CORRELATION OF GENETIC AND MICROBIAL CHANGES IN INFLAMMATORY BOWEL DISEASE

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ABSTRACT

CORRELATION OF GENETIC AND MICROBIAL CHANGES IN INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) is complex, multifactorial, polygenic diseases without a certain cure. Patients require lifelong surveillance, expensive treatments, risky surgeries, have increased cancer and other systemic disease risks. IBD consists of two groups as Ulcerative colitis (UC) and Crohn's disease (CD). Although pathogenesis of IBD is not clear, a dysregulated immune response to environmental factors (intestinal microbiota) develops in genetically susceptible individuals. As early diagnosis is hard, most patients are diagnosed at a later stage, however, they suffer from colon damage and lose the chance of early treatment.

This thesis aimed to identify biomarkers that can analyze IBD patient risk profiles to control disease and reveal individuals at risk before the disease progresses further. Firstly, mutations in Turkish IBD patients were investigated in candidate regions of innate immunity (*NOD2*), adaptive immunity (*IL-23R*), and autophagy (*ATG16L1*) genes. Then, microbiota profiles of patients were revealed by NGS and qPCR. Finally, to identify relationship between mutations and microbiota, statistical analyses were performed with non-parametric methods. As a result of this study, Shannon index showed significant reduction of bacterial diversity in IBD patients versus the control group (p-value, 0.048). At the species level, relative abundance of *F. prausnitzii* decreased while abundance of *E. coli* increased in IBD patients. Significant relationship (p-value, 0.034) was found between rs11209026 genotype and *F. prausnitzii* reduction in CD but not in UC. In conclusion, converting significant observations into a molecular test kit can provide a molecular guide that helps gastroenterologists in planning personalized treatments in IBD patients.

ÖZET

İNFLAMATUVAR BAĞIRSAK HASTALIĞI'NDA GENETİK VE MİKROBİYAL DEĞİŞİKLİKLERİN KORELASYONU

İnflamatuvar Bağırsak Hastalığı (İBH) günümüzün sık rastlanan, kesin bir tedavisi olmayan, etkilediği kişilerde hayat boyu takibi, pahalı tedavileri ve riskli ameliyatları gerektiren, kanser riski ve diğer sistematik rahatsızlıkları artıran, ciddi iş gücü ve yaşam kalitesi kayıplarına yol açan karmaşık, multifaktöriyel, multigenik bir hastalıktır. İBH temel olarak Ülseratif kolit (ÜK) ve Crohn hastalığı (CH) olarak iki grupta incelenir. Hastalığın patogenezi net olarak bilinmemekle birlikte, genetik olarak yatkın bireylerde, çevresel faktörlere (intestinal mikrobiyota) karşı disregüle immün yanıt oluşmaktadır. Erken tanısında zorluklar yaşandığı için hastalar, hastalığın ilerlemiş safhalarında bir uzman tarafından teşhis bulmakta, zaman kaybından dolayı bağırsak dokusunda zararlar artmakta, erken tedavi şansı kaybolmaktadır.

Bu tezde, hastalığı kontrol altına almak ve hastalık daha fazla ilerlemeden risk altındaki bireylerin ortaya çıkarılması için İBH hasta risk profilini analiz edecek biyobelirteçlerin belirlenmesi amaçlanmıştır. İlk olarak doğal immünite (*NOD2*), adaptif immünite (*IL-23R*) ve otofaji (*ATG16L1*) genlerinin aday bölgelerinde Türk IBD hastalarına özgü mutasyonlar araştırıldı. Ardından hastaların mikrobiyota profilleri, amplikon dizileme ve qPCR ile ortaya çıkarıldı. Son olarak, Türk IBD hastalarında, mutasyonlar ve mikrobiyota arasındaki ilişkiyi belirlemek için parametrik olmayan yöntemlerle istatistiksel analizler yapıldı. Çalışma sonucunda, Shannon indeksi, kontrol grubuna kıyasla IBD hastalarında bakteri çeşitliliğinde önemli bir azalma olduğunu göstermiştir (p-değeri, 0.048). IBD hastalarında tür düzeyinde *F. prausnitzii* bakterisinin göreceli bolluğu azalırken *E. coli* bakterisinin arttı. UK'in aksine, rs11209026 genotipi ile CH' deki *F. prausnitzii* azalması arasında anlamlı ilişki (p-değeri, 0.034) bulundu. Sonuç olarak, tüm bu gözlemlerin moleküler bir test kitine dönüştürülmesi, gastroenterologların İBH hastalarında kişiselleştirilmiş tedaviler planlamasına yardımcı olacak bir altyapı sağlayabilir.

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CHAPTER 1

INTRODUCTION

1.1. Inflammatory bowel diseases (IBD)

Inflammatory bowel diseases (IBD), which arise from chronic and uncontrolled inflammation of the GI (gastrointestinal) tract, are mainly examined in two groups as Ulcerative colitis (UC) and Crohn's disease (CD). Although there is no definite cause of IBD; epidemiological and family studies have shown that microbiota, genetics, and environmental factors predispose to IBD (Albenberg et al., 2012). IBD diagnosis according to clinical, endoscopic, and histopathological characteristics, but none of the findings are definitive, and even some patients' differential diagnoses cannot be made. Major symptoms seen in the IBD are fever, uveitis, oral lesions, inflammation of the joints, weight loss, frequent and bloody diarrhea, anemia, intestinal obstruction, malabsorption, and abdominal pain. IBD is seen especially in between 15-30 and 50-70 years old. However, the incidence of IBD has been gradually increasing in recent years, especially in the pediatric group and adolescents. Moreover, about 25% of patients are diagnosed in childhood (Cakir et al., 2015). Since the course of IBD in childhood is different from adults, specific application of diagnosis and treatment approaches to the age group will provide more effective results.

Ulcerative colitis is characterized by recurrent exacerbations of inflammation. Inflammatory involvement is confined to the mucous layer yet is most commonly seen in the rectum. It shows continuous involvement in the colon without leaving a healthy area in between. It usually characterized by bloody diarrhea and small intestine involvement is not observed (Oktar and Alican, 2000). UC divided into four groups as mild, moderate, severe and more severe (fulminant) according to the severity of the symptoms. In the mild subgroup, involvement is usually limited to the rectum while in the moderate subgroup,

the part up to the splenic flexure is usually involved. There is widespread colonic involvement in severe and fulminant subgroups.

Unlike the UC, Crohn's disease can influence anywhere in the GI tract however usually occurs in the terminal ileum (Ünal, 2012). The most important feature that makes the CD different from UC is transmural inflammation that causes narrowing of the lumen, signs of intestinal obstruction, or the development of abscesses and fissures (Ersoy and Hamzaoğlu, 2013). All layers of the intestine are affected by the disease. Crohn's disease has three different clinical phenotypes: inflammatory, penetrating (fistulizing), and structuring (Akpınar et al., 2017). Diarrhea and abdominal pain are the two furthest common indications of CD in adults. Other widespread indications in the CD are weight loss and malnutrition (Van Assche et al., 2010). At the end, all the damage that takes place in the intestine makes the patient a candidate for colon cancer. Therefore, when intestine damages, rising cancer risk, the negative impact on quality of life, health care costs, and lost workdays have taken into account the IBD pathophysiology in Turkey are needed to be studied in detail.

1.2. Epidemiology of Inflammatory Bowel Disease

IBD has become a worldwide disease with 6.8 million patients (Alatab et al., 2020). The incidence and prevalence of IBD differ according to geographical regions. However, as its incidence and prevalence have been increasing in different regions of the world over time, it has become a global disease. The biggest incidence ratios have been reported in Northern Europe and North America for CD and UC (Table 1. 1) (Burisch and Munkholm, 2015). A comprehensive epidemiological study reports that the disease has increased approximately 5 times in Turkey in the last 10 years. Besides the disease is more prevalent in industrialized countries worldwide, the high incidence and prevalence rates have been attributed to the western lifestyle. In a Turkish study, the incidence rates have been found to be 23/100.000 for UC and 15/100.000 for CD; prevalence rates are reported to be 160/100.000 for UC and 63/100.000 for CD (Can et al., 2019). In an epidemiological study based on a hospital-based questionnaire conducted with the participation of 12 centers in Turkey, the incidence in the referral population (UC n=661,

CD n=216 patients) is found 4.4/100.000 in UC and 2.2/100.000 in CD (Tozun et al., 2009). These rates are lower than the incidence rates reported in Europe but close to the rates reported in the Middle East.

Table 1. 1. The incidence and prevalence of IBD.

Per 100.000/year	**CD incidence**	**UC incidence**	**CD prevalence**	**UC prevalence**
Europe	0.0-11.5	0.9-24.0	1.5-213	2.4-294
North America	0-20.2	0-19.2	25.9-318.5	37.5-248.6
Turkey	63	15	160	23

Chronic diseases such as IBD that persist for life cause a significant decrease in quality of life, loss of work force and productivity, and increased health expenses with expensive drug treatments (especially usage of biological agents such as anti-TNF), permanent organ damage, hospitalizations and operations. IBD has important social, psychological and financial implications as well as the deterioration of health-related quality of life. This disease impresses a personal life and brings significant economic burdens, such as time lost from work and reduced productivity at work. In Europe, it has been stated approximately 3 million people are suffering from IBD, and the cost of healthcare services is almost 5 billion Euros per year (Burisch et al.,2013). The economic burden created by the permanent loss of workforce brought about by the disease can be much higher than this price. The financial burden can even higher as IBD also affects individuals at an early age. 10-25% of IBD patients in Europe encounter unemployment or part-time employment problems (Burisch et al., 2013). Considering that the unemployment rate in Turkey is 13.7% as of 2019, we can remark that IBD causes serious loss of functionality, especially in the productive period (Turkish Statistical Institute (TSI), 2020). As the incidence of IBD is increasing worldwide, there will be an ever-increasing economic burden on the healthcare system and economy. Considering all the costs that appear, researches on interventions such as achieving disease control and

stopping the progression of the disease by changing the course of the disease to prevent IBD are of great importance.

1.3. Etiopathogenesis of Inflammatory Bowel Disease

Genetic, immune responses, microbial and environmental factors cause constant stimulation of the immune system by damaging the intestinal epithelial barrier therefore active inflammation and tissue damage are seen. Although the innate and adaptive immunity pathways participate in body defense by assuming different roles, the process cannot be controlled easily due to the multifactorial triggers.

1.3.1. Environmental Factors

Many studies have identified various risk factors that are thought to affect the phenotype of IBD. Environmental factors, especially eating habits, are considered to be effective in the emergence of the IBD (Brunner et al., 2007). Nutrients provide potential antigens or may influence the content of the intestinal microbiota. Inadequate and unbalanced nutrition is more frequent in CD than UC (Türker and Günaldı, 2016). It is known that fast-food consumption, a long-term diet rich in fat and sugar enhances the risk of IBD. On the other hand, it has been reported that consumption of fiber-rich foods and fruits decreases CD while vegetable consumption reduces UC (Kurtaran, 2021). In addition to dietary habits, the presence of anxiety and depression in IBD patients has been found more than twice as compared with the healthy population (Graff et al., 2010). Depression is related to disrupted cellular immunity and incremented inflammation (*IL-6*, *TNF-α*, *CRP*) (Blume et al.,2011; Yanartaş et al., 2014). Therefore, the vita quality and psychosocial functionality are reduced in IBD cases.

Smoking is another risk factor that contributes to the development of IBD. Even so, smoking has contrary effects on UC and CD. Smoking has a protective efficacy in UC patients, and smokers have a better prognosis than non-smokers. Dissimilar to UC,

disease complications are more vigorous in CD patients who proceed to smoke (Johnson et al., 2005). Additionally, in a meta-analysis carried out with 245 articles, a total of 22 articles have been found to highlight the relationship between IBD and smoking, and it has been proven that smoking has different effects on UC and CD (Mahid et al., 2006).

1.3.2. Genetic Factors

Genetic changes between individuals can lead to differences in disease onset, complications, or responses to treatments. That's why genetic factors are really important risk factor in IBD pathogenesis. The similarity of symptoms between relatives in family studies have been shown that genetic factors are responsible not only for predisposition but also for the phenotype of the disease (Özkan, 2013). There is a familial predisposition in both diseases; 40% in CD and 20% in UC so the risk is more in CD than in UC. In studies conducted with monozygous twins, it has been found that the relative CD risk is increased by 800 times (Brant, 2011). Classical Mendelian inheritance pattern is not seen in IBD, so there is polygenic inheritance. The number of risk loci statistically related to IBD has increased substantially with various studies and meta-analyses (Ellinghaus et al., 2015).

Over 200 risk loci implicated in susceptibility to IBD have been identified (Momozawa et al., 2018). Genetic studies have accelerated by the determination of the relationship between Human Leukocyte Antigens (HLA) and IBD. *DRB1*01* and *DRB1*07* (Danze et al., 1996) alleles have been shown to be associated to CD while *HLA-DRB1*1502* (Futami et al., 1995) has related with UC. In another study, *DR2*, *DR9*, and *DRB1*0103* alleles are found related to UC while the *DR7* and *DRB3*0301* alleles related to CD. Also, it has been stated that HLA-DR molecules can play a threefold greater role in the pathogenesis of UC than CD (Stokkers et al., 1999). *NOD2*, the first CD-related gene was discovered in 2001 (Hugot et al., 2001). The *NOD2* gene, which is found 27-39% mutated in CD patients and 12-14% in UC patients, cannot recognize lipopolysaccharide belonging to bacteria and intestinal inflammation develops (Göksel, 2009). The link among *IL-23R* and IBD was found by a genome-wide association study (GWAS) in 2006 (Duerr et al., 2006). One year later, *ATG16L1* was discovered to be

connected with CD (Hampe et al., 2007). Today, after developing useful technologies and many meta-analyzes, it has been revealed that over 200 single nucleotide polymorphisms (SNP) have been related to the pathogenesis of IBD (Ye and McGovern, 2016). *NOD2*, *ATG16L1* and *IL-23R* genes are the most significant genes that increase IBD risk (Silverberg et al., 2009). However, in two different studies conducted with Turkish IBD patients, any relationship between *NOD2/CARD15* mutations and IBD has not been found (Ince et al., 2008; Özen et al., 2006). On the other hand, the G908R mutation in the *NOD2* gene increases the susceptibility to Crohn's disease in the Turkish population (Uyar et al., 2006). Finding different results regarding the relationship of the same gene with IBD in Turkish society reveals the necessity of more comprehensive studies.

1.3.3. Immune Response

The immune system can be categorized into two groups as innate and adaptive immunity that are not independent from each other. Dysregulations in innate and adaptive immunity make a predisposition for both Crohn's disease and Ulcerative colitis. However, it is thought that adaptive immunity plays a more effective role in the pathogenesis of IBD (Kmieć et al., 2017). Furthermore, the autophagy mechanism plays an effective role in defense against pathogens by acting as a bridge between innate and adaptive immune systems.

1.3.3.1. Innate Immunity

The protective mechanisms that defend the body against pathogens and further enable the activation of adaptive immunity constitute innate immunity. Innate immune responses are also present when not encountering antigens, and their protective effect is not pathogen-specific. Mucus barriers, phagocytic cells in blood and tissues (macrophages, neutrophils, eosinophils), acute phase proteins (C-reactive protein), natural killer cells are the main components of innate immunity (Abdulkhaleq et al., 2018; Basset et al., 2003). They function indiscriminately and each time respond with the same

intensity. Specialized cells such as Paneth cells located in the mucous layer secrete antimicrobial peptides that inhibit bacterial growth to protect the body against bacterial attack. Pathogenic microorganisms are detected by macrophages and dendritic cells (DC) thanks to pattern recognition receptors (PRRs) and the inflammatory response is initiated by the secretion of cytokines and chemokines (Figure 1. 1) (Yuan and Walker, 2004).

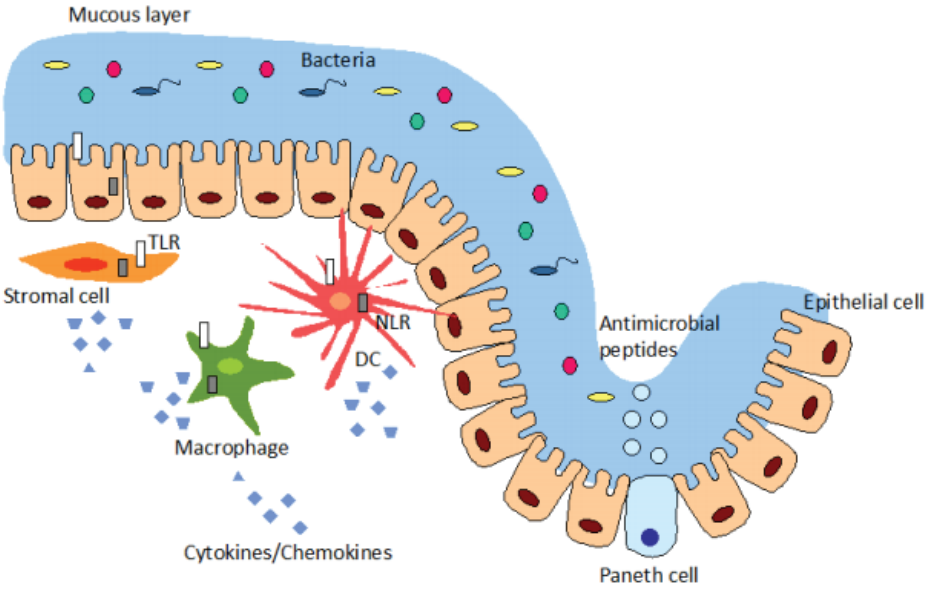

Figure 1. 1. Innate immunity responses in the intestine

(Source: adapted from Geremia et al., 2014).

Making discrimination of microbe-associated molecular patterns (MAMPs) like lipopolysaccharide, peptidoglycans, bacterial DNA from normal antigens is one of the characteristic properties of innate immunity. Toll-like receptors (TLRs) and Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) are important innate immune sensors found in macrophage, neutrophil, dendritic, and intestinal epithelial cells in mammals (Purchiaroni et al., 2013). These receptors act as radar in the cell membrane against any stimulus that may come from outside. Initiation of innate immunity depends on the recognition of MAMP molecules by those receptors. Since innate immune responses are an important defensive system to protect us against infections and tissue injury, receptors' expression increase in the presence of pathogens.

TLRs are type 1 transmembrane proteins in the innate immunity that play a key role in initiating inflammatory response by recognizing the structure of pathogens (Kundakci and Pirat, 2012). Owing to this function, they provide the intestinal homeostasis by shaping the microbiota. 10 members of the TLR family have been defined, from *TLR1* to *TLR10* in human (De Nardo, 2015). Some TLRs are localized on the cell surface (*TLR1*, *TLR2*, *TLR4*, *TLR5*, *TLR6*, *TLR10*) while others are found in endosomes (*TLR3*, *TLR7*, *TLR8*, *TLR9*) (Özbek et al., 2017). Each TLR type is specialized for a different stimulus. TLRs consist of a leucine rich repeats (LRR) responsible for ligand recognition at the extracellular domain (Akira, 2003). In inflammatory bowel disease, varied TLR mutations have been encountered. Among the mutations, *TLR4* polymorphism (Asp299Gly) has the strongest influence on the IBD (Oostenbrug et al., 2005). Besides *TLR4* mutation, it is found that *TLR9* polymorphism (−1237C) is correlated with Crohn's disease while *TLR2* polymorphism (R753Q) is linked with Ulcerative colitis (Fukata et al., 2009).

NLRs are cytoplasmic receptor that one of the member of PRRs recognizing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Kim et al., 2016). Activated NLRs lead to the formation of inflammasomes, signal transduction by initiate nuclear factor kappa B (NF-κB), and mitogen-activated protein kinase (MAPK) signaling pathways, autophagy, or transcription activation (Saxena and Yeretssian, 2014). In humans, 22 NLR receptors have been identified not only participate in innate immunity but also responsible for reproduction and embryonic development (Zhong et al., 2013). Unlike TLR mutations, NLR polymorphisms are more related to inflammatory disorders. Particularly, SNPs in *NOD1*, *NOD2*, and *NLRP3* receptors have been shown highly linked with IBD (Davis et al., 2014). Although both *NOD1* and *NOD2* linked with IBD pathogenesis, *NOD2* is a more general receptor rather than *NOD1* because of muramyl dipeptide (MDP) recognition property (Kim et al., 2016). MDP, a component of the peptidoglycan cell wall of both Gram-positive and Gram-negative bacteria. Even so, *NOD1* is sensitive to γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) that seen in only Gram-negative bacteria (Jakopin et al., 2013).

1.3.3.1.1. Nucleotide Binding Oligomerization Domain Containing 2 (*NOD2/CARD15*)

NOD2 is one of the member of the NLR protein family contributes in innate immunity in our body. It is located on chromosome 16p21 and produces a ternate structure protein that contains nucleotide-binding domain (NBD), leucine-rich repeats (LRR), and caspase recruitment domains (CARD) shown as in Figure 1. 2 (Sidiq et al., 2016). The LRR is located in the C-terminal of *NOD2* protein and responsible for the sensing of MDP (Girardin et al., 2003). Recognition of MDP leads to a conformational change in *NOD2*. After this structural change, the NATCH domain-containing NBD binds to ATP and initiates *NOD2* oligomerization (Philpott et al., 2014). Finally, the CARD domain responsible for protein interaction recruits receptor-interacting serine/threonine-protein kinase 2 (RIPK2) then NF-κB and MAPK signaling pathways are activated (Canning et al., 2015). Apart from bacterial component recognition, *NOD2* can initiate an antiviral response by recognizing viral ssRNA (single-stranded RNA) (Feki et al., 2018). Also, *NOD2* triggers autophagy by gathering *ATG16L1* into the area where pathogens present (Galluzzi et al., 2010).

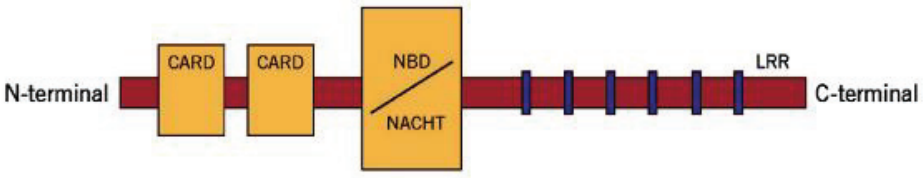

Figure 1. 2. Protein structure of *NOD2*

(Source: Yao, 2013).

NOD2 is the first identified gene related to susceptibility to Crohn's disease. It has been determined that 3 important mutations (R702W, G908R and 1007fs) in the *NOD2* gene are associated with CD (Ince et al., 2008) (Table 1. 2). These mutations located in the LRR region of *NOD2* (Figure 1. 3), which recognizes the microorganism and causes amino acid change, are associated with the susceptibility to Crohn's Disease

(Uyar, 2009). Eventually, NF-κB activation and the innate immune response to bacterial stimuli are decreased due to the impaired function of the mutated receptor. The mutant *NOD2* protein also poses a problem in initiating MDP-dependent autophagy (Mo et al., 2012). Importantly, 30% and 50% of Crohn's patients possess *NOD2* mutation in at least one allele (Noguchi et al., 2009). Loss of *NOD2* function is also associated with autoimmune diseases like Blau syndrome and Rheumatoid arthritis besides Crohn's disease (Dugan et al., 2015; Ospelt et al., 2009).

Table 1. 2. Most common SNPs in *NOD2* gene.

Gene	**Rs number**	**SNPs**	**Position**	**Genetic Variation**	**Type of Mutation**
NOD2	rs2066844	R702W	Exon 4	C/T	missense
	rs2066845	G908R	Exon 8	G/C	missense
	rs2066847	1007fs	Exon 11	Insertion of C	frameshift

The R702W is an SNP in the *NOD2* gene is also known as rs2066844 or Arg702Trp. This SNP is located in exon 4 of *NOD2* and results in an amino acid substitution (Glas et al., 2010). The C allele encodes the arginine (R) whereas the mutated T allele encodes the tryptophan (W). The G908R is known as rs2066845 or Gly908Arg. This SNP is encoded by exon 8 in the *NOD2* gene and like R702W, leads to an amino acid change (Feki et al., 2018). The G allele encodes the glycine (G) while the unwanted C allele encodes the arginine (R). The 1007fs located in exon 11 is identified as rs2066847 or leu1007fsinsC. This mutation stems from the deletion of the C base causes a partial deletion of the LRR domain in the *NOD2* gene (Noguchi et al., 2009).

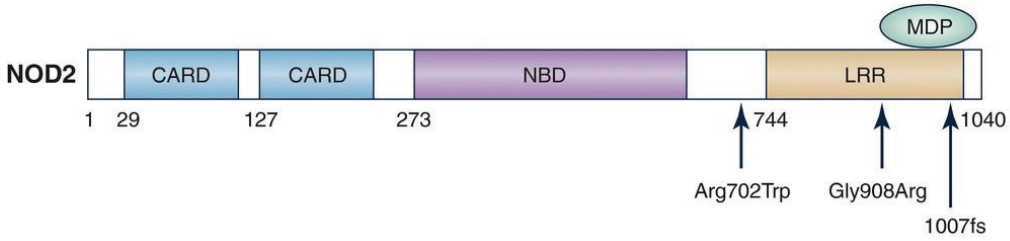

Figure 1. 3. Location of IBD related mutations in *NOD2* gene

(Source: adapted from Goethel et al., 2018).

1.3.3.2. Adaptive Immunity

Stimulation of innate immune cells gathers them together and adaptive immune activation is initiated (Fukata et al., 2009). Therefore, adaptive and innate immunity closely related. Adaptive immunity is initiated pathogen-specific in contrast to innate immunity. Although it takes days for immune cells to become active due to a complex maturation and development process, it provides long-term immunity. B (humoral responses) and T (cellular immune responses) lymphocytes are the main players of adaptive immune responses. Dendritic cells that initiate T cell activation have important roles in both innate and adaptive immune responses. Cytokines have a role in the differentiation and proliferation processes of lymphocytes following the recognition of the antigen in adaptive immunity. The production of antibodies against antigens via B cells is the main function of the humoral immune responses. Antibodies prevent colonization of pathogen microorganisms by attaching to their cell wall. Also, they neutralize and eliminate toxins.

Dendritic cells, B cells, and macrophages in the innate immune system act as antigen-presenting cells (APC) (Gaudino and Kumar, 2019). T cell activation is carried out by binding and signal transduction of the antigen presented to the T cell receptor (TCR) by APCs (Baskan, 2013). In the case of active inflammation, when naive T cells (Th0) encounter antigen, they can transform into T helper cells (Th1, Th2, Th17, regulatory T cells (Treg)) that differ according to the cytokine produced. Th1 cells participate in cellular immunity by activating macrophages, and produce pro-

inflammatory cytokines such as interferon-gamma (*IFN-γ*), tumor necrosis factor-alpha (*TNF-α*), interleukin-1 (*IL-1*), *IL-2*, *IL-6* (Kunkl et al., 2020). Cellular immunity is important in protecting against intracellular infectious agents such as viruses, bacteria and fungi. Th2 cells produce interleukin-13 (*IL-13*), which leads to increasing intestinal permeability and stimulates epithelial apoptosis and also other inflammatory factors, such as *IL-4*, *IL-5* (Huang and Chen, 2016). Th17 cells produce *IL-17A*, which plays a role in the movement of neutrophils to the active inflammation areas. They also secret *IL-21*, which induces MMPs that cause extracellular matrix degradation. It has been thought that Th1 responses related to CD pathogenesis whereas Th2 responses associated with UC (Wallace et al., 2014).

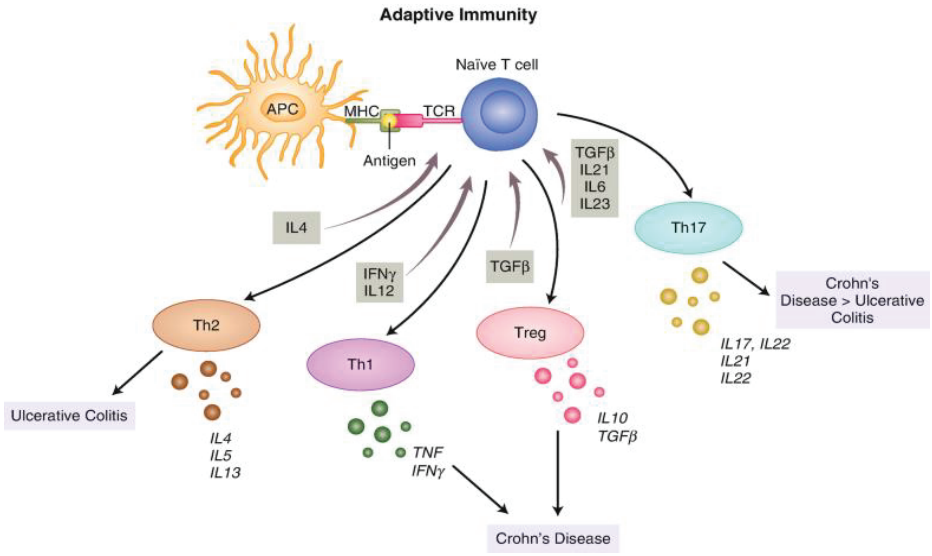

Figure 1. 4. Representation of the role of adaptive immunity in the pathogenesis of IBD (Source: adapted from Connelly and Koltun, 2016).

Immune responses are crucial in the pathogenesis of IBD. In patients with IBD, as a result of the initiating abnormal mucosal immune reactivity against enteric bacteria, intestinal damage is occurred. These dysregulations in innate immunity lead to an exaggerated adaptive immune response (Adışen and Ali Gürer, 2013). Because the activity of epithelial cells to induce regulatory T cells is reduced, T cell activation increases. Activation of immune cells leads to the release of excessive cytokines and inflammatory mediators, which increase tissue damage and cause further inflammation (Figure 1. 4). Increased cytokine concentration across the mucosa leads to matrix

degradation (Göral et al., 2006). It has been observed that serum *IL-22* expression increases in Crohn's patients and disease activity together with *IL-23R* polymorphisms affect the expression level (Schmechel et al., 2008). Conversely, in active UC, the presence of T cells that do not express *IL-22* has been detected (Broadhurst et al., 2010). Further, Treg cells are highly expressed in IBD patients because of its responsibility for gut mucosal homeostasis (Saruta et al., 2007). Unlike UC, there are increased dendritic cell activation that secrete *IL-12* and *IL-6* in CD (Hart et al., 2005). While it is emphasized that the significant increase in *IL-2Rsp*, *IL-6*, *IL-8*, and *IL-10* cytokines in Ulcerative colitis is responsible for the pathogenesis, it is stated that cytokines such as *TNF-α* and *IL1-beta* have less importance (Göral et al., 2006). Since *IL-10* participates in cell-mediated and humoral immune responses, it has both anti-inflammatory and pro-inflammatory effects. In a study, increased *IL-10* mRNA levels have been detected in both the large and small intestine colon of Ulcerative colitis patients (Melgar et al., 2003). All in all, it is thought that cytokines, which have different importance in the pathogenesis of both UC and CD, may be useful parameters in the diagnosis, treatment, follow-up, and prognosis of diseases.

1.3.3.2.1. *IL-23* Receptor (*IL-23R*)

Interleukin 23 (*IL-23*) is a disulfide-linked heterodimeric pro-inflammatory cytokine composed of p40 (*IL-12β*) and p19 (*IL-23A*) subunits, synthesized by dendritic cells and macrophages. The *IL-23R* (*IL-23* receptor) gene is located on the 1p31 chromosome and with the proteins it encodes form a receptor for *IL-23* together with the *IL-12Rβ1* chain (Parham et al., 2002). Interaction of *IL-23* with its receptor triggers signal transduction via different molecules (Figure 1. 5). *IL-23* is essential for the proliferation, survival, and effector functions of highly pathogenic Th17 (*IL-17* producing T helper) cells. Additionally, it is responsible for mucosal immunity and gut homeostasis.

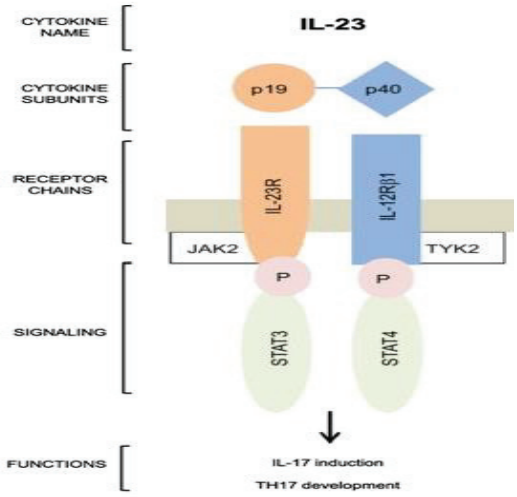

Figure 1. 5. Structural and functional features of *IL-23*

(Sources: adapted from Belladonna and Grohmann, 2013; Jauregui-Amezaga et al., 2017).

IL-23 is an important player for innate and adaptive immunity due to the fact that it increases the release of *IL-17* for both infection control from neutrophils and inflammatory response from T cells (Figure 1. 6). The transformation of $CD4^+$ T cells into Th17 cells is stimulated by *IL-1*, *TGF-β*, *IL-6*, and *IL-23* and these cytokines trigger *IL-17A* and *IL-23R* expression. Subsequently, the presence of *IL-23* increases the production of *IL-17A* as well as the production of *IL-17F*, *IL-21*, *IL-22* (Álvarez-Salamero et al., 2020; Teng et al., 2015). *IL-17A* serves in defense of barrier regions such as skin and intestinal mucosa. Linking of *IL-23* to its receptor induces the activation of JAK (Janus family of tyrosine kinases) and STAT (Signal Transducers and Activators of Transcription) molecules. This linkage mostly leads to phosphorylating and activating JAK2 and STAT3. Afterward, STAT3 enhances the expression of transcription factor RORγt (retinoid-related orphan receptor) which is required for *IL-17* formation (Zhou et al., 2007).

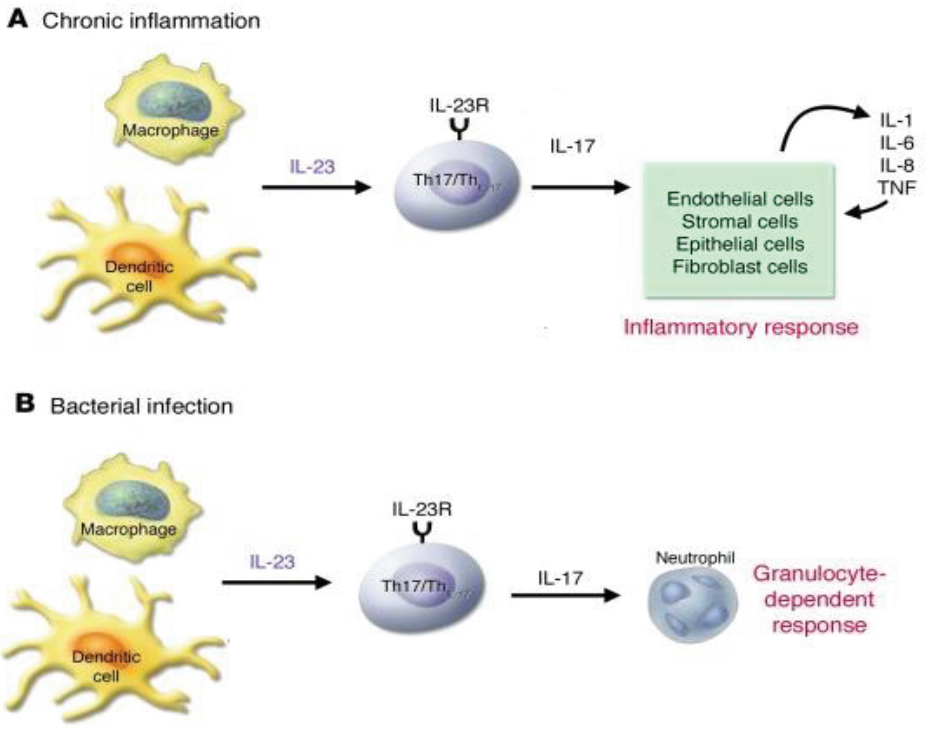

Figure 1. 6. The role of the *IL-23/IL-17* both in adaptive (A) and innate (B) immunity (Source: adapted from Iwakura and Ishigame, 2006).

The *IL-23/IL-17* pathway plays a key role in the pathogenesis of IBD. Th17 cells induced by *IL-23* have an important role in mucosal inflammation in the pathogenesis of IBD (Zanello et al., 2014). *IL-17A*, a proinflammatory cytokine synthesized by Th17 cells, is known to play a role in the pathogenesis of many autoimmune diseases and chronic inflammation (Boniface et al., 2008). It is seen increased *IL-17* expression in different autoimmune disorders (Chen et al., 2011; Zhang et al., 2006). *IL-23* levels have increased in biopsies collected from Ulcerative colitis and Crohn's patients. Intestinal macrophages regulate immune responses in recognizing and removing bacteria. In a study, it is observed that the amount of CD14 macrophages increased in CD patients and increased *IL-23* secretion stems from these cells. Furthermore, it has been shown that bacteria such as *Enterococcus faecalis* and *Escherichia coli* trigger the increase in *IL-23* (Kamada et al., 2008). Because of the presence of dendritic cells and macrophages in intestinal lamina propria (LB) which under the mucosal epithelium, increased mRNA expression of *IL-23R* has been found in LB of UC and CD (Kobayashi et al., 2008).

As a result of the increase in receptor sensitivity because of the polymorphic regions in the *IL-23R* gene, inflammation takes place that influences the *IL-23/IL-17* pathway (Gül, 2020). The risk of *IL-23R* variants in IBD progress is higher in Crohn's disease than in Ulcerative colitis (Jostins et al., 2012). In 2006, a variant in the exon 9 of *IL-23R* gene with a protective impact for Crohn's disease was detected (Duerr et al., 2006). This SNP, known as rs11209026 (R381Q-Arg381Gln), causes a loss of function in the *IL-23R* gene. rs11209026 leads a substitution from arginine (R) to glutamine (Q) and results in a decrease in signal transduction. The location of the rs11209026 is in the cytoplasmic region of the *IL-23R*. Unlike G wild-type allele, protective A allele causes decreasingly secretion of pro-inflammatory cytokines by changing the interaction among *IL-23R* and its related JAK2 kinase (Figure 1. 7). In human cell lines transfected with Arg381Gln-*IL-23R*, a reduction of STAT3 phosphorylation and depends on this, a decrease in Th17 and *IL-17* levels have been observed (Sarin et al., 2011). Unlike Ulcerative colitis, heterozygous polymorphism in Crohn's patients is related to the disease (Lakatos et al., 2008). R381Q is also associated with disease pathogenesis in children as well as adults (Dubinsky et al., 2007). On that account, it is supported by many studies that obstructing the *IL-23* pathway can improve host health in autoimmune diseases such as IBD (Allocca et al., 2018; Sandborn et al., 2008; Venegas et al., 2008).

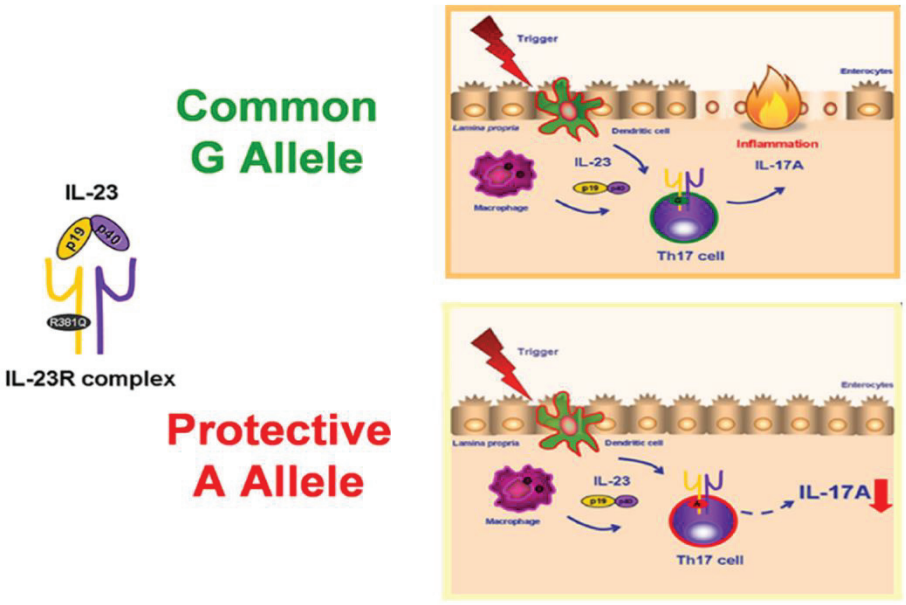

Figure 1. 7. Role of wild-type (G) and mutant (A) alleles of rs11209026

(Source: adapted from Di Meglio et al., 2011).

1.3.3.3. Autophagy

Autophagy or cellular self-digestion is an evolutionarily conserved mechanism, resulted in the degradation of cellular contents transported to the lysosome (Cadwell et al., 2008). It is the primary pathway to broken cell contents and recycled. Non-selective autophagy helps sustain cellular homeostasis by recycling cytoplasmic contents in the lack of stress (Murrow and Debnath, 2013). In the selective self-digestion, which is rapidly activated in the removal of components of the cell, such as damaged organelles, long-lived or misfolded proteins, provides the nutrients necessary for the cell. In this way, by enhancing the cells' resistance to stress, it maintains cells' survival (Karadağ, 2016). Also by removing intracellular pathogens, it contributes to the host defense (Mehrpour et al., 2010). Autophagy is divided into 3 groups: microautophagy and macroautophagy, which happen both selectively and non-selectively, and chaperone-mediated autophagy that is selective (Levine and Kroemer, 2008). While each mechanisms result in lysosomal degradation; they differ in terms of substance transport and regulation mechanisms. Microautophagy causes the cytoplasm to be engulfed in the lysosome by inducing directly inward collapse of the lysosomal membrane. Unlike microautophagy, macroautophagy provides the formation of a double-membrane vesicle called the autophagosome to transport contents that will be degraded (Arslan et al., 2011). Chaperone-mediated autophagy involves the selective transport of cytosolic proteins containing the KFERQ motif to lysosomes, with recognition by heat shock cognate 71 kDa protein (HSC70) without the need for vesicular transport (Kirchner et al., 2019).

Macroautophagy is the main mechanism referred as autophagy. Autophagy is initiated by the formation of an expanding membrane called the phagophore (Figure 1. 8) but the source of the membrane is still not fully understood (Liman and Suna, 2017). Then, the target to be degraded, also called cargo, is surrounded by the phagophore, and a double-membrane vesicle called the autophagosome is formed. Autophagosome is responsible for delivering the cargo to the lysosomes, for this purpose the autophagosome moves toward the lysosome. Afterward, the outer membrane of the autophagosome fuse with the lysosomal membrane, and autolysosome is constituted. Cargo is broken down into macromolecules such as amino acids, carbohydrates, and lipids by lytic enzymes, hydrolases. The released molecules are used as an energy source that induces the cells

metabolic processes (Pavel and Rubinsztein, 2017; Xie and Klionsky, 2007). The critical role of autophagy has been elucidated with studies showing that autophagy is not induced in mice deficient *ATG3*, *ATG5*, *ATG7*, *ATG9*, *ATG16L1* genes, and also these mice die immediately after birth in the absence of nutrients (Arslan et al., 2011; Komatsu et al., 2005; Sou et al., 2008; Takamura et al., 2011).

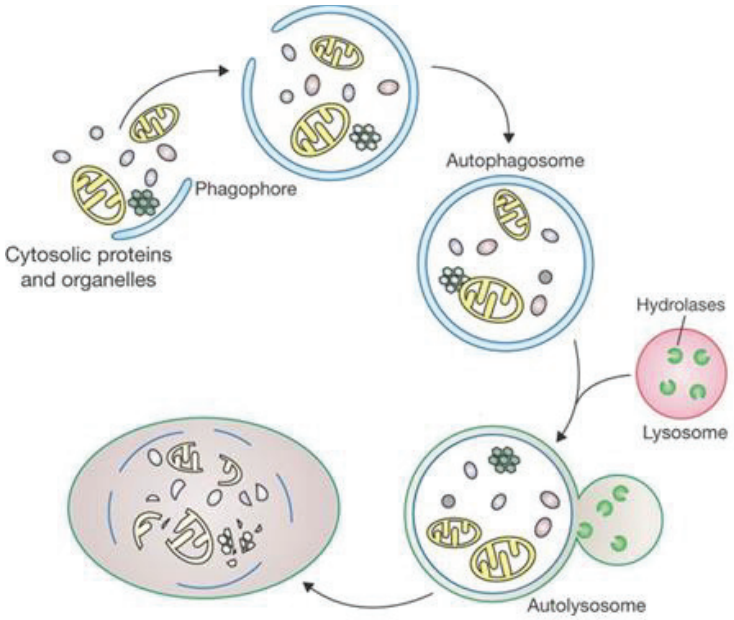

Figure 1. 8. Schematic description of autophagy stages

(Source: Xie and Klionsky, 2007).

Many autophagy-related proteins (ATG) are involved in autophagy regulation like biogenesis of autophagosome, fusion with lysosomes and degradation. ATG genes have been discovered in yeast (*Saccharomyces cerevisiae*) and most of them have mammalian orthologues (Ohsumi, 2014). Even though there are more than 30 ATG genes have been identified, it is known that 18 ATG genes are necessary for autophagosome formation (Suzuki et al., 2013). Most of the proteins involved in autophagy are involved in the formation of autophagic membranes and their elongation. To initiate the autophagy, the first step is the biogenesis of phagophore in this respect Ulk, and class III PI3K complexes are required. Complexes composed of PI3K (phosphatidylinositol 3-kinase) and *Vps34* that encourage autophagy are formed by Beclin 1 known as *ATG6* so lack of the *Beclin1* results in autophagy deficiency. After the cargo recognition, the next step is elongation.

ATG12–ATG5-ATG16L1 and microtubule-associated protein 1 light chain 3 (LC3) are two of the ubiquitin-like conjugation systems and they essential for phagophore elongation (Salem et al., 2015). Binding to phosphatidylethanolamine (PE) on the autophagosomal membrane is carried out by LC3 that is a cytosolic protein and one of the members of the *ATG8* family. The conjugate system of *ATG12–ATG5-ATG16L1* provides the lipidation of LC3 and after autophagosome shutting, LC3 is separated from PE (Weidberg et al., 2011). Most of the proteins involved in the formation of the phagophore and autophagosome are left back to the cytosol for reuse. Fusion of the autophagosome to the lysosome is accomplished via lysosome-associated membrane protein 2 (LAMP-2) and small guanosine triphosphatase Rab7 (Liman and Suna, 2017). Autophagy contributes to the activation of innate and adaptive immunity by sending microbial genetic materials that exist from lysosomal degradation to Toll-like receptor or MHC class II-loading compartments (MIICs) (Levine and Deretic, 2007).

Autophagy disorders can result in many diseases such as cancer, infectious diseases, neurodegenerative diseases (Nixon et al., 2005), and ischemic diseases (Levine and Kroemer, 2019). *Beclin1* gene is monallelically deleted in most cancers such as breast, ovarian and prostate (Qu et al., 2003). Tumor cells can also use the recycling characteristic of autophagy as a food source (White, 2012). The relationship between SNPs in genes functionary in autophagy such as *ATG16L1* (Lavoie et al., 2019), *IRGM* (Lu et al., 2013), *PTPN2* (Glas et al., 2012), *LRRK2* (Liu and Lenardo, 2012), and pathogenesis of IBD has been the subject of study for many years. When taking into account the importance of autophagy in microorganism clearance or proinflammatory cytokine secretion, it can say any deficiency upon autophagy may construct IBD pathogenesis. However, some bacteria like *Staphylococcus aureus*, *Brucella abortus*, and *Porohyromonas gingivalis* can block the fusion of the autophagosome with the lysosome to avoid lysosomal elimination (Burman and Ktistakis, 2010). Even if this fusion is not sabotaged by microorganisms, autophagolysosomal fusion requires lysosomal pH change (Levine and Kroemer, 2019). At this point, it has been shown that mutations in the presenilin 1 (*PS1*) gene, which is involved in the regulation of lysosomal acidification, lead to the early onset of Alzheimer's. Thereby, focusing on the autophagy mechanism may help to develop an appropriate therapeutic strategy for some diseases (Sahin and Yildiz, 2019).

1.3.3.3.1. ATG16L1

ATG16L1 is located on chromosome 2q37.1 and consist of three main parts that *ATG5*-binding domain located N-terminal, middle region containing a coiled-coil domain (CCD), and seven WD40 domains located in C-terminal shown as in Figure 1. 9 (Gammoh, 2020). It contains two different sites at its N-terminus that are necessary for *ATG5* binding and *ATG8* (also called helix 2) lipidation (Lystad et al., 2019; Otomo et al., 2013). CDD domain in the middle region participates in protein-protein and lipid-protein interactions such as *WIPI2* (*ATG18*), *FIP200* (*ATG17*), and *Rab33*. *WIPI2* and *Rab33* contribute to the elongation of autophagosomes by interact with *ATG16L1*. WD40 domains located in the C-terminus have been shown to play a role in mediating interaction with various factors for inflammatory control (Bajagic et al., 2017). *ATG16L1* is one of the core autophagy components. Although the *ATG6L1* gene has versatile activity in autophagy, the most important of these are the steps that provide the conjugation of *ATG8*. *ATG16L1* contributes to phagophore growth by localize to endocytic vesicles. With this situation, *ATG8* lipidation is triggered and autophagosome maturation is stimulated. The participation of *ATG16L1* in autophagosome formation depends on the constitution of PI3P by type III phosphatidylinositol 3-kinase VPS34 (Fletcher et al., 2018).

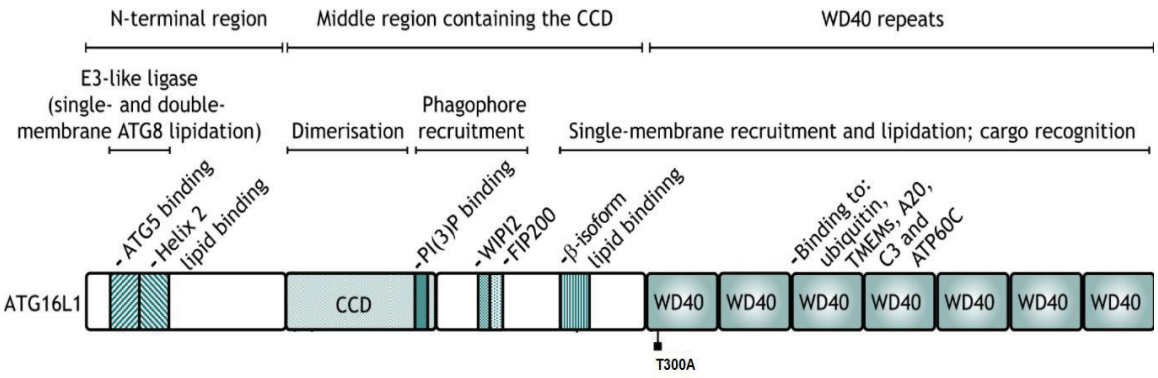

Figure 1. 9. Structure of *ATG16L1* gene

(Source: adapted from Gammoh, 2020).

Mutations in each domain of the *ATG16L1* gene will affect the mechanisms related to autophagy. Genome-wide association studies reveals that single nucleotide polymorphism in the *ATG16L1* gene leads to susceptibility to Crohn's disease. The mostly known of these are rs13412102, rs12471449, rs6431660, rs1441090, rs2289472, rs2241880, rs2241879, rs3792106, and rs4663396 (Salem et al., 2015). Among those 9 variants, the most widespread SNP related to Crohn's disease is known as T300A or rs2241880 and causes a substitution at amino acid position 300 from threonine to alanine (Hampe et al., 2007). This SNP displayed Figure 1. 9, is located in the WD40-domain of *ATG16L1* (exon 9). Due to the T300A variant's localization in the cleavage site of endoprotease enzyme CASP3 (caspase 3), making the *ATG16L1* sensitive to CASP3, accelerates its degradation (Murthy et al., 2014). That's why, T300A leads to a reduction in selective antibacterial autophagy (xenophagy) impairing the downstream processes (Gao et al., 2017). Also, the localization of pathogenic bacteria gradually increases and causes inflammation on the intestinal surfaces, leading to increased immune response (Figure 1. 10).

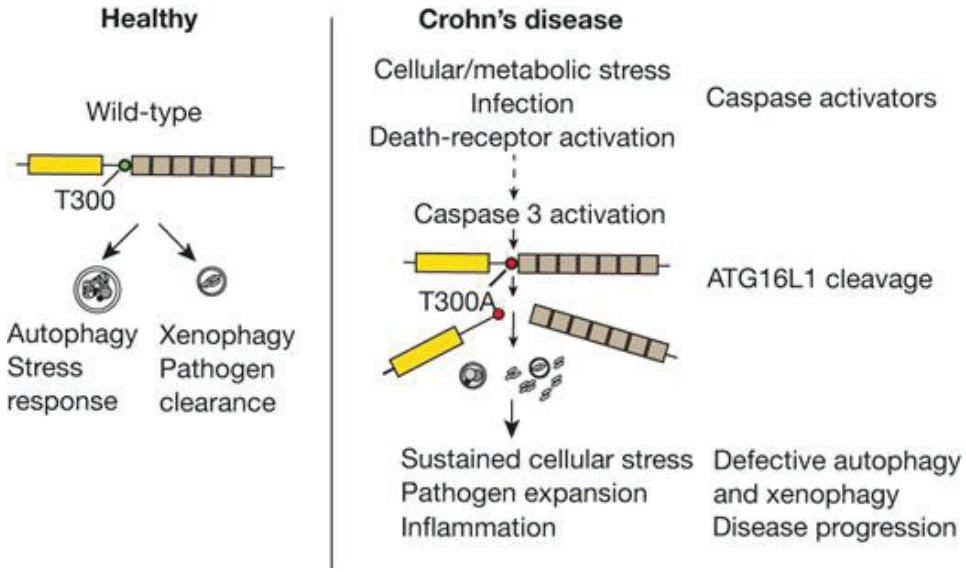

Figure 1. 10. The role of T300A variant to Crohn's disease pathogenesis

(Source: Murthy et al., 2014).

Besides the immune response, the *ATG16L1* gene provides an important infrastructure for epithelial stem cell function in the intestine by providing the protection of the integrity of Paneth cells. Paneth cells contain lysozyme enzymes in their granules that digest the bacterial cell wall and protect the intestinal flora through its phagocytosis ability (Cadwell et al., 2008). The *ATG16L1* (T300A) polymorphism is known to cause abnormalities in Paneth cells lysozyme granules in CD patients (VanDussen et al., 2014). A study has reported an increase in various lysozyme-sensitive bacteria, including *Ruminococcus gnavus*, which is effective in Crohn's pathogenesis in mouse models whose *Lyz1* gene (Paneth cell lysozyme) is deleted or ectopically expressed (Yu et al., 2020).

Imperfections in autophagy also affect either innate or adaptive immune responses. In our body defense against pathogens, *NOD2* stimulation by the bacterial pathogens gathers *ATG16L1* to the site of microorganisms, and the formation of autophagosomes is induced (Mo et al., 2012). Therefore, variants that may occur in *NOD2* will impair the ability to recruit *ATG16L1* to the bacterial infection site, thus affecting the autophagy mechanism (Gammoh, 2020). The sensitivity of *NOD2* to the presence of MDP is an effective inducer of autophagy, but it has been shown that T300A inhibits MDP-mediated autophagy of *Salmonella* in human epithelial cells (Homer et al., 2010).

1.3.4. Microbiota

Microbiota is a complex and dynamic ecosystem formed by a large number and diversity of microorganisms. The human microbiota colonize in the skin, genitourinary system, respiratory system and mostly the gastrointestinal system. The formation of intestinal microbiota in the fetus starts with birth and personalized by being shaped by the effect of nutrition and environmental factors. Studies have shown that the microbiota of the newborn changes even depending on the mode of delivery (Mayer et al., 2015). The gut microbiota consists of nearly 10^{14} microorganisms that specific for each individual and also have differences among people (Purchiaroni et al., 2013). Since the functions of the intestinal segments differ, the microbiota composition change accordingly. Several factors such as diet, age, stress, and drugs can affect the gut microbiota and cause it to

change throughout life. Although the gut microbiota has a dynamic composition consisting of bacteria, viruses, yeast and fungi, bacteria constitute a high percentage. Bacteria have immunoregulatory impacts and hereby lend to intestinal host defenses through interaction with the immune system. In the intestine microbiota; there are anaerobes, facultative anaerobes, and aerobic bacteria. It is known that most of the intestinal microbiota is composed of the bacterial phyla Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria (Sasaki and Klapproth, 2012).

Microbiota contributes to the maturation of the gastrointestinal tract epithelium from infancy. Epithelial cells of the gastrointestinal system are covered by a thick and chemically complex mucus layer. *Bacteroides, Bifidobacterium, Streptococcus, Enterobacteria, Enterococcus, Lactobacillus* and *Ruminococcus* are found on the mucus layer and participate in the structure of the feces. On the other hand, *Clostridium*, *Lactobacillus* and *Enterococcus* can be colonized deeper in the layer (Çelebi and Uygun, 2013). In a healthy person, the microbiota interacts symbiotically with the intestinal mucosa and provides major metabolic, immunological, and protective functions in the gut. Microbiota provides most of its nutrients from carbohydrates. In addition, they have an effect on nutrition by taking part in lipid and protein metabolism. They synthesize vitamins (B_1, B_2, B_6, B_{12} and K) and contribute to drug elimination and metabolizing xenobiotics. By regulating the body's response to stress, they are also effective on the endocrine system. Importantly, they modulate the immune responses by activate either innate or adaptive immunity. Therefore, intestinal microbiota is defined as a new "metabolic organ".

Although genetic factors have primary importance for the pathogenesis of IBD, not only genes are not sufficient but also environmental factors are important in the development of the disease. In the pathogenesis of IBD, genetically sensitive individuals have an inappropriate, persistent inflammatory response to common gut microbiota. Unlike healthy people, IBD patients have significant changes in their microbiota called dysbiosis. The microbiota transforms from a normal state to an abnormal state that can be associated with the disease (Kang et al., 2010). Hereby, dysbiosis can cause diseases by altering intestinal permeability. In addition to the role in the absorption of ions, water, and other nutrients, the intestinal barrier acts as a wall and prevents bacteria from entering the intestinal lumen. However, its permeability increases particularly during the acute

stages of Crohn's disease and promotes local inflammation with the displacement of bacteria through mucus. Decreasing in common flora bacteria such as *Bifidobacterium*, *Lactobacillus*, whereas increasing in pathogenic bacteria leads to deterioration of intestinal microflora. Enteric bacteria, viruses or fungi cause chronic intestinal inflammation by disrupting the balance of beneficial and harmful bacteria in the digestive tract. Then, inflammation is induced by disruption of epithelial cell metabolism as a result of the secretion of enterotoxins or immunosuppressive proteins by microorganisms. Enterotoxins enhance intestinal permeability and allow the translocation of immune activating antigens across the intestinal epithelium (Köseler, 2016).

In many studies, statistically significant differences have been revealed between the microbiota of IBD patients and healthy groups by PCR, qPCR, and NGS analyses. In 2007, the Human Microbiome Project (HMP) was initiated as a continuation of the Human Genome Project (HGP) to test how the human microbiome has changed in relation to health or disease (Peterson et al., 2009). In IBD pathogenesis, there is a reduction in bacteria belonging to phyla of Firmicutes *(Faecalibacterium), Clostridium*, and Bacteroidetes (D'Haens et al., 2014). On the contrary, there is a significant increase observed in Proteobacteria and Actinobacteria phylum. Because butyrate producer bacteria mostly belong to the Firmicutes phylum, decreasing in butyrate level due to Firmicutes abundance in IBD cases results in chronic inflammation and mucosal damage which the main characteristics of IBD. Specifically, *Faecalibacterium prausnitzii* (*F. prausnitzii*) is decreased in IBD patients whereas *Escherichia coli* (*E. coli*) is mostly increased in patients versus healthy individuals (Mylonaki et al., 2005). Interaction among pathogenic *E.coli* strains and host cells results in inflammatory responses as activation of NF-kB pathway, upregulation of interleukin, and migration of leukocytes (de Chambrun et al., 2008). Besides *E.coli*, many studies show the harmful effect of *Bacteriodes vulgatus* in IBD (Marteau et al., 2004). Another pathogen *Clostridium difficile* (Gram positive), which usually occurs in hospitalized patients, plays an important role in the pathogenesis of diarrhea by secreting cytotoxic and pro-inflammatory toxins (Navaneethan et al., 2010). Dissimilar to pathogens, some microorganisms like *Bifidobacteria* and *Lactobacilli* are thought to be a protective role in IBD (Marteau et al., 2004). Those bacteria are effective in the development of intestine mucosa. Also, by directly interact with pathogens or trigger the immune system, they contribute to the prohibition of pathogen attack. Additionally, variants in genes cause loss of function,

making the host vulnerable to pathogens (Homer et al., 2010). Taken into account the effect of SNPs onto microbiota abundance; it has been indicated that people carrying rs2066847 have more community of Bacteroidetes and Firmicutes phyla (Rehman et al., 2011). Mostly, *NOD2* initiates mucosal inflammation by recognizing peptidoglycan. Besides humans, researches have shown that *NOD2* is a significant regulator of the intestine microbiota in mice. Also, *NOD2* deficiency significantly impairs bacteria-killing abilities of mice and therefore mice cannot prevent pathogenic bacteria colonization (Petnicki-Ocwieja et al., 2009).

1.3.4.1. Next generation molecular techniques for microbiota studies

With the utilizing of DNA-based technologies since the 1980s, a new era was initiated in microbiota research. In this technologies, ribosomal RNA (*rRNA*) gene region which includes genetic alterations is targeted. Towards the 21st century, culture-independent techniques that do not require live bacterial cells have come to the fore, following modern developments such as sequencing of the *16S rRNA* gene. Unlike culture-dependent methods, molecular methods aim to identify amount of DNA and RNA within the sample for identification and classification of microorganisms. These methods, which highly specific and sensitive, provide an extra advantage especially for organisms that cannot be produced in-vitro.

In last years, by development of the next generation sequencing (NGS) methods such as targeted amplicon sequencing (16S/18S/ITS) or whole metagenomic shotgun sequencing, significant contributions to microbiota studies has been promoted. Shotgun sequencing provides a comprehensive definition of microbiota than targeted amplicon sequencing, as well as reveals functional characterization. Thus, it elucidates the functional relationships among host and species. Although shotgun sequencing is powerful, its usage is limited due to the high cost and more demanding bioinformatics analysis necessities (Boers et al., 2019).

To discrimination of microbiota based on taxonomically and phylogenetically, the most preferred NGS method is amplicon sequencing analysis. Because it is impractical to fully sequence each genome in the sample, molecular markers that can easily

distinguish different genomes have been identified for identification of microbial community. To elucidate the microbial community within a sample, specific marker genes like *16S rRNA* for bacteria and ITS (Internal Transcribed Spacer) for fungi, is targeted, amplified and sequenced (Massart et al., 2015). Ribosomal RNA (*5S*, *16S* and *23S rRNA*) is highly conserved among bacterial species. Among three rRNA genes, *16S rRNA* gene is more preferred for phylogenetic identification due to its content of conserved and variable regions. The variable regions provide for phylogenetic separation and classification of bacteria at the species and subspecies level. Analyzing marker genes instead of Sanger sequencing is advantageous in terms of shortening of analysis time, larger number of readings in a single run, enabling in-depth sequence, cost-effectively.

Prokaryotic ribosomes have 30S and 50S subunits, and the *16S rRNA* gene is located in the 30S ribosome subunit. Being present in nearly whole bacteria and highly conserved among species, with a length of 1500 bp long enough for research, makes *16S rRNA* an important target gene (Patel, 2001). Because *16S rRNA* gene sequences entried in databases are constantly increasing, provides the accessibility of gene sequences in bacterial diagnosis and classification. *16S rRNA* gene contains both constant and 9 different variable (V1-V9) regions (Figure 1. 11). Among those variable regions, frequently V3-V4 region is targeted because of its 67% bacterial coverage. Therefore, the V3-V4 region was chosen for our samples analysis.

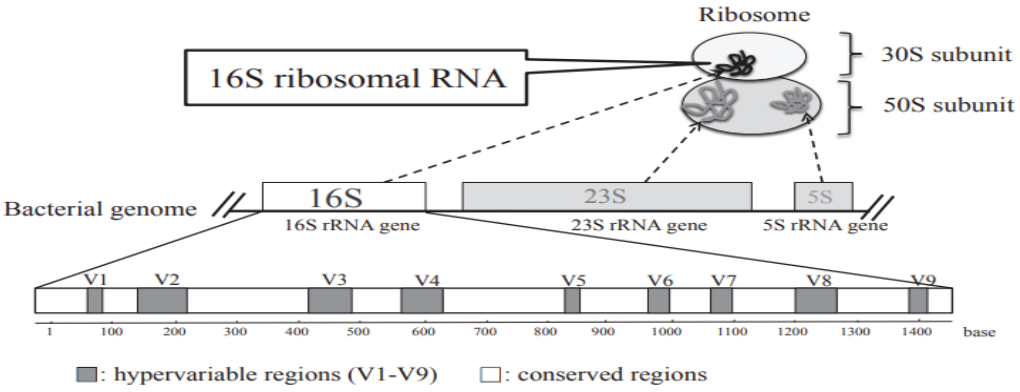

Figure 1. 11. Regions of the *16S ribozomal RNA* gene

(Source: adapted from Fukuda et al., 2016).

The first step for the amplicon sequencing is DNA isolation from samples. Stool samples are generally used in intestinal microbiota studies. Stool samples are frequently used in gut microbiota studies, as thought to reflect variations in the intestine microbiota. From the purity checked DNAs, PCR amplification is performed with *16S rRNA* specific primers (Cox et al., 2013). Created libraries are analyzed on new generation sequencing devices such as Illumina Miseq, Hiseq. High quality sequence data are obtained by applying the filtering process on the obtained raw sequence data. These data are compared with reference databases to define Operational Taxonomic Units (OTUs). The most used databases are Greengenes (DeSantis et al., 2006), Ribosomal Database Project (RDP) (Cole et al., 2005), SILVA (Pruesse et al., 2007) and NCBI. RDP includes bacterial and archaeal *16S rRNA* sequences and fungal *28S rRNA* sequences and updates periodically. SILVA contains small (*16S/18S*) and large subunit (*23S/28S*) rRNA sequences and like RDP, it is regularly updated. Although the Greengenes database also contains *16S rRNA* sequences, lastly it was updated in 2013. The OTUs used to define each bacterial representation are determined by sequence similarity. Although 97% sequence similarity is mostly used for identification at the species level and 95% similarity level at the genus level, in some bacteria, phylogenetic grouping can only be made at the family level (Cox et al., 2013). Until this step for data processing QIIME (Quantitative Insights Into Microbial Ecology) (Caporaso et al., 2010) or Mothur (Schloss et al., 2009) softwares are widely used. After identifying the bacterial community inside of the samples, further analyses (bioinformatics) are required to elucidate any differences both within and among samples. By performing alpha and beta diversity analyzes, dissimilarities can be determined. The workflow for amplicon sequence analysis described above is shown in Figure 1. 12.

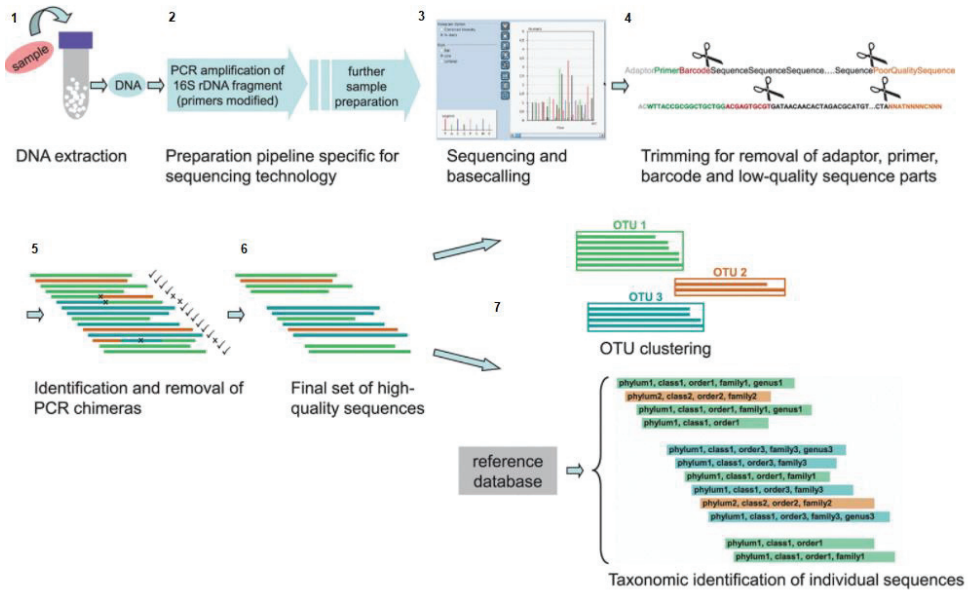

Figure 1. 12. Workflow of *16S rRNA* amplicon sequencing

(Source: Yang et al., 2013).

1.4. Role of the Biomarkers in IBD

Currently, there is no single and simple method for demonstrating and monitoring intestinal inflammation. For this reason, the clinical picture that consisting of anamnesis, laboratory, endoscopic, histological, and radiological tests of the patient is used in the determination and differential diagnosis of the IBD (Uslu et al., 2011). Since many factors play a role in the formation and progression of the disease, diagnosis can sometimes difficult despite all these data. Today, unfortunately, a "gold standard test" that will neither make the diagnosis of IBD nor distinguish CD from UC is not available. An erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), fecal calprotectin, antineutrophil cytoplasmic antibody (ANCA), and anti-*Saccharomyces cerevisiae* antibody (ASCA) are commonly used laboratory test parameters. However, these tests only indicate the presence of infection, and their detection in several diseases limits their specificity (Alatab et al., 2020; Uslu et al., 2011). Given this, it is predicted that the future of the management of IBD diseases will be made with personalized treatment approaches by identifying clinically reliable biomarkers (D'Haens et al., 2014).

The course of the disease varies individually. While some patients have mutations in genes that activate the immune system or are responsible for bacterial recognition, in some these mutations are rare. Also, microbiota resembles normal composition in some patients, whereas, pathogenic microorganisms are abundant in some, so impaired intestinal microbiota is observed compared to normal. All these factors cause changes in disease severity and differences in the type of treatment to be applied. In this case, choosing the biomarkers that will analyze the microbiota and genetic profile of the people becomes important both for the control of the disease and to reveal the individuals at risk before the disease progresses further. Treatments applied to patients differ from less aggressive to more aggressive. Since the patient profile is unknown, less aggressive drug applications are applied for each patient. However, in high-risk patients, the disease continues to progress rapidly because the treatment applied to deal with the disease will not be sufficient. Analyzing the patient profile is crucial at this point. Since the administration of more aggressive drugs will be provided early for high-risk patients, the right treatment will be applied to the right patient at the right time before it is too late.

Mutations in genes increase the severity of the disease by making the gut more susceptible to invasion by pathogenic bacteria. In many studies, statistically significant differences have been revealed between microbiota of UC-CD patients and non-IBD control groups with molecular techniques such as *16S ribosomal RNA* analysis, polymerase chain reaction (PCR), Real-Time PCR analysis (qPCR) and next generation sequencing. Amid them, nowadays, elucidating the ratio of beneficial-harmful bacteria in our intestinal microbiota with NGS technology has become quite popular. However, this application is not specific to IBD, and bacteria analysis is not performed at the species level specific to IBD. Because of its expensiveness, the adaptation of this method in clinical is difficult in terms of financial and practical. Intestinal microbiota enzymatically affects drug bioavailability and immune response. Analysis of the patient's microbiota profile becomes a significant criterion for us, as fecal microbiota transplantation (FMT) and probiotic supplementation are treatments aimed at improving the patient's microbiota. It is important to apply microbiota manipulation in the early stages of life in order to be effective especially on the immune system and to provide therapeutic benefit because become older, the effect of microbiota on immune system maturation decreases. Probiotics by increasing beneficial bacteria and reducing pathogenic bacteria in the gut, help maintain and regeneration the natural balance of bacteria (Kalip and Atak, 2018).

Probiotics reduce the accumulation of lipids and thus regulates metabolic and inflammatory processes (Palacios et al., 2017).

As a consequence, IBD is a life-long disease with its complex pathogenesis. Because the severity of the disease changes individually, the treatments to be applied should be personalized and adjusted during the course of the disease. For this purpose, finding biomarkers that will show the patient risk profile will help gastroenterologists to predict the course of the disease in IBD patients and, based on this, can guide the intensity of follow-up or therapeutic elections. Determining patients that will benefit from different treatment regimens will enable effective personalized treatments and save money for the healthcare system.

1.5. Aims of the Thesis

Due to social reasons such as changing lifestyle and nutrition habits, IBD will negatively affect Turkish society's health and economy at an increasing rate in the 21st century. In this study, we focused to identify molecular biomarkers to determine patients who may require an optimized personalized treatment plan to reduce the disease severity. Based on the information given, and the biology and clinical findings of IBD, there are three aims in this thesis.

The first aim of the thesis was to investigate the mutations/variations in the main genetic risk factors in a selective 3 most common representing adaptive immunity, innate immunity, and autophagy in Turkish IBD patients. Genes of *NOD2* representing innate immunity and *ATG16L1* representing autophagy, responsible for the recognition of bacteria and elimination. *IL-23R* a member of the adaptive immunity participates in the production of helper T cells that activate our immunity in intestinal inflammation. These genes and their variants that mostly contribute pathogenesis of IBD were analyzed and results compared among the CD and UC. Also, besides the target SNPs, the relationship between different variants obtained sequencing and IBD was assessed.

Microbiota is another decisive factor of the IBD pathogenesis so that the second aim of the thesis was to reveal the microbiota profile observed in Turkish IBD patients by next generation sequencing. Because NGS is not cost-effective, microbiota scanning in all samples was performed with the selected species through Real-Time PCR. By revealing the microbiota profile of patients, we will complement the existing pharmacogenetic approaches currently used in IBD treatment and help determine the personalized treatment approach.

Finally, to determine the relationship between mutations/variations and microbiota in Turkish IBD patients, statistical analyses were performed via our data. Based on our findings and literature, it is aimed to develop a rapid, cheap, quantitative molecular method that analyzes genetic and microbiota profiles in individuals to control IBD more effectively. This method developed based on Real-Time PCR, will reveal the personal risk profile of IBD patients and guide gastroenterologists in planning the most appropriate personal treatment for their patients. qPCR is more advantageous than PCR analysis in terms of providing qualitative and quantitative data, and it is cost-effective than NGS. The proposed method will be able to identify microbial and genetic changes occurring in the intestine in the early stages of the disease and will fill needed deficiencies in terms of diagnosis, treatment, and prognosis. This method will be transformed into a molecular test kit with commercial potential over time.

CHAPTER 2

MATERIALS AND METHODS

2.1. Human Samples

The thesis consisted of 3 main groups as patients with Crohn's Disease, Ulcerative colitis, and non-IBD (healty) control group. Blood and stool samples were collected from each individual on the ground that Dokuz Eylul University Clinical Research Ethics Committee Approval. Samples of IBD patients diagnosed according to international guidelines (like clinical, laboratory, endoscopy, histopathology, and radiological examinations) were collected in the Gastroenterology Department of Dokuz Eylul University (Bemelman et al., 2018; Stange et al., 2008).

All stool samples were gathered into the fecal containers; all blood samples were collected into blood collection tubes. Then samples were frozen instantly and kept at -20 ^{o}C until the experiments.

2.2. Microbiota Studies

The purpose of this section; to examine the main intestinal bacterial species and to reveal their distributions between healthy groups and Turkish IBD patients (Crohn's Disease, Ulcerative colitis).

2.2.1. DNA isolation from stool samples

DNA isolation was performed from 22 stool samples (6 of UC, 12 of CD, and 4 of control) using the QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the following protocol (Putignani et al., 2021).

First of all, approximately 200 mg of a frozen stool sample for each sample was transferred to 2 ml microcentrifuge tubes in a biological safety cabinet. 1.4 ml lysis buffer (ASL Buffer) was added to the tubes and vortex was done for homogenization for 1 minute. Then tubes were incubated for 5 minutes at 70-95^{o}C. After the incubation, the tubes that were vortexed for 15 seconds were centrifuged at 14,000 rpm for 1 minute. Stool samples have a complex composition that can be obstructed enzymatic reactions in PCR. In order to eliminate PCR inhibitors and substances that can damage DNA, InhibitEX Tablet was added to supernatants and incubated for 1 minute at room temperature. After that, all the supernatant from the samples centrifuged at 14,000 rpm for 3 minutes was transferred to a new tube and again centrifuged at 14,000 rpm for 3 minutes. 15 µl proteinase K was added to new 1.5 ml microcentrifuge tubes and 200 µl of the supernatant obtained after centrifugation in the previous step. 200 µl AL Buffer was added to the tubes, vortexed for 15 seconds and incubated at 70^{o}C for 10 minutes. After incubation, 200 µl of 96-100% ethanol was added to the tubes and mixed with the vortex. Collection tubes as many as the sample number were prepared and a filter tube were placed in each. All the mixture obtained in the previous step was transferred to filter tubes and centrifuged at 14,000 rpm for 1 minute. Nextly, collection tubes were discarded and filter tubes were placed in new collection tubes. 500 µl of AW1 Buffer was added into tubes and centrifuged for 1 minute at 14,000 rpm. After centrifugation, collection tubes were discarded and filter tubes were placed in new collection tubes. 500 µl AW2 Buffer was added to them and centrifuged for 3 minutes at 14,000 rpm. After centrifugation, collection tubes were discarded and filter tubes were placed in new collection tubes. To dry tubes, centrifuged for 1 minute at 14,000 rpm. Following centrifugation, filter tubes were placed in 1.5 ml sterile microcentrifuge tubes and 200 µl AE Buffer was added to center of each column matrix and stood for incubation at room temperature for 1 minute. After incubation, they were centrifuged at 14,000 rpm for 1

minute. After centrifugation, the sample DNA was collected in 1.5 ml microcentrifuge tubes. The purified DNA samples obtained were stored at -20ºC for the next analysis.

The concentration (ng/µL) and purity (A260/A280 and A260/A230) of the DNA samples obtained were determined by measuring the absorbance at 260 and 280 nm wavelengths in the Nanodrop 8000c Spectrophotometer (Thermo Fisher Scientific, USA). Typically, the ratio of absorbance at A260/280 should be 1.8-2.0 for the pure DNA samples.

2.2.2. Next generation sequencing (NGS)

Next generation sequencing was performed based on the amplicon sequencing. In nine stool DNA samples (3 samples for each UC, CD and control groups), the *16S rRNA* variable V3-V4 region was targeted and amplified with primers 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) each attached to an adapter sequence by PCR. Afterward, for PCR products quantification and qualification, agarose gel electrophoresis was performed. Because nearly 500 bp products can be obtained by PCR performed with V3-V4 primers, samples with bright main strip in gel electrophoresis between 450-500 bp were chosen for further experiments. Behind the purification of PCR products, sequencing libraries generated with Nextera XT DNA Library Preparation Kit (Illumina, USA). Concentration of the libraries generated by qPCR was measured and normalized by diluting to 4nM. Normalized samples were pooled and sequenced by Illumina NovaSeq 6000 as paired-end (2x250 bp). Amplicon sequencing analysis was performed by BM Labosis laboratory in Ankara. The workflow of data analysis were shown in Figure 2. 1.

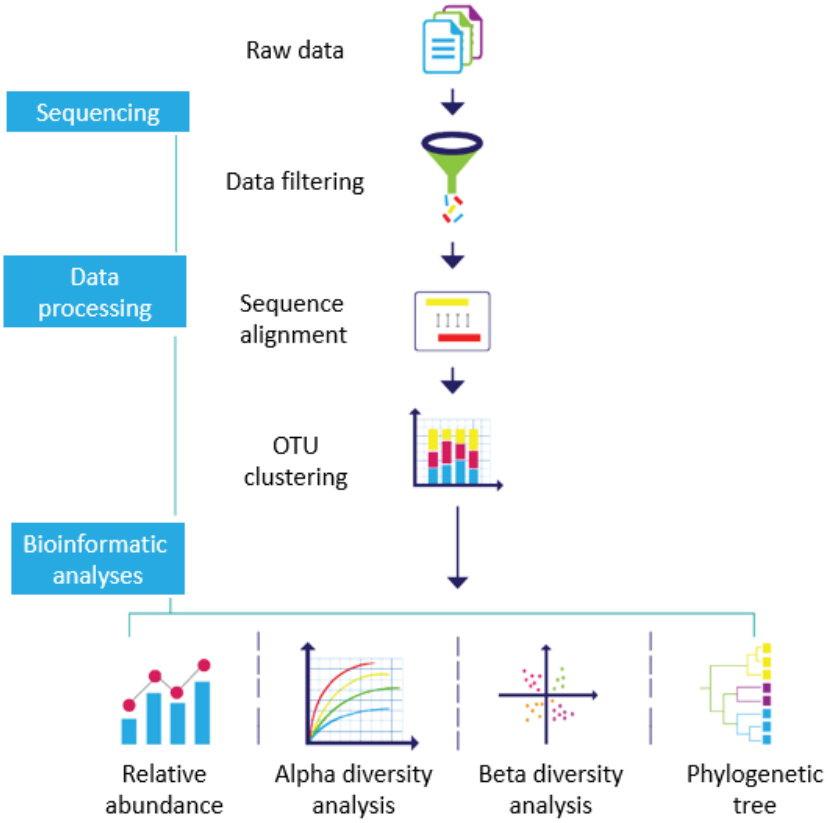

Figure 2. 1. Workflow of the data processing after amplicon sequencing.

2.2.3. Bioinformatic and statistical analyses for microbiota

According to amplicon sequencing, amplicons were sequenced on Illumina platform to generate raw reads. In data mining, raw data was filtered and effective tags were obtained. Using 97% similarity, Operational Taxonomic Units (OTUs) were generated to elucidate species diversity in each sample. With constituted OTU clustering, bioinformatics analyses were performed. Relative abundance were displayed for each sample after normalization of the OTUs abundance information. Alpha diversity and beta diversity analyses were done by this normalized data. Alpha diversity was calculated to analyze of species diversity within a sample through 5 indices, including Observed-species, Chao1, Shannon, Simpson, and ACE and plotted in boxplots. Beta diversity analysis was used to explain differences between groups in species complexity. A distance matrix of weighted UniFrac between samples were calculated and Principal Coordinate Analysis (PCoA) also as known Multi-Dimensional Scaling Analysis (MDS)

was performed to visualize as multidimensional data. All statistical analyzes were performed with R software (Version 4.0.5) (https://www.r-project.org). In the R software, "phyloseq" package was utilized. To visualized relative abundance of phylum, the "ggplot2" package was used.

2.2.4. Real-Time PCR (qPCR) analysis

Real-Time PCR analysis commonly uses for quantifying interested bacterial species as well as total bacteria in the stool samples as it increases the clinical applicability by making the analysis fast, highly sensitive, specific, reproducible, and reduce contamination (Ott et al., 2004). To corroborate the amplicon sequencing results, qPCR analysis were performed. Based on the our NGS results and microbiota studies in the literature includes common information obtained from approximately 20 articles in PubMed (https://pubmed.ncbi.nlm.nih.gov/), 2 different bacterial species were selected. The bacterial species selected were *Faecalibacterium prausnitzii* and *Clostridium difficile*, and these microorganisms dispersion known to differ between UC and CD. Considered that dysbiosis has an important role in the pathogenesis of IBD, a beneficial bacteria (*F. prausnitzii*) and pathogen bacteria (*C. difficile*) were selected.

2.2.4.1. Primer Preparation

The DNA sequences for *F. prausnitzii*, *C. difficile*, and total bacteria *16S rRNA* specific primer pairs, which are thought to be influential in the pathogenesis of UC and CD, were shown in Table 2. 1. The specificity of the primers was checked with NCBI (https://www.ncbi.nlm.nih.gov/), BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers were purchased from Macrogen (Korea).

Table 2. 1. Primers used in Real-Time PCR analysis.

Target microorganism	DNA sequences of the primers (5'-3')		Annealing Temperature (ºC)	Product Size (bp)	References
Faecalibacterium prausnitzii	Forward	GGAGGAAGAAGGTCTTCGG	60	248	(Fujimoto et al., 2013)
	Reverse	AATTCCGCCTACCTCTGCACT			
Clostridium difficile	Forward	TTGAGCGATTTACTTCGGTAAAGA	61	157	(Balamurugan et al., 2008)
	Reverse	CCATCCTGTACTGGCTCACCT			
Total bacteria	Forward	TCCTACGGGAGGCAGCAGT	51	466	(Balamurugan et al., 2008)
	Reverse	GGACTACCAGGGTATCTAATCCTGTT			

***Main stock*:** Primers were diluted by adding the required amount of water for 100 µM (i.e. 100 pmol/µl) specified in the user manuals. The intermediate stocks, clarified below for each forward (F) and reverse (R) primers from the diluted primers were prepared for Real-Time PCR analysis.

Interim stock: 10 µM (10 pmol) intermediate stock was made from forward and reverse main primer stocks. To do that, 10 µl of 100 µM main stock was taken and 90 µl of water was added to it.

2.2.4.2. Performing Real-Time PCR analysis

Using several DNA samples and primers, study protocol optimization was performed. Reactions were completed with the LightCycler® 480 SYBR Green I Master enzyme purchased from Roche Applied Science (Germany). Real-Time PCR analysis was performed with Roche LightCycler® 480 II system in Biotechnology and

Bioengineering Application and Research Center (BIOMER) within İzmir Institute of Technology (IZTECH) Integrated Research Centers.

For a single reaction, as shown in Table 2. 2, 1.9 µl of PCR-grade water, 0.3 µl of microorganism specific forward and reverse primers, and 5 µl of LightCycler® 480 SYBR Green I Master enzyme were mixed into a sterile 0.2 ml PCR tubes and total volume was adjusted to 7.5 µl without the DNA sample.

Table 2. 2. Reaction components of Real-Time PCR.

Components (Per reaction)	**Volume (µl)**
PCR-grade water (ddH_2O)	1.9 µl
Forward Primer (intermediate stock 10 µM)	0.3 µl
Reverse Primer (intermediate stock 10 µM)	0.3 µl
Enzyme Mixture **(**LightCycler® 480 SYBR Green I Master**)**	5 µl
DNA sample	2.5 µl
Total Volume	**10 µl**

Each reaction mixture was prepared separately for 3 primer pairs. This reaction mixture, which was prepared as 7.5 µl per reaction number, was loaded into each 96-well plate to be used in Real-Time PCR analysis. 2.5 µl of sample DNA was loaded on the mixture in the wells and the final volume was completed to 10 µl. The plates were covered with the sealing-foil and centrifuged with the plate-centrifuge (Hettich Rotina 38R, Germany) for 2 minutes. Afterward, the prepared plate was run with the Roche LightCycler® 480 II Real-Time PCR System accordingly the following protocol shown in Table 2. 3. All plates were analyzed with a negative control (no template) for each primer pairs.

Table 2. 3. Run protocol of Real-Time PCR.

Program Name	**Denaturation**	**Amplification**			**Melting Curve**			**Cooling**
Analysis mode	None	Quantification			Melting curves			None
Cycles	1	50			1			1
Target [°C]	95	95	58	72	95	63	97	40
Hold (hh:mm:ss)	00:10:00	00:00:10	00:00:15	00:00:15	00:00:05	00:01:00	00:00:00	00:00:30
Ramp rate (°C/s)	4,8	4,8	2,5	4,8	4,8	2,5	0,11	2,5
Acquisition mode	None	None	None	Single	None	None	Continuous	None

2.2.4.3. Data analysis of Real-Time PCR

Amplification and melting curves for each sample were obtained using Absolute Quantitation/Second Derivative and Tm Calling analysis modes in the LightCycler® 480 II software. Ct values (Cycle Threshold) enounce the number of cycles (threshold value) required to observe the amount of fluorescence signal obtained during Real-Time PCR analysis. These values related to the amount of target sequence in the sample DNA analyzed (Jazmati et al., 2016). The Ct value obtained decreases as the target DNA sequence amount increases. The data analysis was conducted using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). In this method used to calculate the relative gene expression, the expression of the target genes is measured using a housekeeping (reference) gene. Similarly, target microorganisms were considered as a target while total bacteria were as a reference (Navidshad et al., 2012). Comparison of bacterial abundance among IBD patient and control groups were stated as “fold change”.

2.3. Genetic studies

The aim of this part; to research the mutations/variations in innate immunity (*NOD2*), adaptive immunity (*IL-23R*), and autophagy (*ATG16L1*) genes, which increase the risk of Inflammatory Bowel Disease, among the subgroups. Withal, it was aimed to evaluate the effects of mutations on microbiota balance and dysbiosis observed in CD and UC patients.

2.3.1. DNA isolation from blood samples

DNA isolation was performed from 23 blood samples (7 of UC, 12 of CD, and 4 of control) using the Genomic DNA Mini Kit-Blood/Cultured Cell (Geneaid, Taiwan) according to the following protocol.

Firstly, 300 µl of blood samples were taken and transferred to sterile 1.5 ml eppendorf tubes. 900 µl (3X the sample volume) RBC Lysis buffer was added to each sample and left for 10 minutes incubation at room temperature. Tubes were centrifuged at 3,000 g for 5 minutes and then the supernatant was completely removed. After adding 100 µl RBC Lysis buffer and 200 µl GB buffer to the pellet, samples vortexed and incubated for 10 minutes at 60°C. Following incubation, 200 µl of 100% ethanol was added and a short vortex was performed. The GD columns in the kit were placed into the collection tubes and each mixture was transferred to these columns, centrifuged at 16,000 g for 5 minutes. After that, the columns were placed in new collection tubes, 400 µl of W1 buffer was added on it, and for 1 minute at 16,000 g centrifuged. Later, the liquid collected in the collection tubes was discharged and the column was placed back in the collection tubes and 600 µl of Wash Buffer was added on columns; for 1 minute at 16.000 g. centrifuged. After centrifugation, the flow-through was discarded, by placed again the columns in the collection tubes, centrifugation was applied for 3 minutes at 16,000 g to dry the matrix. The dried columns were located in sterile 1.5 ml eppendorf tubes and 100 µl of Elution buffer, previously heated at 60°C, was added to the center of the columns. Tubes were incubated at room temperature for 3 minutes, then centrifuged at 16,000 g for

30 seconds to elute the purified DNA. The obtained DNAs were stored at –20°C for the next analysis.

The concentration (ng/µL) and purity (A260/A280 and A260/A230) of the DNA samples were measured by Nanodrop 8000c Spectrophotometer (Thermo Fisher Scientific, USA).

2.3.2. Primer design for the PCR analysis

rs2066844 (Arg702Trp), rs2066845 (Gly908Arg), rs2066847 (Leu1007insC) mutations for the *NOD2* gene; rs11209026 (Arg381Gln) for the *IL-23R* gene and rs2241880 (Thr300Ala) mutation were been targeted for the *ATG16L1* gene. Targeting DNA sequences for each gene were obtained from NCBI (https://www.ncbi.nlm.nih.gov/), ENSEMBL (https://www.ensembl.org/index.html) and UCSC Genome Browser (https://genome.ucsc.edu/cgi-bin/hgGateway) databases. Using those sequences, primers were designed by Primer 3 (http://primer3.ut.ee/) and IDT SciTools (https://www.idtdna.com/pages/tools) (Table 2. 4). Oligonucleotide properties, melting temperature, hairpins, dimers, and mismatches were identified by IDT SciTools OligoAnalyzer 3.1 (https://www.idtdna.com/calc/analyzer) software (Owczarzy et al., 2008) and specificity of primers were confirmed with BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers were synthesized by Macrogen (Korea).

Table 2. 4. Primers for SNP analysis.

Target gene and mutation (SNP) names		**DNA sequences of the primers (5'-3')**	
IL-23R	rs11209026	Forward	CATGTAGCCCAACTTTCTCAAAC
		Reverse	AGAGAGTTTGGCATGGGTAAG
ATG16L1	rs2241880	Forward	CATGTGCTGGCTCTCTTTCT
		Reverse	TGACTGGCAACTCACTCTAAAC
NOD2	rs2066844	Forward	TTTCTCTTGGCTTCCTGGTG
		Reverse	TTGAGGTGCCCAACATTCA
	rs2066845	Forward	TTAAGGGTTAGGGCTGGGTA
		Reverse	CGAGTGTCCTCTCTTGAAATCC
	rs2066847	Forward	ACTGATGGTACTGAGCCTTTG
		Reverse	CCTGCTCCTAACCTGTGTAATC

2.3.3. Polymerase Chain Reaction (PCR) analysis

First of all, lyophilized primers were diluted to 100 µM (main stock) according to user manuals. Then 10 µM intermediate stock was made for each primer pairs. With primers designed for the specified gene regions, PCR analysis was performed using FastStart High Fidelity PCR System, dNTPack kit purchased from Roche Applied Science (Germany). Entire reactions were completed using the ABI SimpliAmp ThermalCycler machine.

Before preparing the mixtures, each reagent was vortexed and centrifuged. Reaction mixes were made separately for *IL-23R*, *NOD2*, and *ATG16L1* genes using 17.25 µl PCR-grade water, 0.5 µl forward ve reverse primers, 0.5 µl PCR Grade Nucleotide Mix, 2.5 µl FastStart High Fidelity Reaction Buffer, 0.5 µl dimethyl sulfoxide (DMSO), and 0.25 µl FastStart High Fidelity Enzyme Blend as much as the number of reactions. The mixtures were mixed completely and a volume of 22 µl was transferred

into each 0.2 ml PCR tube. 3 µl of sample DNA was added in the PCR tubes and the final volume was completed to 25 µl (Table 2. 5).

Table 2. 5. Reaction components of PCR.

Components (Per reaction)	**Volume (µl)**
PCR-grade water (ddH_2O)	17.25 µl
Forward Primer (intermediate stock 10 µM)	0.5 µl
Reverse Primer (intermediate stock 10 µM)	0.5 µl
PCR Grade Nucleotide Mix	0.5 µl
FastStart High Fidelity Reaction Buffer (10x-with 18 mM $MgCl_2$)	2.5 µl
DMSO	0.5 µl
FastStart High Fidelity Enzyme Blend (5 U/µl)	0.25 µl
DNA sample	3 µl
Total Volume	**25 µl**

PCR tubes were placed in the thermal cycler, and the reaction was performed by the protocol mentioned Table 2. 6.

Table 2. 6. Protocol of PCR analysis.

Program Name	**Initial Denaturation**	**Amplification**			**Final Elongation**	**Cooling**
		Denaturation	Annealing	Elongation		
Cycles	1	35			1	1
Temperature [°C]	94	94	57	72	72	4
Time	10 min.	2 min.	30 sec.	1 min.	7 min.	Unlimited time

2.3.4. Agarose Gel Electrophoresis

Agarose gel electrophoresis is one of the popular methods to analyze DNA or RNA in molecular biology studies. Nucleic acids are fractionated throughout an electric field according to their size. Depending on the pore size of agarose, small fragments move quickly while large fragments move slowly. Also, the concentration of the gel, applied voltage, type of running buffer can affect the migration of nucleic acids. Mostly, agarose is preferred as a gel structure in the analysis. For visualization of the DNA, generally, ethidium-bromide (EtBr) is used as an intercalating agent. Unfortunately, EtBr is highly cancerogenic so GelRed is a beneficial alternative due to is a nonmutagenic safe dye. For DNA electrophoresis, TAE (Tris/Acetate/EDTA) and TBE (Tris/Borate/EDTA) are used to gel-loading buffers (Dash et al., 2020).

Following the PCR analysis, agarose gel electrophoresis was performed to observe band formation in PCR samples. First to prepare stock 10X TBE running buffer, 108 g of Tris base (Sigma-Aldrich), 55 g of boric acid (Sigma-Aldrich), and 7.5 g of EDTA (Sigma-Aldrich) were dissolved in 800 ml of dH_2O until obtaining a homogeneous mixture (Cotreanţi et al., 2016). By adjusting the pH to 8.0 with a pH-meter (S220-K, Mettler Toledo, USA) the total volume was completed to 1000 mL. To prepare 0.5X TBE from stock solution, 50 ml of 10X TBE stock buffer was taken, and added 950 ml of dH_2O. Agarose gel was prepared at 1.4% concentration. A mixture consisting of 100 mL 0.5X TBE and 1.4 g agarose was heated in a microwave. 1 mL GelRed® Nucleic Acid Gel Stain (Biotium, USA) was added then the mixture was poured into the gel tray placed gel comb, and froze at room temperature. The buffer tank was filled with 0.5X TBE and frozen gel placed into the electrophoresis tank by removing the comb. Into the first sample well, 6 µl 100 bp DNA Ladder (Geneaid, Taiwan) was loaded. Next wells were filled with a mixture of 1 µl 6x loading dye and 5 µl sample. The prepared gel was run at 100 V for 70 minutes until the dye line approach nearly the end of the gel. After the analysis, the power was turned off, and the gel removed from the gel box. Using UV light (Uvitec, UK), DNA fragments named as bands were visualized. DNA ladder (a mixture of DNA fragments of known lengths) was used as a guideline to predict sample fragment size.

2.3.5. Sanger Sequencing Analysis

Sanger sequencing is used as a gold standard to identify nucleic acid sequences in the genome. Therefore, this technology is also utilized to verify PCR results (Crossley et al., 2020). First of all, sequencing PCR was performed from the PCR products obtained as a single band. For a single reaction as totally of 10 µl, as displayed in Table 2. 7, 2 µl BigDye Ready Reaction Mix, 1 µl 5x buffer, 1 µl primer (3.2 pmol/µl), 4 µl ddH_2O, and 2 µl PCR products were mixed. The PCR amplification was as follows: initial denaturation of 1 min at 96°C, 30 cycles of 10 sec at 96°C, 5 sec at 50°C, 4 min at 60°C, and cooling at 4°C.

Table 2. 7. Reaction components of sequencing PCR.

Components (Per reaction)	**Volume (µl)**
BigDye Ready Reaction Mix	2 µl
5x Buffer	1 µl
Primer (3.2 pmol/µl)	1 µl
PCR-grade water (ddH_2O)	4 µl
DNA (PCR product) sample	2 µl
Total Volume	**10 µl**

Secondly, after the sequencing PCR, obtained PCR products were purified by Sephadex spin column. For this purpose, 1 gram of Sephadex G50 (Sigma-Aldrich) and 15 ml of distilled water were added into 50 ml falcon tubes. After the prepared mixture was shaken by hand for a few minutes, 600 µl was transferred to the empty spin columns. The filled spin columns were incubated for 10-30 minutes after vortexing. The plugs at the ends of the columns were removed and centrifuged at 5400 rpm for 2 minutes. After centrifugation, water in the collection tubes was poured and columns were transferred to the new collection tubes. Then the PCR products were added in the middle of the

Sephadex columns. After being centrifuged at 5400 rpm for 2 minutes, the cleaned PCR products were obtained.

Finally, purified samples were analyzed by ABI Prism 3130xl Genetic Analyzer. Sequence analysis was done in BIOMER, IZTECH. The data (ABI chromatograms) obtained after sequence analysis were analyzed using BioEdit 7.2.6.1 and Unipro UGENE v.33 programs. Targeted SNPs in all 3 target genes were determined by comparing IBD patients and control samples with multiple sequence alignment using the ClustalW algorithm (Rose et al., 2019).

2.4. Statistical analyses

Association of candidate genes' alleles and genotypes with disease status were assessed by Chi-square categorical analyses. Normality assumption was violated for all continuous variables, so non-parametric tests were performed. Distribution of candidate bacteria amounts among the IBD and control groups were checked by non-parametric Kruskal-Wallis and pairwise Wilcoxon rank-sum tests. P-value less than 0.05 was assessed as statistically significant. All statistical analyzes were performed with R software (Version 4.0.5) (https://www.r-project.org) using "gmodels", "ggpubr", "stats", and "graphics" packages.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Next Generation Sequencing

16S rRNA amplicon sequence analysis was performed to determine bacterial community in stool samples of Turkish IBD patient and control groups. Firstly, raw data was merged and filtered to get clean data. Quality control checks of raw data (fasta format) was performed by FastQC and reads quality control was checked by QIIME2. To remove sequencing errors; primer and barcode sequences, chimeric reads and readings with Phred Score less than 20 were filtered by DADA2 thus, effective tags were obtained. By utilizing the effective tags, representative sequence for each OTU was acquired with 97% similarity against reference databases like Greengenes and SILVA. Each OTU were analyzed by blast with QIIME2 to determine species annotation at taxonomic order (kingdom, phylum, class, order, family, genus, species). OTU table showing the family diversity in each sample can be found in Appendix A.

In this thesis, a broad view of the Turkish IBD patients gut microbiota was provided. A total of 315,707 amplicon sequencing reads were obtained from the 9 fecal samples (derived from 3 CD, 3 CD and 3 control). Relative abundance values were shown at the phylum level for CD, UC and control groups in Figure 3. 1. A total of 8 phyla, 15 classes, 23 orders, 43 families, 96 genus and 233 species were represented. At the phylum level, the most abundant in Crohn's disease were Firmicutes (36.22%), Proteobacteria (29.22%), Verrucomicrobia (25.79%), Bacteroidetes (8.68%), Actinobacteria (0.07%), and equally Fusobacteria and Synergistetes (0.01%). In Ulcerative colitis, phylum abundancy were determined as Proteobacteria (45.63%), Firmicutes (29.21%), Bacteroidetes (10.14%), Fusobacteria (9.98), Actinobacteria (2.80%), and Verrucomicrobia (2.25%). Unlike CD, no phylum of Synergistetes was observed in UC. The most common phylum in the control group was Bacteroidetes (62.63%). This was

followed by Firmicutes (31.47%), Proteobacteria (4.01%), Actinobacteria (1.81%), equally Fusobacteria and Lentisphaerae (0.04%), Verrucomicrobia (0.01%).

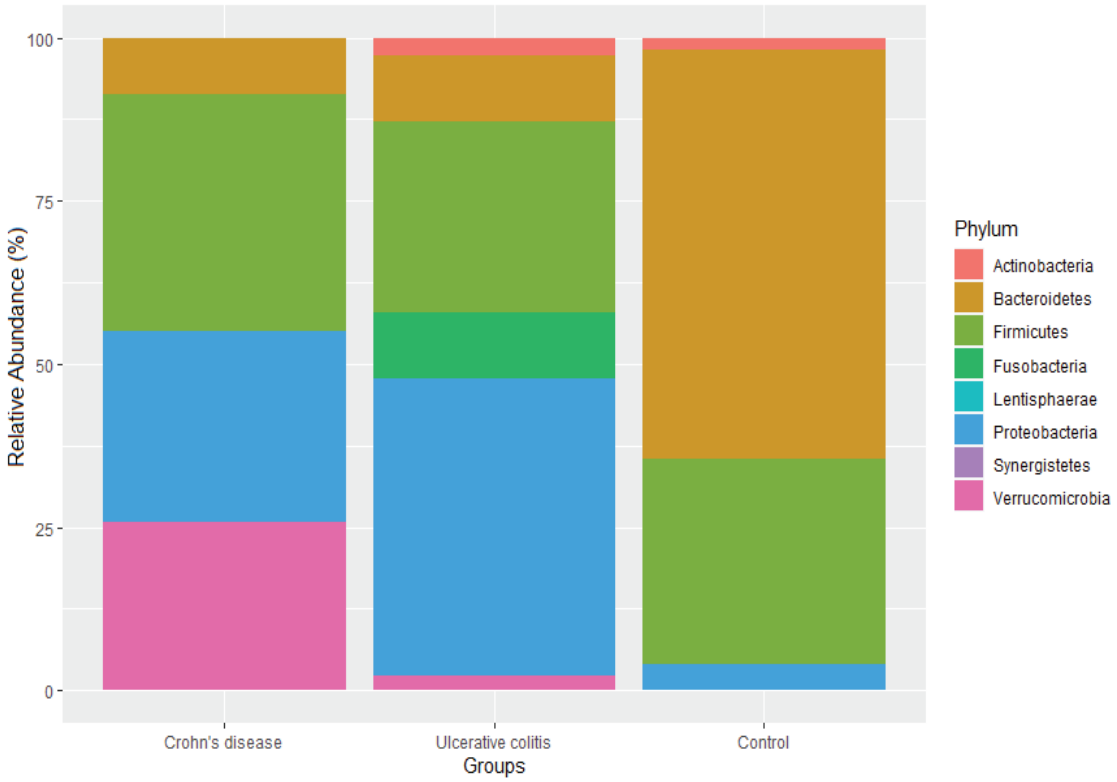

Figure 3. 1. Species relative abundance in phylum within Turkish IBD patients and control groups.

Alpha diversity (α-diversity) is an indicator of the microbial diversity of each sample. It shows how various a single sample considering the count of dissimilar species obtained. There are different alpha diversity indices expressing the decreased or increased diversity between samples. From samples analyzed by NGS, number of Observed taxa (OTUs) and Chao1, ACE (Abundance-based Coverage Estimator), Shannon, Simpson indexes were calculated by "phyloseq" package and represented using the "ggplot2" package in R software (Figure 3. 2). The number of different taxa observed within samples provides a clear representation of the diversity. The numbers of observed taxa (richness), generally states at the species level. Expectedly, among the three groups, the highest bacterial diversity was found in the control (healthy) group. Even if the UC group

richness was found close to control, as particular species dominated in the patient group, this dominance is made it less diverse. One of the diversity indices of alpha diversity mostly use in ecological studies is the Shannon index (H) which estimator for species richness and evenness. Simpson index (D) is another diversity indices and mostly focuses on common species. Because the Shannon index is more sensitive to rare taxa within a sample, it is more advantageous for determining actual diversity. Similarly diversity indices, ACE and Chao1 are nonparametric richness estimators through expected OTUs in the sample (Kim et al., 2017).

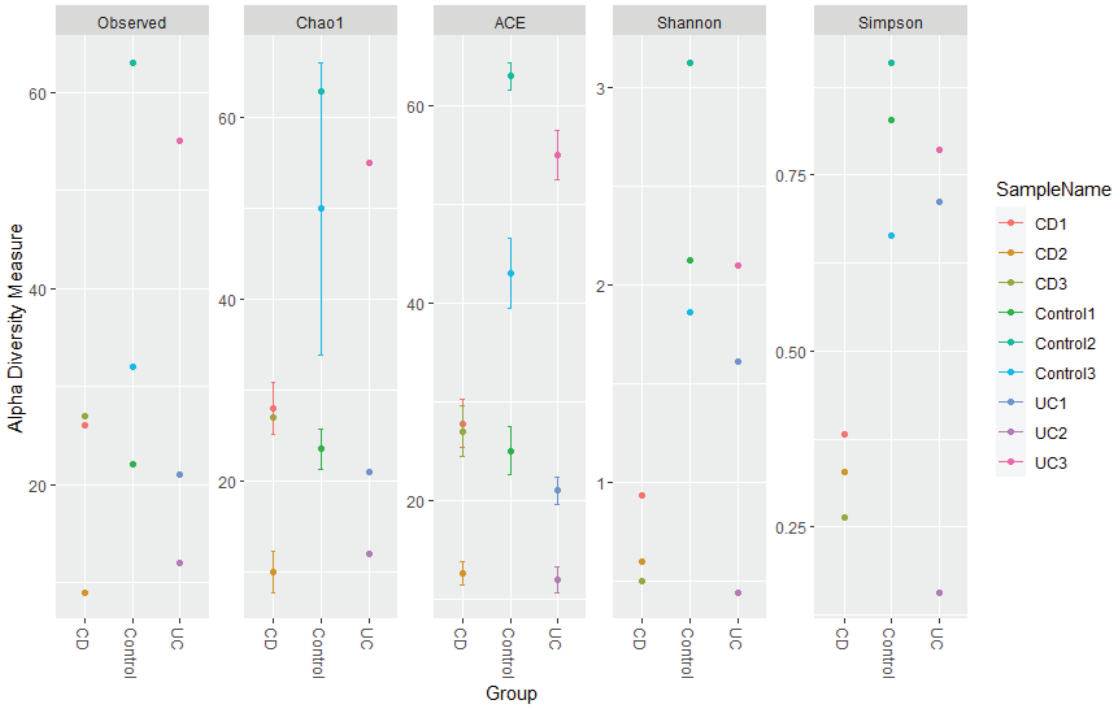

Figure 3. 2. Alpha diversity indices statistics.

Initially, the differences between IBD patients and control groups were tested. As shown in Figure 3. 3 (A), the number of observed taxa (OTU) were decreased unsignificantly in IBD patients versus control group, with p-value = 0.26, using the Wilcoxon rank-sum test. As shown in Figure 3. 3 (B), species diversity between IBD and control groups did not show a statistically significant difference (p-value = 0.095) in terms of Simpson index. On the other hand, according to the Shannon index, species diversity

was found to be significantly lower in the IBD patients against control group (p-value = 0.048).

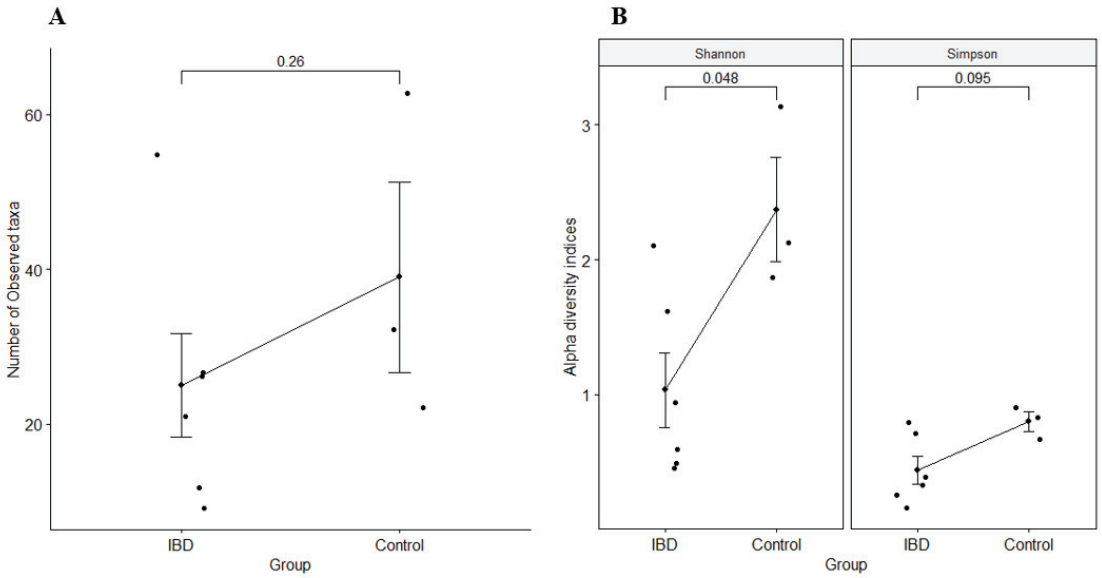

Figure 3. 3. Results of richness (A) and Shannon and Simpson diversity indices (B) between IBD and control groups.

In the next step, it was determined whether there was any discriminative relationship between UC-CD, UC-control, and CD-control groups in terms of bacterial diversity. In contrast to Ulcerative colitis patients, total bacterial richness in Crohn's patients was remarkably reduced. In pairwise comparison with the Wilcoxon rank-sum, no statistical differentiation ($0.05 < p$) was observed in neither richness nor diversity indices (Figure 3. 4). Likewise, the Kruskal–Wallis test was not indicated a statistically significant variation among the three groups in all measured indices (Shannon, p-value = 0.099; Simpson, p-value = 0.15; Observed OTUs, p-value = 0.43).

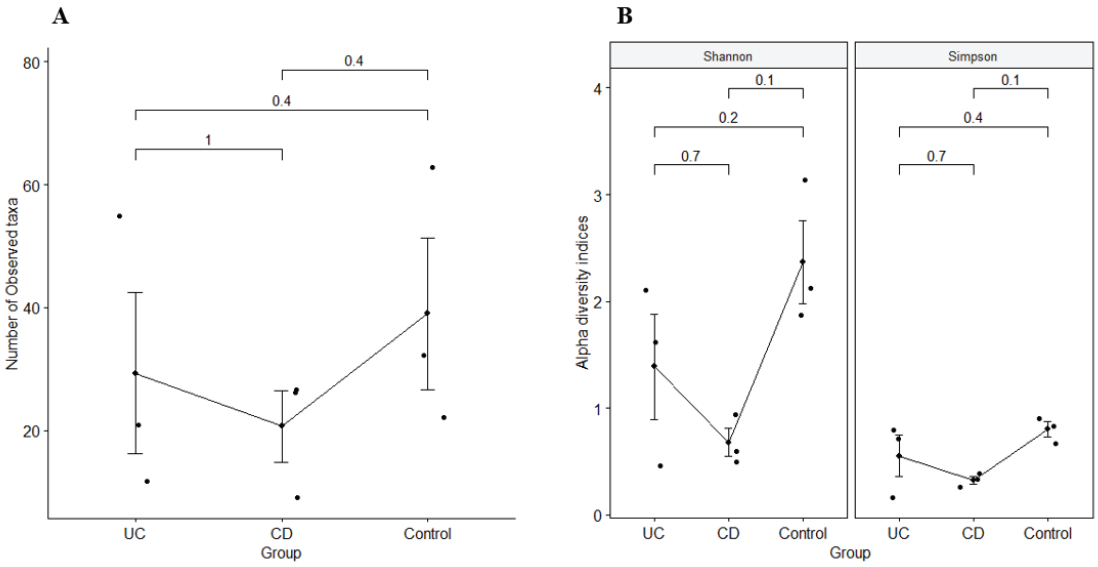

Figure 3. 4. Results of richness (A) and Shannon and Simpson diversity indices (B) between UC, CD and control groups.

Beta diversity is a term utilized to explain differences in microbial diversity among groups. It helps researchers interested in microbiome studies to see whether there are distinction between different groups such as patient and control. There are several ways to evaluate and visualize beta diversity. Between groups, square matrix of distance or dissimilarity can calculate using different approaches such as Bray-Curtis dissimilarity, Jaccard Distance, Unweighted UniFrac and Weighted UniFrac. To visualize the matrix, heat maps, Multi-Dimensional Scaling (MDS) also known as Principal Coordinates Analysis (PCoA), Principal Component Analysis (PCA) or Unweighted Pair group Method with Arithmetic Means (UPGMA) methods are generally preferred.

In this thesis, first of all, heatmaps were generated to visualized microbiota profiles at phylum (Figure 3. 5) and family (Appendix B) levels of each individuals belonging to IBD patients and control groups. While control groups differed from IBD patients in terms of microbiota profile, one Crohn's patient sample had a similar profile to controls (Figure 3. 5).

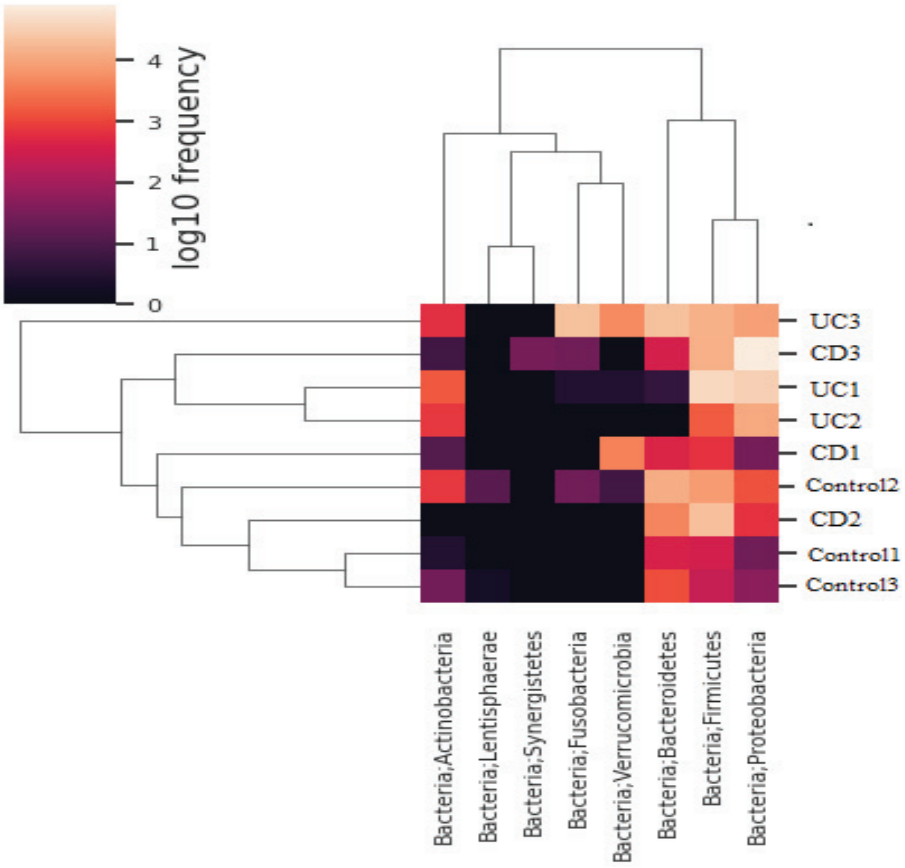

Figure 3. 5. Heatmap of phylum levels between UC, CD and control samples.

After the heatmaps, MDS analysis was performed with three dimensions and visualized by "ggplot2" package in R. Both the abundance and the phylogenetic relationship of bacterial taxa were used to calculate a special distance matrix called Weighted UniFrac distance matrix. Once the distance matrix between the samples was computed, this distance matrix was visualized using MDS analysis. In the MDS plot, samples closer to each other show more similar bacterial diversity. As shown in Figure 3. 6, it was demonstrated the two-dimensional distance among groups by MDS plot. The first dimension of the MDS explained the OTU diversity with a percentage of 55% among the samples. Moreover, this axis was able to separate control samples from IBD patient groups, but one CD patient was seen as close the control groups. The second dimension in MDS separated the CD and UC patients from each other mostly.

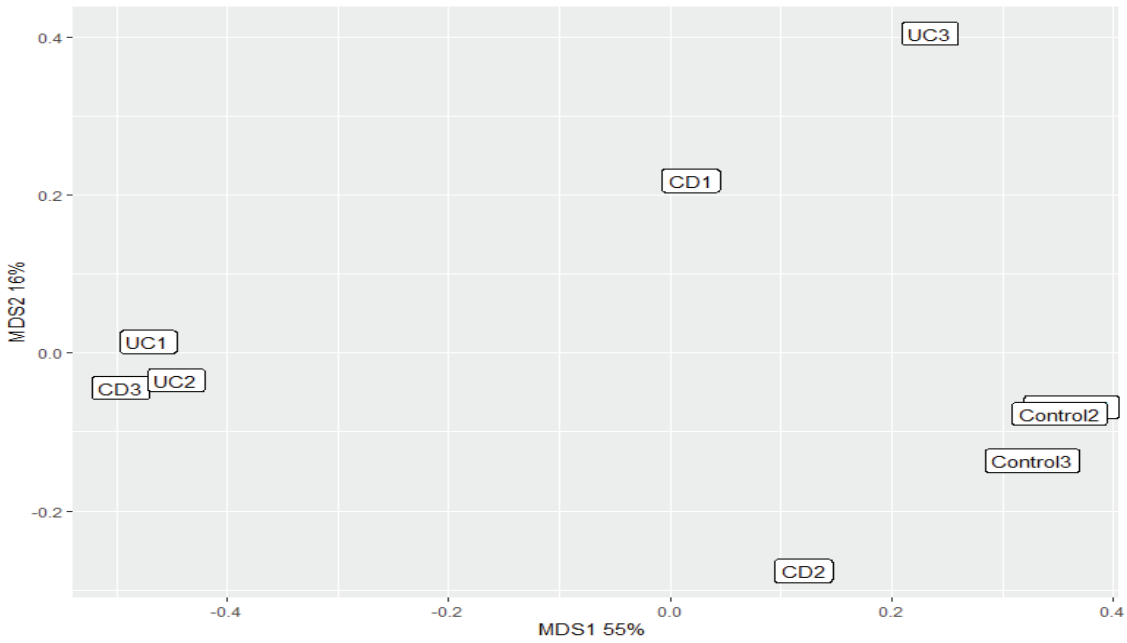

Figure 3. 6. Multi-Dimensional Scaling (MDS) plot between UC, CD and control groups.

3.2. Comparison of abundance of selected bacteria between IBD and control groups

After NGS analysis, 7 different bacterial species (*F. prausnitzii, E.coli, A. muciniphila, B. dorei, B. vulgatus, B. uniformis,* and *S. thermophilus*) that showed the most change between UC, CD, and control groups were selected and the changes of these bacteria abundance between both patient-control and 3 different groups were examined.

One of the most abundant beneficial bacteria in the intestine *F. prausnitzii* was compared between IBD and control groups. *F. prausnitzii* abundance was significantly decreased in IBD patients compared to the control group (Wilcoxon rank sum test, p-value = 0.022) (Figure 3. 7 (A)). Kruskal Wallis rank-sum test (p-value = 0.052) as shown a marginal significance among 3 groups for *F. prausnitzii* abundance (Figure 3. 7 (B)). On the other hand, *E. coli* abundance was higher in the IBD patients group versus the control group (Wilcoxon rank-sum, p-value =0.048). As shown in Figure 3. 7 (B), the IBD group was partition into CD and UC and a three-group comparison was performed.

Although a similar trend was observed, the results were not as statistically significant (Kruskal Wallis rank-sum test, p-value = 0.11). For *A. muciniphila* bacterium, significant results in both the paired group comparison (Wilcoxon rank-sum, p-value =0.67) and the triple comparison (Kruskal Wallis rank-sum test, p-value = 0.81) were not obtained.

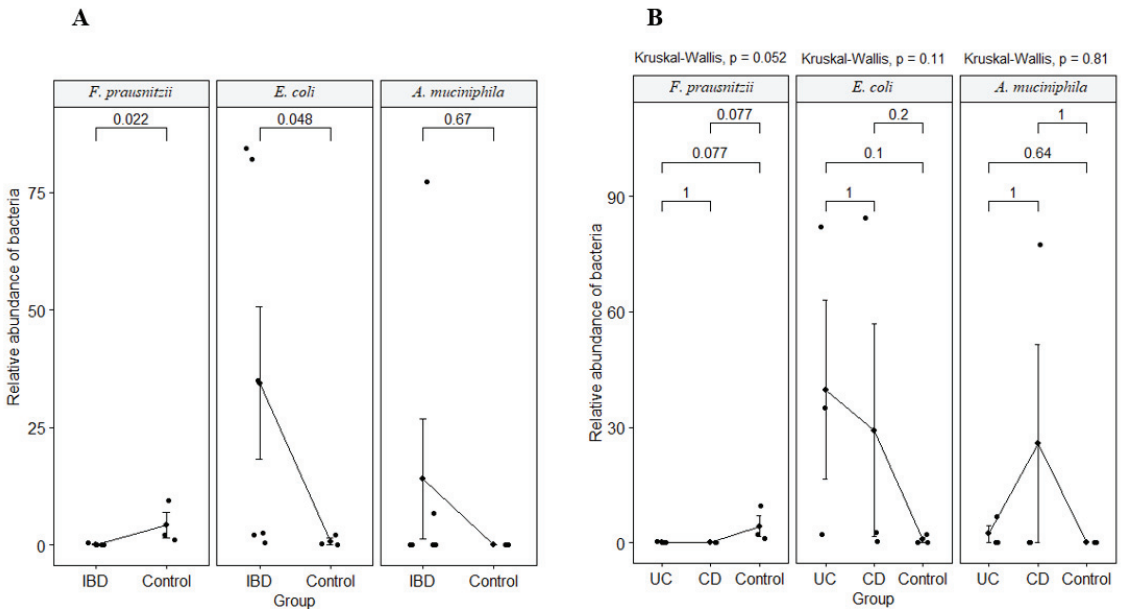

Figure 3. 7. Relative abundance of *F. prausnitzii*, *E.coli* and *A. muciniphila* between IBD-control (A) and UC-CD-control groups (B).

Comparisons were continued with three bacteria belonging to Bacteroides phylum (Figure 3. 8). With the Wilcoxon rank-sum tests, *Bacteroides dorei (B. dorei)* was significantly decreased in IBD control versus control group alike *Bacteroides uniformis (B. uniformis)* (*B. dorei*, p-value = 0.016; *B. uniformis*, p-value = 0.022). In the triple comparison performed by Kruskal Wallis rank-sum test (Figure 3. 8 (B)), *B. dorei* was significantly (p-value = 0.035) but *B. uniformis* was marginal significantly (p-value = 0.052) differed among 3 groups. When IBD patients were divided into UC and CD, the results became less significant as the number of samples within the group decreased.

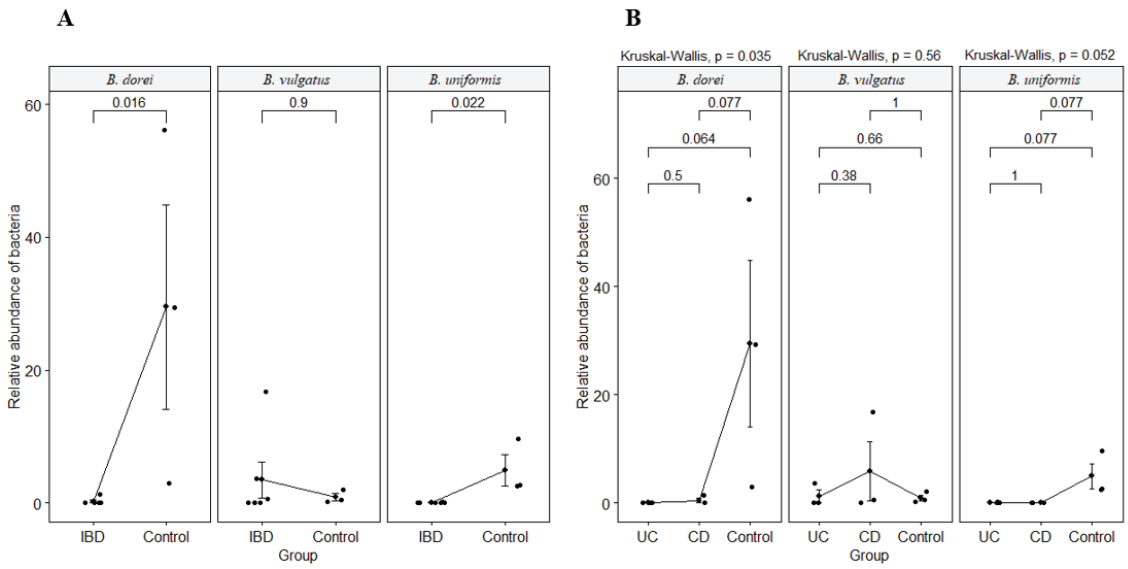

Figure 3. 8. Relative abundance of *B. dorei*, *B. vulgatus* and *B. uniformis* between IBD-control (A) and UC-CD-control groups (B).

Finally, the abundance of *Staphylococcus thermophilus* (*S. thermophilus*) beneficial bacteria, also used as yogurt starter culture, was compared between IBD-control groups and IBD subtypes and control group (Figure 3. 9). This bacteria was reduced in IBD patient group against the control group but not significantly (Wilcoxon rank-sum, p-value = 0.3). Similarly, no significant difference was found in the comparison between the three groups for *S. thermophilus* (Kruskal Wallis rank-sum test, p-value = 0.49).

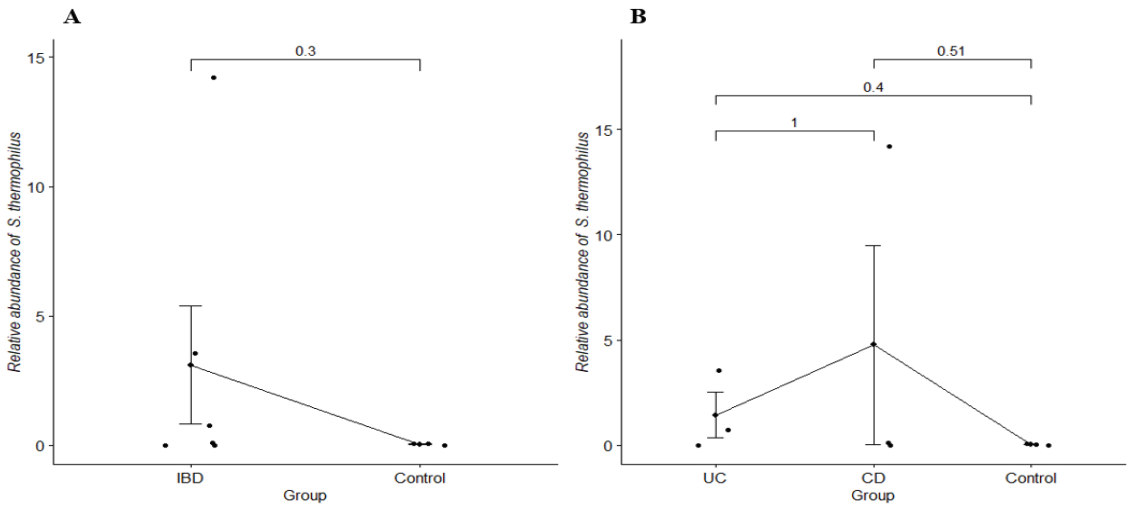

Figure 3. 9. Relative abundance of *S. thermophilus* between IBD-control (A) and UC-CD-control groups (B).

3.3. Verification of Selected Bacteria through Quantitative Real-Time PCR (qPCR)

To verify the amplicon sequencing results, two bacteria were selected according to the NGS data and literature, then were analyzed with qPCR analysis. The amplification plot and melting curve obtained for each primer pair using Absolute Quantitation Second Derivative and Tm calling analysis modes in the LightCycler® 480 II software were shown in Figure 3. 10 and Figure 3. 11, respectively.

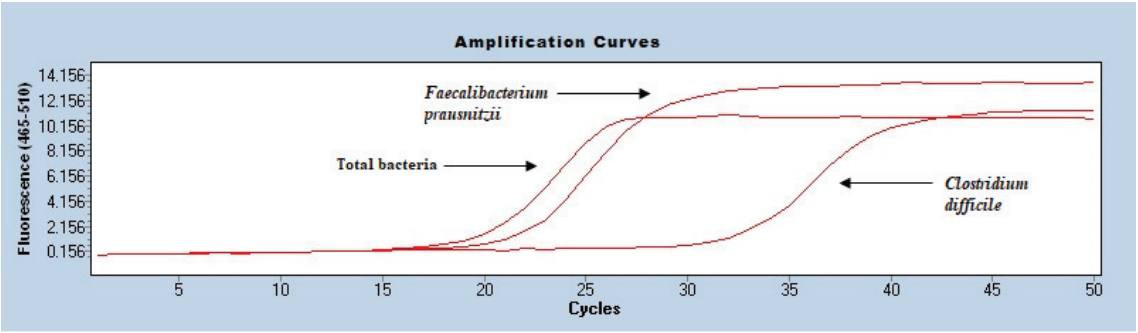

Figure 3. 10. Amplicon curves for each primer set.

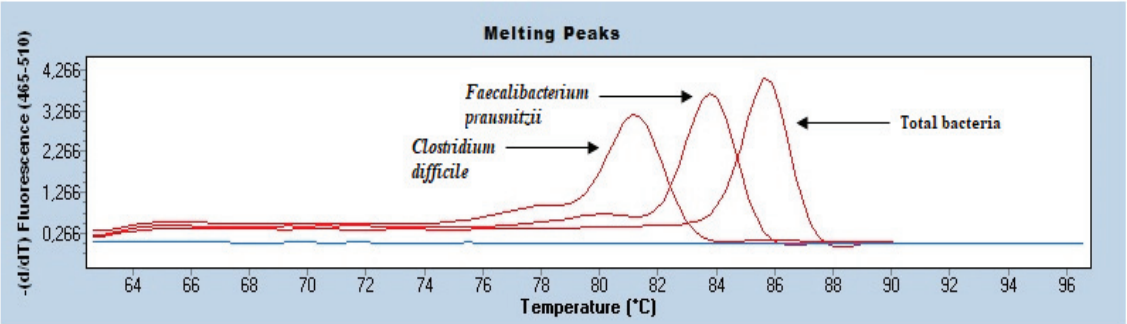

Figure 3. 11. Melting picks of each primer set.

Microbiota profiles of CD, UC and control groups in terms of beneficial bacteria *F. prausnitzii*, pathogenic bacteria *C. difficile* and total bacteria were examined by qPCR analysis. Total bacterial Ct values are expected to have the lowest value, since the amount of total bacterial DNA in the samples will be much more than the targeted 2 other bacterial DNA. The total bacterial Ct values indicated in the amplification curve in Figure 3. 10, confirmed this hypothesis. Total bacteria and *F. prausnitzii* were detected in all samples. However, *C. difficile* was detected in only one patient with Crohn's disease with Ct value of 32.7. Ct values obtained for *F. prausnitzii* were shown among IBD patients and control groups and also individually in 3 groups in Figure 3. 12. As mentioned in chapter 2, lower Ct values represent the high abundance of the target. Therefore *F. prausnitzii* was mostly observed in the control group with $p > 0.05$ by Wilcoxon rank-sum test (Figure 3. 12 (A)). When the 3 groups were evaluated separately as shown in Figure B, the reason for the increase in Ct of beneficial bacteria in the IBD group was seen to be UC patients. However, there was no significant difference between the 3 groups (Kruskal-Wallis, p-value = 0.44) (Figure 3. 12 (B)).

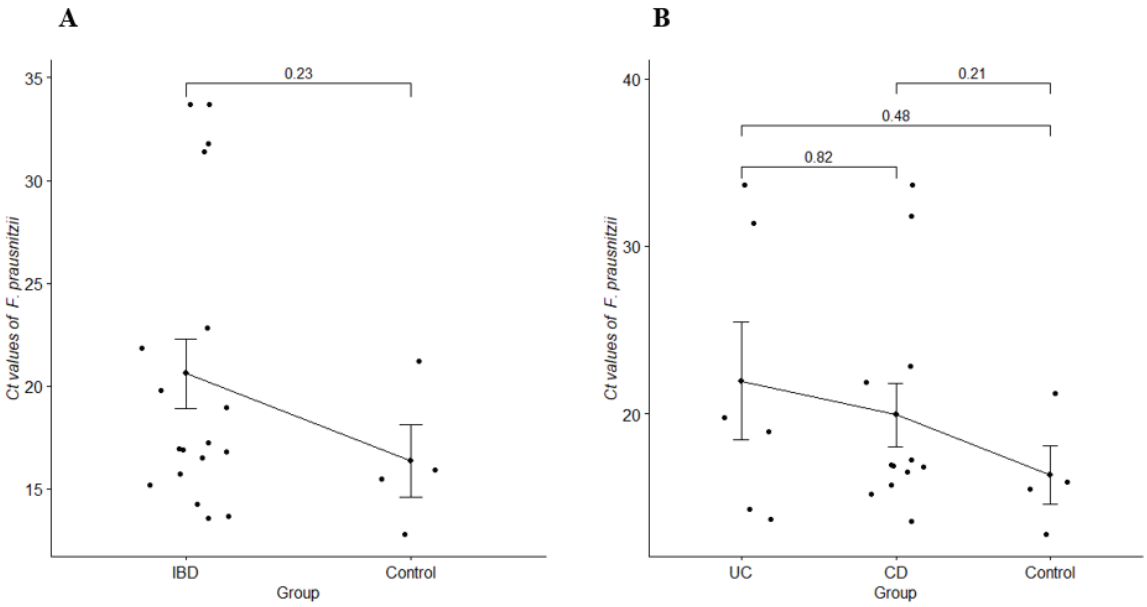

Figure 3. 12. Ct distribution of *F. prausnitzii* between IBD-control (A) and UC-CD-control groups (B).

Although the Ct values are related to the amount of target DNA, further analysis is required to show the relative abundance (fold changes) of *F. prausnitzii* drop between the patient groups. Analysis of qPCR data was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). While analysis, *F. prausnitzii* were considered as a target while total bacteria were as a reference. Then, the $\log_2 2^{-\Delta Ct}$ formula was used to indicate the correlation of target microorganisms among the UC and CD groups (Feng et al., 2010). Firstly, the percentage of *F. prausnitzii* decrease in the samples performed amplicon sequencing and the fold decrease values obtained after qPCR analysis of these samples were compared (Table 3. 1). After amplicon sequencing, *F. prausnitzii* percent of CD, UC and control groups were found to be 0.04, 0.14 and 4.22, respectively. The percentage of beneficial bacteria decreased in both disease groups compared to the control group, but it was observed that the decrease in the CD group was greater. To verification these results, qPCR analysis was performed and beneficial bacteria was seen decreased in NGS samples (UC = 3, CD = 3) compared to control (n = 3). While the decrease was mostly in CD patients with a 15.55 fold change, a 13.88 fold decrease was observed in UC patients.

Table 3. 1. Comparison of NGS and qPCR data in terms of *F. prausnitzii* abundancy.

IBD Subtypes	**Amplicon sequencing abundance (%)**	**qPCR log_2 fold change**
Crohn's disease	0.04	15.55
Ulcerative colitis	0.14	13.88

When making the comparison over all samples (UC = 6, CD = 12), again a reduction for *F. prausnitzii* was observed in both IBD groups but the decrease was at distinct rates within the UC and CD groups (Figure 3. 13). As indicated in Figure 3. 13 (A), *F. prausnitzii* significantly reduced (Wilcoxon, p-value = 0.0072) in IBD patients. Both CD (p-value = 0.017) and UC (p-value = 0.011) patients were showed significant reduction compared to controls using the Wilcoxon rank-sum test. In comparison UC, CD, and control groups by the Kruskal-Wallis test, three groups were separated from each other significantly (p-value = 0.013) (Figure 3. 13 (B)).

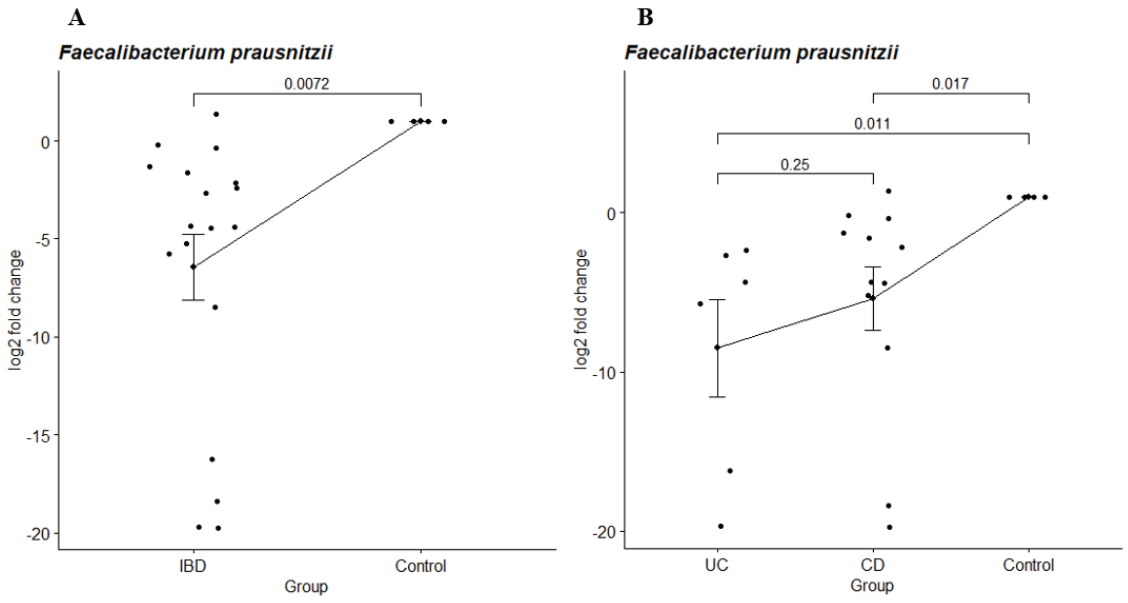

Figure 3. 13. Comparison of relative *F. prausnitzii* abundance between IBD-control (A) and UC-CD-control groups (B).

3.4. Presence of SNPs in *NOD2*, *IL-23R*, *ATG16L1* genes among the IBD

Before sequence analysis, all PCR products were analyzed by agarose gel electrophoresis, and Sanger sequencing analysis was performed from single band samples indicated target base-pair on the gel (Figure 3. 14). After checking the quality of each PCR product on agarose gel, the 652 base-length portion of *ATG16L1*, the 489 base-length portion of *IL-23R*, and the 565, 513, 659 base-length portions of *NOD2* that include rs2066844, rs2066845, rs2066847 variations respectively, were sequenced.

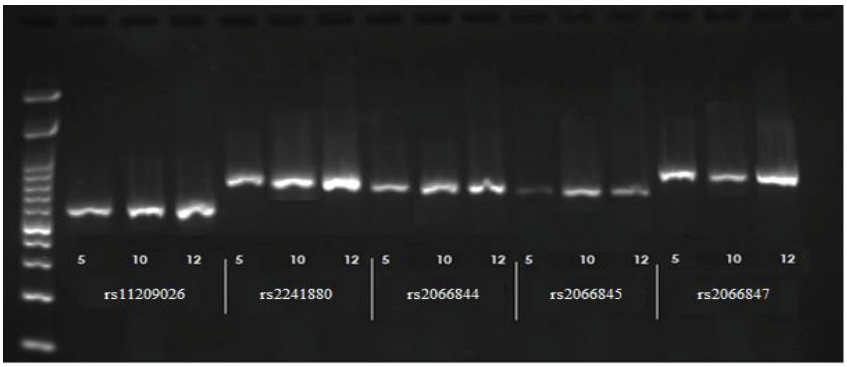

Figure 3. 14. Analysis of PCR products by *IL-23R*, *ATG16L1* and *NOD2* primers. (First column is ladder, following, samples (5, 10, 12) result for each primer (rs11209026, rs2241880, rs2066844, rs2066845, rs2066847)).

To amplified 652 base-length portion of the *ATG16L1* gene, nucleotide sequence of exon 9 and intronic sequence at the beginning and end of the exon 9 were taken from the NCBI (NG_023038.1) and ENSEMBL (ENSG00000085978) databases. By designing primers, PCR and Sanger sequencing analyses were performed. FASTA and AB1 data obtained after sequencing were visualized by BioEdit and UGENE programs. As display in Figure 3. 15, multiple sequence alignment was performed using the ClustalW algorithm, and nucleotide sequences were determined for the rs2241880 variant of each sample.

ATG16L1 Ref. NCBI: https://www.ncbi.nlm.nih.gov/gene/55054

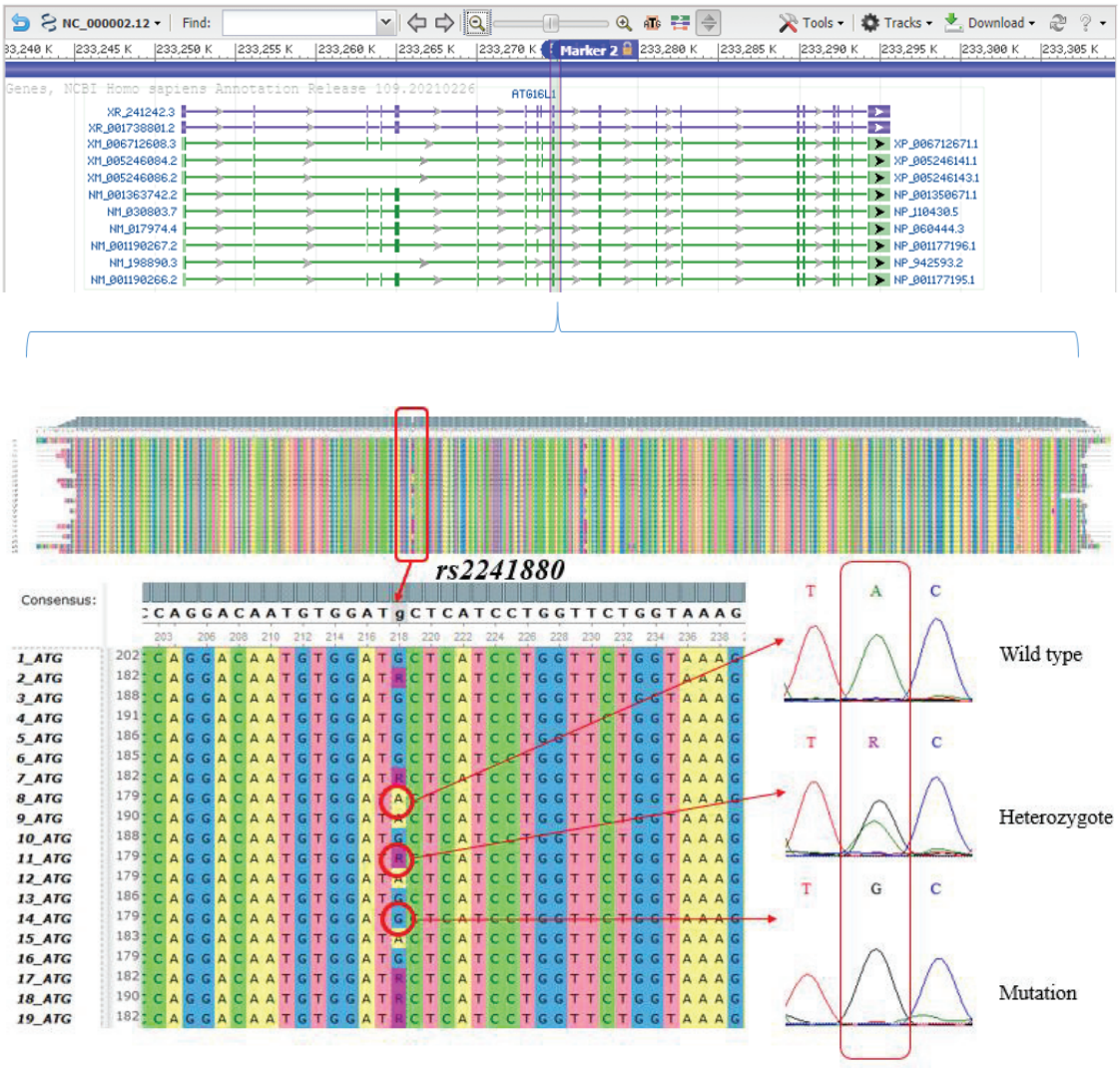

Figure 3. 15. Sequencing and multiple alignment of the *ATG16L1* gene and chromatogram images of rs2241880 variant.

According to reference sequences downloaded from NCBI (NG_011498.1) and ENSEMBL (ENSG00000162594) databases, the 489 base-length portion of *IL-23R* were amplified with primers then be sequenced. The rs11209026 obtained after multiple alignment of the sequence data with the ClustalW algorithm shown in Figure 3. 16. In fasta data, although the position of rs11209026 was given as N in two patients, by visualization of chromatograms, N base confirmed and accepted heterozygote mutation. The rs11209026 variant was detected in only two Crohn's patients. Additionally, an

intronic variant of rs11465816 in heterozygous state was seen in one of the Crohn's patients carrying the rs11209026.

Figure 3. 16. Sequencing and multiple alignment of the *IL-23R* gene and chromatogram images of rs11209026 variant.

Analysis of rs2066844, rs2066845, rs2066847 SNPs located in the NOD2 gene was performed on 3 separate primer pairs covering exon 4, 8, and 11 respectively. The reference sequence for *NOD2* was obtained from the NCBI (NG_007508.1) and ENSEMBL (ENSG00000167207) databases. Primers were designed for amplifying 565, 513, and 659 base pairs. Alignment results obtained after sequencing are shown in detail in Figures 3. 17, 3. 18, and 3. 19.

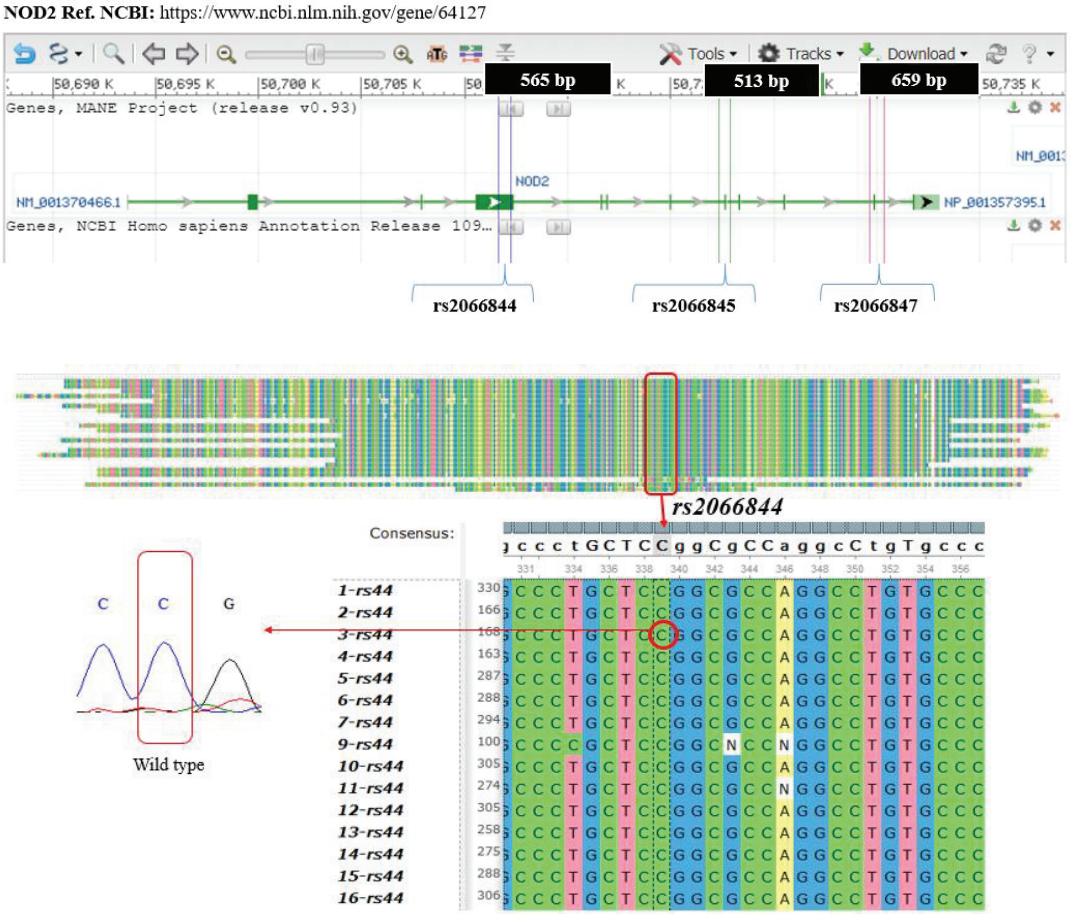

Figure 3. 17. Sequencing and multiple alignment of the *NOD2* gene and chromatogram images of rs2066844 variant.

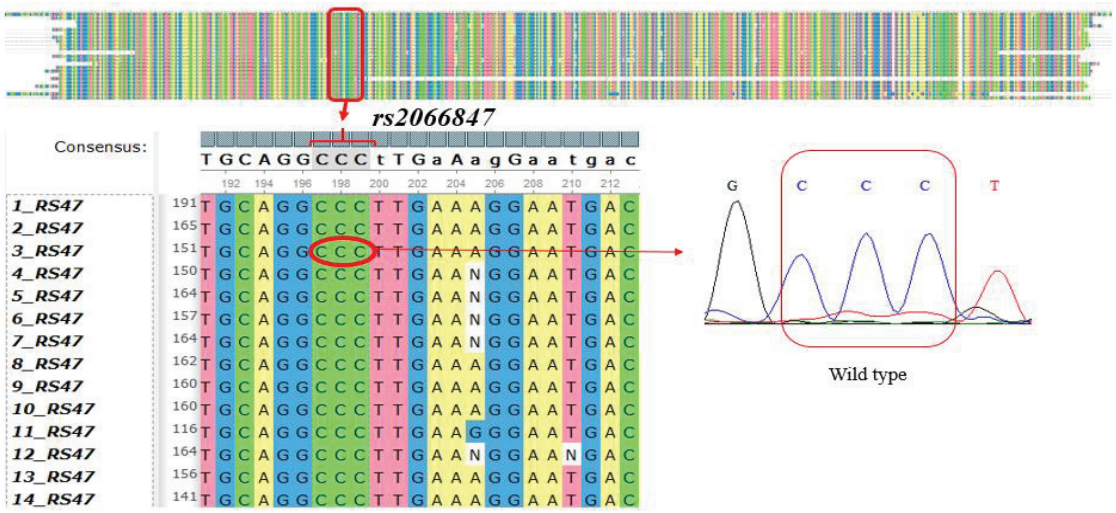

Figure 3. 18. Sequencing and multiple alignment of the *NOD2* gene and chromatogram images of rs2066847 variant.

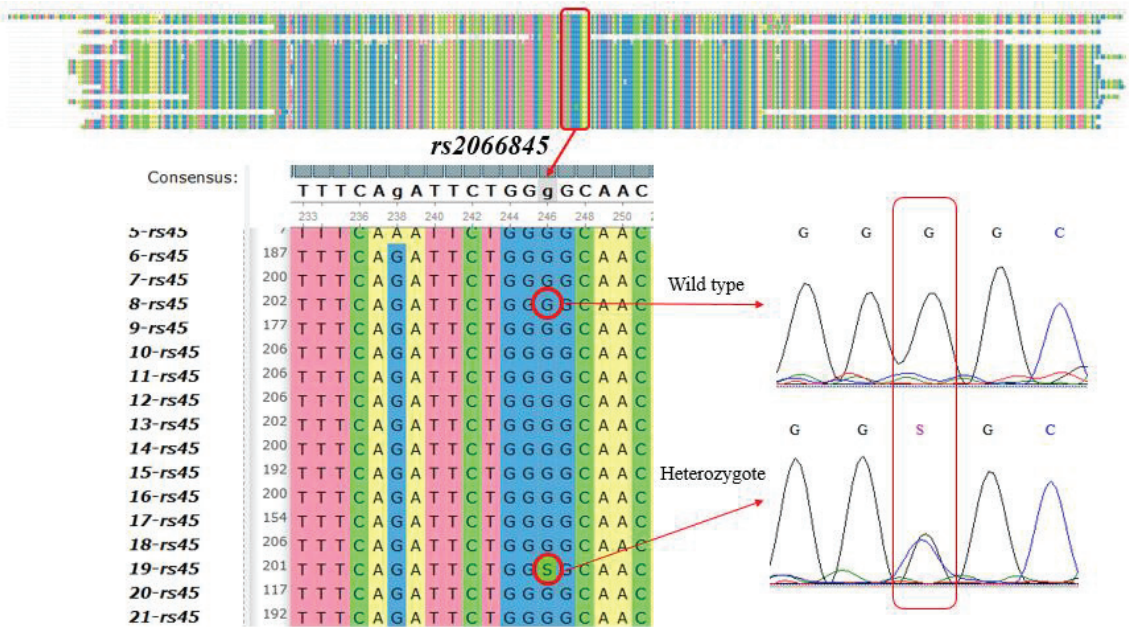

Figure 3. 19. Sequencing and multiple alignment of the *NOD2* gene and chromatogram images of rs2066845 variant.

As shown in Table 3. 2, no homozygous mutations were observed in the control and IBD patient groups for neither the rs2066844 nor the rs2066847 variants of *NOD2* gene. Unlike these two variants, the rs2066845 variant was observed in just a control sample.

Table 3. 2. Genotypic and allelic frequencies of *NOD2* variations.

Name of SNP		**Genotypic frequencies %**			**Allelic frequencies %**	
rs2066844		CC Wild type	CT Heterozygote	TT Mutation	Normal (C)	Mutant (T)
	Crohn's Disease (n=12)	12 (100)	0	0	100	0
	Ulcerative colitis (n=7)	7 (100)	0	0	100	0
	Controls (n=4)	4 (100)	0	0	100	0

(cont. on next page)

Table 3. 3. (cont.).

		GG Wild type	GC Heterozygote	CC Mutation		
rs2066845	Crohn's Disease (n=12)	12 (100)	0	0	100	0
	Ulcerative colitis (n=7)	7 (100)	0	0	100	0
	Controls (n=4)	4 (75)	1 (25)	0	87.5	12.5
		Wild type	Heterozygote	Mutation		
rs2066847	Crohn's Disease (n=12)	12 (100)	0	0	100	0
	Ulcerative colitis (n=7)	7 (100)	0	0	100	0
	Controls (n=4)	4 (100)	0	0	100	0

The heterozygous mutant genotype (GA) for *IL-23R* gene was obtained in two CD patients. Similar to the *NOD2* gene, no homozygous mutant genotype was observed in all three groups for rs11209026 variant (Table 3. 3).

Table 3. 4. Genotypic and allelic frequencies of *IL-23R* variation.

SNP		**Genotypic frequencies %**			**Allelic frequencies %**	
rs11209026		GG Wild type	GA Heterozygote	AA Mutation	Normal (G)	Mutant (A)
	Crohn's Disease (n=12)	10 (83.3)	2 (16.7)	0	91.6	8.4
	Ulcerative colitis (n=7)	7 (100)	0	0	100	0
	Controls (n=4)	4 (100)	0	0	100	0

In *ATG16L1* gene, opposite the candidate two genes (*NOD2* and *IL-23R*), homozygous mutation (GG) was seen in six CD, and 3 UC patients. Also, one control individual had the mutant genotype. However, allelic frequencies of mutant (G) allel in both IBD patient groups was higher than control group (Table 3. 4).

Table 3. 5. Genotypic and allelic frequencies of *ATG16L1* variation.

SNP		**Genotypic frequencies %**			**Allelic frequencies %**	
		AA Wild type	AG Heterozygote	GG Mutation	Normal (A)	Mutant (G)
rs2241880	Crohn's Disease (n=12)	3 (25)	3 (25)	6 (50)	37.5	62.5
	Ulcerative colitis (n=7)	1 (14.2)	3 (42.9)	3 (42.9)	35.6	64.4
	Controls (n=4)	1 (25)	2 (50)	1 (25)	50	50

3.5. Association Between Genetic and Microbiota

Since there was no significant change in *NOD2* variants among the three genes sequenced, genotype association analyzes were continued with *ATG16L1* and *IL-23R* gene variants. The effect of candidate genes (*ATG16L1* and *IL-23R*) associations over 8 different phyla (Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, Verrucomicrobia, Synergistetes, Lentisphaerae), alpha diversity indices (observed taxa, Shannon and Simpson indexes), qPCR results (Ct values and $\log_2$ fold change of *F. prausnitzii*) and abundances of selected bacteria (*E.coli, F. prausnitzii, A. muciniphila, B. dorei, B. vulgatus, B. uniformis, S. thermophilus*) have been tested. After examination effects of genotypes on all the aforementioned factors, only for the *IL-23R* (rs11209026) genotype, there was a significant relationship with $\log_2$ fold change (Figure 3. 20 (A)) and Ct values (Figure 3. 20 (B)) for *F. prausnitzii* with 0.034 and 0.017 p-values respectively. As shown in Figure 3. 20, the heterozygous phenotype of rs11209026 caused a statistically significant reduction of the *F. prausnitzii* beneficial bacteria.

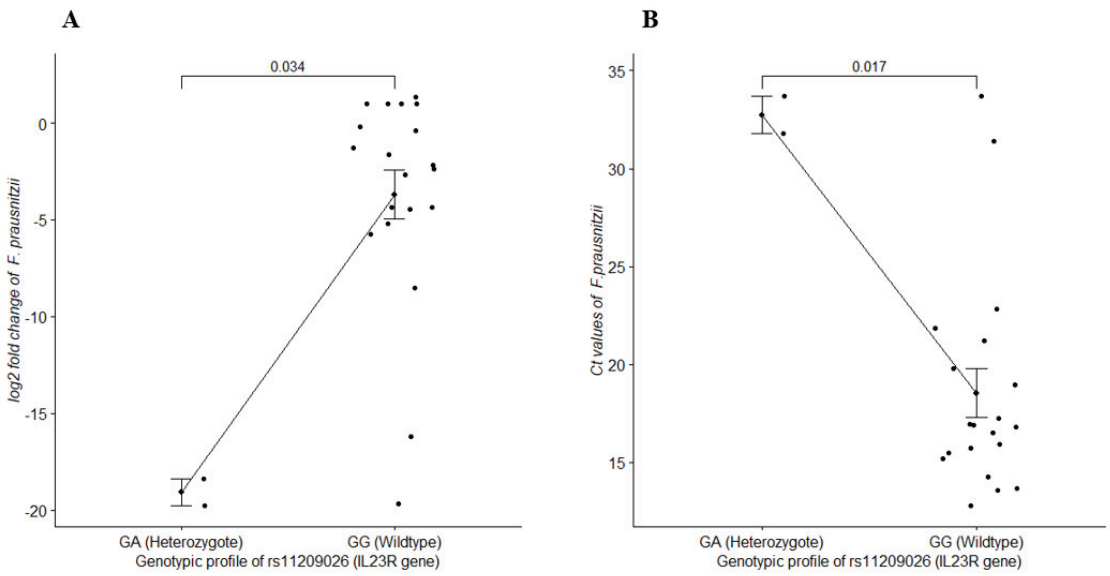

Figure 3. 20. Relationship of rs11209026 variant genotype with log_2 fold change (A) and Ct values (B) of *F. prausnitzii*.

3.6. Discussion

Changes in the intestinal flora are often clinically silent but are associated with many diseases. The main one is Inflammatory Bowel Disease. Although bacterial, fungal, and viral dysbiosis have been identified in IBD patients, it is not yet known whether dysbiosis is the cause or the result of the disease. Microbiota studies indicate that there is an imbalance between "anti-inflammatory" and "pro-inflammatory" bacteria in IBD patients. In many studies using IBD samples, decrease in bacterial diversity therein Firmicutes and Bacteroidetes pyhla; on the contrary, an increase was observed in the Proteobacteria phylum especially Enterobacteriaceae family bacteria (Chassaing and Darfeuille–Michaud, 2011; Manichanh et al., 2006; Rehman et al., 2010). Along with dysbiosis, the overall bacterial diversity and richness were reduced. In this thesis, using fecal samples, it was aimed to revealed bacterial diversity of both Turkish CD and UC patients by 16S rRNA amplicon analysis.

With respect to the number of OTUs and alpha-diversity findings, bacterial richness and diversity reduced in Turkish CD and UC patients ($p > 0.05$). Different studies showing that microbial diversity was decreasing also supported our findings (Danilova et al., 2019; Ott et al., 2004; Vester-Andersen et al., 2019). After amplicon sequencing, the top 8 phyla were examined among the IBD patients and control groups but for increase or decrease statistically significant changes were not obtained except Actinobacteria. UC and CD disease groups were separated from each other in the Actinobacteria phylum with Kruskal Wallis rank-sum test, p-value of 0.04. Additionally, even if no significant differences (p-value > 0.05) among IBD patients and control groups were found in the Simpson index, the Shannon index separated these two groups significantly (p-value = 0.048).

The phylum of Proteobacteria (Gram-negative) was showed the highest increase in both UC and CD disease groups versus healthy control subjects. Also, the enhancement was greater in the UC patients group. Proteobacteria are one of the disease-related phylum that contain many pathogens such as *Escherichia, Shigella, Salmonella, Helicobacter*. For this reason, similarly our results, it has been observed that their abundance has increased in IBD patients according to many studies (Rizzatti et al., 2017; Vester-Andersen et al., 2019). Most of the Proteobacteria identified by the amplicon sequencing in IBD patients was *Escherichia coli* pro-inflammatory bacteria belonging to the Gammaproteobacteria class (p-value = 0.048). Although Enterobacteriaceae family consist of seven *Escherichia* genus, gastrointestinal disease related specie is only *E. coli* (Mukhopadhya et al., 2012). Due to the invasive and adherent properties of *E. coli*, it invades epithelial cells and by escaping from the host defense, causes dysbiosis, one of the main characteristics of IBD. Similarly, after 16S rRNA analysis performed with IBD tissue samples, Gophna et al. found the most common phylotype in both disease groups was *E. coli* (Gophna et al., 2006). Moreover, many bacterial diversity studies have been supported our findings in terms of the *E. coli* increase in IBD patients (Mirsepasi-Lauridsen et al., 2019; Pilarczyk-Zurek et al., 2016; Sasaki et al., 2007).

Some genera like *Faecalibacterium* can preserve the intestinal mucosa from unsuitable inflammatory responses (Manichanh et al., 2012). For instance, *Faecalibacterium prausnitzii* anti-inflammatory bacteria neutralizes the pro-inflammatory impacts of *E. coli* so that maintains intestine homeostasis. *F. prausnitzii*

(Firmicutes) produces short-chain fatty acids (SCFA) such as butyrate and has been shown to be reduced in IBD patients (Aleksandrova et al., 2017). The metabolic effects of SCFAs are to provide energy supply to the mucosa, increase intestinal cell proliferation, stimulate water and sodium absorption, and increase mucus production (Kurtaran, 2021). In this thesis, *F. prausnitzii* was found significantly decreased in the IBD patients group by both qPCR (p-value = 0.0072) and NGS (p-value = 0.022) results. Even though decreasing relative abundance of *F. prausnitzii* was not significant in both UC and CD groups compared to the control, the $\log_2$ fold change of *F. prausnitzii* was significantly lower in UC versus controls. The decline of this bacteria leads to reduction in the amount of butyrate and thus to inhibits the maturation of the epithelial cells. Since butyrate strengthens the intestinal barrier, its reduction brings chronic inflammation and mucosal damage, which are characteristic of inflammatory bowel disease. Although according to the literature, Firmicutes were expected to decrease both in CD and UC patients, in this study Firmicutes abundance was decreased in UC patients but increased in CD patients against controls. Specifically, two bacteria can be responsible for this contrary observation: *Megamonas funiformis* (*M. funiformis*) and *Streptococcus thermophilus* (*S. thermophilus*). Along with the observation *M. funiformis* in two CD patients, interestingly it was formed one CD patient's bacterial microbiota with a percentage of 80%. Unlike *M. funiformis*, *S. thermophilus* was seen in all samples except one CD and one control individuals. Even though microbial diversity was low in Crohn's disease, it was seen that these results made the CD group rich in Firmicutes. Interestingly, *M. funiformis* was detected only in individuals heterozygous for the rs11209026 (IL-23R gene) variant. However, further analysis with more samples is required to understand whether this bacterium has an association with the IL-23R gene protective variant. Interestingly, *M. funiformis* was one of the five previously unreported bacterial species that was suggested to have a role in the etiology of IBD especially Crohn's disease (Wingfield et al., 2018). Additionally, in 2021, Zhang et al. observed that *M. funiformis* was increased in obese individuals suggesting a possible role in triggering obesity by inducing the host for inflammatory response and insulin resistance (Zhang et al., 2021).

Yogurt is known as traditional Turkish food and one of the most consumed dairy products in Turkey. *S. thermophilus* is an anti-inflammatory lactic acid bacterium used as a starter culture for the production of yogurt (Linares et al., 2016). Consumption of yogurt has beneficial effects on people. *S. thermophilus* has anti-oxidative and anti-

inflammatory activity (Ito et al., 2008; Ogita et al., 2011). Due to its ability to use lactose, it helps reduce gastrointestinal symptoms such as bloating, stomach pain, and vomiting. *S. thermophilus* has been shown to significantly reduce weight loss and the blood in feces in the IBD mouse model (Bailey et al., 2017). It is also effective in the treatment of acute diarrhea caused by pathogenic bacteria (Kluijfhout et al., 2020). Also used as a probiotic supplement, this bacteria preserves mucosal barrier unity and reduces inflammation by preventing tight cell junction destruction caused *E. coli*. As a result, we assume that the high presence of *S. thermophilus* ($p > 0.05$) may have a higher consumption of yogurt in IBD patients as part of Turkish culture.

In *16S rRNA* amplicon analysis, we observed that Verrucomicrobia were represented by only *Akkermansia muciniphila* (*A. muciniphila*). *A. muciniphila* is a mucin-degrading gram-negative anaerobe and anti-inflammatory bacterium. The mucus layer increases the protective properties of the intestinal lumen. Mucin, which is the main component of mucus, consists of amino acids and oligosaccharides, thus it is used as a food source by intestinal bacteria. *A. muciniphila*, which has the enzyme necessary for the breakdown of oligosaccharide chains of mucins, degrades mucin (Kostopoulos et al., 2020). It also contributes to immune modulation by supporting the production of mucosa from goblet cells. The positive modulation of the intestinal barrier integrity by *A. muciniphila* is thought to confer probiotic character to this bacteria. Studies have shown that the increase in the number of *A. muciniphila* indicates positive correlation with human health (Ottman et al., 2017; Ouyang et al., 2020). Therefore, it has been observed to decrease in IBD patients (Lopez-Siles et al., 2018; Png et al., 2010; Presti et al., 2019). Unlike to the literature, we observed a high abundance ($p > 0.05$) of *A. muciniphila* in 2 IBD patients (in one CD patient and one UC patient). 7% and 77% of bacterial diversity in UC and CD patients respectively were constituted by this bacteria. On the other hand, in a study to determine the main markers of dysbiosis in IBD, *A. muciniphila* was significantly increased in CD patients compared to the control group (Danilova et al., 2019). Also, a larger amount of *A. muciniphila* was indicated in one CD patient in another research (Wills et al., 2014). Probiotic, prebiotic supplements, FMT application are known to cause an increase in *A. muciniphila*. Probiotics arise antimicrobial factors and strengthen the barrier functions of the mucosa. As well FMT treatment is effective in correcting intestinal microbiota. That's why, we hypothesis that patients with a high abundance of *A. muciniphila* may have received FMT treatment. Since metformin used

in the treatment of type 2 diabetes causes an increase in *A. muciniphila* (Ouyang et al., 2020); usage of this drug by patients may also be another reason of increment.

Bacteroides (Gram-negative) is one of the phyla associated with IBD. Particularly in the active phase of IBD, the level of Bacteroides was reduced in both CD and UC patients (Takahashi et al., 2016; Zhou and Zhi, 2016). Likewise but not significantly (p-value = 0.43), Bacteroides were reduced in UC and CD patients versus control groups in this thesis. However, in some studies, Bacteroides were found to increase because of their ability to secretion of proinflammatory cytokines like *IL-23*. Prevotella genus (anaerobic Gram-negative) is member of the Bacteroidetes phylum and its higher abundance is associated with IBD. Prevotella causes mucosal inflammation through the production of Th17 cytokines by antigen-presenting cells, including *IL-23* (Larsen, 2017). In this thesis, it was observed that Prevotella increased in one UC and one CH patient compared to controls ($p > 0.05$) with an agreement previous studies (Kabeerdoss et al., 2015; Kleessen et al., 2002; Lucke et al., 2006). The altered levels in some species belong Bacteroides have been reported. For example, *Bacteroides vulgatus* (*B. vulgatus*) is one of the pro-inflammatory bacterium with the highest concentration in the intestinal flora of IBD patients (Özden, 2006; Zafar and Saier Jr, 2021). According to *16S rRNA* amplicon sequencing results, *B. vulgatus* abundance was increased in IBD patients against the control group (p-value = 0.9). Also, in animal models *B. vulgatus* has been shown to induce severe Ulcerative disease (Bloom et al., 2011). In another animal model study, it was indicated preventing *E. coli* induced colitis by *B. vulgatus* (Waidmann et al., 2003). *Bacteroides uniformis (B. uniformis)* is another butyrate-producing bacterium and has been demonstrated a significantly decreasing level of this bacteria in CD patients (Takahashi et al., 2016). Our results were shown significantly (p-value = 0.022) decrease of *B. uniformis* in IBD patients versus the control group. Even if no difference was found between UC and CD, a marginally significant (p-value = 0.052) difference was obtained between the 3 groups with the Kruskal-Wallis test. Moreover, the decrease in *B. uniformis* and increase of *B. vulgatus* has also been shown with studies of CD twins (Dicksved et al., 2008).

After *16S rRNA* amplicon sequence analysis, validation was performed on two selected bacteria by Real-Time PCR analysis. qPCR analysis was done with bacteria specific primers for one beneficial (*F. prausnitzii*) and one harmful (*C. difficile*) species.

C. difficile was detected in only one Crohn's patient, while *F. prausnitzii* was seen in all samples. When *F. prausnitzii* Ct values were tested both between IBD-control groups and between 3 groups (UC-CD-control), no significant difference was found. The Ct values obtained for *F. prausnitzii* were normalized with the total bacteria Ct values in each sample and $\log_2$ fold change values were calculated. Fold change values indicate how much beneficial bacteria increased or decreased in the patient samples compared to the control group accepted as 1. *F. prausnitzii* showed increase ($\log_2$ fold change = 1.38) in only one CD patient compared to the controls, while reduction was observed in all the remaining patient samples. After that, in paired comparisons made with Wilcoxon rank-sum test, statistically significant difference was obtained between IBD-control groups (p-value = 0.0072) and UC-control groups (p-value = 0.011). In the triple comparison with the Kruskal-Wallis test; UC, CD and control groups were found to be significantly diverse from each other (p-value = 0.013).

In the genetic studies part of thesis, the most effective mutations in the pathogenesis of IBD in 3 different genes representing innate immunity, adaptive immunity and autophagy pathways were examined in Turkish IBD patients. Regions containing target mutations in all 3 genes were amplified in PCR analysis with specific primers and sequenced by Sanger sequencing analysis. In agreement with previous studies, based on the analyses of mutations we selected in 3 candidate genes, there was no significant association was observed between each mutation genotype and IBD subtypes and control group (Mahurkar et al., 2011; Roberts et al., 2007).

3 different SNPs (rs2066844, rs2066845, rs2066847) in the *NOD2* gene representing innate immunity were analyzed. Among the SNPs, all samples for the rs2066844 and rs2066847 variants displayed a wild type profile. However, in the rs2066845 variant, heterozygous genotype was observed in only one control sample. As a result, *NOD2* mutation was not detected in any of the IBD patients. *NOD2*, which is defined as the first susceptibility gene in Crohn's disease, is involved in the identification of intracellular bacteria. Therefore, mutations in the *NOD2* gene disrupt the bacterial recognition function and increase the likelihood of an individual to be Crohn's disease. Buner et al. researched the reason of increased intestinal permeability within the first relatives with CD and they revealed that rs2066847 was associated weakened barrier function as a result of frameshift mutation (Buhner et al., 2006). In a study performed

with Turkish IBD patients, among the investigated rs2066844, rs2066845, rs2066847 mutations, similarly our results, no statistically significant change was found for rs2066847 and rs2066845. However, the rs2066844 mutation was found to be statistically significantly lower in IBD patients compared to controls (Ince et al., 2008). On the other hand, the fact that polymorphisms were not associated with CD or UC as a result of examining 3 mutations (R702W, G908R and 3020insC) in the *NOD2/CARD15* gene in Turkish IBD patients supports our results (Özen et al., 2006). Our findings suggest that examined *NOD2* gene polymorphisms have no effect on the disease phenotype in Turkish IBD patients and are also supported by previous studies conducted with Turkish IBD patients (Ince et al., 2008; Tekin et al., 2009) and in Asian countries (Guo et al., 2004; Inoue et al., 2002).

After bacteria are recognized by *NOD2*, *ATG16L1* mediated autophagy is induced. Therefore, possible mutations in the *ATG16L1* gene, one of the genes responsible for destruction of recognized bacteria, play an active role in IBD pathogenesis. For example, Kuballa and coworkers shown that in epithelial cells with the T300A variant have a low ability to capture *Salmonella* by autophagosomes (Kuballa et al., 2008). In this thesis, rs2241880 in the *ATG16L1* gene representing autophagy pathway was analyzed. This SNP has been known increases the risk of CD by the percentage of 38% (Grigoras et al., 2015). After Sanger sequencing, wild-type (AA), heterozygous mutant (GA) and homozygous mutant (GG) genotypes were observed for all three groups. However, no significant difference was found between the three groups (p-value = 0.66). For control group, though allelic frequencies was calculated equally (50%), the frequency of G allele (mutant) was obtained higher than A allele in both CD and UC patient groups. In a GWAS performed in the Crohn's disease with 19,779 coding SNPs, it has been found rs2241880 statistically related with susceptibility of Crohn's disease. Even though in the study all exons, splice sites and the promoter region have been sequenced for *ATG16L1*, none of variants has been found except rs2241880 that associated with CD (Hampe et al., 2007). Also, a significant relationship was not reported between G risk allele of rs2241880 and CD (Lauriola et al., 2011). In Turkish IBD patients, it was shown that T300A polymorphism increased the development of CD unlike UC and the mutant allele frequency was determined to be 42% in the controls (Kabaçam and Törüner, 2011). The study investigating the effect of T300A polymorphism in Turkish IBD patients is quite limited, but it has been determined that the variant is effective in susceptibility to the

disease in different ethnic groups (Glas et al., 2008; Lakatos et al., 2008; Roberts et al., 2007; Van Limbergen et al., 2008).

The last analyzed polymorphism was rs11209026 in *IL-23R* gene which represents adaptive-immunity. R381Q amino acid change resulted in rs11209026 has a protective effect against IBD development. Also, this protective relationship has been proven by many studies (Duerr et al., 2006; Zhu et al., 2020). In our results, no homozygous mutation (AA) was observed in any of the samples, but heterozygous (GA) genotype was detected in only two CD patients. rs11209026 has been reported reduced the risk of CD by 54% (Grigoras et al., 2015). Similarly, in Turkish IBD patients, it was not found homozygous mutation, but *IL-23R* polymorphism was found to have a protective effect for both UC and CD (Kabaçam, 2011). In this thesis, alike with a study involves Turkish IBD patients, neither genotypic nor phenotypic association between rs11209026 polymorphism and study groups was found (Can et al., 2016). Interestingly, in New Zealand Caucasian IBD patients, the relationship of *IL-23R* with IBD pathogenesis was associated only in the absence of *CARD15* mutations (Roberts et al., 2007). Although previous studies revealed protective effect of *IL-23R* gene polymorphism in CD patients, we could not identify such differentiations in our samples. To summarize, this complex dynamic seen in gene polymorphisms may make further clear with more samples.

IBD is not the result of a single factor, but the complex interaction of many environmental and genetic factors. For this reason, in the last part of thesis, we examined the correlation between the microbiota profiles we revealed in each IBD patient and the mutation profiles of 3 genes. Heterozygous mutation in *IL-23R* was significantly caused increase of Ct values (p-value = 0.017) and decrease $\log_2$ fold change (p-value = 0.034) for *F. prausnitzii* beneficial bacteria. Zakrzewski et al. supports our results by showing that *NOD2* (R702W, G908R, Leu1007fsinsC) and *ATG16L1* (rs2241880) variants have no effect on microbial diversity in IBD patients (Zakrzewski et al., 2019). Even if for *NOD2* and *ATG16L1* mutations there was no association with microbial composition in this study, Knights et al. confirmed the increasing relative abundance of Enterobacteriaceae was caused by the *NOD2* risk allele (Knights et al., 2014). The observation of low abundance of *F. prausnitzii* in two CD patients carrying rs11209026 heterozygous (GA) genotype shows that intestinal microbiota can be influenced by host genetics.

CHAPTER 4

CONCLUSION

The highly heterogeneous nature of IBD makes it difficult to set the diagnosis, treatment, and follow-up criteria in a systematic structure. Genetic is one the important regulator factor of IBD development. Today, studies on how innate or adaptive immunity and autophagy mechanisms work, how they are regulated and controlled by which signal pathways are still continuing. A better understanding of these mechanisms clinically will lead to the invention of new drugs, diagnosis, or treatment tools and to develop new, conscious and molecular-based solutions for important diseases that threaten human health.

When the effective factors in pathogenesis are investigated, it is assumed that the variations in both genetics and phenotypes may be determinative in terms of treatment, and an individualized treatment approach can be implemented by concerning to these features. With this thesis aimed at determining the genetic and microbial factors involved in the pathogenesis of IBD in Turkish society, important pathophysiological pathways related to the development of the disease were tried to be elucidated. The association of *NOD2*, *ATG16L1* and *IL-23R* gene polymorphisms with Turkish IBD patients were investigated, then the correlation of these polymorphisms with microbiota changes specific to IBD patients was presented for the first time.

Same treatment approach to every patient has started to give way to personalized treatment, which is suitable for each patient's genetic characteristics. In this way, the disease can be controlled or prevented with the right treatment at the right time to the right person. Our results showed the presence of molecular biomarkers that can be measured in blood and feces in order to help the gastroenterologists to generation a personal treatment plan of their patients. The microbiota is one of the important determinants of IBD pathogenesis. Thus, identifying specific microorganisms that are effective in disease development, can provide a unique infrastructure for treatment approaches such as FMT, probiotics, and antibiotics. Based on the results, we thinking

that the *F. prausnitzii* beneficial bacterium and the *IL-23R* gene may be potential biomarkers that can be used to determine the personal treatment plan in IBD patients. Also we anticipate that these findings can be transformed into a test kit as a commercial product with verification analysis to be carried out with more samples.

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APPENDIX A

OTU TABLE

Table A. 1. OTU Table of CD, UC and control samples in family level.

#OTU Table (Relative abundance)	CD1	CD2	CD3	UC1	UC2	UC3	Control1	Control2	Control3
Bacteria;Actinobacteria;Acidimicrobiia;Acidimicrobiales;Ilumatobacteraceae	0	0	0	0	0	0	0	0	0
Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Actinomycetaceae	0	0	0	0.11	0	0.05	0	0	0
Bacteria;Actinobacteria;Actinobacteria;Bifidobacteriales;Bifidobacteriaceae	0.15	0	0	1.84	5.59	0.03	0.25	1.98	1.57
Bacteria;Actinobacteria;Actinobacteria;Micrococcales;Micrococcaceae	0	0	0	0.04	0	0	0	0	0
Bacteria;Actinobacteria;Coriobacteriia;Coriobacteriales;Coriobacteriaceae	0.06	0	0	0	0	0.71	0	1.38	0.20
Bacteria;Actinobacteria;Coriobacteriia;Eggerthellales;Eggerthellaceae	0	0	0	0	0	0.02	0	0.05	0
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Bacteroidaceae	8.16	16.95	0.05	0	0	25.59	46.57	36.32	65.25
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Barnesiellaceae	0	0	0	0	0	0	0.50	2.43	0.66
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Odoribacteraceae	0.04	0	0	0	0	0.10	0	3.32	0.59
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae	0	0	0.34	0.01	0	4.65	0	0.45	0
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Rikenellaceae	0.42	0	0	0	0	0.07	5.12	11.90	9.11
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Tannerellaceae	0.08	0	0.01	0	0	0	0	1.80	3.87
Bacteria;Firmicutes;Bacilli;Lactobacillales;Aerococcaceae	0	0	0.04	0	0	0	0	0	0
Bacteria;Firmicutes;Bacilli;Lactobacillales;Carnobacteriaceae	0	0	0.09	0.08	0	0.03	0	0	0
Bacteria;Firmicutes;Bacilli;Lactobacillales;Enterococcaceae	0	0	0	1.39	2.23	0	0	0	0
Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae	0.02	0.05	0.14	0.39	6.60	0.02	15.98	0.16	0.07
Bacteria;Firmicutes;Bacilli;Lactobacillales;Streptococcaceae	0.21	0	14.43	4.29	3.42	0.12	0	0.06	0.07
Bacteria;Firmicutes;Clostridia;Clostridiales;Clostridiaceae	0	0	0	19.59	0	0.08	0	0.23	0
Bacteria;Firmicutes;Clostridia;Clostridiales;Clostridiales incertae sedis	0.02	0	0	0	0	0	0	0	0
Bacteria;Firmicutes;Clostridia;Clostridiales;Eubacteriaceae	0	0	0	0	0	0.67	11.86	2.10	0.72
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae	0.30	0.24	0.03	0	0	5.53	1.25	10.59	0.46
Bacteria;Firmicutes;Clostridia;Clostridiales;Oscillospiraceae	0.85	0	0	0	0	0	0	0.86	0
Bacteria;Firmicutes;Clostridia;Clostridiales;Peptostreptococcaceae	0	0	0.01	0.30	0	2.00	0	0.06	0
Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae	9.96	0	0.12	0	0	1.63	8.99	14.76	11.02
Bacteria;Firmicutes;Erysipelotrichia;Erysipelotrichales;Erysipelotrichaceae	0	0	0	0	0	0.31	0	0.23	0

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Table A.1. (cont.)

#OTU Table	CD1	CD2	CD3	UC1	UC2	UC3	Control1	Control2	Control3
Bacteria;Firmicutes;Negativicutes;Acidaminococcales;Acidaminococcaceae	1.82	0	0	0	0	0	6.37	1.78	3.08
Bacteria;Firmicutes;Negativicutes;Selenomonadales;Selenomonadaceae	0	80.12	0.06	0	0	0	0	0	0
Bacteria;Firmicutes;Negativicutes;Veillonellales;Veillonellaceae	0	0	0.13	29.46	0	7.24	0	3.73	0
Bacteria;Firmicutes;Tissierellia;Tissierellales;Peptoniphilaceae	0	0	0	0	0	2.26	0	0	0
Bacteria;Fusobacteria;Fusobacteriia;Fusobacteriales;Fusobacteriaceae	0	0	0.01	0	0	29.90	0	0.11	0
Bacteria;Fusobacteria;Fusobacteriia;Fusobacteriales;Leptotrichiaceae	0	0	0.02	0	0	0.04	0	0	0
Bacteria;Lentisphaerae;Lentisphaeria;Victivallales;Victivallaceae	0	0	0	0	0	0	0	0.06	0.07
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae	0	0	0	0	0	0.11	0	0	0
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Oxalobacteraceae	0	0	0	0	0	0	0	0.25	0
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Sutterellaceae	0.11	0	0.01	0	0	0.10	1.00	5.04	3.28
Bacteria;Proteobacteria;Betaproteobacteria;Neisseriales;Neisseriaceae	0	0	0	0.28	0	0.17	0	0	0
Bacteria;Proteobacteria;Gammaproteobacteria;Aeromonadales;Aeromonadaceae	0	0	0	0	0	0.25	0	0	0
Bacteria;Proteobacteria;Gammaproteobacteria;Aeromonadales;Succinivibrionaceae	0	0	0	0	0	0	0	0.17	0
Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Enterobacteriaceae	0.42	2.62	84.49	36.59	82.13	2.39	2.12	0.16	0
Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales;Morganellaceae	0	0	0	0	0	0.51	0	0	0
Bacteria;Proteobacteria;Gammaproteobacteria;Pasteurellales;Pasteurellaceae	0	0	0	5.62	0.03	8.70	0	0	0
Bacteria;Synergistetes;Synergistia;Synergistales;Synergistaceae	0	0	0.03	0	0	0	0	0	0
Bacteria;Verrucomicrobia;Verrucomicrobiae;Verrucomicrobiales;Akkermansiaceae	77.38	0	0	0	0	6.74	0	0.03	0

APPENDIX B

HEATMAP ANALYSIS

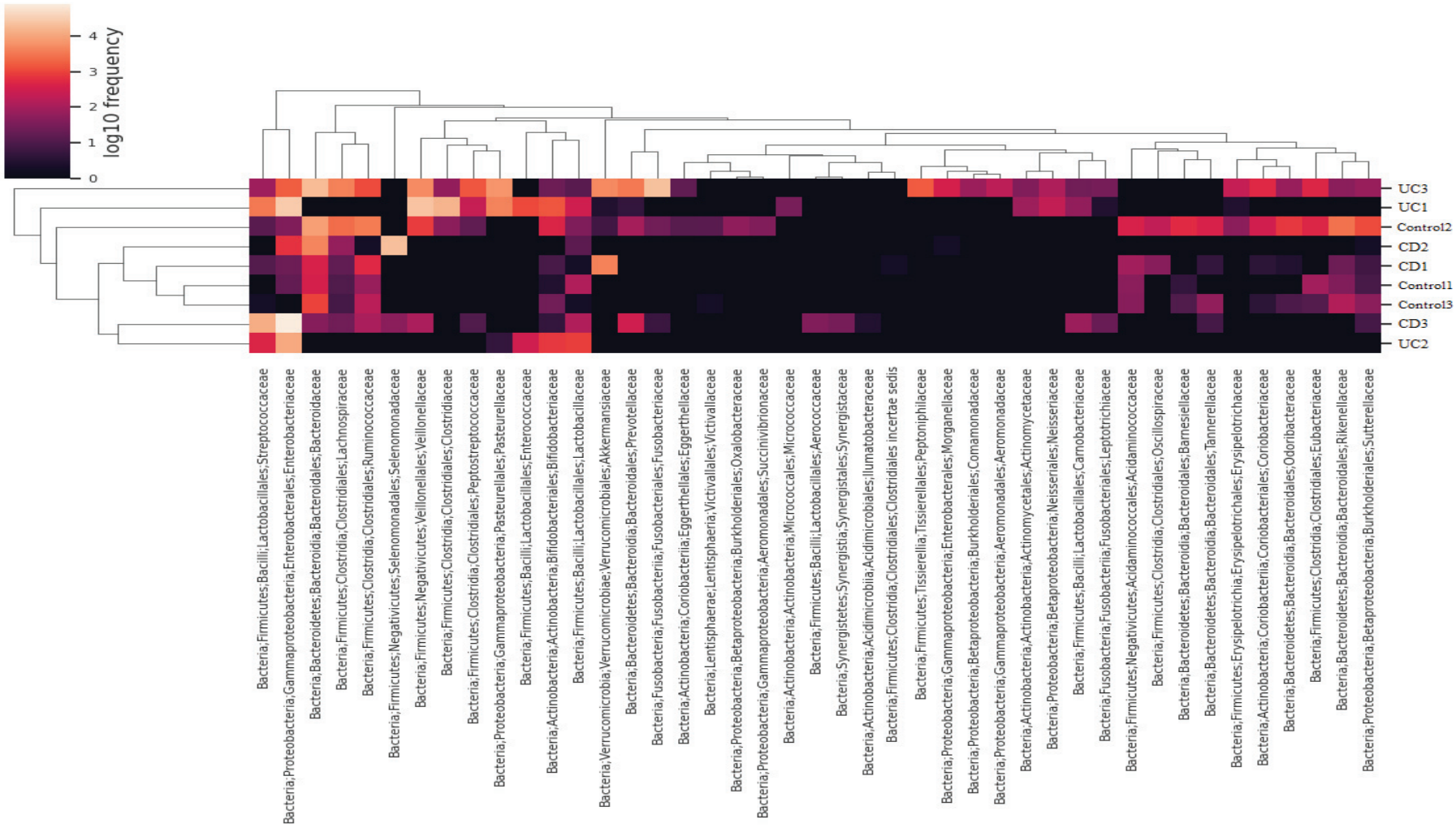

Figure B. 1. Heatmap of family levels between UC, CD and control samples