Comparison of Intestinal, Oral, Skin and Eye Microbiomes in Patients with Behçet's Disease

by

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Comparison of Intestinal, Oral, Skin and Eye Microbiomes
in Patients with Behçet's Disease

Koç University
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To my mentors, shaping my path ahead;

To family & friends, supporting me every step...

ABSTRACT

Introduction

Behçet disease (BD) is a chronic inflammatory disorder with multisystemic involvement. Various factors have been implicated in its pathogenesis, including dysregulation of both the innate and adaptive immune systems. Moreover, alterations in microbiota composition across different body sites have been associated with several chronic diseases, including BD. The aim of our study was to investigate microbiota changes in skin, fecal, oral, and ocular samples of BD patients during both attack and remission phases.

Materials and Methods

A total of 20 patients diagnosed with Behçet disease were included in this longitudinal follow-up study, conducted over a period of two years. Patients were monitored during both attack and remission periods. Demographic data and clinical findings were collected throughout the study. For microbiota analysis, samples were obtained from the antecubital and popliteal fossae (skin), feces, oral cavity, and conjunctival surfaces (eye). DNA was extracted from all samples, and 16S rRNA gene amplification was performed using V1–V9 primers, followed by sequencing. Microbial diversity was assessed using alpha and beta diversity indices, and taxonomic composition was evaluated through relative abundance analysis.

Results

In fecal samples, a statistically significant increase in the Firmicutes to Bacteroidetes ratio was observed during the attack phase (77% vs 84% for Firmicutes, and 19% vs 4% for Bacteroidetes), indicating gastrointestinal dysbiosis. At the species level, Faecalibacterium prausnitzii —a beneficial butyrate-producing bacterium—was reduced in attack samples (3% vs 14%). At the genus level, Catenibacterium was more abundant in remission samples (32% vs 23%), while Collinsella was relatively higher in attack (10% vs 7%).

Regarding the skin microbiome, at the genus level, Staphylococcus was the most dominant taxon in both control and attack samples; however, its abundance was lower in the attack group (53.0%) compared to controls (62.0%). Similarly, Cutibacterium was reduced in the attack samples (2.1%) compared to controls (4.5%). In contrast, Staphylococcus was more abundant in attack samples than in remission (45% vs 27%).

In the oral microbiota, dominant genera included Streptococcus, Haemophilus and Veillonella. A notable decrease in Haemophilus was found during attacks (3% vs 12%), while Prevotella was relatively increased in remission samples.

In eye samples, Staphylococcus and Corynebacterium were less abundant in the attack group (9% vs 21% and 12% vs 19%, respectively). Conversely, Pseudomonas was markedly higher in the attack group (69% vs 39%). At the species level, Pseudomonas aeruginosa was significantly more abundant in attack samples. Staphylococcus epidermidis was reduced in attack (5% vs 15%), while Cutibacterium acnes remained stable (8% in both groups). In remission, Pseudomonas aeruginosa abundance decreased (60% vs 71%) and Staphylococcus capitis abundance increased (6% vs 1%).

Conclusion

Our study demonstrated that during the attack phase in BD patients, an increased Firmicutes to Bacteroidetes ratio in fecal samples might reflect intestinal dysbiosis. Short-chain fatty acid–producing bacteria were reduced during active disease and recovered in remission. In the skin microbiota, fluctuations in Staphylococcus suggested a disrupted cutaneous microenvironment during disease activity. In oral samples, decreased Haemophilus and increased Prevotella might reflect local mucosal dysregulation. Ocular microbiota of BD patients during attacks showed increased Pseudomonas and could be associated with ocular inflammation. Overall, our findings highlighted site-specific microbial shifts associated with disease state and suggested a potential role of microbiota in the pathophysiology of Behçet disease.

Keywords: Behçet's Disease, Microbiota, Oral, Fecal, Skin, Eye

ÖZETÇE (TÜRKÇE ABSTRACT)

Giriş

Behçet Hastalığı pek çok sistemin etkilendiği multisistemik bir hastalıktır. Doğuştan gelen ve edinsel immün sistem aralarında olmak üzere çeşitli faktörler hastalığın patogenezini açıklamada ileri sürülmüştür. Bunun yanında, vücudun farklı bölgelerinde mikrobiyota değişiklikleri, Behçet Hastalığı dahil olmak üzere, kronik hastalıklarda bildirilmiştir. Bu çalışmanın amacı, bu doğrultuda, atak ve remisyonda Behçet Hastalığında görülen deri, fekal, oral ve göz mikrobiyom değişikliklerinin saptanmasıdır.

Yöntem

İki yıl longitudinal takiple, 20 adet Behçet hastası çalışmaya dahil edilmiştir. Hastalar atak ve remisyon periyotları boyunca takip edilmiş, demografik veriler ve klinik bulgular ayrıca kaydedilmiştir. Mikrobiyom analizi için örnekler antekubital ve popliteal fossalar (deri), gaita/dışkı, oral kavite ve konjonktival yüzeylerden (göz) elde edilmiştir. Tüm örnekler DNA ekstraksiyonu, V1-V9 primerleri aracılığıyla gen amplifikasyonu ve sekanslamaya tabii tutulmuştur. Mikrobiyal diversite analizi alfa çeşitliliği ve beta çeşitliliği (alpha/beta diversity) ile gerçekleştirilmiş, taksonomik kompozisyon ise relative abundance analizi (göreceli bolluk) ile irdelenmiştir.

Bulgular

Fekal örneklerde, Firmicutes/Bacteroidetes oranında istatiksel olarak anlamlı bir artış saptanmıştır. Atak vs kontrol Firmicutes için %77 vs %84, Bacteroidetes için %19 vs %4 bulunmuş olup gastrointestinal disbiyozu destekler niteliktedir. Species (tür) incelemesinde, Faecalibacterium prausnitzii'de —butirat üreten yararlı bir bakteri— atak örneklerinde azalma görülmüştür (%3 vs %14). Genus (cins) incelemesinde, Catenibacterium'un remisyon örneklerinde (%32 vs %23), Collinsella'nın ise atakta (%10 vs %7). daha çok olduğu izlenmiştir.

Deri mikrobiyomu incelemesinde, hem atak hem remisyon örneklerinde genus düzeyinde en büyük grup Staphylococcus bulunmuştur; ancak relative abundance atak örneklerinde (%53.0), sağlıklı kontrollerden (%62.0) daha az saptanmıştır. Benzer şekilde, Cutibacterium atak örneklerinde (%2.1), sağlıklı kontrollere (%4.5) kıyasla azalmıştır. Buna karşın, Staphylococcus atak örneklerinde remisyondan fazla saptanmıştır (%45 vs %27).

Oral mikrobiyotada baskın genera Streptococcus, Haemophilus ve Veillonella bulunmuştur. Atak örneklerine Haemophilus'ta belirgin bir azalma (%3 vs %12) saptanırken, Prevotella'nın remisyon örneklerinde yüksek olduğu görülmüştür.

Göz örneklerinde, Staphylococcus (%9 vs %21) ve Corynebacterium'un (%12 vs %19) ataklarda daha az olduğu ortaya çıkmıştır. Buna karşılık, Pseudomonas atak örneklerinde belirgin olarak daha yüksektir (%69 vs %39). Species düzeyinde, Pseudomonas aeruginosa atak örneklerinde anlamlı ölçüde yüksektir. Staphylococcus epidermidis ataklarda azalmış (%5 vs %15), Cutibacterium acnes sabit kalmıştır (%8). Remisyonda, Pseudomonas aeruginosa azalmış (%60 vs %71), Staphylococcus capitis artmıştır (%6 vs %1).

Sonuç

Çalışmamız, Behçet hastalarında atak döneminde fekal örneklerde artmış Firmicutes/Bacteroidetes oranını ve olası bir disbiyozu ortaya koymuştur. Aktif hastalıkta kısa zincirli yaş asidi üreten bakterilerin azaldığını, remisyonda ise yeniden arttığını göstermiştir. Deri miktobiyomunda görülen Staphylococcus değişimleri, kutanöz mikroçevrenin bozulduğuna işaret etmiştir. Benzer şekilde oral örneklerde, azalmış Haemophilus ve artmış Prevotella lokal mukozal disregülasyonu düşündürmüştür. Atak döneminde oküler mikrobiyomda görülen artmış Pseudomonas'ın oküler inflamasyonla ilgili olabileceği düşünülmüştür. Sonuç olarak çalışmamız Behçet hastalığı ilişkili spesifik mikrobiyom değişikliklerini aydınlatmış ve hastalık patofizyolojisinde mikrobiyomun sahip olabileceği rolü ortaya koymuştur.

Anahtar Kelime: Behçet Hastalığı, Mikrobiyota, Oral, Fekal, Deri, Göz

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ABBREVIATIONS

BD	Behçet’s Disease
BPB	Butyrate-Producing Bacteria
CD	Cluster of Differentiation
DNA/(m)RNA	Deoxyribonucleic Acid/(messenger) Ribonucleic Acid
eNOS	Endothelial Nitric Oxide Synthase
ERAP	Endoplasmic Reticulum AminoPeptidase
FMF	Familial Mediterranean Fever
HLA	Human Leukocyte Antigen
ICAM	Intercellular Adhesion Molecule
ICBD	International Criteria for Behçet's Disease
IFN	Interferon
IL	Interleukin
ISG	International Study Group
KUISCID	Koç University İşbank Center for Infectious Diseases
LFA	Lymphocyte Function-associated Antigen
MBL	Mannose-Binding Lectin
MHC	Major Histocompatibility Complex
NET	Neutrophil Extracellular Trap
NO	Nitric Oxide
PCoA	Principal Coordinates Analysis
RAS/RAU	Recurrent Aphthous Stomatitis/Recurrent Aphthous Ulcers
ROR	RAR-related Orphan nuclear Receptor
SRB	Sulphate-Reducing Bacteria
Th	T helper cells
T-reg	Regulatory T cell
TCR	T-Cell Receptor
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor

CHAPTER 1
INTRODUCTION

This section will serve as an introduction towards Behçet's disease (BD). To better illustrate the concept of this study, clinical properties of the entity will be scrutinized first, followed by a separate section dedicated to the underlying pathophysiology of the disease process.

1.1. Behçet's disease from a clinical perspective

1.1.1. Epidemiology

Although described meticulously by Hulusi Behçet in the 20th century, Behçet's disease dates further back in history, possibly to the times of Hippocrates[1]. During many decades, the disease has been described among people from the Silk Route, spanning from Eastern Asia to Mediterranean[2]. Current prevalence studies rank countries located in the corresponding geographic region among the ones with highest disease incidence, as represented in Figure 1.1[3]. Turkey in this regard leads the list with 17-42 per 10.000, closely followed by Iran and Iraq[3-5].

In other regions such as western Europe, although the disease prevalence is relatively low, the ancestry of a significant portion of the patients can be traced back to silk route countries (i.e. history of immigration). In a French study, the prevalence of Behçet's disease was calculated as 7,1 per 100.000 people in Paris. Detailed ancestry analysis showed prevalence was higher in people of North African (34,6 per 100.000) or Asian (17,5 per 100.000) descent, compared to Europeans (2,4 per 100.000)[6]. Other studies focusing outside of the silk route argued the disease prevalence to be 8.9-10.6 per 100,000 in the American Southwest[7] and 5.2 per 100,000 in North[8].

Although the disease is prototypically described in young to middle aged men (20-40 years), larger cohorts demonstrate that both sexes may be effected and age range is actually wide, also covering the pediatric population[9,10]. According to certain authors, the onset of Behçet's disease coincides with childhood in almost one fifth of cases[11]. Compared to

sporadic cases that constitute the majority of clinical practice, studies describing multiple members of the same family affected from the disease exist, and genetic factors are argued behind the familial aggregation[12,13].

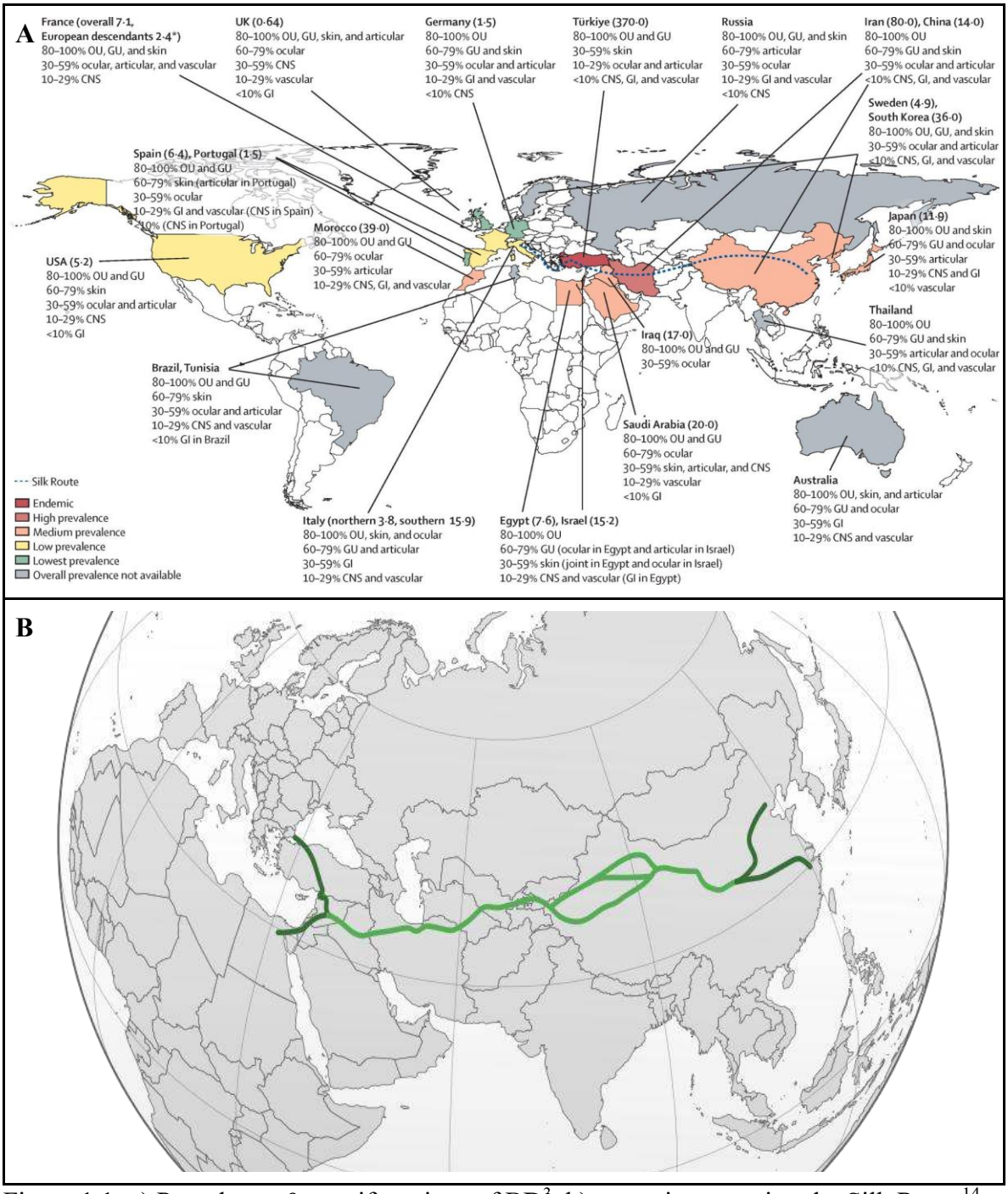

Figure 1.1: a) Prevalence & manifestations of BD[3], b) countries spanning the Silk Route[14].

1.1.2. Clinical Signs & Symptoms

Although the disease is referred to as a vasculitis that can affect vessels - both arteries and veins - of practically any size, clinical findings can be grouped according to organs/systems involved that necessitate clinical care[15].

1.1.2.1. Oral ulcers

In most patients, oral ulcers are among the first findings of the disease. Commonly referred to as canker sores, the ulcers are similar to aphthous stomatitis in many ways. These ulcers are painful and in severe instances may interfere with daily activities of speaking, chewing and swallowing, depending on the location. Ulcers of Behçet's disease are oftentimes multiple in nature and affect the mouth more extensively compared to common oral ulcers. The lesions, as represented in Figure 1.2, are characterized by shallow ulcerations with well-defined rounded contours, and are called minor and major ulcers, regarding the size limit of 1 cm. Although single lesions last around a week or two, occasionally new lesions develop before previous ones fade and patients continuously suffer from ulcers for longer times. Ulcers heal spontaneously, however treatment with oral phosphodiesterase 4 inhibitors are approved in certain countries such as the United States and Japan[16].

1.1.2.2. Genital ulcers

Similar to oral ulcers, genital ulcers are among the cardinal features of Behçet's disease, arguably the most specific finding. The ulcers are painful, and are located commonly in the vulva in female and in the scrotum in male patients and heal by scarring. Ulcers raise functional and esthetic concern and might interfere with sexual intercourse. Other than ulcers, epididymo-orchitis and varicoceles are also described in Behçet's disease patients among urogenital problems[17]. Coexistence of genital and oral ulcers is common in patients during initial evaluation for Behçet's disease[18], also seen in Figure 1.2. Yet, diagnosis of Behçet's disease requires more findings, as oral-genital ulcers can be seen in different syndromes such as PFAPA (periodic fever, aphthosis, pharyngitis, and adenitis) or overlap syndromes such as MAGIC (mouth and genital ulcers with inflamed cartilage)[19,20].

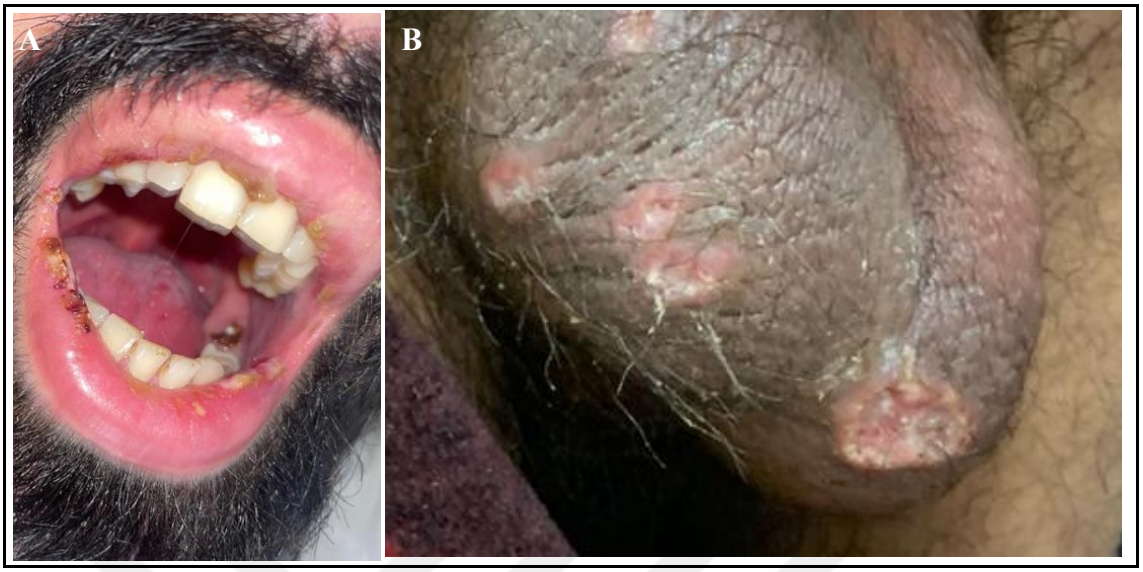

Figure 1.2: Oral and genital ulcers in a patient with Behçet's disease.
Courtesy of Mehmet Engin Tezcan, MD

1.1.2.3. Ocular lesions

Ocular involvement usually represents posterior uveitis or pan-uveitis (anterior and posterior uveitis) and is the first finding in 20% of patients[21,22]. Isolated anterior uveitis is rare and hypopyon is usually accompanied by involvement of the posterior as well. Uveitis in the setting of Behcet's disease is typically bilateral and non-granulomatous[23]. Vision impairment is common and permanent, in case of untreated retinal arteritis or optic neuritis. Examples can be appreciated in Figure 1.3 (insets a and b).

1.1.2.4. Cutaneous lesions

Present in at least half of the patients at some stage during the course of the disease, cutaneous manifestations of Behçet disease deserves special attention[24]. There exists multiple lesions that have been described in the setting of Behçet's disease, however none of them by itself is pathognomonic for the disease and may be seen in other clinical entities as well. Papulopustular lesions are argued to be the most common skin manifestation of Behçet's disease and are characterized by development of acne-like papules on an erythematous base that develop into pustules in the following 1-2 days. Folliculitis-like lesions can also be seen in BD, as seen in Figure 1.3 (inset c).

Erythema nodosum-like lesions are also common during the course of disease and have been described in female patients. Lesions are mostly seen in lower extremities and heal in 21 days. Although clinically similar to classical erythema nodosum lesions, histologically those in the setting of Behçet's disease differ by the presence of vasculitis[25]. Superficial thrombophlebitis also occurs in Behçet's disease and sometimes creates confusion with erythema nodosum. Thrombophlebitis is typically seen in the extremities of male patients and clinically can be described as linearly distributed, painful nodules that can be palpated superficially[26]. Lastly, extragenital skin ulceration are described among the manifestations of the disease and these recurrent, scarring ulcers have been argued to be rare in diseases other than Behçet's disease[24].

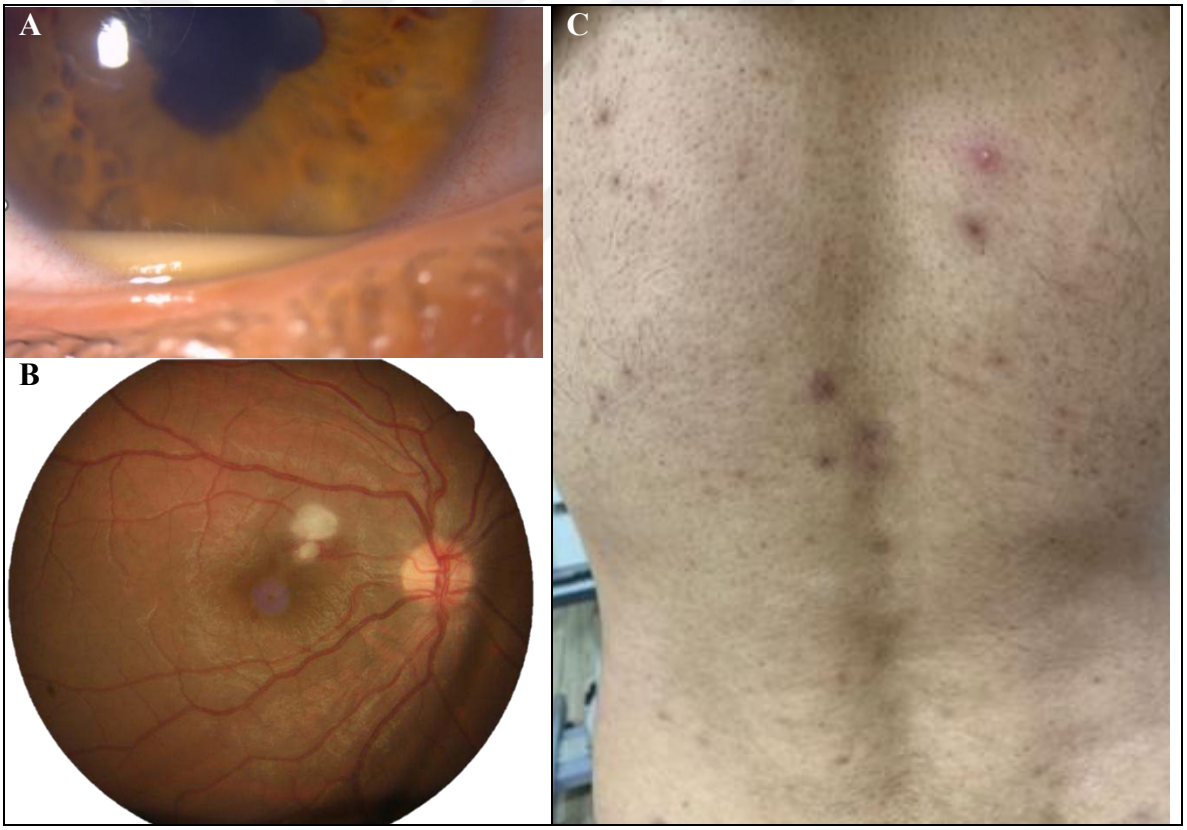

Figure 1.3: Ocular and cutaneous manifestations of Behçet's disease.
a) hypopyon and posterior synechia; b) retinitis; c) folliculitis.
Courtesy of Nilüfer Zorlutuna Kaymak, MD (a,b) and Mehmet Engin Tezcan, MD (c).

1.1.2.5. Vascular disease

Behçet's disease is unique among other diseases associated with vasculitis, in the sense that it can affect vessels of various size (i.e. from large caliber vessels to smaller ones), both in the arterial and venous side of the circulation. Besides the large repertoire of symptoms that can cause clinical confusion, the vascular pathologies deserve emphasis as they cause significant morbidity and mortality[27].

Regarding its prevalence, vascular lesions are rare compared to ocular, genital or cutaneous manifestations of Behçet's disease. In a large Turkish cohort (n=2319), they were present in around 15% of patients, typically in young-middle aged patients (mean age: 30+/-8 years) with a male to female ratio of 5.26:1[28]. Superficial vein thrombophlebitis, also described in the previous section, was the most common vascular manifestation, followed by deep vein thrombosis, seen in 50% and 30% of patients with vascular involvement, respectively. In that cohort, arterial pathologies were much rarer, around 3-4% of patients.

Since many vessels can be affected, findings in the venous side range from superficial thrombophlebitis to large vessel obstruction, such as vena cava occlusion or Budd-Chiari syndrome[29]. In the arterial side, besides the more common small vessel vasculitis, serious morbidity ensues upon aneurysms or thrombosis affecting the pulmonary arteries and intracardiac thrombosis[30,31]. Examples from literature can be seen in Figure 1.4[3,30,32].

1.1.2.6. Neurologic disease

Neurologic involvement in Behçet's disease, also known as Neuro-Behçet syndrome, is rare compared to other manifestations[15]. Yet, similar to the previous section on vascular involvement, neurologic impairments cause significant impairment for patients, potentially being the reason for death. Besides vascular pathologies involving the brain, brainstem or the spinal cord; parenchymal pathologies involving directly these structures are also described in Neuro-Behçet syndrome. Psychiatric disturbances, cognitive impairments, focal neural deficits, vision abnormalities and intracranial hypertension have been described in patients with Behçet's disease[15].

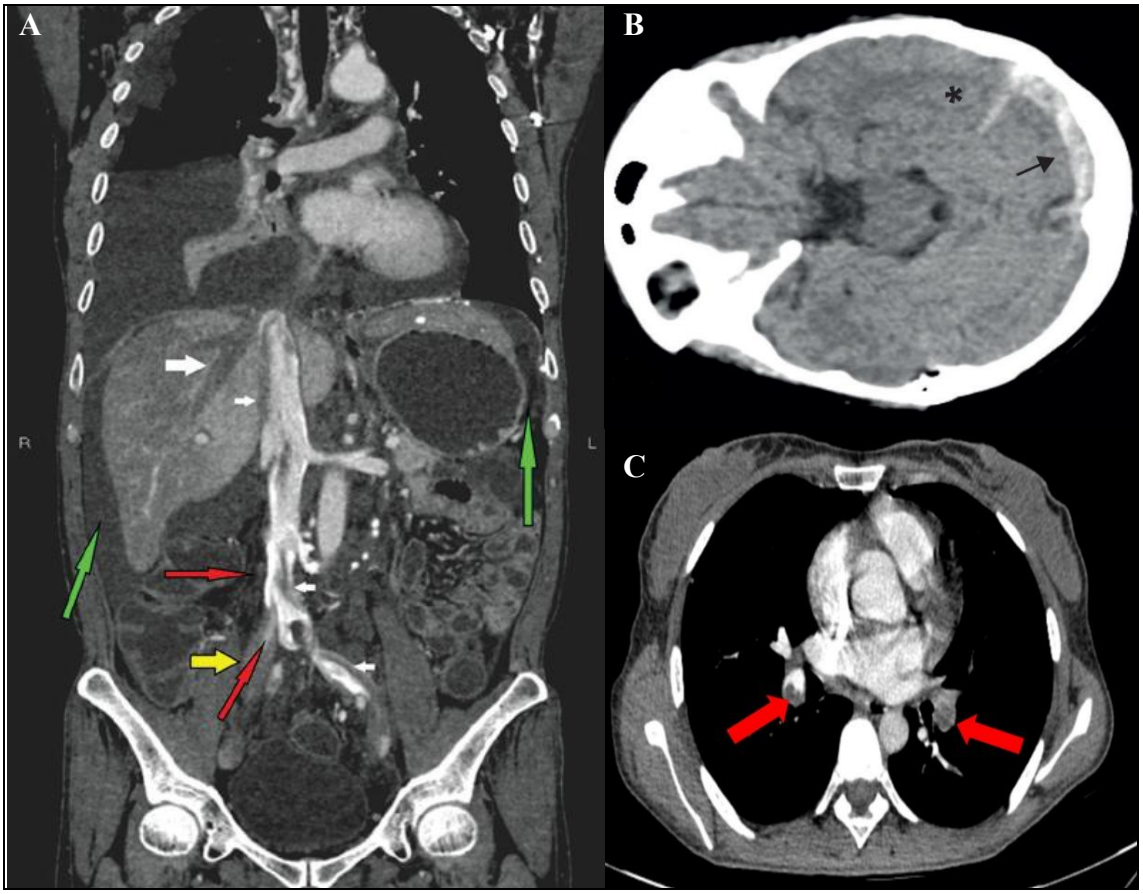

Figure 1.4: Vascular involvement of BD, examples from literature.

a) Budd-Chiari Syndrome with impaired flow through hepatic vein due to thrombus[32]; b) Thrombosis of left transverse sinus[3]; c) Pulmonary artery thrombosis with filling defect[30].

1.1.2.7. Involvement of other systems

Gastrointestinal involvement in Behçet's disease can be evaluated in two groups: mucosal inflammation and intestinal ischemia, due to small and large-caliber vascular involvement respectively[33,34]. Well-demarcated, deep-seated ulcers in the ileocecal region and volcano-shaped ulcers in the colon have been described, creating confusion with inflammatory bowel disease ulcers. Symptom-wise, patients describe a wide range of symptoms including bloating, diarrhea/constipation, abdominal pain, nausea/vomiting and weight loss. More serious, potentially fatal complications including fistulas and bowel perforation have also been reported[35].

Renal involvement has been partially attributed to vasculitis in Behçet's disease, while parenchymal mechanisms have also been mentioned, including amyloidosis (AA type) as the major cause of renal failure[36]. Glomerulonephritis and less commonly, interstitial nephritis are other manifestations described in Behçet's disease[37].

Behçet's disease has a propensity for larger joints such as wrist, knee and ankle. Symptoms peak typically developing during disease flare-ups and characteristically follows an oligoarticular pattern[38]. Although non-deforming, arthritis-related pain and functional impairment have been described as debilitating by patients[39].

As explained previously, Behçet's disease is a multisystemic disease[15], necessitating holistic care from multiple different specialties. Patients in general require follow-up for a long time and goals include alleviating symptoms during acute exacerbations, prolonging remission times and preventing new attacks in the future.

1.1.3. Diagnosis

As the patterns of systemic involvement are diverse and no finding per se is sufficient for a diagnosis of Behçet's disease, diagnosis requires coexistence of multiple findings associated with Behçet's disease[15]. Diagnostic approach varies depending on the clinician and the context. However, in general, guidelines suggested by international experts are used and clinical suspicion is often higher in patients from endemic countries, spanning middle east and mediterranean basin.

1.1.3.1. International Study Group (ISG) Diagnostic Criteria

The ISG approach is the most widely used system in diagnosis of Behçet's disease. It is used worldwide and is developed upon the initial criteria suggested in 1990[40]. In this approach, the cardinal feature is presence of recurrent aphthous ulcers. It is defined as having 3 attacks (or more) in a year and can be discovered by the physician or simply recounted by the patient. It should not be better explained by another systemic disease, i.e. should be idiopathic.

Among those with recurrent oral ulcers, presence of any 2 of the 4 findings explained below is sufficient for the diagnosis of Behçet's disease:

1. Recurrent genital ulcers, as explained in Section 1.1.2.2.
2. Eye involvement, as explained in Section 1.1.2.3.
3. Skin findings, as explained in Section 1.1.2.4.
4. Positive pathergy test, see Section 1.1.3.3.

1.1.3.2. The International Criteria for Behçet's Disease (ICBD)

With the goal of increasing sensitivity compared to ISG, the ICBD was first created in 2006 and re-evaluated afterwards[41]. Although it is less accepted today compared to ISG Diagnostic Criteria, it deserves mention here, as multiple studies have proved its validity[42,43].

ICBD relies on getting a score of at least 4 points using the following algorithm[15]:

- **Oral ulcers : 2 points**
- **Genital ulcers : 2 points**
- **Ocular involvement : 2 points**
- Skin involvement : 1 point
- Positive pathergy test : 1 point
- Vascular involvement : 1 point
- Neurologic involvement : 1 point

1.1.3.3. Pathergy test

Pivotal in the diagnosis of Behçet's disease, pathergy phenomenon was first described in 1937[44,45]. Back then, it was mentioned as an abnormal response to minute trauma. During the diagnostic procedure that is used in the evaluation of possible Behçet's disease today, sterile 20-gauge needles are used to simulate minor trauma to the skin. The area is observed for the next 48 hours to see if a papule develops. Occasionally, pustules develop as well. The latter are sterile and hence different from bacterial diseases or folliculitis.

In BD, pathergy phenomenon reflects a broader concept of hypersensitivity and hence can be encountered in distinct organs/systems other than the skin, e.g phlebitis or synovitis

after invasive procedures involving the vessels and joints. Although also described in other dermatologic diseases including pyoderma gangrenosum and Sweet syndrome, pathergy test is still a reliable tool in diagnosis of Behçet's disease[45]. Examples of a negative pathergy test and positive tests with papule and pustule formation are illustrated in Figure 1.5[46].

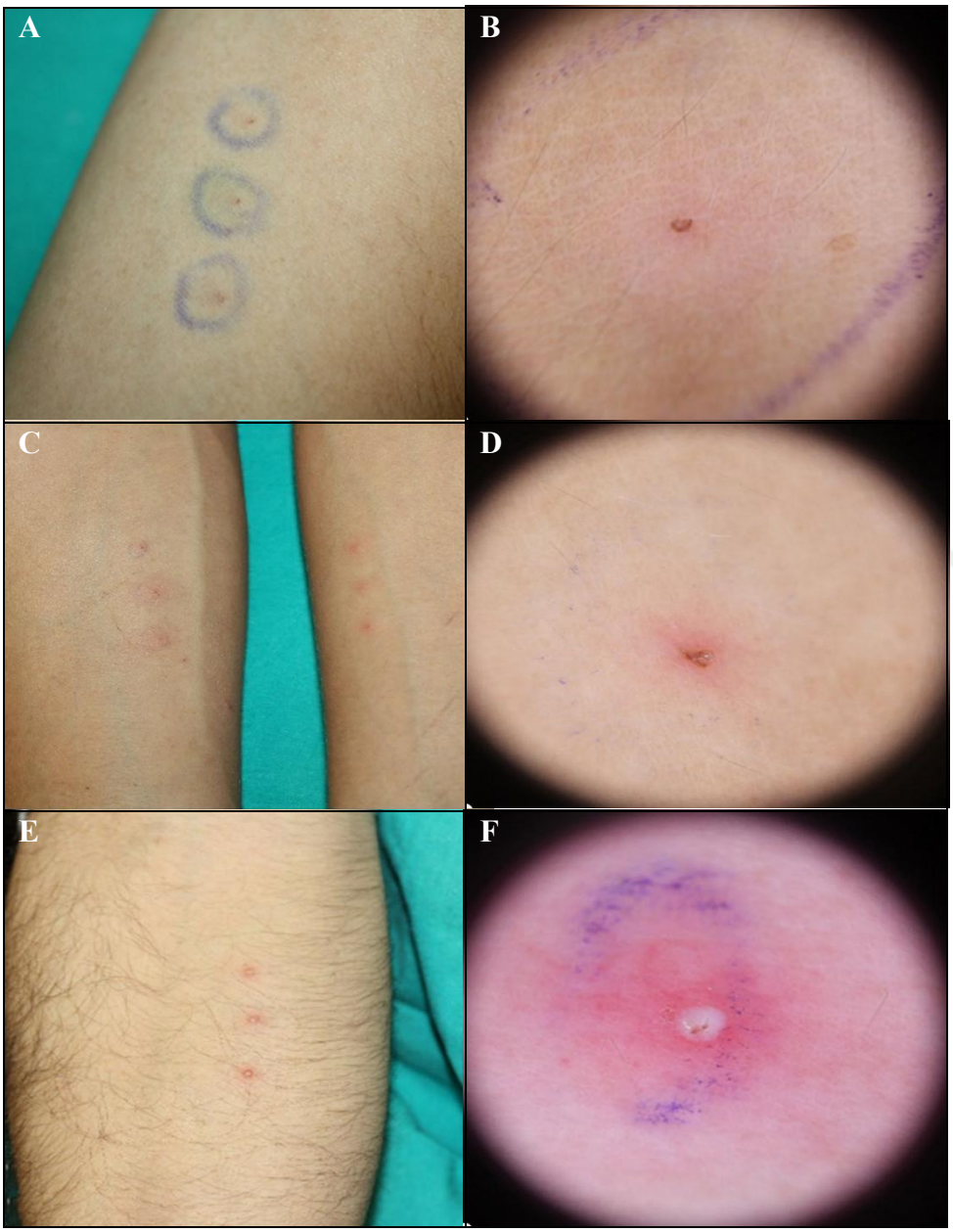

Figure 1.5: Different outcomes in pathergy test.

(a, b) Negative result, (c,d) papule and (e,f) pustule formation, seen with the naked eye (left) and via dermatoscopy (right). Adapted from Kecici AS. et al.[46]

1.2. Etiology & Pathogenesis of Behçet's disease

It has not been possible to draw a causative effect between a certain etiologic factor and development of Behçet's disease over the years. Multiple factors have been attributed to disease development, with underlying pathophysiology not fully elaborated. Behçet's disease is centered in between autoimmune and autoinflammatory diseases and has been repeatedly explained by vasculitis as the core event[47]. In this section, factors reported in Behçet's disease will be listed.

1.2.1. Human Leukocyte Antigen B*51

Similar to many other autoimmune diseases, specific Human Leukocyte Antigen (HLA) types have been evaluated for disease development and severity. Regarding Behçet's disease, studies have found HLA-B*51 to be common both among patients and endemic countries. Takeno et al. reviewed HLA-B*51 prevalence in this scope and have found that 75% of Behçet's disease patients in Turkey have HLA-B*51[48]. Similar findings have been found in other silk route countries and are summarized in Table 1.

Table 1.1: Prevalence of BD and frequency of HLA-B∗51. Adapted from Takeno, 2021[48].

	Prevalence (/100.000)	HLA-B*51(%) in patients with BD	HLA-B*51(%) in controls
German	0.6-1.47	57.6	12.3
Italy	3.8	57.4	19.2
Spain	5.6-7.5	36.2	19.6
Japan	7.0-14.6	58.9	13.8
Iran	16.7-80.0	61.9	28.7
Saudi Arabia	19.5	76.9	22.2
Turkey	**80.0-421.0**	**75.0**	**24.7**

Cytosolic proteins are broken into smaller pieces by proteases before they enter the endoplasmic reticulum by TAP transporter. Inside, they are loaded on major histocompatibility complex (MHC) class 1 molecules and eventually are delivered to the cell surface to interact with T cell receptor (TCR) of CD8+ T lymphocytes. During the transit in endoplasmic reticulum, enzymes encoded by endoplasmic reticulum aminopeptidase (ERAP) 1/2 cleave either free peptides inside endoplasmic reticulum or peptides that are bound to MHC class 1. This process is important in creation of the peptide repertoire on MHC class 1 molecules (peptidome).

From a simplistic approach, the effects of having a specific HLA allele might seem straightforward, i.e. having certain alterations on the antigen binding groove of major histocompatibility complex and hence increased or decreased affinity to different proteins. This phenomenon is also described for HLA-B*51 in Behçet's disease[49], however there are additional layers in the etiopathogenetic machinery. In people harboring HLA-B*51, a low activity variant of ERAP1 has been described in literature, known as HAP10. Changes in ERAP activity in turn change the MHC-associated peptidome and induce pathologic immune reactions[50,51].

Moreover, the effects of having the HLA-B*51 allele are indeed more complicated. The presence of specific combinations of certain genes in non-random proportions among members of a population is linkage disequilibrium. Although controversial in Behçet's disease, linkage disequilibrium has been mentioned in the scope of HLA-B*51. Located close to the former, the *TNF*, *MICA* and *lymphotoxin* genes have been argued to be affected by having the B*51 allele[52-54].

All these factors might come in play in understanding the higher incidence of HLA-B*51 among people with Behçet's disease and higher severity of the disease in patients carrying the allele[47].

1.2.2. Innate Immune System

Neutrophil function and activation has been described to be altered in Behçet's disease[47]. Under physiologic conditions, when bacterial or other cellular insults cause tissue-resident cells to secrete certain cytokines (IL-1, TNF), they act on surrounding endothelial cells by inducing expression of selectins on cell surface. Specific carbohydrate molecules on neutrophils bind to selectins of endothelial cells. The interactions are strong enough to slow the flow of neutrophils down with the bloodstream, but weak in the sense that blood flow can still detach the neutrophils from endothelial cells, causing cycles of binding and releasing between the two, as the neutrophils roll over the endothelial cells. Endothelial cells also display chemokines on their surface to the chemokine receptors of neutrophils, causing internal changes in the latter to switch the leukocyte integrins (e.g. LFA-1) from low-affinity to high affinity states. As high affinity integrins bind to ligands (ICAM-1) on endothelial cells, strong bonds form between neutrophils and endothelial cells (adherence). Skeletal proteins undergo reorganisation and neutrophils migrate through endothelial cells, following a gradient of chemoattractants (e.g. IL-8) secreted by cells of the inflamed tissue. Once in close contact, neutrophils try to destroy harmful microorganisms in two ways, they either ingest & digest them intracellularly or they damage them via secretions extracellularly. The first way (respiratory burst) involves phagocyte oxidase and other enzymes that convert free oxygen to reactive oxygen species. The second way that also ensues when neutrophils die consists of releasing toxic granules and nuclear contents outside the cells, producing neutrophil extracellular traps (NETs).

Studies on BD have identified alterations in almost all of the steps described above[47]. Sarı et al. demonstrated that serum levels of soluble E-selectin are higher in patients with Behçet's disease compared to healthy controls[55]. Similarly, increased ICAM-1 levels are demonstrated to be higher in BD and are argued to correlate with disease severity[56]. Cytoskeletal changes reflected by increased truncated actin levels are described as well[57]. Overall, these changes involving E-selectin, ICAM-1 and actin represent increased capacity of neutrophil rolling, adherence and migration, respectively. Known among the most potent chemoattractants, IL-8 levels are found to be increased in active BD patients[58]. Furthermore, neutrophil activation, represented by myeloperoxidase and reactive oxygen species, is shown

to be increased in BD and is correlated with disease severity[59]. Similarly, antioxidant enzymes catalase and superoxide dismutase are reduced in BD[60]. In parallel, neutrophils of people with BD are shown to release more NETs compared to healthy individuals[61]. This pathology is also argued in development of vasculitis in BD[62,63].

Besides neutrophils, a limited number of studies evaluated Mannose-binding lectin (MBL) in people with Behçet's disease. MBL has a configuration similar to C1q, and hence is an important mediator in the innate immune system. Binding to the surface of bacteria, it mediates complement pathway activation, imitating the classical complement pathway. Inanç et al. demonstrated decreased levels of blood MBL in people with Behçet's disease and also correlated low MBL levels with disease severity[64]. On the other hand, Kim et al. evaluated blood MBL levels and *MBL2* gene polymorphism and found no difference between patients and controls[65]. It has also been argued that decreased MBL levels might predispose patients to certain bacterial infections such as S. mutans, which in turn might trigger Behçet's disease, creating an impression of direct causality between MBL and Behçet's disease[66].

1.2.3. Adaptive Immune System

Both cellular and humoral immunity have been researched in the scope of Behçet's disease and revelations are described in this section respectively. Studies that focused on T Helper (Th) cells described Th1, Th2 and Th17-predominant changes in BD patients compared to controls and in flare-ups during disease course[47].

Upon interaction with an antigen presenting cell (i.e. dendritic cell), the polarisation of naive CD4+ T cell to Th1 cell is mediated via IL-12, IL-18 and interferon (IFN) gamma. Upon commitment, the master transcription factor and cytokine in Th1 are Tbet and IFN-ɣ, respectively and the Th1 lineage act predominantly on intracellular pathogens[67]. Th1 lymphocytes produce interleukins (IL-2, IL-6, IL-8, IL-12, IL-18), IFN-ɣ and TNF-α, and they are reported to be increased in BD. Hamzaoui et al. demonstrated higher levels of IL-12 and IFN-ɣ in BD patients, both active and in remission, compared to healthy controls[68]. Frassanito et al. argued that increased levels of IL-12 might contribute to disease pathogenesis by preventing spontaneous and FAS-induced killing of Th1 lymphocytes[69].

In comparison to Th1, the Th2 commitment is boosted via IL-4 and is required mostly in the setting of extracellular pathogens including helminths and allergy. The central transcription factor is Gata3 and major cytokines that are produced are IL-4, IL-10 and IL-13[67]. Although the underlying mechanism is not fully elucidated, increased levels of Th2 cytokines have been described in Behçet's disease. Ben Ahmed et al. described a 75-fold increase in the expression levels of IL-10 in BD, while no change was seen in other Th2 cytokines[70]. Similarly, in the study of Raziuddin et al., peripheral blood mononuclear cells of BD patients secreted higher levels of Th2 ILs[71].

Although less studies compared to Th1 and Th2, Th17 cells have been implicated in mucosal immunity, immunity against intracellular pathogens, neutrophil-mediated immune responses. Mediated by IL-6 and TGF-β, Th17 polarization is characterized by transcription factor ROR-γδ and cytokine mediators IL-17A and IL-22[67]. In their study, Kaabachi et al. demonstrated higher levels of IL-26 in serum, cerebrospinal and bronchoalveolar lavage fluids of patients with active BD and associated higher IL-26 levels with disease severity[72]. In a separate study, Geri et al. demonstrated IL-21 promoted polarization towards Th17 while decreasing regulatory T cells in BD, hence they argued IL-21 as a new therapeutic target[73].

Antibodies against self-proteins and antigen-driven expansion of B lymphocytes have been described in BD. Suh et al. specifically evaluated synovial cell from patients with BD and described an expansion of B cells, argued to be antigen-driven and oligoclonal[74]. To discover auto-antibodies in BD, studies evaluated photoreceptor S-antigen and alpha-tropomyosin and demonstrated antibodies against them. While antibodies against retinal S-antigen have been found to correlate with ocular involvement in BD[75], tropomyosin has been argued as the culprit self-antigen in both ocular and skin manifestations of BD[76].

1.2.4. Vascular Dysfunction

As Behçet's disease is mentioned among diseases with predominant vasculitis, related pathophysiology deserves special attention. There are two pillars in this regard, vascular inflammation and coagulative disarray, both of which are induced by endothelial activation.

Nitric oxide (NO) is a key mediator in endothelial cell functioning. It is produced from arginine by endothelial nitric oxide synthase (eNOS) in endothelial cells, and the production is increased by shear stress and other chemical mediators, including acetylcholine. It acts on surrounding smooth muscle cells of the vascular wall where it promotes dilatation, and also is released directly to blood. In Behçet's disease, NO levels are found higher in blood and also synovial fluid of joints and aqueous humor of the eye[77-79]. In parallel with increased NO, endothelin-1 and homocysteine levels are also found elevated in BD and correlated with disease activity[80]. Moreover, increased vascular endothelial growth factor (VEGF) is reported in BD and higher levels are associated with increased risk of developing ocular disease[81].

Often in conjunction with endothelial activation and vasculitis, tendency to thrombosis is frequently described in Behçet disease. However, besides the factors described above, the hypercoagulable state is also attributed to changes in levels of other molecules involved in coagulation and fibrinolysis. In their study, Probst et al. showed higher levels of factor VIII, factor XI and fibrinogen in BD compared to healthy controls[82]. Similarly, Navarro et al. demonstrated lower thrombomodulin and activated protein C levels in BD, potentially predisposing them to venous thromboembolism[83]. Other studies on tissue-type plasminogen activator and plasminogen activator inhibitor-1 also mentioned a pro-coagulative tendency in BD[84,85]. Lastly, elevated homocysteine and increased neutrophil activity, as described in previous sections, may further contribute to thrombosis episodes during the course of Behçet's disease.

1.3. Exploring the Microbiota: Addressing the Gap in Behçet's Disease

Behçet disease is described to be multifactorial in the current literature, with effort given to HLA & non-HLA genes (ICAM-1, VEGF, IL-10, TNF, NOS) and correlation to innate & adaptive immune system to predict disease occurrence[47,48,86-90]. Epigenetic modifications including DNA methylation, histone modification and non-coding RNAs have been scrutinized in the scope of BD, however human or animal studies failed to illuminate the disease pathophysiology per se, shifting the attention to other parameters that might provide better explanation[91-95].

In this regard, antibacterial host response and microbiota have been of interest. Hypersensitivity towards streptococcal antigens, salivary colonization of S.mutans, MBL deficiency and seropositivity for *H.pylori* CagA are shown to differ from healthy controls in limited studies on patients with Behçet's disease[66,96,97]. However, the findings have not been reproduced in larger populations in multi-center studies yet.

Intestinal microbiota is studied among the etiologic factors of BD with emphasis on increase in sulfate-reducing bacteria (SRB) and lactic acid-producing bacteria or decrease in methanogenic and butyrate-producing bacteria (BRB) - specific studies will be elaborated in the following paragraphs[98]. Briefly, the authors argue that changes in intestinal metabolism characterized by decreased butyric acid induce an increase in mucosal permeability. This triggers an imbalance of proinflammatory cytokines including IFN-ɣ and IL-17 over anti-inflammatory cytokines (mainly IL-10). Suppression of regulatory T cells, together with stronger Th1 and Th17 polarization is thought to induce a systemic immune dysregulation[99,100].

In a key study on microbiota changes in Behçet's disease, researchers evaluated an Italian cohort (Florence). Consolandi et al, compared fecal microbiota of people with BD and healthy controls. Of interest, they detected a statistically significant decrease in *Roseburia* and *Subdoligranulum* genera, with an ensuing decrease in butyric acid production[101]. Yet in another study based in Japan, Shimizu et al. demonstrated compositional alteration of fecal microbiota in favor of the phylum *Actinobacteria*, the family *Lactobacillaceae*, and the genus *Bifidobacterium* in BD[102]. In contrast with the predominance of the phylum *Firmicutes* and the class *Clostridia* seen in health controls, alterations in people BD were meaningful for the researchers, as they claimed loss of short-chain fatty acid-producing bacteria (*Clostridia*) might induce an imbalance between Th17 and regulatory T cells[103].

In a Chinese cohort, fecal and salivary samples from people with BD with ocular involvement were collected to compare with healthy individuals. Ye et al. described increase in *Bilophila*, *Parabacteroides* and *Paraprevotella*; together with decrease in *Clostridium*, *Methanoculleus* and *Methanomethylophilus*. When they transferred these fecal samples

enriched in sulfate-reducing and depleted in methanogenic bacteria into mice in an experimental uveitis model, they observed an increase in uveitis severity[104]. Indeed, other animal studies on experimental autoimmune uveitis have also suggested alterations in intestinal microbiome as an important trigger in disease development, both in BD and Vogt-Koyanagi-Harada syndrome[105,106].

In their study based in Istanbul, Turkey; Oezguen et al. evaluated gut microbiota of patients suffering from multiple sclerosis and Neuro-Behçet's disease and compared them with healthy controls. They described *Prevotella* and *Bacteroides* as predominant in both disease and healthy controls, however *Clostridiales* was found to be increased in Neuro-Behçet's disease group upon stratification[107]. In yet another study based in Eskişehir, Turkey; Bilge et al. evaluated fecal samples of 27 patients with BD with mucocutaneous, ocular and vascular symptoms. *Dialister*, *Lachnospiraceae NK4A136* and Gemella genera were predominant in mucocutaneous, ocular and vascular BD groups, respectively[108].

The studies mentioned above did not yield identical results. We believe this might have stemmed from population-based differences in microbiome. However, there are two additional major points to highlight:

1) Studies in general focused only on fecal microbiota. While this may initially seem straightforward as many similar studies on other diseases also focus on gastrointestinal microbiome. However, we believe that additional work up on the microbiota of other sites (e.g. skin, oral/salivary, eye) could also yield important findings, as the disease's etiopathogenesis is currently incompletely understood.
2) Studies compare the microbiome of BD patients with those of healthy controls. We believe having the baseline microbiome composition of patients and comparing it with the one during disease exacerbation might yield additional results that would improve our understanding of the disease. Similarly, evaluations after disease remission can be of interest.

Studies have evaluated the microbiota either by shotgun metagenomic analysis or by 16S rRNA sequencing (via V3-V4 hypervariable region)[101,103,104]. Each method has certain

advantages and disadvantages; and selection among them should be made based on the study's aim, budget and the amount of genetic material available. The advantages of metagenomics include higher taxonomic resolution (strain level) and accompanying functional profiling. 16S rRNA sequencing on the other hand stands out with less interference with host DNA (i.e. less false positivity), lower cost and necessity of a smaller amount of nucleic acid[109].

Evaluation of microbiota can also have treatment-focused implications, besides shedding light on disease etiology. For example, in a major ongoing study, via lacto-ovo-vegetarian, Mediterranean or butyric acid-enriched diets, researchers aim to create a shift in the gastrointestinal microbiota in people with BD to improve clinical outcomes[110]. As current literature fails to illustrate a specific microorganism as the sole culprit of the disease, current efforts shift towards implementing dietary changes and providing prebiotic supplementations, which are rather indirect ways of improving the microbiome content[111].

Using intestinal microbiome to predict disease exacerbation has been recently suggested as a new approach and preliminary researchers have tried to implement a risk stratification in prospective and cross-sectional studies. This approach has been of interest, especially in the setting of psoriasis, where Dei-Cas et al. profiled (16S rRNA) fecal microbiota of 55 patients who have psoriasis[112]. In their study, microbial changes of the patients have been correlated to their clinical disease severity (e.g. mild, moderate, severe) and compared with health controls to formulate a “Psoriasis Microbiota Index”. Hence, they suggested respective microbiome signatures in psoriatic patients of various severity[112].

In this study, we aim to implement a similar approach in the setting of Behçet’s disease. We aspire to evaluate the microbiota changes of people with Behçet’s disease. In parallel with healthy controls, we believe comparing samples taken during disease exacerbation and remission would create a unique aspect in our study. Last but not least, we expand the focus from fecal microbiota to a broader scope where we simultaneously study changes in gastrointestinal, skin, salivary (oral) and eye microbiota.

CHAPTER 2
MATERIALS AND METHODS

The study protocol was written in detail by our team and was approved by Koç University Committee on Human Research, with the protocol number 2023.121.IRB2.029.

2.1. Patient Selection

Behçet's disease patients treated at Kartal Dr. Lütfi Kırdar Şehir Hastanesi were evaluated for the study. They were included in the study upon meeting the inclusion criterion, i.e. adult patient with mucocutaneous and/or ocular involvement of BD. If patients had received antibiotic treatment in the preceding 2 months or have serious concomitant/other systemic disease that might interfere with microbiome, they were excluded from the study.

Patients were disclosed about the purpose and the methodology of the study by their rheumatologist and written informed consents were taken upon agreement. No alterations were made in the medical care of the patients based on whether they were included to or excluded from the study.

Briefly, the key concept was having microbiota samples during disease exacerbation and remission, as details will be described in the following section (see 2.2. Sample Collection). However, besides the microbiota samples, patients were assessed extensively regarding their disease history and treatment protocols. Responsible clinicians from the rheumatology service evaluated each patient via a standardized approach to obtain specific information, as seen in Table 2.1.

Table 2.1: Information collected during sample collection

Information on Demographics & General Health	**Information on Attack/Exacerbation Sample**	**Information on Remission Sample**
Age	Date of sample collection	Date of sample collection
Sex	Fecal sample present: Y/N	Fecal sample present: Y/N
Contact information	Oral sample present: Y/N	Oral sample present: Y/N
Comorbidity, if any	Skin sample present: Y/N	Skin sample present: Y/N
Date of initial diagnosis	Eye sample present: Y/N	Eye sample present: Y/N
Findings in previous attacks	Findings in current attack	Response to treatment
Previous treatment for BD	Current treatment for BD	Current treatment for BD
Drugs used for other causes	Last time of antibiotics use	Last time of antibiotics use
Family history of BD	Informed consent: Y/N	Informed consent: Y/N
		Interval between 2 samples
***If present, any other relevant information	***If present, any other relevant information	***If present, any other relevant information

The cohort was followed by ophthalmology service during and after attacks to evaluate remission, residual disease and flare ups. Remission was defined by disappearance of symptoms via rheumatologic evaluation and ophthalmologic evaluation, if relevant. Regarding the latter, angiographic evidence of absent vascular leak and absent optic disc hyperfluorescence was accepted as remission. Representative evaluations from attack and remission is seen in Figure 2.1 and Figure 2.2, respectively.

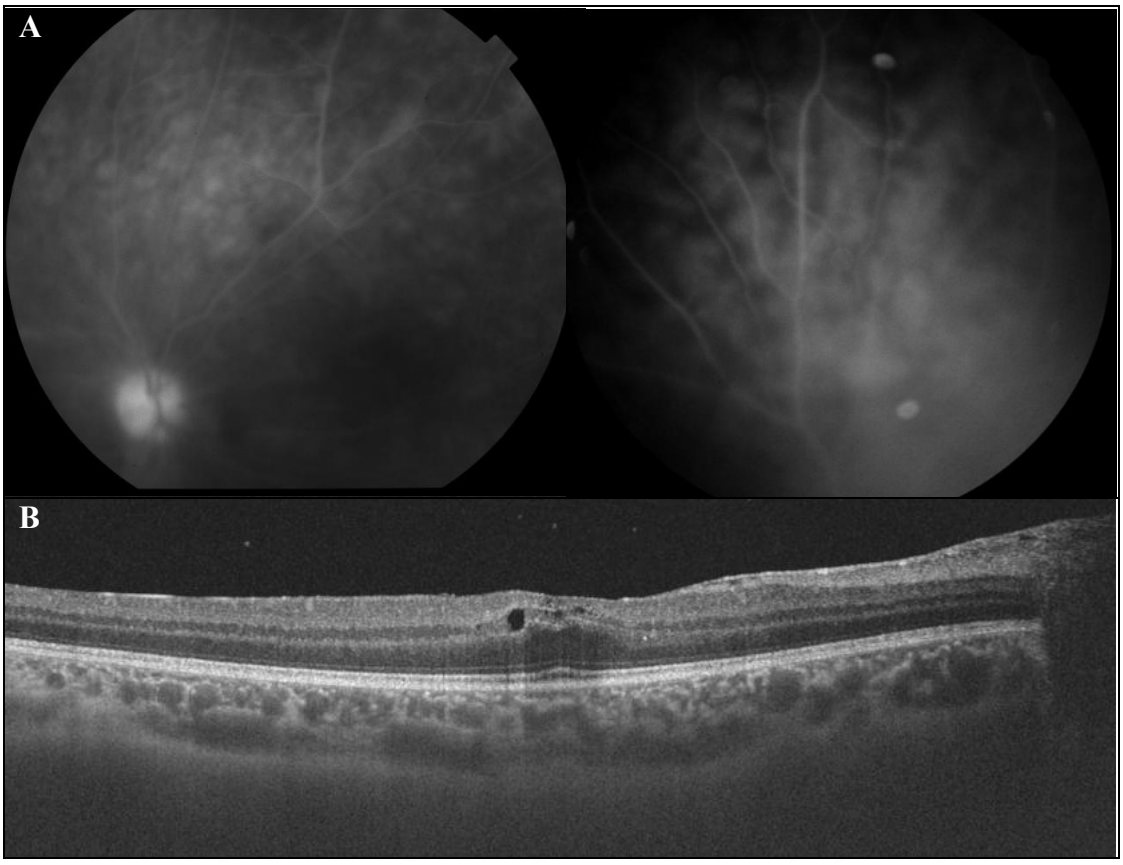

Figure 2.1: Fluorescein angiography & optical coherence tomography at BD episode.

a) Fluorescein angiography of reveals fern-like leak and optic disc hyperfluorescence.

b) Optical Coherence Tomography demonstrates macular edema, seen as intraretinal cyst.

Patient Code: #5. Courtesy of Nilüfer Zorlutuna Kaymak, MD.

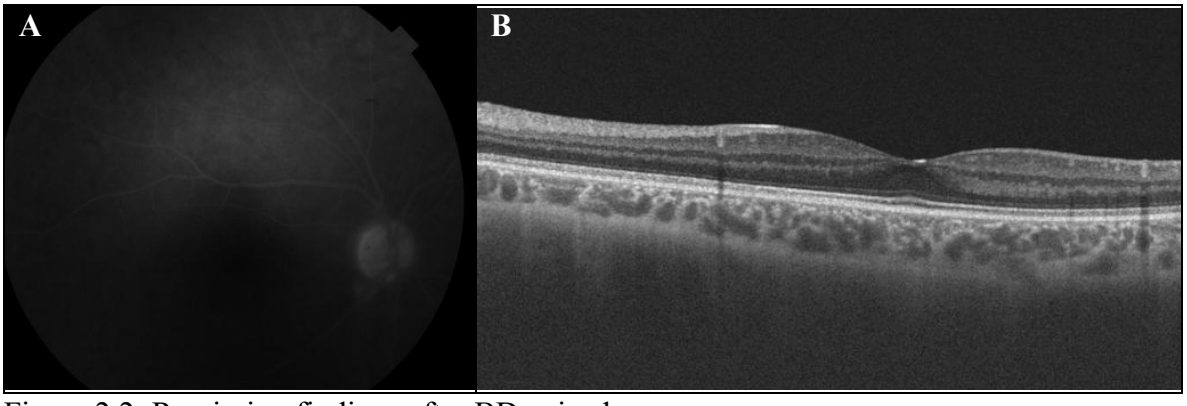

Figure 2.2: Remission findings after BD episode.

a) Fluorescein angiography of reveals no fern-like leak or optic disc hyperfluorescence.

b) Optical Coherence Tomography demonstrates no hypercellularity or macular edema.

Patient Code: #5. Courtesy of Nilüfer Zorlutuna Kaymak, MD.

Once the patient cohort was established and samples were collected, effort was made to find age- and sex-matched healthy controls to include in the study. In contrast with the attack and remission samples taken from Behçet's disease patients, only a single set of samples were taken from healthy controls. As explained above, antibiotic use in the preceding 2 months and concomitant systemic disease were exclusion criteria in this group as well. Informed consents were taken prior to inclusion to study.

2.2. Sample Collection

In order to obtain a standardized procedure, an educational meeting was carried out initially, where details were explained to every study member that will be involved in sample collection. Besides, to make sure key information is available at all steps of sample collection, a specific manual was written, describing the procedure step-by-step and copies were distributed to each researcher in the study. Detailed explanation for each sample is available in the following sections below.

2.2.1. Skin

Skin samples were collected from non-overlapping areas of the dry volar forearms and moist antecubital and popliteal fossae. The fossae were defined as the region extending ±4 cm from the flexure, while the volar forearm was defined as the area extending from just below the antecubital fossa to 4 cm above the wrist.

Superficial skin scrapings were obtained by making 20 strokes in various directions using a disposable scalpel or glass slides. The skin debris was collected with a sterile swab and transferred into tubes containing 3 mL of ZYMO DNA/RNA Shield Stabilization Solution. Subsequently, a second swab was applied over the scraped area to collect additional samples. This swab was also placed into the stabilization solution tube. All samples were rapidly stored at -80°C[113,114].

2.2.2. Gastrointestinal

Stool samples were collected in sterile fecal collection containers and immediately stored at -80°C[115].

2.2.3. Oral

Before sample collection, the oral cavity was rinsed with clean water. Sterile swabs were then used to collect samples from the inner cheeks. The swabbing process was repeated several times to ensure sufficient sample collection. The swabs were placed into 15 mL Falcon tubes containing 3 ml ZYMO DNA/RNA Shield Stabilization Solution. By mixing the swab against the inner wall of the tube, the sample was thoroughly transferred into the solution. All samples were stored at -80°C[116,117].

2.2.4. Eye

The conjunctival swab sampling method was used. Both the upper and lower conjunctival sacs of each eye were wiped using opposite sides of the same sterile swab. The head of the swab, now containing the sample, was placed into a tube containing ZYMO DNA/RNA Shield Stabilization Solution. Samples were promptly stored at -80°C[118,119].

2.3. Sample Transfer and Preservation

The samples were collected at Kartal Dr. Lütfi Kırdar City Hospital and stored at -80°C until the time of transfer. On the day of transfer, they were rapidly transported to the Koç University İşbank Center for Infectious Diseases (KUISCID) reference center using dry ice. All samples were properly labeled and stored at -80°C until analysis.

To ensure standardization across subsequent procedures, no further processing was performed at this stage until the collection of all patient samples—both during attack and remission phases—was completed.

2.4. DNA Isolation

For DNA isolation, sample preparation was applied to skin, oral, and eye samples. Fecal samples were directly processed using the Zymo Quick-DNA™ Fecal/Soil Microbe Microprep Kit according to the manufacturer's instructions.

For the skin, oral, and eye samples, the tubes containing the stabilization solution were thawed on ice and homogenized by vortexing. The homogenates were centrifuged at

20,000 × g for 30 minutes. After centrifugation, the supernatant was discarded. The resulting pellet was resuspended in 1 mL of PBS. A 250 µL aliquot of this suspension was stored at -80°C, while the remaining 750 µL was used for DNA isolation using the same Zymo Quick-DNA™ Fecal/Soil Microbe Microprep Kit, following the manufacturer's protocol.

For skin and eye samples, the final elution volume was reduced by evaporation to concentrate the DNA. DNA quantification for all samples was performed using the Qubit dsDNA High Sensitivity kit (Invitrogen).

2.5. 16S rRNA (V1–V9) Library Preparation and Sequencing

DNA extracts were first normalized to a concentration of 10 ng in 15 µL of nuclease-free water prior to amplification. The normalized samples were then amplified and barcoded using the ONT 16S V1-V9 Barcoding All-in-One Kit (Oxford Nanopore Technologies, SQK-16S024), following the manufacturer's protocol, with a maximum of 25 PCR cycles.

PCR amplification was carried out under the following conditions: an initial denaturation at 95 °C for 3 minutes; 5 cycles consisting of 95 °C for 15 seconds, 55 °C for 15 seconds, and 72 °C for 30 seconds; followed by 30 additional cycles at 95 °C for 15 seconds, 62 °C for 15 seconds, and 72 °C for 30 seconds; and a final extension step at 72 °C for 1 minute.

Following amplification, the DNA products were purified using AMPure® XP beads (Beckman Coulter) and quantified with a NanoDrop® 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For sequencing library preparation, 100 ng of DNA was incubated with 1 µL of Rapid Adapter at room temperature for 5 minutes. Subsequently, 11 µL of the prepared DNA library was combined with 34 µL of Sequencing Buffer, 25.5 µL of Loading Beads, and 4.5 µL of nuclease-free water. The final mixture was loaded onto a FLO-MIN106 (R9.4) flow cell and sequenced using the MinION™ Mk1B platform. Data acquisition was managed via MINKNOW software (v1.11.5, Oxford Nanopore Technologies).

2.6. Bioinformatics and Statistical Analysis

Basecalling of MinION™ sequencing data (FAST5 format) was performed using Albacore software (v2.3.4, Oxford Nanopore Technologies), generating high-quality FASTQ files with mean quality scores above 7. Adapter and barcode sequences were removed using the EPI2ME Fastq Barcoding workflow (v3.10.4, Oxford Nanopore Technologies).

The fast5 files were base-called demultiplexed, and adapter-trimmed with Dorado v1.0.1. Read qualities were assessed with NanoPlot v.1.44.1 and raw reads were filtered for min. quality = 10, min. length=1000 , max. length=2000 with NanoFilt v2.8.0. Filtered reads were used for taxonomic identification with the EMU v3.5.1 using its pre-built database. Alpha and beta diversities were calculated with skbio.diversity package in Python.

Bacterial identification was conducted by aligning each read to a curated database of 5,850 representative bacterial genomes (GenomeSync database). The species corresponding to the highest alignment score was assigned to each read. In cases of equal top scores for multiple species, reads were classified at the lowest shared taxonomic level. Taxonomic assignments were made using the NCBI taxonomy database. Reads representing taxa with a relative abundance below 0.01% of the total were excluded from the final analysis.

Group comparisons were carried out using one-way ANOVA followed by Dunnett's post hoc test for multiple comparisons. Alpha-diversity comparison (Shannon's diversity) was achieved via Mann Whitney U test. Pearson correlation coefficients were calculated to evaluate the consistency of bacterial composition across different sequencing approaches. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were conducted using Prism 10 software (GraphPad Software, Inc., La Jolla, CA, USA).

CHAPTER 3
RESULTS

3.1. Characteristics of Cohort

We evaluated 20 patients with BD. Mean age of the patient during the attack was 32 years (range 21-51) with a male to female ratio of 17:3. 19 of patients already had a diagnosis of BD coined before being included in the study (median: 5 years, range 4 months-20 years). 3 patients had at least 1 family member with BD in their family, while one had a family member with FMF.

During the time of inclusion to the study, patients presented with oral ulcers (5/20, 25%), genital ulcers (2/20, 10%), skin findings i.e. papulopustular or erythema nodosum-like lesions (5/20, 25%), uveitis (11/20, 55%), arthritis (4/20, 20%), and/or vasculitis (1/20, 5%, deep venous thrombosis).

Checking from medical records, patients previously had other BD attacks/complaints involving oral ulcers (18/20, 90%), genital ulcers (10/20, 50%), skin findings (15/20, 75%), uveitis (15/20, 75%), arthritis (5/20, 25%), vasculitis (2/20, 10%, n=1 with deep venous thrombosis, n=1 with thrombophlebitis), and/or epididymitis (1/20, 5%).

In terms of treatment, patients already received various combination of regimens including azathioprine, cyclosporine, colchicine, methylprednisolone, hydroxychloroquine, infliximab and adalimumab, depending on the symptoms and organs involved. Patients also used other medications such as levothyroxine, antihypertensives and proton-pump inhibitors, depending on their comorbidities. Detailed regiments before the attack and during the management of attack are provided in the following tables.

During follow up, with regular rheumatologic and ophthalmology visits, 6 patients entered in remission. Time until remission ranged between 2 and 13 months (mean: 7.15 months, medians: 5 and 8 months). All the information summarized in this part can be appreciated in detail in Table 3.1.

Table 3.1: Characteristics of cohort

Patient Code	Age	Sex	Comorbidity	Diagnosed with BD for	Pertinent family history	Symptoms on attack						Previous symptoms						
						Oral ulcer	Genital ulcer	Skin	Eye	Joint	Vasculitis	Oral ulcer	Genital ulcer	Skin	Eye	Joint	Vasculitis	Epididymitis
1	23	M	none	first attack	*Mother: BD *Aunts: BD	+	+	+	+	//								
2	36	M	none	7 months	*Mother: Arthritis *Aunt: FMF					+		+			+	+	+	
3	31	M	none	12 years	none					+		+		+		+		
4	30	M	none	7 years	none			+		+		+	+	+	+	+		+
5	21	M	none	8 months	none				+			+		+	+	//		
6	28	M	none	3 years	none				+			+		+	+			
7	34	M	none	7 years	none	+	+			//		+	+	+	+	+		
8	40	M	none	15 years	*Sister: BD				+			+	+	+	+			
9	23	M	none	3 years	none				+			+	+	+	+			
10	44	F	HBV (carrier)	19 years	*Father: BD	+		+		+		+	+	+		+		

Abbreviations: F: Female, M: Male; HT: Hypertension; BD: Behçet's Disease, FMF: Familial Mediterranean Fever; //: arthralgia; mg: milligrams; m: month, y: year.

Table 3.1: Characteristics of cohort (continued)

Patient Code	Age	Sex	Comorbidity	Diagnosed with BD for	Pertinent family history	Symptoms on attack						Previous symptoms						
						Oral ulcer	Genital ulcer	Skin	Eye	Joint	Vasculitis	Oral ulcer	Genital ulcer	Skin	Eye	Joint	Vasculitis	Epididymitis
11	37	M	Polycystic kidney disease	9 years	none				+			+	+	+				
12	34	F	none	5 years	none	+		+				+	+	+	+			
13	26	M	none	3 years	none	+			+			+	+	+	+			
14	35	M	glaucoma, cataracts	10 months	none				+						+			
15	31	M	chronic pharyngitis	unknown	none				+			+		+	+			
16	27	M	none	4 months	none				+			+			+			
17	51	M	none	20 years	none						+	+	+				+	
18	39	F	Hypothyroidism HT, proteinuria	19 years	none			+				+		+	+			
19	26	M	none	3 years	none				+			+		+	+			
20	22	M	none	unknown	none							+	+	+	+			

Abbreviations: F: Female, M: Male; HT: Hypertension; BD: Behçet's Disease, FMF: Familial Mediterranean Fever; //: arthralgia; mg: milligrams; m: month, y: year.

Table 3.1: Characteristics of cohort (continued)

Patient Code	Treatment prior to attack	Treatment during attack	Other medication	Last use of antibiotics	Remission time	Last use of antibiotics (interval)
1	none	*azathioprine 50 mg 2*1, *methylprednisolone 48 mg, *cyclosporine 100 mg 2*1, *steroid eyedrop 5*1	none	3 y		
2	*azathioprine 50 mg 2*1, *cyclosporine 100 mg 2*1, *colchicine 2*1	*azatiopurin 50 mg 2*1, *cyclosporine 100 mg 2*1, *colchicine tb 3*1	none	3 y	13 m	2 m
3	*colchicine 1*1	*colchicine 1*1	none	at least 3 m		
4	*colchicine 2*1	*colchicine 3*1 *betamethason (single dose)	none	at least 3 m		
5	*azathioprine 50 mg 3*1, *cyclosporine 100 mg 2*1, *methylprednisolone	*azathioprine 50 mg 3*1, *cyclosporine 100 mg 2*1, *methylprednisolone *infliximab	none	7 m	8 m	none
6	***no drug use during attack *azathioprine 50 mg 2*1, *cyclosporine 100 mg 2*1, *hydroxychloroquine200 mg 1*1, *methylprednisolone	*methylprednisolone *infliximab	*proton pump inhibitor	3 m	5 m	multiple times
7	*infliximab 600 mg/m	*infliximab 600 mg/month *colchicine 3*1	none	2 w	3 m	none
8	*colchicine 2*1 *prednisolone	*colchicine 3*1 *prednisolon 48 mg, *cyclosporine 100 mg 2*1,	none	3 m		
9	***Only occasional use of azathioprine and colchine	*azathioprine 50 mg 3*1 *prednisolone 16 mg	none	3 m	12 m	none

Abbreviations: F: Female, M: Male; HT: Hypertension; BD: Behçet's Disease, FMF: Familial Mediterranean Fever; //: arthralgia; mg: milligrams; m: month, y: year.

Table 3.1: Characteristics of cohort (continued)

10	*colchicine 2*1	*colchicine 3*1	not known	at least 3 m	2 m	1 w
11	*azathioprine 50 mg 3x1 *colchicine 3*1	*azathioprine 50 mg 3x1 *colchicine 3*1 *adalimumab	none	3 m		
12	*colchicine 2x2	*colchicine 2x2 *methotrexate 10 mg/week	none	2 m		
13	*azathioprine 50 mg 2*1, *adalimumab 40 mg/2 weeks	*methylprednisolone 1 gr *azathioprine 50 mg 2*1, *adalimumab 40 mg/2 weeks	*proton pump inhibitor	2 m		
14	*adalimumab 40 mg/week	*infliximab 300 mg	*eye drops	1 w		
15	*steroid	*azathioprine 50 mg tb 3*1	none	at least 3 m		
16	*prednisolone 48 mg	*prednisolone 1 gr (3 days only) *infliximab 400 mg/month	*eye drops	at least 3 m		
17	*colchicine 3*1	*prednisolone 48 mg/day *azathioprine 50 mg tb 2*1, *colchicine 3*1	none	at least 3 m		
18	*azathioprine 150 mg, *adalimumab 40 mg/12 weeks	*azathioprine 150 mg, *adalimumab 40 mg/12 weeks *colchicine 3*1	*Levothyroxin, *Irbesartan/amlodipine/ hydrochlorothiazide	at least 3 m		
19	none	*azathioprine 100 mg, *cyclosporine 100 mg 2*1, *prednisolone 1 gr (3 d), then 48 mg	none	12 m		
20	none	Local treatment only: *prednisolone 5*1, *dexamethason 1*1, *oxytetracycline&polymyxin B 2*1, *levofloxacin 8*1 *tropicamide 3*1	none	6 m		

Abbreviations: F: Female, M: Male; HT: Hypertension; BD: Behçet's Disease, FMF: Familial Mediterranean Fever; //: arthralgia; mg: milligrams; m: month, y: year.

3.2. Initial Evaluation of Samples

The study initially enrolled 20 patients. Attack-phase samples were collected from all 20 participants. Additionally, remission-phase samples were obtained from 6 of these patients. One patient provided a second attack-phase sample after remission.

Of the 20 remission samples collected, 2 were excluded due to recent antibiotic use, reducing the cohort to 18 patients. Among the 6 patients who provided remission samples, 3 were excluded from remission-phase analyses because they had received antibiotics during the follow-up period. Consequently, the final analysis included 3 patients with valid remission samples.

Fecal samples were not provided by 10 participants, and thus these samples were excluded from further analysis. In addition, one oral sample was removed during the quality control process.

To serve as a control group, samples from 5 healthy individuals matched were included in the study with mean age 31.6 years (range 23-49 years) and male to female ratio of 4:1. Detailed illustration of the cohort can be seen in Table 3.2.

Table 3.2: Summary of samples included in the study

	Patient Code : Sample Code	Skin	Oral	Eye	Fecal
Initial Samples	01:01	+	+	+	+
	02:01	+	+	+	+
	03:01	+	+	+	+
	04:01	+	+	+	+
	05:01	+	+	+	+
	06:01	+	+	+	+
	07:01	Excluded			
	08:01	+	+	+	NA
	09:01	+	+	+	+
	10:01	+	+	+	+
	11:01	+	+	+	+
	12:01	+	+	+	+
	13:01	+	+	+	NA
	14:01	Excluded			
	15:01	+	+	+	NA
	16:01	+	+	+	NA
	17:01	+	+	+	NA
	18:01	+	+	+	NA
	19:01	+	+	+	NA
	20:01	+	NA	+	+

Sample codes (:0X) meaning: 1: First Attack, 2: Remission, 3: Second Attack; +: Sample present, NA: Not Available.

Table 3.2: Summary of samples included in the study (continued)

Follow up Samples	02:02	+	+	+	NA
	05:02	+	+	+	+
	06:02	Excluded			
	06:03	+	+	+	NA
	07:02	Excluded			
	09:02	+	+	+	NA
	10:02	Excluded			
Control Samples	C1	+	+	+	+
	C2	+	+	+	+
	C3	+	+	+	+
	C4	+	+	+	+
	C5	+	+	+	+
Total Samples		**27**	**26**	**27**	**17**

Sample codes (:0X) meaning: 1: First Attack, 2: Remission, 3: Second Attack; +: Sample present, NA: Not Available.

Summary tables presenting the DNA concentrations of all skin, oral, eye and fecal samples following the isolation procedure are provided in Tables 3.3, 3.4, 3.5 and 3.6, respectively.

Table 3.3: DNA Concentrations of Skin Samples

Sample Name	Details	DNA Concentration		Sample Name	Details	DNA Concentration
01:01 S	P/A	0,0524		02:02 S	P/R	0,0428
02:01 S	P/A	0,0179		05:02 S	P/R	0,017
03:01 S	P/A	0,0508		09:02 S	P/R	0,0522
04:01 S	P/A	0,0416				
05:01 S	P/A	0,0542		C1-S	Control	0,0628
06:01 S	P/A	0,0595		C2-S	Control	0,102
06:03 S	P/A	0,0608		C3-S	Control	0,102
08:01 S	P/A	0,0266		C4-S	Control	0,0615
09:01 S	P/A	0,0937		C5-S	Control	0,0522
10:01 S	P/A	0,0376				
11:01 S	P/A	0,0395				
12:01 S	P/A	0,0078				
13:01 S	P/A	0,0283				
15:01 S	P/A	0,024				
16:01 S	P/A	0,0148				
17:01 S	P/A	0,0442				
18:01 S	P/A	0,069				
19:01 S	P/A	0,0568				
20:01 S	P/A	0,0311				

S: Skin, P/A: Patient on Attack, P/R: Patient on Remission.

Table 3.4: DNA Concentrations of Oral Samples

Sample Name	Details	DNA Concentration		Sample Name	Details	DNA Concentration
01:01 O	P/A	1,1		02:02 O	P/R	0,879
02:01 O	P/A	0,972		05:02 O	P/R	1,65
03:01 O	P/A	0,349		09:02 O	P/R	0,277
04:01 O	P/A	0,285				
05:01 O	P/A	1,06		C1-O	Control	2,21
06:01 O	P/A	0,805		C2-O	Control	0,806
06:03 O	P/A	2,07		C3-O	Control	0,845
08:01 O	P/A	0,0351		C4-O	Control	0,0504
09:01 O	P/A	0,356		C5-O	Control	0,278
10:01 O	P/A	1,42				
11:01 O	P/A	0,56				
12:01 O	P/A	0,384				
13:01 O	P/A	0,517				
15:01 O	P/A	0,604				
16:01 O	P/A	0,246				
17:01 O	P/A	0,991				
18:01 O	P/A	0,164				
19:01 O	P/A	2,07				

O: Oral, P/A: Patient on Attack, P/R: Patient on Remission

Table 3.5: DNA Concentrations of Eye Samples

Sample Name	Details	DNA Concentration		Sample Name	Details	DNA Concentration
01:01 E	P/A	0,0432		02:02 E	P/R	0,119
02:01 E	P/A	0,196		05:02 E	P/R	0,116
03:01 E	P/A	0,0553		09:02 E	P/R	0,014
04:01 E	P/A	0,0399				
05:01 E	P/A	0,132		C1-E	Control	0,041
06:01 E	P/A	0,125		C2-E	Control	0,0329
06:03 E	P/A	0,113		C3-E	Control	0,0808
08:01 E	P/A	0,0172		C4-E	Control	0,0378
09:01 E	P/A	0,037		C5-E	Control	0,0619
10:01 E	P/A	0,168				
11:01 E	P/A	0,0601				
12:01 E	P/A	0,249				
13:01 E	P/A	0,231				
15:01 E	P/A	0,0812				
16:01 E	P/A	0,078				
17:01 E	P/A	0,123				
18:01 E	P/A	0,0685				
19:01 E	P/A	0,0552				
20:01 E	P/A	0,0534				

E: Eye, P/A: Patient on Attack, P/R: Patient on Remission.

Table 3.6: DNA Concentrations of Fecal Samples

Sample Name	Details	DNA Concentration		Sample Name	Details	DNA Concentration
01:01 F	P/A	12		05:02 F	P/R	121
02:01 F	P/A	8,66				
03:01 F	P/A	114		C1-F	Control	18,1
04:01 F	P/A	12,8		C2-F	Control	25,8
05:01 F	P/A	24,0		C3-F	Control	34,8
06:01 F	P/A	10,6		C4-F	Control	28,1
09:01 F	P/A	117		C5-F	Control	18,9
10:01 F	P/A	9,75				
11:01 F	P/A	13,9				
12:01 F	P/A	4,32				
20:01 F	P/A	14,4				

F: Fecal, P/A: Patient on Attack, P/R: Patient on Remission.

3.3. Alpha Diversity & Beta Diversity

A total of 2,995,505 high-quality reads were used to identify taxonomic groups (958,375 reads for control samples). The mean number of sequences obtained per sample was 39414.5 (47918.8 for controls). Between 97.80-99.97% of the reads were successfully mapped on to the 16S rRNA gene sequences, identifying a total of 1094 species, belonging to 393 genera, representing 16 phyla, across all samples (including controls).

The characteristics of bacterial communities during the disease's active (attack) and remission phases were assessed by analyzing both alpha and beta diversity. We evaluated species richness and alpha diversity using the Shannon Diversity Index across two group

comparisons: (1) control vs attack and (2) attack vs remission, in each distinct body sites (skin, eye, fecal, and oral microbiota).

Our analysis indicated that the Shannon Diversity Index did not show statistically significant differences between the control and attack groups across all sample types (skin, eye, fecal, and oral; $p > 0.05$). Similarly, no significant differences were observed between the attack and remission phases within the same regions ($p > 0.05$) (Table 3.7 and Table 3.8).

Beta diversity was assessed using the Bray–Curtis dissimilarity index and visualized through UniFrac-based Principal Coordinates Analysis (PCoA). The PCoA plots showed no clear separation between the groups across all sampling sites, further supporting the lack of significant compositional changes in microbial communities during different disease phases (Figure 3.1 and Figure 3.2).

Table 3.7: Alpha-diversity comparison between control and attack samples (Shannon's diversity). Mann Whitney U test was used for statistical analysis.

	Controls	**Patients (Attack)**	**P value**
Skin	2.35058659	2.302754985	Non-Significant
Oral	3.397564921	3.962874964	Non-Significant
Eye	2.231901861	1.621065869	Non-Significant
Fecal	3.966694505	3.877094334	Non-Significant

Table 3.8: Alpha-diversity comparison between attack and remission samples (Shannon's diversity). Mann Whitney U test was used for statistical analysis.

	Attack	**Remission**	**p**
Skin	3.086063965	2.664052858	0.7671
Oral	3.383683997	3.326697487	0.2571
Eye	1.66750975	1.725583492	0.2177
Fecal	2.960665682	3.037855362	0.2674

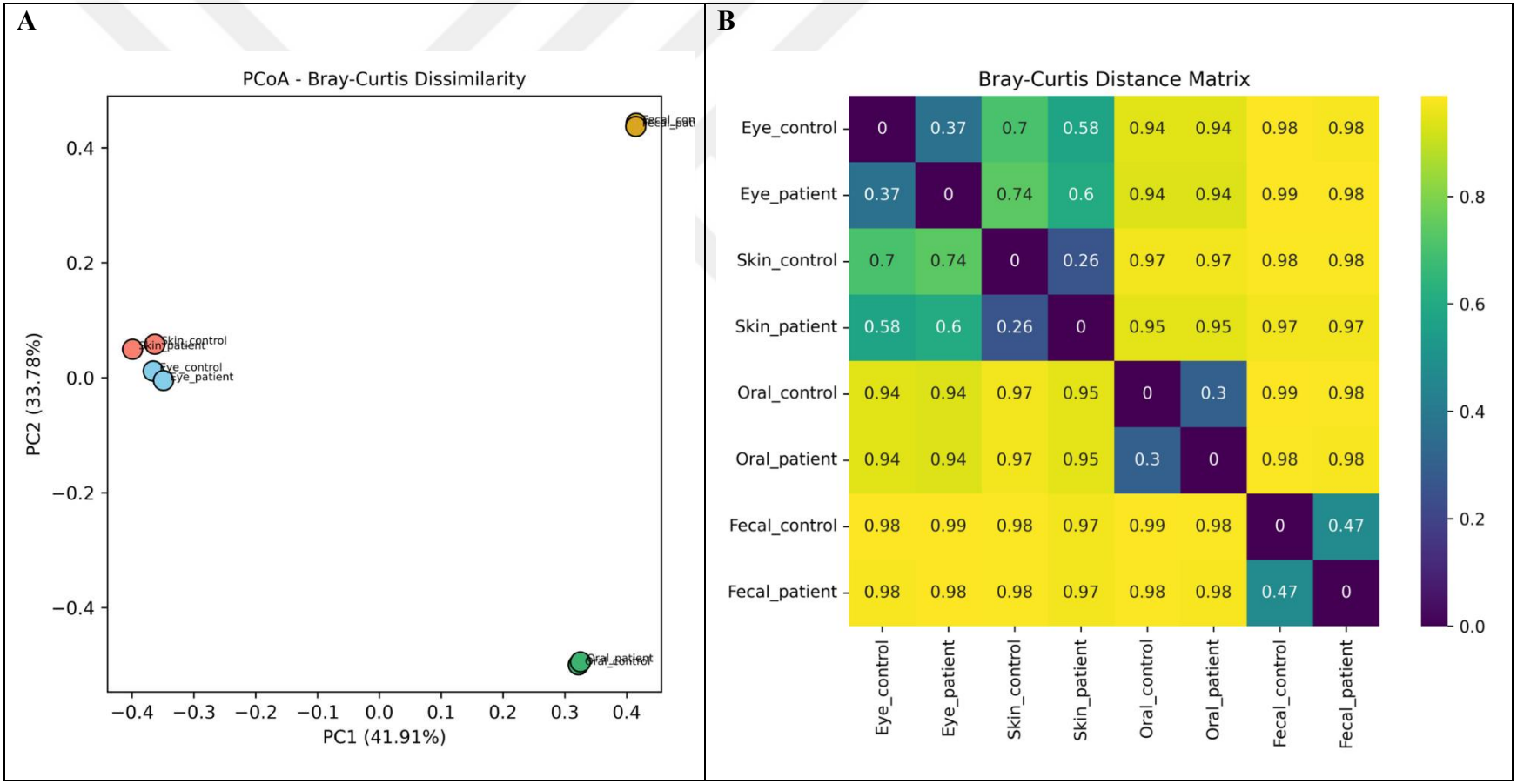

Figure 3.1: Beta diversity between the control and attack groups.

a) Principal coordinates analysis (PCoA) plot of UniFrac distances. Red circles represent the skin, blue circles represent the eye, yellow circles represent the fecal and green circles represent the oral samples.

b) Heat map of Bray Curtis Distance Matrix.

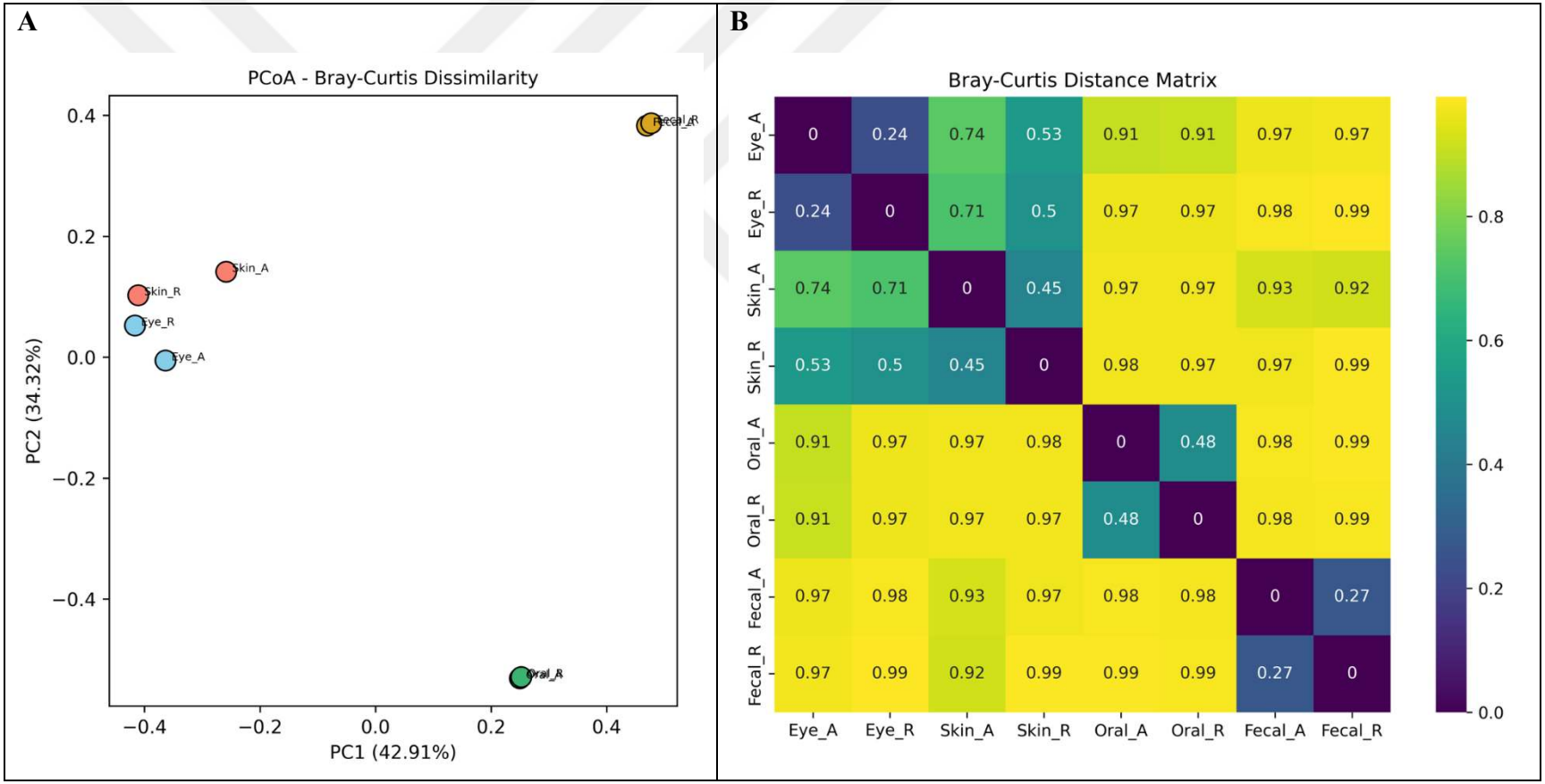

Figure 3.2: Beta diversity between the attack and remission groups.

a) Principal coordinates analysis (PCoA) plot of UniFrac distances. Red circles represent the skin, blue circles represent the eye, yellow circles represent the fecal and green circles represent the oral samples.

b) Heat map of Bray Curtis Distance Matrix.

3.4. Relative Abundance Analysis & Taxonomic Composition

We analyzed the differences in relative abundance across two group comparisons: (1) control vs attack and (2) attack vs remission, in all distinct body sites (skin, eye, fecal, and oral microbiota). Findings for each site are described in detail in the following sections below.

3.4.1. Skin Microbiome

In skin samples, the three dominant bacterial phyla identified were Firmicutes, Actinobacteria, and Proteobacteria.

3.4.1.1. Control vs Attack Comparison

In the comparison between control and attack samples, Firmicutes was the most predominant phylum, with an average relative abundance of 67.1% in controls and 62.6% in attack-phase samples. Actinobacteria had lower relative abundance in attack samples compared to the control group (16.0% in controls vs 10.0% in attack). See Figure 3.3.

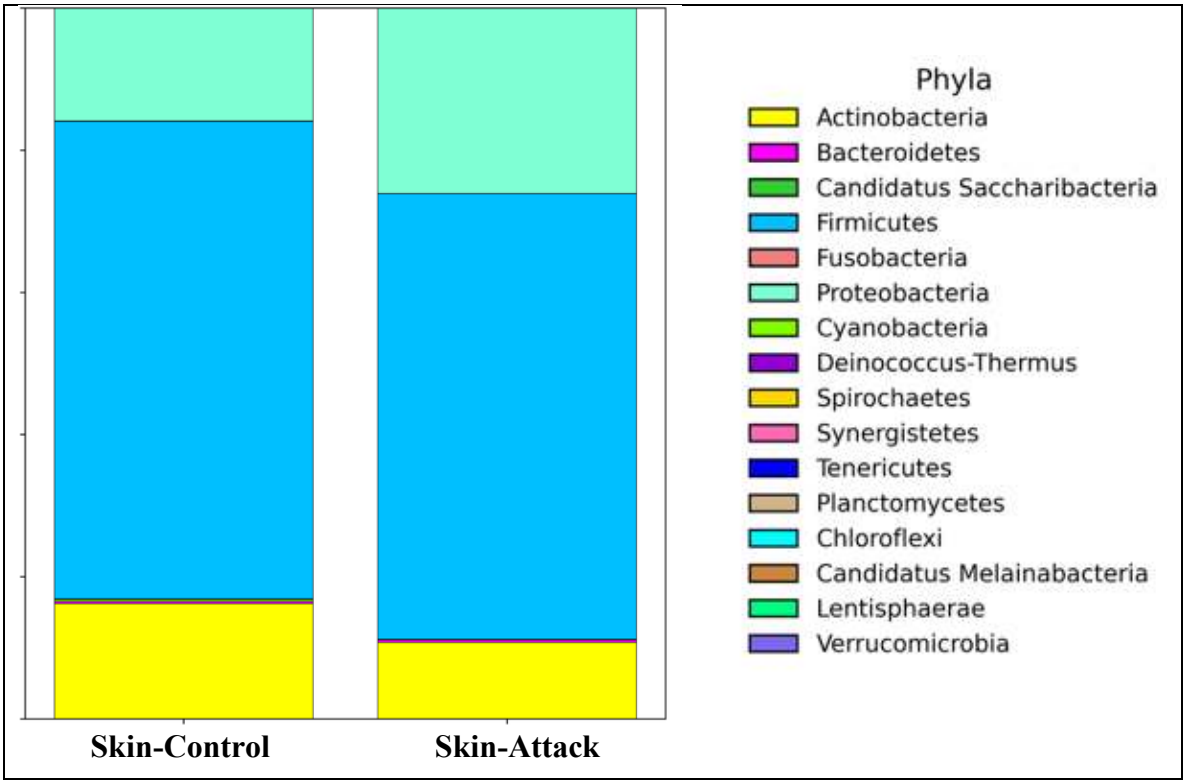

Figure 3.3: Skin Microbiota - Control vs Attack Comparison (Phylum)

At the genus level, Staphylococcus was the most dominant taxon in both control and attack samples. However, its relative abundance was lower in the attack group (53.0%) compared to the control group (62.0%). Similarly, the abundance of Cutibacterium was lower in the attack samples (2.1%) relative to controls (4.5%). See Figure 3.4.

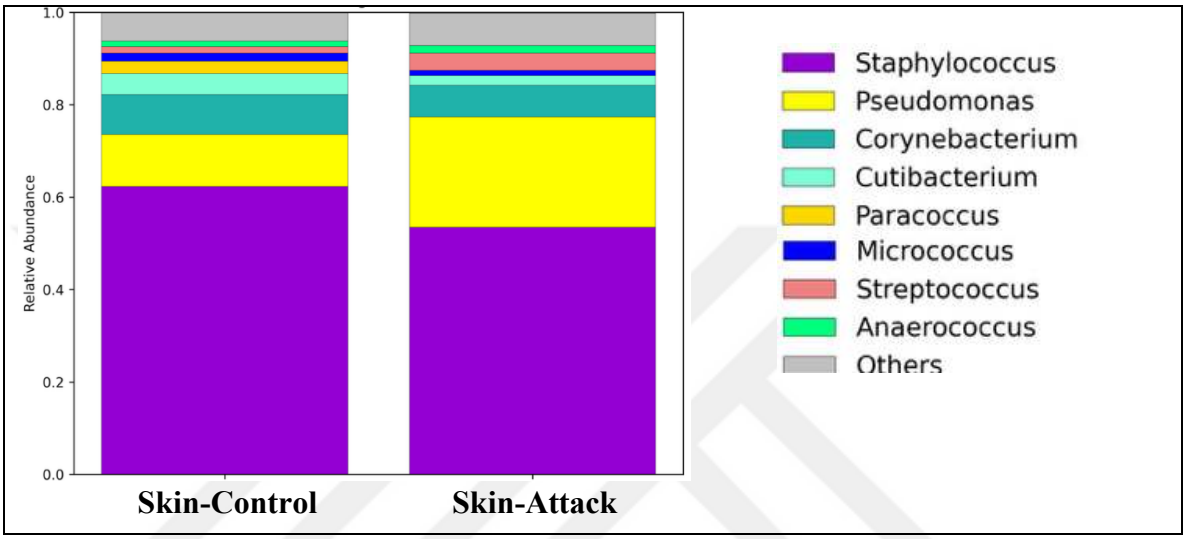

Figure 3.4: Skin Microbiota - Control vs Attack Comparison (Genus)

At the species level, Staphylococcus hominis (51% in controls vs 45% in attack), Cutibacterium acnes (4% vs 2%), and Corynebacterium tuberculostearicum (4% vs 2%) had lower relative abundance in the attack samples. See Figure 3.5.

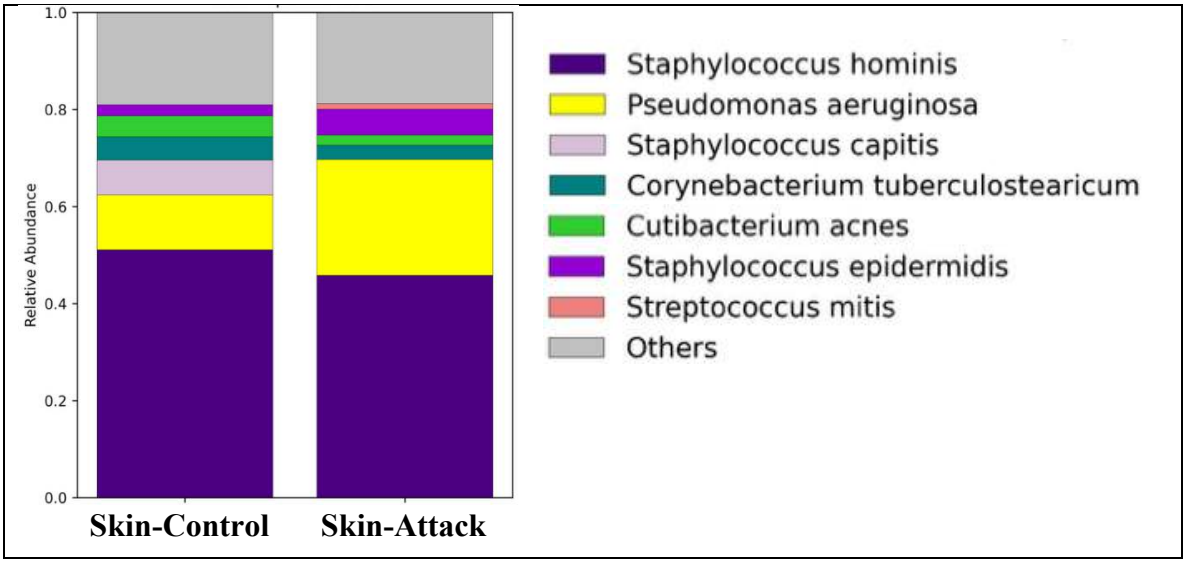

Figure 3.5: Skin Microbiota - Control vs Attack Comparison (Species)

3.4.1.2. Attack vs Remission Comparison

At the phylum level, Firmicutes was the most abundant phylum in attack and Proteobacteria was the most abundant phylum in the remission samples. However, relative abundance of Firmicutes was markedly higher during the attack phase (66.3%) compared to the remission phase (37.7%). See Figure 3.6.

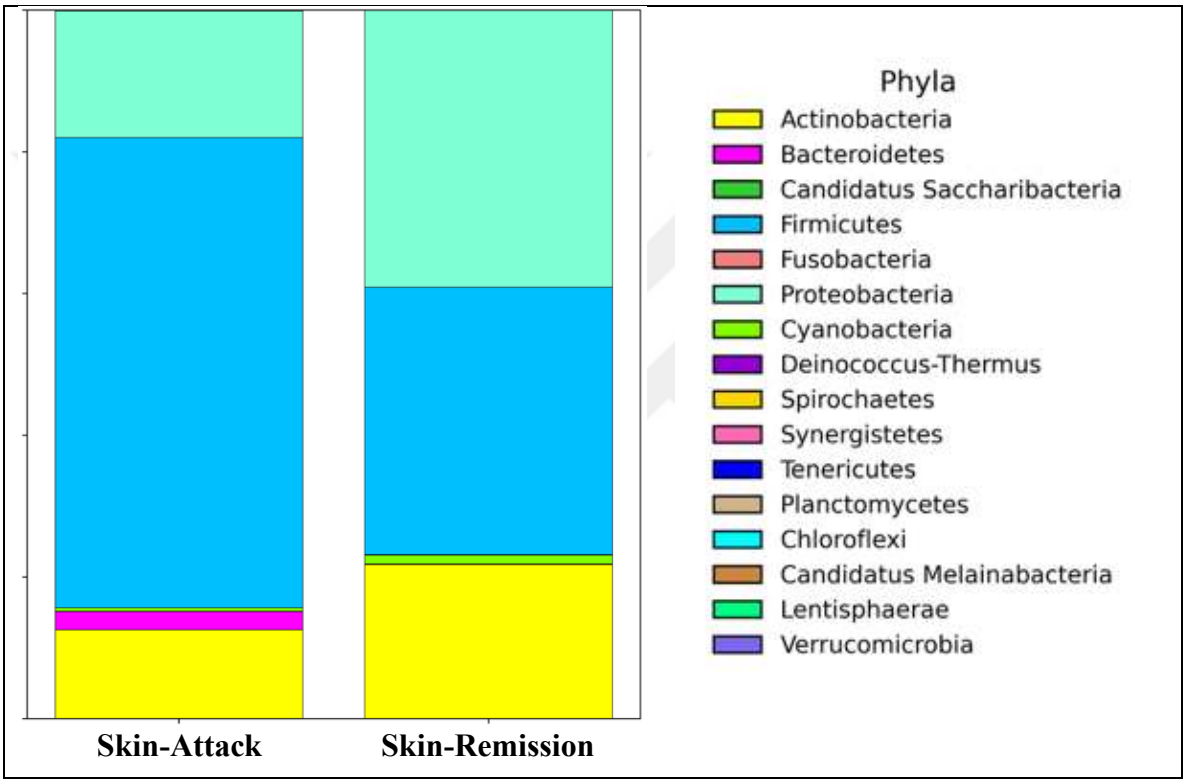

Figure 3.6: Skin Microbiota - Attack vs Remission Comparison (Phylum)

At the genus level, Staphylococcus and Corynebacterium were the most abundant genera in both phases. The relative abundance of Staphylococcus was higher during the attack phase (45%) compared to the remission phase (27%), while Corynebacterium showed the opposite trend, being lower in the attack phase (8%) and higher in remission (13%). See Figure 3.7.

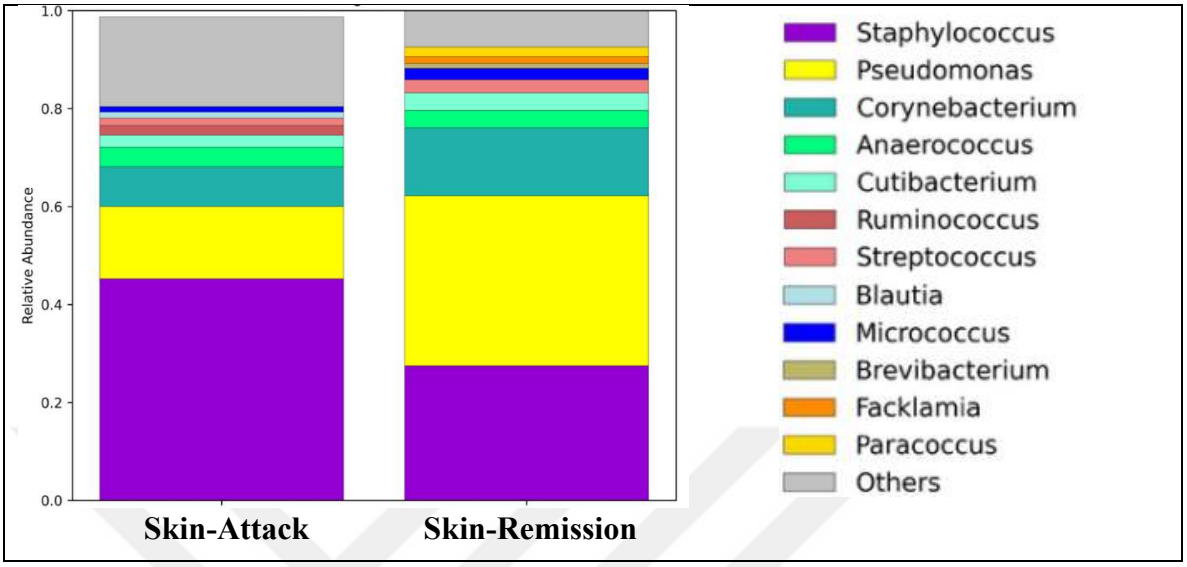

Figure 3.7: Skin Microbiota - Attack vs Remission Comparison (Genus)

In terms of species composition, the relative abundances of Staphylococcus hominis (35% vs 22%) and Staphylococcus epidermidis (8% vs 3%) were both higher in remission samples than in attack. Conversely, Cutibacterium acnes was higher in the remission samples (3%) compared to attack (2%). See Figure 3.8.

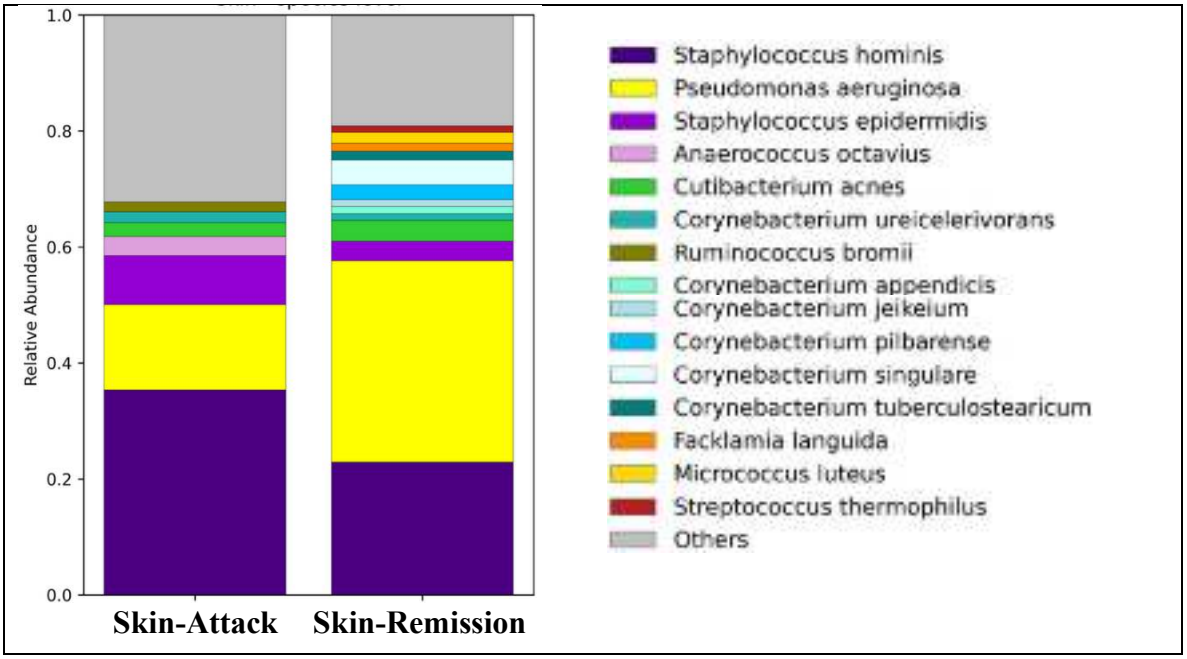

Figure 3.8: Skin Microbiota - Attack vs Remission Comparison (Species)

3.4.2. Oral Microbiome

In oral samples, the two most dominant phyla were Firmicutes and Proteobacteria.

3.4.2.1. Control vs Attack Comparison

At the phylum level, Firmicutes and Actinobacteria showed similar relative abundances in both groups (73% control vs 75% attack and 2% vs 3%, respectively). However, Proteobacteria had less relative abundance in the attack group (10%) compared to controls (19%), while Bacteroidetes higher in the attack samples (8% vs 3%). See Figure 3.9.

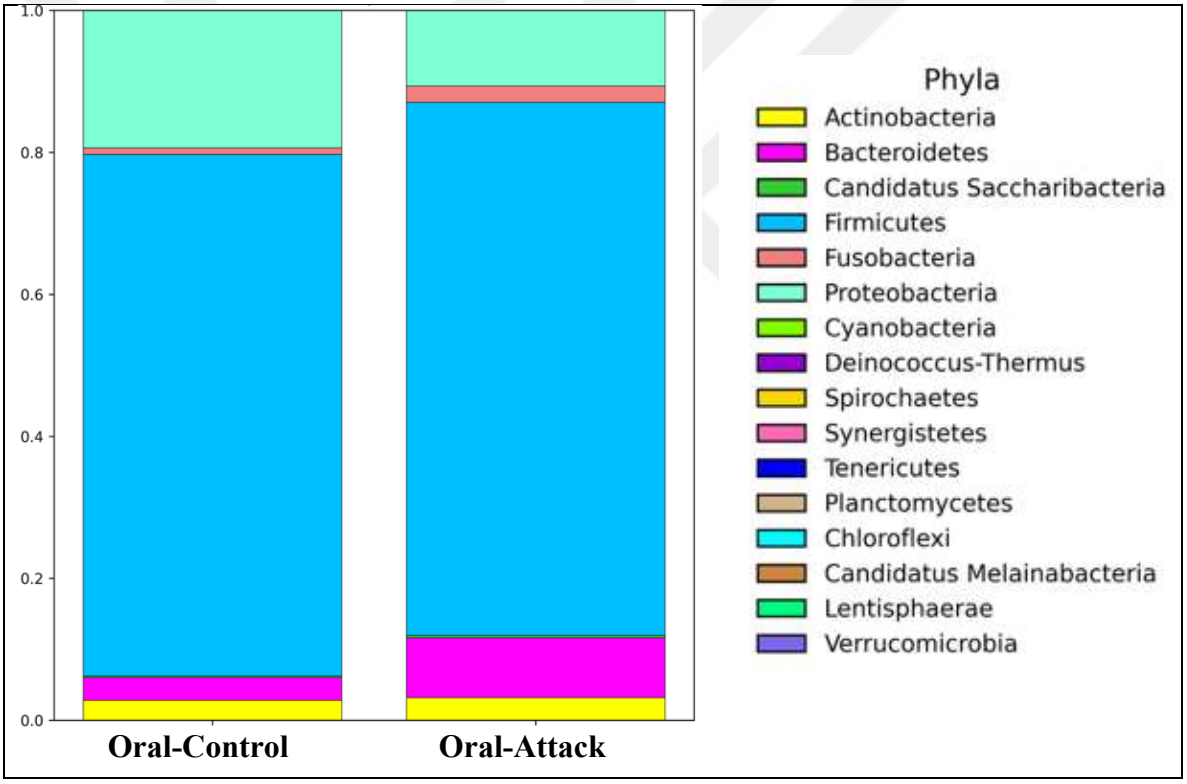

Figure 3.9: Oral Microbiota - Control vs Attack Comparison (Phylum)

At the genus level, the most dominant genera were Streptococcus, Haemophilus, and Veillonella. Streptococcus showed comparable levels in both groups (56% vs 54%), while Haemophilus exhibited a low abundance in the attack samples (3% vs 12%). See Figure 3.10.

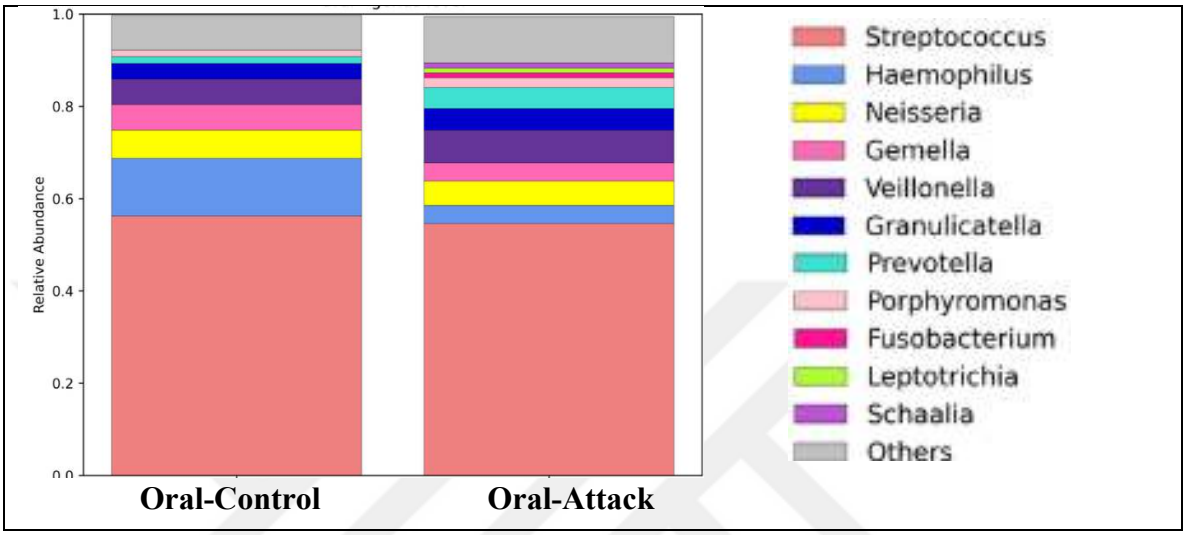

Figure 3.10: Oral Microbiota - Control vs Attack Comparison (Genus)

At the species level, Streptococcus mitis was less abundance in the attack group (15% vs 24%), whereas Streptococcus oralis was more abundant (10% vs 8%). Additionally, Streptococcus salivarius was detected at 6% in attack samples. See Figure 3.11.

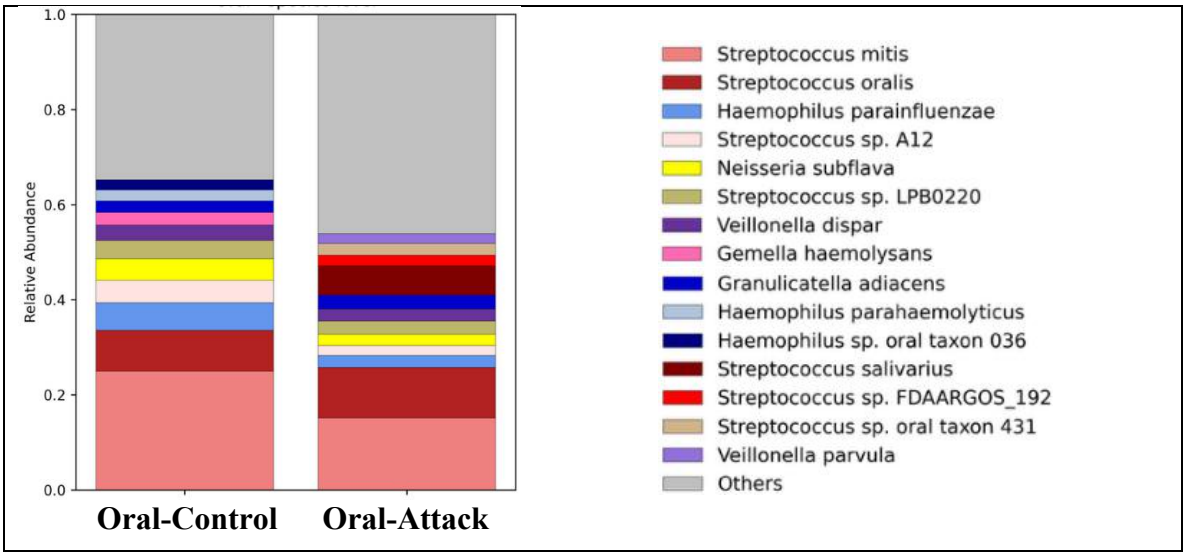

Figure 3.11: Oral Microbiota - Control vs Attack Comparison (Species)

3.4.2.2. Attack vs Remission Comparison

At the phylum level, Firmicutes, Proteobacteria, and Bacteroidetes remained the dominant taxa. Their relative abundances were similar between the two groups: Firmicutes (86% vs 85%) and Proteobacteria (3% vs 3%). Actinobacteria had slightly higher abundance in remission samples (2%) compared to the attack group (1%). Notably, Prevotella exhibited a higher abundance in remission (4% vs 1%). See Figure 3.12.

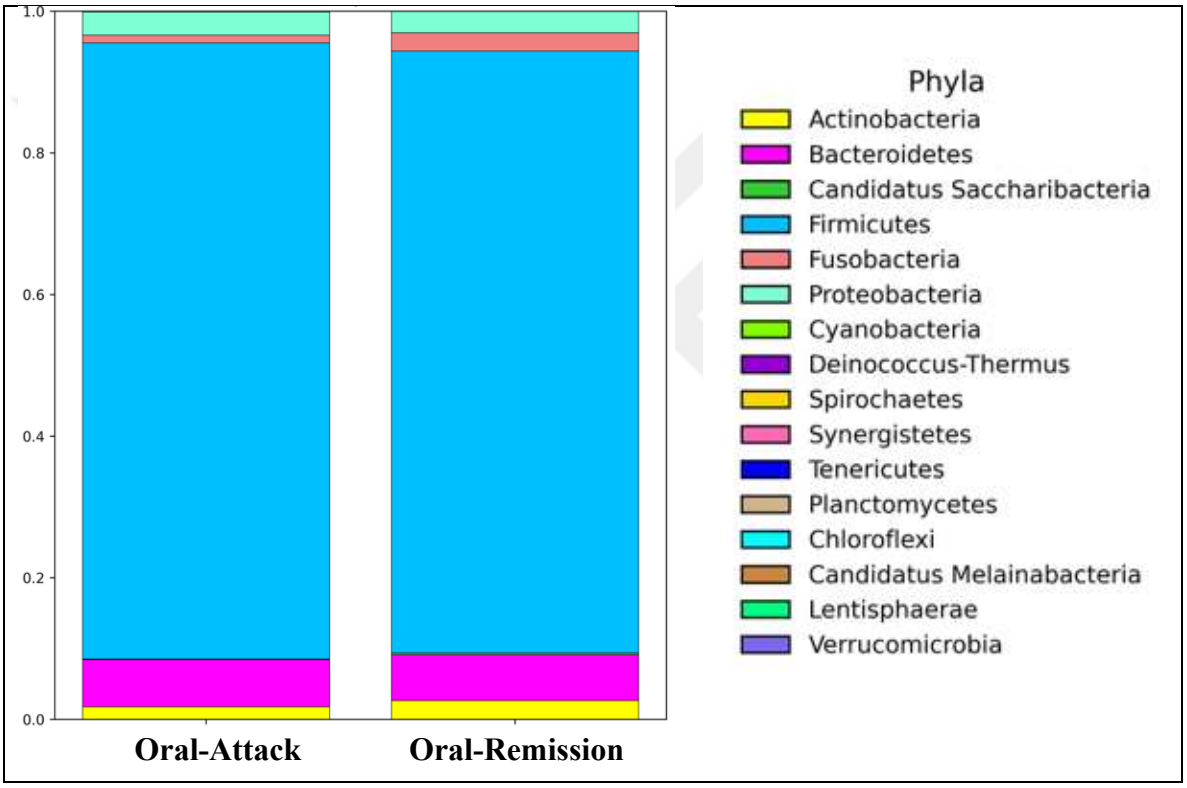

Figure 3.12: Oral Microbiota - Attack vs Remission Comparison (Phylum)

At the genus level, Streptococcus, Veillonella, and Prevotella displayed relatively similar abundances between attack and remission samples: 69% vs 66%, 10% vs 10%, and 4% vs 5%, respectively. Haemophilus was present at a relative abundance of 1% in attack-phase samples, whereas it was undetectable in remission-phase samples See Figure 3.13.

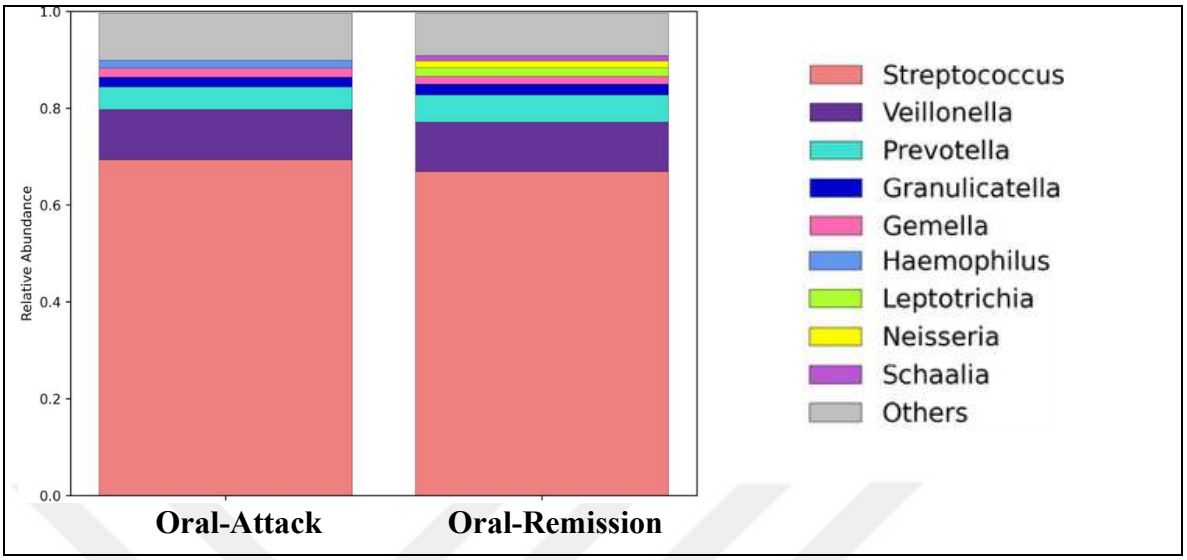

Figure 3.13: Oral Microbiota - Attack vs Remission Comparison (Genus)

At the species level, both Streptococcus mitis (22% vs 8%) and Streptococcus oralis (16% vs 7%) showed less abundance in remission samples. In contrast, Streptococcus salivarius demonstrated a high abundance in remission compared to the attack phase (25% vs 6%). See Figure 3.14.

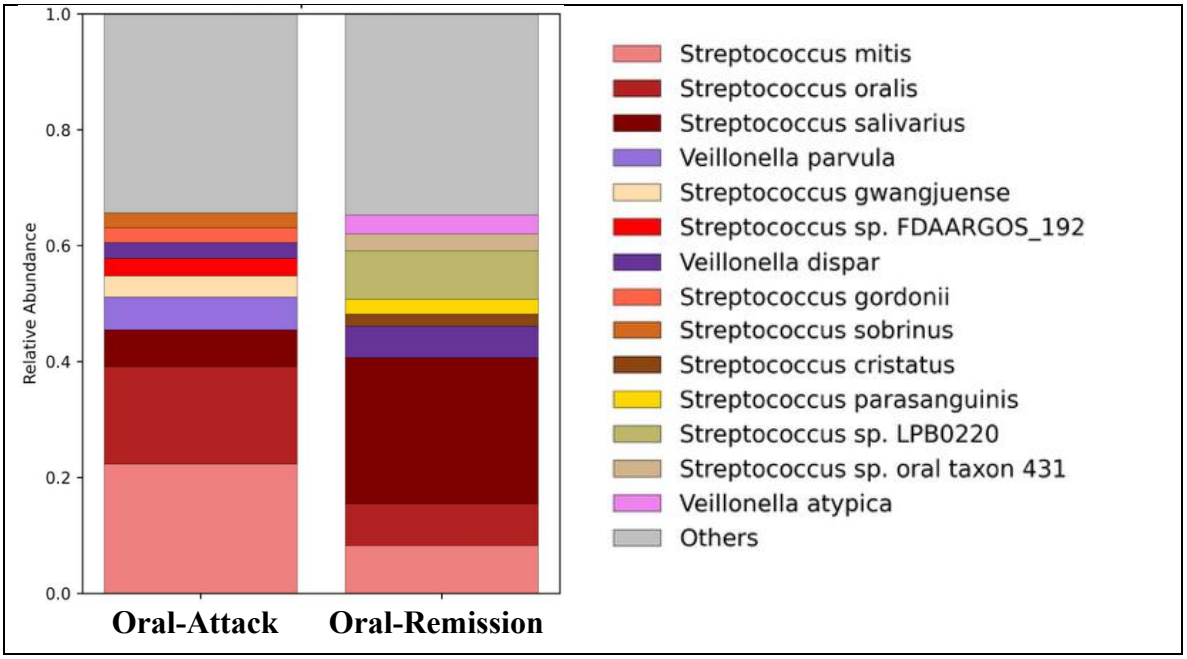

Figure 3.14: Oral Microbiota - Attack vs Remission Comparison (Species)

3.4.3. Eye Microbiome

In eye samples, the three most dominant phyla were Proteobacteria, Firmicutes, and Actinobacteria.

3.4.3.1. Control vs Attack Comparison

At the phylum level, Proteobacteria was markedly higher in the attack group compared to controls (72% vs 42%). In contrast, Actinobacteria (10% vs 28%) and Firmicutes (16% vs 28%) were both lower in the attack samples. See Figure 3.15.

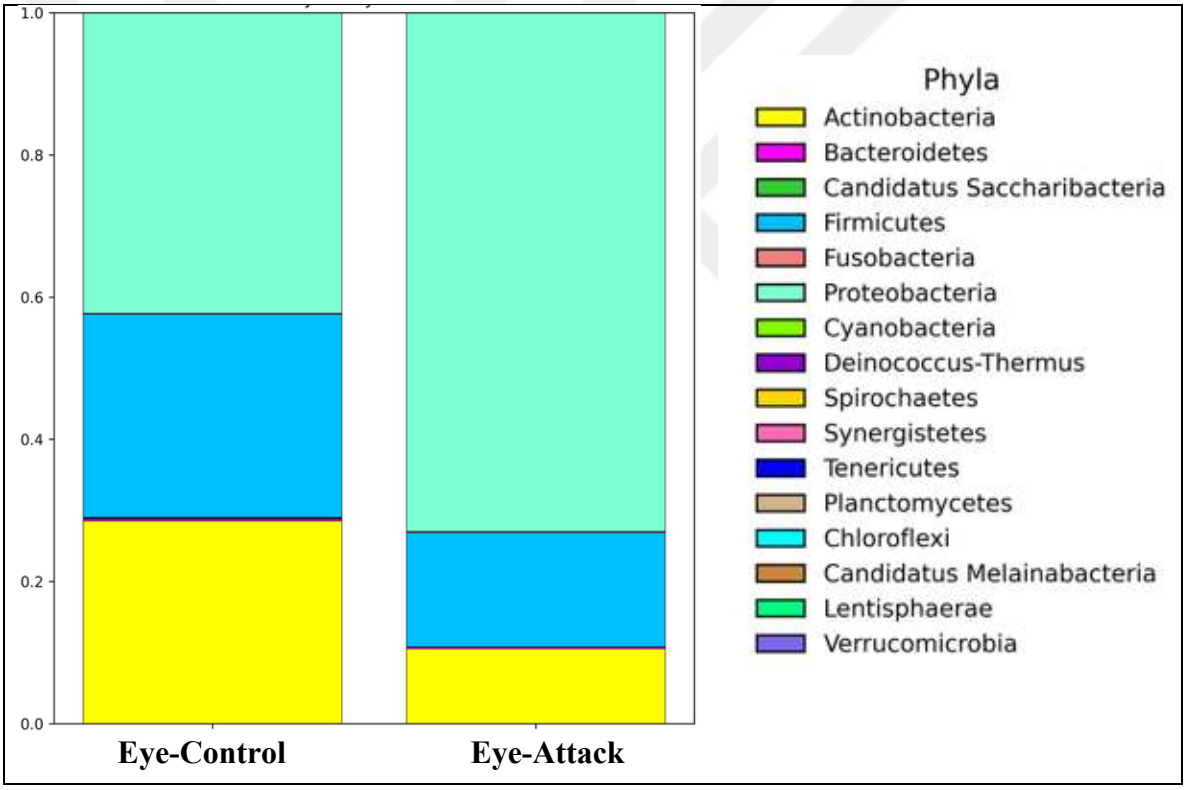

Figure 3.15: Eye Microbiota - Control vs Attack Comparison (Phylum)

At the genus level, Staphylococcus and Corynebacterium were less abundant in the attack group (9% vs 21% and 12% vs 19%, respectively). Conversely, Pseudomonas showed higher abundance in the attack samples (69% vs 39%). Specifically, the relative abundance of Pseudomonas aeruginosa was higher in the attack group (69%) compared to the control group (39%). Staphylococcus epidermidis was also lower in the attack samples (5% vs 15%), whereas Cutibacterium acnes maintained a similar abundance in both groups (8% vs 8%). See Figure 3.16 and Figure 3.17.

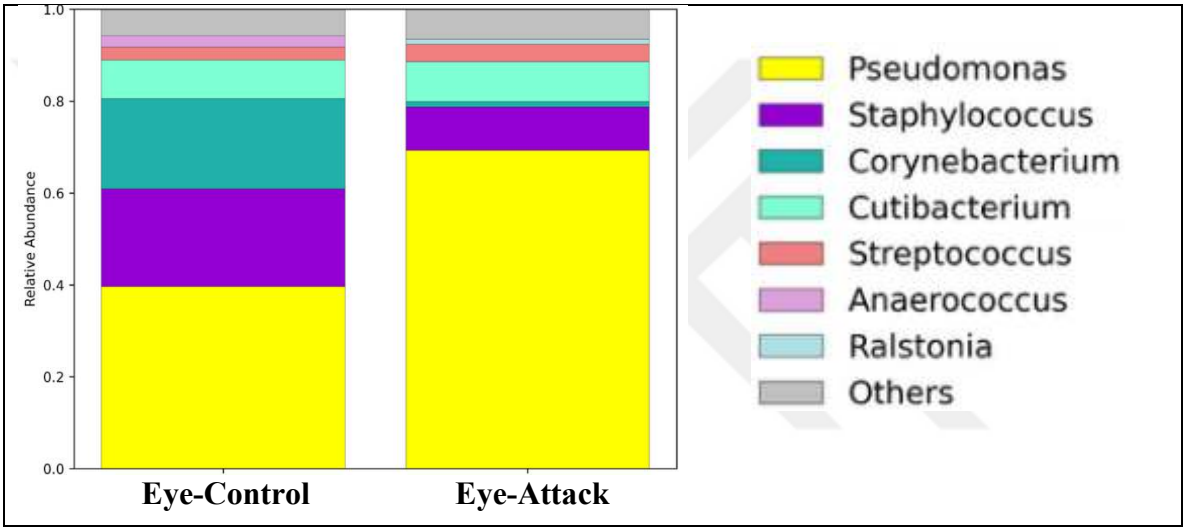

Figure 3.16: Eye Microbiota - Control vs Attack Comparison (Genus)

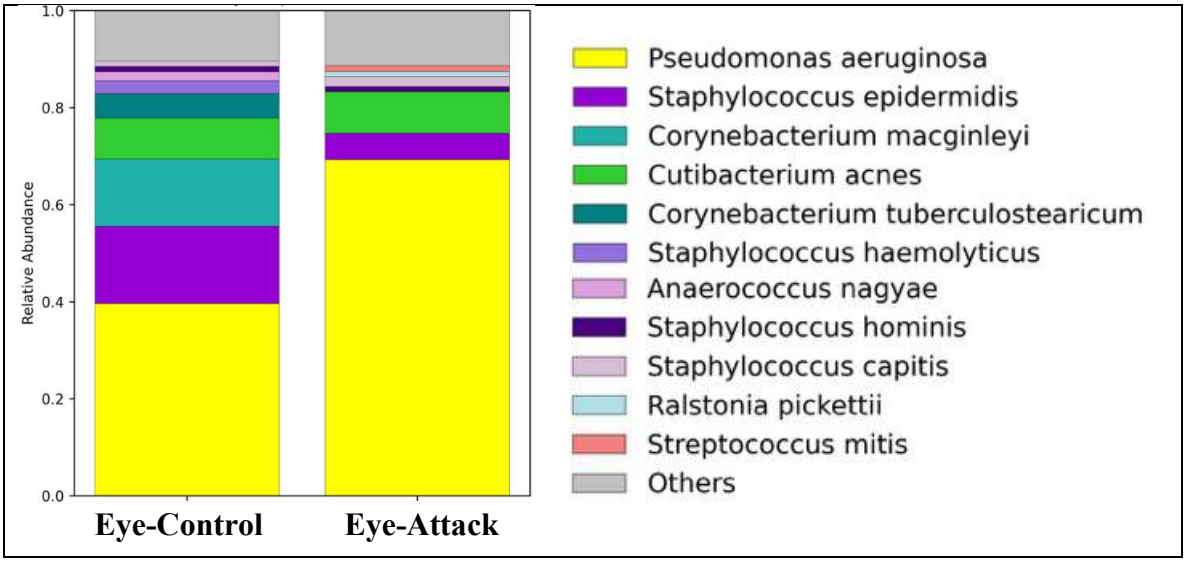

Figure 3.17: Eye Microbiota - Control vs Attack Comparison (Species)

3.4.3.2. Attack vs Remission Comparison

At the phylum level, Proteobacteria was less abundant in remission samples (65% vs 77%), while Actinobacteria (17% vs 7%) and Firmicutes (17% vs 14%) showed higher relative abundances in remission samples. See Figure 3.18.

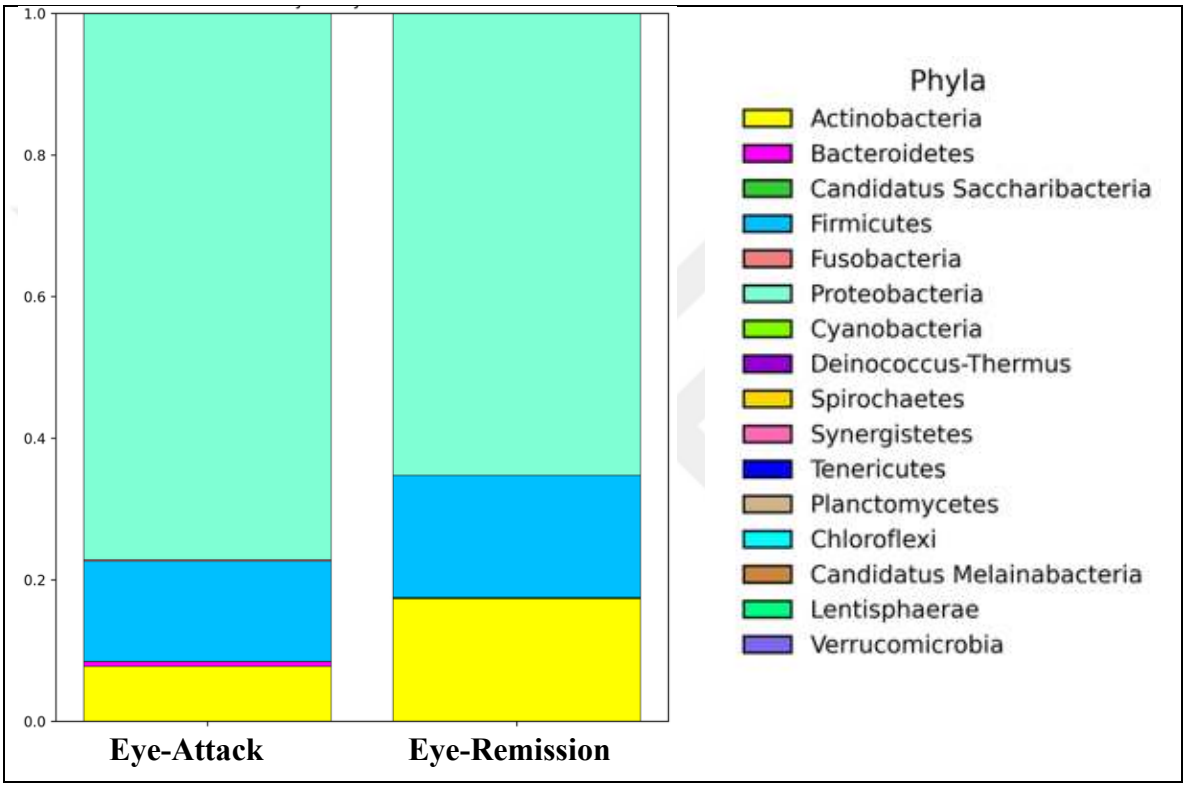

Figure 3.18: Eye Microbiota - Attack vs Remission Comparison (Phylum)

At the genus level, Pseudomonas and Streptococcus were less abundant in remission samples compared to the attack group (60% vs 71% and 2% vs 5%, respectively). Cutibacterium was higher relative abundance in remission samples (12% vs 5%).

In contrast, Cutibacterium acnes (12% vs 5%) and Staphylococcus epidermidis (5% vs 1%) were more abundant in remission samples. Likewise, Pseudomonas aeruginosa showed less abundance in remission samples (60%) compared to attack samples (71%). Additionally, Staphylococcus capitis was more abundant in remission samples (6% vs 1%). Findings are summarized in Figure 3.19 and Figure 3.20.

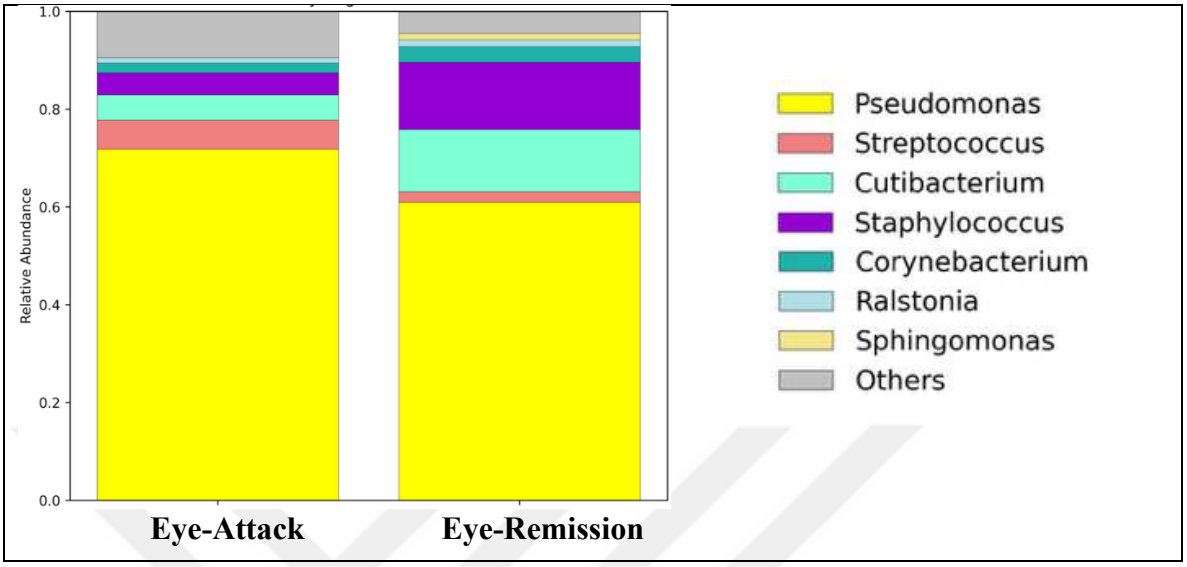

Figure 3.19: Eye Microbiota - Attack vs Remission Comparison (Genus)

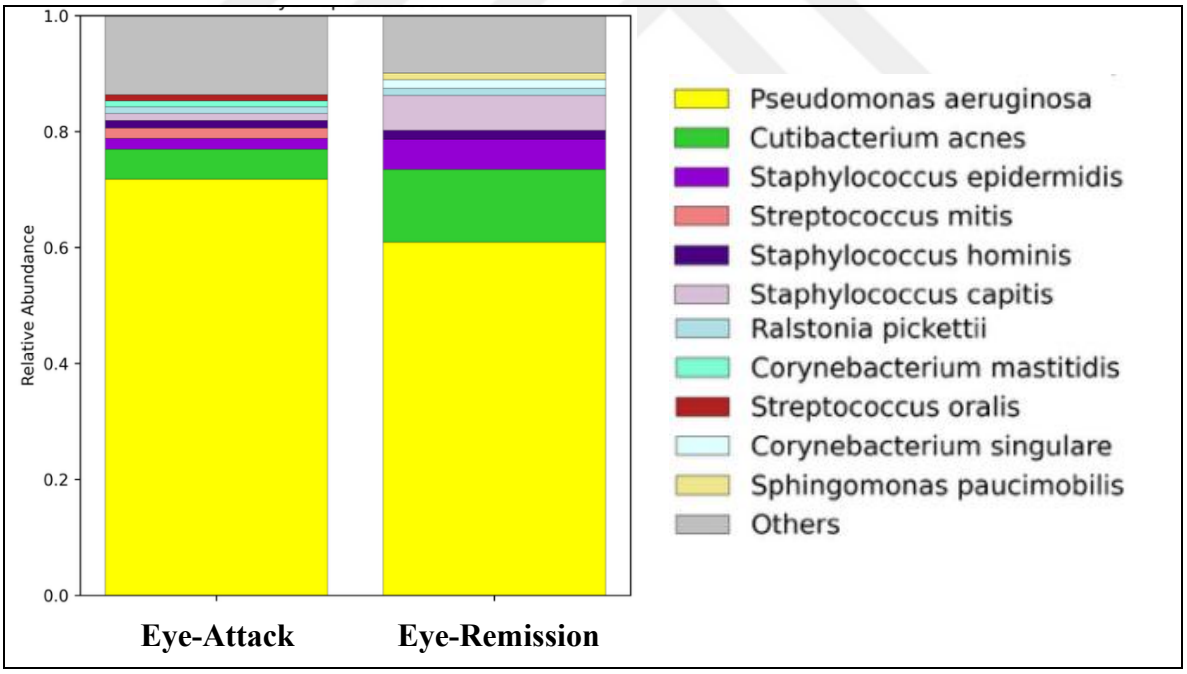

Figure 3.20: Eye Microbiota - Attack vs Remission Comparison (Species)

3.4.4. Fecal Microbiome

The fecal samples were predominantly composed of the phyla Firmicutes and Bacteroidetes, which were consistently observed as the dominant taxa.

3.4.4.1. Control vs Attack Comparison

At the phylum level, Firmicutes was higher in the attack group compared to the control group (77% vs 84%), while Bacteroidetes exhibited a lesser relative abundance in the attack samples (19% vs 4%). See Figure 3.21.

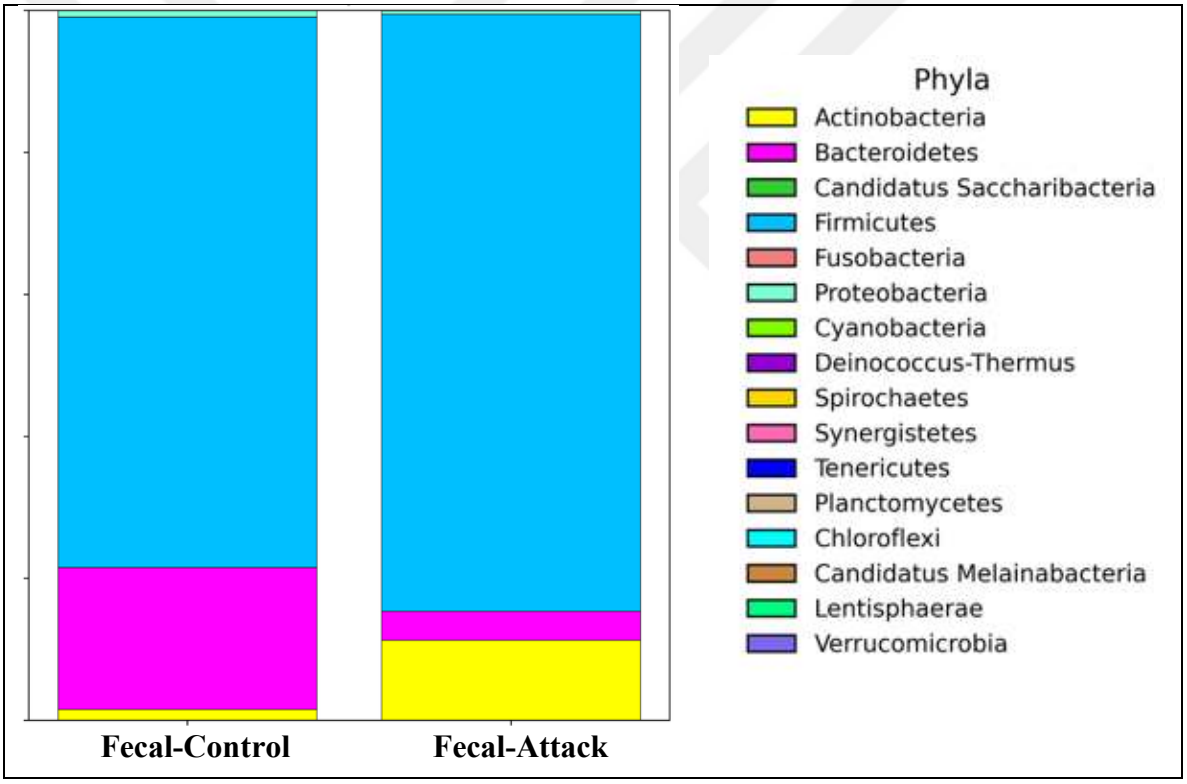

Figure 3.21: Fecal Microbiota - Control vs Attack Comparison (Phylum)

At the genus level, several genera demonstrated higher relative abundance in the attack group, including Collinsella (9% vs 1%), Blautia (25% vs 10%), and Dorea (4% vs 1%). In contrast, Prevotella (2% vs 13%) and Faecalibacterium (3% vs 14%) showed less relative abundance in the attack samples. Genera such as Anaerobutyricum and Anaerostipes were also more abundant in the attack samples. See Figure 3.22.

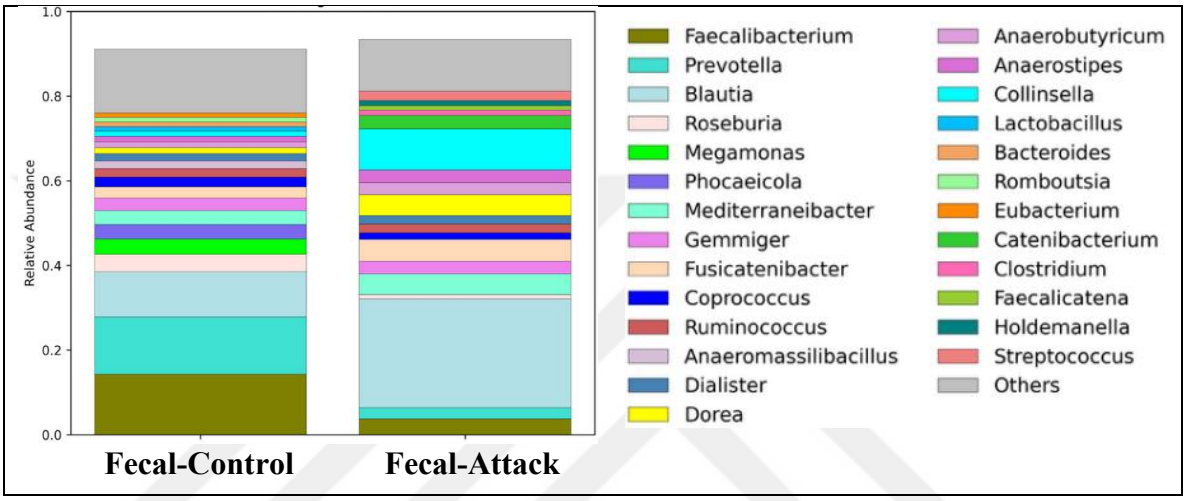

Figure 3.22: Fecal Microbiota - Control vs Attack Comparison (Genus)

At the species level, Faecalibacterium prausnitzii (3% vs 14%) and Prevotella copri (2% vs 13%) exhibited less abundance in the attack group compared to controls. Conversely, Blautia sp. SC05B48 showed a high abundance (11% vs 3%), and Collinsella aerofaciens was seen exclusively in the attack samples with a relative abundance of 9%. See Figure 3.23.

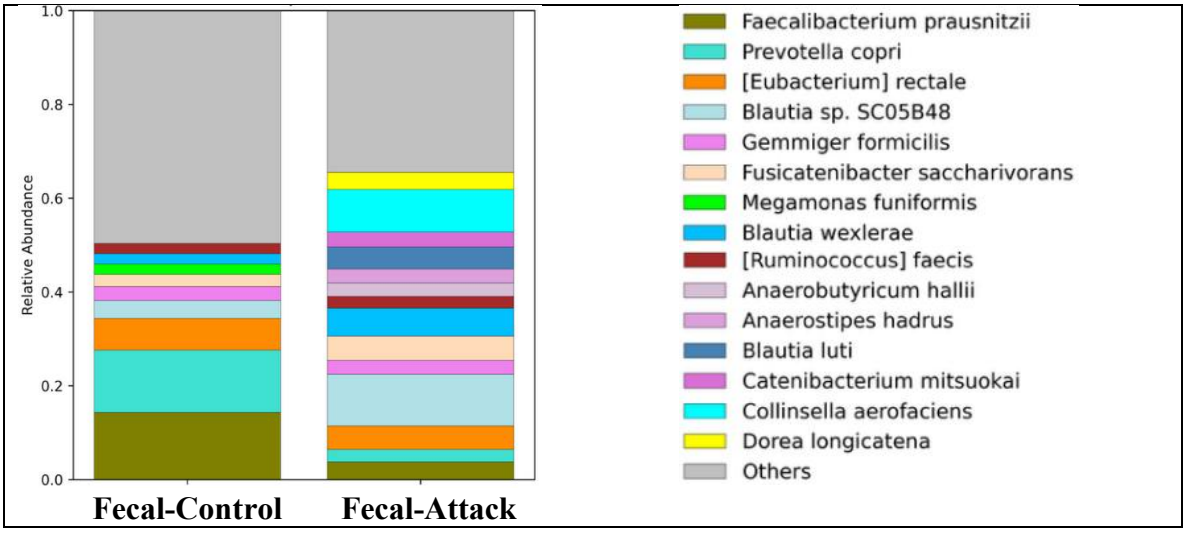

Figure 3.23: Fecal Microbiota - Control vs Attack Comparison (Species)

3.4.4.2. Attack vs Remission Comparison

In both groups, Firmicutes (85% attack vs 88% remission) and Actinobacteria (12% vs 11%) were the most dominant phyla, with comparable relative abundances. See Figure 3.24.

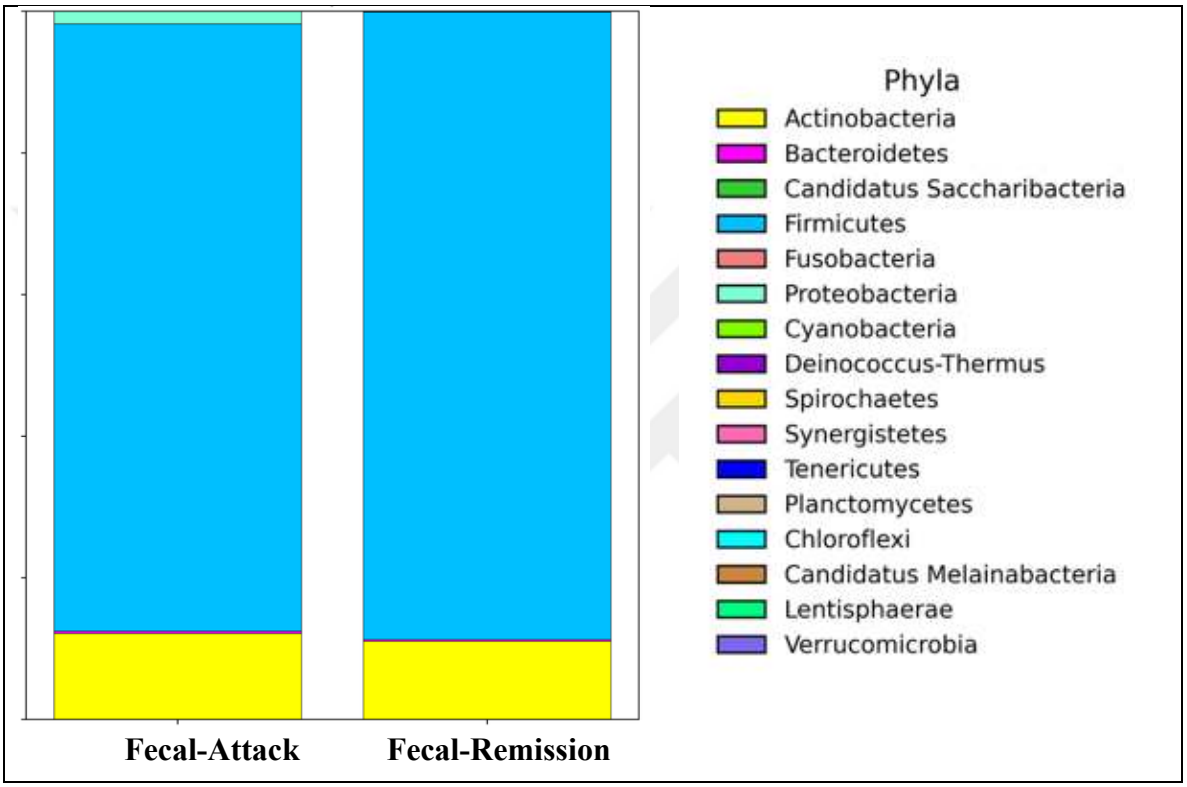

Figure 3.24: Fecal Microbiota - Attack vs Remission Comparison (Phylum)

At the genus level, Catenibacterium had higher abundance in remission samples (32% vs 23%), while Faecalibacterium displayed similar levels between the two groups (4% vs 3%). Collinsella was observed at a relative abundance of 10% in the attack group and 7% in the remission group. See Figure 3.25.

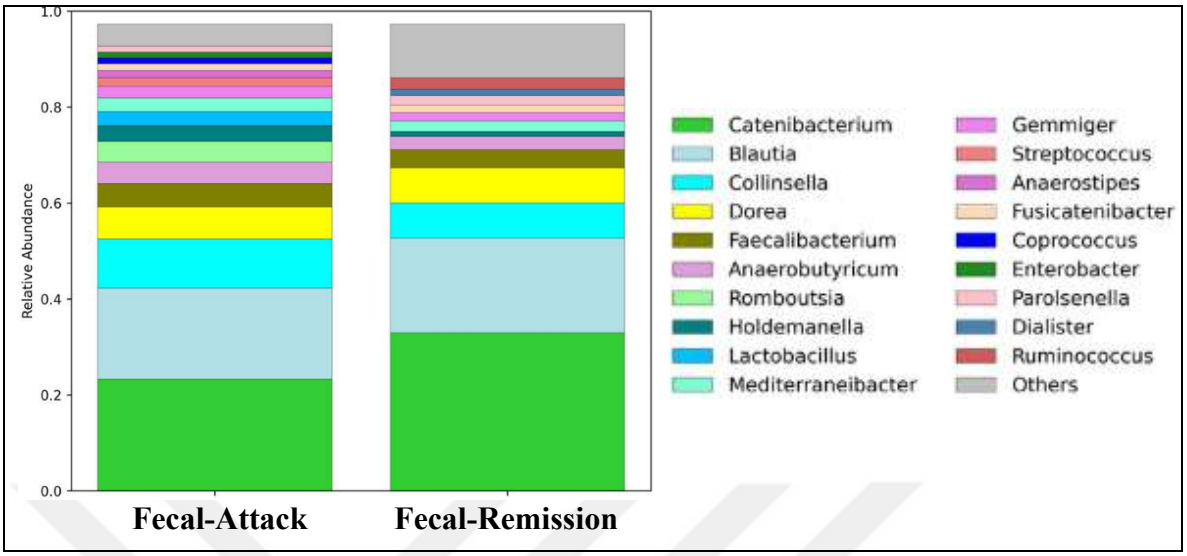

Figure 3.25: Fecal Microbiota - Attack vs Remission Comparison (Genus)

Collinsella aerofaciens decreased from 10% in the attack group to 7% in remission. Similarly, Catenibacterium mitsuokai showed a higher abundance in remission samples (32%) compared to attack (23%). See Figure 3.26.

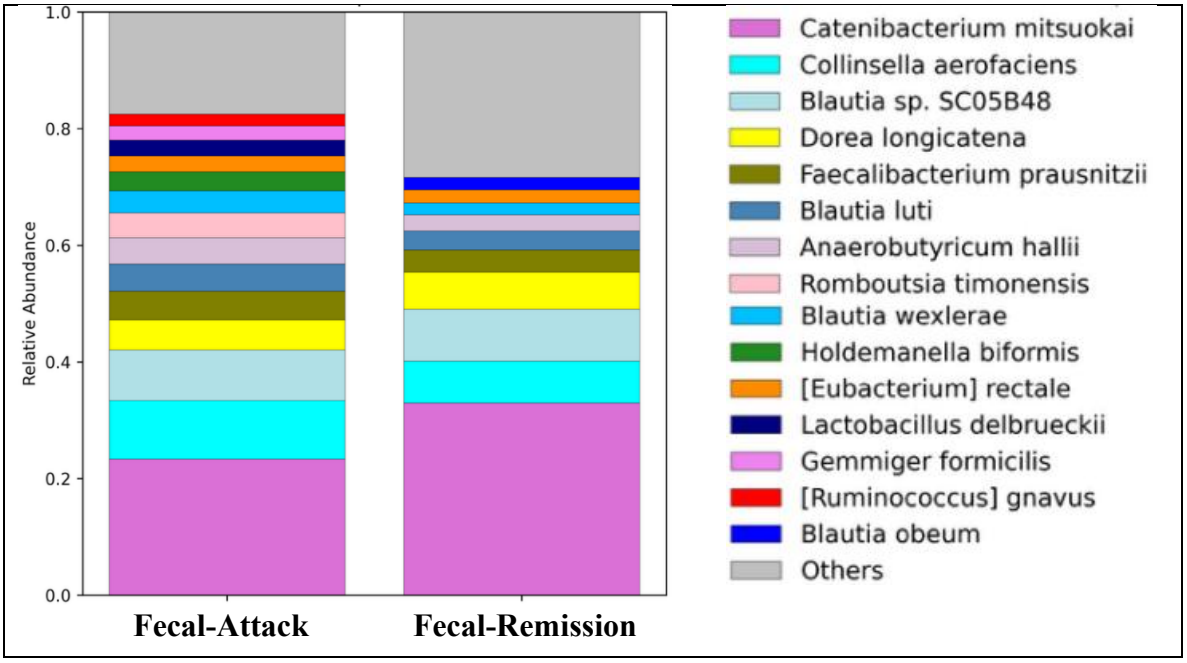

Figure 3.26: Fecal Microbiota - Attack vs Remission Comparison (Species)

CHAPTER 4
DISCUSSION

Behçet's disease (BD) is a chronic, multisystem inflammatory disorder in which genetic susceptibility, environmental triggers, and immune dysregulation are thought to interact. Growing evidence suggests that the microbiota may influence BD pathogenesis, yet studies comparing multiple body sites and different disease phases remain scarce. In this study, we investigated how Behçet's disease (BD) influences the microbiota across multiple body sites, including the skin, fecal, oral, and ocular niches, and how these microbial communities change between the attack and remission phases. Overall, alpha diversity, as measured by the Shannon Diversity Index and beta diversity analyses using the Bray–Curtis dissimilarity index revealed no distinct clustering patterns. Nevertheless, taxonomic profiling at the phylum, genus, and species levels uncovered notable compositional shifts in specific microbial taxa, particularly within the gastrointestinal compartment.

In the Fecal microbiota analysis, we observed that a statistically significant increase in the Firmicutes-to-Bacteroidetes ratio in BD (i.e. attack samples) compared to healthy controls, driven by a proportional rise in Firmicutes (77% to 84%) and a sharp reduction in Bacteroidetes (19% to 4%). This shift was consistent with patterns of gut dysbiosis reported in other inflammatory conditions, including inflammatory bowel disease and rheumatoid arthritis[120]. The elevated Firmicutes-to-Bacteroidetes ratio has been associated with metabolic imbalance and immune dysregulation[120]. Altered composition of GI microbiome has been argued to induce changes in microbial fermentation capacity and short-chain fatty acid (SCFA) production, both of which are important for mucosal immune homeostasis[121]. In the comparison between attack and remission phases of BD patients, the gut microbiota composition remained relatively stable at the phylum level, with Firmicutes and Actinobacteria dominating both conditions (85% vs. 88% and 12% vs. 11%, respectively; Figure 3.24).

Notably, the relative abundance of *Faecalibacterium prausnitzii*—a butyrate-producing bacterium with potent anti-inflammatory properties—was markedly reduced during attacks, compared to healthy controls (3% vs. 14%), consistent with prior reports linking its depletion to increased intestinal permeability and systemic inflammation in autoimmune diseases[122]. At the genus level, *Catenibacterium* was more abundant during remission (32% vs. 23%), whereas *Collinsella* was enriched during attacks (10% vs. 7%), the latter having been implicated in pro-inflammatory metabolic pathways and modulation of gut barrier function[120]. These findings suggest that while global microbial diversity remains stable, disease flares in BD are accompanied by specific taxonomic shifts.

Looking into studies from literature, Consolandi et al. compared the fecal microbiota of 22 patients with Behçet Disease with those of 16 healthy controls with similar lifestyle (i.e. diet) in an Italian cohort[119]. Pyrosequencing of 16S rRNA (V3-V4) showed decrease in relative abundance of *Subdoligranulum* and *Roseburia*. The former decreased from 3.28% (controls) to 1.98% (BD) while the latter moved from 10.45% (controls) to 4.97% (BD). The group later on carried out extracted short chain fatty acids from fecal samples of the cohort and correlated the previous findings with decreased butyrate production. The authors hence argued a peculiar dysbiosis in BD, revolving around diminished T-reg responses.

Shimizu et al. performed a metagenomic analysis in a Japanese cohort, comparing the fecal microbiome of healthy controls (n=12) with BD patients (n=12)[102]. The group demonstrated an increase in *Eggerthella* and *Bifidobacterium* and a decrease in *Prevotella* and *Megamonas* in BD compared to controls. Their findings are illustrated in Figure 4.1, where increased bacterial taxa are represented in red and green in BD and controls, respectively.

In a subsequent study, Shimizu et al. polished their previous findings on compositional alterations of gut microbiome with addition of a functional analysis via PICRUSt software[103]. This time in a larger Japanese cohort of 13 BD and 27 controls, the group drew parallels between dysbiosis in Behçet's disease (i.e. abundant *Lactobacillus* and *Eggerthella* in BD while abundant *Megamonas* and *Butyrovibrio* in controls) and metabolic

alterations (prominent pentose phosphate pathway and inosine monophosphate biosynthesis in Behcet's Disease).

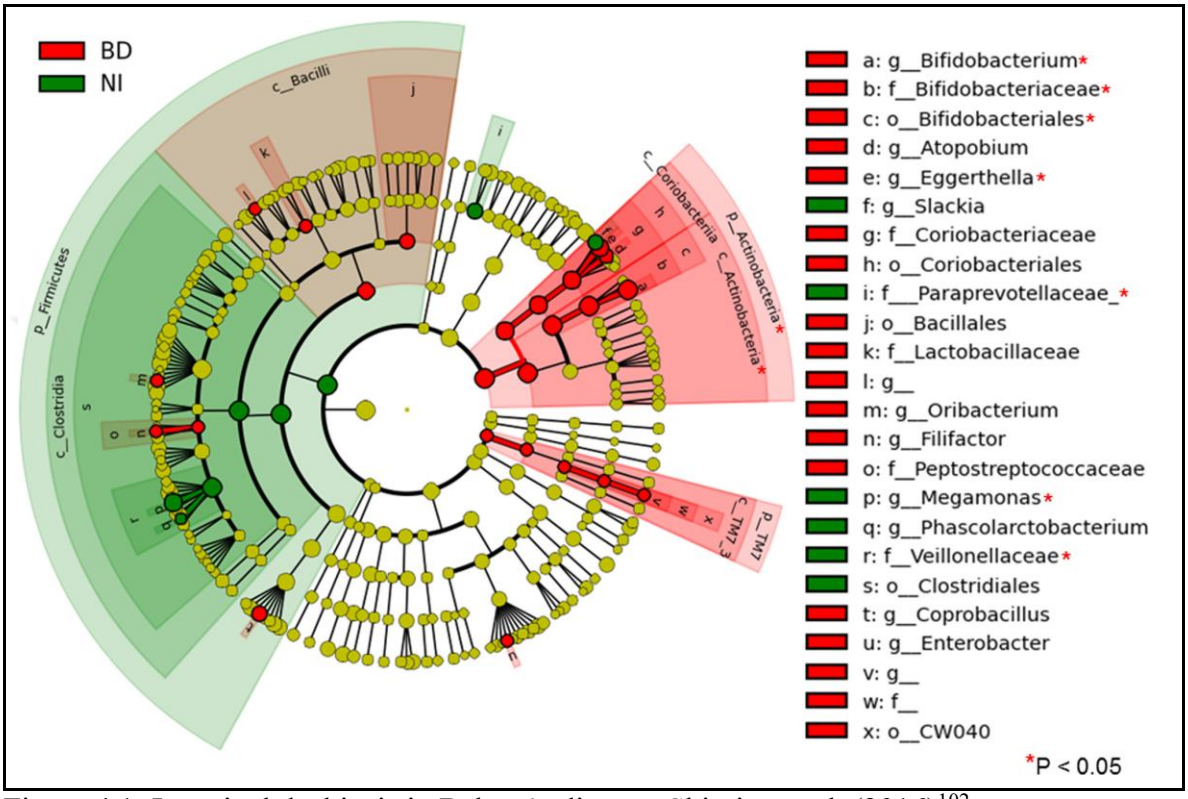

Figure 4.1: Intestinal dysbiosis in Behçet's disease, Shimizu et al. (2016)[102].

Ye et al. evaluated microbiome changes in BD via a Chinese cohort of 32 patients with BD and 74 controls[104]. DNA extracted from fecal samples were used in metagenomic analysis, while 16S rRNA sequencing was performed for saliva. Using a similar analogy with the previous studies, Ye reported an increase in *Bilophila*, *Paraprevotella* and *Parabacteroides* in BD and decrease in *Clostridium*. In parallel with the previous studies, relative enrichment in SRB over BRB was hence observed in their cohort as well.

Further analysis in Ye's Chinese cohort revealed positive correlation between sulphate-reducing and opportunistic bacteria and a negative correlation with butyrate-producing and methanogen bacteria. To suggest a causative effect between the afore-mentioned findings and BD, the group performed fecal transplant in mice, which yielded an aggravation in proinflammatory response[104].

Oezguen et al. examined fecal microbiome changes in patients with Behçet's Disease in a Turkish cohort, where 13 patients were compared with 14 controls[107]. Analysis upon 16S rRNA sequencing yielded an increase in *Clostridiales* in Behçet's Disease, better seen after *Prevotella*-stratification. It is crucial to highlight here that all of the BD patients in this cohort had indeed Neuro-Behçet's Disease.

Tecer et al. in their Turkish cohort included 7 patients with Behçet's disease, 12 patients with Familial Mediterranean Fever, 9 patients with Crohn's disease and 16 controls[123]. 16S rRNA sequencing of fecal samples stratified the cohort in 4 different microbiome signatures, as seen in Figure 4.2. In short, BD patients had higher amounts of *Succinivibrionaceae* and *Veillonellaceaea*, and were depleted in *Bacteroidaceae*. Similarly, it is worth mentioning here that all of the BD patients in this cohort had uveitis as part of their disease profile.

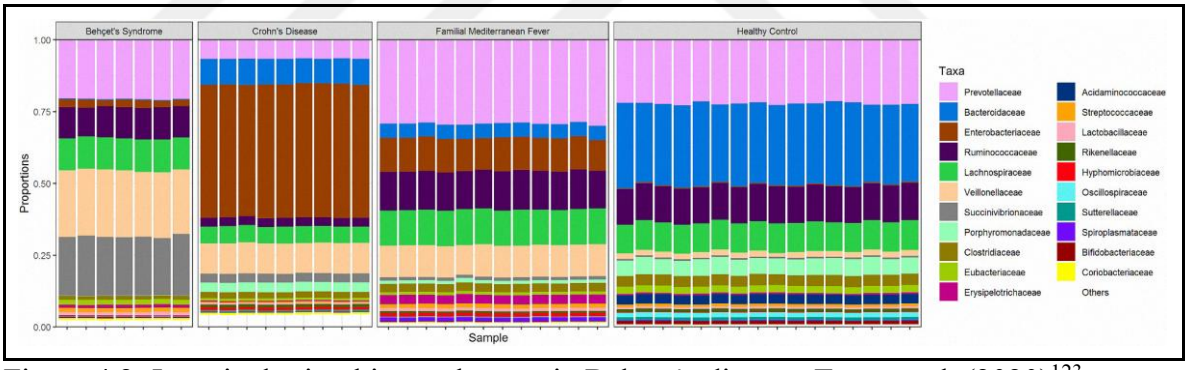

Figure 4.2: Intestinal microbiome changes in Behçet's disease, Tecer et al. (2020)[123]

Yet in another Turkish cohort, Bilge et al. compared BD with 3 distinct clinical profiles with healthy controls. In their study, BD patients (n=27) were stratified based on mucocutaneous (n=10), eye (n=7) and vascular (n=10) involvement[108]. Fecal samples were compared with those of healthy controls (n=10). Although alpha and beta microbial diversity showed no statistically significant difference between BD patients and controls, *Collinsella* and *Eggerthella* among others were found to be increased in BD, while *Lachnospira* and *Akkermansia* were decreased in BD.

Although the sample size was small to make solid deductions, the group described significant changes in microbiome according to distinct clinical profiles of BD as well, highlighting *Marvinbryantia*, *Dialister* and *Intestinomonas* for the mucocutaneous, *Gemella* in the vascular involvement and *Lachnospiraceae NK4A136* in the eye involvement groups[108]. We must emphasize here that our cohort was not suitable to segregate patients in distinct clinical groups as such, since the majority of our patients had more than one system involved without any clear-cut organ-based distinction.

Van der Houwen et al. evaluated the microbiome analysis with a different point-of-view and constructed two cohorts, one in Netherlands and one in Italy, to underscore the generalizability or population-centered nature of their findings[124]. They included 19 BD patients with 17 controls in the Netherlands and 13 BD patients with 15 controls in Italy cohorts. Fecal sample analysis illustrated a decrease in unclassified *Barnesiellaceae* and *Lachnospira* in both cohorts. The difference was statistically significant in the Italian cohort, while it was only a trend in the Dutch cohort. Moreover, the group performed oral microbiome analysis (limited to Dutch cohort) and noted an increase in *Dethiosulfovibrionaceae* and *Spirochaetaceae*.

In the oral microbiota, we observed that the dominant genera across all groups were *Streptococcus*, *Haemophilus*, and *Veillonella*, consistent with typical composition of a healthy oral ecosystem described in literature[125]. A notable decrease in *Haemophilus* was observed in BD, compared to healthy controls (3% vs. 12%) Within the BD cohort, Haemophilus was detected at a relative abundance of 1% during the attack phase but was undetectable in remission samples. In fact the decrease in BD comparted to contrls was also reflected at the species level by decreased abundance of *Haemophilus parainfluenzae,* which is a commensal species associated with oral health and immune modulation[126]. In fact, reduction of *Haemophilus* has been associated with an anaerobe-dominated community, which may in turn perpetuate a pro-inflammatory state in oral microbiome[127]. Conversely, *Prevotella* was relatively enriched in remission samples, in line with studies showing that *Prevotella* species, although part of the normal oral flora, can act as

opportunistic pathogens under inflammatory or dysbiotic conditions[128]. We believe this might be valid for Behçet's disease pathogenesis as well.

Similarly, Kim et al. performed fecal and salivary microbiome analysis, this time in a Korean cohort[129]. Their cohort included patients with BD (n=9), recurrent aphthous ulcers (n=7) and controls (n=16). 16S rRNA gene sequencing demonstrated that alpha diversity was not different between BD and matched controls, while a tendency of clustering was observed for beta diversity. Furthermore, increased *Bacteroides uniformis* was observed in fecal samples of patients with BD. Similar to our setup, the cohort was followed for remission, and in fact *Bacteroides uniformis* levels were higher during disease flares compared to remission. Regarding salivary microbiome analysis, in contrast with other studies (that will follow shortly), there was no statistically significant difference in terms of diversity between active BD, inactive BD and controls. Yet the group mentioned decreased *Veillonella* in BD patients with uveitis, compared to BD patients without uveitis[129].

Seoudi et al. focused on oral microbiome in BD in their UK-based cohort[130]. They compared 54 patients with BD, 8 patients with recurrent aphthous stomatitis (RAS) and 25 controls and noted that at the ulcer sites, patients with BD had increased *Streptococcus salivarius* and *Streptococcus sanguinis* compared to RAS patients and controls, respectively. Meanwhile, non-ulcer sites yielded increased levels of *Rothia denticariosa*. On the other hand, controls had higher *Veillonella* and *Neisseria*. The authors implied that the level of evidence was not enough to argue a causative relationship (instead of reactive) between oral microbiome changes and development and BD.

Coit et al. examined oral microbiome changes in Behçet's disease in a Turkish cohort where 31 patients were compared to 15 controls via 16S rRNA V4 high-throughput sequencing[131]. The group reported increased amounts of *Haemophilus parainfluenzae* and decreased *Alloprevotella rava* in BD and a less diverse microbiome compared to controls. Interestingly, the group had post-periodontal treatment samples for a subset of patients (n=9), however before-after comparison demonstrated no direct effect on oral microbiome, despite clinical improvement of periodontal symptoms.

To better summarize the literature, characteristics of the cohorts mentioned in this section are illustrated in Table 4.1, adapted from the contemplative review of Joubert et al.[98].

Table 4.1: Summary of characteristics of the cohorts. Adapted from Joubert et al.[98].

Authors	Population	Cohort	Microbiome	Reference
Consolandi et al.	Italian	BD (n=22) Control (n=16)	Intestinal	[101]
Shimizu et al.	Japanese	BD (n=12) Control (n=12)	Intestinal	[102]
Shimizu et al.	Japanese	BD (n=13) Control (n=17)	Intestinal	[103]
Ye et al.	Chinese	BD (n=32) Control (n=74)	Intestinal	[104]
Oezguen et al.	Turkish	BD (n=13) Control (n=14)	Intestinal	[107]
Tecer et al.	Turkish	BD (n=7) FMF (n=12) Crohn (n=9) Control (n=16)	Intestinal	[123]
Bilge et al.	Turkish	BD (n=27) Control (n=10)	Intestinal	[108]
Van der Houwen et al.	Dutch	BD (n=19) Control (n=17)	Intestinal	[124]
	Italian	BD (n=13) Control (n=15)	Intestinal	
Kim et al.	Korea	BD (n=9) RAU (n=7) Control (n=16)	Intestinal Oral	[129]
Seoudi et al.	UK	BD (n=54) RAS (n=8) Control (n=25)	Oral	[130]
Coit et al.	Turkish	BD (n=31) Control (n=15)	Oral	[131]

Regarding the skin microbiome, *Staphylococcus* was the most dominant genus in both control and attack samples, although its relative abundance was lower in the attack group (53.0%) compared to controls (62.0%). This finding aligns with previous reports showing that *Staphylococcus* species, particularly *S. epidermidis*, are key commensals involved in maintaining cutaneous immune balance, and that their reduction can be associated with skin barrier dysfunction and inflammation[132]. Similarly, *Cutibacterium* − a genus known for its role in maintaining skin homeostasis through the production of antimicrobial peptides and modulation of immune responses−was reduced in attack samples (2.1% vs. 4.5%), consistent with studies showing its depletion in inflammatory skin conditions[133]. Interestingly, when comparing attack and remission phases, Staphylococcus abundance was higher during the attack phase. This increase may indicate a shift toward more inflammation-associated Staphylococcus species, which are known to exacerbate inflammatory pathways in susceptible hosts[134]. These compositional fluctuations suggest that while core genera remain dominant in BD skin microbiota, disease activity is accompanied by shifts that may influence local immune responses and lesion formation.

Commenting on our findings, what we observe in skin diverge from patterns observed in some other inflammatory skin conditions such as atopic dermatitis. In atopic dermatitis, Staphylococcus aureus is known to expand during disease flares, which may contribute to inflammation and disease severity[135,136]. In contrast, we see a progressive decline we see in our cohort in total Staphylococcus abundance from healthy controls through the attack phase and into remission, with no rebound toward control levels. Moreover, a relatively similar downward trend was seen at the species level for S. hominis. The sustained depletion of commensals during attacks can imply they may be collateral victims of inflammation or disruption of a “healthy skin microenvironment”. Interestingly, the failure of Staphylococcus to recover in remission can possibly indicate a long-term loss of protective commensals or even a persistent niche disturbance.

Applying a similar conceptual framework, other skin commensals, such as Cutibacterium acnes and Corynebacterium tuberculostearicum also decline during attack phases. In fact, these exhibit partial recovery in remission, consistent with their argued

protective roles in maintaining skin barrier integrity and producing antimicrobial metabolites[137]. Corynebacterium at the genus level also demonstrates an increase in remission.

In our cohort, these changes display that Behçet-related skin inflammation is characterized by a loss of beneficial commensal bacteria. We could not clearly demonstrate an overgrowth of inflammatory pathogens. Although “absence of evidence” does not necessarily imply “evidence of absence”, this dysbiosis profile is different from other commonly-studied inflammatory dermatosis (i.e. psoriasis or atopic dermatitis), where pathogenic organisms including staphylococci proliferate in flare-ups[135,136]. The depletion of protective microorganisms – if permanent – may escalate to an impaired barrier function or dysregulated immunity. We prefer to call this a “disturbed skin microenvironment”, as the term dysbiosis in skin is not as clear as it is in gastrointestinal system.

As we mentioned before, studies specifically examining the skin microbiome in BD are limited. Yet, a prior report mentioned shifts in staphylococcus between patients with positive and negative pathergy reactions[138]. Without confirmatory studies, we accept the implications remain unclear. We believe our findings provide a more detailed picture, emphasizing depletion of key commensals may favor a unique microbial signature of BD skin pathology.

The composition of the oral microbiome displayed notable shifts as well, between control and disease phases, with disruption of commensal equilibrium in BD attacks and partial reorganization during remission. In contrary with the skin microbiome, where protective organisms were progressively depleted, the oral microbiota showed a more dynamic reshuffling among dominant commensals. Despite the dominant phyla remaining relatively stable, important fluctuations were seen in key genera and species.

Prevotella abundance was notably increased during the attack phase, compared to controls, while Haemophilus showed the opposite trend. Although it is not specifically analyzed in BD, Prevotella has been argued to be increased in other systemic inflammatory

conditions, including rheumatoid arthritis[120,128]. On the other hand, there is partial contrast between our results and a previous study which reported increased Haemophilus parainfluenzae in BD. That study compared saliva of BD with healthy controls and found increased Haemophilus, though they did not distinguish between attack and remission phases[131].

In our cohort, Streptococcus salivarius was found in higher abundance in attack samples compared to controls. Streptococcus salivarius is in fact a commensal bacterium that is highly studied in patients suffering from allergic rhinitis. Studies demonstrated higher abundance in nasal microbiome of the patients[139]. Relevance in BD is not fully clear, however, one study found increased Streptococcus salivarius abundance in salivary microbiome of BD patients, compared to people with RAS[130]. From another point of view, this time focusing on RAS, a separate study reported decreased Streptococcus salivarius in microbiome of patients with RAS[140].

In ocular surface samples, *Staphylococcus* and *Corynebacterium* were less abundant in BD compared to controls (9% vs. 21% and 12% vs. 19%, respectively), consistent with previous studies showing that these genera constitute part of the healthy ocular microbiome and may contribute to immune homeostasis[141]. Conversely, *Pseudomonas* was markedly higher in BD compared to controls (69% vs. 39%), with *Pseudomonas aeruginosa* being more abundant at the species level. This opportunistic pathogen is known for its capacity to exploit compromised epithelial barriers and induce robust inflammatory responses on the ocular surface[142]. *Staphylococcus epidermidis*, a commensal species with protective roles against ocular pathogens, was reduced in BD compared to healthy controls (5% vs. 15%), potentially diminishing colonization resistance[143]. Remission samples showed decreased *Pseudomonas aeruginosa* (60% vs. 71%) abundance and increased *Staphylococcus capitis* (6% vs. 1%), changes that may reflect a partial restoration of ocular surface microbial balance during disease quiescence.

We should note that our findings do not necessarily mention Pseudomonas as the cause of BD. From another perspective, Behçet's flares may instead lead to ecological niches

favoring P. aeruginosa proliferation. In fact, studies argue loss of commensal bacteria in the eye might predispose to Pseudomonas overgrowth or even keratitis[144].

Note that the partial reduction of Pseudomonas in our remission samples may correlate with some restoration of the local microbial balance, however the levels are in fact still elevated relative to healthy controls. Our findings may suggest a persistent ocular dysbiosis, even after noticeable clinical quiescence. Our data can also be read as an underlying susceptibility to flare recurrence or ongoing subclinical inflammation.

Last but not least, concerning fecal microbiota, our cohort parallels key findings in prior studies on BD: severe depletion of butyrate-producing taxa (e.g., Faecalibacterium prausnitzii and Prevotella copri) during attack, accompanied by enrichment of non-butyrate Firmicutes such as Blautia, and Dorea. This mirrors the Italian cohort's reduction in Roseburia and Subdoligranulum[101], Japanese findings of elevated Bifidobacterium with reduced Prevotella and Megamonas[102,103], and the Chinese cohort's enrichment in sulfate-reducing bacteria with reduced Clostridium[104]. These studies are explained in greater detail previously. Notice that specific agents change from study to study, potentially representing population-based changes, however the trend is identical. Therefore, to better illustrate this trend, we prefer providing a summary table by Louis and Flint on propionate and butyrate production capacities of commensal gut microbiome, see Figure 4.3[145-147].

The Firmicutes to Bacteroidetes ratio provides an integrated index of this dysbiosis: our attack-phase samples show elevated Firmicutes (~84%) with severely suppressed Bacteroidetes (~4%, far below that seen in controls, 19%). We believe this exaggerated imbalance may reflect severe microbiota disruption. Similar to other microbiomes discussed above, even in remission, many disturbances persist or display only partial recovery, for instance Collinsella drops from 10% to only 7%. To us, this indicates incomplete restoration toward a healthy microbiome.

In conclusion, our study identified distinct, site-specific microbial alterations associated with Behçet's disease. In the gut, the attack phase was characterized by an elevated

Firmicutes-to-Bacteroidetes ratio and a reduction in short-chain fatty acid‑producing taxa, changes indicative of intestinal dysbiosis that appeared to partially normalize in remission. The skin microbiota displayed fluctuations in *Staphylococcus* abundance in active disease and remission, suggestive of a disrupted cutaneous microenvironment. In the oral cavity, decreased *Haemophilus* and increased *Prevotella* during different disease phases reflected local mucosal immune dysregulation. On the ocular surface, increase in *Pseudomonas* (particularly *P. aeruginosa*) during attacks and decrease in remission demonstrated BD's connotation with inflammation-associated taxa. Collectively, these findings emphasize that while overall microbial diversity remained relatively stable, Behçet's disease is associated with taxonomic shifts at multiple body sites, supporting the hypothesis that microbiota composition may contribute to the pathophysiology and clinical manifestations of the disease.

Taken together, findings of our study strengthen a unifying model in Behçet's disease: gut dysbiosis marked by an essential loss of anti-inflammatory taxa and overabundance of metabolic fermenters. We agree this skews host metabolism and immunity towards inflammatory phenotypes, especially during clinical BD flares. Appreciation of this phenomenon may have implications in the close future such as microbial-targeted therapies or dietary interventions aimed at regaining butyrate-producing commensals to restore the microbial balance.

Phylum (family)	Species	Butyrate[a]	Propionate[b]
Bacteroidetes (Bacteroidaceae)	*Bacteroides uniformis*	–	+ (Suc)
	Bacteroides vulgatus	–	+ (Suc)
Bacteroidetes (Prevotellaceae)	*Prevotella copri*	–	+ (Suc)
Bacteroidetes (Rikenellaceae)	*Alistipes putredinis*	–	+ (Suc)
Firmicutes (Lachnospiraceae)	*Eubacterium rectale*	+ (CoAT)	–
	Roseburia inulinivorans	+ (CoAT)	+ (Pdu)
	Roseburia intestinalis	+ (CoAT)	–
	Dorea longicatena	–	–
	Eubacterium hallii	+ (CoAT)	+ (Pdu)
	Anaerostipes hadrus	+ (CoAT)	–
	Ruminococcus torques	–	–
	Coprococcus eutactus	+ (ButK)	–
	Blautia obeum	–	+ (Pdu)
	Dorea formicigenerans	–	–
	Coprococcus catus	+ (CoAT)	+ (Acr)
Firmicutes (Ruminococcaceae)	*Faecalibacterium prausnitzii*	+ (CoAT)	–
	Subdoligranulum variabile	+ (ButK)	–
	Ruminococcus bromii	–	–
	Eubacterium siraeum	–	–
Firmicutes (Veillonellaceae)	*Dialister invisus*	–	+ (Suc)
Firmicutes (Acidaminococcaceae)	*Phascolarctobacterium succinatutens*	–	+ (Suc)
Firmicutes (Erysipelotrichaceae)	*Eubacterium biforme*[c]	+ (CoAT)	–
Actinobacteria (Bifidobacteriaceae)	*Bifidobacterium adolescentis*	–	–
	Bifidobacterium longum	–	–
Actinobacteria (Coriobacteriaceae)	*Collinsella aerofaciens*	–	–
Verrucomicrobia (Verrucomicrobiaceae)	*Akkermansia muciniphila*	–	+ (Suc)

Figure 4.3: Propionate and butyrate production capacities of commensal gut microbiome, Louis et al. (2016)[145-147].

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APPENDIX

Appendix 1: Plagiarism Check Report

Plagiarism Report from Turnitin stated similarity index as 17%.

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