التي استنفدت في الاشخاص الاصحاء، وهي *Veillonella dispar* و*Megasphaera stantonii* و*Prevotella jejuni* و*Dolosigranulum pigrum* و*Lactobacillus backii*. تم التأكد من أن صحة الفم لها أهمية قصوى في اختلال التوازن الميكروبي للجراثيم الفموية حيث إن أهم السمات هي مسببات التسوس مثل *Streptococcus mutans* أو مسببات امراض التهاب اللثة مثل *Fusobacterium periodonticum*. تم إظهار فقدان التنوع لأصناف البكتيريا من خلال عدة مؤشرات تنوع ألفا، بالإضافة إلى الاختلافات بين عينات مرض كرون التي تم تفسيرها من خلال مقاييس التنوع بيتا. كان لنشاط المرض ومدته وانتكاس الأعراض أيضًا تأثيرات كبيرة على تحول أو اضطراب التوازن الطبيعي لميكروبات الفم. ومن المثير للاهتمام أن العلاج البيولوجي أدى إلى ظهور نوع جديد من البكتيريا يسمى *Simonseilla muelleri*. عند استخدام العقاقير المعدلة للمناعة مع العوامل البيولوجية، تم التعرف على أنواع من البكتيريا المسببة للأمراض مثل السالمونيلا المعوية والإشريكية القولونية والكلبسيلا الرئوية والزائفة الزنجارية. أخيرًا، تم تحليل المؤشرات الحيوية الالتهابية أيضًا لتأكيد الارتباط مع كونها مؤشرات حيوية لمرض التهاب الأمعاء وتقليل التنوع في الميكروبيوم الفموي.

في الختام، تمكنا من فك شفرة الميكروبيوم اللعابي لمرضى كرون وإثبات أن تفاعل العوامل المختلفة ساهم في خلل التوازن الميكروبي. يبدو أن كل عامل له تأثير فريد على ميكروبيوم الفم. ومع ذلك، وجد أن حالة صحة الفم لها التأثير الأعظم. يساهم ضعف صحة الفم في الإصابة بخلل التوازن الميكروبي في الفم، وبالتالي يمكن أن يؤدي إلى التهاب الأمعاء، خاصة في وجود أمراض اللثة الفموية مثل التهاب دواعم الأسنان التي من الواضح أنها حالة التهابية. كان لصحة الفم التأثير الأكبر وفقًا لفرضية ابتلاع كمية هائلة من اللعاب كونها خزانًا لأنواع جرثومية مختلفة (مسببة للأمراض أو انتهازية)، مما يساهم في خلل التوازن الميكروبي لدى مرضى كرون. بالإضافة إلى ذلك، كان لأدوية الـ IBD تأثير مكافئ مثل صحة الفم من حيث خلل التوازن الميكروبي. يمكن استخدام اللعاب كأداة للكشف عن اختلال التوازن الميكروبي الجرثومي ودرجة معينة من الالتهاب، لأنه أقل توغلاً وأكثر ملاءمة.

تعتبر دراستنا فريدة من نوعها لأن هذا النوع من التحليلات المتعمقة للميكروبيوم اللعابي في مرض كرون تم إنشاؤه لأول مرة في الإمارات العربية المتحدة، باستخدام تقنية التسلسل الجيني بدقة عالية تمكن من توصيف الميكروبات وصولاً إلى مستوى الأنواع، بالإضافة إلى المشاركة من العوامل المتعددة التي أضافت إلى تفرده.

مفاهيم البحث الرئيسية: داء كرون، مرض الأمعاء الالتهابي، الجراثيم، الميكروبيوم، اختلال التوازن الميكروبي، عقاقير IBD، التنوع، المؤشرات الحيوية الالتهابية، بروتين سي التفاعلي (CRP) وكالبروتكتين (CAL).

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I would like to begin with an expression of gratitude to my advisor Dr. Farah Al-Marzooq whom the completion of this masters would not have been possible without her guidance, support, and assistance.

There must be an extraordinary thanks to my husband Dr. Ahmed Godat for his endless encouragement. Furthermore, I value the love and patience of my kids. Finally, I'd like to express my gratitude to my parents, siblings and especially my father Dr. Ali Elzayat for his continued advice and wise counsel.

Dedication

To my beloved parents, husband, kids, family, and friends

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List of Abbreviations

B	Biologicals
bp	Base pairs
C	Caries
CD	Crohn’s Disease
°C	Degree Celsius
CDAI	Crohn's Disease Activity Index
CAL	Calprotectin
CRP	C-reactive protein
DNA	Deoxyribonucleic Acid
ELISA	Enzyme linked immunosorbent assay
EDTA	Ethylenediaminetetraacetic acid
fmoles	femtomole (10^{-15} moles)
GIT	Gastrointestinal tract
H	Healthy
HC	Healthy controls
I	Immunosuppressants
IBD	Inflammatory bowel disease
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimole
NGS	Next generation sequencing
NaCl	Sodium chloride
ng	Nano gram
P	Periodontal disease
pg	Pico gram
PCR	Polymerase chain reaction
pH	Potential Hydrogen, measure of acidity or basicity of a solution
RNA	Ribonucleic acid

rpm	Revolutions per minute
S	Steroids
UAE	United Arab Emirates
UC	Ulcerative colitis
µg	Micro-gram
µl	Micro-litre
X g	Gravity relative centrifugal force

Chapter 1: Introduction

1.1 Inflammatory Bowel Disease (IBD)

Inflammatory bowel disease (IBD) is a chronic, relapsing, progressive, and potentially debilitating inflammatory illness that affects the Gastrointestinal tract (GIT). The incidence and prevalence of IBD are growing at an alarming rate over the world, notably in the Middle East, African nations, and Asia Pacific. Despite recent discoveries of the increase in the prevalence of IBD in Arab nations, there is inadequate data on IBD patients' features and disease progression in the Arab globe (Mosli et al., 2021). Rising trends have recently been discovered in populations previously believed to have low prevalence and incidence of IBD (Al-Mofarreh & Al-Mofleh, 2013). IBD is characterized by periods of abdominal discomfort, diarrhea, bloody stools, and weight loss, IBD includes two primary types of chronic inflammatory intestinal disorders: Crohn's disease (CD), and Ulcerative colitis (UC) (Gajendran et al., 2018).

CD is a chronic idiopathic inflammatory bowel illness characterized by skip lesions and transmural inflammation that can affect the whole gastrointestinal system from the mouth to the anus (Gajendran et al., 2018b). CD most commonly affects the terminal ileum, cecum, perianal area, and colon, but it can affect any region of the intestine in a random pattern. UC, on the other hand, affects the rectum and can affect a portion of the colon or the entire colon in a continuous pattern. CD and UC present in distinct ways. CD is distinguished by granulomatous inflammation that can affect any portion of the GIT and involve all mucosal layers, whereas UC inflammation is restricted to the colon and only affects the mucosa and superficial submucosa. CD has thickened submucosa, transmural inflammation, fissuring ulceration, and granulomas, whereas UC has mucosa and submucosa inflammation with cryptitis and crypt abscesses (Guan, 2019). Figure 1 shows the major differences between CD and UC.

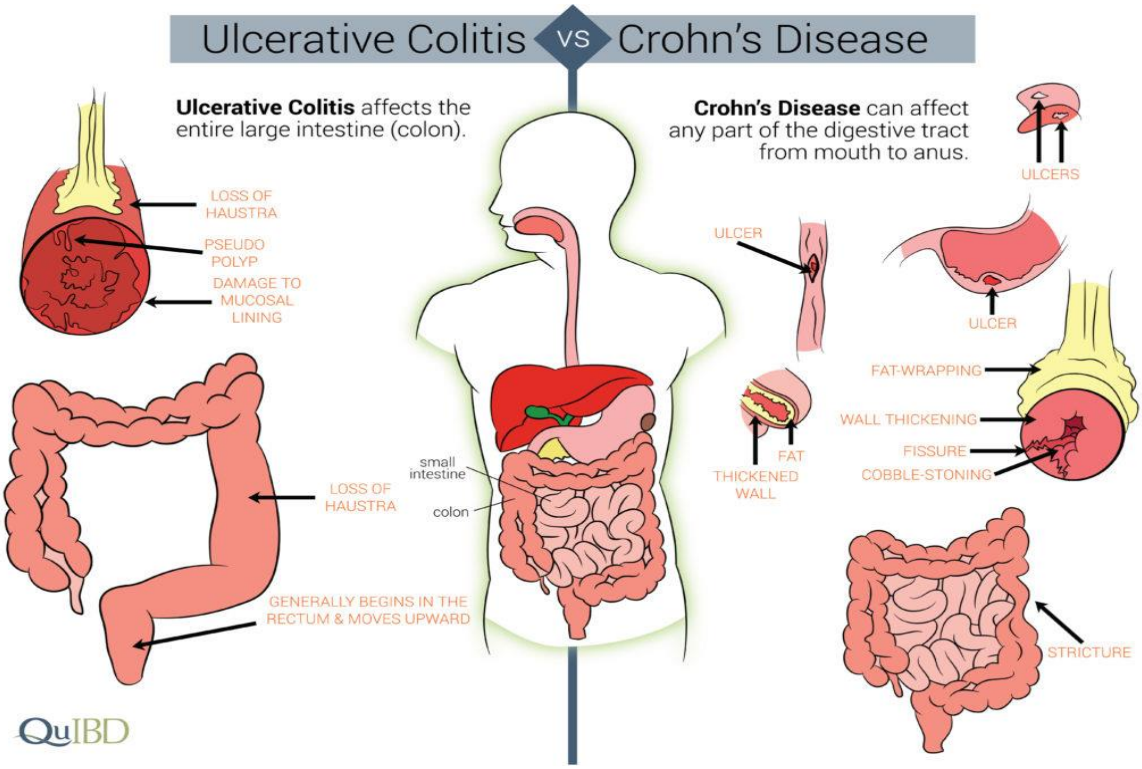


Figure 1: Differences Between Crohn's Disease and Ulcerative Colitis (QuIBD, 2022).

1.2 Clinical Manifestations of CD

Because of the heterogeneity in location of CD lesions in the gut and the transmural nature of CD, patients come with a wide range of symptoms. Diarrhea, gastric discomfort, nausea, and vomiting are among the symptoms that may be present. In severe cases, patients may present with systemic symptoms such as fever, anorexia, and weight loss. In addition to the underlying disease pattern, up to one-third of individuals have perianal involvement. Endoscopic and/or radiologic evidence are often used to make the diagnosis (Feuerstein & Cheifetz, 2017).

There are several sorts of disease phenotypes, including inflammatory, stricturing, and penetrating. During their condition, individuals may have one or more of these disease phenotypes, and patients frequently advance from one phenotype to the other. CD, like most immune-mediated illnesses, has a chronic, indolent course with intervals of recurrence. The natural course of the disease is marked by alternating periods of remission and relapse.

Disease flares occur at random and, for the most part, are currently unpredictable. To facilitate treatment decisions and avoid overtreatment, predictors of a favorable or unfavorable clinical course are required (Liverani et al., 2016).

For the assessment of the activity of the disease, Crohn's Disease Activity Index (CDAI) is used. CDAI is the sum of scores from a list of eight elements and multiplied by weighting factors for each item to characterize the severity of "disease activity" in CD patients. The CDAI is essentially a numerical estimate of a physician's assessment of a patient's symptoms. Index values of 150 or below were related with dormant or inactive illness (i.e., remission). Values more than 150 indicated active illness, and values greater than 450 indicated highly severe disease (Freeman, 2008). Alternatively, the Montreal classification is used to divide CD patients into subgroups based on age of onset, disease site, and disease behavior (Zhang et al., 2020).

It is believed that roughly 47% of IBD patients have Extraintestinal manifestations (EIMs), which most commonly affect the skin, eyes, joints, liver, biliary tract, and lungs. Surprisingly, the existence of one EIM has been demonstrated to increase the likelihood of the creation of other EIMs (Woo, 2015). According to some research, oral symptoms in CD are an excellent indication of IBD and can be used to make a diagnosis (Elmaghrawy et al., 2021).

1.3 Oral Manifestations in Crohn’s Disease

Oral CD typically manifests at a young age and is most commonly found in teenagers and young adults (Woo, 2015). Persistent lip swelling, cobblestoning of the oral mucosa, mucogingivitis, deep linear or serpiginous ulcerations surrounded by epithelial hyperplasia, and tissue tags or polyps are all pathognomonic for CD (Nijakowski & Surdacka, 2020). Based on the presence of granulomas noted on histopathology reports, oral manifestations of CD can be specific or non-specific. The buccal mucosa, gingiva, lips, vestibular, and retromolar areas of the mouth are the most affected. Cobblestoning is defined as fissured, swollen buccal mucosa with corrugation and a hyperplastic appearance of the mucosa. These lesions are typically found in the posterior buccal mucosa and are sometimes associated with succulent mucosal folds with normal epithelium. In CD, the gingiva can become edematous, granular, and

hyperplastic, with or without ulceration. The entire gingiva, up to the mucogingival line, could be affected. Patients with IBD and other EIMs are more likely than others to experience recurrent aphthous stomatitis (Lankarani et al., 2013). Table 1 summarizes the possible oral manifestations of CD.

Table 1: Oral Manifestations in Crohn's Disease (Woo, 2015).

Lesion	Site(s)	Characteristics
Persistent mucosal swelling	Lips, buccal mucosa	Labial enlargement, firm to palpation, typically painless
Cobblestoning of mucosa	Buccal mucosa, vestibule	Mucosal edema with or without fissuring
Mucogingivitis	Attached gingiva, alveolar mucosa	Patchy erythematous macules or plaques with or without hyperplasia
Linear ulcerations	Vestibule, buccal mucosa, tongue, palate	Deep ulcerations with or without hyperplastic margins
Mucosal tags or polyps	Buccal mucosa, vestibule	Hyperplasia of mucosa, firm or boggy to palpation

1.4 IBD Pathogenesis

For a long time, investigations of mucosal immunity, particularly the T cell response, have dominated the exploration of IBD pathophysiology. Evidence shows that innate and adaptive immune pathway dysfunctions lead to abnormal intestinal inflammatory response in IBD patients. Most of the research over the last two decades has concentrated on the role of aberrant adaptive immune responses in the pathogenesis of IBD. The emphasis on the adaptive immune response has eventually led to the idea that the two major kinds of IBD reflect clearly separate forms of intestinal inflammation. CD has long been thought to be caused by a Th1 response, whereas UC is thought to be caused by a non-conventional Th2 response (Zhang & Li, 2014).

There is growing acknowledgment of single gene disorders that underpin a fraction of IBD patients, particularly those with early-onset disease, such as primary immunodeficiencies associated with early-onset IBD (Kelsen & Sullivan, 2017).

Inflammatory bowel illness is frequently a polygenic condition involving the gut microbiota, barrier dysfunction, and dysregulated host responses to microbial stimulation. IBDs are genetically connected to host pathways that suggest an underlying role for abnormal immune responses to intestinal microbiota (Gevers et al., 2014).

1.5 Pathophysiology of Crohn's Disease

Based on epidemiological, genetic, and immunological evidence, CD is thought to be a heterogeneous condition with a complex etiology, in which genetics and environment combine to cause the disease (Gajendran et al., 2018a).

1.5.1 Genetics

In recent years, there has been a significant increase in information regarding genetic variables that are crucial for CD, and multiple susceptibility genes have been linked to the disease (Dicksved et al., 2008). The transition to an inflammatory state in CD is thought to be produced by changes in the gut flora and the host's mucosal response, both of which are controlled by genetics and immunology. To date, the most important independent risk factor for developing CD is a positive family history. Over a decade of genome-wide association studies and other genetic investigations, IBD has been related to genetic loci that indicate an abnormal immune response to the gut microbiota. Recently, genome-wide association studies have discovered more than 30 loci linked to CD (Guan, 2019).

The genetic variants that modify adaptive immunity, as well as the mutations linked with insufficient bacterial monitoring by the intestinal mucosa, may be significant. The combined effect of these genetic mutations may result in the development of aberrant immunological tolerance to intestinal antigens. The improper mucosal inflammatory response is most likely the outcome of immune system dysfunction. Immunologic factors reported to be implicated in CD include dysfunction of the innate immune system, resulting in a persistent proinflammatory milieu in the

intestines; excessive activation and differentiation of T-cell subsets against mucosal antigens; and abnormal cytokine production. The cytokine IFN-γ appears to play an important role in sustaining the inflammatory environment in the gut. Such discoveries might be important in the development of more focused CD treatments (Guan, 2019).

1.5.2 Environmental variables

Environmental variables have been linked to the pathophysiology of CD. These include sociodemographic variables like economic growth, income increases, and urbanization, geographic factors like exposure to northern temperatures, and lifestyle elements including tobacco smoking, oral contraceptive usage, food, and psychological stress. In North America, the incidence of CD ranges from 3.1 to 14.6 instances per 100,000 person-years, with the first peak happening in early adulthood and the second peak occurring between the ages of 50 and 70 (Guan, 2019).

1.5.3 Microbiota and dysbiosis

The human microbiome is a reciprocal network of microbes that spans various organ systems. Bacteria dominate the variety of human microbiota, but fungi, viruses, and protists should not be overlooked. Microbial cells are considered to outnumber host cells in the human body. A growing body of research suggests that the "microbial signature" is host-specific and rather stable through time. As our understanding of the human microbiome and its link to host health grows, it is becoming obvious that many, if not most, chronic illnesses have a microbial component.

The human digestive system microbiome is made up of hundreds of bacterial and fungal species, and these microorganisms have 150 times more genes than the human genome. The gut microbiota, the body's richest reservoir of bacteria, coexists with its host in varying densities throughout the GIT, peaking in the colon at 10^{11} or 10^{12} cells/g luminal contents (Kostic et al., 2014).

This ecosystem performs a variety of important services for the host, such as digesting substrates that are inaccessible to host enzymes, teaching the immune system, and suppressing the proliferation of dangerous microbes. The composition, function, and metabolites of microbiota have a significant impact on host physiology; thus, gut

microbiota plays an important role in metabolic physiology and host physiology. The gut microbiota is important for human health and has been linked to nutritional absorption, mucosal barrier strengthening, xenobiotic metabolism, angiogenesis, and postnatal intestinal development. Moreover, the human microbiota increases immunity (both innate and adaptive) and plays a crucial role in immune system development. Furthermore, research in germ-free mice indicates that gut bacteria impact body fat accumulation, metabolism, and immunological function (Kostic et al., 2014).

Genetics, nutrition, age, pharmacological treatment, smoking, and potentially many other variables can all influence the composition of the microbial gut population. The proportional impact of each of these elements is unknown, however some are directly or indirectly related to illness condition (Kostic et al., 2014).

The function of the microbiome in the pathogenesis of IBD is still being contested; however, the illness involves a significant inflammatory response that can be induced by acquired infection or alterations in the host's own microbiome. A growing body of research shows that a combination of host genetics and gut microbiota makeup is crucial in the development of CD. The most acknowledged mechanism of CD pathogenesis is inflammation caused by an altered host immune response in conjunction with ongoing stimulation by the resident gut flora. Several studies indicate that intestinal inflammation is caused by an unbalanced mucosal immune response to commensal bacteria in genetically sensitive people (de Alencar Junior et al., 2020). Many studies have also demonstrated that the gut microbiota of IBD patients differs significantly from that of healthy controls, a condition known as dysbiosis (Said et al., 2014). Dysbiosis is the consequence of disruptions in the microbiome, which is described as changes in the organization of a microbial community that are harmful to its host. The resulting dysbiosis manifests as an altered balance of gut microbiota elements. This may impede key microbiome functions, including resistance to harmful bacteria. Possible causes of dysbiosis are shown in Figure 2.

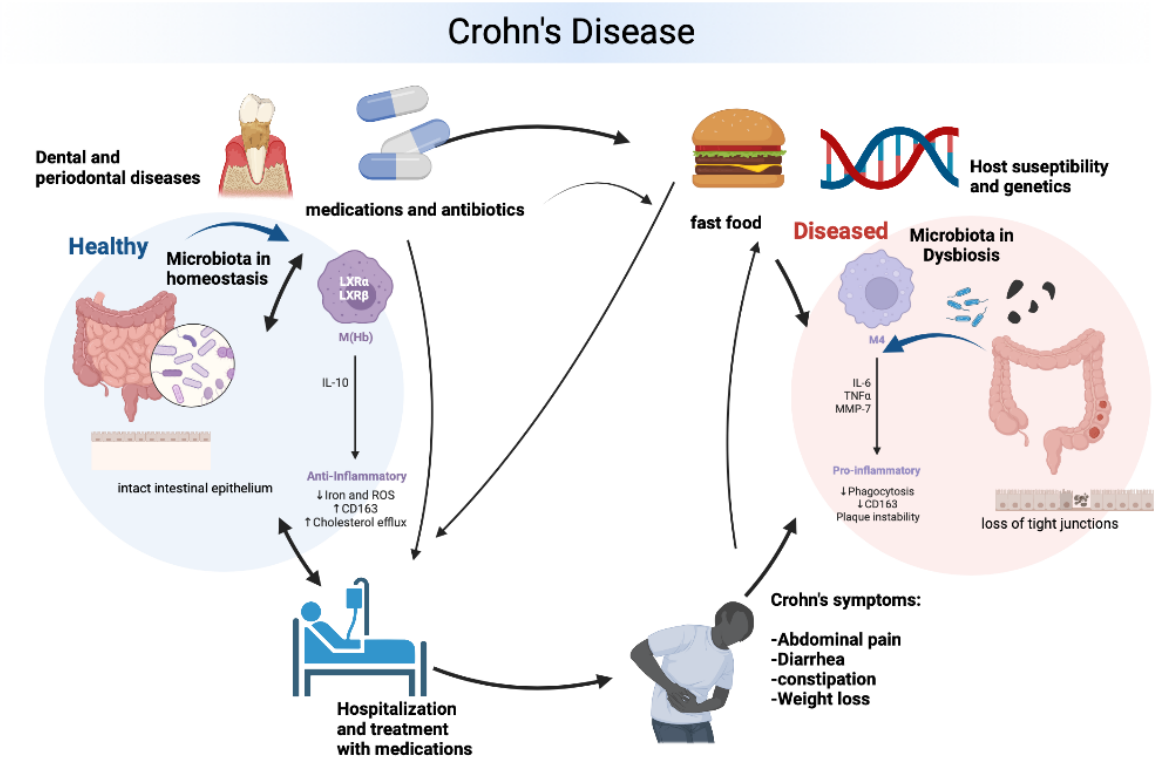


Figure 2: Possible causes of Dysbiosis as a Consequence of Disruptions in the Microbiome.

The investigation of the involvement of microbial communities in the development of IBD has reached a significant milestone. Improved technology can help us better understand the interactions between the host and its resident microbiota, as well as their involvement in IBD, from both a substantial pathway perspective and at the metabolic level (Kostic et al., 2014).

The extensive use of low-resolution surveys of microbial community structure in the past, as well as renewed efforts using next-generation sequencing for a high-resolution description of composition, function, and ecology, have improved our overall understanding of the role of the gut microbiota in health, which is required for the study of disease-related dysbiosis. Sequencing of the bacterial 16S rRNA gene has improved our understanding of the bacterial makeup in various body regions as well as the complex bacterial communities in IBD. Various changes in the gut microbiota have been detected in CD patients using next-generation sequencing technologies.

Despite some discrepancies, the most common effects include decreased bacterial diversity, which is associated with loss of *Firmicutes* and increases in *Proteobacteria*.

However, there is some evidence that certain infections, such as *Mycobacterium avium* subspecies paratuberculosis and adhesive-invasive *E. coli*, have a role in CD, it is more likely that an imbalance in the total microbiota is more significant for the development of CD (Kostic et al., 2014).

Furthermore, certain gut microorganisms, such as *Faecalibacterium prausnitzii*, appear to exhibit protective benefits; *F. prausnitzii* concentration is reduced in colitis patients, and its mechanisms of protective activity, as well as its potential therapeutic implications, are being investigated. Another intriguing finding is that the gut microbiota in CD patients is more unstable than in healthy persons. However, it is unclear whether the observed dysbiosis is the cause or a result of the intestinal inflammation in CD, with most evidence pointing to the hypothesis that dysbiosis is directly connected to hereditary and environmental variables (Nikitakis et al., 2017).

Although the processes of gut microbiota interaction with the immune system have lately been emphasized, the link between the oral microbiota and host immunity in IBD is less well understood (Qi et al., 2021).

1.6 Oral Microbiome and its Role in CD

The complex microbiota that inhabits the mouth, which includes more than 700 prominent species, contributes greatly to the host's oral and extra-oral health. The oral cavity is connected to the GIT; therefore, oral health may be directly related to gut health. Although the host–microbe interaction has been linked to the pathophysiology of CD in genetically predisposed hosts, little is known about oral microorganisms in CD. It is speculated that the microbiology of the oral cavity may differ in IBD patients (Docktor et al., 2012).

W.D. Miller, a pioneering oral microbiologist, postulated in the 1890s that bacteria in the mouth cavity and their products might have a dramatic impact on several illnesses, both local and general, owing to dental bacteremia, which was dubbed "oral focal infection hypothesis"(Xun et al., 2018).

Even though the mouth is constantly exposed to a bombardment of host and environmental toxins, the oral microbiome in healthy people remains reasonably consistent over time.

Given this, alterations in the oral microbiota profile may give correlative insight into illness initiation, progression, and recurrence. Recent microbiome research in IBD suggests that translocation of oral bacteria to the gut is a frequent characteristic of microbial dysbiosis that is a hallmark of CD (Xun et al., 2018). Figure 3 shows the possible ways of translocation of oral microbiota from the mouth to the gut in IBD patients.

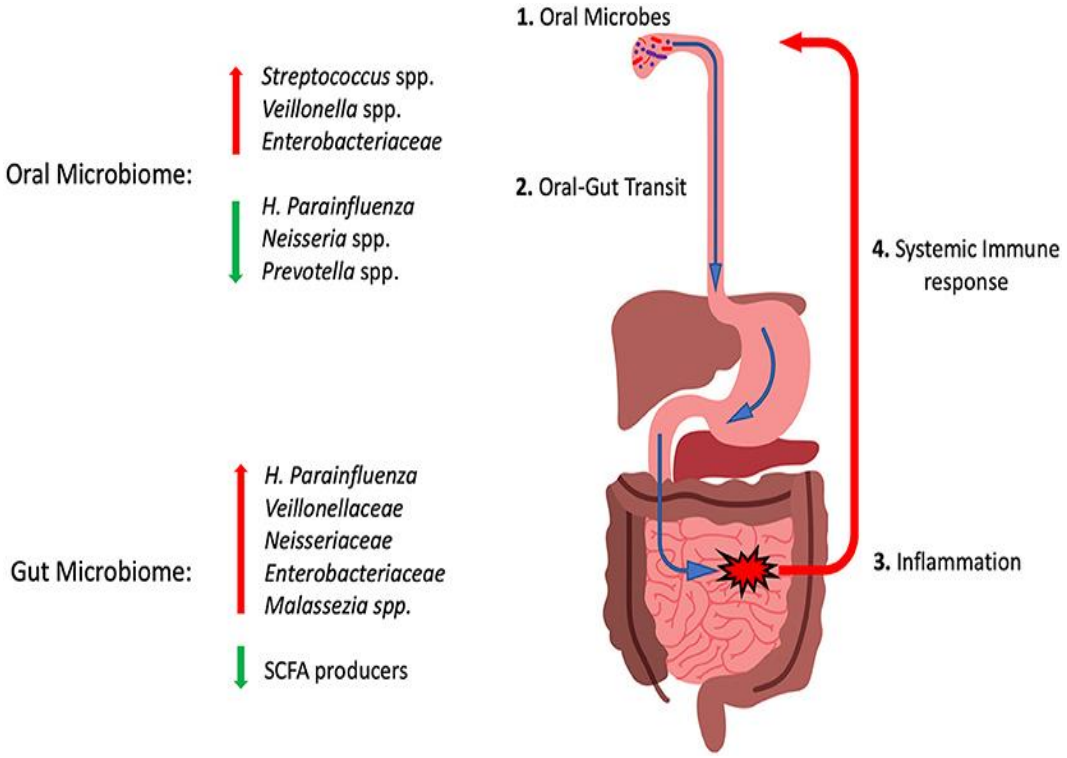


Figure 3: The Journey of Microbiota from the Oral cavity to the Gut (Elmaghrawy et al., 2021).

As shown in Figure 3, translocation of oral microbiota to the lower GIT may cause inflammation, indicating a mechanistic connection to the development of IBD. In contrast, other studies have found that dysbiosis of the oral microbiome may arise, potentially as a result of inflammatory reactions, and that it could be a helpful source of indicators of GI health (Elmaghrawy et al., 2021). The finding of significant amounts of

oral taxa in the guts of IBD patients was a common characteristic of prior research. The reciprocal flow of bacterial pathogens provides more evidence of commonalities between the oral and gut microbiomes (Kodukula et al., 2017).

Microbiome disruptions in the oral cavity induced by excessive sugar intake or poor oral hygiene may result in dental caries and inflammatory gum disease, respectively. The oral microbiome has been well-characterized in terms of its role in oral diseases (caries, and periodontitis), but its members have also been implicated as contributing factors in a variety of non-oral diseases such as colorectal cancer, diabetes mellitus, cardiovascular disease, bacteremia, and preterm birth. Few studies have looked specifically at the influence of IBD on the oral microbiota, and relation to oral health (Elmaghrawy et al., 2021). A common extraintestinal sign of CD is oral pathology. Oral signs found in CD patients imply a link between oral microbiota and such manifestations; nevertheless, little is known about the oral microbiota of CD patients (Said et al., 2014).

The significant advances in scientific techniques for microbial detection, identification, and classification in recent decades, particularly the emergence of high-throughput next-generation sequencing (NGS) technologies, have led to enhanced understanding of microbiomes (Said et al., 2014). The GIT, and particularly the oral and gut microbiomes, contain most of the microbial biodiversity in the human microbiome. However, these two groups are significantly different in terms of makeup. The oral cavity is dominated by facultative, sugar fermenting organisms (e.g., *Streptococcus* and *Actinomyces* spp.), whereas the gut is dominated by a metabolically varied population of anaerobic bacteria (e.g., *Clostridium* and *Bacteroides spp*.). *Bacteriodetes, Firmicutes, Proteobacteria, Synergistetes, Fusobacteria, Spirochaetes, Actinobacteria, SR-1,* and *TM-7* are among the nine most prevalent bacterial phyla discovered in the oral cavity, using Human Oral Microbe Identification Microarray (Qi et al., 2021) .

It has previously been reported that particular oral bacteria, such as a subset of *Porphyromonas gingivalis, Streptococcus mutans, Fusobacterium nucleatum, Campylobacter concisus,* and *Klebsiella pneumoniae*, may aggravate inflammation in

IBD (Qi et al., 2021). Ectopic colonization by these oral bacteria may breakdown the intestinal epithelial barrier, generate excessive release of inflammatory cytokines, disturb the host immune system, promote immunological escape, and create gut microbiota dysbiosis, exacerbating chronic intestinal inflammation. Understanding the pathophysiology of CD requires research into dysbiosis of the oral microbiota. Previous research focused only on changes in the makeup of the oral microbiota, while ignoring the relationship between oral microbiota dysbiosis and the inflammatory state in the gut (Qi et al., 2021). Although the makeup of the communities in the mouth and gut differs, the amount of species richness in both settings is comparable, and a single individual may house over 100 unique species at each site (Qi et al., 2021). Surprisingly, the oral microbiome has less interindividual variance than the gut microbiome. The increased interindividual variance reported in gut microbiomes appears to be connected to the larger influence on these communities of variables such as nutrition and antibiotic use, whereas the oral microbiome appears to be more robust to similar challenges(Qi et al., 2021).

Regardless of the presence of oral symptoms, the oral mucosa is an immunologically active surface, with higher cytokine generation in CD. Dysbiosis or divergence from this core has revealed different changes in the intestinal microbiota of CD patients. When compared to UC and health, there is a marked and severe reduction of diversity in the oral microbiota of CD. A significant reduction in both total microbial diversity and particular phylum levels was found in CD. Furthermore, the loss of certain phyla such as *Fusobacteria* and *Firmicutes* has been demonstrated in investigations of the gut microbiome in CD. The oral microbiota is changed in IBD patients, particularly in CD (Docktor et al., 2012) .Remarkably, patients with CD and oral symptoms had significantly greater anti-*Saccharomyces cerevisiae* antibody (ASCA) titers than those without oral signs (Docktor et al., 2012).

Salivary microbiota dysbiosis is associated with inflammatory responses in IBD patients, indicating that it is probably related to gut microbiota dysbiosis (Zhang et al., 2020). Since the oral and gastrointestinal tract microbiome account for the majority of the overall human microbial load, it offers unique prospects for improving human

health prognosis, diagnosis, and medication development (Kodukula et al., 2017).

Table 2 summarizes the findings of previous studies on salivary microbiome in CD patients, the sequencing techniques used are mostly of the short read sequencing such as Illumina and pyrosequencing.

Table 2: Salivary Microbiome in Crohn's Disease Patients, with Techniques Used in Different Studies.

Bacteria	Finding: increased/decreased	Technique used	Reference
Veillonella, Klebsiella	Increased	DNA microarray analysis	(Elmaghrawy et al., 2021)
Actinobacteria Proteobacteria	Increased	Shotgun sequencing	(Hu et al., 2022)
Firmicutes Bacteriodetes	Decreased		
Firmicutes	Decreased	Shotgun and metagenomic sequencing	(Nikitakis et al., 2017)
Proteobacteria	Increased		
Phyla: *Fusobacteria, Firmicutes*	Decreased	Molecular microbial diagnostics	(Yoshizawa et al., 2013)
Fusobacteria, Firmicutes	Decreased	Human Oral Microbe Identification Microarray	**(**Docktor et al., 2012)
Spirochaetes, Synergistetes	Increased		
Bacteroidetes	Increased		
Genera: *Streptococcus, Prevotella, Veillonella* and *Haemophilus*	Increased with elevated levels of fecal lysozyme	ELISA	(Nijakowski & Surdacka, 2020)
Fusobacterium nucleatum Heamophilus parainfluenzae Veillonella parvula	Increased	Illumina MiSeq platform	(Gevers et al., 2014)

Table 2: Salivary Microbiome in Crohn's Disease Patients, with Techniques Used in Different Studies (continued).

Eikenella corrodens Gemella moribillum			
Bacteroides vulgatus Bacteroides caccae	Decreased		
*Neisseria (*phy. *Proteobacteria.)* and *Gemella (*phy. *Firmicutes)*	Decreased	Pyrosequencing	(Said et al., 2014)
Bacteroidetes and *Prevotella*	Increased		
Proteobacteria, Neisseria Haemophilus	Decreased		
Firmicutes, Bacteroidetes, Proteobacteria Genus*: Streptococcus, Neisseria, Prevotella, Haemophilus*, and *Veillonella*	Increased 5 Most abundant taxa	Illumina MiSeq platform	(Zhang et al., 2020)

1.7 Management in CD and its Relation to Microbiota

Unfortunately, CD has no cure, and most patients require at least one surgical resection. The objective of medical therapy is to achieve clinical and endoscopic remission in order to avoid complications and surgery (Feuerstein & Cheifetz, 2017). The treatment of CD is determined by the location and activity of the illness, as well as the occurrence of complications.

The most often used pharmaceuticals in the treatment of CD are:

1. Anti-inflammatory drugs such as aminosalicylates and steroids
2. Immunosuppressants or immunomodulators such as thiopurines and methotrexate
3. Biologic agents such as infliximab

For patients with mild-to-moderate CD localized to the ileocecal region, the standard "step-up" protocol recommends oral corticosteroid therapy, and for patients with moderate-to-severe small bowel disease and relapsing or steroid-refractory disease, a combination of oral corticosteroids and immunosuppressants. Biologic drugs are advised for individuals who do not react to or cannot tolerate normal therapy and are contraindicated for corticosteroids. Surgery is normally avoided for as long as feasible because it is not considered therapeutic and may result in several functional problems and disease recurrence.

Recently, there has been a movement toward using biologic treatments like infliximab in newly diagnosed CD patients, a so-called "top-down" strategy. It is thought that introducing biologics early in the illness may disrupt the normal progression of CD from the inflammatory stage to the later phases, which are often less susceptible to pharmacologic treatment and are more frequently linked with the development of comorbidities. A 5 mg/kg intravenous infusion is the standard dosage for IFX induction, which is administered at weeks 0, 2, and 6 and then every 8 weeks after that. To optimize a patient's therapy, this dose regimen can be altered in a variety of different ways. Intensifying IFX (infliximab) dose during maintenance therapy in patients with low IFX trough levels (and absence or low-titre anti-drug antibodies) can enhance clinical outcomes and boost the proportion of patients who get a clinical response. You can accomplish this by either increasing each injection to 10 mg/kg or by cutting the time between doses to 4 or 6 weeks. Decisions about dose adjustment should ideally be made by TDM (Therapeutic drug monitoring), which includes anti-drug antibody measurement. This is because dose escalation is less justified in the clinical situations that are frequently encountered. As an illustration, active disease brought on by the emergence of high-titre antibodies with sub-therapeutic trough levels (immune-

mediated pharmacokinetic failure) or adequate trough levels in the absence of antibodies (mechanistic/pharmacodynamic failure) may call for a change in therapy rather than dose intensification (Samaan et al., 2019).

1.7.1 Steroids and immunosuppressive agents

The effectiveness of corticosteroids in the context of CD maintenance treatment has never been firmly shown. Based on the currently available data from controlled trials, it would be important to determine whether chronic corticosteroid therapy is beneficial in patients with quiescent CD or if there is a distinct subset of CD patients who might benefit from such treatment, such as those who cannot successfully taper therapy (Steinhart et al., 2003).

Even though we have been in the era of biologic therapy for several decades, the use of immunomodulators (primarily thiopurines [azathioprine and mercaptopurine] and, to a lesser extent, methotrexate) remains an important component of IBD pharmaceutical arsenal. Thiopurines can maintain long-term remission in a significant proportion of patients who have frequent relapses and are or have become mesalazine and/or corticosteroid intolerant or refractory to mesalazine and/or corticosteroid intolerant or refractory to mesalazine and/or corticosteroid intolerant or refractory to mesalazine and/or corticosteroid intolerant. Methotrexate, when combined with a decreasing dosage of corticosteroids, is an effective treatment for active luminal CD (Mantzaris, 2017).

Many patients who receive biologic therapy have previously received or continue to use concomitant therapies to which the biologics are added. It is critical to recognize that not all toxicity issues in these patients are caused by biologic therapy, but rather by concomitant therapies in IBD, most commonly corticosteroids and/or immunomodulators. Furthermore, the underlying disease itself can cause complications. Examples include dysplasia and cancer in long-term UC patients, and intestinal strictures in CD patients (D'Haens, 2007).

1.7.2 Biologic agents

Infliximab was the first monoclonal antibody to be licensed for the treatment of moderately to highly active CD and UC in pediatric and adult patients. It has been found to produce and sustain clinical remission as well as mucosal healing in patients with IBD who have been ineffective or resistant to traditional therapy (Hemperly & Vande Casteele, 2018).

Patients with CD who respond to a first dosage of infliximab are more likely to be in remission at weeks 30 and 54, to cease corticosteroids, and to sustain their response for a longer length of time if infliximab treatment is continued every 8 weeks (Hanauer et al., 2002).

Biologic therapies have significantly improved outcomes for patients with inflammatory bowel disease in the two decades since the introduction of infliximab (IFX) for the treatment of CD. They not only alleviate symptoms (resulting in demonstrably improved quality of life), but they also resolve inflammation, as measured objectively by endoscopic, radiological, or biochemical measures. Furthermore, biologic therapies have significantly altered the way perianal CD is managed and are now our most effective pharmacological class of drugs for this particularly debilitating manifestation (Samaan et al., 2019).

1.7.3 Microbiome-modulating therapies

Like probiotics, it's likely that FMT's different degrees of efficacy are influenced by the make-up of the donor feces and the correction of the microbiome abnormalities linked to IBD. Results have not been consistent or impressive in patients with IBD to date, despite studies showing that probiotics can alter the mucosal immune system through Toll-like receptors to promote TH1 cell differentiation, improve intestinal barrier function, increase bacterial diversity, and inhibit the growth of potentially pathogenic bacteria. In the future, probiotics designed to target the precise microbial changes associated with a particular IBD phenotype may be developed. There has been a lot of interest in the potential advantages of microbiome-modulating therapies in the treatment of IBD, such as probiotics, prebiotics, antibiotics, fecal microbiota transplantation, and gene editing (Glassner et al., 2020a)

1.7.4 Do antibiotics cure or cause Crohn's Disease?

To answer this question, two problems must be clarified. The first is whether antibiotic medication is useful in CD, and the second is whether antibiotic usage predisposes people to CD. These are two distinct difficulties that may or may not be related. Except for some evidence that metronidazole can improve the course of ileal or ileocolonic CD and that it or a related compound could improve or reduce the likelihood of disease recurrence after surgical resection of ileal or ileocecal CD, there is no solid evidence that antibiotic therapy improves the course of CD, particularly inflammatory CD. There is some evidence that antibiotic usage may raise the likelihood of CD development. Research found that when people with IBD were compared to controls, the IBD group was more likely than the control group to have had antibiotic prescriptions a few years before the IBD diagnosis. The findings do not imply cause and effect; rather, they show a link between antibiotic usage and the development of IBD (Bernstein, 2013).

The first evidence that antibiotic-induced changes in intestinal microflora may contribute to the etiology of CD came from two case control studies that found a link between increased antibiotic use and CD diagnosis in children. Both studies relied on recall of antibiotic use, which was assessed many years after the initial diagnosis. In studies where the onset of disease may influence recall, such recall of exposures is known to be biased. In both studies, the authors regarded the discovery as an artifact rather than a cause (Card et al., 2004).

1.8 Diagnosis of CD and Role of Biomarkers

CD is distinguished by a variety of endoscopic and microscopic findings. The identification of "skip" lesions, which are strongly delineated pockets of illness surrounded by perfectly normal mucosa, is regarded as a critical step in the diagnosis of CD. Once identified, the patient must go through further testing, including as imaging scans, to determine the location and extent of lesions, as well as the existence of complications, such as strictures and cancer (Woo, 2015). Periodic colonoscopic monitoring is thus an essential component of therapy.

Laboratory findings are frequently non-specific, but they may indicate GI malabsorption (e.g., low albumin, calcium, folate, iron, and red blood cell count), elevated erythrocyte sedimentation rate (ESR), elevated platelet counts, anemia, and increased acute phase reactants such as C-reactive protein (CRP). Non-invasive measures such as CRP and fecal proteins can give baseline information to establish the existence of intestinal inflammation and should be utilized as a first step in identifying individuals who require additional study (Woo, 2015).

1.8.1 C-reactive protein (CRP)

CRP is a five-monomer pentameric protein that is one of the most essential acute phase proteins in humans. In response to an acute phase stimulation, such as inflammation, hepatocytes quickly increase CRP synthesis under the effect of interleukin (IL-6), tumor necrosis factor α (TNF-α), and IL-1β, reaching peak levels of 350–400 mg/ L. CRP values of 10–40 mg/ L are often reported in situations of moderate inflammation or viral infections. CRP values of 50–200 mg/ L and very high levels of are usual in severe active inflammation or bacterial infection. Only under extreme circumstances and burns are concentrations of 200–250 mg/L reported (Vermeire et al., 2006).

Because of its short half-life, CRP is a valuable marker for identifying and monitoring CD activity. It can be utilized as a very accurate index of disease activity as well as an independent predictor of short and medium-term clinical recurrence in patients with high CRP levels at diagnosis (D'Incà & Caccaro, 2014).

CRP levels are often elevated in individuals with active disease, and there is a considerable connection with CDAI readings. It has been hypothesized that systematic CRP testing during remission is also prognostically important, with the potential to predict clinical recurrence (Sostegni et al., 2003).

1.8.2 Fecal biomarkers in CD including calprotectin.

The fact that faces are easily accessible in IBD patients is one apparent reason to look for fecal indicators. Furthermore, serum markers can be elevated by illnesses other than gastrointestinal inflammation, therefore fecal markers would have a greater

specificity for IBD in the absence of gastrointestinal infection. Furthermore, if fecal indicators are indicative of mucosal inflammation in the gut in IBD patients, endoscopic investigations may be avoided (Vermeire et al., 2006).

Calprotectin is a calcium-binding neutrophil protein that is stable during intestinal transit. It accounts for up to 60% of total neutrophil cytosol proteins. Calprotectin is strongly correlated with endoscopic and histological CD activity ratings in ileocolonic or colonic illness, but not in ileocolonic disease. Calprotectin outperformed CRP and CDAI in distinguishing between various levels of intestinal inflammation according to the simple endoscopic score for CD. Once disease activity has been demonstrated, fecal calprotectin can be utilized to track the disease's progression and response to medical therapy (D'Incà & Caccaro, 2014).

Increased levels of calprotectin (> 50 mg/L) during remission have been shown to be a good predictor of relapse within 1 year; the sensitivity and specificity of calprotectin in predicting relapse are 90% and 83%, respectively, with a relative risk of relapse of 10.6 in patients with calprotectin levels higher than 50 mg/L (Sostegni et al., 2003).

Fecal calprotectin has approximately 80% sensitivity and accuracy in predicting imminent clinical relapse in individuals with established, generally asymptomatic IBD. A patient with silent IBD who has a high calprotectin level has an 80% risk of having a clinical relapse in the next 6 months, but only 20% of individuals with a low calprotectin level would have a clinical relapse (Bjarnason, 2017).

1.8.3 Salivary biomarkers in CD

Saliva is primarily regarded as a vital component of the digestive process because it initiates the breakdown of fats and carbohydrates via endogenous enzymes. Saliva, as a biological fluid, offers tremendous promise for non-invasive diagnosis of a variety of systemic illnesses. It includes a wide range of molecular and microbiological analytes. The "holy grail" of biomarker development based on oral sample is still some time away, but it would be a more practical, accessible, and acceptable source for patients and physicians than existing blood or stool specimen-based tests (Elmaghrawy et al., 2021).

As previously stated, specific saliva-based biomarker profiles can be linked to specific illnesses and may give vital information about an individual's present physiologic condition. Identifying, verifying, and comprehending saliva-based biomarkers might play a significant role in establishing oral fluids as a reliable diagnostic biofluid (Yoshizawa et al., 2013).

Our understanding of salivary secretions and the oral cavity has shifted substantially in recent years. Furthermore, several researchers claim that these salivary ingredients can be used to detect both local and systemic diseases. Oral inflammation can alter the content of saliva and disrupt the expression of certain diagnostic proteins. Dental caries and periodontal disorders are two of the most frequent oral illnesses. Surprisingly, research has demonstrated that salivary bacteria, particularly those shed from dental caries, can be used as diagnostic biomarkers of illness in diagnosis, monitoring, and general health evaluation. With this in consideration, much effort has been expended on defining the human oral microbiome (Yoshizawa et al., 2013).

The oral cavity may be a potential source of biomarkers for diagnosing and monitoring therapy results in IBD patients. Much of the available research on the oral microbiota in IBD comes from patients who are already on different therapies. The impact of persistent therapies or times of elevated disease activity on oral microbial profiles have not yet been thoroughly investigated.

Saliva calprotectin has been proposed as a potential index of active IBD in a recent study. Calprotectin concentrations in stimulated whole saliva are up to three times higher in IBD patients than in healthy controls, and saliva calprotectin concentrations are higher in IBD patients than in controls. However, while it increased in both unstimulated and stimulated CD patients, it only increased in stimulated UC patients. Calprotectin levels are elevated in acute-phase inflammatory reactions and are linked to elevated CRP levels (Finamore et al., 2020).

Salivary calprotectin levels are higher in IBD, indicating that IBD manifests as subclinical inflammatory reactions in the oral cavity. Calprotectin is also found in saliva, where elevated levels have been documented in individuals with periodontitis and Sjögren's disease (Majster et al., 2019). Despite the involvement of the oral cavity

in IBD and discoveries of heightened levels of pro-inflammatory cytokines in saliva during IBD, there have been no previous attempts to validate the analysis of calprotectin in saliva, nor to study the levels in IBD patients, to the best of our knowledge. As a result, the goal is to confirm the analysis of calprotectin in saliva under various settings, as well as to compare the levels in a small group of IBD patients with active illness before and after therapy to controls with no intestinal inflammation The findings show that salivary calprotectin levels are much higher in IBD patients with active illness, particularly in newly diagnosed CD patients, indicating the presence of intestinal inflammation (Majster et al., 2019). Given that CRP is a sensitive indicator of the condition of inflammation, the rise in its level is not unexpected (Finamore et al., 2020).

1.9 Statement of the Problem

Inflammatory bowel diseases are multifactorial, and no single specific reason can currently be identified as the definite cause of the disease. The reasonable concentration of IBD research to date on the intestinal microbiota has left a lot to be discovered about the oral microbiome in CD patients. There are very limited previous research studies in the past decade on the relationship of salivary microbiome to CD and on how it can be linked to gut dysbiosis playing a role in CD pathogenesis (Han et al., 2022). More focus was on fecal samples to explain gut dysbiosis, but saliva being away from the gut was the question, where is the association? In our current study, we propose to examine multiple variables in patients with CD, and effect on the oral microbiome. Focusing on multiple variables such as oral health, the current use of IBD medications, the activity of the disease, the duration of the disease, the frequency of relapse of symptoms is what makes this study unique. What makes our research of paramount significance is the inclusion of different aspects that might lead to a better understanding of CD pathogenesis.

Our hypothesis was that the oral cavity is a reservoir of tremendous number of bacteria; some being normal commensals and many others being pathogenic due to cavities in the teeth or periodontal diseases which cause destruction of the attachment between the gums and teeth. Firstly, saliva can get translocated from the oral cavity into

the gut by the action of continuous ingestion throughout the day, secondly thin mucosa (loss of integrity) from the periodontal destruction makes it permeable to passage of bacteria into the systemic circulation leading to bacteremia. The presence of oral bacteria in the circulation may trigger inflammation. Since periodontitis is already an inflammation, it can contribute to systemic inflammation, via circulating inflammatory mediators that can reach the GIT (Xun et al., 2018). Furthermore, the novelty in the sequencing technique which we used to detect the microbial profiles in saliva, gave us credit of having pioneering results in the field of salivary microbiome, as a few studies used long read sequencing to explore the oral microbiome (Al Kawas et al., 2021a). All taxonomic levels were revealed from the kingdom and down to the final level which is species. Other studies using different sequencing techniques such as pyrosequencing (Said et al., 2014), stopped at the phylum or genus since only short reads were possible to obtain by other technologies, unlike our third generation sequencing which produces long reads. Finally, this study was the first of its kind in the United Arab Emirates, as limited research has been conducted on IBD, which again adds to the uniqueness of our research.

1.10 Research Objectives

1. Characterize the compositional changes in the salivary microbiota of patients with CD compared to healthy controls.
2. Compare CD patients for salivary microbiome complexity and diversity according to different variables, including oral health, IBD drug use, disease duration, activity of the disease and relapse of symptoms.
3. Explore any possible correlation between oral health (such as periodontal disease or caries) and the salivary microbiota profiles that may contribute to the pathogenesis of CD.
4. Investigate the correlation between the inflammatory biomarkers (CRP in serum and CAL in stool) with their levels in saliva, and any possible link to oral dysbiosis.

Chapter 2: Methods

2.1 Study Design and Settings

This is a case-control study including 80 subjects composed of 40 CD patients and an equal number of Healthy controls (HC). Subjects were recruited from Sheikh Shakhbout Medical City (SSMC) in Abu Dhabi and Tawam hospital, Al Ain in the period between August 2021 and February 2022. Data collection workflow is summarized in Figure 4.

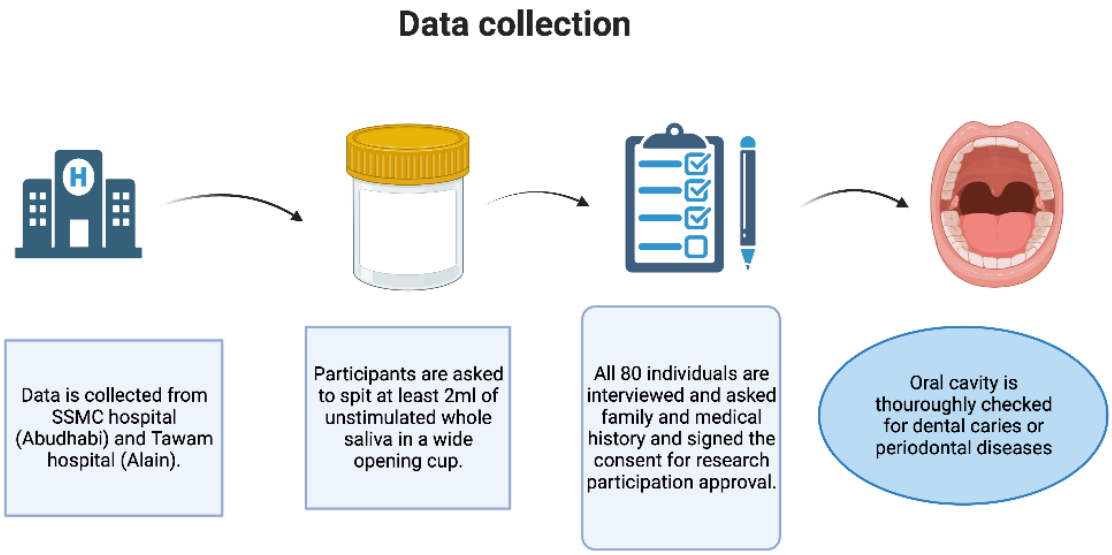


Figure 4: Schematic diagram of data collection steps.

2.2 Ethical Approvals and Consent Form

Ethical approvals have been obtained from the Department of Health in Abu Dhabi (Reference number: DOH/CVDC/2020/2470), as well as the approval from Sheikh Shakhbout Medical City (SSMC) in Abu Dhabi and Tawam hospital in Al Ain. A consent form (in English and Arabic languages) was read or explained carefully then signed by the patient to ensure that he/she understood the purpose of the study and fully agreed to participate in the research.

2.3 Recruitment of Participants

In the department of gastroenterology, CD patients were approached to participate in the research after the follow up appointments in the clinic, and some before or after infusion of the medication in the infusion clinic. Controls were healthy individuals coming to the hospital for other complaints, but with no history of IBD. Some controls included healthy volunteers from the community. Controls were age and gender-matched with the cases.

Inclusion criteria: Adults (≥18 years old), of any gender (male or female) who agreed to voluntarily participate in the study and signed the informed consent were recruited into 2 groups:

- Cases: patients with CD attending to two hospitals: Tawam hospital; Al Ain, and Sheikh Shakhbout Medical City; Abu Dhabi, UAE. The diagnosis of CD was based on clinical symptoms, endoscopic characteristics, radiological findings, and histological features, as verified by the treating clinician.

- Controls: healthy subjects with no history of chronic inflammatory disorder including IBD and current gastrointestinal symptoms. Subjects with a known history of medical or systemic diseases, including medical conditions that can influence nutrient intake, or bowel health were excluded from the healthy control group.

Exclusion criteria: Individuals receiving antibiotics in the past ninety days were also excluded from the study as this is expected to alter the composition of the oral microbiome (Bernstein, 2013).Pregnant and lactating ladies, as well as all participants having serious systemic diseases are excluded from the study.

2.4 Data Collection

A questionnaire (appendix no. A) was used to obtain a thorough history from the participants. Demographic data (age, and gender) were obtained. In addition, information on CD including the onset of the disease, how the disease has been diagnosed, presence of any accompanying diseases such as diabetes, hypertension, cardiovascular disease, thyroid, or any other medical problems were recorded. The family history was also included to inquire if the same disease or any other IBD were

reported in the family. Any previous or awaiting operations to treat the bowel disease were also recorded. Moreover, any investigation and diagnostic tests such as colonoscopy reports were reviewed. Additional information and further details about the medical records including the laboratory data, radiography findings, treatment received, clinical outcomes, and previous surgeries were also retrieved and reviewed from the hospital's electronic database. The history of any medication prescribed was taken carefully, including the name of the medication, dose, and duration. Details about the history of medications that affect immunity were focused on such as steroids, and immunosuppressants. It is of great importance that patients with CD take all their prescribed medications properly, so the patient was asked about the medication adherence. Results such as hemoglobin levels, hematocrit, C-reactive protein, fecal calprotectin were recorded.

2.5 Crohn's Disease Activity and Related Bowel Symptoms

The clinical activity and severity of CD were judged and scored using Crohn's Disease Activity Index (CDAI). CDAI is widely adapted for scoring CD based on patients' clinical symptoms including:

1. Subjective reporting of the degree of abdominal pain, stool pattern, and general well-being
2. Presence of extraintestinal manifestations, such as fever, arthritis, rash, and uveitis
3. Physical examination findings
4. Weight and height
5. Hematocrit, erythrocyte sedimentation rate, and serum albumin

Calculation of CDAI was done using the free online tool. CDAI scores can range from 0 to 600. CDAI ≤ 150 was regarded as a remission phase while CDAI > 150 was regarded as an active phase. CDAI scores of 150–219 has been labelled as mildly active disease, scores of 220–450 as moderately active disease, and > 450 as a very severe disease (Freeman, 2008).

Symptoms of CD can be classified as mild, moderate, or severe depending on how many symptoms the patient has.

The patient was asked about the signs and symptoms of active CD which are: fever, abdominal pain, blood in stool, weight loss, diarrhea or diarrhea that wakes the patient up at night, joint pain, new skin rashes or sores in the mouth or if there were any other signs mentioned by the patient.

The area of the gut which is affected by CD was noted, to determine if the disease affected the small intestine, large intestine, or any other part of the digestive tract. The patient was asked about the frequency of relapse of symptoms throughout the year. Last symptoms of bowel disease requiring doctor visit or hospital admission were recorded. Details about any CD complications were taken into consideration such as anemia, liver problems, skin, eye, or joints.

2.6 Dental Examination

Dental examination included a general checkup using a tongue depressor to look for any abnormalities or color changes. The gingiva was also checked for signs of inflammation or ulcerations. Some detailed dental information was obtained from the electronic records, if the patient had any recent visits to the dentist. A Dental examination sheet is shown in appendix no.B.

2.7 Saliva Sample Collection

Saliva samples were collected from both CD patients and controls. Samples were obtained just once during the study's enrollment. Participants were instructed prior to giving the sample on how to give the right amount, they should rinse the mouth and encouraged to provide unstimulated saliva (Al-Rawi & Al-Marzooq, 2017). One hour before the sample collection, participants were requested to refrain from drinking, eating, and cleaning their teeth. To eliminate any food residue, a mouth rinse with tap water was performed first, followed by the collection of a sample. Participants were provided with a sterile container with a wide opening to make the process easier and comfortable.

At least 2 ml of unstimulated whole saliva were collected from the participants. The container was clearly labelled and temporarily placed in a cooler box with ice packs, then transported to the lab for storage at -80°C freezer until testing.

2.8 Saliva Sample Processing, DNA Extraction and Quality Assessment

Saliva samples previously stored in -80°C were allowed to thaw at room temperature. The processing area was well-disinfected with 70% ethanol, in the designated hood with all racks and disposable sterile pipettes. Samples were transferred into sterile Eppendorf tubes clearly marked with patient code. A volume of 1ml saliva was centrifuged at 12,000 rpm speed for 10 mins to pellet the cells then the supernatant was removed carefully without disturbing the pellet using a disposable 1 ml pipette. The supernatant obtained after centrifugation was transferred to sterile Eppendorf tubes and stored at -80°C to be used to test the salivary calprotectin and CRP, subsequently (Al-Rawi & Al-Marzooq, 2017). Saliva processing steps summarized in Figure 5.

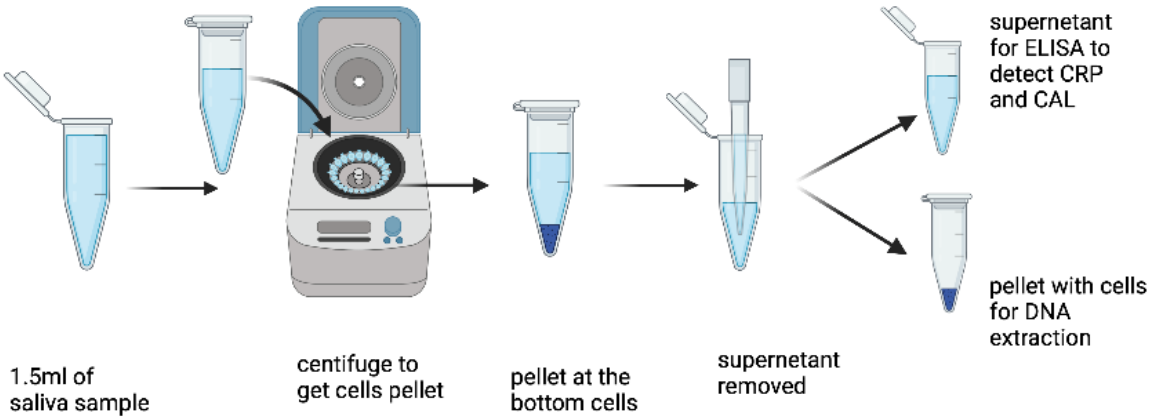


Figure 5: Saliva processing steps.

Salivary DNA was extracted using the HMW DNA extraction kit (Promega, USA) which is designed for isolation of high molecular weight DNA from bacteria found in saliva. The salivary pellet with some of supernatant was vortexed till homogenized, then boiled at 100°C in a heating block (Thermo scientific, USA) for 10 mins, then chilled on ice for two mins (Al-Rawi & Al-Marzooq, 2017).

Lysozyme (Thermo scientific, USA) at a concentration of 3 mg/ml (100 µl) was added to the boiled pellet, then incubated in a heating block for 30 mins at 37°C (Eppendorf, Thermomixer compact, USA).

A volume of 500 µl of HMW lysis buffer A was added to the samples after the appropriate incubation period using a wide bore pipette tip, then the solution was mixed five times to lyse the cells. This was followed by incubation in the heating block for another 5 mins at 80°C to make sure all the cells were lysed then cooled to room temperature. A volume of 3µl of RNase A solution was added to break down the cells and destroy RNA hence the name. Solution was mixed by inverting the tube 5-7 times then incubated at 37°C for 15 minutes.

Following this step, 20 µl of Proteinase K Solution was added to each sample and mixed by inverting the tubes 10 times then incubated at 56°C for 15 mins. Samples were cooled to room temperature or chilled on ice for 1 minute. Next, 200 µl of Protein Precipitation Solution were added to the cell lysate using a 1,000 µl wide bore pipette then mixing the solution five times. Small protein clumps were visible after mixing. Samples were incubated for five minutes on ice, then centrifuging was done to pellet the cells, at 13,000 X g speed for 10 minutes at room temperature. A protein pellet was clearly visible including all the cellular debris to release intracellular DNA. The supernatant was slowly transferred to a clean Eppendorf tube and the remaining pellet was discarded, making sure that only clean liquid was removed not contaminated with the pellet. A volume of 600 µl of isopropanol (Sigma Aldrich, USA) was added to the supernatant, and gently mixed by inverting the tube eight times. This was followed by another centrifuging at 13,000 X g speed for 2 minutes room temperature. The DNA was visible as a small white pellet. The supernatant was decanted carefully, and DNA pellet was then washed with 600 µl of 70% Ethanol (Sigma Aldrich, USA). Tubes were inverted several times to ensure that the DNA was properly washed. This was followed by centrifuging for 2 minutes at 13,000 X g. Ethanol was finally aspirated, and remaining Ethanol droplets were air dried and placed open in a sterile biosafety cabinet for few minutes till no more ethanol was left. A volume of 20 µl of DNA Rehydration

Solution was added to dissolve the DNA, which was then checked for quality and quantity. Concentration of DNA was explored by nanodrop (ND-1000 Spectrophotometer, Thermo scientific USA). Purity of the DNA samples is assessed using A260/A280 and A260/A230 ratios. Samples that have A260/A2280 and A260/A230 values >1.8 were considered pure (Volarić et al., 2021).

The Qubit dsDNA HS (high Sensitivity) Assay Kit (Invitrogen, USA) was used for DNA quantification before sequencing. Concentrated test reagent, dilution buffer, and prediluted DNA standards are included in the kit. The test is highly selective for double-stranded DNA (dsDNA) and accurate for sample concentrations ranging from 10 pg/ µl to 100 ng/ µl.

The test was carried out at room temperature. The Qubit working solution was prepared by diluting the Qubit dsDNA HS Reagent 1:200 in Qubit dsDNA HS Buffer. For one sample, 1 µl of the DNA was mixed with 199 µl of the Qubit working solution in the prelabelled 0.5 ml PCR tubes, to make a final volume in each tube up to 200 µl. For standards, 190 µl of Qubit working solution was added to each of the tubes used for standards, and 10 µl of each Qubit standard was added to the appropriate tube. All the reaction tubes were then mixed for 2-3 seconds. All tubes were allowed to incubate at room temperature for 2 mins. Then, tubes with the standards and DNA from samples were read for the concentration with the Qubit Fluorometer (Qubit 2, Invitrogen, USA). Concentration (ng/ µl) were used to calculate the required volume needed to prepare the library for next generation sequencing.

2.9 Next Generation Sequencing for Microbiome Profiling

The salivary microbiome was studied by sequencing the full (1,500 bp) bacterial 16S rRNA gene with the Oxford Nanopore sequencer Mk1C (Oxford Nanopore, UK). Barcoding kit 1-24 (SQK-16S024) was used as it contained 24 distinct barcodes, which allowed combining up to 24 different samples in a single sequencing session. The DNA is amplified by PCR using specific 16S primers (27F and 1492R) that contain 5’ tags which facilitate the ligase-free attachment of Rapid Sequencing Adapters, as shown in Figure 6.

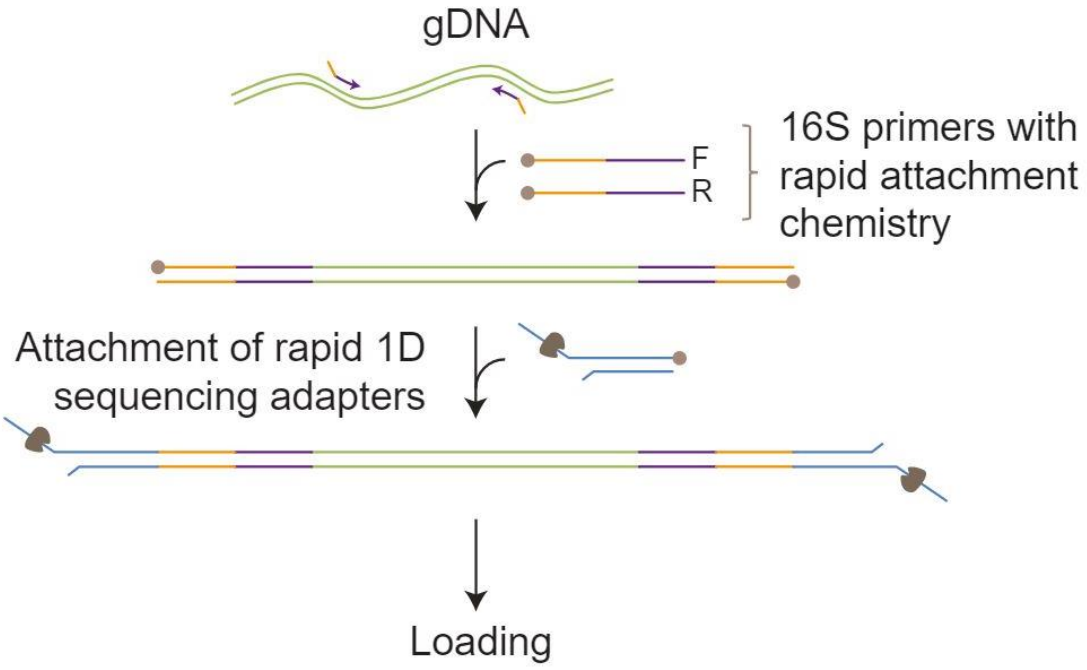


Figure 6: Amplification and Barcoding of Bacterial 16S rRNA Gene Before Sequencing (Oxford Nanopore, UK).

Library preparation was done following the manufacturer's protocol, with the following steps:

1- DNA preparation

DNA in nuclease-free water was prepared by transferring 10 ng genomic DNA into a DNA LoBind Eppendorf tube and adjusting the volume to 10 µl with nuclease-free water. Tubes were mixed thoroughly by flicking, to avoid unwanted DNA shearing.

2- PCR

The following mixture was prepared in separate 0.2 ml thin-walled PCR tubes.

- 5 µl Nuclease-free water
- 10 µl input DNA (10 ng)
- 25 µl LongAmp Hot Start Taq 2x Master mix (NEB, UK)
- 10 µl of each 16S barcode

Mixing was done gently by flicking the tube and spinning down. PCR was done in Veriti 96 well thermal cycler (Applied Biosystems, USA). Amplification was done using the following cycling conditions):

- Initial denaturation 1 min at 95°C - 1 cycle
- Denaturation 20 sec at 95°C
- Annealing 30 sec at 55°C
- Extension 2 mins at 65°C

(25 cycles)

- Final extension 5 mins at 65°C - 1 cycle
- Hold at 4°C

3- DNA purification

Each sample was transferred to a separate 1.5 ml DNA LoBind Eppendorf tube. The AMPure XP beads (Beckman Coulter, USA) were resuspended by vortexing then added to the reaction mix by pipetting. The volume of beads added to each sample was 30 µl, then samples were incubated while the tubes were rotating on the Hula mixer (Invitrogen, USA) for 5 mins at room temperature.

DNA on the beads was pelleted on a magnetic rack (Invitrogen, USA), then the supernatant was pipetted off. The beads were washed with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. The ethanol was then removed, and washing was repeated twice. Samples were allowed to dry for ~30 seconds, without reaching the point where the pellet is cracking. The tubes were removed from the magnetic rack and the pellet was resuspended in 10 µl of 10 mM Tris-HCL pH 8.0 with 50 mM NaCl, then incubated for 2 mins at the room temperature. The beads were pelleted on the magnetic rack until the eluate was clear, then 10 µl of the elute was removed and retained which contains the DNA.

4- Samples Pooling and Loading for Sequencing

The Qubit Fluorometer was used again to quantify the DNA after performing the same steps mentioned above. The last step is to pool all barcoded libraries in the desired ratios to a total of 50-100 fmoles in 10 µl of 10 mM Tris-HCL pH 8.0 with 50mM NaCl. Then, 1 µl of RAP was added to the barcoded DNA and mixed by flicking.

The reaction was incubated for 5 minutes at room temperature. The prepared library was then loaded into the MinION flow cell. Sequencing was carried out for 24 hrs to obtain an adequate number of reads for bioinformatic analysis.

5- Bioinformatic Analyses

MinKNOW software (Oxford Nanopore Technologies, UK) was used for live base calling and data acquisition. Raw data were converted into FASTQ format using Guppy, followed by demultiplexing, removal of nanopore and adaptor sequences with default minimum Q score of 7 (Al-Marzooq et al., 2022). Preliminary bacterial identification was done via 'What's in my Pot?' (WIMP) workflow provided by Oxford Nanopore Technologies, UK38. Reads assigned to all targets were re-analyzed by Kraken taxonomic sequence classification system (version 2.0.8-beta) using Partek Genomics Suite software (Partek Inc., St. Louis, MO, USA). The numbers of reads assigned per taxon were counted and the relative abundance of reads per taxon were used for separate downstream analysis (Al Kawas et al., 2021). Microbiome Analyst platform was used for comprehensive statistical analysis of microbiome data (Chong et al., 2020). Operational Taxonomic Units (OTUs) at phyla, genus and species were analyzed. Linear discriminant analysis effect size was used to detect biomarkers of microbial profiles. Furthermore, log transformed counts, relative abundance, alpha and beta diversity were interpreted. Figure 7 summarizes next generation sequencing workflow.

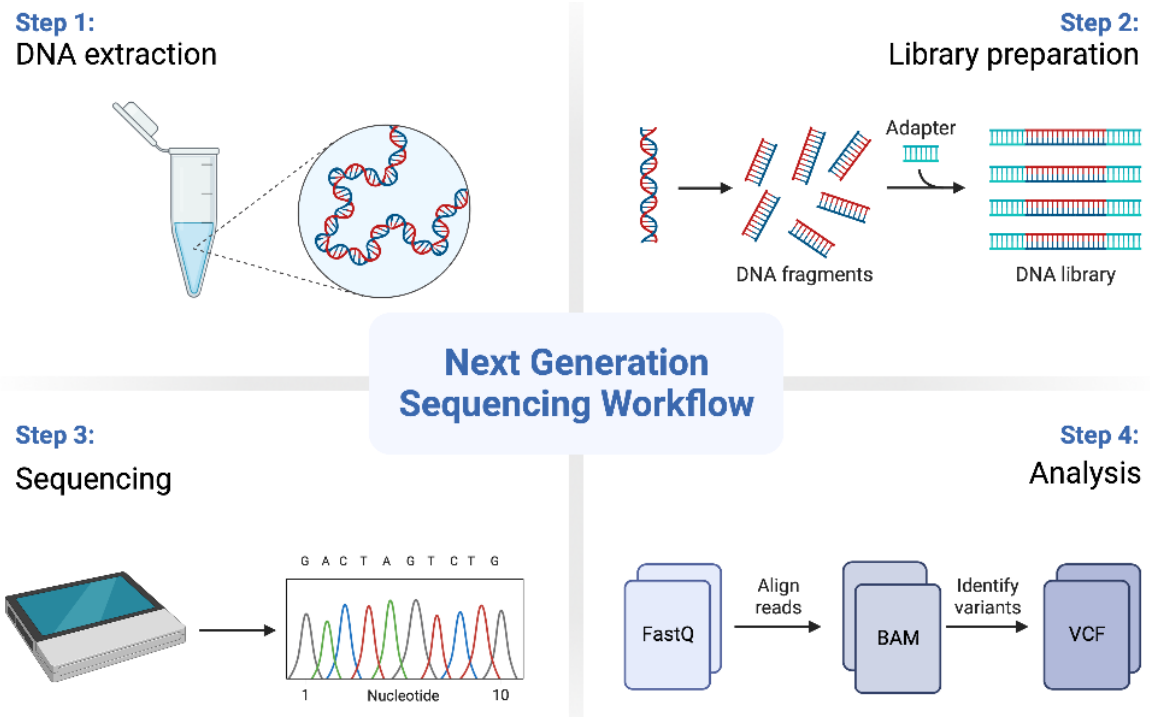


Figure 7: Next generation sequencing workflow

2.10 Enzyme-Linked Immune-Sorbent Assay (ELISA) for Salivary Biomarkers of Inflammation

ELISA was used for the quantification of salivary CRP and calprotectin. The kit used for CRP was Human CRP (C-Reactive Protein) ELISA Kit (Catalog No: MBS2505217 MyBioSource, USA) with detection range of 0.39-25 ng/ml. The kit used for calprotectin was Human Calprotectin ELISA Kit (Catalog No.: MBS7606803; MyBioSource, USA) with detection range of 0.156-10 ng/ml. Sandwich enzyme-linked immune-sorbent assay technology was used in both kits for both biomarkers. Briefly, 96-well plates were pre-coated with capture antibody (the first antibody). The second antibody is the detection antibody, which is biotin conjugated, and the targeted biomarker (antigen) is sandwiched between both antibiotics. The steps followed in each assay are listed below:

2.10.1 C-reactive protein

1. The standards, samples or blank (100 µl) were added to each well and incubated for 90 mins at 37°C. Standards were prepared by serial dilution in the provided assay buffer.
2. The liquid was removed, and 100 µl of the Biotinylated Detection antibodies were added and incubated for 1 hour at 37°C.
3. Aspiration and washing with wash buffer using automated ELSIA plate washer (HydroFlex, Tecan washer, USA) was performed three times.
4. HRP conjugate (100 µl) was added and incubated for 30 mins at 37°C.
5. Aspiration and washing were performed five times.
6. The substrate reagent (90 µl) was added and incubated for 15 mins at 37°C. Only the wells containing human CRP, biotinylated detection antibody, and avidin-HRP conjugate will be blue.
7. The stop solution (50 µl) was added. When stop solution was added to the enzyme-substrate reaction, the color turned yellow.
8. The optical density (OD) was measured using a 96 well micro-plate reader (infinite M200 pro, Tecan, USA) at 450 nm.
9. Calculation of CRP concentration: The concentration of CRP in the samples were calculated by comparing the OD of the samples to the OD of the standards by generating a standard curve using the free online analysis tool.

2.10.2 Calprotectin

1. The plate was washed 2 times before adding the standard, sample, and control (blank) wells.
2. The standards, samples or blank (100 µl) were added to each well and incubated for 90 mins at 37°C.
3. Aspiration and washing with wash buffer using automated ELSIA plate washer (HydroFlex, Tecan washer, USA) was performed twice.
4. Biotin- labeled antibody working solution (100 µl) was added to each well and incubated for 60 mins at 37°C.
5. Aspiration and washing were performed three times.

6. HRP-Streptavidin Conjugate (SABC) working solution (100 µl) was added into each well and incubated for 30 mins at 37°C.
7. Aspiration and washing were performed five times.
8. TMB Substrate solution (90 µl) was added and incubated for 10-20 mins at 37°C.
9. The stop solution (50 µl) was added. When stop solution was added to the enzyme-substrate reaction, the color turned yellow.
10. The optical density (OD) was measured using a 96 well micro-plate reader (infinite M200 pro, Tecan, USA) at 450 nm.
11. Calculation of CRP concentration: The concentration of calprotectin in the samples were calculated by comparing the OD of the samples to the OD of the standards by generating a standard curve using the free online analysis tool. Figures 8 and 9 summarize the steps used in ELISA assays.

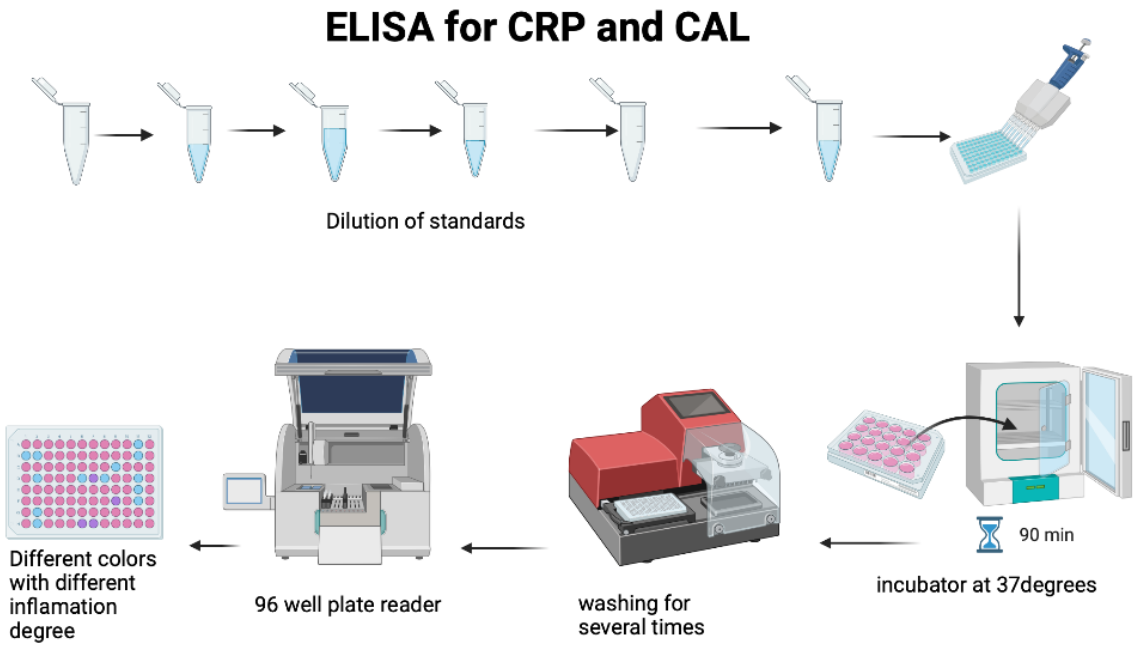


Figure 8: Schematic Diagram for Steps of ELISA Testing for C-Reactive Protein (CRP) Calprotectin (CAL) in the Saliva.

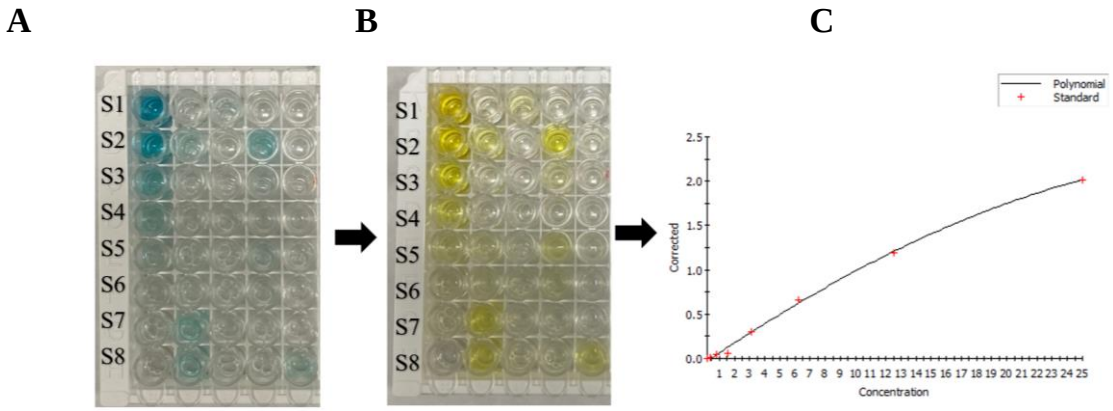


Figure 9: Color Change in Wells before (A) and After (B) Adding the Stop Solution, then OD was Measured to Generate a Standard Curve (C) to Calculate the Concentration in each Sample. S1-S8 are the Standards.

2.11 Statistical Analyses

Continuous variables were presented using mean ± SD. Statistical comparison of clinical, demographic, microbiota relative abundance, and alpha diversity were made using Mann–Whitney U or Kruskal–Wallis tests to compare samples grouped based on different factors. Correlations were tested using Spearman correlation coefficients (SPSS software, version 26). All statistical tests were two-sided. A p-value < 0.05 was considered statistically significant (Al-Marzooq et al., 2022). Venn diagrams were generated to show the shared and unique Operational Taxonomic Units (OTUs) among groups (CD vs HC and CD patients grouped based on multiple factors) using Venny bioinformatic tool (version 2.1) (Al-Marzooq et al., 2022).

Chapter 3: Results

3.1 Participants' Characteristics, Demographic, and Clinical data

The total number of participants was 80, including 40 CD patients and 40 HC. Males with CD were more than females (60% and 40%, respectively). The age of CD patients was matching to controls ±2 years difference. The age range was between 16-52 years for CD (mean ± SD = 32.75±10), while for HC the age range was between 18-54 (mean± SD = 33.37 ± 9.67). Crohn's disease participants' characteristics are shown in Table 3.

Table 3: Crohn's Disease Participants' Characteristics (n=40).

Characteristics	**Frequency (n)**	**Percentage (%)**
Duration of the disease		
Newly diagnosed	6	15
1-10 years	21	52.5
More than 10 years	13	32.5
Number of relapses		
0-1 times per year	29	72.5
2-4 times per year	11	27.5
Activity of the disease		
Active (CDAI ≥ 150)	10	25
Inactive (CDAI < 150)	30	75
Oral Health		
Caries	5	12.5
Periodontal disease	8	20
Caries and periodontal disease	16	40
Good oral hygiene	11	27.5
IBD drugs		
Biologicals alone	25	62.5
Biologicals + Steroids	9	22.5
Biologicals + Immunosuppressants	3	7.5
Biologicals + Steroids + Immunosuppressants	3	7.5

For data analysis, subjects were divided into those with active disease (score ≥150), or inactive disease (score <150). As shown in Table 3, the greatest percentage of patients were classified as having 1-10 years of disease duration (52.5%), followed by more than 10 years disease duration (32.5%) and the least were newly diagnosed with CD (15%). The frequency of relapse of symptoms was divided into 2 categories, up to once per year (72.5%) and more than twice per year (27.5%). The question was asked directly to the patient and the response was according to his/her recurrence of symptoms of CD which are abdominal pain, blood in stool, diarrhea, and weight loss. Most of the patients had CD in the small intestine (terminal ileum) after confirmation of diagnosis via colonoscopy (n=31). Twenty five percent (n=10) of the patients had active disease and seventy five percent (n=30) were inactive.

Active patients are those having CDAI score ≥ 150, while inactive patients are patients with CDAI scores < 150.

The critical part of the study is questioning the history and current medication taking since it plays a tremendous part in understanding the cause of dysbiosis in the GIT. As for antibiotic use, few patients (n=2; 5%) were currently consuming antibiotics at the time of treatment for CD with other medication, while the rest (n=38 ;95%) did not mention such consumption. Some (n=3;7.5%) of the patients mentioned history of intake of several antibiotic courses prior to the final diagnosis via colonoscopy.

Since most of the patients were interviewed in the infusion clinic, this indicates that majority were receiving the intravenous medications, particularly biologicals (monoclonal antibodies) mainly infliximab (62.5%), but few used other biologicals such as vedolizumab, ustekinumab and adalimumab. Other medication including steroids (prednisolone, hydrocortisone, and budesonide) and immunosuppressants (azathioprine and mercaptopurine) were taken concurrently to reduce other symptoms. This includes biologicals and steroids (22.5%), biologicals and immunosuppressants (7.5%) and the three types of medications together (biologicals, steroids and immunosuppressants; 7.5%), as shown in Table 3.

As for oral health (Figure 10), most of CD patients had poor oral hygiene (n=29; 72.5 %) compared with HC (n=19; 47.5%). In CD, the poor oral health is further

classified into having caries (C) (n=5; 12.5%), caries and periodontal disease (C + P) (n=16; 40%), or periodontal disease (P) (n=8; 20%), while some CD patients had good oral hygiene (n=11; 27.5%). As for HC, most of them had good oral hygiene (n=21; 52.5%), while some had caries (n=2; 5%), caries and periodontal disease (n=13; 32.5%), or periodontal disease (n=4; 10%).

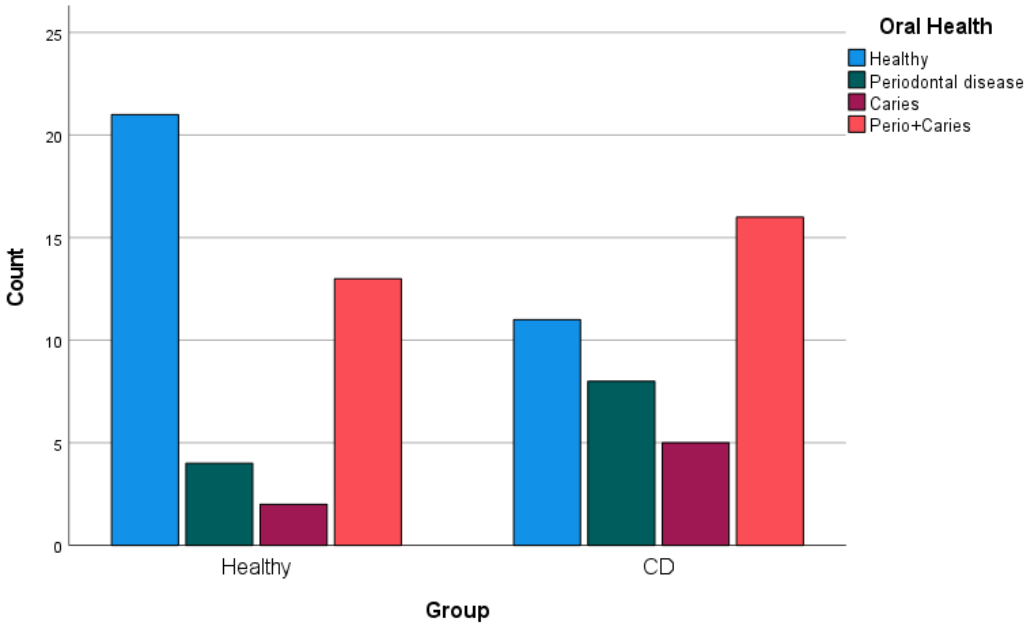


Figure 10: Distribution of the Study Participants Based on Oral Health.

3.2 Microbiome Analyses

3.2.1 Sequencing statistics

To investigate the oral microbial features, bacterial 16S rRNA gene was sequenced in the salivary samples from 80 participants. Average read length was 1542.68 ± 24.9 bp which is equivalent to the length of the bacterial 16S rRNA gene.

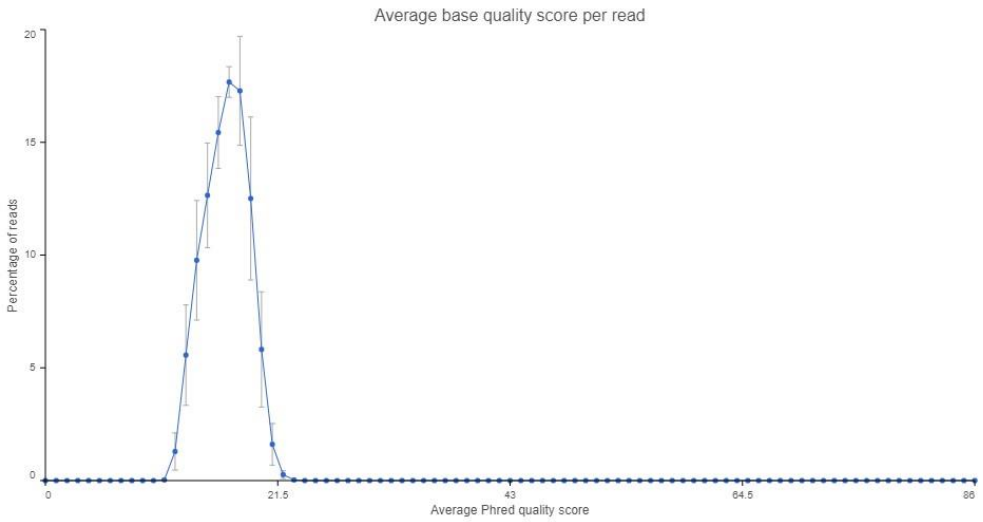
3.2.2 Average quality score

A mean quality score of 20 (Q20) was obtained (as shown in Figure 11-A), representing an error rate of 1 in 100, with a corresponding base call accuracy of 99%.

3.2.3 Library size

Figure 11 (B) shows the depth of sequencing represented by the rarefaction curves of HC and CD samples. Rarefaction curve analysis for HC and CD samples show species richness for the sequences and confirms the adequacy of sequencing reads for valid microbiota analysis. Average number of reads obtained from the libraries was 348173.99 reads and number of reads for each library is shown in Figure 11 (C) presenting the minimum and maximum number of reads in the libraries prepared from each sample, which help to identify the potential outliers due to under sampling or sequencing error.

A.



B.

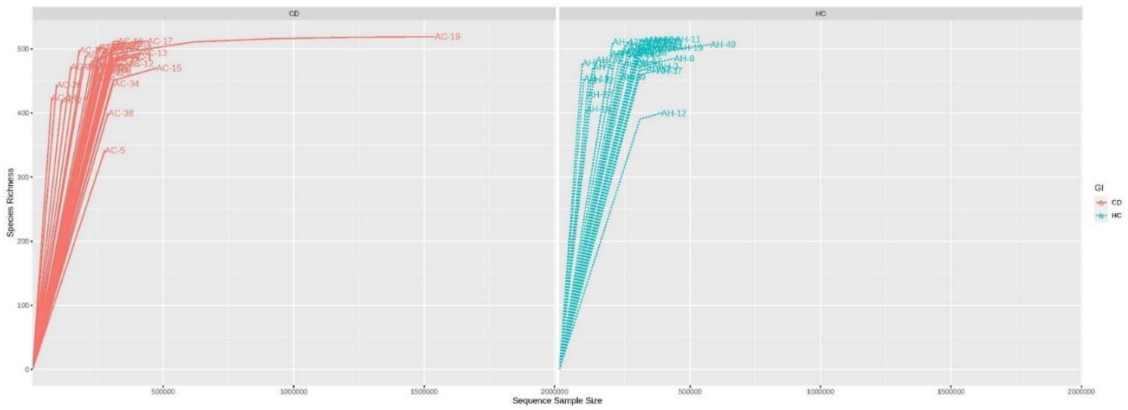


Figure 11: Sequencing Statistics: (A) Quality Score of the Samples Analyzed in this study, (B) Rarefaction Curve Analysis for HC and CD Samples.

C.

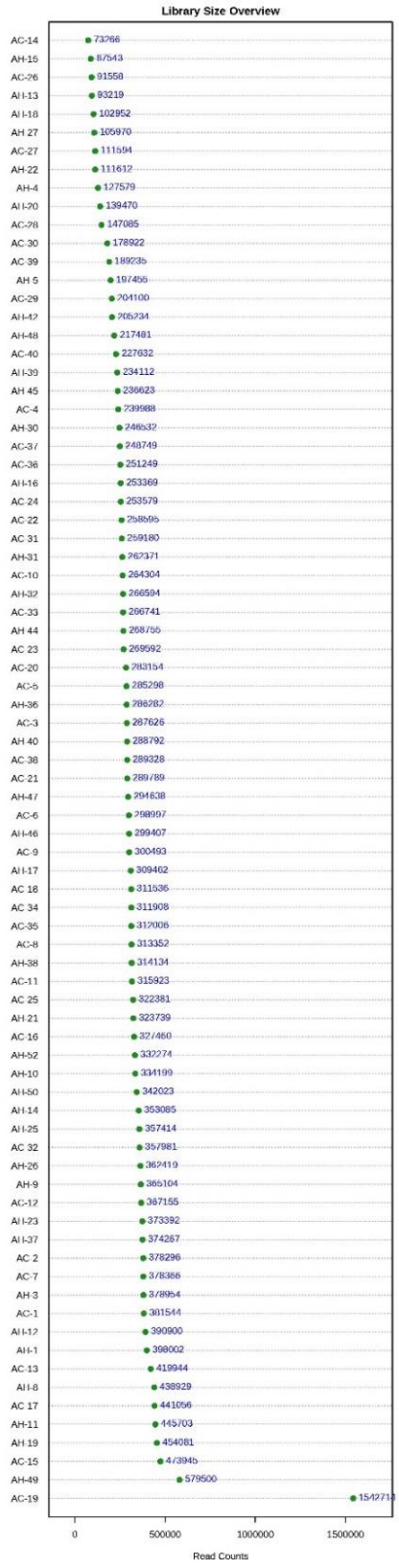


Figure 11: Sequencing Statistics: (C) Minimum and Maximum Number of Reads in the Libraries Prepared from Each Sample (continued)

3.2.4 Microbial counts

Bacterial counts in different taxonomic levels from phyla down to the species were identified. The focus of the analysis was on 3 levels, including phyla, genera, and species.

Overall, the number of bacterial taxa (mean ± SD) in each taxonomic level were 19.475± 2.938 for phyla, 31.16 ± 4.17 for class, 72.57 ± 9.914 for order, 139.88 ± 21.19 for family, 318.675± 54.49 for genus, and 780±112.42 for species. Comparison of the number of taxa detected in CD patients and HC is shown in Figure 12, which revealed non-significant differences in numbers of phyla to genera between the 2 groups; however, CD patients had slightly less counts of bacteria in each taxonomic level compared to HC. For species, there was a significant difference between HC and CD, as the latter had significantly less species.

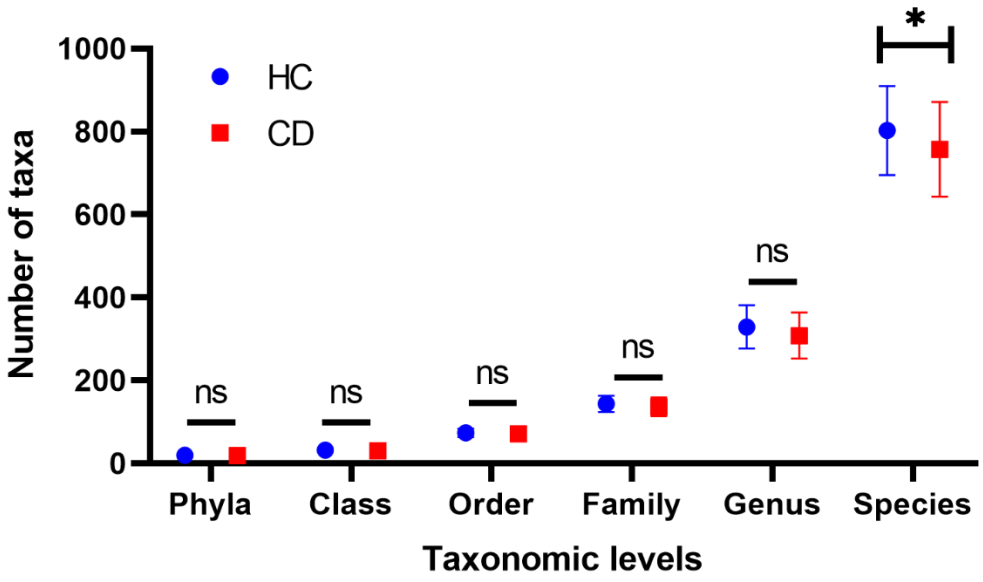


Figure 12: Analysis for the 6 Taxonomic Levels Comparing CD with HC. ns= non-significant, * = significant.

3.3 Microbiota in CD vs HC

Examples of the microbiota detected at different taxonomic levels are shown in Figure13.

A.

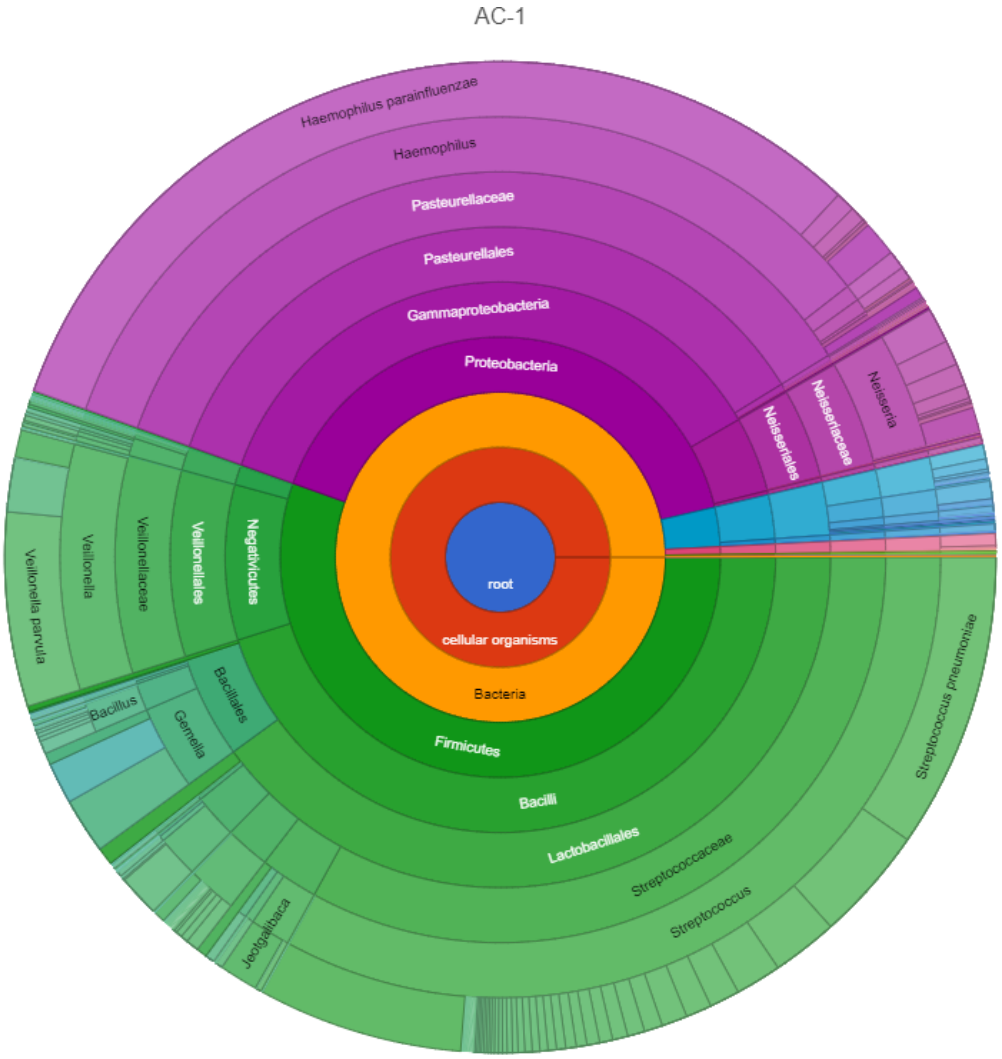


Figure 13: Krona Pie Chart Showing an Example of Different Taxa in (A) CD (sample AC-1).

B.

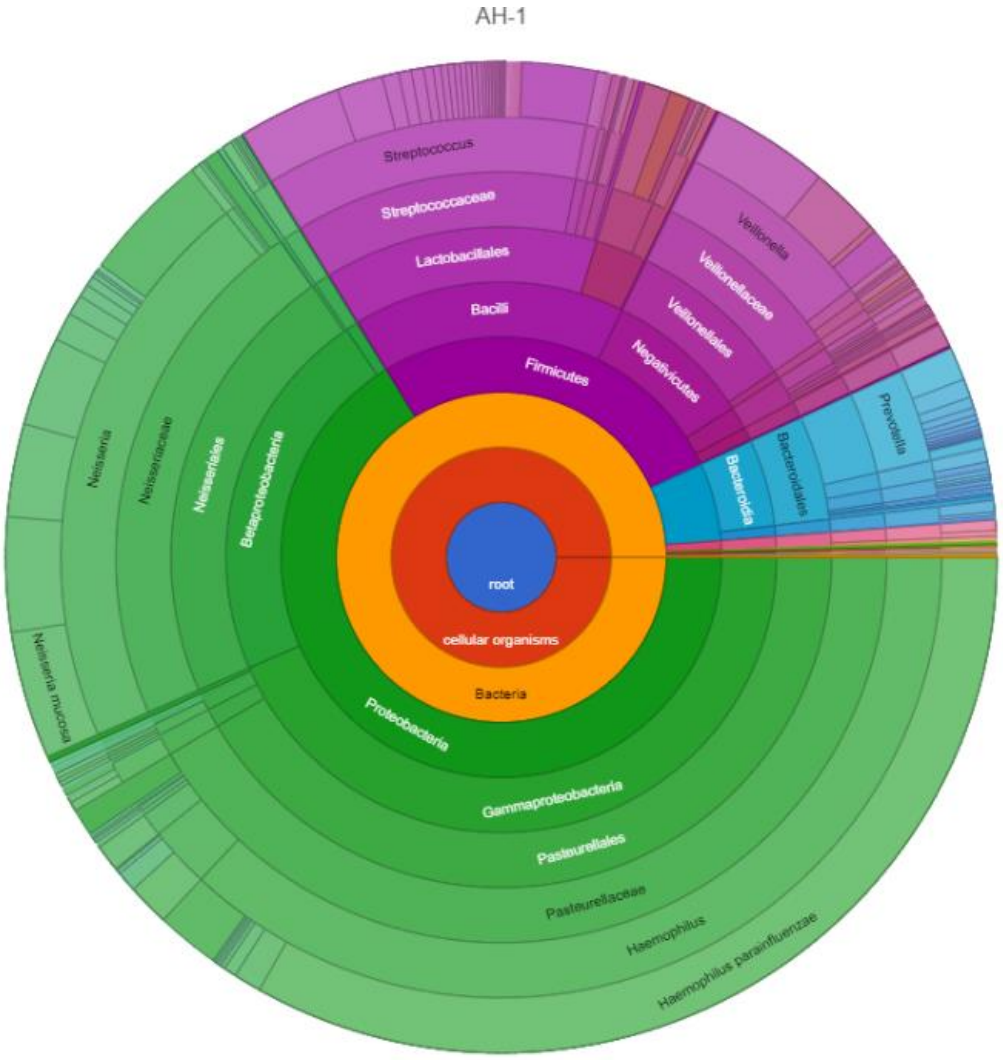


Figure 13: Krona Pie Chart Showing an Example of Different Taxa in (B) HC (sample AH-1) (continued).

3.3.1 Relative abundance of microbiota in CD Vs HC at 3 taxonomic levels

Relative abundance of microbiota in CD Vs HC at three taxonomic levels (phyla, genera, and species) were identified (shown in Figures 14, 16 and 18) with the identification of the core microbiome which represents the common microbial taxa within a host population. The bacterial taxa with the highest detection threshold (relative abundance), are in red color (Figures 15, 17 and 19).

Figure 14 shows the relative abundance of microbiota in CD and HC at the phylum level. *Firmicutes* had the highest abundance followed by *Proteobacteria* and *Bacteroidetes.* Figure 15 shows the core microbiome in CD (A) and HC (B) with phyla prevalence in each group. *Firmicutes, Proteobacteria* and *Bacteriodetes* had the highest percentage of relative abundance. HC had similar abundance of phyla.

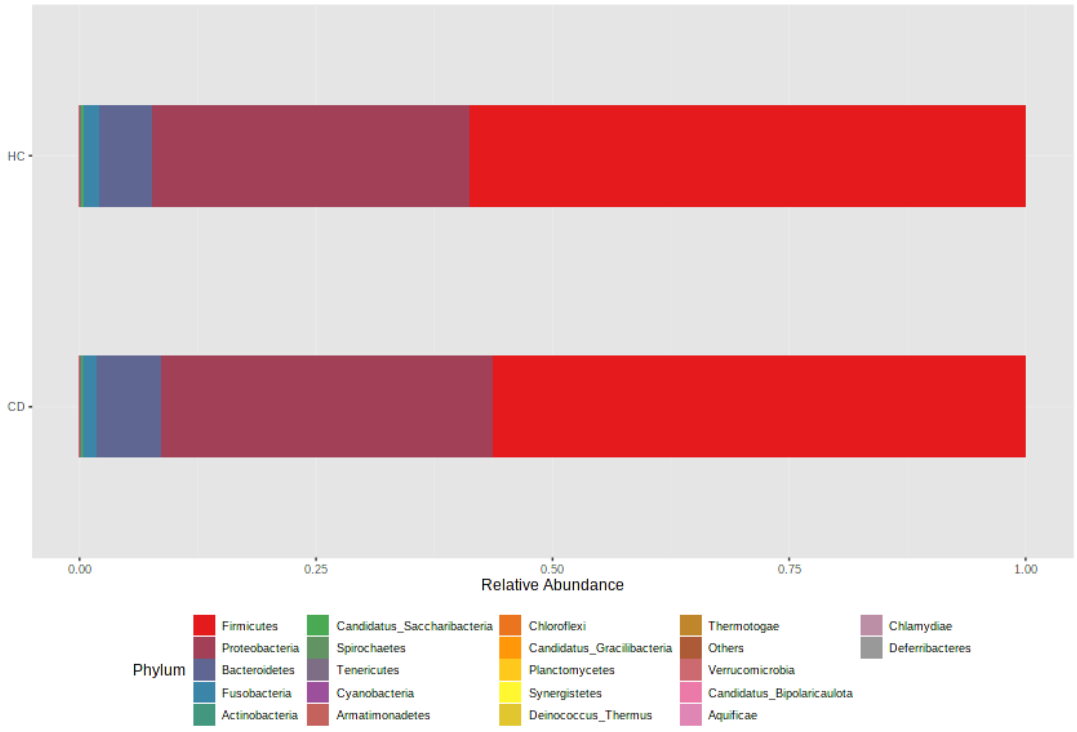


Figure 14: Relative Abundance of Microbiota in CD Vs HC at the Phylum Level.

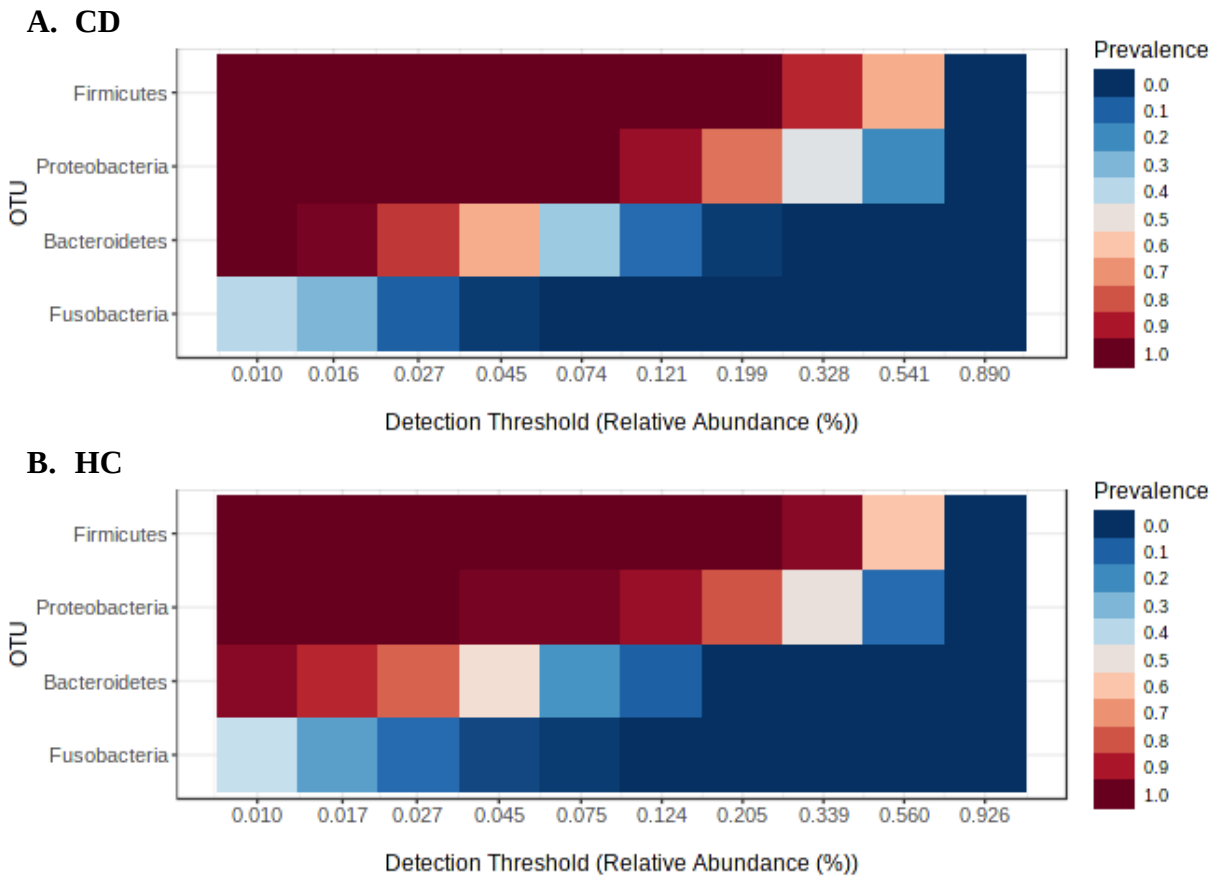


Figure 15: The Core Microbiome in CD (A) and HC (B) Showing Phyla Prevalence in each Group.

At the genus level (Figures 16 and 17), the highest percentage of genera in CD were *Streptococcus,* followed by *Haemophilus*, *Veillonella*, *Neisseria* and *Prevotella.* While HC had the highest percentage of *Streptococcus,* followed by *Haemophilus, Veillonellla, Neisseria* and *Gemella.* The relative abundance of the prevalent phyla and genera were less in CD.

At the species level (Figures 19 and 20), the shared microbiota between HC includes: *Haemophilus parainfluenzae, Veiollonella parvula, Streptococcus pneumoniae, Streptococcus pyogenes Veillonella dispar,* and *Streptococococcus salivariu*s. The bacterial species with the highest detection threshold (relative abundance), are in red color. While in CD the highest percentage of species were *Haemophilus parainfluenzae, Veillonella parvula, Veillonella dispar, Streptococcus pneumoniae, Streptococcus pyogenes,* and *Streptococococcus salivarius.*

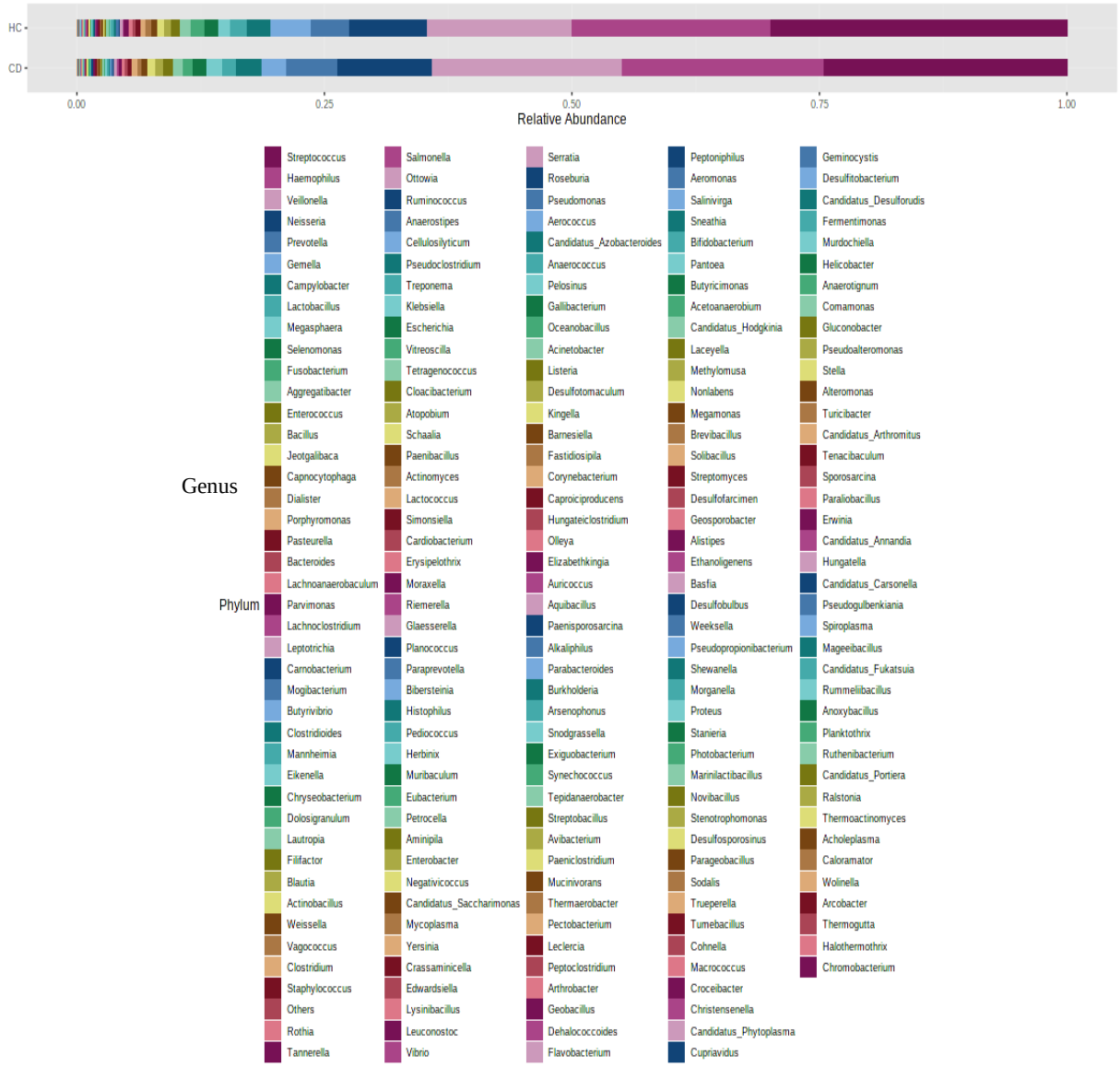
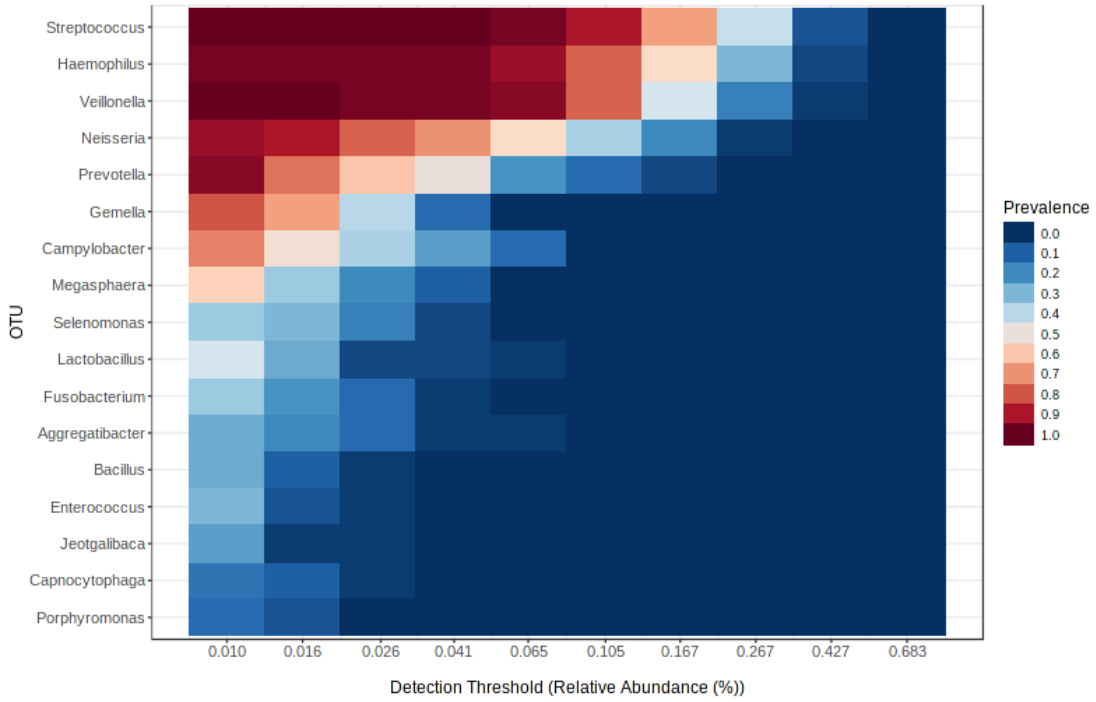


Figure 16: Relative Abundance of Genera in CD and HC.

A. CD



B.HC

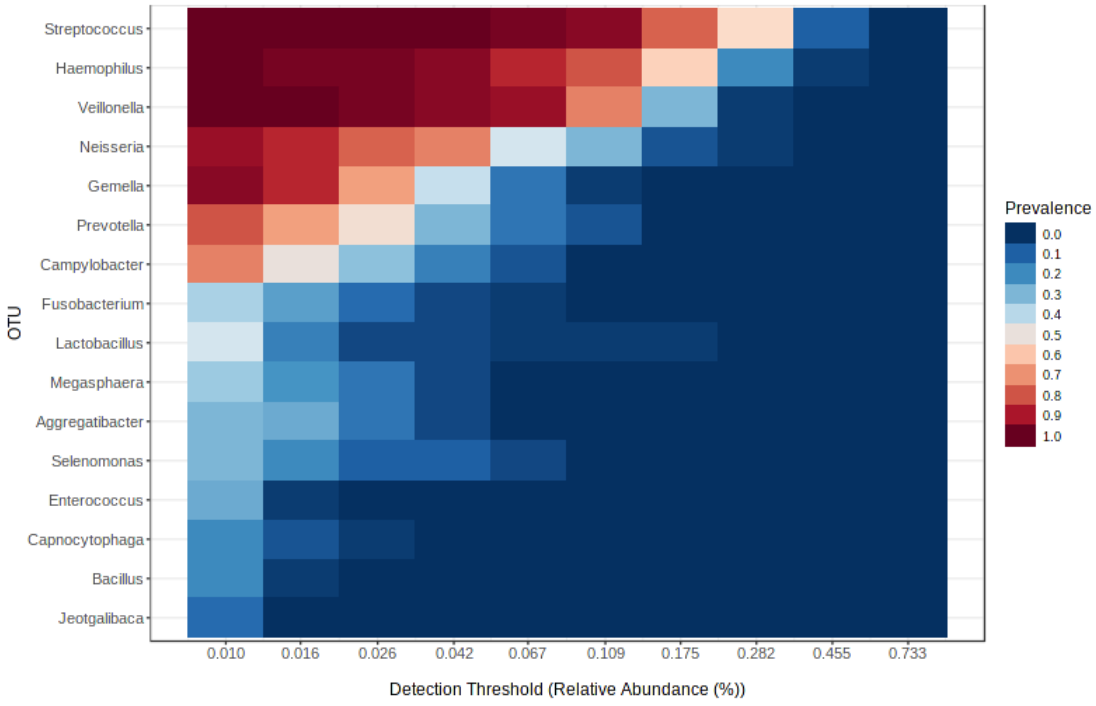


Figure 17: The Core Microbiome in CD (A) and HC (B) at the Genus Level.

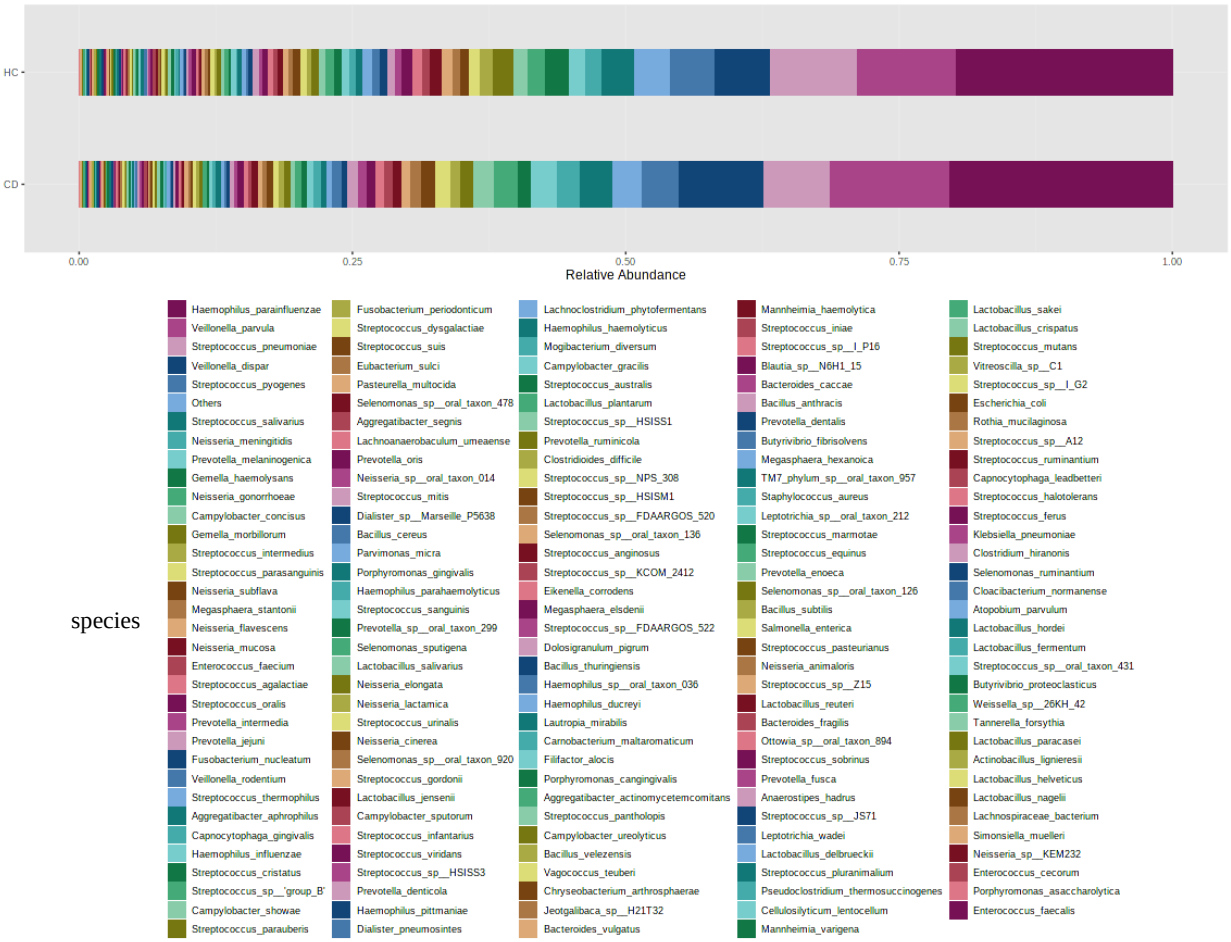


Figure 18: Relative Abundance Showing of Species in CD vs HC.

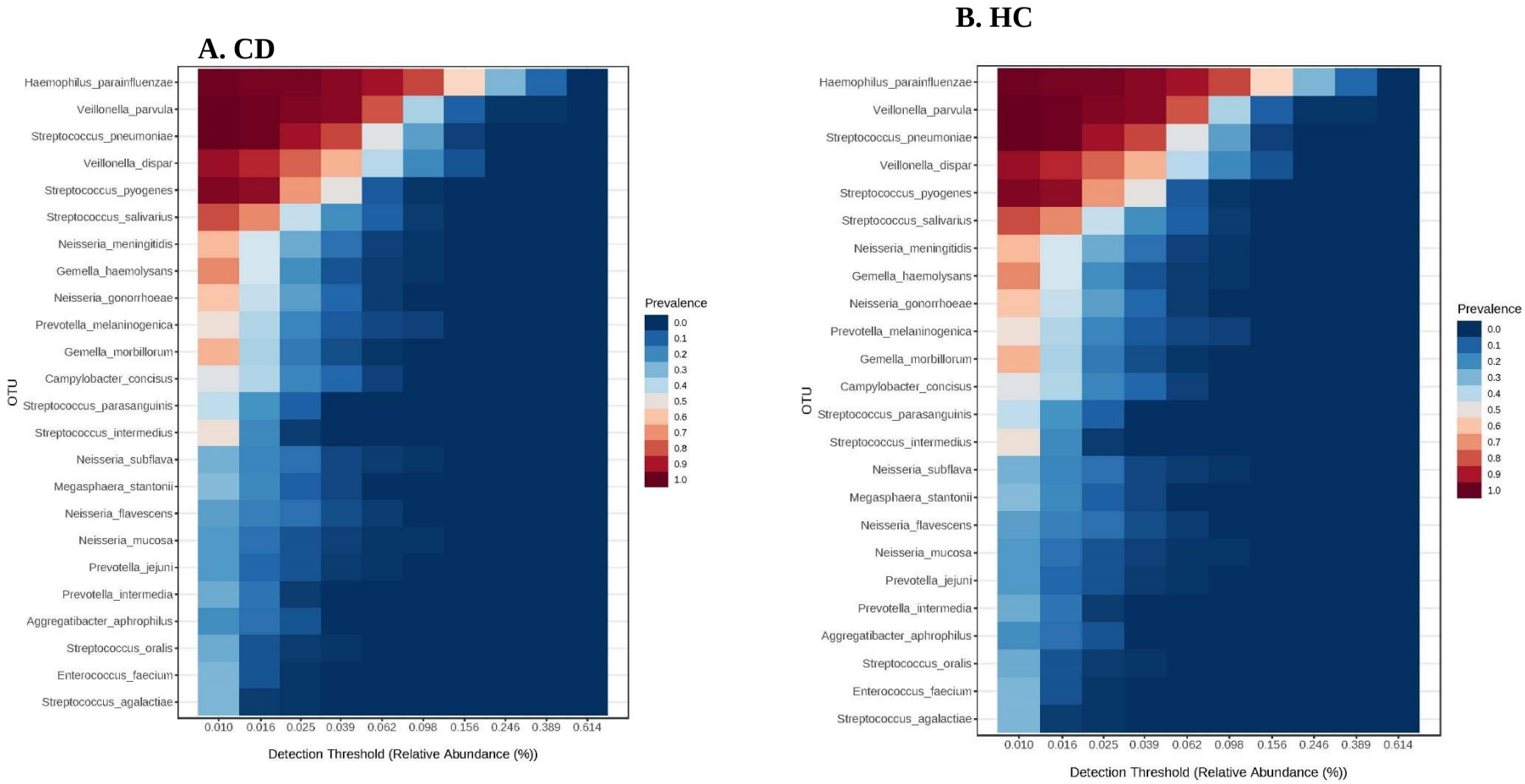


Figure 19: The Core Microbiome in CD (A) and HC (B) at the Species Level.

3.3.2 *Comparison of the microbial profiles in CD and HC using Linear Discriminant analysis (LDA) Effect Size (LEFSe) at three taxonomic levels (phylum, genus, and species)*

The linear discriminant analysis, which is a measure of potential biomarkers of bacteria, was used to test the 80 samples to compare CD patients and HC at three taxonomic levels (phylum, genus, and species). The results indicate significant differences between CD and HC (Figure 20).

At the phylum level, *Tenericutes* and *Spirochetes* were significantly more in HC and depleted in CD. At the genus level, only one genus, *Dolosigranulum* was significantly higher in CD and depleted in HC, while 16 significant features were significantly higher in HC and depleted in CD. At the species level, LDA analysis clearly demonstrated a total of 65 significant features, 5 of which were significantly higher in CD with higher LDA scores reaching up to 5, and the remaining 60 are bacteria significant in HC and depleted in CD.

Figure 19: The Core Microbiome in CD (A) and HC (B) at the Species Level.

The five dominant species enriched in CD are *Veillonella dispar, Megasphaera_stantonii, Prevotella jejuni, Dolosigranulum pigrum* and *Lactobacillus backii*. These bacterial species are not detected in HC instead other microbial profiles are present such as *Mucinivorans hirudinis, Streptococcus mitis, Fusobacterium sp oral taxon 203* and *Streptococcus viridans.*

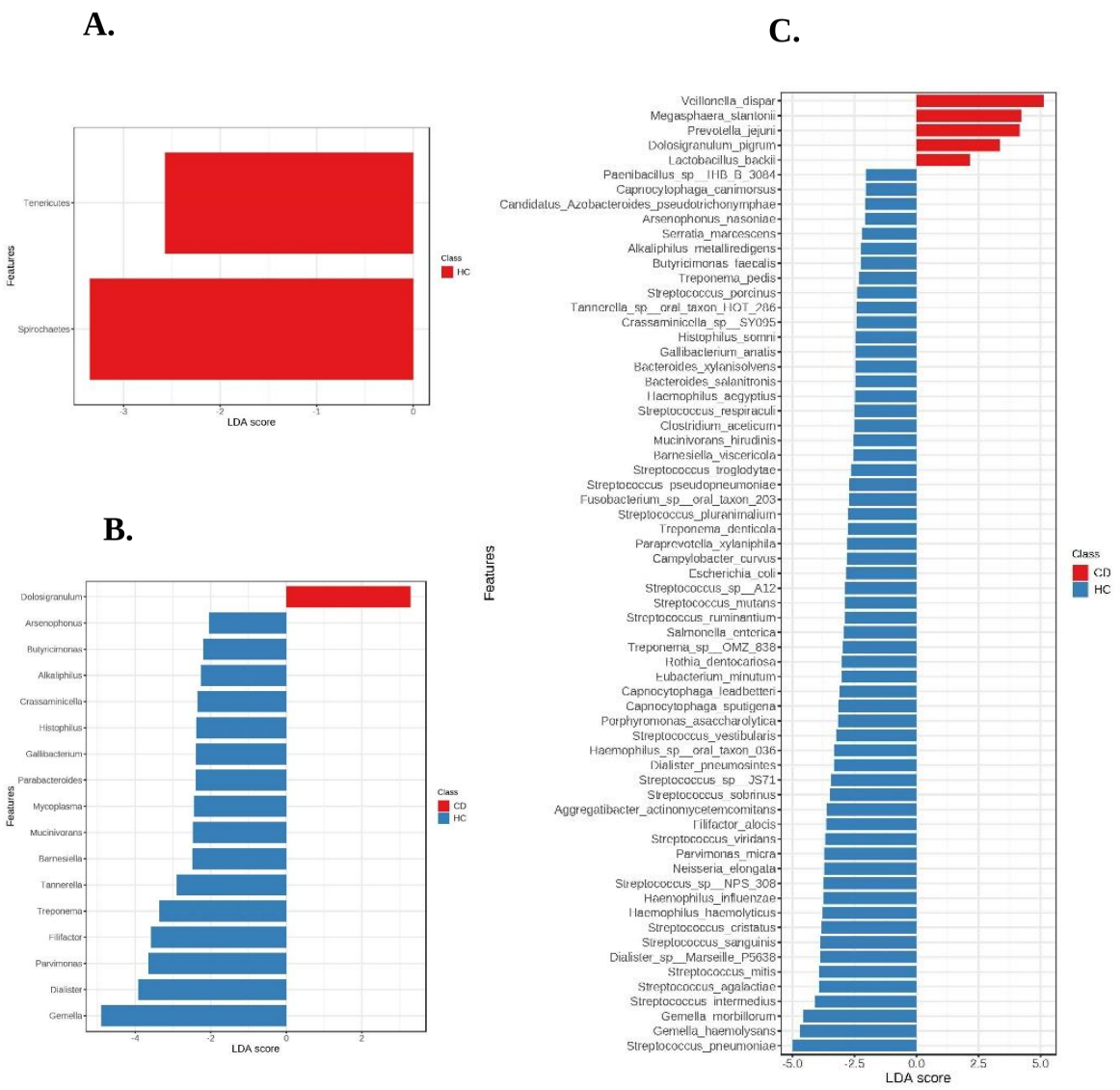


Figure 20: Significant features (A) phyla, (B) genus, and (C) species detected using LDA in CD vs HC.

The log transformed salivary microbial count of the 5 five distinct species of CD are shown in Figure 21. The figures clearly show that the abundance of these species was significantly higher in CD compared to HC.

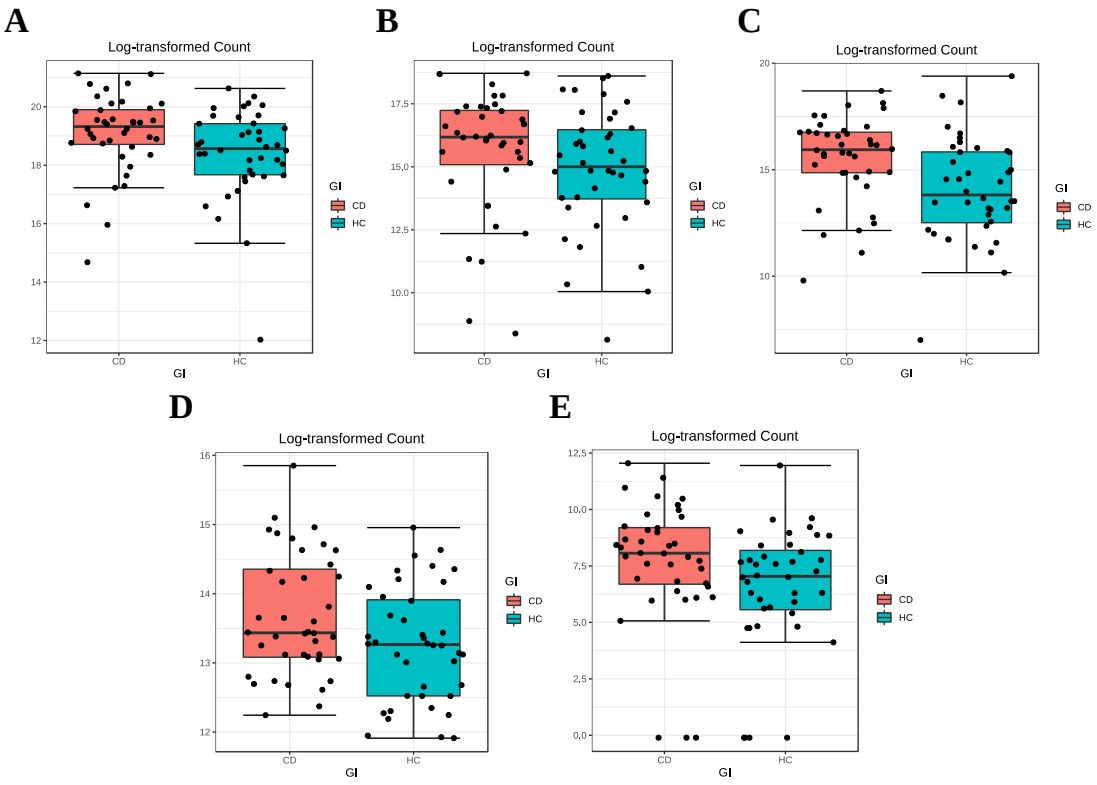


Figure 21: Significant Bacterial Species Detected in the Saliva of CD Patients, (A) *Veillonella dispar*, (B)*Megasphaera stantonii*, (C) *Prevotella jejuni (*D) *Dolosigranulum pigrum*, and (E) *Lactobacillus backii*.

3.4 Effect of Oral Health on the Salivary Microbiome

3.4.1 Comparison of HC and CD with respect to their oral health status

Relative abundances of bacterial taxa were compared in subjects with CD and HC with respect to the oral health status including caries (C), periodontal disease (P), periodontal disease and caries (P+C) and good oral hygiene or healthy (H), as shown in Figures 22-24. The figures clearly show variations in the microbiome in both CD and HC in subjects with different oral health conditions.

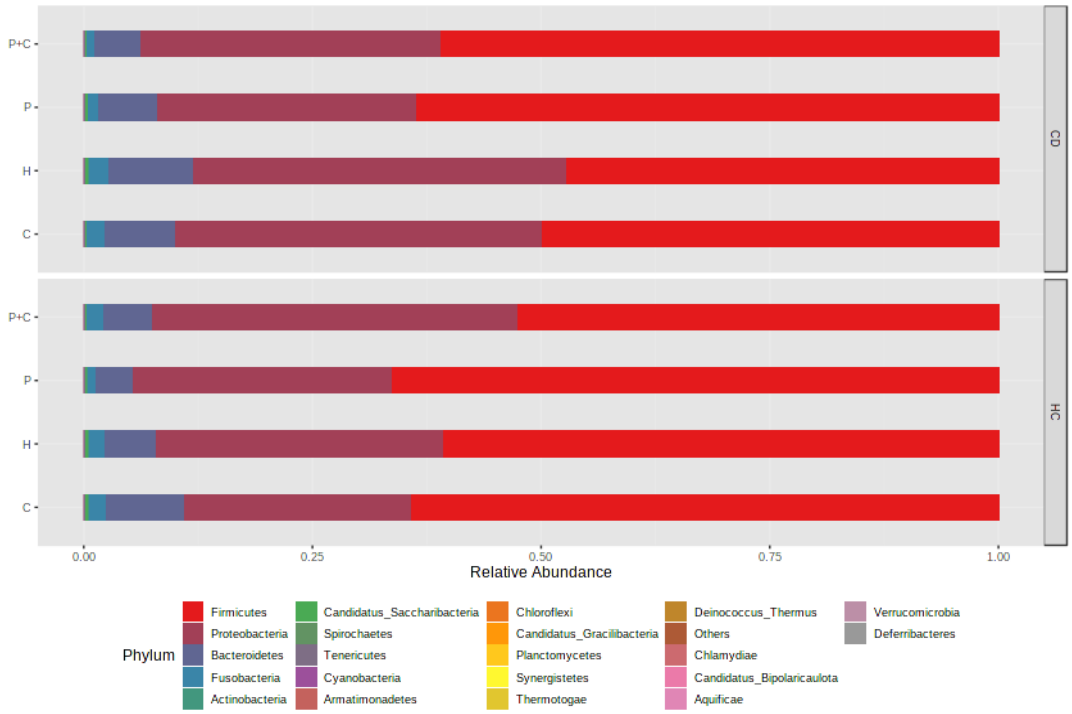


Figure 22: Relative Abundance of Phyla in CD vs HC Based on Oral Health.

Figure 23: Relative Abundance of Genera in CD vs HC Based on Oral Health.

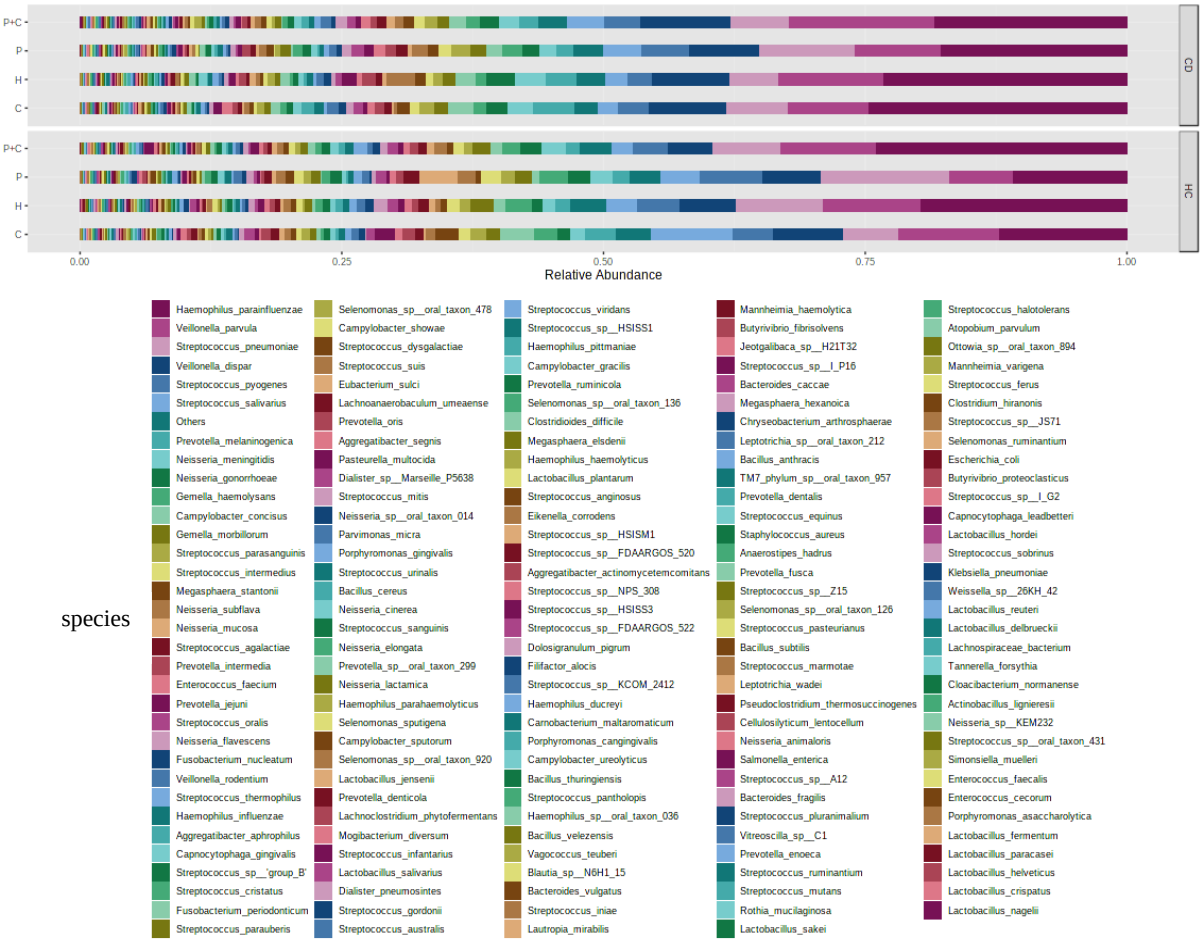


Figure 24: Relative Abundance of Species in CD vs HC Based on Oral Health. Healthy (H), Caries (C), Periodontitis (P) and Periodontitis and Caries (P+C).

3.4.2 Linear Discriminant analysis (LDA) Effect Size (LEFSe) of CD and HC with oral health as the experimental factor

First, all 80 subjects were compared for microbiota based on their oral health status. Subjects were classified into healthy (H), caries (C), periodontitis (P) and periodontal disease + caries combined (P+C).

At the phylum level, as shown in Figure 25, *Fusobacteria* was the only biomarker *in C* while in H only *Actinobacteria wa*s detected. No significant features were detected in other groups such as P and P+C.

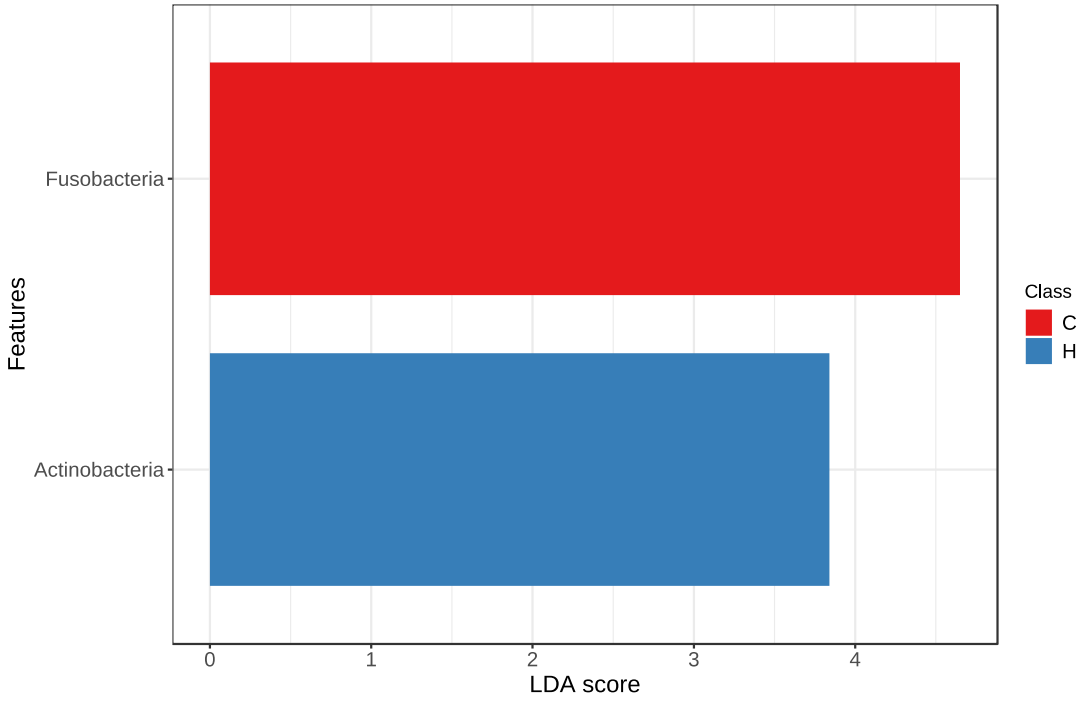


Figure 25: Linear Discriminant Analysis (LDA) Effect Size (LEFSe) of CD and HC with Oral Health as the Experimental Factor. (H) Healthy, (C) Caries.

At the genus level, as shown in Figure 26, the bacterial biomarkers in C were *Fusobacterium, Actinobacillus, Salmonella* and *Escherichia.* Two genera were found in patients with P, including *Dolosigranulum* and *Pediococcus*. For H patients, three bacterial biomarkers, *Mucinivorans, Candidatus Azobacteroides* and *Pectobacterium* were found.

At the species level, no significant features were detected in subjects with P+C. As shown in Figure 27, the results indicate that the bacterial biomarkers in healthy individuals as in those having no caries or periodontal diseases are *Prevotella enoeca, Prevotella dentalis*, and *Bacteroides intestinalis.* Patients with caries show bacterial biomarkers of *Fusobacterium periodonticum, Salmonella enterica,* and *Escherichia coli.* Patients with periodontitis or periodontal diseases show bacterial biomarkers of *Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus oralis*, and *Streptococcus mutans.*

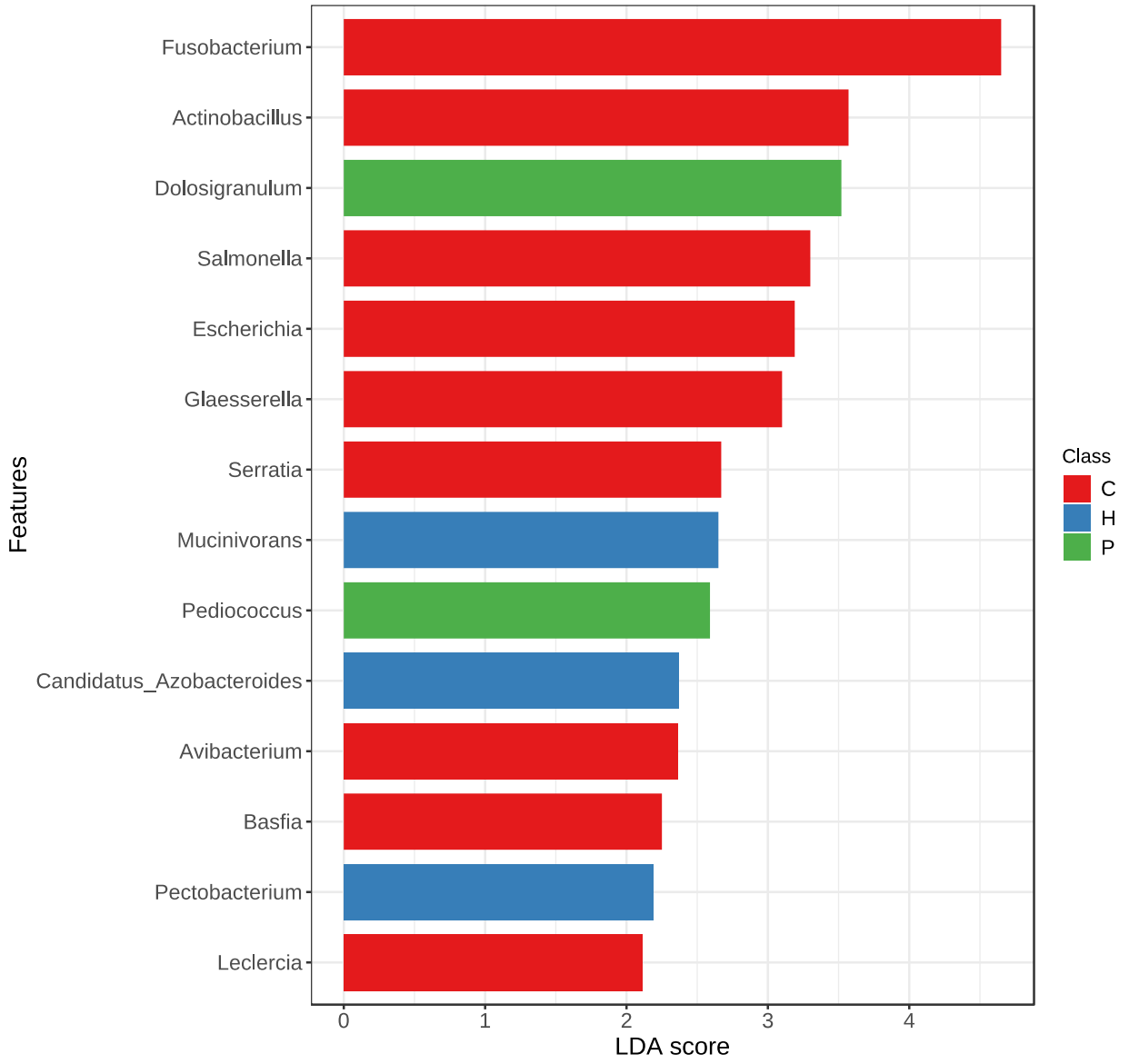


Figure 26: Linear Discriminant Analysis (LDA) Effect Size (LEFSe) of CD and HC at the Genus level with Oral Health as the Experimental Factor. (H) Healthy, (C) Caries and (P) Periodontitis.

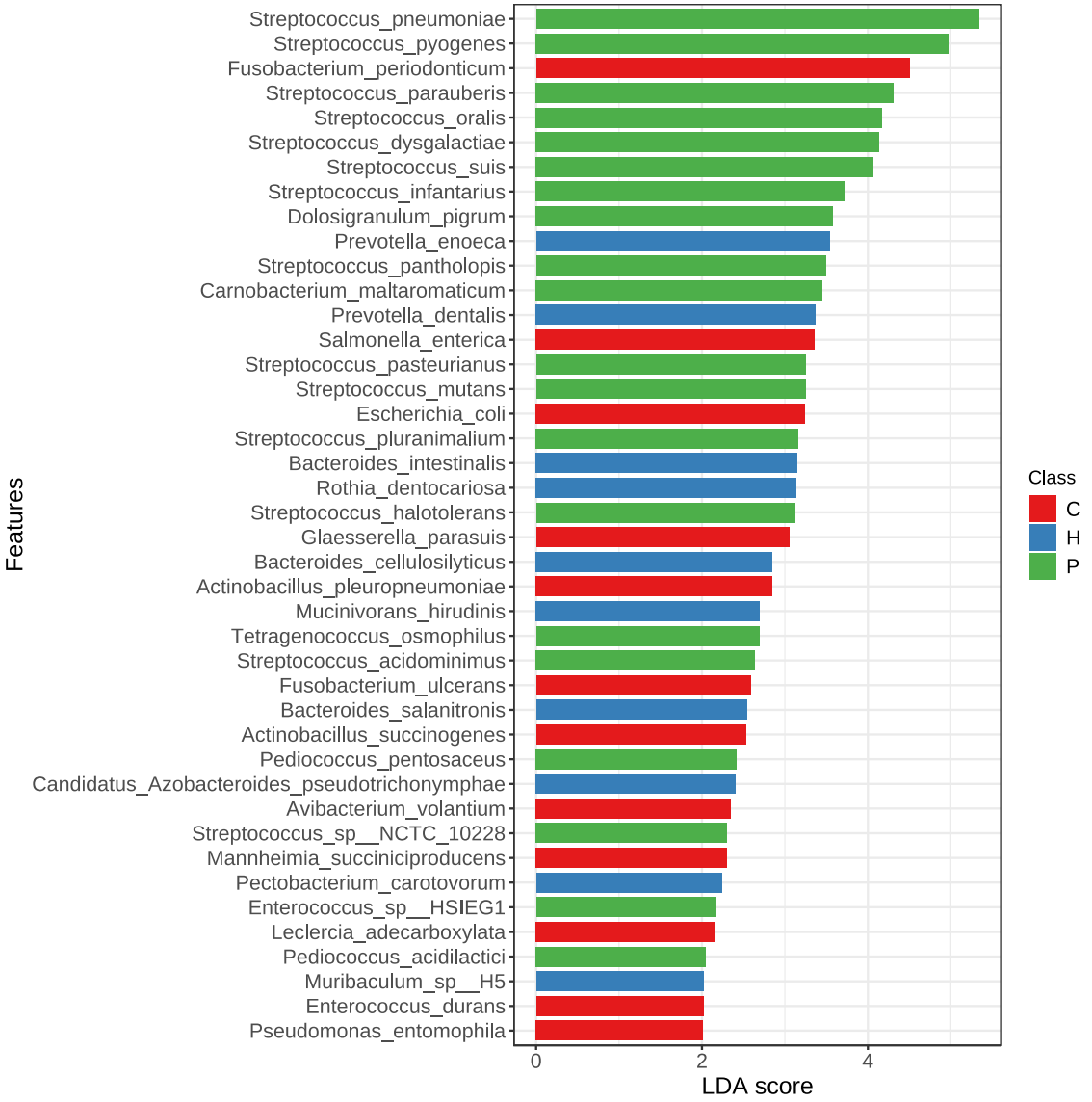


Figure 27: Linear Discriminant Analysis (LDA) Effect Size (LEFSe) of CD and HC at the Species Level with Oral Health as the Experimental Factor. (H) Healthy, (C) Caries and (P) Periodontitis.

The log transformed counts of significant features (species) when oral health was used as an experimental factor are shown in Figure 28.

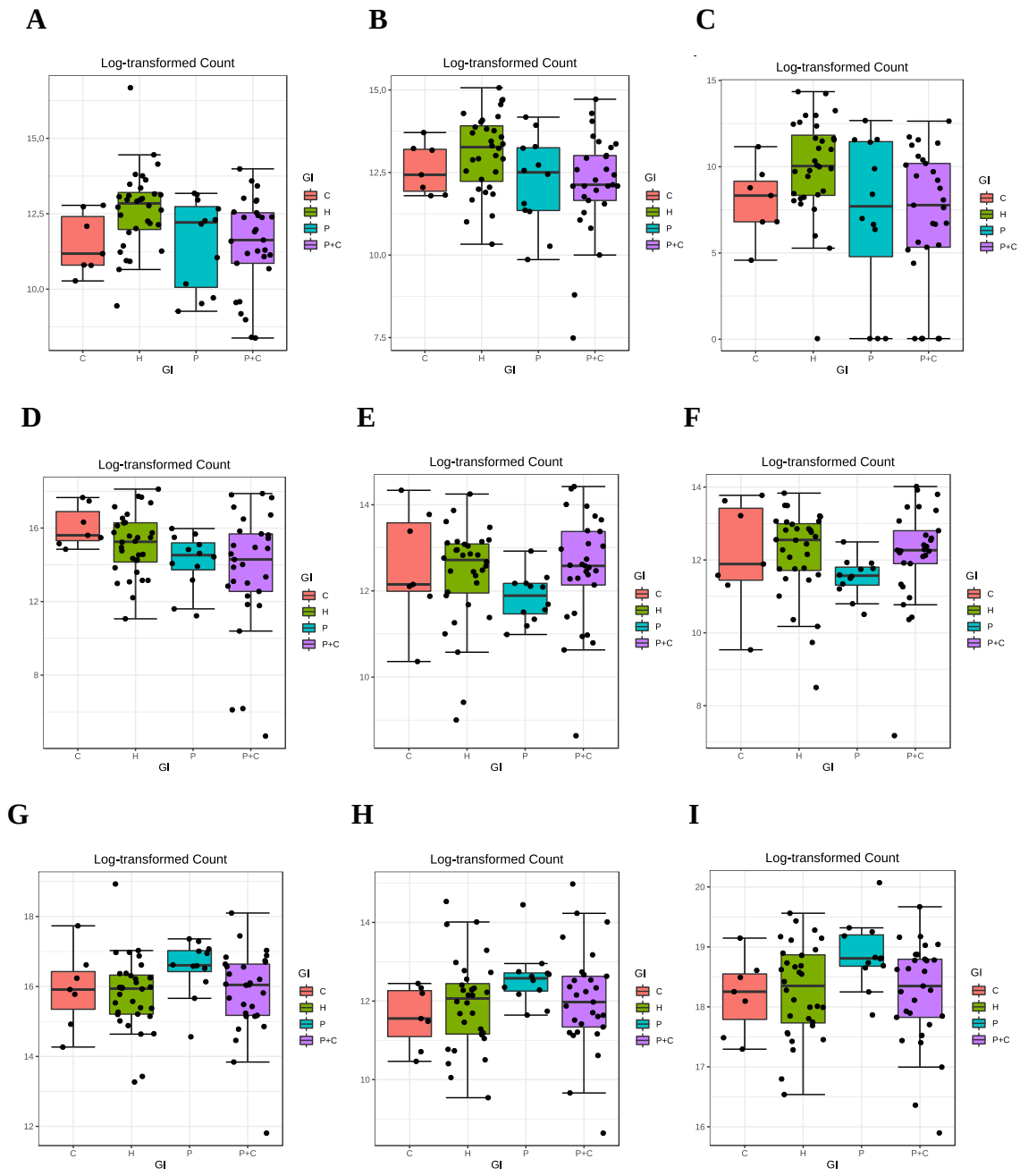


Figure 28*:* The Log Transformed Count in the 9 Significant Species Present in the Saliva of Different Oral Health Categories.

Healthy: *(A) Prevotella enoeca, (B) Prevotella dentalisv, (C) Bacteroides intestinalis.* Caries: (*D) Fusobacterium periodonticum, (E) Salmonella enterica, (F) Escherichia coli;* Periodontal disease: *(G) Streptococcus oralis, (H) Streptococcus mutans, (I) Streptococcus pyogenes*.

To identify significant features in each of CD and HC based on oral health, and if any features are unique in each of these groups, linear discriminant analysis (LDA) effect size (LEFSe) was tested at three taxonomic levels (phylum, genus, and species) for HC and CD subjects grouped based on oral health.

3.4.3 Linear discriminant analysis (LDA) effect size (LEFSe) was tested at three taxonomic levels (phylum, genus, and species) for HC based on oral health.

No significant features were detected at the phylum level when HC subjects were compared according to their oral health status. Figure 29 shows the 22 significant features that were detected in HC at the genus level based on oral health. A total of 4 bacterial genera were detected in individuals with caries, namely *Lachnoclostridium, Actinomyces, Cardiobacterium* and *Nonlabens.* Only one genus *Croceibacter* in periodontal disease and one genus *Mucinivorans* were found in individuals with good oral hygiene. The remaining were detected in individuals with periodontal disease and caries combined, such as *Pasteurella, Salmonella, Escherichia, Klebsiella* and others.

At the species level, 42 significant features were detected in HC based on oral health. The highest LDA score was for *Haemophilus parainfluenzae* in individuals with periodontal disease and caries combined followed by *Streptococcus salivarius* in individuals with caries, *Streptococcus mitis* in periodontal disease cases and those with good oral hygiene had *Streptococcus gordonii* and *Lactobacillus acidophilus.*

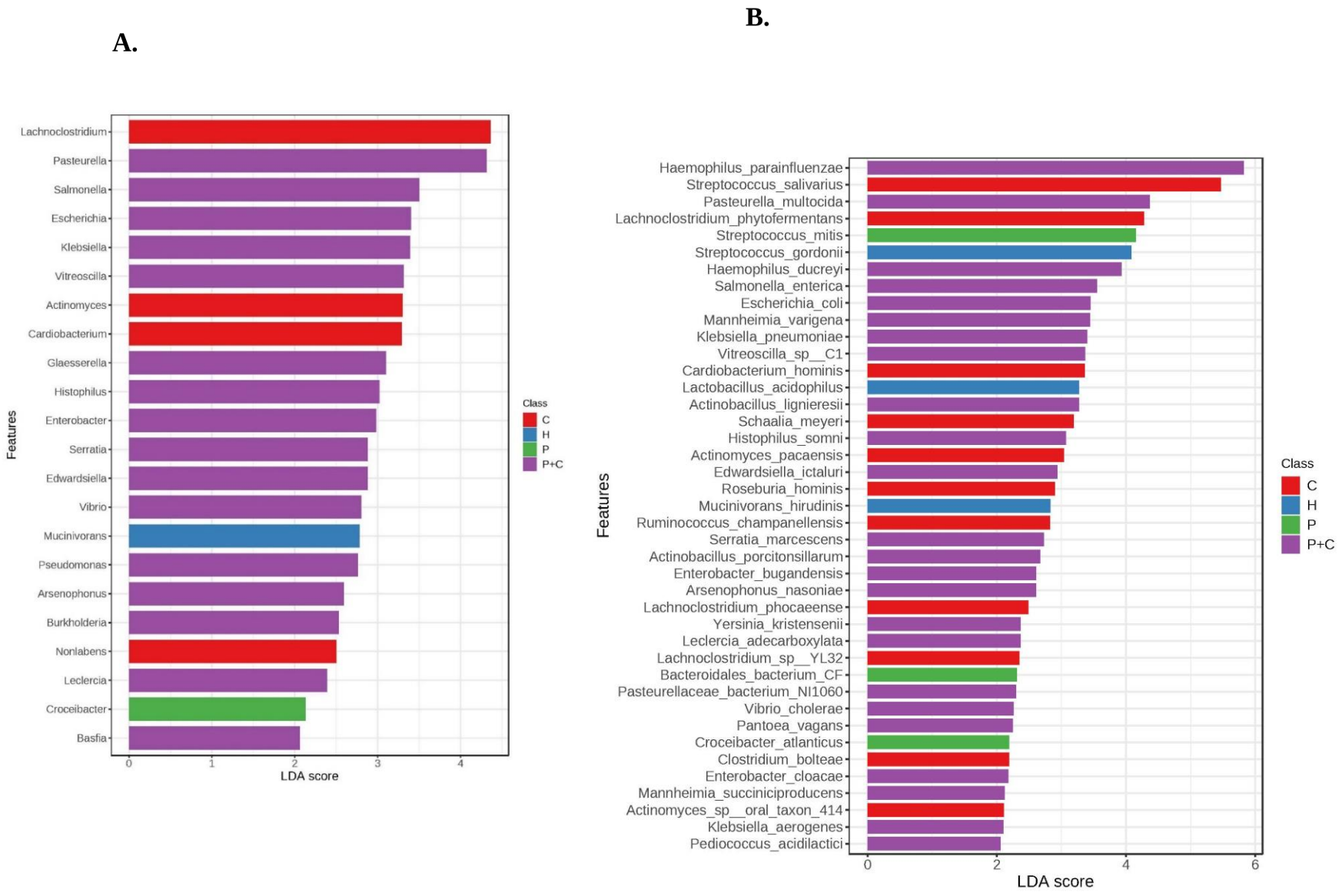


Figure 29: Significant features Detected in HC at the Genus (A) and Species (B) level Based on Oral health.

3.5 Factors that Might Contribute to Dysbiosis in CD

Patients with CD (n=40) were compared for microbiota composition based on different factors that might contribute to dysbiosis. These factors are:

1- Oral Health

Figures 30-32 show significant bacterial features at the phylum, genus, and species levels, respectively using linear discriminant analysis (LDA) effect size when oral health is used as an experimental factor in CD.

At the phylum level, CD had two significant bacterial features at the phylum level, *Fusobacteria* in good oral health and *Actinobacteria* in periodontal disease (Figure 30).

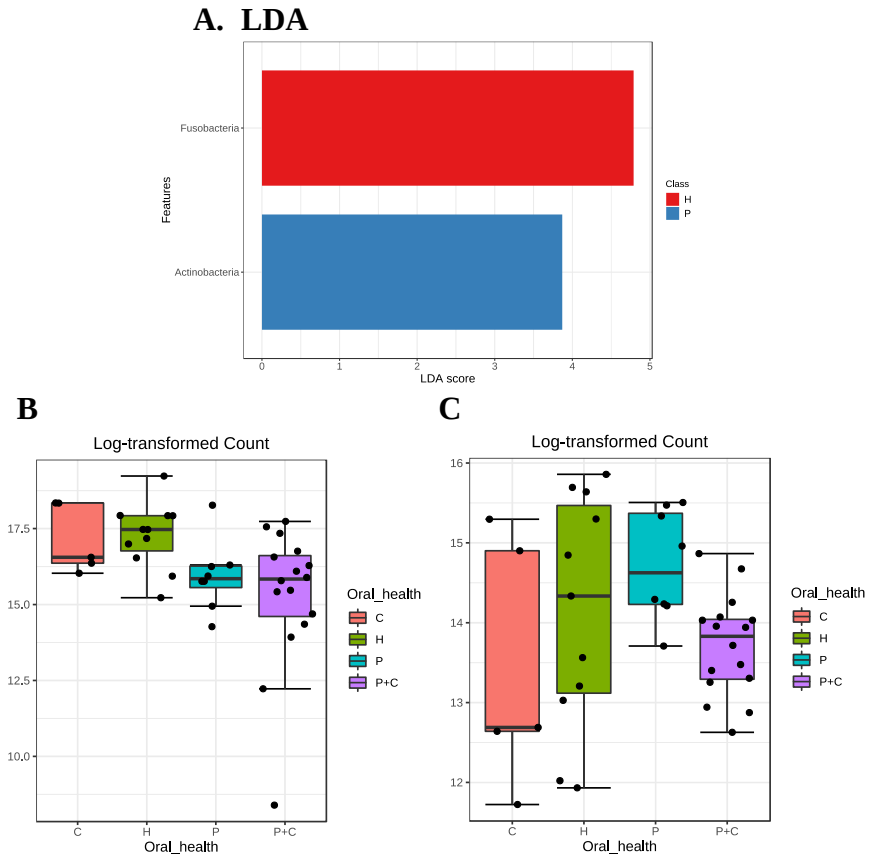


Figure 30: Significant Features Detected in CD at the Phylum Level Based on Oral Health (A). The Log Transformed Counts of Bacteria, (B) *Fusobacteria*, and (C) *Actinobacteria.*

At the genus level as shown in Figure 31, a total number of 28 features were detected at the genus level when oral health was considered. CD patients with good oral hygiene had the phylum *Bacteroides* as the most abundant. Patients with caries had the phylum *Fusobacterium* as the most abundant genera, *Streptococcus* in patients with periodontal disease and *Lactobacillus* in patients with both caries and periodontal disease.

Bacteroides (good oral hygiene), *Fusobacterium* (caries), *Streptococcus* (periodontal disease) and *Lactobacillus* (periodontal disease and caries) are the main features present with highest LDA scores and p value of less than 0.05.

At the species level, a total of 121 significant features were recognized at the special level utilizing the linear discriminant analysis (Figure 32). In patients with good oral hygiene, the significant bacteria were *Neisseria subflava, Prevotella jejuni, Porphyromonus gingivalis, Prevotella dentalis, Bacteroides fragilis, Prevotella enoeca, Tanerella forsythia,* and *Bacteroides intestinalis.* In patients with dental caries, bacteria of the genus *Fusobacterium* such as *Fusobacterium periodonticum* and *Fusobacterium ulcerans* were detected. In patients with periodontal disease, the genus *Streptococcus* is dominant such as *Streptococcus mutans*, *Streptococcus pyogenes*, *Streptococcus oralis*, and *Streptococcus viridans*. Patients with both periodontal disease and caries have the bacteria which is naturally present in the human oral microbiota such as *Streptococcus mutans* along with both *Lactobacillus fermentum* and *Lactobacillus acidophilus*.

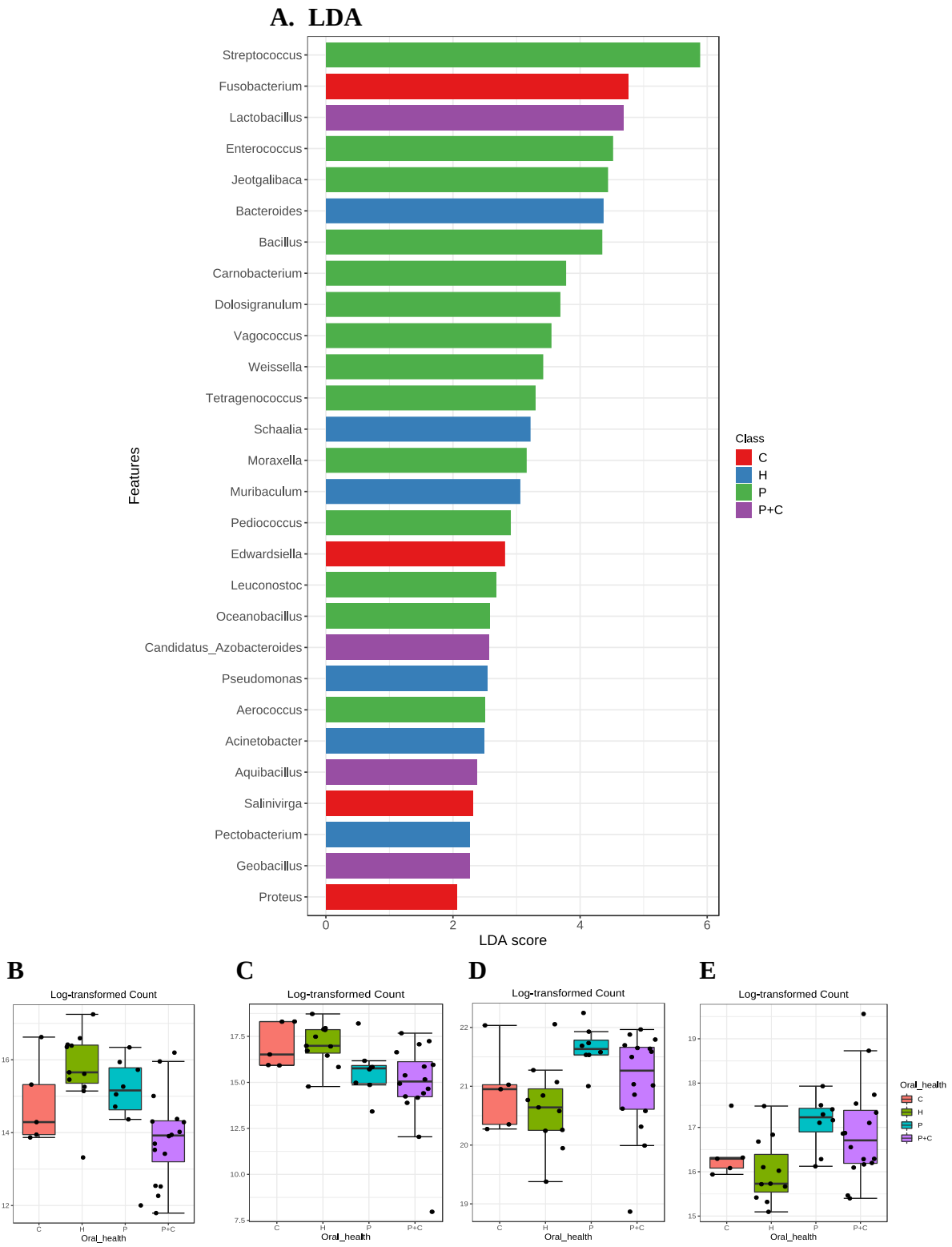


Figure 31: Significant Features Detected in CD at the Genus Level Based on Oral Health (A) Log Transformed counts of Dominant Genera (B) *Bacteroides*, (C) *Fusobacterium*, (D) *Streptococcus* and (E) *Lactobacillus*.

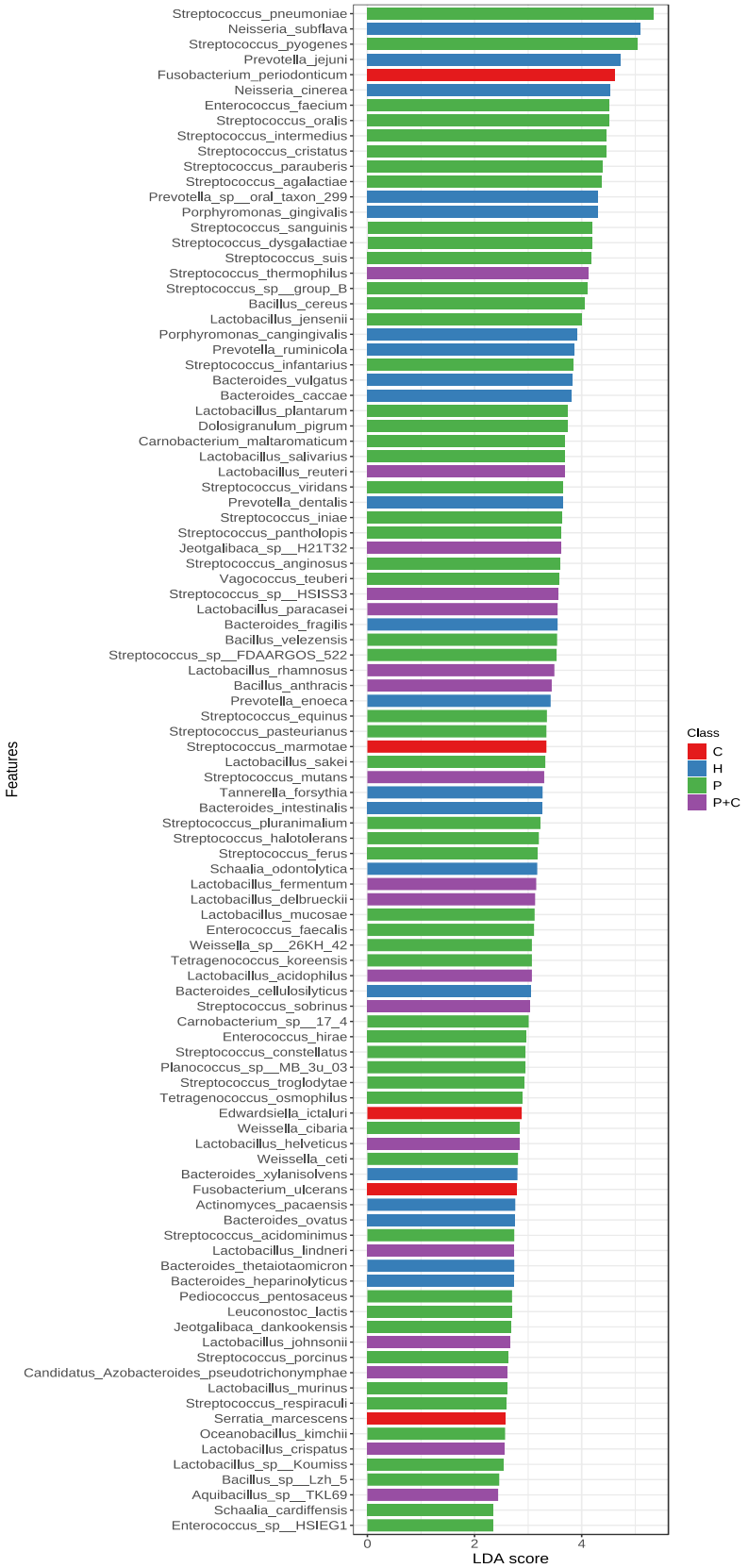


Figure 32: Significant Features Detected in CD at the Species Level Based on Oral Health.

2- IBD Medications

At the phylum level, the only significant bacteria detected was *Proteobacteria* that had an LDA score of 6.11 in patients receiving 3 medications (biologicals, steroids and immunosuppressants). No other significant bacteria were detected with other medications at the phylum level. The log transformed count of *Proteobacteria* in different groups of CD consuming different medications is shown in Figure 33.

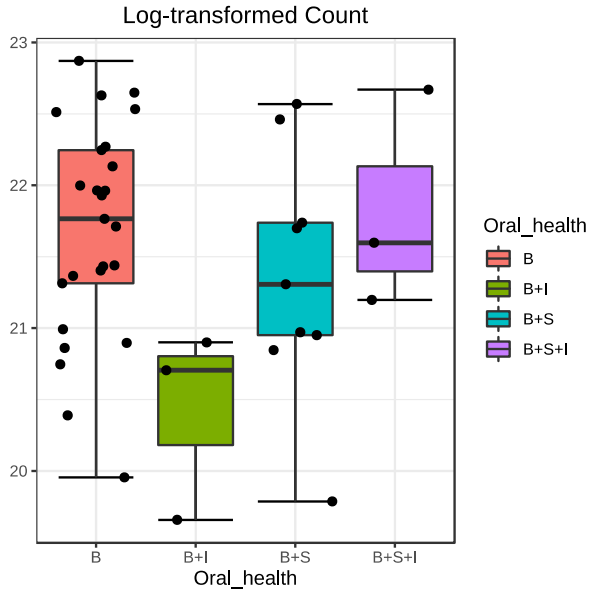


Figure 33*: Proteobacteria,* in IBD Drug Consumption was the Highest with the Use of 3 Medications (Biologicals, Steroids and Immunosuppressants).

At the phylum level, significant features were detected in patients grouped based on oral health and IBD drug use (B+S+I). Other experimental factors such as IBD activity, relapse of symptoms and duration did not show any significant bacterial features at this level.

At the genus level, a total of 21 significant bacterial features at the genus level were detected in CD receiving IBD drugs. Interestingly, a sole and significant bacteria appeared in patients receiving only biologicals as the main treatment for CD which was the genus *Simonsiella*. The other 20 bacterial profiles were miscellaneous. Patients receiving 3 types of medications (biologicals, steroids and immunosuppressants) also have notable genera such as *Haemophilus*, *Aggregatibacter*, *Actinobacillus*, *Salmonella*,

Escherichia, and *Pseudomonas. Haemophilus* had the highest abundance with an LDA score of 6 (Figure 34).

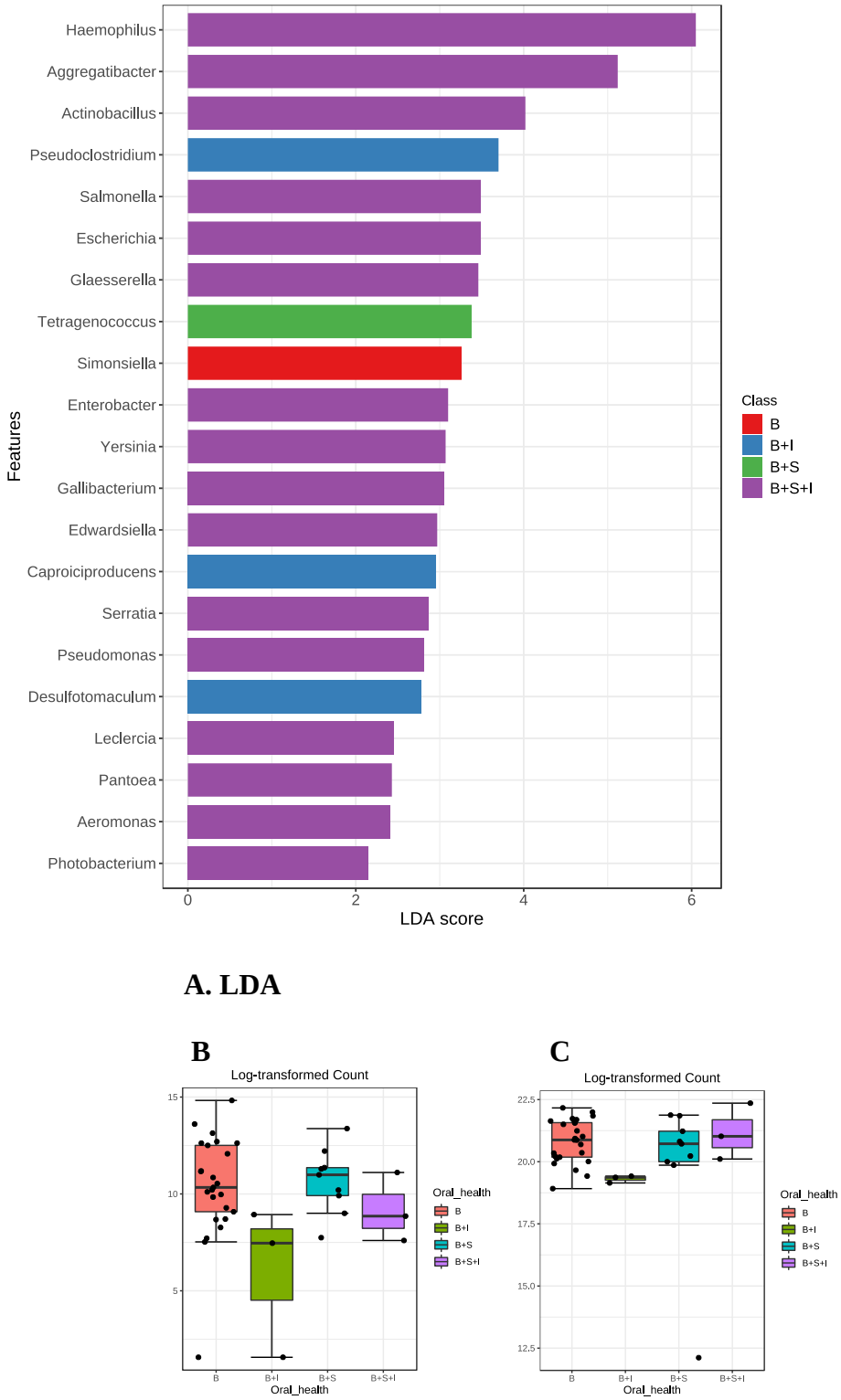


Figure 34: Significant Features Detected in CD at the Genus Level Based on IBD Drugs use (A) Log Transformed Counts of Dominant Features in IBD Drugs at the Genus Level: (B) *Simonseilla*, biologicals only (C) *Haemophilus*, Combination of the 3 Medications.

At the species level, a total of 45 significant features were detected at the species level in patients receiving IBD medications (Figure 35). The distinctive bacteria of great interest (*Simonseilla muelleri*) appeared in patients receiving only biologicals with no other medication. Other eye-catching bacteria detected were in patients receiving 3 types of medications, biologicals, steroids and immunosuppressants concurrently. These bacterial features are *Escherichia coli, Salmonella enterica, Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* Significant bacteria such as the sole emergence in biologicals users and the pathogenic bacteria in the 3 types of medications are summarized in Table 4.

Table 4: Significant Bacteria such as the Sole Emergence in Biologicals Users and Pathogenic Bacteria when the 3 Types of Medications are Used.

IBD Drugs	**species**
Biologicals Alone	*Simonsiella muelleri*
Biologicals+ Immunosuppressants	*Pseudoclostridium thermosuccinogenes* *Lactobacillus mucosae* *Desulfotomaculum reducens*
Biologicals + Steroids	*Streptococcus australis* *Lactobacillus jensenii* *Enterococcus faecalis* *Lactobacillus bombii*
Biologicals, Immunosuppressants and Steroids	*Haemophilus parainfluenzae* *Aggregatibacter aphrophilus* *Aggregabacter segnis* *Aggregatibacter actinomycetemcomitans* *Salmonella enterica* *Escherichia coli* *Klebsiella pneumoniae* *Pseudomonas aeruginosa*

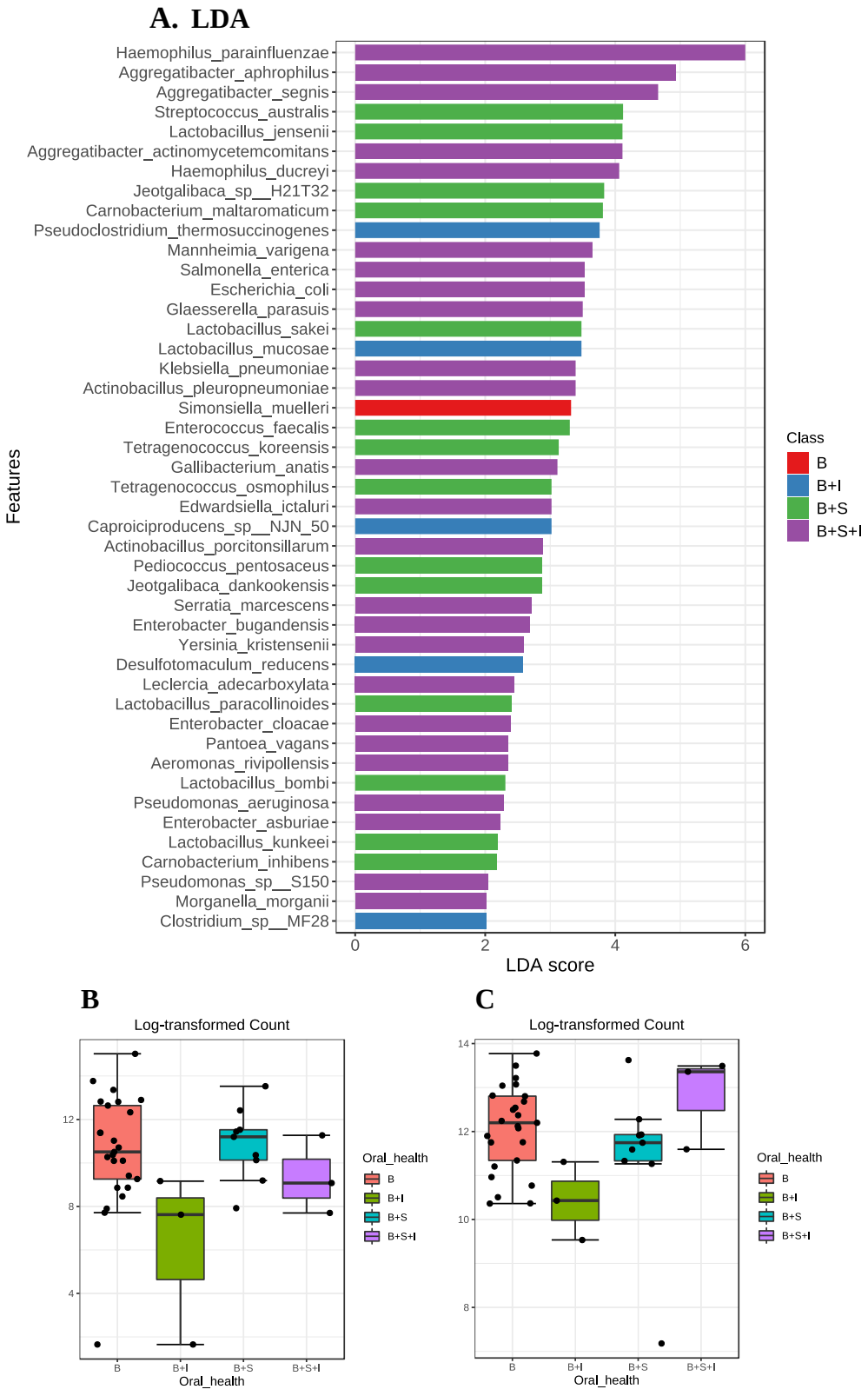


Figure 35: Linear Discriminant Analysis (LDA) Effect Size According to IBD Drugs as an Experimental Factor at the Species Level. Log Transformed Counts of Dominant Features in IBD Drugs at the Species Level, (B) *Simonseilla muelleri*, (C) *Escherichia coli.*

3- IBD Activity

At the phylum level, no significant bacterial features were detected when CD patients were compared based on disease activity. At the genus level, six dominant features were detected two of which are *Acetoanaerobium* and *Mycoplasma* in the active disease patients and four bacteria: *Schaalia, Cardiobacterium, leptotrichia,* and *Capnocytophaga* were significant in patients with inactive CD (Figure 36 A). At the species level, a total of 22 significant features were detected, 8 of which were in patients with active disease and 13 in patients with inactive CD (Figure 36 B).

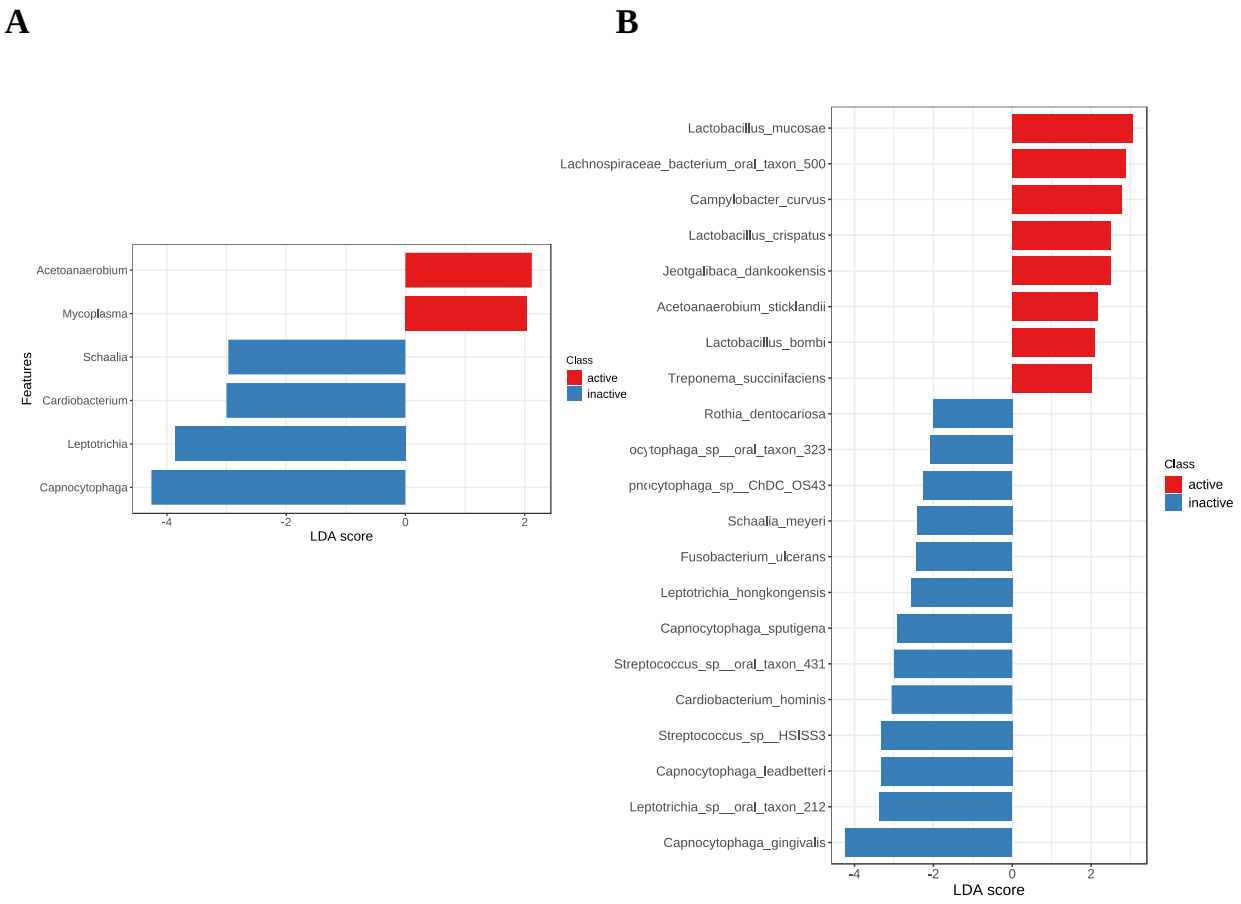


Figure 36: Detection of Significant Features at the Genus Level (A) and Species Level (B) When CD Activity was Considered.

4- Frequency of Relapse of Symptoms

At the phylum level, no significant bacterial features were detected when CD patients were compared based on frequency of relapse of symptoms. At the genus level, 15 significant features were detected (Figure37), Most common are *Eikenella*, *Simonsiella* and *Kingella* in patients who had relapses more than twice a year. *Peptoniphilus*, and *Anaerococcus* were found in patients who had relapses up to once per year (Figure 37).

At the species level (Figure 38), 11 bacterial species were found in patients having a relapse of more the twice a year, the most common species are *Prevotella oris*, *Prevotella jejuni* and *Simonsiella muelleri*. In patients with relapse of symptoms up to once per year, 21 significant bacterial species were found, some of them might be of interest such as *Treponema denticola* and *Lactobacillus backii.*

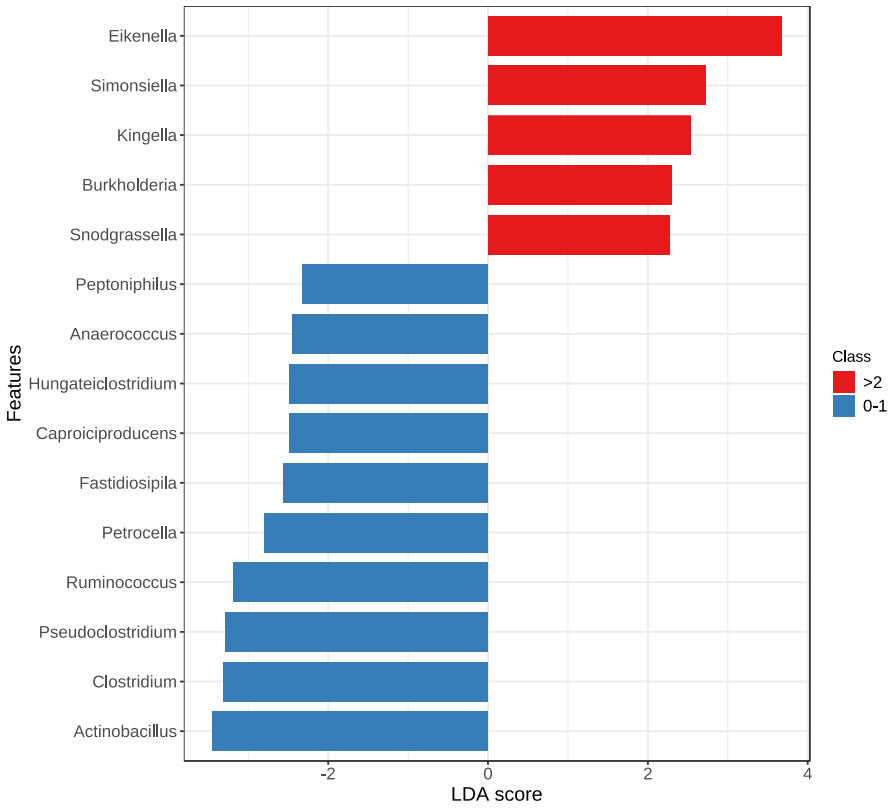


Figure 37: Summary of Significant Bacteria Detected at the Genus Level when CD Patients were Compared for the Frequency of Relapse of Symptoms.

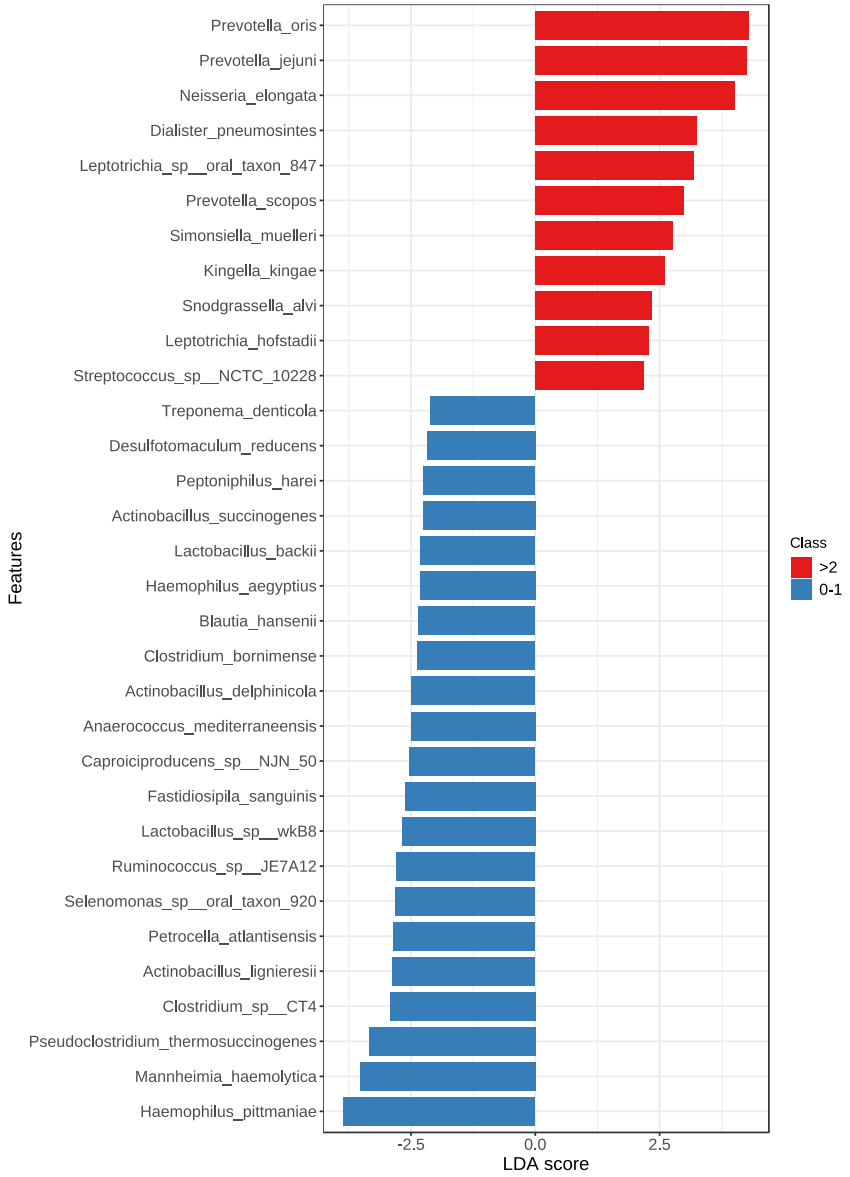


Figure 38: Summary of Significant Bacteria Detected at the Species Level when CD Patients were compared for the Frequency of Relapse of Symptoms.

5- Duration of Disease

At the phylum level, no significant bacterial features were detected when CD patients were compared based on the duration of the disease.

At the genus level, a total of 8 significant features were detected, using the linear discriminant analysis. *Porphyromonas* is the dominant genus in newly diagnosed patients, while *Pasteurella* is the genus dominant in patients having the disease for more than a decade (Figure 39).

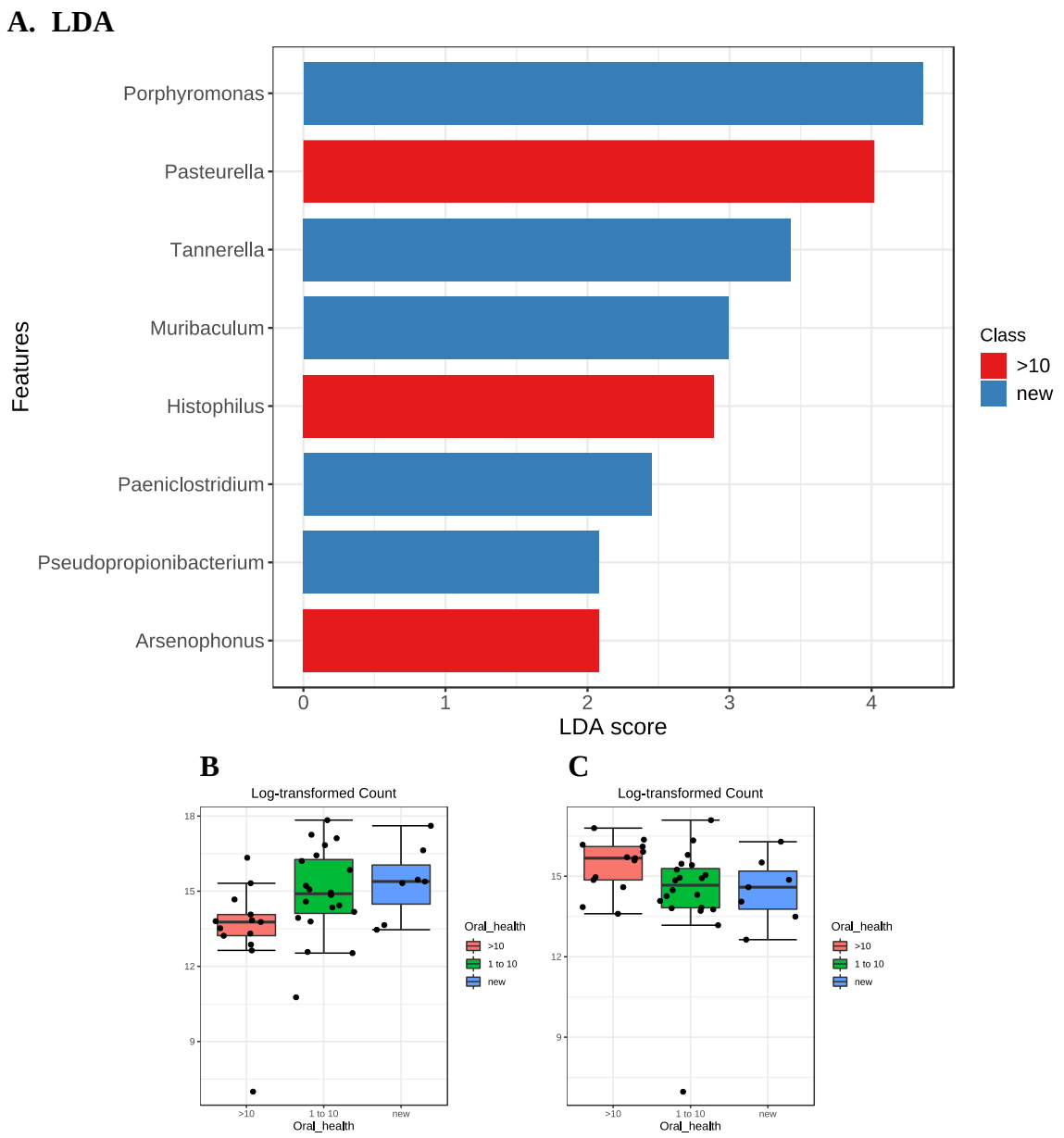


Figure 39: A Summary of Significant Bacteria Detected at the Genus level (A)when CD Patients were Compared According to the Disease Duration. Log Transformed Counts of Dominant Features when the Duration of the Disease is Used as an Experimental Factor at the Genus Level, (B) *Porphyromonus* (C) *Pasteurella*.

At the species level (Figure 40), thirteen bacteria were in patients that are newly diagnosed with CD, bacteria were *Prophyromonas gingivalis*, *Streptococcus urninalis* and *Streptococcus viridans*. Only 4 species are detected in patients having the disease lasting from 1-10 years; one of them is *Lactobacillus crispatus.* The 9 species detected

were in patients having CD for more than 10 years, *Campylobacter helveticus* and *Klebsiella pneumoniae* are 2 of them.

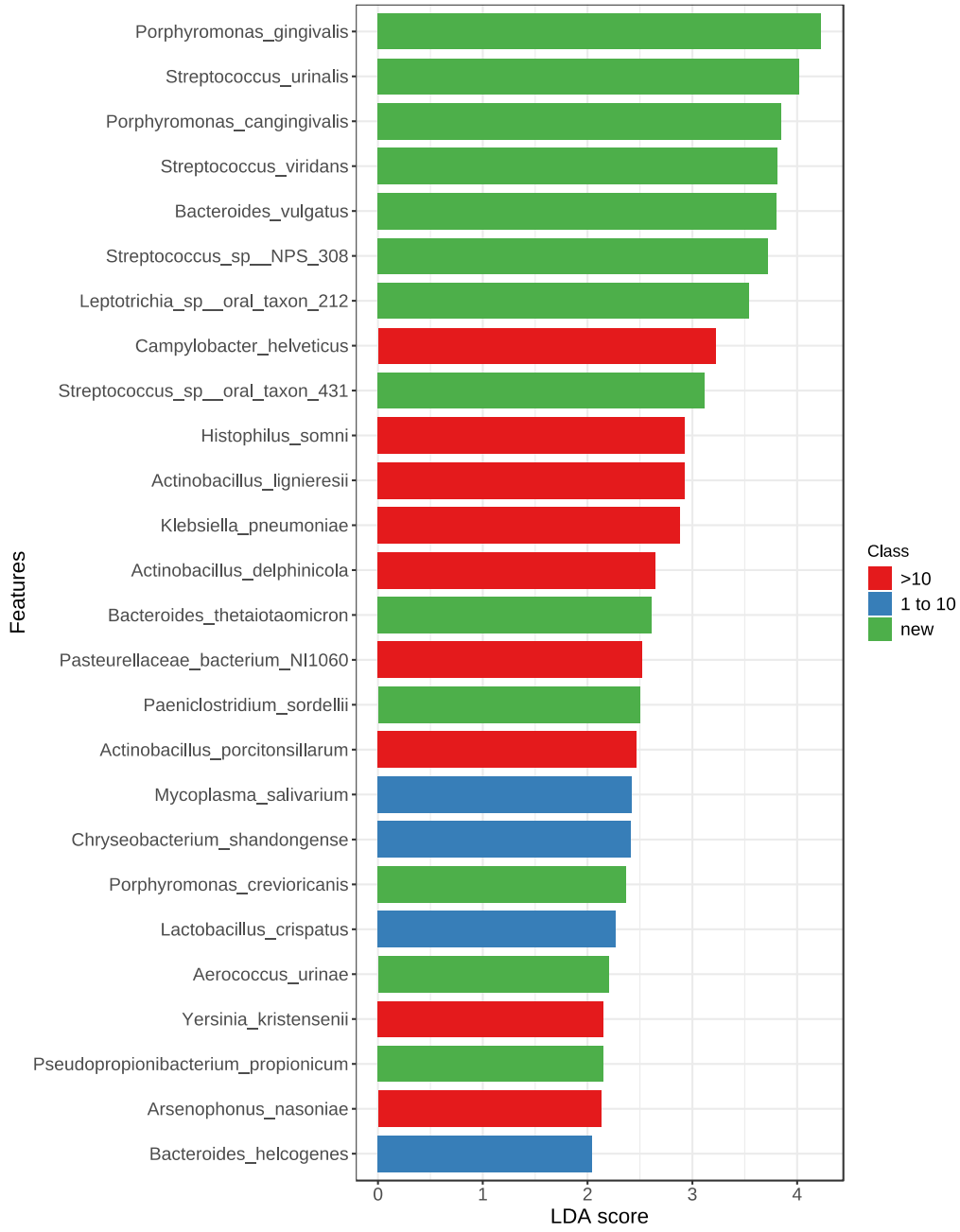


Figure 40: A Total of 26 Significant Features were Detected at the Species Level Using the Linear Discriminant Analysis when CD Patients were Compared According to the Disease Duration.

3.6 Microbiome Diversity

3.6.1 Alpha diversity in CD vs HC

When different indices of alpha diversity were compared between CD and HC, significant difference were detected using observed species index, Chao 1 index and ACE index (p values 0.049, 0.022 and 0.048, respectively), while Simpson's and Shannon indices were not significantly different (p values 0.268 and 0.129, respectively). The results show significant reduction in alpha diversity in CD compared to HC (Figure 41).

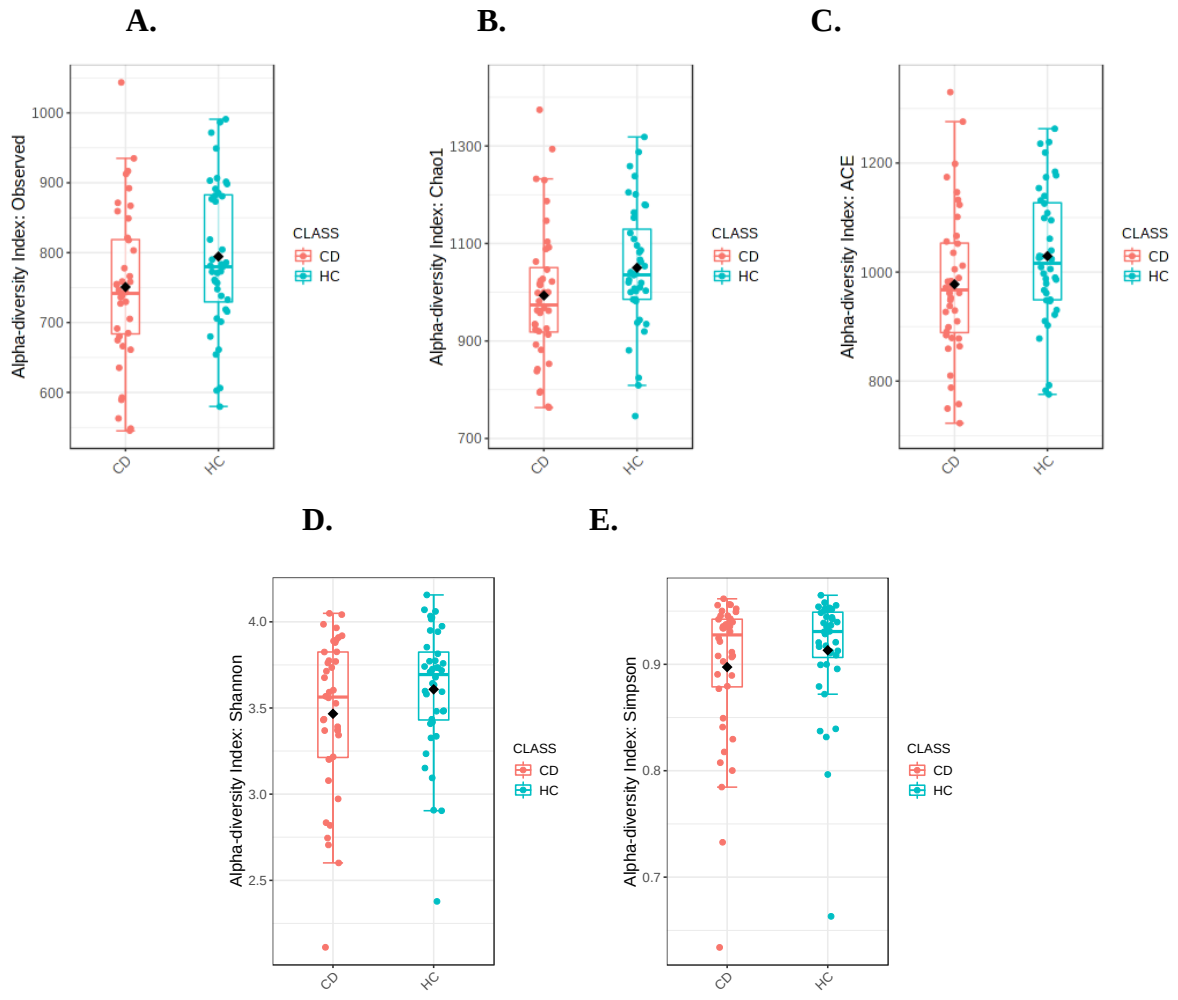


Figure 41: Alpha diversity Indices used are (A) Observed, (B) Chao 1, (C) ACE (D) Shannon and (E) Simpson.

3.6.2 Beta Diversity in CD vs HC

Beta diversity of CD and HC was compared and plotted by the principal coordinates analysis (PCoA) using the statistical analysis PERMANOVA, using the Bray-Curtis as the diversity measure. It demonstrates the dissimilarity across CD and HC with a p-value of 0.067 which is statistically non-significant (p>0.05). The samples overlap with a wider range of the diseased samples and just a few scatter outside the overlapped area (Figure 42).

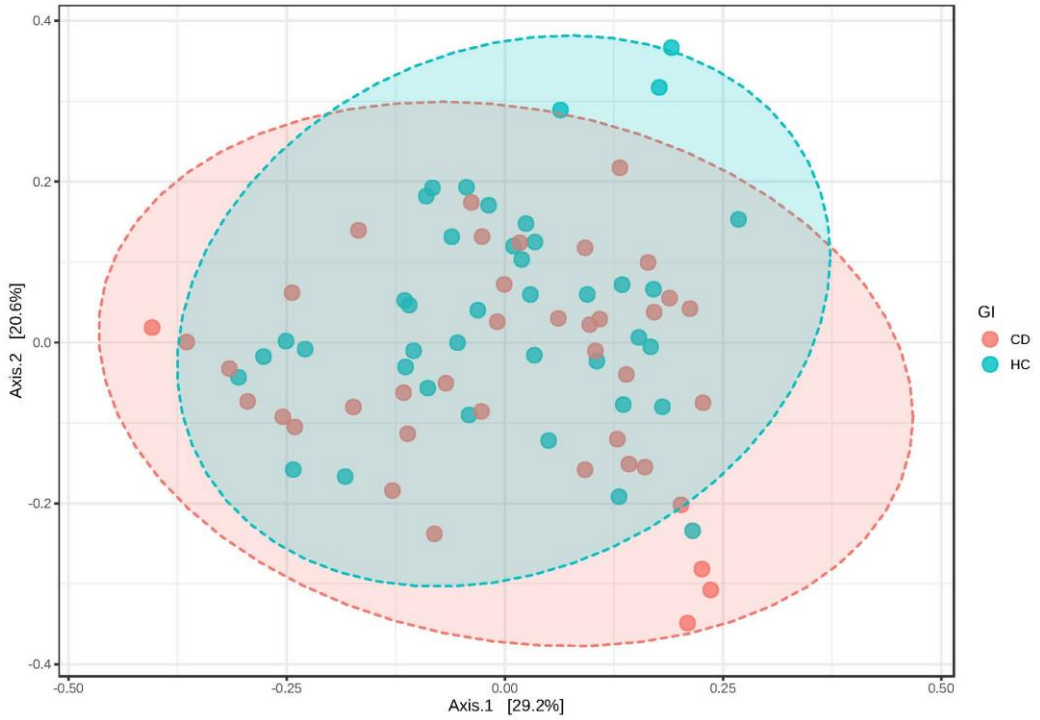


Figure 42: Principal Coordinates Analysis for Beta Diversity in CD vs HC.

Beta diversity indices were also plotted as dendrograms (Figure 43) to show the similarity of HC and CD samples. It is visible from the color and dendrogram clustering patterns that some samples are related to each other, and they belong to the same group (either HC or CD), although some samples from both groups may be related.

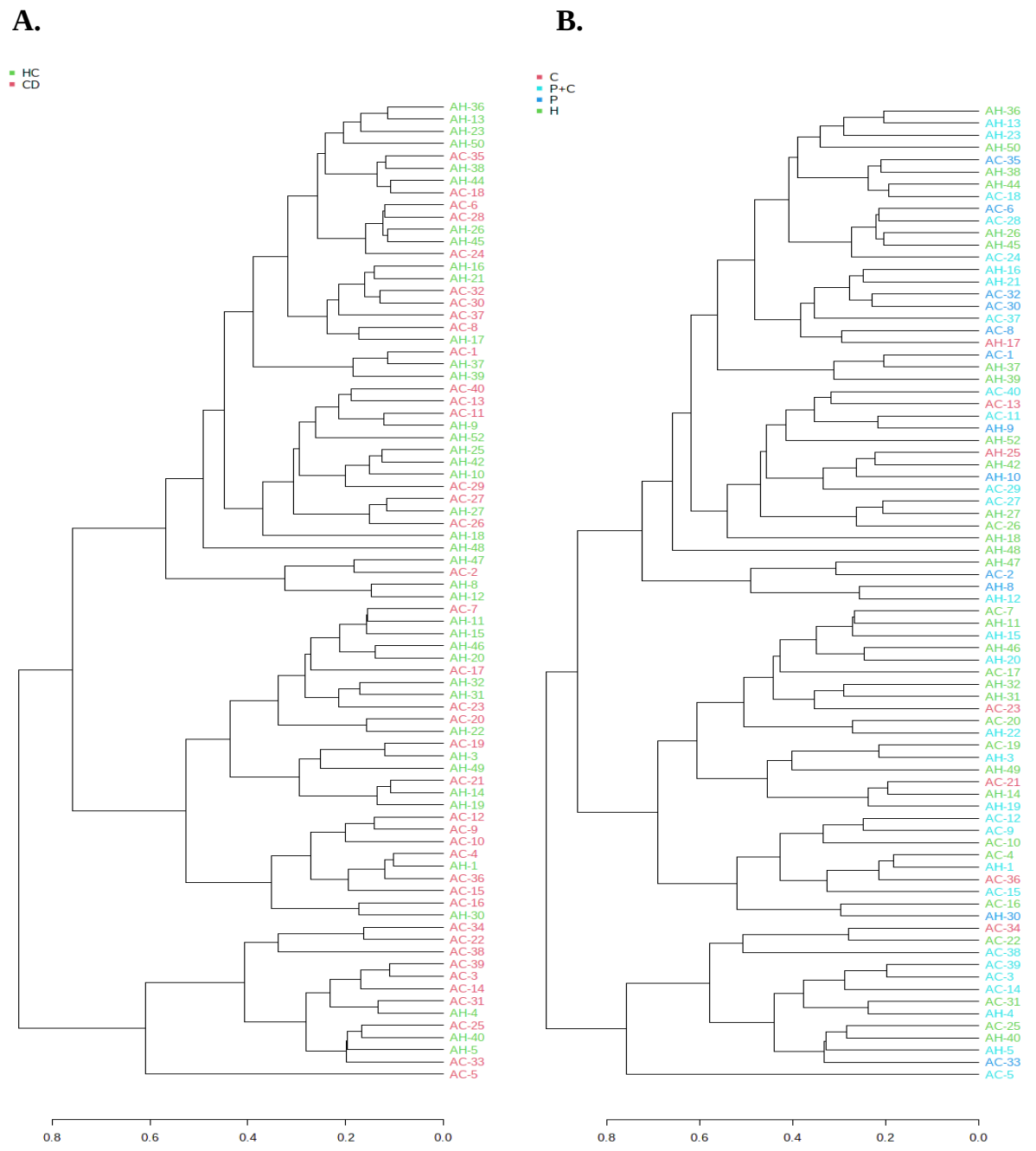


Figure 43: Dendrogram Analysis Based on Beta Diversity Mercies (Jaccard and Bray Curtis), (A) Showing the Samples Belonging to CD (AC) and HC (AH) at the Genus Level (B) Based on Oral Health.

3.7 Identification of Shared Significantly Different Species Between CD and HC Grouped Based on Their Oral Health Status Using Venn Diagrams

Venn diagrams were generated to illustrate the shared species among the microbial communities of CD and HC when they were grouped based on their oral health status (Figure 44). The goal was to identify if the effect of oral health on the oral microbiome was the same in CD vs HC.

As shown in Figure 44, significantly altered species were unique in both CD (n=100) and HC (n=103). Only 4 species (*Fusobacterium varium, Olsenella sp GAM18, Enterococcus durans* and *Aeromonas hydrophila)* were found common (shared) in both CD and HC. This means that these 4 microbial species were considered to have affected the two groups when comparing CD to HC.

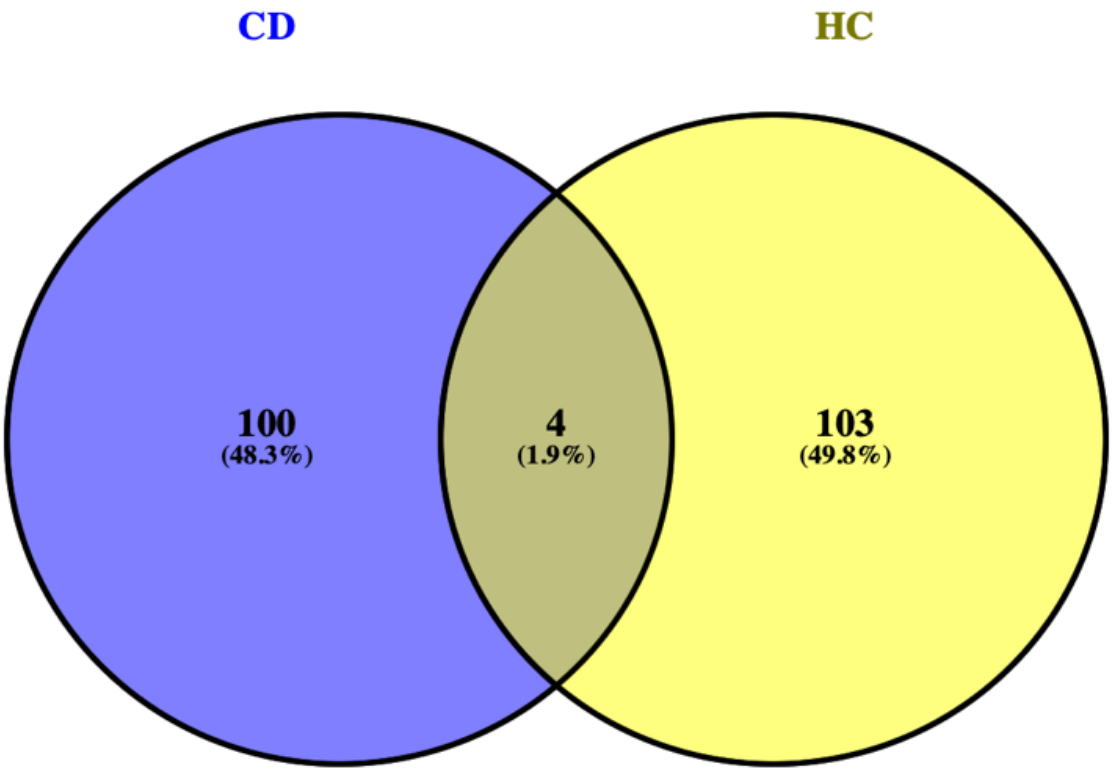


Figure 44: Venn diagram of Exclusive and Shared Taxonomically Unique Microbiota at the Species Level Between CD and HC. The Blue Represents CD and the Yellow is for HC, the Overlapped Part Shows the Species Shared Among Both Groups.

3.8 Interaction Between Different Factors Contributing to Dysbiosis in CD Using Venn Diagrams

Separate Venn diagrams (Figures 45 and 46) were generated to illustrate the shared species in CD patients grouped according to different factors that may affect the microbiome, including oral health, IBD drug use, activity of the disease, relapse of symptoms and duration of the disease.

As shown in Figure 45, significantly altered species were unique as different species were detected exclusively in each group (76 species for oral health ;12 species for IBD drug use and 10 species for the activity of the disease). There were 4 (3.8%) common species including: *Enterococcus faecalis, Lactobacillus ensenii, Lactobacillus paracollinoides,* and *Vibrio parahaemolyticus,* which represent the shared altered species by the three factors. So, these 3 microbial species were present in samples of all patients considering these 3 experimental factors. Furthermore, 3 (2.9%) common species (*Fusobacterium ulcerans, Rothia dentocariosa,* and *Lactobacillus crispatus*) were altered by the effect of both factors (oral health and CD activity). When only these two factors were combined 3 bacterial species were found to affect these patients.

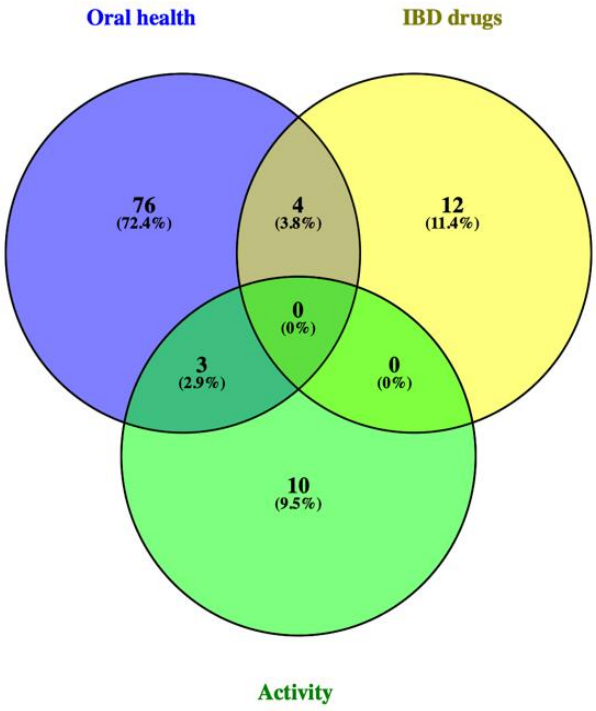


Figure 45: Venn diagram of Exclusive and Shared Taxonomically Unique Microbiota at the Species Level Based on Oral Health, IBD Drug Use and Activity of the Disease. Overlapped Parts Show the Species Shared Among Groups.

We also tested the possible interaction between factors related to the disease including duration, IBD drugs, frequency of relapse, and activity to detect any shared species which might be altered by the combined effect of these factors. As shown in Figure 46, significantly altered species were unique as different species were detected exclusively in each group (12 species for duration; 14 species for IBD drug; 12 species for relapse of symptoms and 11 species for activity of the disease). Only one species (*Caproiciproducens sp NJN 50*) was common for both relapse and IBD drugs, another species (*Yersinia kristensenii*) was common for both duration and IBD drugs, and 2 species were common for both duration and activity, including *Streptococcus sp oral taxon 431 and Leptotrichia sp oral taxon*_212 which were the overlapping species between the factors. No species were shared among the four factors; thus, no species were altered by the combined effect of these factors. This confirms that each experimental factor is exclusive and unique to its contribution to dysbiosis and the pathogenesis of CD.

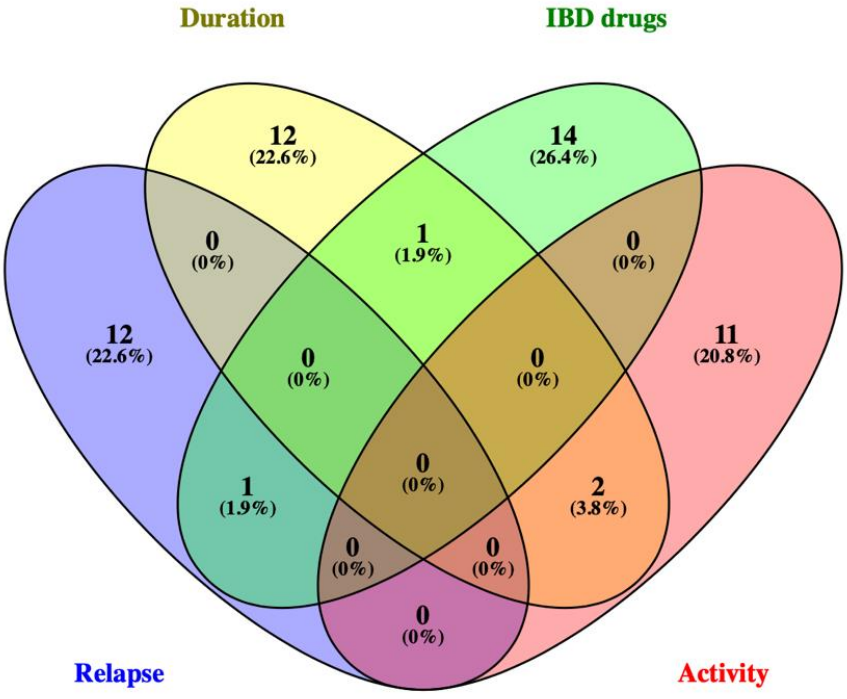


Figure 46: Venn Diagram of Exclusive and Shared Taxonomically Unique Microbiota at the Species Level Based on 4 Experimental Factors, Duration, IBD Drugs, Relapse, and Activity. Overlaps are also shown that demonstrated the shared microbial species within the groups.

3.9 Alpha Diversity in CD with Different Factors that Might Contribute to Dysbiosis in CD

1- Oral Health

When different indices of alpha diversity were compared in CD subjects based on oral health, significant difference were detected using observed species index, Chao 1 index and ACE index (p value: 0.048, 0.025 and 0.025 respectively), while Simpson's and Shannon indices were not significantly different (P values: 0.57 and 0.49, respectively), as shown in Figure 47.

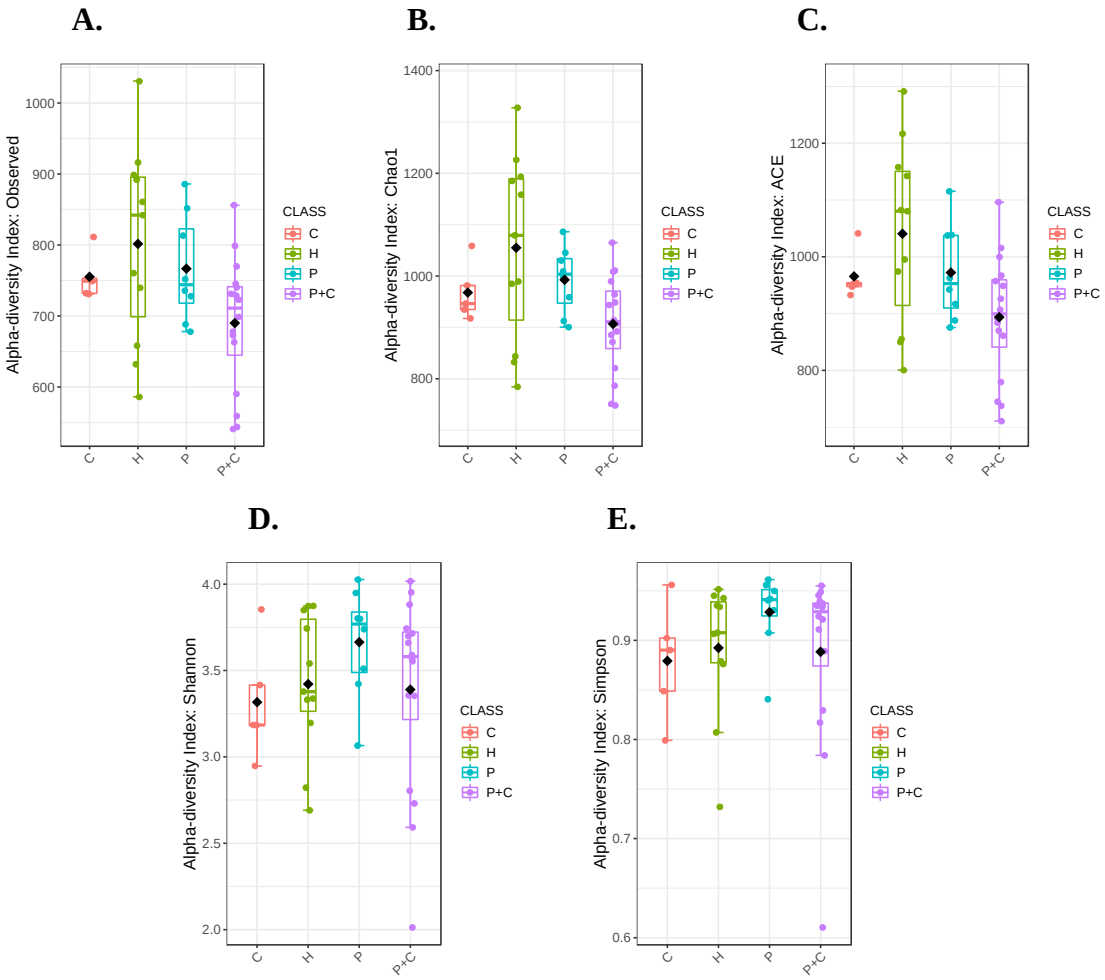


Figure 47: Alpha Diversity Indices for Oral Health: (A) Observed, (B) Chao 1 and (C) ACE (D) Shannon and (E) Simpson.

2- IBD Medications

When different indices of alpha diversity were compared in CD subjects based on IBD drug use. The p values were not significant, Chao1, Simpson, Shannon, observed and ACE indices (p value: 0.91829, 0.81776, 0.53979, 0.6 and 0.638 respectively), as shown in Figure 48.

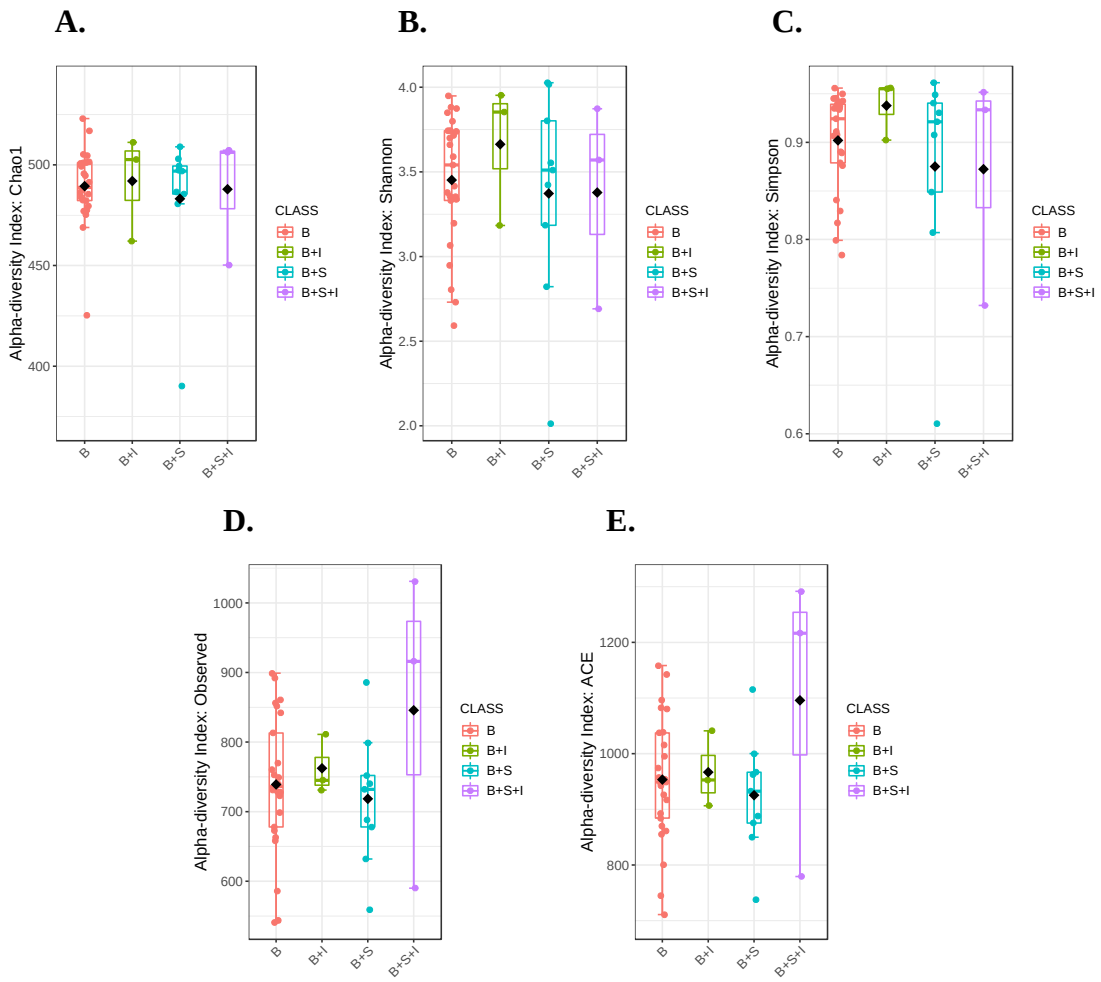


Figure 48: Alpha Diversity Indicies for IBD Medications: (A) Chao1 (B) Shannon and (C) Simpson (D) Observed and (E) ACE.

3- Activity of Disease

When different indices of alpha diversity were compared in CD subjects based on the activity of the disease. The p values were not significant, Chao1, Shannon, Simpson,

observed and ACE indices (p value: 0. 823, 0.44036, 0.265, 0.97 and 0.849 respectively), as shown in Figure 49.

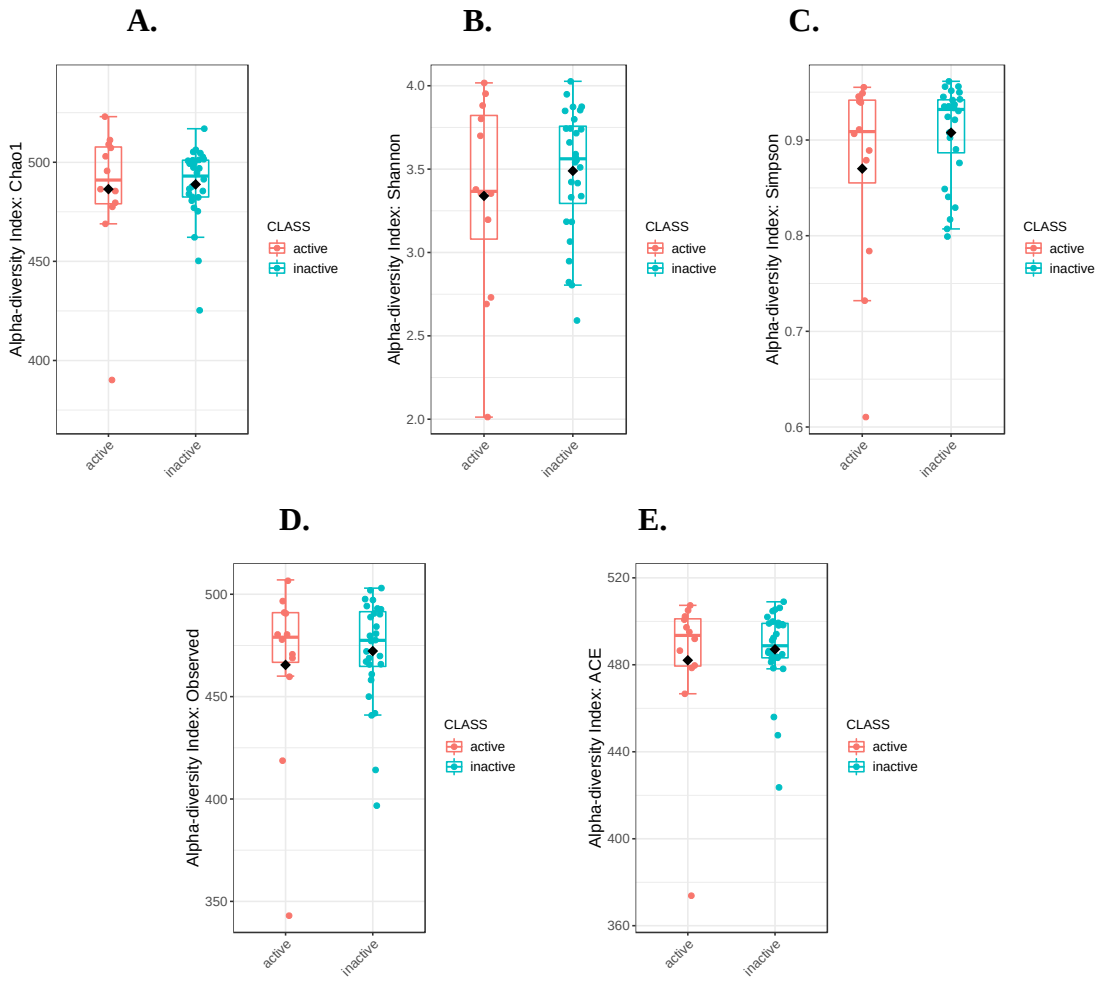


Figure 49: Alpha Diversity Indicies for Activity of Disease (A) Chao1 (B) Shannon (C) Simpson Observed and (E) ACE.

4- Frequency of Relapse of Symptoms

When different indices of alpha diversity were compared with CD subjects based on the relapse of symptoms. The p values were not significant, Chao1, Shannon, Simpson, observed and ACE indices (p value: 0.534, 0.898, 0.9926, 0.915 and 0.88 respectively), as shown in Figure 50.

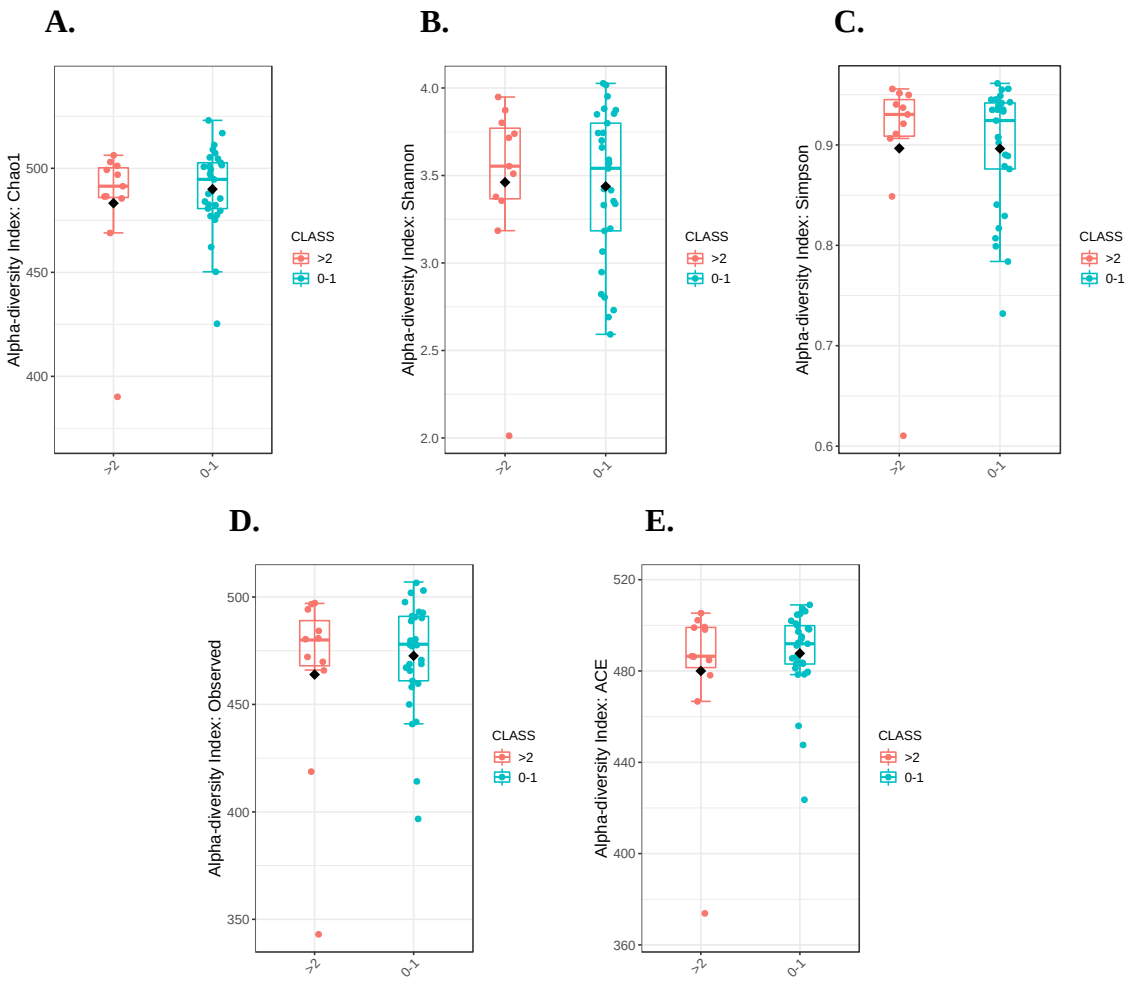


Figure 50: Alpha Diversity Indicies for Frequency of Relapse of Symptoms (A) Chao1 (B) Shannon (C) Simpson (D) Observed and (E) ACE.

5- Duration of Disease

When different indices of alpha diversity were compared CD subjects based on the duration of the disease. The p values were not significant, Chao1, Shannon, Simpson, observed and ACE indices. (p value: 0.763, 0.754,0.428, 0.53and 0.659 respectively), as shown in Figure 51.

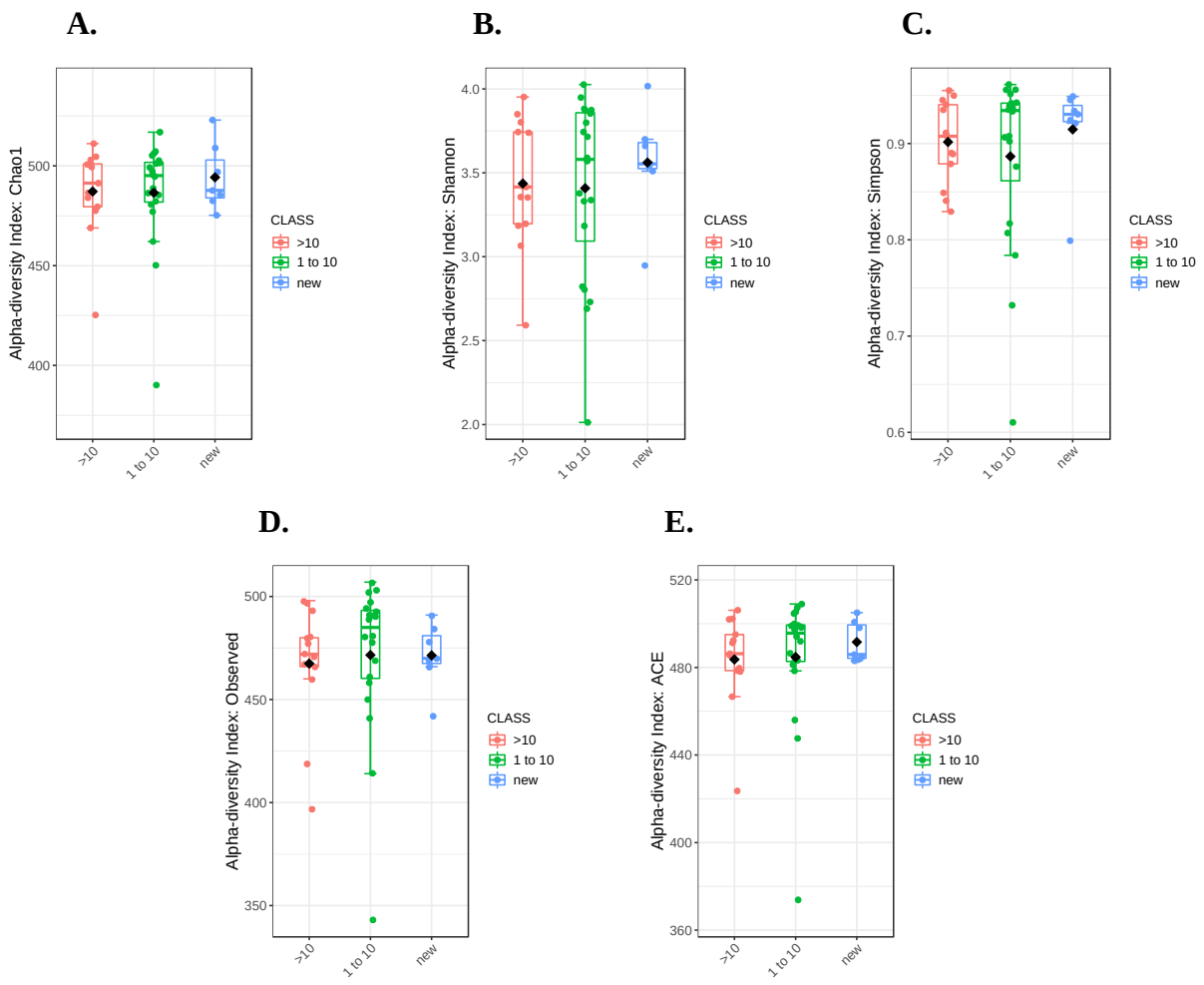


Figure 51: Alpha diversity Indicies for Duration of Disease (A) Chao1 (B) Shannon (C) Simpson (D) Observed and (E) ACE.

3.10 Beta Diversity in CD Patients with Different Factors that Might Contribute to Dysbiosis in CD.

Bray Curtis and Jaccard were used as the distance methods. Clustering was demonstrated using principles coordinates analysis (PcoA) as a data comparison technique to visualize sample similarity based on different factors such as:

1- Oral Health (Figure 52A)

P value (0.276) indicates non-significant difference between the groups. PCoA shows clustering of samples mostly within periodontal disease, then periodontal disease and caries, with a very wide circle of caries, indicating high variability.

2- IBD Drugs (Figure 52B)

P value (0.379) indicates non-significant difference between the groups. All samples are grouped in the biologicals circle since most of patients were consuming this type of medication, but 2 samples were out of the red circle and into the blue circle of biologicals and steroids.

3- Activity of the Disease (Figure 52C)

P value (0. 886) indicates non-significant difference between the groups. All samples except one overlapped, the outcast one is a patient with active disease.

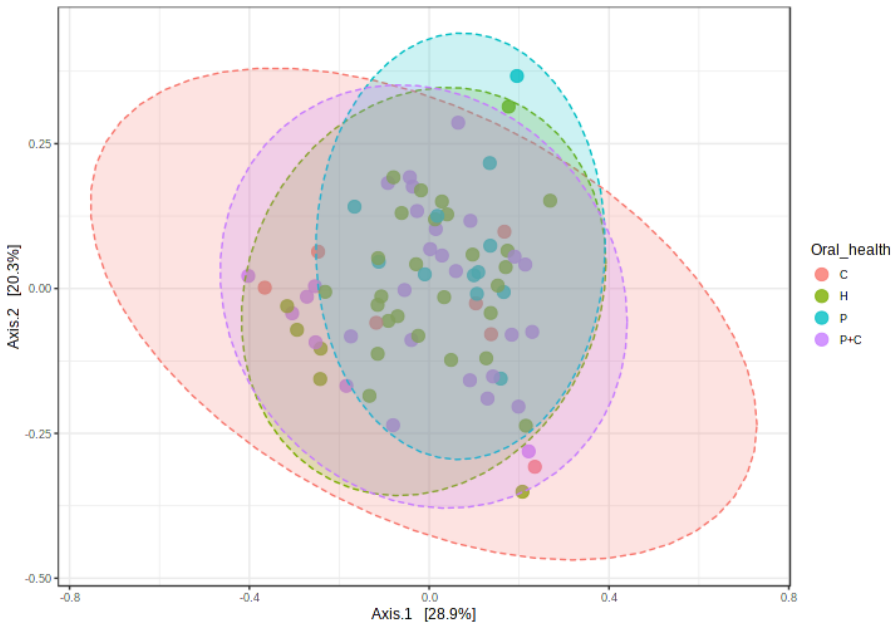
4- Frequency of Relapse of Symptoms (Figure 52D)

P value (0.4) indicates non-significant difference between the groups. Samples are gathered with some samples of patients within 0-1 relapses per year outside and only one away for a patient with a relapse of more than 2 times per year.

5- Duration of the Disease (Figure 52E)

P value (0.753) indicates non-significant difference between the groups. Only two samples cluster apart which are patients with duration of the disease lasting 1-10 years, while the rest overlapped with disease duration of more than 10 years.

A.



B.

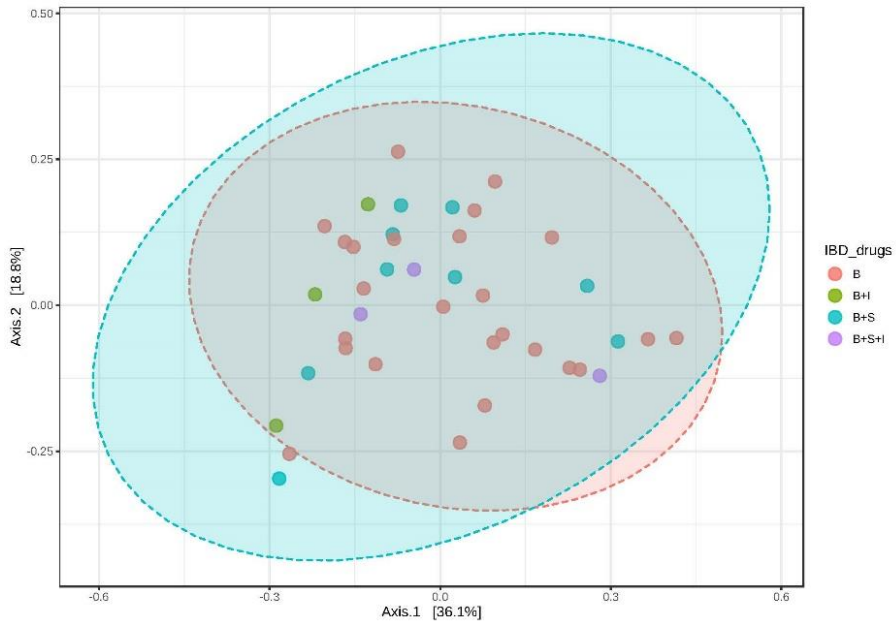
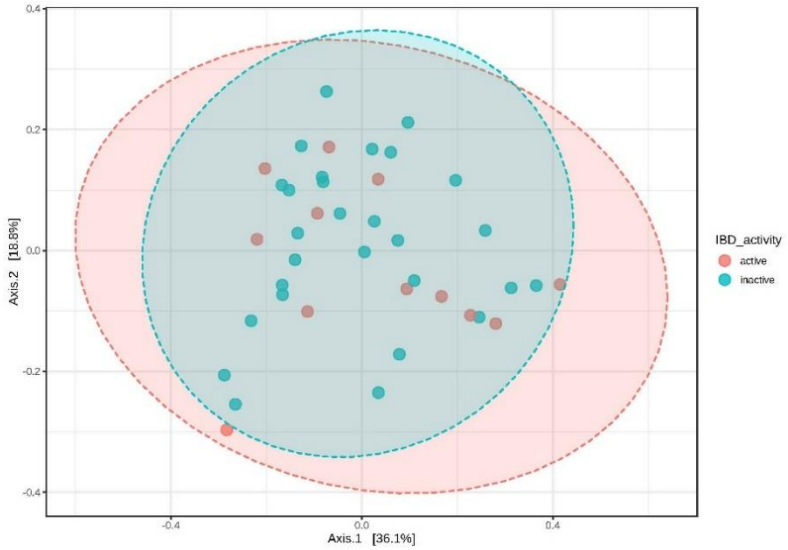


Figure 52: Beta Diversity using PERMANOVA as the Statistical Method. (A) Oral (B) IBD Drugs.

C.



D .

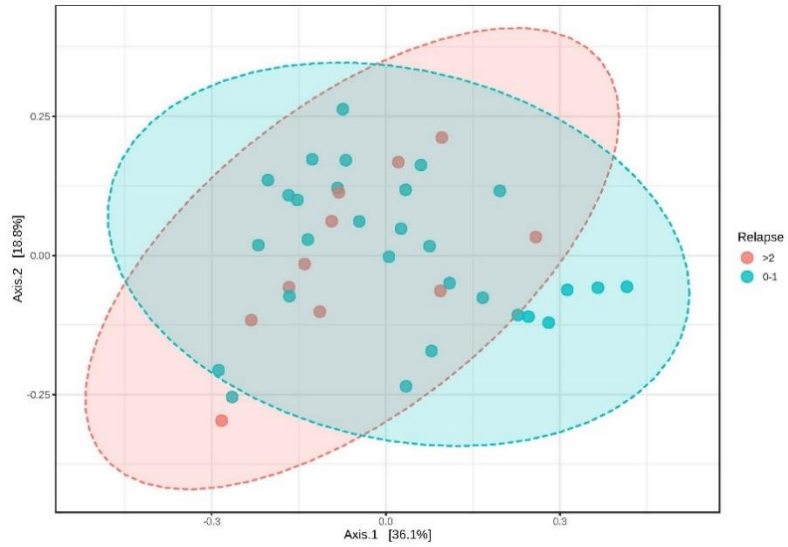


Figure 52: Beta Diversity using PERMANOVA as the Statistical Method (C) Activity of the Disease, (D) Frequency of Relapse of Symptoms (continued).

E.

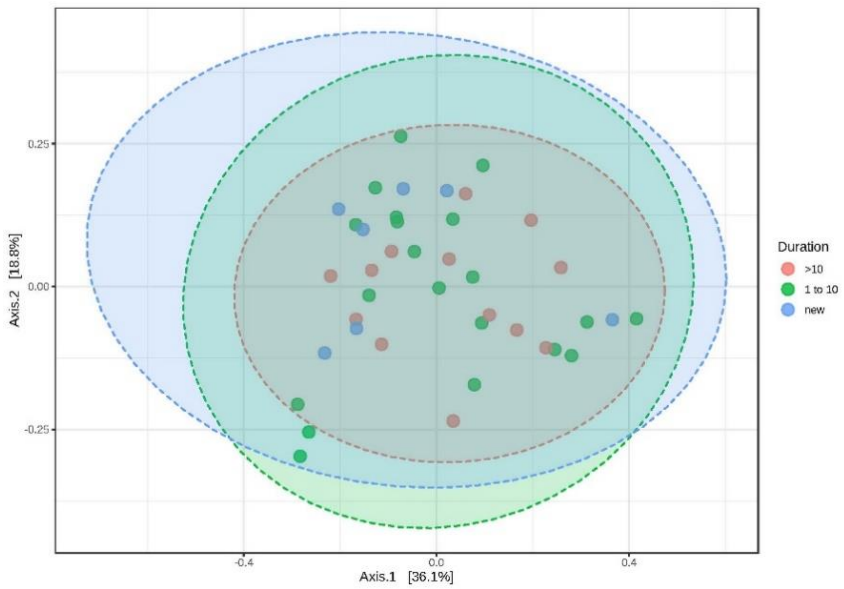


Figure 52: Beta Diversity using PERMANOVA as the Statistical Method. (E) Duration of the Disease (continued).

3.11 Salivary Biomarkers of Inflammation

Inflammatory biomarkers including C-reactive protein (CRP) and calprotectin (CAL) were measured in saliva of all the 80 subjects. CRP saliva had a mean ± SD =1.12 ± 1.714 ng/ml and CAL saliva had a mean ± SD =3.48 ±3.22 ng/ml.

Furthermore, levels of CRP is serum, and CAL in stool for CD pateitns were obtained from the patients' records. CRP serum had a mean ± SD = 12.117 ± 20.15 mg/l; and CAL stool had a mean ± SD =384.55 ± 522.573 μg/mg.

No signfication correaltion was detected between serum CRP with salivary CRP, and fecal CAL with salivary CAL. Same for salivary CAL and CRP, as they were not signficantly correalted (p-values > 0.05).

3.11.1 Salivary biomarkers in Crohn's patients compared to healthy controls.

Salivary CRP and CAL were compared in CD patients and HC, using non-parametric Mann-Whitney U test. The diffrence was not statistically significant (p-values > 0.05), but CD patients had higher CAL values. In CD, CRP saliva had a mean ± SD =0.8650 ± 0.7826 ng/ml and CAL saliva had a mean ± SD =3.9445 ± 3.856 ng/ml. In HC, CRP saliva had a mean ± SD =0.1.375 ± 0.2.282 ng/ml and CAL saliva had a mean ± SD = 2.6186 ±2.266 ng/ml, as shown in Figure 53.

A B

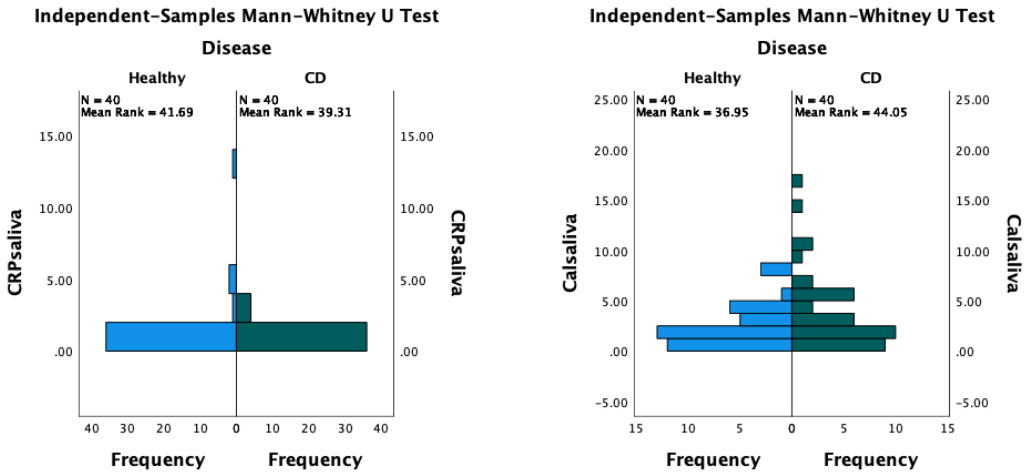


Figure 53: Comparison of Salivary CRP (A) and CAL (B) in CD vs HC.

3.11.2 Comparison of CRP and CAL in CD patients based on different factors.

Salivary CRP and CAL were compared in CD patients based on different experimental factors. Non-parametric data analysis was performed using Mann-Whitney U and Kruskal-Wallis tests. Except for the oral health, there was non-significant diffrence among CD patietns (p-values > 0.05) for mutiple variables such as IBD drugs, disease activity, disease duration, and relapse of symptoms of the disease.

1- Oral Health

Salivary CRP showed non-significant diffence (p>0.05) among patietns with different oral health conditions. In good oral hygiene patients (H), CRP saliva had a mean ± SD =0.956 ± 0.927 ng/ml and CAL saliva had a mean ± SD =5.1224 ± 4.986 ng/ml. In C, CRP saliva had a mean ± SD =0.454 ± 0.0279 ng/ml and CAL saliva had a mean ± SD =6.39 ± 4.478 ng/ml. In P, CRP saliva had a mean ± SD =1.243 ± 0.984 ng/ml and CAL saliva had a mean ± SD =2.336 ± 2.896 ng/ml. In P+C, CRP saliva had a mean ± SD =0.741 ± 0.64 ng/ml and CAL saliva had a mean ± SD =3.173 ±2.792 ng/ml. For CAL, there was a significant diffence between patietns with periodontal disease (P) and caries (C) with P value = 0.009, but the diffence was non-significant for the other groups, as shown in Figure 54.

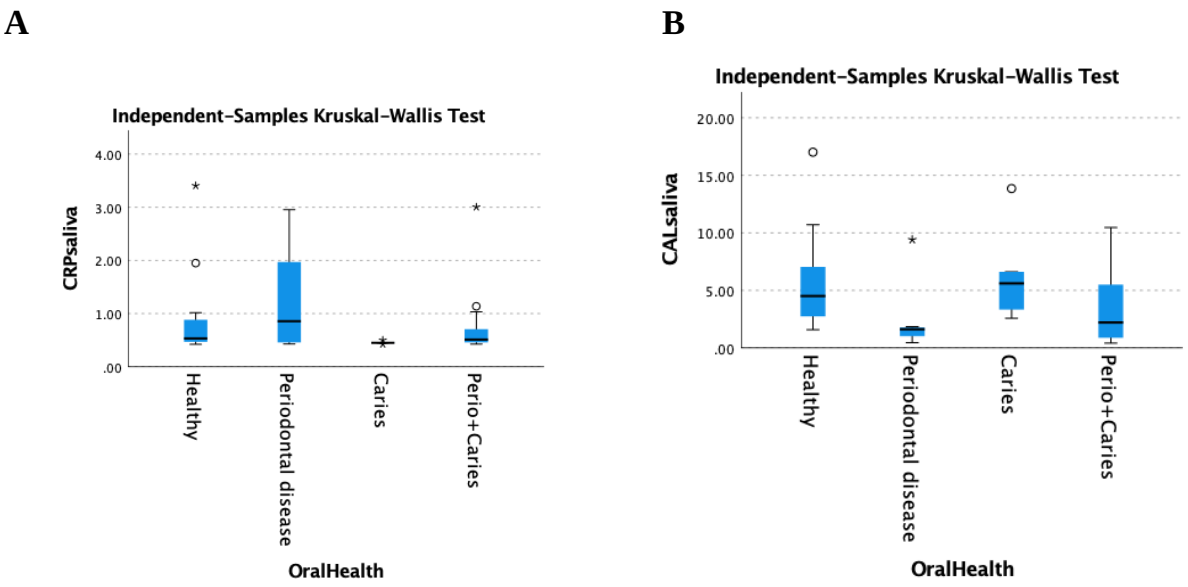


Figure 54: Comparison of Salivary CRP (A) and CAL (B) when Oral Health was Used as an Experimental Factor.

2- IBD

As shown in Figure 55, there was non-significant difference (p>0.05) in salivary CRP and CAL results based on the variable IBD drug use including biologicals (B), biologicals and steroids (B+S) and biologicals, steroids and immunosuppressants (B+S+I). In B, CRP saliva had a mean ± SD =0.936 ± 0.851 ng/ml and CAL saliva had a mean ± SD =3.638 ±3.967 ng/ml, In B+S, CRP saliva had a mean ± SD =0.8406± 0.836

ng/ml and CAL saliva had a mean ± SD =4.1924 ±4.079 ng/ml. In B+I, CRP saliva had a mean ± SD =0.687± 0.391ng/ml and CAL saliva had a mean ± SD =4.905 ±2.09 ng/ml. In B+S+I, CRP saliva had a mean ± SD =0.5174 ± 0.03 ng/ml and CAL saliva had a mean ± SD =4.791 ±5.122 ng/ml.

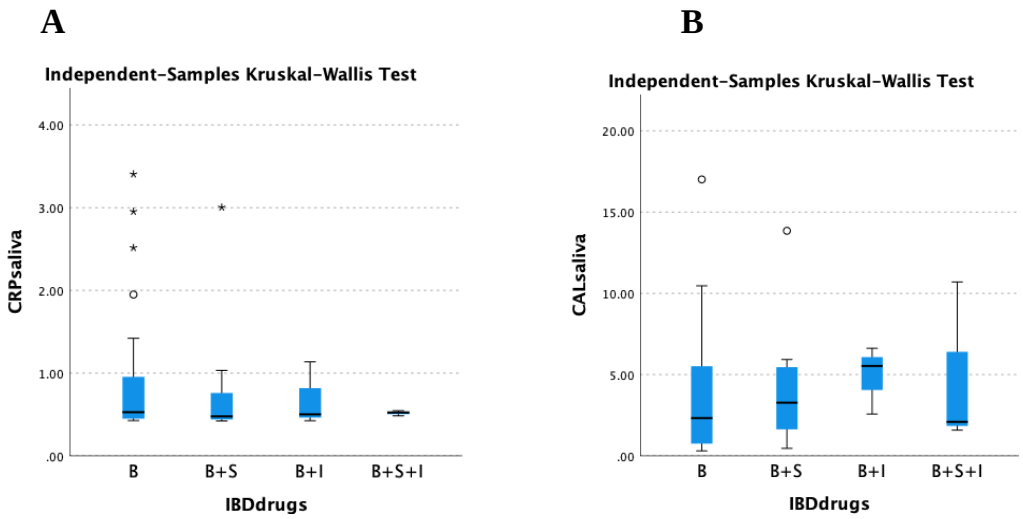


Figure 55: Comparison of Salivary CRP (A) and CAL (B) when IBD Drugs was used as an Experimental Factor.

2- Activity of the Disease

As shown in Figure 56, there was non-significant difference ($p>0.05$) in salivary CRP and CAL results based on the activity of the disease. Nevertheless, patients with active disease demonstrated higher CAL levels. In active patients CRP saliva had a mean ± SD =0.811 ± 0.797 ng/ml and CAL saliva had a mean ± SD =4.148 ±3.24 ng/ml, while in inactive patients CRP saliva had a mean ± SD =0.883 ± 0.790 ng/ml and CAL saliva had a mean ± SD =3.876 ±4.089 ng/ml.

A **B**

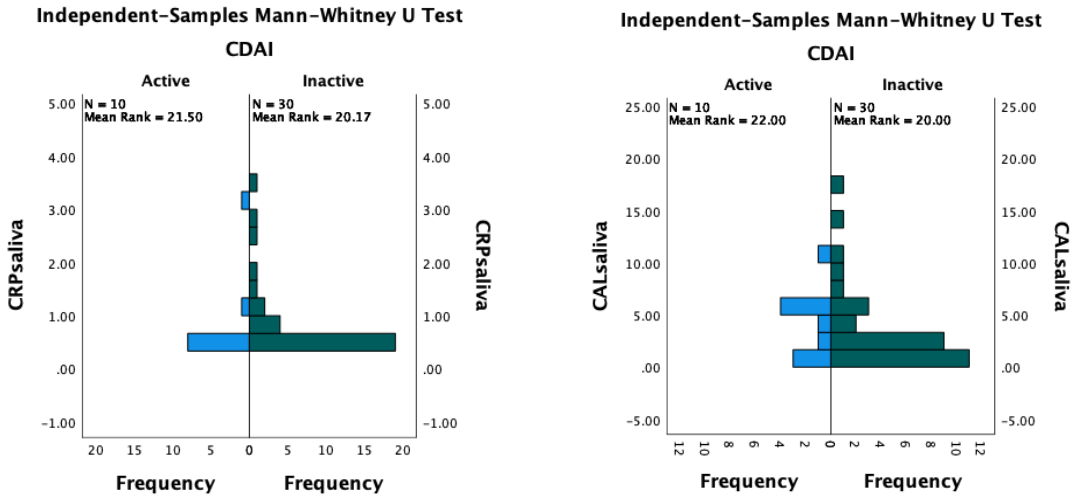


Figure 56: Comparison of Salivary CRP (A) and CAL (B) when the Activity of the Disease is Used as an Experimental Factor.

3- Frequency of Relapse of Symptoms

As shown in Figure 57, there was non-significant difference ($p>0.05$) in salivary CRP and CAL results based on the frequency of relapse of symptoms. Nevertheless, patients with more frequent relapse (2-4 times per year) demonstrated higher CRP and CAL levels, as CRP saliva had a mean ± SD =1.019 ± 0.99 ng/ml and CAL saliva had a mean ± SD =5.467 ±5.53 ng/ml, while patients with a relapse of 0-1 times per year had CRP saliva of mean ± SD = 0.806 ± 0.699 ng/ml and CAL saliva of mean ± SD =3.366 ±2.917 ng/ml.

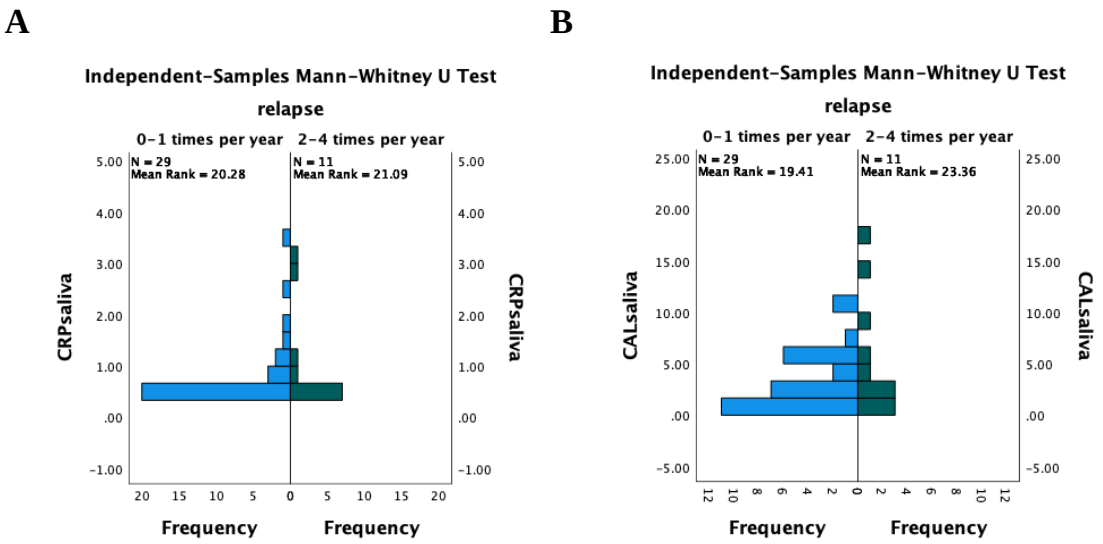


Figure 57: Comparison of Salivary CRP (A) and CAL (B) when the Relapse of Symptoms was Used as an Experimental Factor.

4- Duration of the Disease

As shown in Figure 58, there was non-significant difference ($p>0.05$) in salivary CRP and CAL results based on the duration of the disease. In newly diagnosed patients, CRP saliva had a mean ± SD =0.734± 0.597 ng/ml and CAL saliva had a mean ± SD =4.10 ±3.969 ng/ml. In patients of 1-10 years duration of CD, CRP saliva had a mean ± SD =0.856± 0.752 ng/ml and CAL saliva had a mean ± SD =4.097 ±3.938 ng/ml. In patients with CD diagnosed more than 10 years ago, CRP saliva had a mean ± SD =3.62± 3.969 ng/ml and CAL saliva had a mean ± SD =3.62 ±3.969 ng/ml.

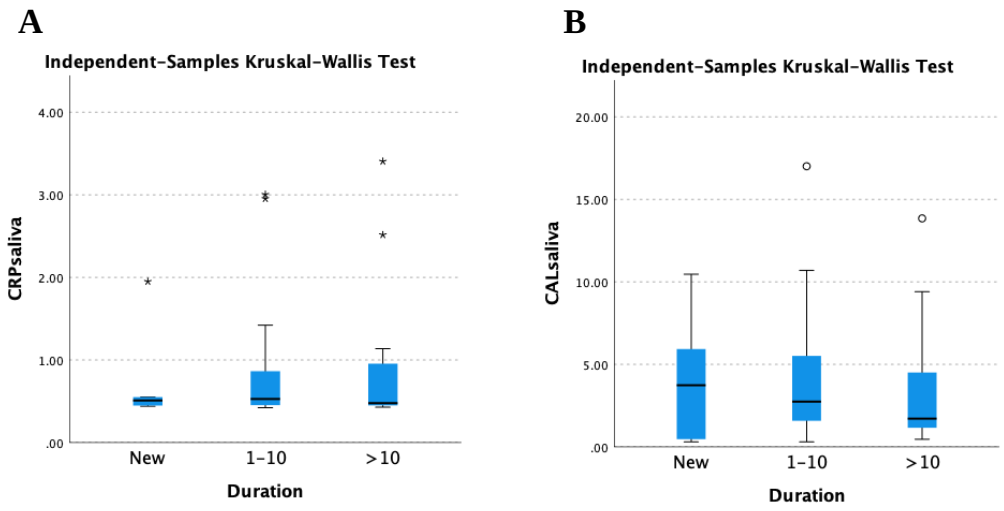


Figure 58: Comparison of Salivary CRP (A) and CAL (B) when the Duration of the Disease was Used as an Experimental Factor.

3.12 Relation Between Microbiota Diversity and Inflammatory Biomarkers in CD

Correlation analysis revealed a negative relation between all alpha diversity indices and both CAL level in the stool and CRP levels in the serum of CD patients. The correlation was not significant, except for Shannon index as the correlation between its level and fecal CAL was statistically significant (p 0.038; correlation coefficient: -0.329).

Also, a significant positive correlation was found between Simpson index and CRP level in the saliva of CD patients (p 0.027; correlation coefficient: 0.349).

Chapter 4: Discussion

Crohn's disease is a chronic relapsing inflammatory bowel condition, with main symptoms including weight loss, bloody stool, abdominal pain, constipation, and diarrhea. In this study, we have recruited 40 patients with CD, with females being more than males (n=24, 60% and n=16,40% respectively). CD usually affects both genders. This information was supported by an epidemiology study in that gender has no bearing on the total incidence of UC in Europe, North America, and Oceania (Mak et al., 2020). In the case of CD, results have been less consistently reported, with some cohorts revealing a female predominance in the prevalence of CD and others failing to discover any gender difference at all (Mak et al., 2020). Females predominated among CD patients from adolescence to middle age, according to an aggregated analysis of research conducted in the West (Mak et al., 2020). The condition typically manifests at age 30, with its two peaks occurring between ages 20 and 30 and at around 50, respectively (Feuerstein & Cheifetz, 2017). Similar findings were seen in our study; in which our age range was 16-52 years. A regional epidemiology review, including 1,627 UC patients and 1,588 CD patients, stated that there were 16 studies that looked at IBD in Saudi Arabia, Egypt, Kuwait, the United Arab Emirates, Bahrain, Lebanon, and Oman. The included studies ranged in time from the early 1990s to the late 2010s (Mosli et al., 2021). Few studies on CD were performed in the UAE or the Arab countries in general specifically focusing on the microbiome. Thus, this study was conducted to fill the knowledge gap on CD in the UAE and to explore the salivary microbiome of CD patients with focus on factors leading to dysbiosis.

Our study aimed to investigate the differences in the salivary microbiome between CD and HC, then to identify the differences among CD patients based on different factors that might have an impact on the oral microbiome. Oxford nanopore technology was used to characterize the microbiota by sequencing the entire 16S rRNA gene. Our data is considered superior to the previous research work on microbiome as we used an advanced technology with third generation sequencing allowing exploration of long reads opposed to most previous research, which used short read sequencing (Said et al., 2014). Third-generation sequencing technologies address the shortcomings of next

generation sequencing. While the Sanger and short read sequencing approaches have read length limits of 1 kilobase pair, third-generation sequencing technologies have read length limits of 5 to 30 kilobase pairs (Adewale, 2020). Thus, sequencing of the entire 16S rRNA gene was successful in this study, and the data generated was of high quality (~ Q20) allowing a thorough analysis of the microbiota in our samples. Bioinformatic analysis of the data revealed a plethora of significant bacterial features at phyla, genera, and species levels. This study is distinctive since the comparison and some discoveries of new bacterial species are based on five factors within CD which include the oral health, IBD medications use, the activity of the disease, the frequency of relapse of symptoms, and the duration of the disease.

A comparison of the microbial profiles in CD and HC was performed using the linear discriminant analysis (LDA) effect size (LEFSe), which is a measure of potential biomarkers of bacteria. A study on the assisted selection of biomarkers by LEfSe in microbiome data stated that, it is essential to find biomarkers with statistical disparities between groups to investigate and reveal the intergroup differences among various samples or environments. The ease of finding genetic biomarkers that describe statistical differences across biological groups was made possible by LEfSe (F. Chang et al., 2022).

The 80 samples collected in this study were analyzed to compare CD patients and HC at the three taxonomic levels (phylum, genus, and species). Our results indicate that there was a difference between microbiota detected in CD and HC. At the phylum level *Tenericutes* and *Spirochetes* were significantly more in HC and depleted in CD. At the genus level only one genus *Dolosigranulum* was significantly more in CD and absent in HC, several other species were abundant in HC and not present in CD. In a previous study, oral gut axis was examined to define and compare the fecal and salivary microbiota of IBD patients and control people (Abdelbary et al., 2022). One of their key findings was the discovery of a signature of the salivary microbiome linked to IBD patients, which was primarily connected to a high abundance of the genera *Prevotella* and *Veillonella* and a depletion of the salivary genera *Streptococcus*, *Haemophilus*, and *Neisseria*, which are linked to the healthy gut state (Abdelbary et al., 2022).

In our study, the findings were different regarding HC since the phyla *Tenericutes* and *Spirochetes* were enriched in this group and depleted in CD while the genus *Dolosigranulum* were depleted in HC and enriched in CD. This was in the phyla and genera level; but clear identification can be discovered when we move to the species level. Coming to the species level, LDA analysis clearly demonstrated a total of 65 significant features, 60 species were found in HC, some are recognized bacteria such as *Treponema denticola, Escherichia coli, Streptococcus mutans* and *Streptococcus viridians* but the majority were *Streptococci*. The five significant bacterial species that are enriched in CD and depleted in HC and can also be considered as biomarkers for CD, since they are only available in the saliva of patients diagnosed with this inflammatory bowel disease. These bacterial species are: *Veillonella dispar, Megasphaera stantonii, Prevotella jejuni, Dolosigranulum pigrum* and *Lactobacillus backii*. *Veillonella dispar* is a Gram-negative anaerobic bacterium, that is well known for its lactate fermenting abilities. It is a normal bacterium in the intestine and oral mucosa as well as nitrate reducing bacterium in the oral cavity which is beneficially antibacterial (Mitsui et al., 2018). *Megasphaera stantonii* is an obligately anaerobic, Gram-negative, coccoid bacteria belonging to the phylum *Firmicutes*. Since most of the previous studies mentioned abundances in the taxa of CD at the phylum and genus level, it might be of a difficulty to identify the exact species, nevertheless our results agreed with the recent previous study in that, *Prevotella* and *Veillonella* abundance was much higher in both UC and CD patients, while *Streptococcus, Haemophilus,* and *Neisseria* abundance was significantly lower, with the latter occurring solely in the CD group (Abdelbary et al., 2022). This agrees with our study since the phyla *Prevotella* and *Veillonella* are similar pathogenic bacteria that are increased in abundance in CD. There is a close relation between the abundance of *Firmicutes* in CD and the elevated level of butyrate producing bacteria. A previous study agreed on that, *Firmicutes* are well known to be associated with IBD and reduced levels of *Firmicutes*, such as the *Clostridium leptum* group and *Faecalibacterium prausnitzii,* are associated with decreased gut microbiota diversity in IBD patients. In our study in CD, the anaerobic Gram positive normal commensal *Faecalibacterium prausnitzii* was depleted. Its presence is of utmost importance since it releases short chain fatty acids (SCFAs) which are byproducts needed by cells of the

colon to maintain its integrity (Baldelli et al., 2021). A previous study confirmed this by reporting that IBD patients have lower concentrations of protective anaerobic commensal bacteria such *Faecalibacterium prausnitzii, Clostridium* spp., and *Bacteroidetes fragilis* in their bodies. SCFAs, which are produced *by F. prausnitzii*, have been demonstrated to have anti-inflammatory effects, including the capacity to influence the host's mucosal immune response (Baldelli et al., 2021). Colonic epithelial cells use SCFAs such acetate, propionate, and butyrate as their main source of energy. Therefore, the positive effects of SCFAs, including as the suppression of pro-inflammatory cytokine expression, the creation of mucin and antimicrobial peptides, and tight junction protein downregulation, are diminished because of this bacterium's underrepresentation in IBDs (Baldelli et al., 2021). *Firmicutes,* particularly *F. prausnitzii*, produce anti-inflammatory substances such as butyrate, which can inhibit Th17 cells in IBD. Changes in *Firmicutes* levels have been shown in studies to be an important marker, even during anti-TNF- α treatment (Park et al., 2022). Bacteria from the species *Prevotella jejuni* are strictly anaerobic Gram-negative rods whsionuich constitute a substantial part of the normal microflora. *Prevotella* species often occur in opportunistic infections and dysbiosis-associated disease, and produce major metabolic end products such as acetic acid and succinic acid (Hedberg et al., 2013). We also found *Dolosigranulu pigrum*, a Gram-positive bacteria associated with upper respiratory tract infections, and *Lactobacillu backii*, which produces D(L)-lactic acid as well as a beer spoiling bacterium. The absorption of metabolic products of the intestinal microbiota into the living body of the host is thought to be the cause of the correlation between host health and intestinal bacteria; this relationship is known as the host-intestinal microbiota metabolic correlation. Research has also shown that increased lactic acid in feces is an indicator of the severity of IBD. As a result, dysbiosis of the intestinal bacterial microbiota caused by oral bacteria intake, as well as variations in organic acid levels, may result in an inflammatory state (Kobayashi et al., 2020). Miller suggested that acid produced by oral microorganisms from sugar in diet was the primary cause of tooth caries in his book "The Micro-organisms of the Human Mouth" (Miller, 1973). *Lactobacillus* species were known to be cariogenic bacteria, but was it appropriate to focus on one specific bacterium as the cause of oral diseases affected by a complex oral microbiome?

He failed to identify cariogenic bacteria and concentrated on acids as bacterial metabolites rather than on the bacteria that produced these metabolites. Additionally, The Micro-organisms of the Human Mouth's descriptions of the link between oral bacteria and overall health served as a reminder of the modern idea of periodontal medicine, which reflects his significant scientific contributions (Yamashita & Takeshita, 2017).

In CD, *Firmicutes* was the most abundant at the phylum level, *Haemophilus* at the genus level and *Haemophilus parainfluenzae* at the species level. In healthy individuals, the genus *Streptococcus* was the most abundant. In a previous study, the genus *Prevotella* was found to be significantly more abundant in the salivary microbiota of IBD patients, with its relative abundance nearly equaling that of reduced *Streptococcus*, which is most abundant in healthy salivary microbiota (Said et al., 2014), which is close to our study in HC having more *Streptococcus* species. Another study demonstrated changes in the microbiota associated with IBD in which there was an increase in *Fusobacterium* species, *Pasturellaceae, Proteobacteria* and a decrease in *Bacteroides* species, *Bifidobacterium* species, and *Faecalibacterium prausnitzii* (Glassner et al., 2020b). A study stated that in immunocompromised population, the entry of a sufficient load of oral opportunistic or pathogenic bacteria into the bloodstream via the oral mucosal barrier during daily oral hygiene or tooth treatment procedures could result in abnormal local and systemic immune and metabolic responses, as well as nutrient digestion, indicating the pathogenic basis of the oral microbiota (Xun et al., 2018). The presence of pathogenic bacteria in the oral cavity of CD is alarming, since it can enter the bloodstream contributing to systemic inflammation (Kobayashi et al., 2020). Other studies also reported increased *Actinomycetota (Actinobacteria)* and *Pseudomonadota (Proteobacteria)* species in the saliva of patients with CD (Hu et al., 2022). This was different from our study in that these two phyla were depleted, or not detected.

In a previous study, patients with CD exhibited decreased bacterial diversity and altered abundance of some taxa, including a decrease in health-promoting microorganisms (e.g., *Faecalibacterium* and *Roseburia spp*.) and an increase in pathogenic microorganisms (e.g., *Escherichia, Fusobacterium,* and *Mycobacterium*

spp.). Moreover, mounting evidence suggests that such dysbiosis may be a causal factor in the emergence of chronic IBD such as CD (Núñez-Sánchez et al., 2022). Numerous studies have shown that the microbiota changes in inflammatory bowel illness, with a decline in stringent anaerobes and specific *Firmicutes* and a burst of *Proteobacteria* (Elson & Cong, 2012). This agrees with our study when CD patients were compared to HC; they demonstrated decreased diversity of microbial profiles with depletion of beneficial bacteria which indicates frank dysbiosis.

In our study, we were keen to discover the association between the oral cavity and the gut; thus, a thorough check up of the oral cavity was performed to classify the oral hygiene into good (H), caries (C), periodontal disease (P), and both caries with periodontal disease (P+C). CD patients demonstrated poorer oral hygiene (n=29; 72.5%) in comparison to healthy controls (n=19; 47.5%), which is in agreement with a previous research study which confirmed this finding by investigating the prevalence of dental caries and periodontal disease in patients with CD compared to the control group (Tan et al., 2021). In the latter study, the IBD group had a significantly higher total DMFT (Decayed missing filled teeth) index indicating poor oral health compared to the healthy subjects. Another study reported that patients with CD frequently experienced oral health issues and have a higher prevalence of oral manifestations such as dental caries and periodontitis than healthy people (Sun et al., 2021). However, the previous studies did not analyze the oral microbiota in CD patients and lack any evidence on oral dysbiosis in these patients. On the contrary, we did this in our study as oral health was considered as an important factor that was closely measured between CD and HC. This factor is considered important according to the hypothesis that bacteria in the oral cavity that is surely translocated into the intestine as each person can ingest 1-1.5 L of saliva per day (Bao et al., 2022). Furthermore, more pathogenic microbes may enter the gut with saliva in patients with periodontitis. As a result, pathogenic oral microbes that are swallowed have the potential to disrupt the balance of the gut microbiota. Microbial metabolites might be also involved as they can be swallowed or absorbed, which can be another hypothesis explaining how oral dysbiosis can affect gut (Bao et al., 2022). Many studies have found that an imbalance in the intestinal microbiome is one of the possible mechanisms that contribute to the pathogenesis of IBD. Accordingly, oral microbiota

may have a great contribution to gut dysbiosis and inflammation, as also explained above (Matsuoka & Kanai, 2015).

When we studied and analyzed the results of the salivary microbiome based on oral health, by performing the LDA analysis, we performed the comparison by differentiating the bacterial biomarkers according to the oral hygiene in the 3 taxonomic levels (phyla, genera, and species) and in C, P, P+C and H groups. When HC were investigated, no significant features were detected at the phylum level when HC subjects were compared according to their oral health, but other levels such as genus and species revealed many. What makes our study strong is the disclosure of specific operational taxonomic levels. The highest abundant species in HC was *Haemophilus parainfluenzae*, specifically in individuals with P+C followed by *Streptococcus salivarius* in C, *Streptococcus mitis* in P and *Streptococcus gordonii* and *Lactobacillus acidophilus* in H. *Haemophilus parainfluenzae*, is a normal inhabitant of the human respiratory tract; also, *Streptococci* and *Lactobacilli* are components of the normal flora. A study on the oral microbiome and human health suggested that the majority of bacteria found in the salivary microbiota, including *Streptococcus, Neisseria, Rothia, Prevotella, Actinomyces, Granulicatella, Porphyromonas, Haemophilus,* and *Porphyromonas* species (Yamashita & Takeshita, 2017). This explains the high abundance of these bacteria in the healthy subjects of our study.

In CD, LDA analysis showed significant features at phylum, genus and species levels. At the phylum level, CD had two significant bacterial features, *Fusobacteria* in H and *Actinobacteria* in P. A previous study had similar findings as the salivary microbial communities of the group with CD and periodontitis compared to another group of periodontitis alone without CD differed in that the CD with periodontitis group had relatively high abundances of *Firmicutes* and *Proteobacteria*, whereas the group with periodontitis alone had relatively high abundances of *Actinobacteria, Bacteroidetes,* and *Fusobacteria* (Sun et al., 2021). The most prevalent genera were *Fusobacterium* in C and *Lactobacillus* in P + C. In CD at the species level, which is a more specific level that our study has reached, the most dominant species are from the anaerobic Gram-negative genus *Fusobacteria,* namely *Fusobacterium periodonticum* and *Fusobacterium ulcerans*

in C group, and from the Gram-positive genus *Streptococcus.* Patients with caries show bacterial biomarkers of *Fusobacterium periodonticum, Salmonella enterica,* and *Escherichia coli.* These bacteria are pathogenic organisms which reside in the intestines; therefore, caries in CD, might be a factor that increase pathogenic bacteria which endanger the patients' health resulting in intestinal inflammation when these bacteria are ingested. While patients with periodontal diseases show bacterial biomarkers of *Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus oralis,* and *Streptococcus mutans.* Most of the remaining bacteria are of the genus *Streptococcus,* Gram positive cocci, such as *Streptococcus pyogenes, Streptococcus salivarius* and *Streptococcus intermedius.* This explains the high abundance of *Veillonella parvula* since it feeds on lactate provided by *Streptococcus* species. *Veillonella parvula* colonizes dental plaque early, it is unable to ferment glucose or most other sugars and must instead rely on lactate excreted by *Streptococci* as a carbon source for growth, hence contribute to caries development (Liu et al., 2020). Our results revealed that in CD with P+C, the species detected were highly caries and periodontal pathogenic, these are *Lactobacillus fermentum, Lactobacillus acidophilus* and *Streptococcus mutans*. A previous study stated that Miller's approach is said to have deceived his successors, who classified the *Lactobacillus* species as cariogenic bacteria in line with Koch's notion (Yamashita & Takeshita, 2017). Another study confirmed the fact that those with poor dental health had more commonly detected pathobionts in their salivary microbiota, including *S. mutans* and *P. gingivalis* (Yamashita & Takeshita, 2017). At the species level, no significant features were detected in subjects with P+C. As shown in the results this indicates that the bacterial biomarkers in H as in those having no caries or periodontal diseases are *Prevotella enoeca,* and *Bacteroides intestinalis,* both are inhabitants of the normal flora, which is a logical explanation that their good oral health did not cause oral dysbiosis. Another study results stated that, *Bacteroides fragilis, Prevotella baroniae, Prevotella enoeca,* and *Prevotella dentasini* were more abundant in the CD with periodontitis and periodontitis alone groups than in the HC group. This evidence supports the hypothesis that dysbiosis of the oral microbiota causes patients with CD to have an unfavorable tolerance to periodontal pathogenic bacteria (Sun et al., 2021). Therefore, it is of paramount importance to investigate the alterations in the salivary

microbiome in patients with CD compared to HC. Since microbiota is necessary to maintain a healthy gut environment, dysbiosis or an imbalance in the normal homeostasis can predispose to CD and other IBD. The role of oral microbiota is still vague; however, a previous study reported that intestinal colonization by bacteria from the oral cavity has been linked to a variety of negative health outcomes, including IBD (Atarashi et al., 2017). A research study in 2020 suggested that administration of human oral bacteria may cause changes to the murine intestinal microbiota and bacterial metabolites, resulting in decreased intestinal immunity and inflammation (Kobayashi et al., 2020). Our findings clearly demonstrates that poor oral health specifically periodontal diseases (periodontitis) which is an inflammatory condition, plays a great role in the pathogenesis of CD since the bacteria found in the oral cavity are pathogenic and destructive to the tissues surrounding the tooth and hence when translocated into the intestine via swallowing lead to inflammation. A previous study's key conclusion was that periodontitis, damaged teeth, and poor oral hygiene were all strongly linked with high bacterial richness in the salivary microbiome (Yamashita & Takeshita, 2017). Another interesting finding was that conditions linked to oral health were significantly correlated with the relative quantity of the prevalent bacteria in saliva. The greater relative abundance of *Prevotella* and *Veillonella* species was linked to poor dental health, a high body mass index, and old age, of the two cohabiting groups of bacteria discovered in the salivary microbiota. These results imply that oral and systemic health are reflected in the salivary microbiome (Yamashita & Takeshita, 2017).

As for the effect of medications, most of the saliva samples were collected from CD patients while they were receiving their intravenous IBD drugs which was mainly the monoclonal antibody (infliximab). Although *Firmicutes, Actinobacteria, Bacterioidetes, Verrucomicrobia,* and *Proteobacteria* are the most abundant phyla in both health and disease, the richness and diversity of these groups change in disease. A previous study reported that the lack of microbial diversity in IBD was caused primarily by the loss of normal anaerobic bacteria (Kowalska-Duplaga et al., 2020). Our results agreed with the statement of these authors especially when only a sole bacterium was found significantly more in patients receiving only infliximab which is *Simonseilla*

muelleri. No previous studies were found on this specific species, but some studies were found mentioning *Simonseilla* spp. not *Simonseilla muelleri,* which gives this study its novelty in the detection of new species related to IBD and the use of medication. The other confirmation of dysbiosis or depletion of normal flora with the intake of other medications in addition to biologicals, such as steroids and immunosuppressants is the emergence of other pathogenic and opportunistic bacteria such as *Haemophilus parainfluenzae, Salmonella enterica, Escherichia coli, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa.* These bacteria are important findings since they are all pathogenic bacteria associated with serious infections, with a possibility of profound loss in diversity and shifts in microbial abundances. As a previous study confirmed that multidrugs resistant *Klebsiella* strains tend to colonize when the intestinal microbiota is dysbiotic and cause a serious gut inflammatory response in genetically vulnerable hosts (Atarashi et al., 2017). They also mentioned that their findings suggest that possible intestinal pathobionts can aggravate intestinal disease that may be stored in the oral cavity , finding members of the healthy gut microbiota can offer colonization resistance against bacteria that are taken orally may open up new possibilities for the creation of potent remedies for multidrug-resistant bacteria and persistent inflammation (Atarashi et al., 2017). A study in 2013 again confirmed our conclusion reporting that in CD patients, the *Klebsiella* bacteria appeared to play a significant role in the beginning and maintenance of the degenerative damage to the gut and joint tissues (Rashid et al., 2013). In the latter study, elevated levels of antibodies against *Klebsiella* in CD patients have been documented by six separate gastroenterology clinics in the UK. On numerous situations, CD patients' antibody responses to *Klebsiella* bacteria were significantly higher than those of healthy individuals. As a result, it is conceivable that CD could be brought on by persistent, subclinical infections of the large bowel with *Klebsiella* microbes, which would then cause inflammations and tissue damage in the bowel and joints as a result of the binding of anti-*Klebsiella* and anti-self-tissue antibodies to the cross reactive targeted antigens (Rashid et al., 2013). Therefore, this interplay proves that the immune response to pathogenic bacteria either in the gut or oral cavity can be implicated in the pathogenies of IBD.

When the activity of CD was considered, we classified patients into having active (relapse or newly diagnosed) or inactive disease (remission). Active patients accounted for a percentage of 25% while inactive patients were 75% since the majority of patients were under treatment, mostly receiving biologicals. Bacteria having high LDA scores were in the patients with active disease such as the genus *Lactobacillus*: *Lactobacillus mucosae, Lactobacillus crispatus* and *Lactobacillus bombi,* which are part of the normal microbiota and are lactic acid producing bacteria. It is reported in a previous study that lactate-producing and lactate-utilizing bacteria coexist in the human intestine. Other lactate producers, such as *Lactobacillus* spp., *Faecalibacterium prausnitzii,* and *Bacteroides* spp., must be responsible for the D-lactate detected at pH 5.2. Furthermore, many other bacterial groups can produce lactate as one of their fermentation products under certain nutritional conditions (Belenguer et al., 2007).

The phylum *Firmicutes* contains species from the orders *Lactobacillales, Clostridiales, Erysipelotrichia,* and *Negativicutes,* are varied to a great extent. Some of the most widely used probiotics are members of the order *Lactobacillales,* which also contains facultative anaerobes. Contrarily, the *Clostridiales* make up a significant portion of the anaerobic bacterial population in the colon and cecum and are obligate anaerobes. It is expected that members of the *Lactobacillales* and *Clostridiales* orders will play a significant role in upcoming efforts to restore the functionality of the microbiota after disruption because significant positive functions have been attributed to these groups (Sorbara & Pamer, 2022). This may explain why we found more *Lactobacilli* in patients with active disease in our study.

A previous study stated different abundance in other salivary bacterial profiles that unlike our findings, during the active phase of the disease, *Prevotella* had the greatest differential abundance. *Prevotellaceae, Bacteroidetes, Bacteroidia, Veillonellaceae, Pedobacter, Megasphaera, Salmonella, Clostridium XI, Solobacterium, Oribacterium, Mogibacterium, Atophobium,* and *Lachnoanaerobaculum* were also found in active phase samples. *Sphingobacteriaceae, Enterobacteriaceae, Coriobacteriaceae,* and *Erysipelotrichaceae* also showed an increase. Differentially abundant bacteria in the remission phase were mostly from the orders *Neisseriales, Fusobacteriales,* and *Rhodobacterales.*

In comparison to their abundance in active phase and control, *Neisseriales* showed the greatest enrichment (Zhang et al., 2020). During the disease inactivity or remission, our results were also slightly different from the active disease it showed abundance in the genus *Capnocytophaga* which is an opportunistic bacterium, also the genus *Streptococcus* and the interesting species *Fusobacterium ulcerans* which is associated with ulcerations and hence its name.

Since most of our findings support that there is a disruption in the normal flora of the oral cavity, specifically related to the activity of the disease, one of the main constituents of the probiotics noticed is *Lactobacillus*, therefore, the logical treatment of IBD is to restore or maintain the normal homeostasis of the gut by the administration of probiotics. A study confirmed this by using probiotics and prebiotics to restore natural flora, prevent pathogenic bacteria infection by producing antimicrobial peptides, and promote intestinal health by stimulating the growth and activity of beneficial bacteria via prebiotic fermentation. Clinical treatment has included the use of *Bifidobacteria*, *Lactobacillus*, VSL#3 (a proprietary mixture of *Lactobacillus acidophilus*, *Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus plantarum, Bifidobacteriium brevis, Bifidobacteriium infantis, Bifidobacteriium longum,* and *Streptococcus salivarius ssp thermophilus* and butyric acid-producing bacteria). Probiotics effectively regulate intestinal flora imbalance, improve the microecological environment, improve intestinal mucosal barrier function, modulate local and systemic immune responses, and provide new treatment options for diseases such as IBD (Guo et al., 2021).

When CD patients were interviewed and asked questions during data collection, one of the main questions was the frequency of relapse of symptoms since CD is characterized by recurrent relapse and remission phases. The main symptoms are abdominal pain, diarrhea, bloody stool, and weight loss. The frequency was classified as once per year (0-1 times per year) or more than twice per year (2-4 times per year). In the category of 2 times and more, our findings demonstrated a raised abundance in the anaerobic Gram-negative bacteria of the *Bacteroidetes* phylum, *Prevotella* examples are *Prevotella oris* and *Prevotella jejuni,* also the species mentioned earlier in IBD drugs specifically biologicals alone (*Simonseilla muelleri*).

A previous study stated that patients with recurrent CD had a microbiota that favored proteolytic-fueled fermentation and lactic acid-producing bacteria, including *Enterococcus* and *Veillonella* spp., whereas those in remission had a microbiota that favored saccharolytic *Bacteroides*, *Prevotella*, and *Parabacteroides spp.*, as well as saccharolytic, butyrate-producing *Firmicutes* (De Cruz et al., 2015).

The duration of CD since its confirmation via colonoscopy was categorized as newly diagnosed, 1-10 years duration and more than 10 years duration.

Surprisingly, the most common species was in the patients that were newly diagnosed with CD which is *Porphyromonas gingivalis* a Gram-negative anaerobe associated with the pathogenesis of periodontal disease. This explains the relation with poor oral hygiene in CD. This also suggests that the longer the duration of the disease, the more effect on oral health, and hence more pathogenic oral bacteria. Other species such as *Campylobacter helveticus* and *Klebsiella pneumoniae* were also detected in the saliva of patients having a duration of disease which exceeds a decade. A previous study had similar results mentioning that the colonization of several oral bacteria, including a subset of *Porphyromonas gingivalis*, *Streptococcus mutans*, *Fusobacterium nucleatum*, *Campylobacter concisus*, and *Klebsiella pneumoniae*, may result in intestinal epithelial barrier destruction, excessive secretion of inflammatory cytokines, disruption of the host immune system, and dysbiosis of gut microbiota (Qi et al., 2022). The duration of the disease gives us an insight on the obvious shift in microbiota throughout the years. This brings us again to the same conclusion that dysbiosis can disrupt the normal balance required to maintain homeostasis.

As for microbiota diversity, different dissimilarity indices were tested such as alpha and beta diversities to compare between CD and HC. Significant differences in alpha diversity were detected using observed species index, Chao 1 index and ACE index (p values were significant <0.05), while in Simpson's and Shannon indices (p values were not significant). Our results show significant reduction in species richness in CD compared to HC. This based on the interpretation of various indices such as observed and Chao1 which represent the true richness of the OTU counts in a sample,

ACE index which is a non-parametric method for estimating the number of species using sample coverage, while Shannon index which represents both the richness and evenness of species in a sample and Simpson only reflects the evenness of the number of species present, and the relative abundance of each sample (Wagner et al., 2018). A previous study focusing on the oral-gut axis reported that the number of detected taxonomic

groups, i.e., the Shannon index, was significantly lower in samples of the IBD group compared to the control group, according to alpha diversity analysis.

This agrees with our study, as alpha diversity measures were lower in CD compared to HC; although the difference was statistically significant for some indices (Abdelbary et al., 2022). When we measured the beta diversity in this study using the principles coordinate analysis as a data comparison technique to visualize inter sample similarity using Bray-Curtis as a distance method and PERMANOVA as the statistical test, all p-values were non-significant (>0.05), although samples from different groups formed distinct clusters. A controversial previous study showed that the beta diversity analysis revealed significant differences between the IBD and control groups and to support this finding, they compared the distances between the two groups using the Bray-Curtis dissimilarity method, which confirmed that the IBD group had the highest beta diversity heterogeneity (Abdelbary et al., 2022).

In our study, CD patients were under control by monoclonal antibodies and anti-TNF-α treatment; thus, we concluded that this might be the cause of non-significant difference in beta diversity when compared to healthy controls. This was supported by a research study focusing on the microbial changes before and after treatment with similar IBD medications, furthermore, there was no significant clustering of salivary microbiota in β-diversity between the study groups (Park et al., 2022). Another study agreed on that information regarding alpha diversity when it reported that the effects of enteral nutrition therapy on gut-microbiome dynamics has revealed a decrease in α-diversity, which has been proposed to favor the long-term restoration of the gut microbiota (Núñez-Sánchez et al., 2022).

However, when different factors were used to compare CD patient, we found that samples from patients belonging to unique groups clustered together. This was obviously seen in the dendrograms generated from the beta diversity indices of samples, and in the PCO graphs. This implies that different factors can contribute to the diversity in CD patients.

When we studied the link between various experimental factors using Venn diagrams, we found few shared species.

In the Venn diagram illustrations, most the overlapped species were marked as 0% indicating that each factor has its distinct and unique impact on the microbiome, which was altered by the combined effect of all the factors collectively contributing to dysbiosis. A related study had similar Venn diagram findings in that the genera found were significantly different between groups in earlier analyses concatenated in the Venn diagram (Davrandi et al., 2022). Based on the findings in the Venn diagrams, there were only four (3.8%) common species including: *Enterococcus faecalis, Lactobacillus ensenii, Lactobacillus paracollinoides,* and *Vibrio parahaemolyticus,* which represent the shared altered species by the three factors; oral health, IBD drug use and activity. *Enterococcus faecalis* is a Gram-positive, commensal bacterium inhabiting the gut but when the host has lowered immunity, for example by these factors, it becomes opportunistic and starts causing the infection. This agrees with a study by Zhou et al. (2016) relating *E. faecalis* to CD activity by finding that *E. faecalis* is a typical opportunistic pathogen that can cause IBD. Our research shows that elevated *E. faecalis* counts are a common finding in people with CD. In CD patients, a considerable rise in *E. faecalis* levels seems to be related to clinically active illness. CDAI score was significantly and positively correlated with high levels of *E. faecalis* colonization based on a previous study (Zhou et al., 2016). Increased *E. faecalis* colonization in CD was favorably correlated with CD activity index, fecal calprotectin, and disease activity. Patients with IBD had significantly higher concentrations of *E. faecalis* and *Fusobacterium spp*., and clinically active CD is linked to higher *E. faecalis* infection (Zhou et al., 2016).

As for inflammatory biomarkers in IBD, C-reactive protein in serum and calprotectin in stool values were obtained from patient's records. CRP is a protein in the serum released by the liver in response to inflammation caused by inflammatory conditions such as in the case of CD and CAL is also a protein present in faeces which detects intestinal inflammation. Calprotectin is a calcium-binding neutrophil protein that is stable during intestinal transit. It accounts for up to 60% of total neutrophil cytosol proteins. Calprotectin is strongly correlated with endoscopic and histological CD activity ratings in ileocolonic or colonic illness (D'Incà & Caccaro, 2014).

We compared these values with the salivary CRP and CAL for CD and HC. Values were also compared to the serum and stool values for CD patients. We were interested to know more about the biomarkers of inflammation, moreover, to know if saliva can be considered a tool to detect inflammation in replacement to blood and stool since it's more convenient and not invasive. Our results showed that salivary CAL is the only significant inflammatory biomarker specifically related to oral health, in CD and HC patients with periodontal disease and caries. Most studies reported that patients with periodontal disease were excluded as the primary cause of oral inflammation. This finding is closely related to our results where we found a correlation between poor oral health and elevated salivary calprotectin. The relation was significant with a p value of less than 0.05. Periodontitis is an oral inflammation, thus may affect salivary biomarkers, since it is a local inflammation of the tissues surrounding the teeth; therefore, the saliva will have higher biomarkers values. The values were higher in CD but the difference was not significant. A recent study also proposed that salivary calprotectin is a potential indicator of active IBD (Finamore et al., 2020). In previous studies, it was reported that fecal calprotectin is considered a useful biomarker that is more specific for intestinal inflammation than serum CRP (S. Chang et al., 2015). Another study stated that because serum markers can be elevated by conditions other than gut inflammation, fecal markers have a higher specificity for IBD in the absence of GI infection (Vermeire et al., 2006). A study demonstrated that salivary CRP levels were higher in patients with active CD than in controls. ROC analysis suggested that salivary CAL could distinguish IBD patients from controls (Nijakowski & Surdacka, 2020).

This was not the case in our study probably as our patients were receiving IBD treatment, limiting the inflammation. The level was higher in CD but not significant. A previous study in 2020 stated that there was a significant positive correlation between serum concentrations and salivary CRP levels (Nijakowski & Surdacka, 2020). In our findings, no significant correlation was found between salivary and serum CRP values, this is probably because most of the patients did not have active inflammation and the values were not variable as much. Another factor affecting our analysis results might be the low sample size. Mainly fecal CAL values were important since it is recognized as a reliable biomarker of inflammation as mentioned earlier. A previous study confirmed this information whereby non-invasive measures such as CRP and fecal proteins can give baseline information to establish the existence of intestinal inflammation and should be utilized as a first step in identifying individuals who require additional study (Woo, 2015).

Interestingly, CAL stool was negatively correlated with Shannon index of alpha diversity, which means that higher levels of gut inflammation are associated with less alpha diversity which might be related to dysbiosis and depletion of beneficial microbiota from the oral cavity of CD patients.

4.1 Limitations

The limitations to our study include low sample size, as we managed to recruit 40 CD patients only, due to the COVID-19 pandemic as various precautional measures caused many restrictions to access the hospitals and patients were concerned about providing saliva samples since it is an infectious liquid. The patients targeted were receiving their medication in the infusion clinic and some in the endoscopy department after the colonoscopy procedure. The patients in the infusion clinic had only 30 minutes to complete the intravenous medication, and the challenge was to be quick and to avoid any disturbance to the patients. While the patients post colonoscopy were exhausted since they had to fast for the procedure, so some of them refused to participate due to dry mouth.

Chapter 5: Conclusion

Crohn's disease is an inflammatory bowel disease previously suggested to have an unknown cause and its main etiology is still a mystery. Nevertheless, our study and findings can give us a hint that there might be many underlying factors that lead or contribute to CD. Our research focuses on the interplay of multiple factors contributing to dysbiosis by studying the salivary microbiome in CD subjects and comparing it to HC group. Our results demonstrated significant bacterial biomarkers in the saliva that are unique in CD when compared to HC. Depletion of beneficial bacteria in CD and enrichment of pathogenic ones was clear. In addition, HC had bacteria that are significant and not present in CD which again confirms dysbiosis.

We were able to decipher the most important factors contributing to dysbiosis in CD patients. Each factor seems to have a unique effect on the oral microbiome, nevertheless, oral health status was found to be the most impactful factor. Poor oral health contributes to oral dysbiosis and hence can induce bowel inflammation, especially oral periodontal disease such as periodontitis which is obviously an inflammatory condition. CD is unfortunately up to this date incurable but with IBD medications, symptoms can be reduced. We explored the effect of medications for the treatment of CD with the monoclonal antibody (infliximab), and other medications like steroids, and immunosuppressants that were concurrently administrated with infliximab to reduce symptoms or hasten recovery. In both cases, these medications can disturb the normal microbiota in addition to the emergence of novel microbial species and even ones that are pathogenic or opportunistic. The activity of the disease also greatly influences the composition of the oral microbiota, so if the disease is active meaning it is not fully treated yet or the inflammation is still in process makes a remarkable variation in comparison to an inactive disease, remission state or when inflammation is nearly resolved. The frequency of relapse of symptoms gave us a deduction of the importance of having this information in mind when looking for bacterial features that correlate to CD. As for the duration of the disease, there was obvious detection of different bacterial species in newly diagnosed patients and patients living with the disease for longer periods, which explains the clear shift in the normal flora. Lower diversity was found in

CD patients due to the depletion of beneficial microbiota. As saliva is a compelling oral secretion; in this research we measured the values of salivary CRP and CAL in healthy subjects and compared them with the salivary values of CD. We concluded that saliva is not only a liquid that can detect similar microbiome to the intestine, but can also detect some degree of inflammation especially the salivary CAL. Therefore, saliva can be used as a tool to detect bacterial dysbiosis and some degree of inflammation, since it is less invasive and more convenient.

5.1 Recommendations and Future Directions

These findings could provide prognostic information, allowing for the identification of patients at high risk of developing the disease or recurrence and laying the groundwork for a more targeted and prophylactic approach or an early therapeutic intervention. Understanding CD pathogenesis and discovering novel biomarkers for CD may be aided by research into oral microbiota dysbiosis. We aim to encourage the maintenance of microbiome homeostasis that is the key to well-being, bringing back the natural balance of normal microbiota. In addition, raising the awareness of oral hygiene is of paramount significant by providing advice from dentists that must work in collaboration with gastroenterologists, improving the oral health to avoid dental caries and periodontal inflammation that will reduce CD pathogenesis.

Future research on microbiota modulation with probiotics, prebiotics and fecal microbiota transplantation is strongly recommended. A novel idea is the checking of the homeostatic level of the normal flora before the administration of the correct microbiota modulation agents to prescribe the correct bacterial combination.

More studies including larger sample size are also recommended in the future. Longitudinal studies may be needed to monitor microbiota changes over time. Functional analysis to detect the metabolites of the bacteria and their relation to inflammation are also recommended in future studies.

Our study is considered unique as this type of salivary microbiome work is established for the first time in the UAE, utilizing sequencing technique down to the species level, in addition to the involvement of multiple experimental factors that adds to its uniqueness.

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Appendices

Appendix A

Data collection Sheet

Study ID:

General information

1. Patient's name:
2. Age:
3. Nationality:
4. Gender: □ Male □ Female
5. Occupation:

Medical and surgical history

History of systemic diseases:

 Cardiovascular diseases:
 Hypertension:
 Diabetes: □ Type 1 □ Type 2
 Thyroid dysfunction:
 Asthma:
 Allergy:
 Any other medical problems:
 Any surgeries:
 Smoking or use of any tobacco products

□ Yes
□ No

Inflammatory bowel disease

1. Type of inflammatory bowel disease:

□ Crohn's Disease
□ Others:

2. When was the condition diagnosed?

Please give month and year:
Age at diagnosis:

3. Family history of inflammatory bowel disease:

4. Do you know what area of your body is affected by Crohn's disease? If so, indicate below.

□ Small intestine
□ Large intestine or colon
□ Perianal region or anorectal region
□ Symptoms outside the digestive tract/in other parts of the body
□ Unknown

5. How often do you experience a relapse of symptoms?

□ 0-1 times per year
□ 2-4 times per year
□ More than 4 times per year
□ Connuous symptoms

6. When were your last symptoms of bowel disease requiring you to attend your doctor?

7. Have your symptoms required admission to hospital?

□ No

□ Yes

If ‘yes’, please give the dates: From To

8. Have you had any complications of your bowel condition, such as anemia, or problems with your liver, skin, eye or joints?

□ No

□ Yes

If ‘yes’, please give details below:

9. Symptoms of Crohn’s disease can be classified as mild, moderate, or severe depending on how many symptoms you have.

Do you have any of the following signs or symptoms of active Crohn’s disease?

□ Fever

□ Abdominal pain

□ Blood in stool

□ Weight loss

□ Diarrhea

□ Diarrhea that wakes you up at nighttime

□ Joint pain

□ New skin rashes or sores in the mouth

□ Others, specify :

Management of inflammatory bowel disease including medications

1. Have you taken or are you taking any **medication** for your bowel condition?

□ No

□ Yes

If ‘yes’, please tell us the **name of the medication prescribed and the period(s) of use below**:

Name of medication, dose and duration

2. How often are you treated with **steroids**?

□ More than twice a year

□ Twice a year

□ Once or less than once a year

□ Never

If you have needed treatment with steroids, when did you last need steroid treatment?

3. Use of antibiotics the last one month: □ Yes □ No

4. Use of any immunosuppressant medications in past 6 months (such as cyclosporine, azathioprine):

□ Yes □ No

5. It is very important that patients with Crohn's disease take all of their prescription medications as prescribed. Which of these statements applies to you?

□ I never miss my medication for Crohn's disease

□ I miss at least three doses of medication per week

□ I miss at least one dose of medication per day

□ I miss multiple doses of medication per day

□ I am not taking my medication for Crohn's disease

6. Have you had an **operation** to treat your bowel condition?

□ No

□ Yes

If 'yes', please tell us the name and date of the operation(s):

7. Are you waiting for an operation to treat your bowel condition?

□ No

□ Yes

If your answer is 'Yes', please tell us the date your operation is due:

Investigations and diagnostic tests

1. How often do you have colonoscopy reviews for your bowel condition?

Result of your latest colonoscopy (can be obtained from the patient's records):

Colonoscopy findings:

2. Do you have any reports or letters from the specialist about your condition?

□ Yes

□ No

Results of laboratory tests (if available)

- Genetic analysis
- Complete blood count (CBC)
- Liver tests
- Iron studies
- C-reactive protein
- Other biomarkers:
- Stool studies
- Osteoporosis screening
- Screening for cancers

Nutrition

1. Are you getting the nutrition you need, including any supplements?

2. Patients who require medication for Crohn's disease have special nutritional requirements.

Please check off which statements apply to you.

□ I eat a healthy and balanced diet

□ My weight is stable

□ I am on calcium/vitamin D supplements

□ I take a multivitamin

Appendix B

Dental Examination Form

Oral Health:

1. Frequency oral hygiene is performed: once daily ☐ twice daily ☐ three times a day ☐ rarely/ not done related to uncooperative behavior
2. Method of oral hygiene: ☐ toothbrush ☐ flossing ☐ inter-dental cleaning

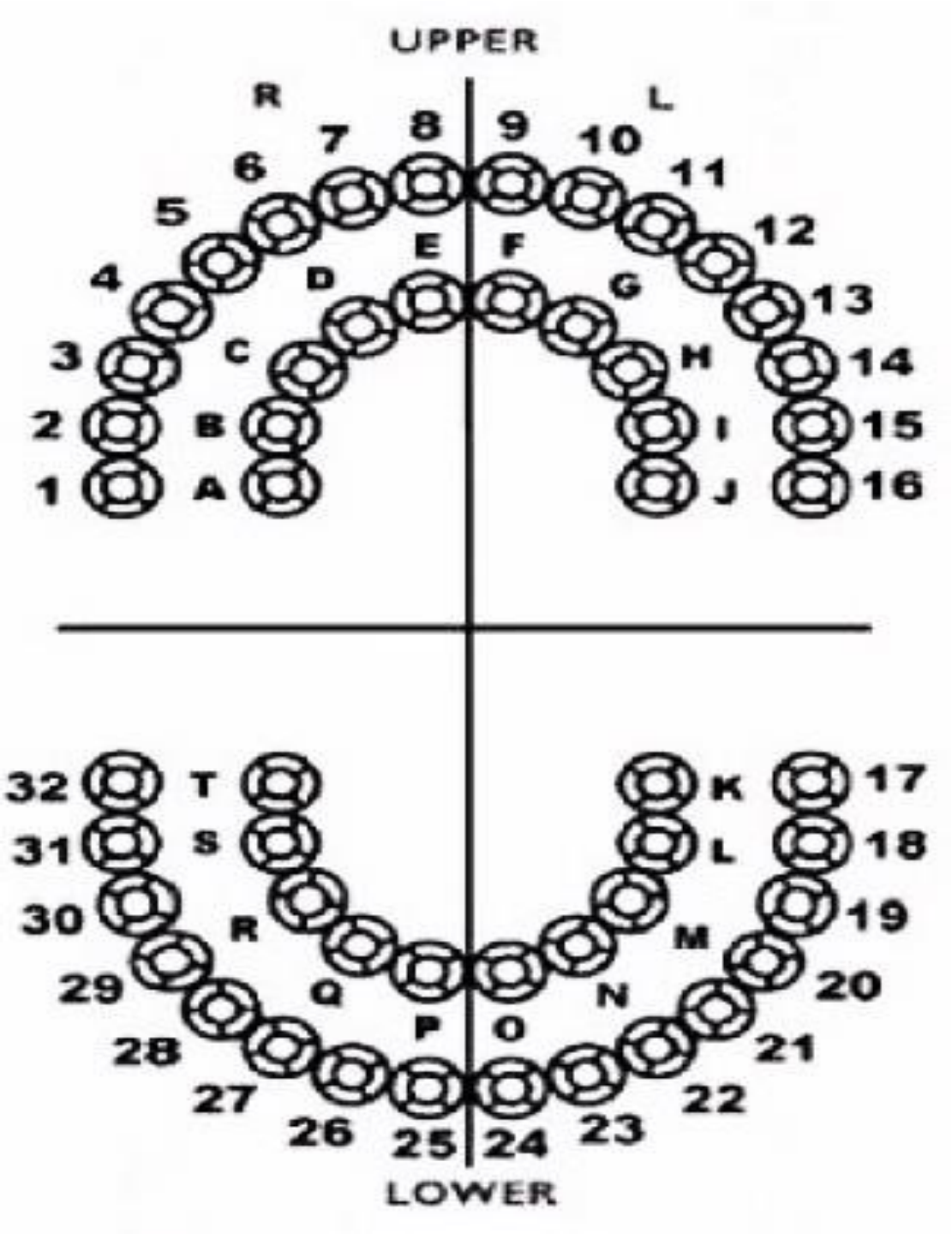
: ☐ **No** bleeding associated with oral hygiene

☐ Bleeding **sometimes** associated with oral hygiene

☐ Bleeding **always** associated with oral hygiene

Oral Findings:	**Oral Conditions:**
Plaque ☐	General _____
Stains ☐	Mouth floor _____
Abrasion/Ulceration ☐	Palate _____
Gingivitis ☐	Cheeks _____
Periodontitis ☐	Lips _____
Cavities ☐	Tongue _____
Tooth infection ☐	Throat/Neck _____
Cracked tooth ☐	Frenum _____
Broken tooth ☐	Ridges _____
Soft tissue ☐	
Bruxism ☐	
TMD ☐	
Calculus ☐	
Occlusion ☐	
Wisdom teeth impacted ☐	
Dry mouth ☐	

3.Gum assessment: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UAE UNIVERSITY MASTER THESIS NO. 2023:18

Microbiota is necessary to maintain a balanced gut environment which is essential for good health. Dysbiosis can predispose to many diseases including CD. The oral cavity has the second largest and most diverse microbiota after the gut harboring over 700 species of bacteria. This study aims to investigate the alterations in the salivary microbiome in patients with CD compared to healthy controls (HC). It also aims to compare CD patients for salivary microbiome complexity and diversity according to different factors that can contribute to dysbiosis, including oral health, IBD drug use, disease duration, activity of the disease and relapse of symptoms.

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