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ABSTRACT

Dysbiosis in the gut microbiota of patients with rheumatoid arthritis

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The gut microbiota is a complex community of diverse microorganisms comprising bacteria, viruses, fungi, and other microbes in the human gastrointestinal tract. The gut microbiota plays a pivotal role in human health and disease through its close relationship with the gut environment. Dysbiosis, an imbalance in the gut microbiota, has been linked to various diseases. This thesis discusses the role of gut bacteria and fungi in metabolic disorders, neurological disorders, immune regulation, and drug metabolism. The gut microbiota is essential for nutrient absorption and energy metabolism; indeed, dysbiosis is a significant driver of the development of cardiovascular and metabolic disorders. In addition, it can induce inflammatory responses that may result in neuronal damage via the gut-brain axis and is associated with immune dysregulation.

The gut microbiota has a critical role in immune regulation, and there is a significant body of research on the interaction between gut microbiota and autoimmune diseases. Rheumatoid arthritis (RA) is a representative autoimmune disease closely associated with gut microbiota. Although the detailed mechanisms have not been fully established, studies indicate that fungal cell wall components may be critical to the pathogenesis of RA. Thus, the composition of the fecal microbiota in patients with RA and healthy subjects was examined to determine potential correlations between RA and changes in the gut microbiota. It was found that changes in the fungal community were more pronounced than those in the bacterial community in patients with RA. Specifically, in patients with RA, the proportion of *Aspergillus* was lower, and that of *Candida* was significantly higher than in healthy subjects. Moreover, the analysis of microbial community structure indicated that the fungal community had a more critical role than the bacterial community in patients with RA. These findings suggest that fungi play a crucial role in the gut microbiota and in the pathogenesis of RA.

The gut microbiota can influence drug efficacy or lead to adverse drug effects. Gut bacteria are reported to impact drug metabolism, and research into personalized therapies to make use of this knowledge is ongoing. In recent studies, efforts have been made to resolve imbalances in the gut microbiota as a means of disease prevention and treatment. For example, in patients treated with prebiotics or probiotics, partial restoration of the gut microbiota was observed and resulted in improved immune regulation and symptom relief.

In this study, the impact of imbalances in the gut microbiota on the disease was explored, providing a basis for research into future treatments. The significant role of gut fungi in RA was confirmed. Therefore, the importance of research into the gut fungal community is proposed to support the development of new therapies for this disease. Multidisciplinary studies of the gut microbiota should afford novel insights into preventing and treating this disease.

Keywords: Gut microbiota, *Candida*, *Aspergillus*, Dysbiosis, Rheumatoid arth ritis

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CONTENTS

LIST OF TABLES

page

CHAPTER I

CHAPTER II

LIST OF FIGURES

page

CHAPTER II

CHAPTER I

Gut Dysbiosis and Its Impact on Human Disease

ABSTRACT

The human microbiome is closely related to one's health, and the gut microbiota plays the most crucial role. This relationship significantly influences various aspects of human physiology, such as the immune system, nutritional status, and metabolic activities. While the ideal composition and function of gut microbiota contribute to a healthy life, dysbiosis (an imbalance in the microbial community) increases the risk of developing various diseases. This review discusses the roles of gut microbiota, both the bacterial and fungal communities, within the human body. The impact of gut dysbiosis on metabolic disorders, neurological diseases, and immune regulation is explored. Furthermore, the influence of gut bacterial communities on drug metabolism is investigated, with evidence indicating that the observed variability in drug responses among individuals is attributed to the gut microbiota. A multifaceted examination of gut microbiota may offer new insights for developing disease treatments.

INTRODUCTION

Humans are exposed to numerous microorganisms that are both beneficial and harmful to our health. The human microbiota refers to both the microorganisms we come into contact with in the external environment and those that reside within our bodies. It comprises bacteria, viruses, and fungi, and their numbers were once believed to exceed 100 trillion, far more than human cells (Bäckhed et al., 2005). However, this figure is now estimated at approximately 39 trillion microorganisms, slightly more than the 30 trillion human cells in our body (Sender et al., 2016). The microbiota exist in different microbiomes and perform various functions, including decomposing inorganic substances such as dietary fibers (Baky et al., 2022), regulating the immune system (Kamada et al., 2013), and producing vitamins (LeBlanc et al., 2013). The composition of the human microbiome differs among individuals and is influenced by age, diet, health status, and the environment (Qin et al., 2010;Consortium, 2012;Yatsunenko et al., 2012). Consequently, the microorganisms comprising the human microbiota have a significant impact on each other's survival and health beyond mere mutualism.

There has been considerable research on the effects of human microbiota on health. The advent of next-generation sequencing technologies led to an explosion of studies yielding significant results. In particular, the Human Microbiome Project (HMP), led by the US National Institutes of Health, collected and analyzed microbial community samples from five sites (oral, skin, digestive, reproductive, and respiratory) in thousands of individuals

(Consortium, 2019). This project reported on the diversity and distribution of human microbial communities, their changes, and their associations with disease onset. The HMP also developed various technologies and methodologies for analyzing human microbial community data, contributing to the advancement of microbial community research by enhancing standardization and the comparability of analyses of microbial communities

The gut is the organ with the highest concentration of microorganisms in the human body. These microorganisms are called gut microbiota, consisting of bacteria, viruses, fungi, and protozoa. Gut microbiota are crucial in maintaining barrier functions and promoting a healthy environment within the gastrointestinal tract (Alam and Neish, 2018). Recent studies have shown that gut microbiota are involved not only in preserving barrier functions and promoting a healthy environment within the gastrointestinal tract but also in a wide range of diseases, including autism spectrum disorder (ASD), depression, peripheral vascular disease, hypertension, obesity, metabolic syndrome, and inflammatory bowel disease (IBD), as well as drug metabolism (Lynch and Pedersen, 2016;Dhurjad et al., 2022). Dietary intake has been shown to significantly impact the composition and functionality of the gut microbiota in humans (Rinninella et al., 2023). In this review, we examine the roles of gut microbiota, classified into bacteria and fungi; the mechanisms underlying the interaction between gut microbiota and diseases; and the impact of gut dysbiosis on disease development and progression.

I. Functions and contributions of gut microbial community

Bacterial communities constitute the majority of gut microbiota in the human body and play a crucial role in nutrient absorption and energy metabolism. Gut bacteria collaborate to digest beneficial components such as dietary fibers and complex carbohydrates, and decompose simple carbohydrates, proteins, and lipids into simple unsaturated fatty acids that serve as an energy source in the body (Rowland et al., 2018). Moreover, they synthesize vitamins, including the vitamin K and B groups (LeBlanc et al., 2013), and produce appetite-regulating hormones, such as leptin and ghrelin, which affect energy balance and body weight management (Han et al., 2021). Thus, gut bacterial dysbiosis may disrupt nutrient absorption and energy metabolism. For example, obese individuals harbor a lower number of microorganisms than healthy individuals, and their gut microbiota is characterized by a higher abundance of *Firmicutes*, which are involved in energy extraction (Kallus and Brandt, 2012). Several studies have demonstrated that providing prebiotics or probiotics to obese patients partially restores nutrient absorption and energy metabolism (Megur et al., 2022). To summarize, the gut bacterial community is crucial for nutrient absorption and energy metabolism in the human body.

The gut bacterial community is pivotal in preserving intestinal microbial homeostasis and has three main functions. First, gut bacteria modulate the growth of beneficial microorganisms and curb that of pathogenic microorganisms. Beneficial microorganisms, such as *Bifidobacterium* and *Lactobacillus*, trigger the immune system and impede the proliferation of pathogenic microorganisms (Turroni et al., 2014;Nishida et al., 2018). In contrast, pathogenic microorganisms such as *Clostridium difficile* may disrupt the gut

microbial balance, leading to gastrointestinal disorders (Samarkos et al., 2018). Second, gut bacteria preserve the intestinal barrier's function. The intestinal barrier protects against the infiltration of pathogenic microorganisms and regulates nutrient absorption, upholding the intestinal milieu. Gut bacteria produce essential nutrients to maintain this barrier function and activate the immune system to reinforce this role (Alam and Neish, 2018). Finally, by metabolizing dietary fibers and various ingested proteins, gut bacteria regulate and preserve intestinal pH, which is critical for managing the growth of beneficial and pathogenic microorganisms (Patterson et al., 2014).

Since the gut bacterial community is crucial for maintaining homeostasis of the gut microbiota, dysbiosis is associated with various diseases. The preservation of an optimal gut bacterial community necessitates the adoption of suitable dietary and lifestyle habits.

II. Association between gut bacterial dysbiosis and diseases

Investigation of the role of the gut bacterial community in the human body has revealed that dysbiosis could lead to the development of diseases. Numerous studies have demonstrated that intestinal dysbiosis is connected with or might be a consequence of the onset of several human disorders (Lozupone et al., 2012;Lynch and Pedersen, 2016). Table 1 summarizes bacteria associated with specific diseases and categorizes the effect of gut bacterial community dysbiosis by disease.

Metabolic disorders

The association between the gut bacterial community and metabolic disorders, such as diabetes, obesity, and hyperlipidemia have been demonstrated. Regarding metabolic disorders, gut bacteria are involved in inducing insulin resistance or promoting insulin sensitivity in metabolic disorders, such as diabetes, obesity, and hyperlipidemia (Caricilli and Saad, 2013;Khan et al., 2014). Maintaining an ideal composition of the bacterial community may increase microbial populations that promote insulin sensitivity, leading to the prevention and treatment of diabetes. Additionally, certain gut bacteria are associated with weight management by regulating genes involved in energy metabolism and modulating metabolic activity, thereby influencing changes in body weight (Donohoe et al., 2011), as well as cholesterol metabolism, which could be a possible mode of prevention and treatment of hyperlipidemia (Vourakis et al., 2021). The gut bacterial community also affects liver metabolism and is associated with liver diseases, such as fatty liver and cirrhosis (Chassaing et al., 2014). Therefore, improving the composition of the gut microbiome may be a promising approach for preventing and treating liver diseases. Vegetarians with a high intake of dietary fiber have been reported to have more abundant gut bacteria and a lower risk of metabolic disorders (Tomova et al., 2019). Thus, detailed investigations are necessary to explore the interaction between metabolic disorders and the gut bacterial community.

Neurological disorders

The gut microbiota synthesizes vitamins, neurotransmitters, and short-chain fatty acids (SCFAs), which regulate human metabolism and brain function (Cryan and Dinan, 2012;Badawy, 2017). These signaling molecules are conveyed to the brain via the vagus nerve and other central nervous system pathways (Kennedy et al., 2017), modulating the interaction between the nervous system and the gut microbiota through the gut-brain axis. Such molecules include redox-active metabolites, SCFAs, and hormone-like molecules. Redox-active metabolites are signaling molecules produced by the catabolism of amino acids like tyrosine, phenylalanine, and tryptophan. They inhibit inflammatory responses in the gut–brain axis and affect mood and behavior (Wu et al., 2022). A variety of SCFAs are produced from the breakdown of dietary fiber by gut microbiota. These regulate appetite and metabolism via the gut–brain axis (Han et al., 2021). The structure of hormone-like molecules produced by the gut microbiota resembles various hormones, such as growth hormone and insulin, and they modulate hormone levels (Clarke et al., 2014).

Gut-brain axis dysfunction can contribute to the development and progression of various brain disorders. This happens when gut bacterial dysbiosis induces inflammatory responses, leading to changes in hormone and signaling molecule levels and subsequent brain function, promoting the onset and progression of depression and anxiety disorders (Foster and Neufeld, 2013;Rogers et al., 2016). Furthermore, gut microbiota dysbiosis has been reported to alter the production of signaling molecules related to neurodevelopment and alterations in brain function in cases of ASD, a developmental disorder of the central nervous system (Fowlie et al., 2018). Children with ASD have been reported to show a

lower ratio of *Akkermansia*, *Bacteroides*, *Bifidobacterium*, and *Parabacteroides* and an increased ratio of *Faecalibacterium* compared with neurotypical children (Xu et al., 2019). However, the exact role of gut bacterial dysbiosis in ASD remains unclear. Two of the most common neurodegenerative diseases, Alzheimer and Parkinson disease, have also been linked to inflammatory responses induced by gut bacterial dysbiosis, which can damage neurons in the brain (Lin et al., 2019;Sochocka et al., 2019). Accordingly, therapeutic approaches that target the gut microbiota are currently under investigation. Notably, the consumption of prebiotics or probiotics has been reported to exert positive effects on emotional and cognitive functions that are closely linked to brain function (Liu et al., 2015;Dahiya and Nigam, 2022).

Immune regulation

The immune system is modulated through a complex with the gut microbiota. First and foremost, gut bacteria activate immune-regulatory cells such as T-helper (Th) 17 cells, regulatory T-cells (Tregs), and Th1 cells, which are vital for maintaining and regulating an appropriate immune response (Stockinger and Veldhoen, 2007;Zhang et al., 2014;Omenetti and Pizarro, 2015;Plitas and Rudensky, 2016;Sun et al., 2018). Additionally, gut bacteria produce immune-regulatory proteins. For example, *Lactobacillus reuteri*, induces toll-like receptor 2 protein in the gut, promoting the generation and activation of Th17 cells (Jia et al., 2020). Furthermore, *Bacteroides fragilis* produces polysaccharide A, an extracellular adhesion molecule that stimulates the generation and activation of Treg cells in the gut (Kayama and Takeda, 2014). *Porphyromonas gingivalis* and *L. rhamnosus* interact with

Treg cells in the gut, playing a significant role in gut immune regulation (Jia et al. 2020). Thus, the gut microbiota performs a crucial function in immune regulation, and the interplay between the gut bacterial community and the immune system provides valuable insights for developing prophylactic and therapeutic interventions for relevant diseases.

Drug metabolism

Emerging evidence suggests that not only individual biological characteristics but also interactions with the gut microbiota determine the effects and side effects of drugs. Thus, the gut bacterial community affects drug absorption, metabolism, and toxicity, and this has been newly proposed as evidence for explaining differences in an individual's response to drugs (Zimmermann et al., 2019a). Gut bacteria can express drug-metabolizing enzymes affecting the activation or inactivation of drugs, which might increase or decrease their effects (Wilson and Nicholson, 2017). Moreover, gut bacteria may also biotransform phytochemicals such as ginsenosides, catechins, and quercetin, leading to differences in the efficacy of botanical drugs based on nationality or race (Santangelo et al., 2019). Additionally, some gut bacteria could facilitate or impede drug absorption, influencing bioavailability and duration of action (Tuteja and Ferguson, 2019). The types of gut bacteria that affect drug metabolism are highly diverse, and representative examples are summarized in Table 2.

Thus far, we have explored the role of the gut bacterial community and how it impacts disease. It is critical in maintaining overall health and well-being. Understanding gut

bacteria interactions and regulating their composition may be helpful for disease prevention and improving health.

Table 1. Alterations in gut bacterial abundance associated with human diseases

Disease		Significant shifts in bacterial community composition	Reference
Class	**Subtype**		
Metabolic disorders	Type 2 diabetes	*Actinobacteria*, *Bacteriodetes*, *Escherichia coli*, *L. acidophilus*, *L. gasseri*, *L. salivarius* ↑ *Lactobacillus*, *L. amylovorus* ↓	Bondy, 2023
	Obesity	*Eubacterium rectale*, *Clostridium coccoides*, *Lactobacillus reuteri*, *Akkermansia muciniphila*, *Clostridium histolyticum*, and *Staphylococcus aureus* ↑	Gomes et al., 2018
		Firmicutes and *Actinobacteria* ↑ *Bacteroidetes*↓	Tseng and Wu, 2019
	Hyperlipidemia	*E. coli* and *Enterobacter*↑ *Lactobacillus*, *Faecalibacterium* and *Roseburia*↓	Moreno-Indias et al., 2016
Neurological disorders	Alzheimer's diseases	*Collisella*, *Alistipes*, *Barnesiella*, *Odoribacter*, *Bilophila*, *Escherichia*, *Shigella*, *Phascolarctobacterium*, *Gemella*, *Blautia*, *and Subdoligranulum*↑	Sochocka et al., 2019

		Bifidobacterium, *Adlercrutzia*, *Clostridium*, *SMB53*, *Cc115*, *Turicibacter*, *Eubacterium*, *Lachnoclostridium*, *and Roseburia*↓	
	Parkinson's diseases	*Lactobacillaceae*, *Barnesiellaceae*, *Enterococcacea*, *Bifidobacteriaceae*, *Christensenellaceae*, *Tissierellaceae*, *Enterobacteriaceae*, *Lachnospiraceae*, *Pasteurellaceae*, *and Verrucomicrobiaceae*↑ *Bacteroidetes*, *Prevotellaceae*, *Erysipelotrichaceae*, *Clostridium-coccoides*, and *Bacteroides fragilis*↓	Elfil et al., 2020
	Autism spectrum disorder (ASD)	*B. fragilis*, *Porphyromonas*, *Clostridium perfringens*, *Roseburia*, *Dorea*, *S. thermophiles*, *Prevotella*, and *Enterobacteriaceae*↑ *Oscillospira*, *Subdoligranulum*, *Turicibacter*, *Dialister*, *Veillonella*, and *Bifidobacterium fragilis*↓	Ho et al., 2020
Autoimmune disease	Type 1 diabetes	*Bacteriodetes* and *E. coli*↑	Bondy, 2023
	Inflammatory bowel disease (IBD)	*Ruminococcus gnavus*, *Enterobacteriaceae*, *E. coli*, *Proteobacteria*, *Fusobacterium*, *Streptococcus*, *Veillonella*, *Peptostreptococcus*, *Campylobacter*, *Klebsiella pneumonia*, *Candida glabrata*, and *Enterococcus*↑ *Faecalibacterium prausnitzii*, *Ruminococcus*, *Cyanobacteria*, *Flavobacterium*, *Oscillospira*, *Roseburia*, *Prevotella copri*, *Coprococcus*, *Dorea*, *Blautia*, and *Eubacterium*↓	Upadhyay et al., 2023

Rheumatoid arthritis	*Bacteroides* sp., *Coprobacillus* sp., *Gardnerella* spp., *Prevotella* spp., *Lactobacillus* sp., *Clostridium asparagiforme*, *Holdemania- filiformis*, *Eggerthella lenta*, *Gordonibacter-pamelaeae*, *Ruminococcus lactaris*, *Bacteroides sartorii*, and *Porphyromonas somerae*↑ *Bacteroides*, *Haemophilus* sp., *Veillonella* sp., *Klebsiella pneumoniae*, *Coprococcus catus*, *Dialister invisus*, *Sutterella-wadsworthensis*, *Megamonas hypermegale*, *Lactobacillus-sanfranciscensis*, and *Bifidobacterium bifidum*↓	Miyauchi et al., 2023
Sjögren's syndrome	*Proteobacteria, Actinobacteria, Bacteroidetes, Escherichia-Shigella, Sardovia, Veillonella, Insteinimonas, Lactobacillales, E.- coli, Lactobacillus phage Sal3, Lactobacillus reuteri, Lactobacillus gasseri, Streptococcus lutetiensis, Streptococcus-mutans, Scardovia wiggsiae, and Fusobacterrium ulcerans*↑ *Firmicutes, Lactobacillales, and Lactobacillus gasseri*↓	Mendez et al., 2020;Wang et al., 2023

Table 2. Biotransformation of pharmaceuticals by gut microbiota

Biotransformation type	Drug	Therapeutic applications	Implicated bacteria	Reference
Prodrug activation	Sulfasalazine	Crohn's disease and Rheumatoid arthritis	*Clostridium* and *Eubacterium spp*.	Peppercorn and Goldman, 1972;Sousa et al., 2014
	Olsalazine	Ulcerative colitis	*Clostridium* and *Eubacterium spp*.	Wadworth and Fitton, 1991
	Balsalazide	IBD	*Clostridium* and *Eubacterium spp*.	Chan et al., 1983
	Lovastatin	Cardiovascular disease and Hyperlipidemia	Not Applicable	Yoo et al., 2014
	Loperamide oxide	Chronic diarrhea	Not Applicable	Lavrijsen et al., 1995
Inactivation	Digoxin	Cardiovascular disease	*Eggerthella lenta*	Haiser and Turnbaugh, 2013
	Deleobuvir	Hepatitis C virus infection	Not Applicable	McCabe et al., 2015

	Metronidazloe	Antibiotic and Antiprotozoal	*Clostridium perfringens*	Koch et al., 1979
	Epacadostat	Cancer	Not Applicable	Boer et al., 2016
Enhanced toxicity	Brivudine	Antiviral drug	*B. thetaiotaomicron*	Zimmermann et al., 2019b
	Diclofenac	NSAID*	*Bacteroides, Clostridium,* and *Bifidobacterium spp*.	Saitta et al., 2014
	Indomethacin	NSAID	*Bacteroides, Clostridium,* and *Bifidobacterium spp*.	Saitta et al., 2014
	Irinotecan	Cancer	*E. coli*	Wallace et al., 2010
	Ketoprofen	NSAID	*Bacteroides, Clostridium,* and *Bifidobacterium spp*.	Saitta et al., 2014
	Nitrazepam	Anxiety and Insomnia	*Clostridium leptum*	Rafii et al., 1997
	Sorivudine	Antiviral drug	Not Applicable	Okuda et al., 1998

*: Non-steroidal anti-inflammatory drugs

III. Human diseases influenced by the gut mycobiota

In recent years, the importance of studying the various fungi that inhabit the human gut has started to receive attention, as research in this area was primarily focused on studying gut bacteria. These fungi, collectively called the gut mycobiome, have been found to exist in more significant quantities within the gut than previously recognized (Hallen-Adams and Suhr, 2017). The interaction between gut fungi and bacteria plays a critical role in maintaining the homeostasis of gut microbiota. After exposure to antibiotics or other drugs, some fungal species can inhibit bacterial growth by blocking the secretion of growth factors or consuming nutrients that bacteria need to thrive (Chin et al., 2020).

Dysbiosis of the gut microbiota may lead to excessive fungal growth and the development of inflammatory diseases. For example, gut fungi dysbiosis has been associated with IBD. Although the exact cause of IBD remains unclear, extensive research has documented the role of dysbiosis in the gut (Sokol et al., 2017;Beheshti-Maal et al., 2021). Excessive proliferation of certain fungal species, such as *Candida albicans*, has been closely linked to the development of IBD. Additionally, *C. albicans* plays a role in promoting the growth and survival of colon cancer cells (Zhu et al., 2021), and its toxin, candidalysin, induces neutrophil and interleukin 17 responses, which may affect various inflammatory diseases (Ho et al., 2021). Furthermore, gut fungal dysbiosis with an increase in the *Candida* genus has also been associated with neurological disorders, such as Rett syndrome, ASD, schizophrenia, and bipolar disorder (Chin et al., 2020). Similar to gut bacteria, gut fungi are also associated with dietary and lifestyle changes. Excessive sugar

intake, low dietary fiber intake, antibiotic use, and stress may lead to gut fungal dysbiosis (Hallen-Adams and Suhr, 2017;Markey et al., 2020;Seelbinder et al., 2020), which has been linked to obesity and metabolic disorders (Mar Rodríguez et al., 2015). However, research on the role of gut fungi and gut fungi dysbiosis in disease is still in its early stages, so the exact mechanisms are not yet well understood. Since the relationship between human gut fungi and disease is complex, specific research on the association of each fungal species and disease is necessary.

PERSPECTIVE

Gut microbiota are crucial in maintaining the balance of intestinal microorganisms and are closely related to health, including nutrient absorption and energy metabolism. Findings from the HMP have provided a basis for understanding the diversity and function of gut microbiota and uncovered the association between gut dysbiosis and several diseases, including metabolic syndrome, neurological disorders, digestive diseases, and immune diseases. Current studies have focused on restoring gut dysbiosis to prevent or treat diseases. Furthermore, the use of prebiotics or probiotics enhances nutrient supply and immune regulation and alleviates various diseases, such as IBD, diabetes, obesity, and neurodegenerative diseases. Collectively, these findings indicate that gut dysbiosis is an attractive target for developing disease therapies. However, studying the gut microbiota has limitations. Most rely on bioinformatics to predict the composition of the gut microbiota, and research on how gut microbiota causes specific diseases needs detailed investigations. Although various mechanistic studies are underway, they are predominantly conducted on animal models and may not be directly applicable to humans. The study of the gut mycobiome may be the solution, as it represents a missing link in the correlation between gut microbiota and human diseases. Thus, it is essential to recognize the importance of the mycobiome in microbiota research and to adopt a multidisciplinary approach in future studies.

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CHAPTER II

Dysbiotic but nonpathogenic shift in the fecal mycobiota of patients with rheumatoid arthritis

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ABSTRACT

Rheumatoid arthritis (RA) is closely associated with the oral and gut microbiomes. Fungal cell wall components initiate inflammatory arthritis in mouse models. However, little is known regarding the role of the fungal community in the pathogenesis of RA. To evaluate the association between RA and the gut microbiome, investigations of bacterial and fungal communities in patients with RA are necessary. Therefore, we investigated the compositions and associations of fecal bacterial and fungal communities in 30 healthy controls and 99 patients with RA. The relative abundances of *Bifidobacterium* and *Blautia* decreased, whereas the relative abundance of *Streptococcus* increased, in patients with RA. The relative abundance of *Candida* in the fecal fungal community was higher in patients with RA than in healthy controls, while the relative abundance of *Aspergillus* was higher in healthy controls than in patients with RA. *Candida* species-specific gene amplification showed that *C. albicans* was the most abundant species of *Candida*. Ordination analysis and random forest classification models supported the findings of structural changes in bacterial and fungal communities. *Aspergillus* was the core fecal fungal genus in healthy controls, although *Saccharomyces* spp. are typically predominant in Western cohorts. In addition, bacterial–fungal association analyses showed that the hub node had shifted from fungi to bacteria in patients with RA. The finding of fungal dysbiosis in patients with RA suggests that fungi play critical roles in the fecal microbial communities and pathogenesis of RA.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease that mainly affects the synovium in joints. Synovial thickening leads to the destruction of joint cartilage and bone (Harris Jr, 1990;Klareskog et al., 2009;Smolen et al., 2018). Subsequently, RA can worsen and affect other joints, thereby increasing the risks of osteoporosis, Sjögren syndrome, heart diseases, and lung diseases (Haugeberg et al., 2000;Solomon et al., 2006;Tsuchiya et al., 2011;He et al., 2013). Although the pathogenesis of RA is incompletely understood, interactions among genetic, environmental, and lifestyle factors have been proposed. A significant genetic risk factor for RA is *HLA-DRB1* (McInnes and Schett, 2011). Genome-wide association studies have shown that *PTN22*, *PADI4*, *STAT4*, and *TRAF1-C5* are associated with the onset of RA (Stahl et al., 2010). Notably, the HLA-DRB1*0405 allele is closely associated with RA severity and susceptibility in Koreans (Bae, 2010;Okada et al., 2014). Clinical and experimental animal studies have shown that infection with *Porphyromonas gingivalis*, *Proteus mirabilis*, Epstein–Barr virus, or mycoplasma contributes to RA pathogenesis (Li et al., 2013). The involvement of microbes in the etiopathogenesis of RA has prompted the investigation of relationships between RA and changes in human-associated microbial communities.

Dysbiosis has been identified in the fecal bacterial communities of patients with RA. Generally, gut microbial diversity is lower in patients with RA than in healthy individuals (Zhang et al., 2015). Differences in the bacterial abundance were also observed. Specifically, the abundances of *Prevotella copri*, *Collinsella, Eggerthella,*

and *Lactobacillus* increased in patients with RA, while the abundances of *Bacteroides*, *Faecalibacterium*, *Veillonella*, and *Haemophilus* decreased in those patients (Wu et al., 2010;Maeda and Takeda, 2019). Among the bacteria affected by RA, *P. copri* is predominantly present in the feces of patients with early RA; this species has been implicated in RA pathogenesis (Maeda et al., 2016). Treatment for RA also affects the composition of the gut bacterial community. For example, etanercept increased the abundances of the *Cyanobacteria* and *Nostocophycideae* classes and the *Nostocales* order; it decreased the abundances of the *Deltaproteobacteria* class and the *Clostridiaceae* family (Picchianti-Diamanti et al., 2018). Patients who received methotrexate (MTX) showed a reduced abundance of Enterobacteriales and partial community restoration, compared with the typical dysbiotic community in patients with RA (Zhang et al., 2015;Chen et al., 2016;Picchianti-Diamanti et al., 2018). Additionally, the bacterial community is affected by the presence of rheumatoid factor or anti-citrullinated protein antibody (ACPA), which are markers used to classify RA. Moreover, the C-reactive protein level and erythrocyte sedimentation rate are associated with gut microbiome dysbiosis in patients with RA (Picchianti-Diamanti et al., 2018;Chiang et al., 2019;Rooney et al., 2021).

Similar to studies of bacteria, an association between fungi and RA pathogenesis has been reported. Intraperitoneal injections of a fungal cell wall component (zymosan or fungal β-glucan) into SKG mice in a specific pathogen-free laboratory resulted in the induction of autoimmune arthritis, whereas injections of an antifungal agent and antifungal cell wall component did not (Yoshitomi et al., 2005). Therefore,

fungi are essential for the initiation of autoimmune arthritis. In a previous study that investigated the gut fungal community of patients with RA in China, the abundance of *Pholiota, Scedosporium*, and *Trichosporon* were lower than in healthy controls. *Suhomyces* and *Trebouxia*, two fungal genera abundant in patients with RA, were positively correlated with RA biomarkers (Sun et al., 2022). However, the effects of the fecal fungal community on RA have been less extensively investigated than the effects of the bacterial community.

Here, we investigated the fecal bacterial and fungal communities of patients with RA. We aimed to i) evaluate the fecal bacterial and fungal compositions and their interkingdom associations, ii) identify key taxa or operational taxonomic units (OTUs) associated with compositional shifts in the fecal bacterial and fungal communities, and iii) examine the effects of medications on the fecal fungal community. The abundance of *Candida* was increased, while the abundance of *Aspergillus* was decreased, in the feces of patients with RA. The abundances of *Candida* and *Aspergillus* showed contrasting correlations with clinical factors used for RA diagnosis. In addition, the hub node, which plays a central role in bacterial–fungal associations, shifted from fungi to bacteria in patients with RA. Finally, the abundance of *Candida albicans* was affected by treatment for RA. Our study provides insight into the crucial roles of the fungal community in pathogenesis of RA.

MATERIALS AND METHODS

I. Sample collection

Healthy controls (HC) (n = 30) were recruited from the Wonju Severance Christian Hospital. HC who had a chronic, systemic autoimmune disease and pregnant or lactating women were excluded. RA (n = 99), who fulfilled the 2010 ACR/EULAR classification criteria (Aletaha et al., 2010), were recruited from the Catholic University of Korea Seoul St. Mary's Hospital. Each individual had been prescribed medication, including non-steroidal anti-inflammatory drugs, corticosteroids, csDMARDs, and biologics. All clinical data were obtained according to established methods, and the DAS28 was used to quantify dis-ease activity.

The Ethics Committees of the Wonju Severance Christian Hospital Ethics Committee (IRB Approval Number: 19–008) and the Catholic University of Korea (IRB Approval Number: KC14TIMI0248) approved this study. Fecal samples were collected from March 2017 to November 2018 and promptly frozen at −20°C. Sequentially collected samples were transported to the laboratory and stored at −80°C before DNA extraction.

II. DNA extraction from feces

DNA extraction was performed using the QIAamp PowerFecal Pro DNA Kit

(Qiagen, Germany), in accordance with the manufacturer's instructions. DNA concentration and purity were determined with a Nanodrop 1000 (Thermo Fisher Scientific, USA). The collected DNA was stored at −20°C before amplification by polymerase chain reaction (PCR).

III. PCR amplification and sequencing

The V3–V4 regions of 16S ribosomal RNA (rRNA) genes were amplified using the Illumina-adapted universal primers 314F/805R. Each PCR reaction contained 12.5 ng of genomic DNA, 2.5 μL of Ex Taq 10× PCR buffer (Takara, Japan), 2.5 μL of dNTP mixture (Takara), 0.125 μL of Takara Ex Taq (Takara), 5 μL of each primer (200 nM final concentration), and distilled water to a total volume of 25 μL. The following thermocycler protocol was used: initial denaturing at 95°C for 3 min; 25 cycles of denaturing at 95°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 30 s; and final extension at 72°C for 5 min. PCR products were purified using AMPure XP beads (Beckman Coulter, USA), then quantified using a KAPA Library Quantification kit (KAPA Biosystems, USA). Sequencing was conducted on the MiSeq platform using a paired-end 2×300 base pairs reagent kit (Illumina, USA).

Subsequently, the fungal internal transcribed spacer 2 (ITS2) region of the 18S ribosomal RNA genes was amplified using the ITS3F/ITS4R primers and i-Starmax II polymerase (Intron Biotechnology, Korea). Each PCR reaction (final volume, 25 μL) contained 2.5 μL of 10× PCR buffer, 2.5 μL of dNTP mixture, 0.31 μL of i-Starmax II polymerase (Intron Biotechnology), 1.25 μL of each primer (500 nM final

concentration), and 50–120 ng of genomic DNA. The following thermocycler protocol was used: initial denaturing at 94°C for 4 min; 35 cycles of denaturing at 94°C for 1 min, primer annealing at 60°C for 1 min, and extension at 72°C for 1 min; and final extension at 72°C for 10 min. PCR products were purified using AMPure XP beads (Beckman Coulter). DNA quality and quantity were measured using an Infinite 200 pro (Tecan, Switzerland). All samples were diluted to the same concentration, pooled into a single library, and concentrated using AMPure beads (Beckman Coulter); the pooled library was subjected to gel purification to remove any residual unwanted PCR products. Finally, the pooled library was sequenced on the Illumina MiSeq platform with a read length of 2 × 300 base pairs at the National Environmental Management Center of Seoul National University.

IV. Sequence processing and filtering

After demultiplexing, overlapping sequences were merged with PEAR, then filtered with the DADA2 plugin (Callahan et al., 2016) using the "denoise-single" command in QIIME2. Subsequently, high-quality sequences were clustered into OTUs using the open reference vsearch algorithm (vsearch cluster-features-openreference) (Rognes et al., 2016) against the Silva 99% OTU representative sequence database (version 132, April 2018) (Quast et al., 2012), then assembled into an OTU table. Bacterial OTUs were clustered into OTUs using the UCHIME-de novo algorithm (Edgar et al., 2011), fungal sequences were checked for chimerism with UCHIME using the June 2017 chimera detection ITS2 database (Nilsson et al., 2015). Next, the taxonomies of nonchimeric OTUs were assigned

using the naïve Bayes algorithm implemented in the q2-feature-classifier, based on the Silva database for the V3–V4 region of the 16S rRNA sequences (Bokulich et al., 2018). Alternatively, eukaryotes were classified using the UNITE database (UNITE version 7 dynamic of January 2017) for the ITS2 region (Abarenkov et al., 2010).

Short bacterial (400 base pairs) and fungal (100–500 base pairs) sequences were used for in-depth analyses. First, OTU tables were imported into R using the readRPM component of the phyloseq package (McMurdie and Holmes, 2013). Next, sequence data were removed for organisms that had been assigned to non-kingdom-level groups (bacterial OTUs: orders "Chloroplast" and "Rickettsiales;" fungal OTUs: kingdoms "Unassigned," "Rhizaria," and "Metazoa"). Subsequently, false positive OTUs were removed from stool samples, while singleton OTUs were eliminated from all samples. This process reduced the total bacterial OTU count from 1346 to 1338 and total fungal OTU count from 1641 to 1595. The remaining 1338 bacterial OTUs and 1595 fungal OTUs were used for further analysis.

V. Statistical analyses and visualization

Statistical analysis was performed using R statistical software, version 3.5.2 (R-Core-Team). After multiple hypothesis tests had been corrected using the false discovery rate method, significant results were determined using a *p*-value threshold of 0.05. First, OTU tables were scaled by cumulative-sum scaling (CSS) and log-transformed (for normalization) using the *cumNum* and *MRcounts* functions in the metagenomeSeq package in R (Paulson et al., 2013). Next, rarefication of bacterial

(5425 reads) and fungal (4256 reads) reads was conducted using the *rarefy_even_depth* function in the Phyloseq package in R; this was followed by calculation of the Shannon and Simpson indices using the *diversity* function in the Vegan (version 2.5–3) package in R. The Wilcoxon rank-sum test and one-way analysis of variance were also used. A Bray–Curtis dissimilarity matrix was produced for use in two separate principal coordinates analyses; canonical analysis of principal coordinates (CAP) was then performed using RA and HC constraints, respectively, with the *capscale* and *ordinate* functions from the Vegan and Phyloseq packages. Permutational analysis of variance (PERMANOVA) using the *adonis* function in the Vegan package (version 2.5–3) was also used for analysis (Oksanen et al.). Subsequently, the core OTUs of RA and HC groups were identified using a prevalence threshold of 85% for bacteria and 70% for fungi. Differentially abundant OTUs between the RA and HC groups were identified using linear discriminant analysis effect size (LEfSe) (https://huttenhower.sph.harvard.edu/galaxy/) (Segata et al., 2011). Differences in OTU abundance were considered significant when p-values were < 0.05.

VI. Microbial correlation networks

Bacterial–fungal networks were constructed to infer hub and complex OTU associations for RA and HC groups. Because the number of participants differed between the RA (n = 99) and HC (n = 30) groups, 30 samples from the RA group were randomly subsampled using the sample function in R software to avoid differences in network properties based on differences in sample size. Thus, we

obtained 1302 OTUs for the HC group and 1428 OTUs for the RA group; these OTUs were used to construct interkingdom co-occurrence networks. In contrast, CSS-normalized OTU abundance tables that included bacteria and fungi were used as an input for SparCC (Friedman and Alm, 2012); significant associations between OTUs were restricted to OTUs with correlations of > 0.3 and < −0.3 ($P < 0.05$) (Kurtz et al., 2015). Co-occurrence networks were visualized with Gephi (version 0.9.2) (Bastian et al., 2009) using the ForceAtlas2 layout. Within the networks, the proportions of inter-kingdom (associations between bacteria and fungi) and intra-kingdom (associations within the same kingdom) links were quantified and displayed in bar graph format (Durán et al., 2018). Specifically, HC and RA networks were compared in terms of degree, betweenness centrality, closeness centrality, and eigenvector centrality; these values were computed using igraph (version 1.2.1) (Csardi and Nepusz, 2006). The hub OTUs of each network were defined as the top 1% of OTUs in terms of degree, betweenness centrality, and closeness centrality. For the RA group, OTUs of degree > 20, betweenness centrality > 0.05343148, and closeness centrality > 0.01229823 were defined as hub OTUs. For the HC group, OTUs of degree > 19.7, betweenness centrality > 0.06493721, and closeness centrality > 0.02258181 were identified as hub OTUs.

VII. Fungal strain cultivation

C. albicans (KCCM 11282) was obtained from the Korean Culture Center of Microorganisms (Korea). *C. albicans* was cultivated and maintained in yeast extract peptone dextrose (YPD) agar plates or YPD broth at 25°C. Viable cell numbers were

determined by spreading serially diluted culture medium on YPD agar plates. Absorbance at 550 nm was measured using a microplate reader (Tecan).

VIII. DNA extraction and qualitative PCR (gel blotting of samples)

Single *C. albicans* colonies were inoculated and cultivated in YPD broth for 24 h. Broth cultures were centrifuged at 12,000 g for 5 min; DNA was then extracted from cell pellets using the QIAamp PowerFecal Pro DNA Kit (Qiagen). Next, genomic DNA was amplified using i-Starmax II polymerase (Intron Biotechnology). Each PCR reaction (final volume, 25 μL) contained 2.5 μL of 10× PCR buffer, 2.5 μL of dNTP mixture, 0.31 μL of i-Starmax II polymerase (Intron Biotechnology), 1.25 μL of each primer (500 nM final concentration), and 50–120 ng of genomic DNA. The following thermocycler protocol was used: initial denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and extension at 72°C for 1 min; and final extension at 72°C for 10 min. The following primers were used: forward, 5′-TTTATCAACTTGTCACACCAGA-3′; reverse, 5′-ATCCCGCCTTACCACTACCG-3′ (Frykman et al., 2015). PCR products were separated by electrophoresis on a 1.5% agarose gel containing SYBR DNA SafeStain (Thermo Scientific Pierce, USA). Bands were visualized using a ChemiDoc device (Thermo Scientific Pierce).

IX. Real-time quantitative PCR to quantify *C. albicans* abundance

Real-time quantitative PCR was conducted with a QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems, USA) using PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific). The primers used for qualitative PCR of *C. albicans* were used for real-time quantitative PCR. Each PCR reaction (final volume, 20 μL) contained 10 μL of SYBR Green Master Mix, 0.8 μL of each primer, and 2 μL of genomic DNA. The following thermocycler protocol was used: denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15s and annealing at 60°C for 60s. Amplification specificity was evaluated by melt curve analysis.

RESULTS

I. Descriptive statistics

The demographic and clinical features of patients with RA and HC are shown in Table 1. The study included 99 RA and 30 HC. Ninety-one samples (91.9%) in the RA group were from women, while 100% of samples in the HC group were from women. The mean participant ages were 57.8 ± 10.1 years in the RA group and 46.9 ± 3.5 years in the HC group. RF and ACPA positivity were detected in 77 (77/93, 82.8%) and 75 (75/98, 76.5%) patients, respectively. Of the RA patients, 87 (87.9%) were prescribed conventional synthetic disease-modifying antirheumatic drugs (csDMARDs), while 40 (40.4%) were prescribed biologics (including 32 patients who were prescribed both biologics and csDMARDs). Medications used by patients with RA are listed in Table 2.

Table 1. Characteristics of study participants.

Characteristics	Healthy controls (*n* = 30)	Patients with RA (*n* = 99)
Demographics		
Mean age (year)	46.9 ± 3.5	57.8 ± 10.1
Female proportion	30 (100%)	91 (91.9%)
BMI	23.9 ± 3.0	22.8 ± 2.7
Disease characteristics		
RF positivity at entry of study	2 (6.7%)	77 (*n* = 93, 82.8%)
Anti-CCP positivity at entry of study	NA	75 (*n* = 98, 76.5%)
Disease duration, median (IQR), year	NA	8.9 (0.1-40)
CRP, median (IQR), mg/dL	NA	0.5 (0.0-5.1)
ESR, median (IQR), mm/hr	NA	13.3 (2.0-70.0)

BMI, body mass index; NA, not applicable; CRP, C-reaction protein; ESR, erythrocyte sedimentation rate

Table 2. The medication for patients with RA.

Medication, n (%)	Healthy controls (*n* = 30)	Patients with RA (*n* = 99)
csDMARDs		
MTX	-	56 (56.6 %)
Leflunomide	-	42 (42.4 %)
Hydroxychloroquine	-	40 (40.4 %)
Sulfasalazine	-	15 (15.2 %)
Biologics		
Eetanercept	-	3 (3.0 %)
Adalimumab	-	3 (3.0 %)
Abatacept	-	17 (17.2 %)
Tocilizumab	-	17 (17.2 %)
Others		
Tofacitinib	-	1 (1.0 %)
Tacrolimus	-	13 (13.1 %)
Glucocorticoids	-	69 (69.7 %)
NSAIDs	-	54 (54.5 %)

II. Fecal microbial community composition

Distinct compositional differences between HC and RA were observed in the fecal bacterial community (Figure 1). *Bifidobacterium*, *Streptococcus*, *Blautia, Lachnospiraceae,* and an unidentified species were abundant in both the RA and HC groups (Figure 2a). However, genera with relative abundances < 0.3% constituted 89.4% of genera in the RA group and 54.2% of genera in the HC group (Figure 2a). Furthermore, the abundances of *Bifidobacterium* and *Blautia* were higher in the HC group than in the RA group; the abundance of *Streptococcus* was higher in the RA group than in the HC group. These differences were statistically significant (*Bifidobacterium*, $P = 0.0299$; *Blautia*, $P = 0.0024$; *Streptococcus*, $P = 0.0195$) (Figure 2b).

The most abundant fungal phyla were *Ascomycota, Basidiomycota*, and *Mucoromycota* (Figure 3). The ratio of *Basidiomycota* to *Ascomycota* was greater in the HC group than in the RA group (5.28%:65.7% in the HC group; 4.55%:75.42% in the RA group), while the proportion of *Mucoromycota* was greater in the HC group than in the RA group (mean relative abundances: 4.35% in the HC group and 1.74% in the RA group). The abundance of *Saccharomycetes* was greater in the RA group (HC, 35.2%; RA, 59.9%), while the abundance of *Aspergillaceae* was greater in the HC group (HC, 23.9%; RA, 10.0%) (Figure 3).

At the genus level, *Candida*, *Saccharomyces*, and *Aspergillus* were the most abundant fungi (Figure 2c, d). The relative abundance of *Candida* ($P = 0.00013$) was significantly greater in the RA group than in the HC group, while the relative abundance of *Aspergillus* ($P = 0.00092$) was significantly greater in the HC group

than in the RA group (Figure 2d). Furthermore, the abundances of the genera *Kazachctania*, *Issatchenkia*, *Penicillium*, and *Mucor* tended to be greater in the RA group, although these findings were not statistically significant (Figure 2c, d). In contrast to the findings in a Western cohort, the abundance of *Saccharomyces* did not differ between the two groups (Figure 2d; mean relative abundances: HC, 12.9%; RA, 14.2%; $P = 0.6204$). Therefore, RA may be associated with compositional shifts in the fecal bacterial and fungal communities.

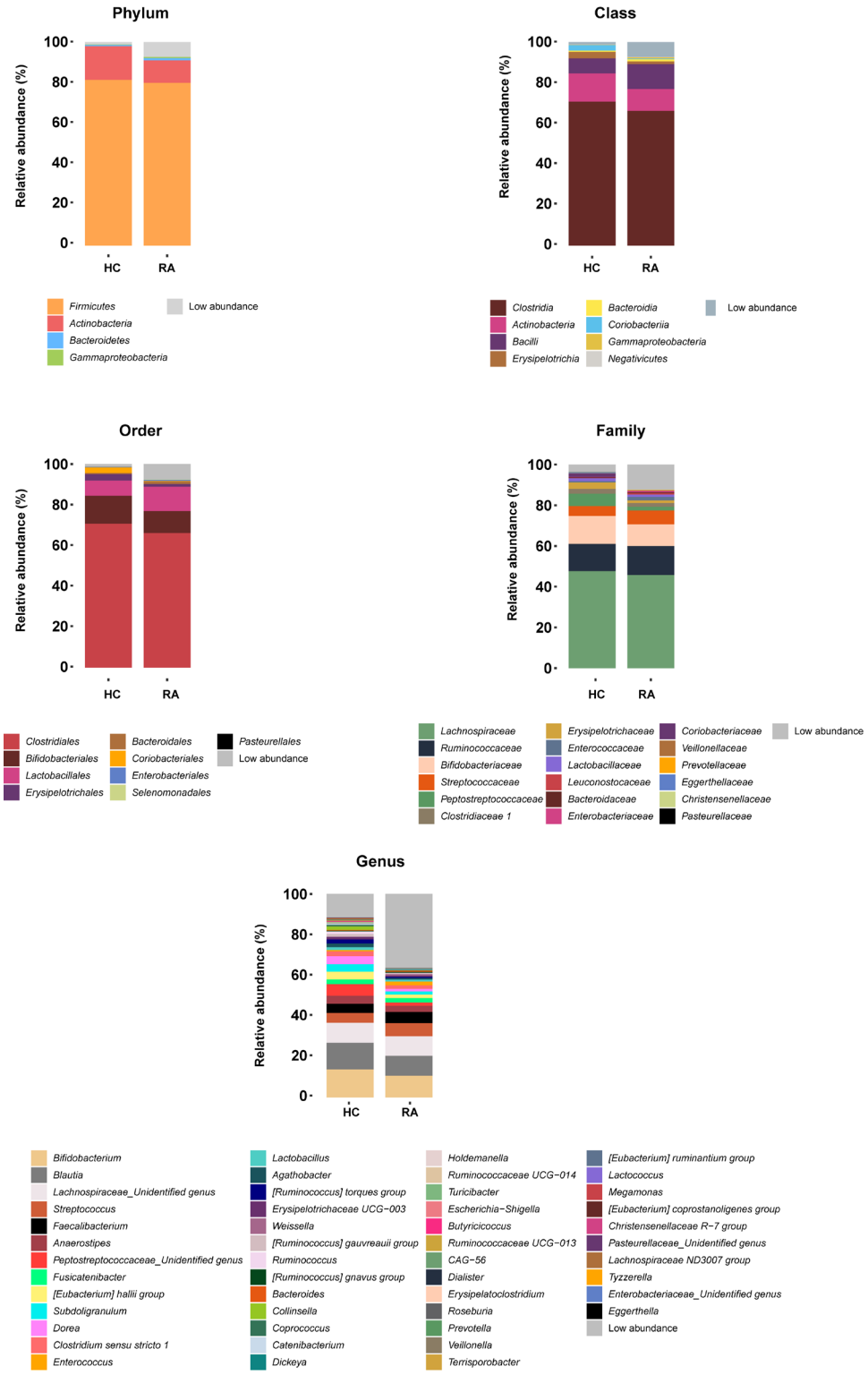

Phylum
Relative abundance (%)
100
80
60
40
20
0
HC
RA
Firmicutes
Actinobacteria
Bacteroidetes
Gammaproteobacteria
Low abundance
Class
Relative abundance (%)
HC
RA
Clostridia
Actinobacteria
Bacilli
Erysipelotrichia
Bacteroidia
Coriobacteriia
Gammaproteobacteria
Negativicutes
Low abundance
Order
Relative abundance (%)
HC
RA
Clostridiales
Bifidobacteriales
Lactobacillales
Erysipelotrichales
Bacteroidales
Coriobacteriales
Enterobacteriales
Selenomonadales
Pasteurellales
Low abundance
Family
Relative abundance (%)
HC
RA
Lachnospiraceae
Ruminococcaceae
Bifidobacteriaceae
Streptococcaceae
Peptostreptococcaceae
Clostridiaceae 1
Erysipelotrichaceae
Enterococcaceae
Lactobacillaceae
Leuconostocaceae
Bacteroidaceae
Enterobacteriaceae
Coriobacteriaceae
Veillonellaceae
Prevotellaceae
Eggerthellaceae
Christensenellaceae
Pasteurellaceae
Low abundance
Genus
Relative abundance (%)
HC
RA
Bifidobacterium
Blautia
Lachnospiraceae_Unidentified genus
Streptococcus
Faecalibacterium
Anaerostipes
Peptostreptococcaceae_Unidentified genus
Fusicatenibacter
[Eubacterium] hallii group
Subdoligranulum
Dorea
Clostridium sensu stricto 1
Enterococcus
Lactobacillus
Agathobacter
[Ruminococcus] torques group
Erysipelotrichaceae UCG-003
Weissella
[Ruminococcus] gauvreauii group
Ruminococcus
[Ruminococcus] gnavus group
Bacteroides
Collinsella
Coprococcus
Catenibacterium
Dickeya
Holdemanella
Ruminococcaceae UCG-014
Turicibacter
Escherichia-Shigella
Butyricicoccus
Ruminococcaceae UCG-013
CAG-56
Dialister
Erysipelatoclostridium
Roseburia
Prevotella
Veillonella
Terrisporobacter
[Eubacterium] ruminantium group
Lactococcus
Megamonas
[Eubacterium] coprostanoligenes group
Christensenellaceae R-7 group
Pasteurellaceae_Unidentified genus
Lachnospiraceae ND3007 group
Tyzzerella
Enterobacteriaceae_Unidentified genus
Eggerthella
Low abundance

Figure 1. Comparisons of the fecal bacterial community composition between HC and RA. Community composition was compared at the phylum, class, order, family, and genus levels, respectively. The columns of different colors represent different taxa, and the height of the bars represents the proportions of each taxon. Genera with abundance <0.3% are grouped as "Low abundance." HC, healthy controls; RA, patients with RA.

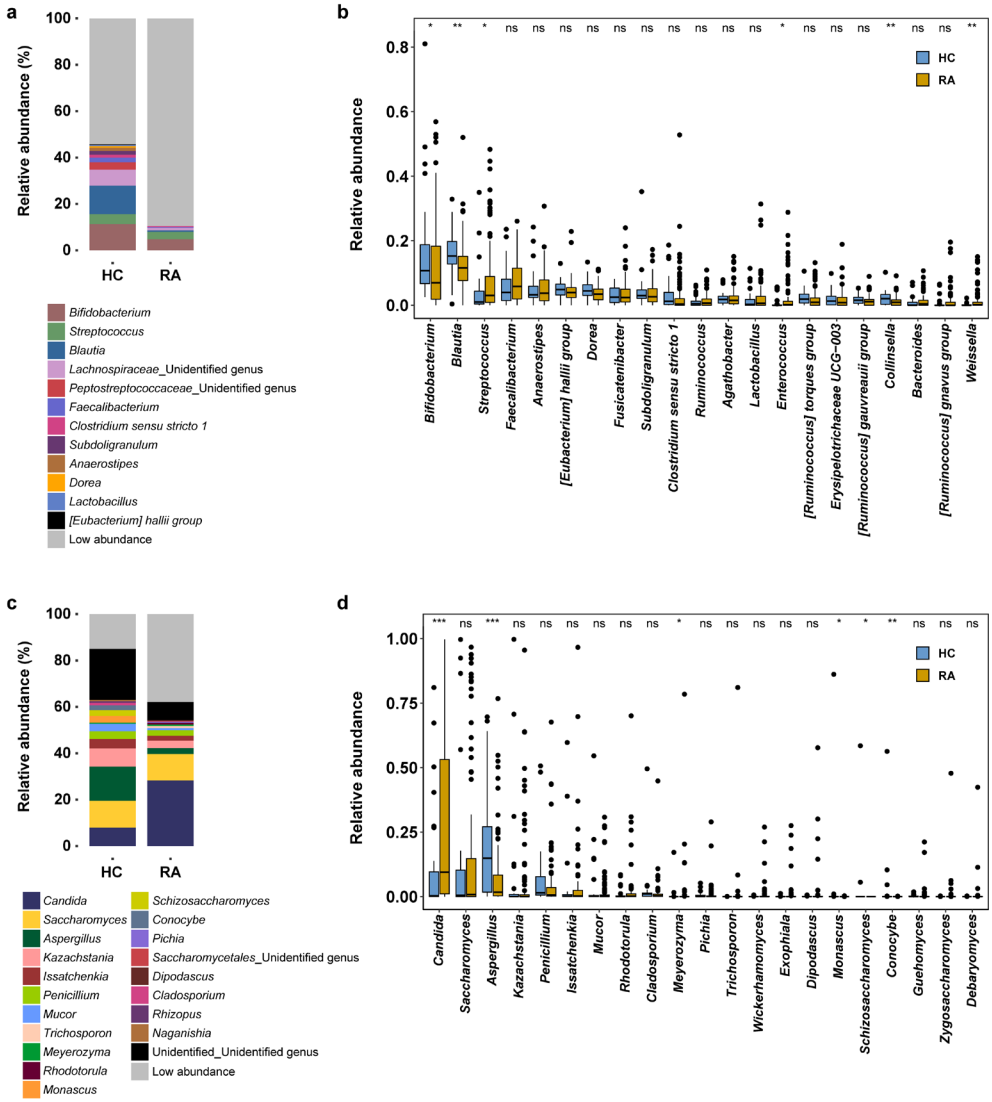
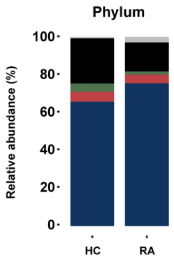
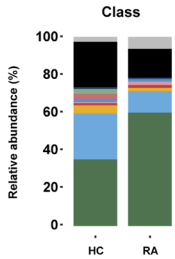
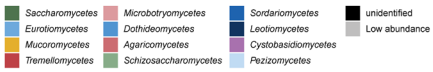

a
Relative abundance (%)
100
80
60
40
20
0
HC
RA
Bifidobacterium
Streptococcus
Blautia
Lachnospiraceae_Unidentified genus
Peptostreptococcaceae_Unidentified genus
Faecalibacterium
Clostridium sensu stricto 1
Subdoligranulum
Anaerostipes
Dorea
Lactobacillus
[Eubacterium] hallii group
Low abundance
b
Relative abundance
0.8
0.6
0.4
0.2
0.0
HC
RA
Bifidobacterium
Blautia
Streptococcus
Faecalibacterium
Anaerostipes
[Eubacterium] hallii group
Dorea
Fusicatenibacter
Subdoligranulum
Clostridium sensu stricto 1
Ruminococcus
Agathobacter
Lactobacillus
Enterococcus
[Ruminococcus] torques group
Erysipelotrichaceae UCG-003
[Ruminococcus] gauvreauii group
Collinsella
Bacteroides
[Ruminococcus] gnavus group
Weissella
c
Relative abundance (%)
100
80
60
40
20
0
HC
RA
Candida
Saccharomyces
Aspergillus
Kazachstania
Issatchenkia
Penicillium
Mucor
Trichosporon
Meyerozyma
Rhodotorula
Monascus
Schizosaccharomyces
Conocybe
Pichia
Saccharomycetales_Unidentified genus
Dipodascus
Cladosporium
Rhizopus
Naganishia
Unidentified_Unidentified genus
Low abundance
d
Relative abundance
1.00
0.75
0.50
0.25
0.00
HC
RA
Candida
Saccharomyces
Aspergillus
Kazachstania
Penicillium
Issatchenkia
Mucor
Rhodotorula
Cladosporium
Meyerozyma
Pichia
Trichosporon
Wickerhamomyces
Exophiala
Dipodascus
Monascus
Schizosaccharomyces
Conocybe
Guehomyces
Zygosaccharomyces
Debaryomyces

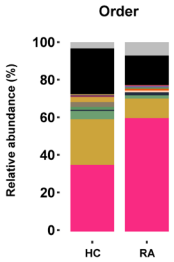
Figure 2. Fecal microbial community composition in healthy controls and patients with RA. (a) Bacterial community composition at the genus level**.** (b) Pairwise comparison of abundant bacterial genera. (c) Fungal community composition at the genus level**.** (d) Pairwise comparison of abundant fungal genera. In panels **b** and **d**, boxes and lines represent the interquartile ranges (Q3-Q1) and medians of relative abundances, respectively. Black dots indicate potential outliers. Lower and upper whiskers show minimum and maximum relative abundances of genera. Statistical significance was estimated by two-sided Mann–Whitney U test. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$ (not significant). HC, healthy controls; RA, patients with RA.

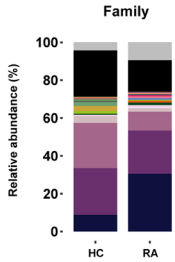

Phylum
Relative abundance (%)
100
80
60
40
20
0
HC
RA

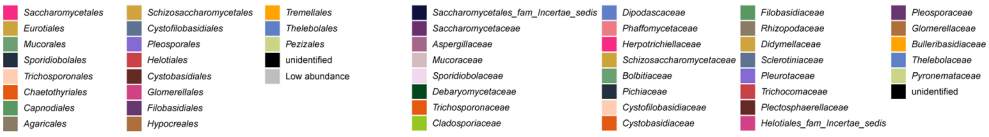
Ascomycota
Basidiomycota
Mucoromycota
unidentified
Low abundance

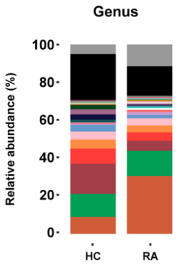

Class
Relative abundance (%)
100
80
60
40
20
0
HC
RA


Saccharomycetes
Eurotiomycetes
Mucoromycetes
Tremellomycetes
Microbotryomycetes
Dothideomycetes
Agaricomycetes
Schizosaccharomycetes
Sordariomycetes
Leotiomycetes
Cystobasidiomycetes
Pezizomycetes
unidentified
Low abundance


Order
Relative abundance (%)
100
80
60
40
20
0
HC
RA


Family
Relative abundance (%)
100
80
60
40
20
0
HC
RA


Saccharomycetales
Eurotiales
Mucorales
Sporidiobolales
Trichosporonales
Chaetothyriales
Capnodiales
Agaricales
Schizosaccharomycetales
Cystofilobasidiales
Pleosporales
Helotiales
Cystobasidiales
Glomerellales
Filobasidiales
Hypocreales
Tremellales
Thelebolales
Pezizales
unidentified
Low abundance
Saccharomycetales_fam_Incertae_sedis
Saccharomycetaceae
Aspergillaceae
Mucoraceae
Sporidiobolaceae
Debaryomycetaceae
Trichosporonaceae
Cladosporiaceae
Dipodascaceae
Phaffomycetaceae
Herpotrichiellaceae
Schizosaccharomycetaceae
Bolbitiaceae
Pichiaceae
Cystofilobasidiaceae
Cystobasidiaceae
Filobasidiaceae
Rhizopodaceae
Didymellaceae
Sclerotiniaceae
Pleurotaceae
Trichocomaceae
Plectosphaerellaceae
Helotiales_fam_Incertae_sedis
Pleosporaceae
Glomerellaceae
Bulleribasidiaceae
Thelebolaceae
Pyronemataceae
unidentified


Genus
Relative abundance (%)
100
80
60
40
20
0
HC
RA


Candida
Saccharomyces
Aspergillus
Kazachstania
Penicillium
Issatchenkia
Mucor
Rhodotorula
Meyerozyma
Saccharomycetales_Unidentified genus
Trichosporon
Cladosporium
Monascus
Exophiala
Schizosaccharomyces
Dipodascus
Wickerhamomyces
Conocybe
Pichia
Debaryomyces
Guehomyces
Cystobasidium
Zygosaccharomyces
Chaetothyriales_Unidentified genus
Rhizopus
Naganishia
Didymellaceae_Unidentified genus
Ciboria
Pleurotus
Dipodascaceae_Unidentified genus
Torulaspora
Talaromyces
Gibellulopsis
Cyberlindnera
Kendrickiella
Alternaria
Glomerellaceae_Unidentified genus
Hannaella
Thelebolus
Agaricales_Unidentified genus
Pyronemataceae_Unidentified genus
Filobasidium
Cutaneotrichosporon
unidentified_Unidentified genus
Low abundance

Figure 3. Comparisons of the fecal fungal community composition between HC and RA. Community composition was compared at the phylum, class, order, family, and genus levels, respectively. The columns of different colors represent different taxa, and the height of the bars represents the proportions of each taxon. Genera with abundance <0.3% are grouped as "Low abundance." HC, healthy controls; RA, patients with RA.

III. Fecal microbial diversity

We next investigated the effects of RA on microbial community diversity. The alpha diversity indices, including OTUs, Shannon, and Simpson indices, of bacteria and fungi did not significantly differ between groups (all $P > 0.05$) (Figure 4). In CAP, the constrained ordination analysis showed that bacterial and fungal communities were clearly separated into HC and RA groups (Figure 5a), although the unconstrained principal coordinates analysis did not show clear clustering of microbial communities according to RA status (Figure 6). PERMANOVA indicated significant compositional differences in the bacterial ($R^2 = 0.01746$, $P = 0.0002$) and fungal communities ($R^2 = 0.0216$, $P = 0.0001$) of the RA group (Table 3). The relative abundances of *Bifidobacterium*, *Streptococcus*, *Aspergillus*, and *Candida* differed between the two groups (Figure 5b). Although RA did not affect the richness or diversity of fecal microbial communities, it significantly affected beta diversity by shifting the taxonomic compositions of the fecal bacterial and fungal communities.

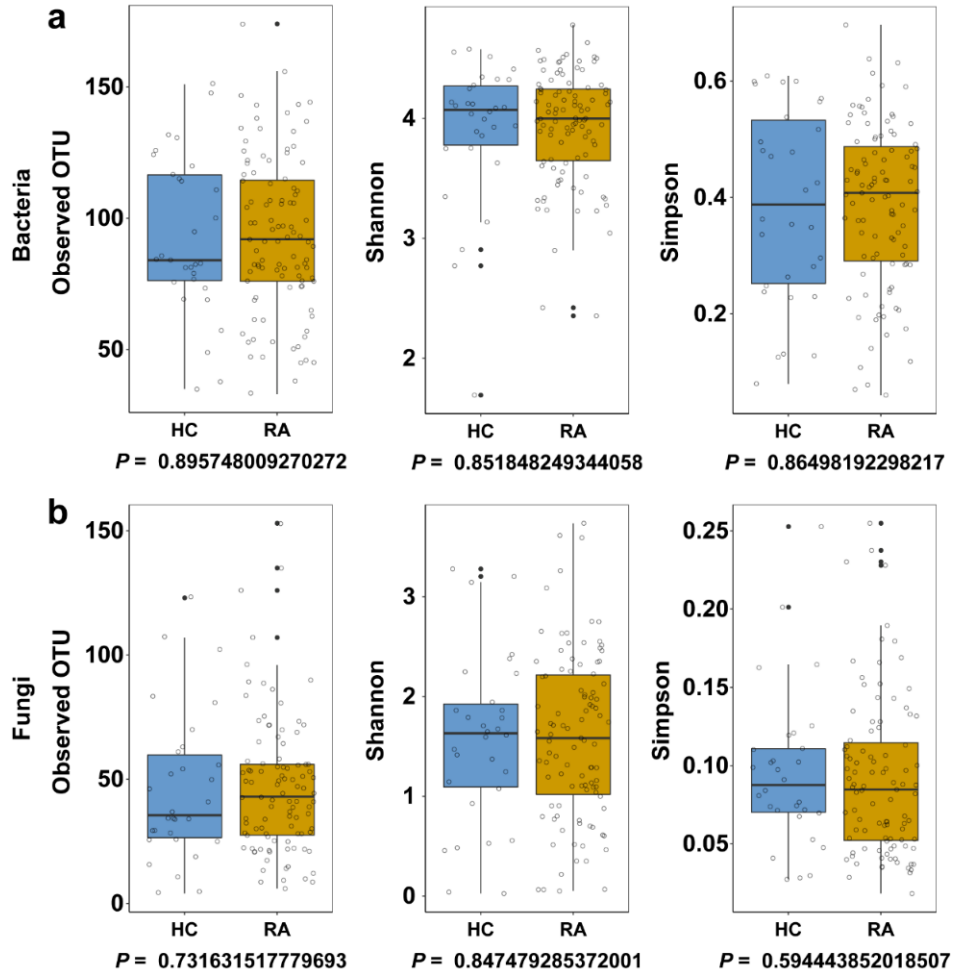

a
Bacteria
Observed OTU
150
100
50
HC
RA
P = 0.895748009270272
Shannon
4
3
2
HC
RA
P = 0.851848249344058
Simpson
0.6
0.4
0.2
HC
RA
P = 0.86498192298217
b
Fungi
Observed OTU
150
100
50
0
HC
RA
P = 0.731631517779693
Shannon
3
2
1
0
HC
RA
P = 0.847479285372001
Simpson
0.25
0.20
0.15
0.10
0.05
HC
RA
P = 0.594443852018507

Figure 4. Differences in alpha diversity between HC and RA. (a) Alpha diversity metrics for the fecal bacterial community**.** (b) Alpha diversity metrics for the fecal fungal community. Boxes and lines in the boxes represent the inter-quantile range (Q3–Q1) and median of diversity values, respectively. Black-filled dots indicate potential outliers. Lower and upper whiskers show minimum and maximum alpha diversity values in each group. The gray dots correspond to the exact values of the diversity indices of each sample. Statistical significance was estimated using a two-sided Mann–Whitney test. HC, healthy controls; RA, patients with RA.

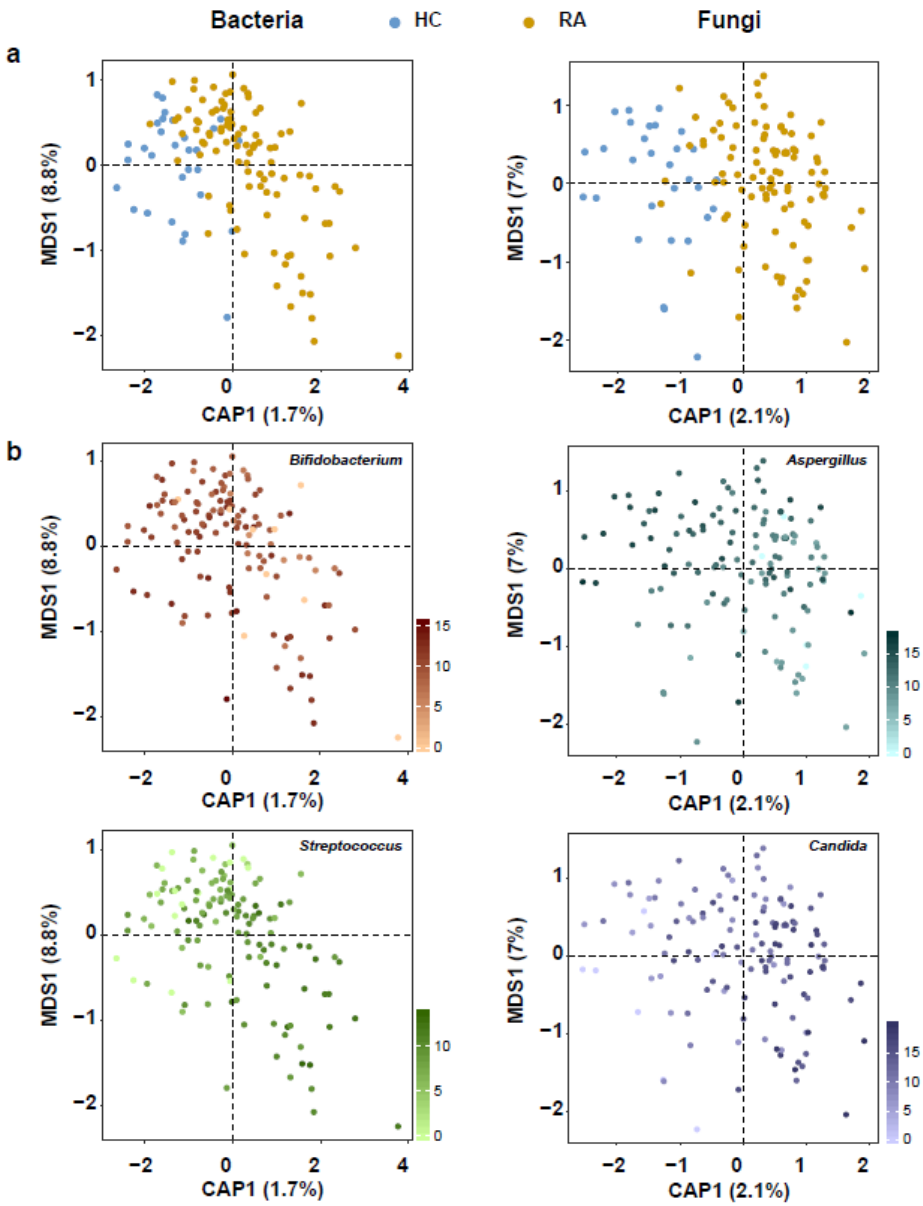

Bacteria
HC
RA
Fungi
a
MDS1 (8.8%)
CAP1 (1.7%)
MDS1 (7%)
CAP1 (2.1%)
b
Bifidobacterium
Aspergillus
Streptococcus
Candida

Figure 5. Ordination analysis of fecal bacterial and fungal communities in healthy controls and patients with RA. Compositional variations among samples were estimated by canonical analysis of principal coordinates (CAP), based on the Bray–Curtis distance metric**.** (a) Changes in composition of fecal bacterial and fungal communities. Healthy control (HC) samples are shown in blue; RA samples are shown in dark yellow. (b) Ordination analysis according to the relative abundances of abundant genera. Greater intensity denotes higher relative abundance. Left and right sides of panels **a** and **b** are bacterial and fungal communities, respectively.

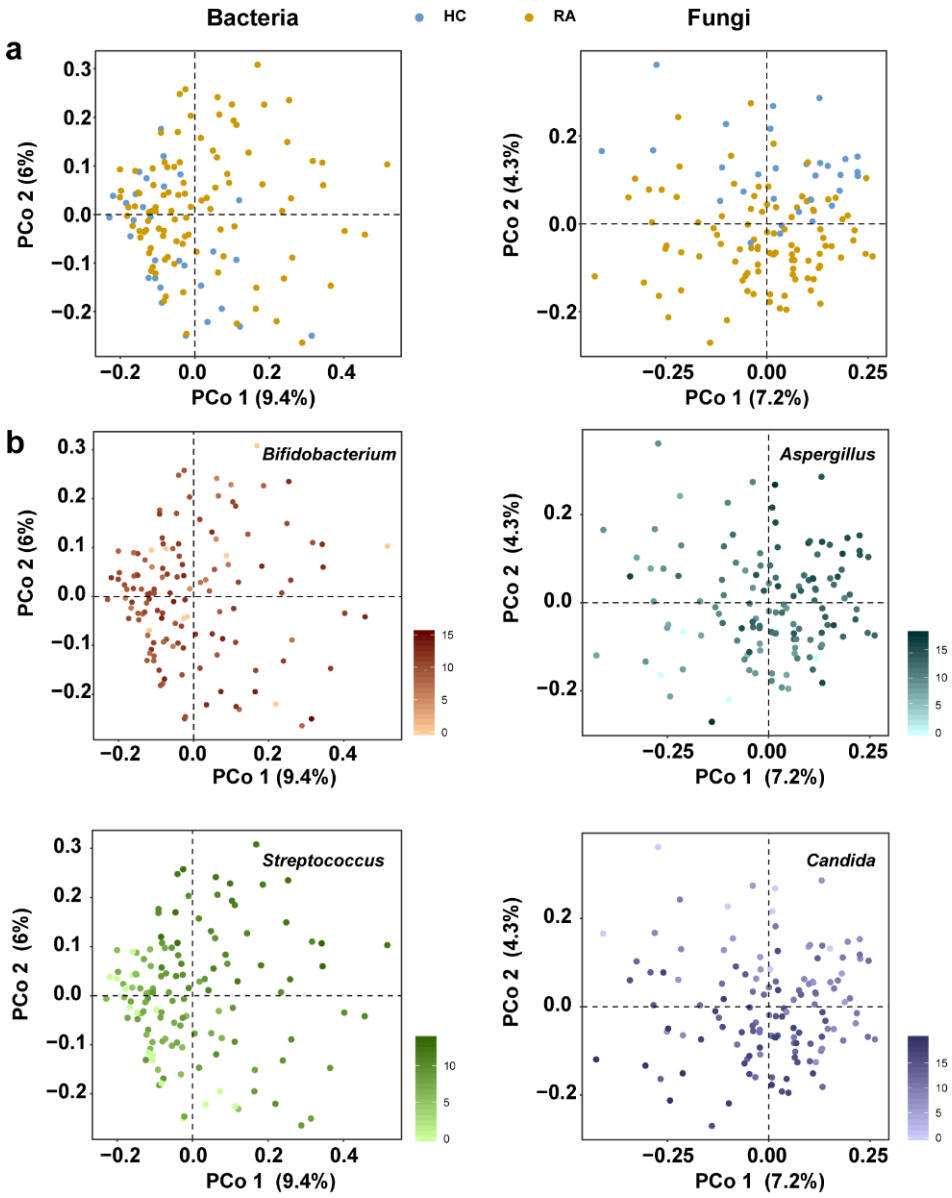

Bacteria
HC
RA
Fungi
a
PCo 2 (6%)
PCo 1 (9.4%)
PCo 2 (4.3%)
PCo 1 (7.2%)
b
Bifidobacterium
Aspergillus
Streptococcus
Candida

Figure 6. Unconstrained principal coordinate analysis (PCoA) of bacterial and fungal communities between HC and RA. (a) The data ordination from the beta diversity metrics for human fecal microbial community structure**.** (b) Ordination analysis indexed based on abundant genera of bacterial (left) and fungal (right) communities. HC, healthy controls; RA, patients with RA.

Table 3. Results on permutational analysis of variance (PERMANOVA)

Bacteria	**Df**	**SumsOfSqs**	**MeanSqs**	**F.Model**	**R^2**	**Pr(>F)**	
Diagnosis	1	0.568	0.56787	2.2563	0.01746	2.00E-04	***
Residuals	127	31.963	0.25168		0.98254		
Total	128	32.531			1		
	Df	**SumsOfSqs**	**MeanSqs**	**F.Model**	**R2**	**Pr(>F)**	
Age	1	0.466	0.46553	1.86272	0.01431	0.002	**
BMI	1	0.343	0.34328	1.37359	0.01055	0.0493	*
Total_cholesterol	1	0.333	0.33307	1.33274	0.01024	0.0705	.
Duration	2	0.574	0.28697	1.14825	0.01764	0.1493	
HDL	1	0.203	0.20323	0.81318	0.00625	0.8217	
Triglyceride	1	0.353	0.35321	1.41331	0.01086	0.0412	*
RA_factor	1	0.167	0.16688	0.66775	0.00513	0.9732	
anti_CCP	1	0.269	0.26944	1.07813	0.00828	0.3117	
CRP	1	0.35	0.35031	1.40172	0.01077	0.0454	*
ESR	1	0.232	0.23171	0.92715	0.00712	0.5988	
Residuals	117	29.24	0.24992		0.89885		
Total	128	32.531			1		

Fungi	**Df**	**SumsOfSqs**	**MeanSqs**	**F.Model**	**R^2**	**Pr(>F)**	
Diagnosis	1	0.885	0.88533	2.8042	0.0216	1.00E-04	***
Residuals	127	40.096	0.31572		0.9784		
Total	128	40.981			1		
	Df	**SumsOfSqs**	**MeanSqs**	**F.Model**	**R2**	**Pr(>F)**	
Age	1	0.583	0.58312	1.84445	0.01423	0.0002	***
BMI	1	0.26	0.26037	0.82356	0.00635	0.8672	
Total_cholesterol	1	0.504	0.50378	1.5935	0.01229	0.0032	**
Duration	2	0.758	0.37907	1.19903	0.0185	0.0546	.
HDL	1	0.319	0.31886	1.00858	0.00778	0.4489	
Triglyceride	1	0.272	0.2715	0.85879	0.00663	0.8059	
RA_factor	1	0.383	0.38336	1.21261	0.00935	0.1014	
anti_CCP	1	0.304	0.30386	0.96113	0.00741	0.56	
CRP	1	0.338	0.33806	1.06932	0.00825	0.3041	
ESR	1	0.271	0.27083	0.85665	0.00661	0.8056	
Residuals	117	36.989	0.31615		0.90259		
Total	128	40.981			1		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

IV. RA patient-associated bacterial and fungal OTUs

We investigated the relative abundances of OTUs in the HC and RA groups by LEfSe analysis. Among 1338 bacterial OTUs and 1595 fungal OTUs, 57 bacterial OTUs and 45 fungal OTUs were differentially abundant (Figures 7a, 8a). In total, 14 bacterial OTUs and 10 fungal OTUs were more abundant in the RA group than in the HC group. The RA-enriched OTUs belonged to the fungal genera *Candida*, *Meyerozyma*, *Penicillium*, *Aurobasidium*, *Xeromyces*, *Coprinopsis*, and *Wallemia*. Furthermore, 43 bacterial OTUs and 35 fungal OTUs were more abundant in the HC group than in the RA group. The HC-enriched OTUs belonged to the fungal genera *Aspergillus*, *Conocybe*, *Monascus*, and *Schizosaccharomyces*.

We investigated RA-associated OTUs via machine learning-based classification. For this analysis, we constructed random forest classification models for bacterial and fungal communities. The random forest models revealed that 70 bacterial OTUs and 70 fungal OTUs were needed to classify HC and RA samples (Figures 7b, 8b). Among these OTUs, 27 bacterial OTUs and 25 fungal OTUs were also identified by LEfSe (7a, 8a, 9). Two bacterial OTUs and four fungal OTUs were more abundant in the RA group, while the remaining OTUs were more abundant in the HC group (Figure 9). These findings imply that decreased abundances of bacterial and fungal OTUs contributed to compositional differences between the HC and RA groups.

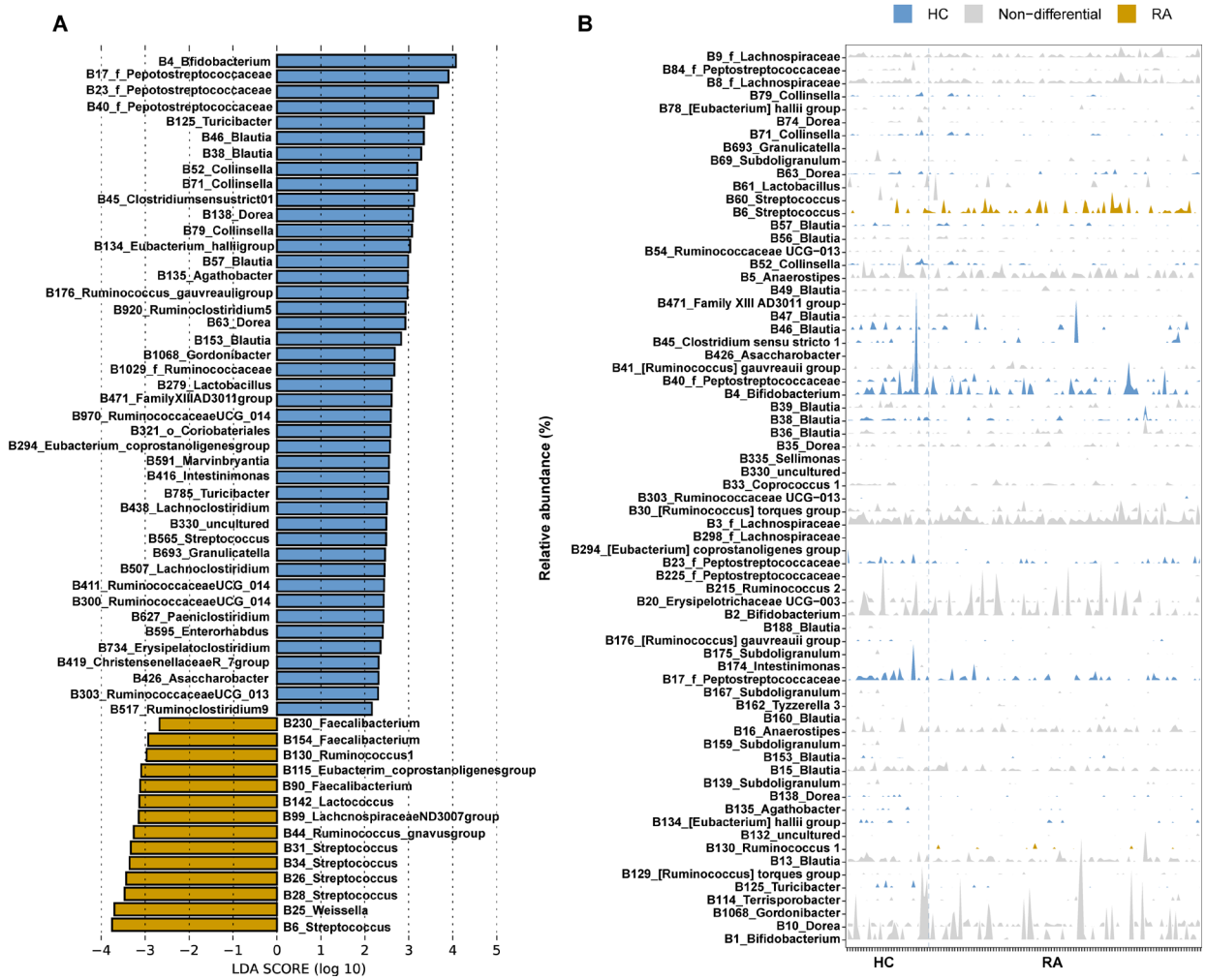

A
B4_Bifidobacterium
B17_f_Peptostreptococcaceae
B23_f_Peptostreptococcaceae
B40_f_Peptostreptococcaceae
B125_Turicibacter
B46_Blautia
B38_Blautia
B52_Collinsella
B71_Collinsella
B45_Clostridiumsensustrict01
B138_Dorea
B79_Collinsella
B134_Eubacterium_hallii group
B57_Blautia
B135_Agathobacter
B176_Ruminococcus_gauvreauii group
B920_Ruminoclostiridium5
B63_Dorea
B153_Blautia
B1068_Gordonibacter
B1029_f_Ruminococcaceae
B279_Lactobacillus
B471_FamilyXIIIAD3011group
B970_RuminococcaceaeUCG_014
B321_o_Coriobacteriales
B294_Eubacterium_coprostanoligenesgroup
B591_Marvinbryantia
B416_Intestinimonas
B785_Turicibacter
B438_Lachnoclostiridium
B330_uncultured
B565_Streptococcus
B693_Granulicatella
B507_Lachnoclostiridium
B411_RuminococcaceaeUCG_014
B300_RuminococcaceaeUCG_014
B627_Paeniclostiridium
B595_Enterorhabdus
B734_Erysipelatoclostiridium
B419_ChristensenellaceaeR_7group
B426_Asaccharobacter
B303_RuminococcaceaeUCG_013
B517_Ruminoclostiridium9
B230_Faecalibacterium
B154_Faecalibacterium
B130_Ruminococcus1
B115_Eubacterim_coprostanoligenesgroup
B90_Faecalibacterium
B142_Lactococcus
B99_LachnospiraceaeND3007group
B44_Ruminococcus_gnavusgroup
B31_Streptococcus
B34_Streptococcus
B26_Streptococcus
B28_Streptococcus
B25_Weissella
B6_Streptococcus
−4 −3 −2 −1 0 1 2 3 4 5
LDA SCORE (log 10)
B
HC
Non−differential
RA
Relative abundance (%)
B9_f_Lachnospiraceae
B84_f_Peptostreptococcaceae
B8_f_Lachnospiraceae
B79_Collinsella
B78_[Eubacterium] hallii group
B74_Dorea
B71_Collinsella
B693_Granulicatella
B69_Subdoligranulum
B63_Dorea
B61_Lactobacillus
B60_Streptococcus
B6_Streptococcus
B57_Blautia
B56_Blautia
B54_Ruminococcaceae UCG−013
B52_Collinsella
B5_Anaerostipes
B49_Blautia
B471_Family XIII AD3011 group
B47_Blautia
B46_Blautia
B45_Clostridium sensu stricto 1
B426_Asaccharobacter
B41_[Ruminococcus] gauvreauii group
B40_f_Peptostreptococcaceae
B4_Bifidobacterium
B39_Blautia
B38_Blautia
B36_Blautia
B35_Dorea
B335_Sellimonas
B330_uncultured
B33_Coprococcus 1
B303_Ruminococcaceae UCG−013
B30_[Ruminococcus] torques group
B3_f_Lachnospiraceae
B298_f_Lachnospiraceae
B294_[Eubacterium] coprostanoligenes group
B23_f_Peptostreptococcaceae
B225_f_Peptostreptococcaceae
B215_Ruminococcus 2
B20_Erysipelotrichaceae UCG−003
B2_Bifidobacterium
B188_Blautia
B176_[Ruminococcus] gauvreauii group
B175_Subdoligranulum
B174_Intestinimonas
B17_f_Peptostreptococcaceae
B167_Subdoligranulum
B162_Tyzzerella 3
B160_Blautia
B16_Anaerostipes
B159_Subdoligranulum
B153_Blautia
B15_Blautia
B139_Subdoligranulum
B138_Dorea
B135_Agathobacter
B134_[Eubacterium] hallii group
B132_uncultured
B130_Ruminococcus 1
B13_Blautia
B129_[Ruminococcus] torques group
B125_Turicibacter
B114_Terrisporobacter
B1068_Gordonibacter
B10_Dorea
B1_Bifidobacterium
HC
RA

Figure 7. Fecal bacterial OTUs affected by the dysbiosis of rheumatoid arthritis. (a) Differentially abundant bacterial OTUs estimated from the LEfse analysis between HC and RA. Blue and yellow bars indicate enrichment of OTUs in HC and RA, respectively. The size of the bars corresponds to the logarithmic discriminant analysis (LDA) score. The threshold of LDA score is 2**.** (b) Bacterial OTUs discriminating between the compositional differences in HC and RA using a random forest classification model. OTUs are colored based on their categorization as "HC-enriched" and "RA-enriched" groups based on their differential abundance test results. Each tick on the x-axis indicates an individual control HC and RA sample. HC, healthy controls; RA, patients with RA.

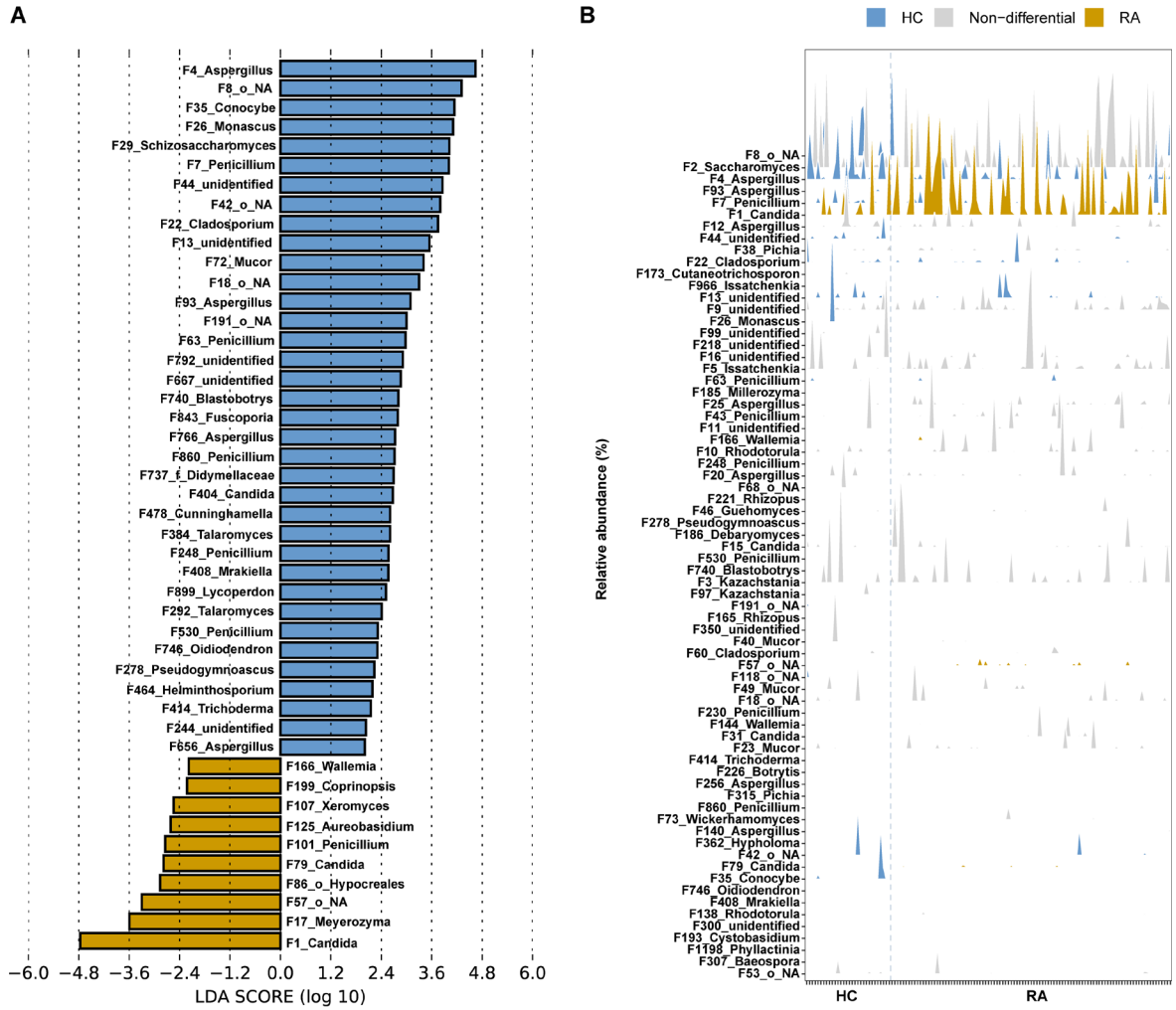

A
F4_Aspergillus
F8_o_NA
F35_Conocybe
F26_Monascus
F29_Schizosaccharomyces
F7_Penicillium
F44_unidentified
F42_o_NA
F22_Cladosporium
F13_unidentified
F72_Mucor
F18_o_NA
F93_Aspergillus
F191_o_NA
F63_Penicillium
F792_unidentified
F667_unidentified
F740_Blastobotrys
F843_Fuscoporia
F766_Aspergillus
F860_Penicillium
F737_f_Didymellaceae
F404_Candida
F478_Cunninghamella
F384_Talaromyces
F248_Penicillium
F408_Mrakiella
F899_Lycoperdon
F292_Talaromyces
F530_Penicillium
F746_Oidiodendron
F278_Pseudogymnoascus
F464_Helminthosporium
F414_Trichoderma
F244_unidentified
F656_Aspergillus
F166_Wallemia
F199_Coprinopsis
F107_Xeromyces
F125_Aureobasidium
F101_Penicillium
F79_Candida
F86_o_Hypocreales
F57_o_NA
F17_Meyerozyma
F1_Candida
−6.0 −4.8 −3.6 −2.4 −1.2 0.0 1.2 2.4 3.6 4.8 6.0
LDA SCORE (log 10)
B
HC
Non−differential
RA
Relative abundance (%)
F8_o_NA
F2_Saccharomyces
F4_Aspergillus
F93_Aspergillus
F7_Penicillium
F1_Candida
F12_Aspergillus
F44_unidentified
F38_Pichia
F22_Cladosporium
F173_Cutaneotrichosporon
F966_Issatchenkia
F13_unidentified
F9_unidentified
F26_Monascus
F99_unidentified
F218_unidentified
F16_unidentified
F5_Issatchenkia
F63_Penicillium
F185_Millerozyma
F25_Aspergillus
F43_Penicillium
F11_unidentified
F166_Wallemia
F10_Rhodotorula
F248_Penicillium
F20_Aspergillus
F68_o_NA
F221_Rhizopus
F46_Guehomyces
F278_Pseudogymnoascus
F186_Debaryomyces
F15_Candida
F530_Penicillium
F740_Blastobotrys
F3_Kazachstania
F97_Kazachstania
F191_o_NA
F165_Rhizopus
F350_unidentified
F40_Mucor
F60_Cladosporium
F57_o_NA
F118_o_NA
F49_Mucor
F18_o_NA
F230_Penicillium
F144_Wallemia
F31_Candida
F23_Mucor
F414_Trichoderma
F226_Botrytis
F256_Aspergillus
F315_Pichia
F860_Penicillium
F73_Wickerhamomyces
F140_Aspergillus
F362_Hypholoma
F42_o_NA
F79_Candida
F35_Conocybe
F746_Oidiodendron
F408_Mrakiella
F138_Rhodotorula
F300_unidentified
F193_Cystobasidium
F1198_Phyllactinia
F307_Baeospora
F53_o_NA
HC
RA

Figure 8. Fecal fungal OTUs affected by the dysbiosis of rheumatoid arthritis. (a) Differentially abundant fungal OTUs estimated from the LEfse analysis between HC and RA. Blue and yellow bars indicate the enrichment of OTUs in HC and RA, respectively. The size of the bars corresponds to the logarithmic discriminant analysis (LDA) score. The threshold of LDA score is 2**.** (b) Fungal OTUs discriminating between the compositional differences in HC and RA using a random forest classification model. OTUs are colored based on their categorization as "HC-enriched" and "RA-enriched" groups according to the results of the differential abundance test. Each tick on the x-axis indicates an individual control HC and RA sample. HC, healthy controls; RA, patients with RA.

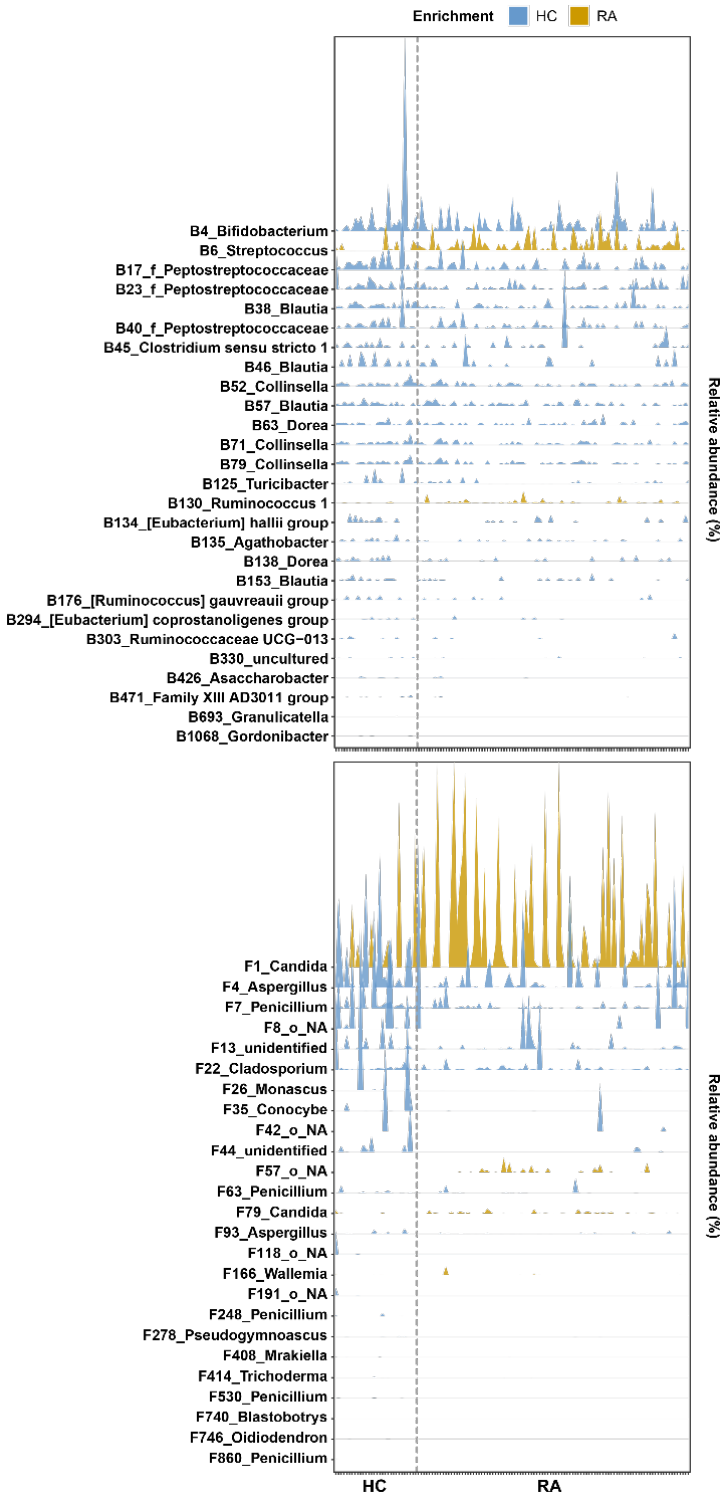

Enrichment
HC
RA
B4_Bifidobacterium
B6_Streptococcus
B17_f_Peptostreptococcaceae
B23_f_Peptostreptococcaceae
B38_Blautia
B40_f_Peptostreptococcaceae
B45_Clostridium sensu stricto 1
B46_Blautia
B52_Collinsella
B57_Blautia
B63_Dorea
B71_Collinsella
B79_Collinsella
B125_Turicibacter
B130_Ruminococcus 1
B134_[Eubacterium] hallii group
B135_Agathobacter
B138_Dorea
B153_Blautia
B176_[Ruminococcus] gauvreauii group
B294_[Eubacterium] coprostanoligenes group
B303_Ruminococcaceae UCG−013
B330_uncultured
B426_Asaccharobacter
B471_Family XIII AD3011 group
B693_Granulicatella
B1068_Gordonibacter
Relative abundance (%)
F1_Candida
F4_Aspergillus
F7_Penicillium
F8_o_NA
F13_unidentified
F22_Cladosporium
F26_Monascus
F35_Conocybe
F42_o_NA
F44_unidentified
F57_o_NA
F63_Penicillium
F79_Candida
F93_Aspergillus
F118_o_NA
F166_Wallemia
F191_o_NA
F248_Penicillium
F278_Pseudogymnoascus
F408_Mrakiella
F414_Trichoderma
F530_Penicillium
F740_Blastobotrys
F746_Oidiodendron
F860_Penicillium
Relative abundance (%)
HC
RA

Figure 9. Microbial signatures associated with RA. A random forest model was used to identify OTUs that explain the gut bacterial (a) and fungal (b) communities. OTUs are colored based on their classification as "HC-enriched" and "RA-enriched," based on the results of differential abundance analysis (Figures S5a, S6a). Random forest models were constructed using a 10-fold cross-validation method. OTUs are arranged along the y-axis according to total abundance. Each mark on the x-axis indicates an individual HC or RA sample. HC, healthy controls; RA, patients with RA.

V. Fecal bacterial–fungal associations

We constructed a correlation-based microbial network to investigate microbial associations. The fecal microbial network of the HC group comprised 701 nodes and 1419 edges (Figure 10a), whereas the fecal microbial network of the RA group comprised 801 nodes and 1679 edges (Figure 10b). Degree and betweenness centrality did not significantly differ between the HC and RA groups (Figure 11). There were more fungal nodes in the HC group than in the RA group, and hub composition differed between groups. Hub nodes were defined as nodes in which degree, betweenness centrality, and closeness centrality were in the top 1%. Based on this criterion, the hub of the bacterial–fungal interkingdom network of the HC group was the fungal OTU F87_*Penicillium* (Figure 10c). In the RA group, the hub node was the bacterial OTU B3_f_Lachnospiraceae in the *Lachnospiraceae* family (Figure 10d). These data suggest that fungi influence the microbial community composition in HC, while bacteria associated with dysbiosis influence the microbial community in patients with RA.

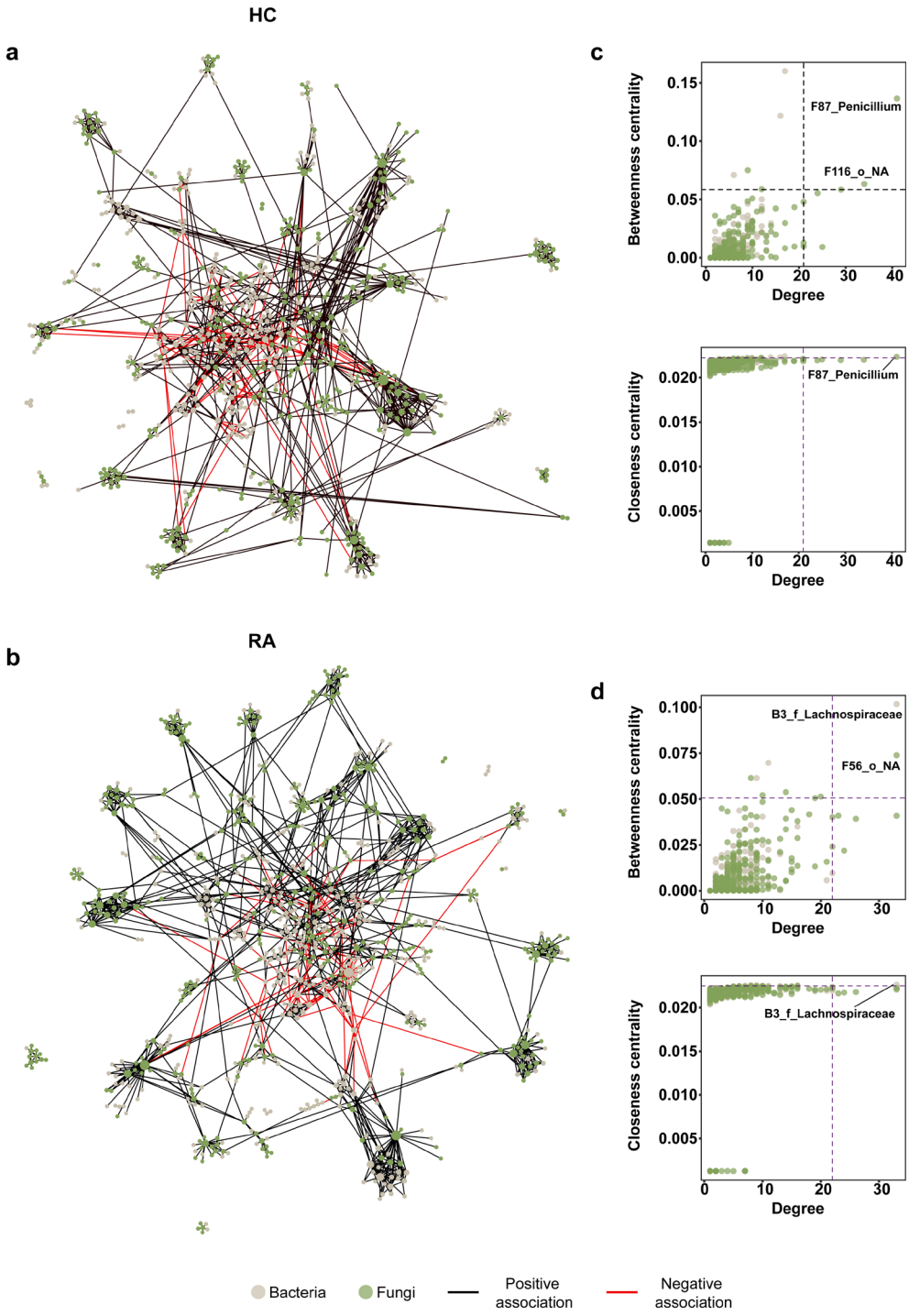

HC
a
c
Betweenness centrality
0.15
0.10
0.05
0.00
F87_Penicillium
F116_o_NA
0
10
20
30
40
Degree
Closeness centrality
0.020
0.015
0.010
0.005
F87_Penicillium
Degree
RA
b
d
Betweenness centrality
0.100
0.075
0.050
0.025
0.000
B3_f_Lachnospiraceae
F56_o_NA
Degree
Closeness centrality
0.020
0.015
0.010
0.005
B3_f_Lachnospiraceae
Degree
Bacteria
Fungi
Positive association
Negative association

Figure 10. Interkingdom co-occurrence networks and hub nodes of fecal microbiota. (a) Interkingdom HC networks. (b) Interkingdom RA networks. In panels **a** and **b**, each node corresponds to an out; edges between nodes correspond to positive (black) or negative (red) correlations inferred from OTU abundance profiles using the SparCC method ($P < 0.05$, correlation values of < -0.3 or > 0.3). OTUs that belong to different microbial kingdoms are indicated by colors (bacteria, ivory; fungi, green), and node size reflects degree of centrality. (c) Hub nodes of microbial HC networks. (d) Hub nodes of microbial RA networks. In panels **c** and **d**, the hub was defined as a node in which degree, betweenness centrality, and closeness centrality were in the top 1%. Dashed lines indicate threshold values of degree, betweenness centrality, and closeness centrality. HC, healthy controls; RA, patients with RA.

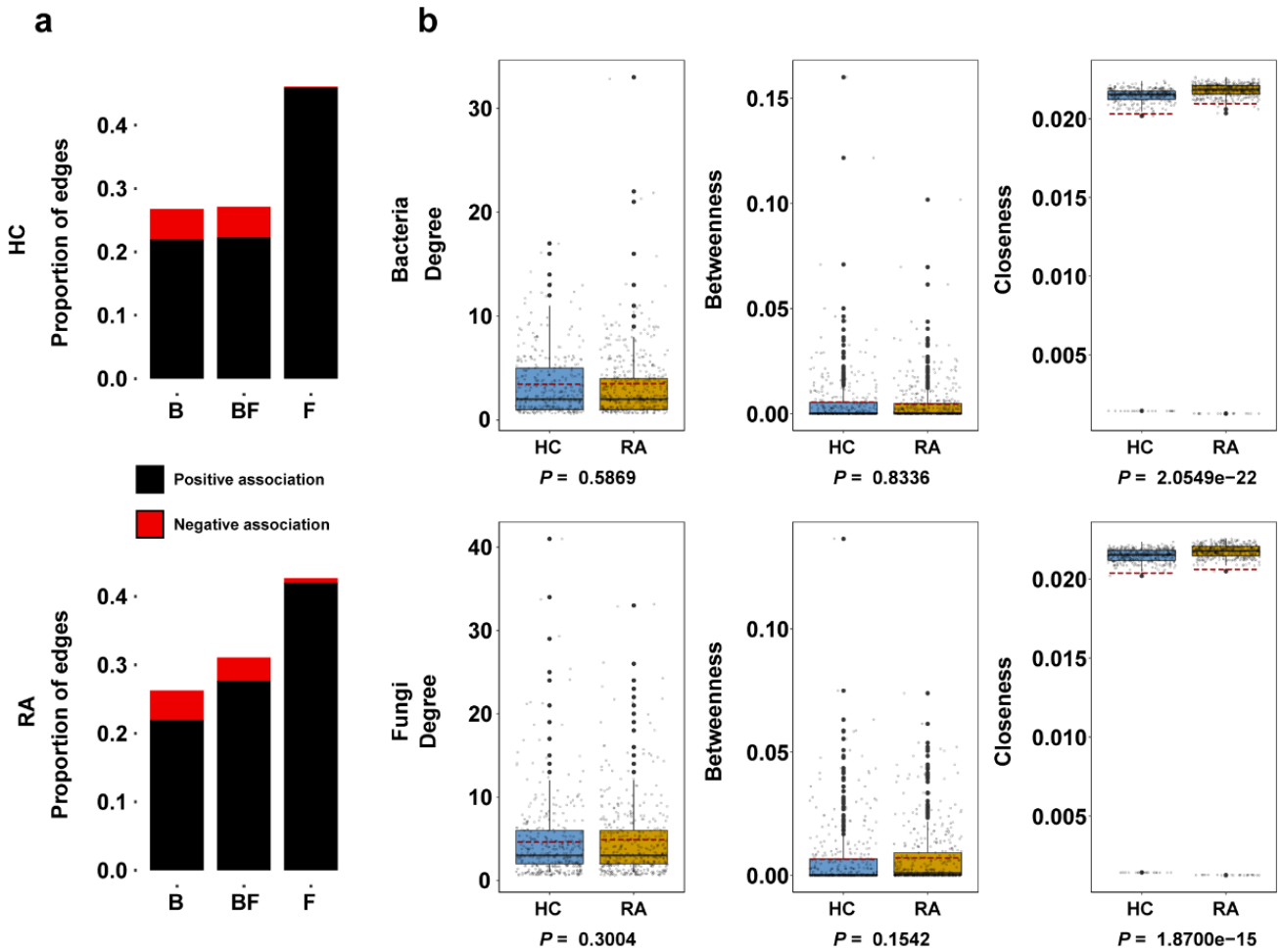

a
b
HC
Proportion of edges
0.4
0.3
0.2
0.1
0.0
B
BF
F
Positive association
Negative association
RA
Proportion of edges
Bacteria
Degree
Betweenness
Closeness
Fungi
Degree
HC
RA
P = 0.5869
P = 0.8336
P = 2.0549e−22
P = 0.3004
P = 0.1542
P = 1.8700e−15

Figure 11. A comparison of the topological properties between microbial HC and RA networks. (a) The proportion of edges (associations) comprising microbial Healthy (upper panel) and RA (bottom panel) networks. The proportion of positive and negative associations is indicated as black- and red-colored bars, respectively. B, bacterial–bacterial association; BF, bacterial–fungal association, F, fungal–fungal association. (b) Comparison of the topological properties of microbial control and RA networks. Pairwise comparison of the topological properties of bacterial nodes is indicated on the upper panel, whereas that of fungal nodes is displayed on the bottom panel. HC, healthy controls; RA, patients with RA.

VI. Changes in the fecal fungal community in response to medication

The use of antirheumatic drugs alters the microbial community. For example, etanercept partially alleviated bacterial dysbiosis in ***patients*** with RA (Picchianti-Diamanti et al., 2018). The gut bacterial community can also determine the responses of RA patients to MTX (Artacho et al., 2021). We examined the effects of RA therapeutics on the fungal community. We stratified the patients into three groups: csDMARDs (patients treated with csDMARDs; n = 55), csDMARDs + biologics (patients treated with csDMARDs and biologics; n = 32), and biologics (patients treated with biologics; n = 8). Because few patients were treated with biologics, the biologics group was excluded from further analysis. The genus *Candida* was more abundant in the csDMARDs and csDMARDs + biologics groups than in the HC group (Figure 12a, b). However, among patients with RA, the relative abundance of *Candida* was lower in the csDMARDs group than in the csDMARDs + biologics group (Figure 12a). Differences in *Candida* abundance within the RA groups were not statistically significant (Figure 12b). Compared with the csDMARDs + biologics group, the relative abundance of *Aspergillus* was decreased in the csDMARDs group, while the relative abundance of *Penicillium* was increased (Figure 12a).

PCR using *Candida*-specific primers was performed on fecal samples randomly selected from the HC and RA groups. *C. albicans* was a fungal species with significantly greater abundance in RA samples. In the RA group, bands of 200 to 300 bp were observed; such bands were absent in the HC group (Figure 13). The amount of *C. albicans* DNA was significantly greater in the RA group than in the HC group

(HC, Ct = 30.4 ± 1.33; RA, Ct = 23.9 ± 3.08). The amount of *C. albicans* DNA was slightly greater in the csDMARDs + biologics group than in the csDMARDs group (csDMARDs, Ct = 24.0 ± 3.02; csDMARDs + biologics, Ct = 23.7 ± 3.19) (Figure 12c).

In terms of clinical factors, the relative abundance of *Candida* was significantly positively correlated with age (Spearman r = 0.29153, P = 0.000802), rheumatoid factor (RF) level (Spearman r = 0.20217, P = 0.021579), C-reactive protein (CRP) level (Spearman r = 0.2927, P = 0.000762), erythrocyte sedimentation rate (ESR) (Spearman r = 0.27676, P = 0.000136), MTX dose (Spearman r = 0.18648, P = 0.034349), and total cholesterol level (Spearman r = 0.24822, P = 0.004563) (Figure 12d). The relative abundance of *Aspergillus* was significantly negatively correlated with those factors (RF level: Spearman r = −0.24993, P = 0.004283; ESR: Spearman r = −0.20742, P = 0.018344; MTX dose: Spearman r = −0.22778, P = 0.00943; and total cholesterol level: Spearman r = −0.238, P = 0.006607).

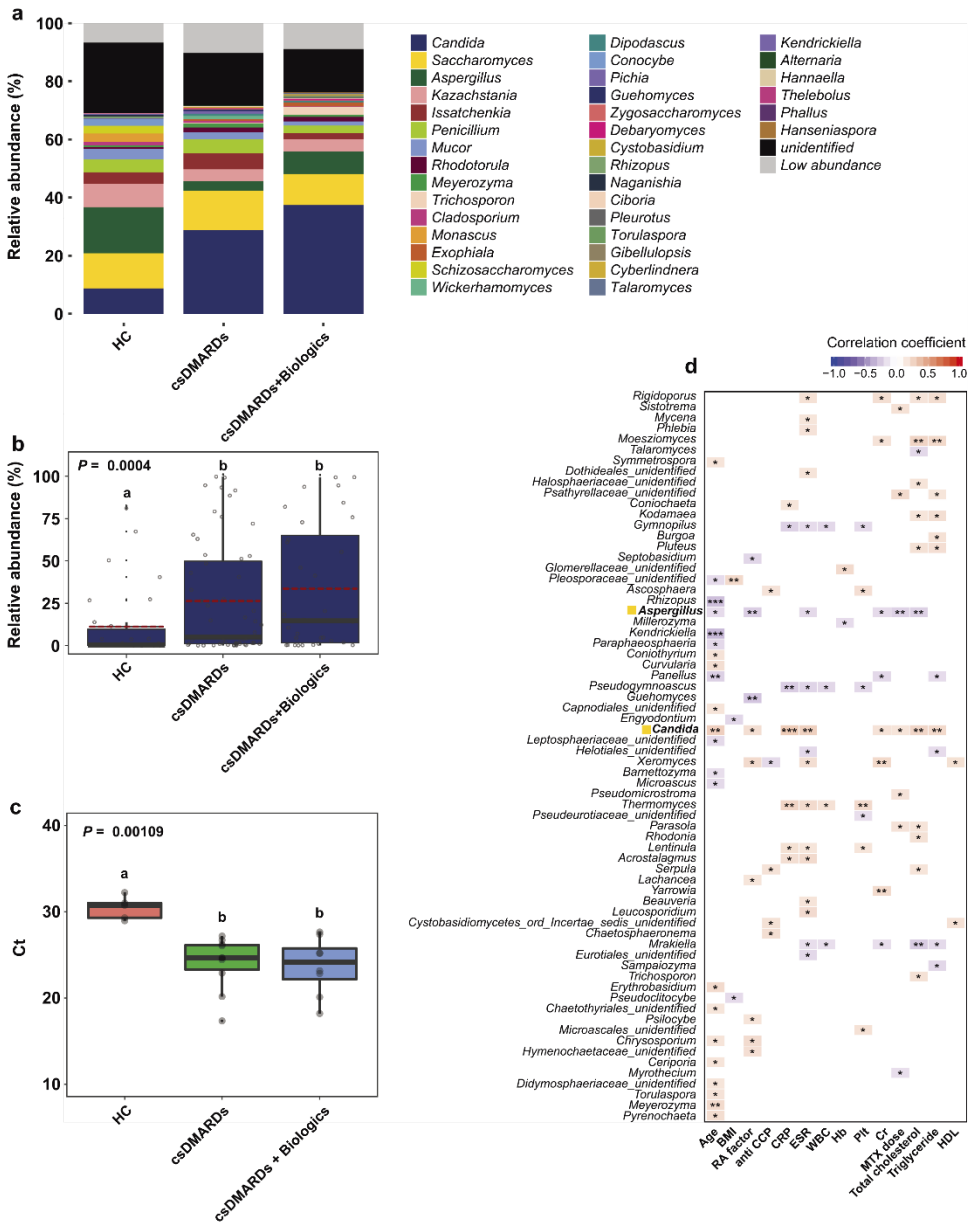

a
Relative abundance (%)
HC
csDMARDs
csDMARDs+Biologics
Candida
Saccharomyces
Aspergillus
Kazachstania
Issatchenkia
Penicillium
Mucor
Rhodotorula
Meyerozyma
Trichosporon
Cladosporium
Monascus
Exophiala
Schizosaccharomyces
Wickerhamomyces
Dipodascus
Conocybe
Pichia
Guehomyces
Zygosaccharomyces
Debaryomyces
Cystobasidium
Rhizopus
Naganishia
Ciboria
Pleurotus
Torulaspora
Gibellulopsis
Cyberlindnera
Talaromyces
Kendrickiella
Alternaria
Hannaella
Thelebolus
Phallus
Hanseniaspora
unidentified
Low abundance
b
P = 0.0004
c
P = 0.00109
Ct
csDMARDs + Biologics
d
Correlation coefficient
Age
BMI
RA factor
anti CCP
CRP
ESR
WBC
Hb
Plt
Cr
MTX dose
Total cholesterol
Triglyceride
HDL
Candida
Aspergillus

Figure 12. Effects of medication on *Candida* abundance. (a) Fecal mycobiota composition according to medication. (b) Relative abundance of *Candida*. Letters indicate statistical significance, as determined by Kruskal–Wallis test followed by Dunn's test. (c) Results of quantitative PCR analysis of *C. albicans*. Letters indicate statistical significance, as determined by analysis of variance followed by Tukey's honestly significant difference test. (d) Correlations between relative abundances of fungal OTUs and quantitative variables. Correlation coefficients were estimated using Spearman's rank correlation. Asterisks indicate statistical significance (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$). Red and blue boxes indicate positive and negative correlations, respectively.

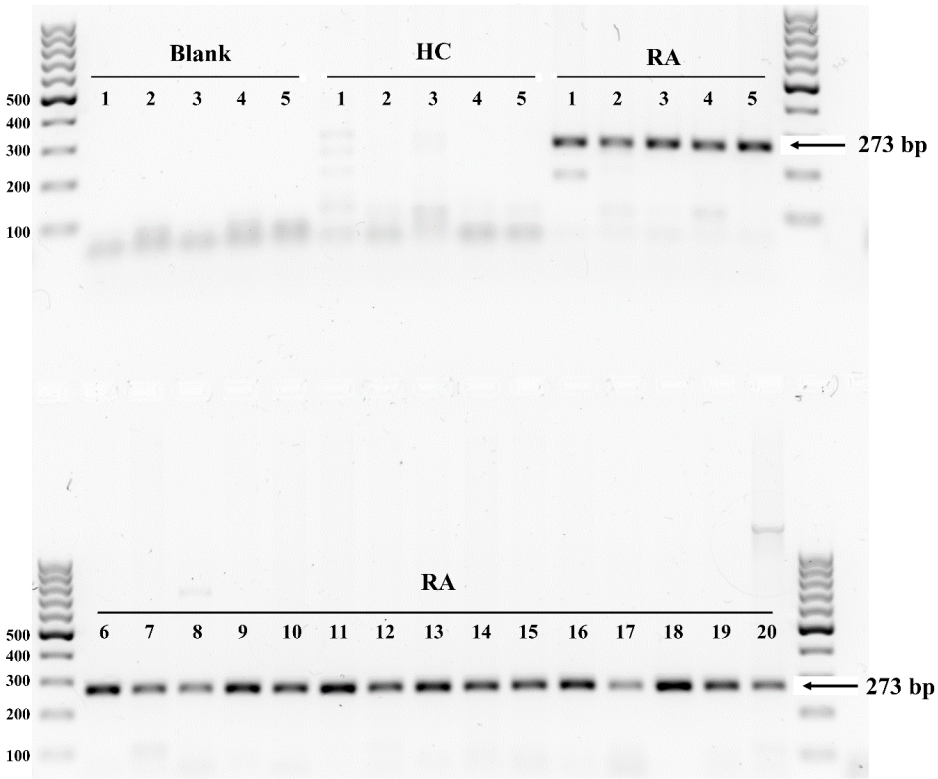

Blank
HC
RA
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
500
400
300
200
100
273 bp
RA
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
500
400
300
200
100
273 bp

Figure 13. Agarose gel electrophoresis image of the *C. albicans* specific PCR products. Control and HC exhibit weak primer bands at the bottom. RA confirmed the presence of *C. albicans* by observing significant bands at 273 bp. Blank, water; HC, healthy controls; RA, patients with RA.

VII. Fungal dysbiosis can be used for characterization of RA

We identified core OTUs (or prevalent OTUs) in fecal samples: core bacterial OTUs were detected in 85% of the 129 fecal samples, while core fungal OTUs were detected in 70% of the fecal samples. Five core bacterial OTUs belonged to *Lachnospiraceae*, whereas five core fungal OTUs belonged to *Candida*, *Aspergillus*, *Issatchenkia*, *Cladosporium*, and an unidentified fungal genus (Figure 14a). Subsequently, three overlapping core fungal OTUs were discovered, but no overlapping core bacterial OTU was identified (Figure 14b, c). Among the bacterial core OTUs, B3_f_Lachnospiraceae, B8_f_Lachnospiraceae, and B9_f_Lachnospiraceae could distinguish between HC and RA groups using a random forest model. However, LEfSe revealed that differences in the relative abundances of these OTUs were not statistically significant (Figures 9, 7). Among the fungal core OTUs, F1_Candida, F4_Aspergillus, and F22_Cladosporium could distinguish between HC and RA groups using both LEfSe and a random forest model (Figures 8, 9, 14c). The association between ACPA and RF, which are serological markers of RA, and the fecal fungal community were investigated. *Aspergillus* and *Candida*, which differed in abundance between the HC and RA groups, were not associated with ACPA. Aspergillus was significantly associated negatively with RF, whereas Candida was correlated positively (Figure 12d). Therefore, changes in the fungal microbial community, particularly involving *Candida* and *Aspergillus*, could be a feature of RA.

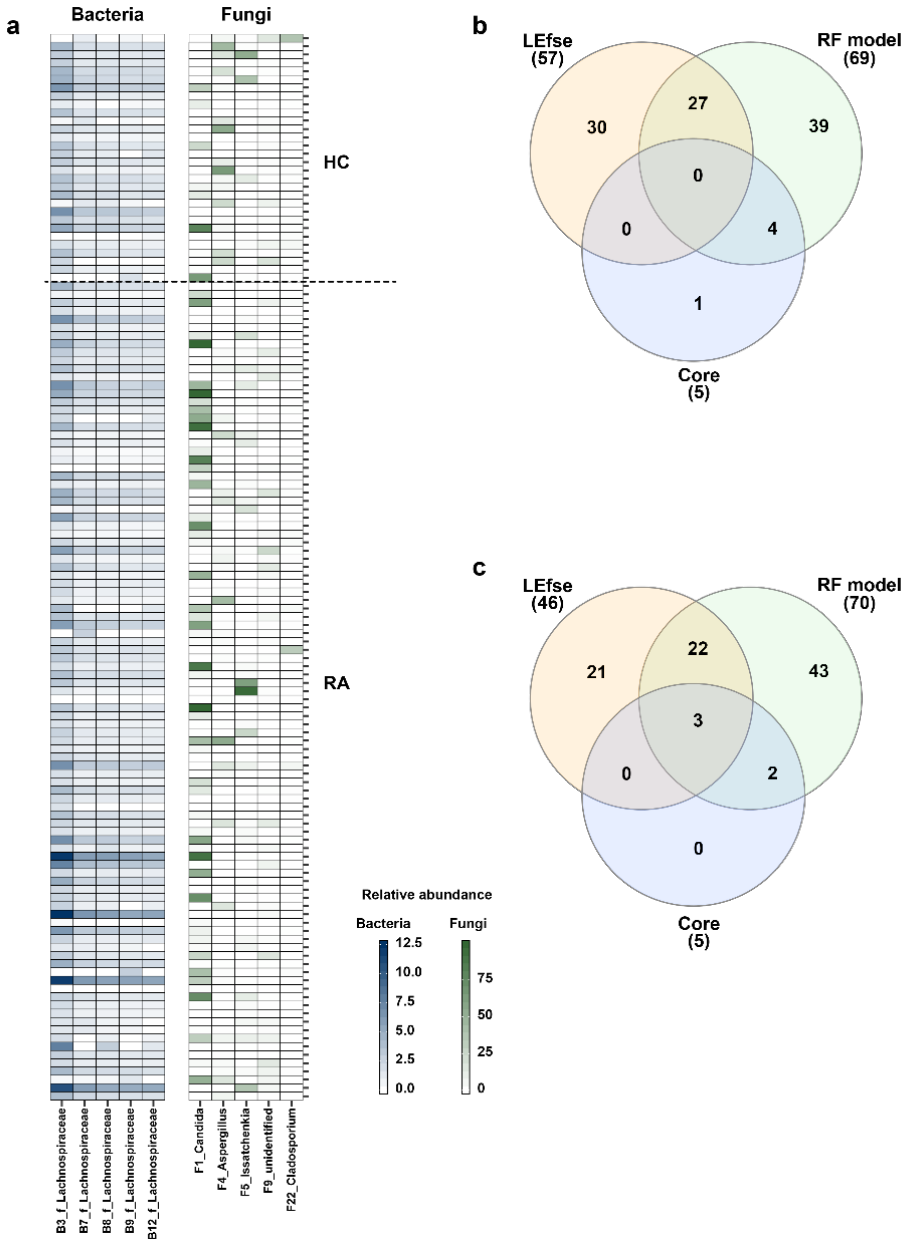

a
Bacteria
Fungi
HC
RA
B3_f_Lachnospiraceae
B7_f_Lachnospiraceae
B8_f_Lachnospiraceae
B9_f_Lachnospiraceae
B12_f_Lachnospiraceae
F1_Candida
F4_Aspergillus
F5_Issatchenkia
F9_unidentified
F22_Cladosporium
Relative abundance
Bacteria
12.5
10.0
7.5
5.0
2.5
0.0
Fungi
75
50
25
0
b
LEfse
(57)
RF model
(69)
Core
(5)
30
27
39
0
0
4
1
c
LEfse
(46)
RF model
(70)
Core
(5)
21
22
43
3
0
2
0

Figure 14. Analysis of core OTUs in fecal microbiota. (a) Core OTUs were identified based on 85% prevalence for bacteria (dark blue) and 70% prevalence for fungi (dark green). Box colors indicate relative abundances of OTUs. Greater color intensity indicates higher relative abundance. Each mark on the y-axis indicates an individual sample. Venn diagrams of the numbers of (b) bacterial and (c) fungal OTUs identified by LEfSe, the random forest model, and core out analysis.

DISCUSSION

The gut or fecal microbiome plays an important role in several human diseases. We found a distinct fecal microbial composition in patients with RA. Although the relative bacterial abundance differed between the RA and HC groups, alpha diversity did not differ (Figures 1, 2, 4a). CAP and random forest analysis revealed that *Bifidobacterium* and *Streptococcus* were representative of the HC and RA groups, respectively (Figures 5, 7). Similar distributions of bacterial genera in fecal samples have been identified in Asian cohorts (Zhang et al., 2015;Chiang et al., 2019;Jeong et al., 2019;Liu et al., 2020).

Fungi affect the composition of the bacterial community (Sam et al., 2017;Deveau et al., 2018;van Tilburg Bernardes et al., 2020). A bacterial–fungal interkingdom network analysis showed that F87_Penicillium was the hub OTU in the HC group, while B3_f_Lachnospiraceae was the hub OTU in the RA group (Figure 10). The *Penicillium* subgenus produces numerous beneficial secondary metabolites, which have antibiotic, antifungal, immunosuppressive, and cholesterol-lowering properties (Kumar et al., 2018). The altered relative abundance of *Penicillium* was restored in RA patients via treatment with csDMARDs alone (Figure 12a). *Lachnospiraceae* are reportedly abundant in ACPA-positive patients (Mangalea et al., 2021;Rooney et al., 2021). Because 75 (76.5%) of our RA patients were ACPA-positive, we hypothesized that the hub OTU shifted from F87_Penicillium to B3_f_Lachnospiraceae in patients with RA. The difference between RA and HC groups was clearer in the fungal community than in the bacterial community. F1_*Candida* and F4_*Aspergillus* were the most differentially abundant fungal genera

(Figure 14).

Aspergillus caused substantial changes in the fungal community. An OTU that belonged to *Aspergillus* (F4_Aspergillus) was a core fungal OTU (Figure 14a); it was more abundant in the HC group than in the RA group (Figure 9). *Saccharomyces cerevisiae* has a beneficial effect on human health (Nash et al., 2017;Wu et al., 2021). Alterations in fecal fungal communities have mostly been studied in Western cohorts. We found a significant difference in *Aspergillus* abundance, rather than *Saccharomyces* abundance, between the HC and RA groups. *Saccharomyces* is reportedly more common among individuals who consume a Western diet (e.g., bread, beer, and dairy products), while *Aspergillus* is more common among individuals with a vegetarian diet (Suhr et al., 2016;Hallen-Adams and Suhr, 2017). In Japan and China, where the diets are similar to the diet consumed in South Korea, *Aspergillus* was more abundant than *Saccharomyces* in the fecal fungal community of healthy adults (Motooka et al., 2017;Qiu et al., 2020). Therefore, based on the dietary proportions of vegetables and fermented soybean foods, *Aspergillus* is an essential member of the fecal fungal community in Koreans (Suhr et al., 2016;Hallen-Adams and Suhr, 2017).

Fungi had a substantial effect on fecal microbial community composition in patients with RA; *Candida* was the most abundant fecal genus (Figure 2b, d). *Candida* spp. are frequently detected in the human gastrointestinal tract (Hallen-Adams and Suhr, 2017) and feces (Gurleen and Savio, 2016); their abundance is increased in patients with inflammatory bowel disease, cystic fibrosis, and vaginal candidiasis (Limon et al., 2017). We found that medications for RA affected the

fungal community composition, such that the abundance of *C. albicans* was enhanced by csDMARDs and biologics (Figure 12c). This is consistent with previous reports of increased *C. albicans* abundance in patients with inflammatory bowel disease (IBD) who were treated with immunosuppressants (Li et al., 2014;Sokol et al., 2017;Imai et al., 2019). During treatment with disease-modifying antirheumatic drugs and tumor necrosis factor-α inhibitors, patients with RA showed an impaired *C. albicans*-specific Th17 response, which led to an increased abundance of *C. albicans*. Although the increased abundances of *C. albicans* in patients with RA and patients with IBD do not exclude the possibility that dysbiosis is caused by disease, they suggest that the dysbiosis is caused by medication. Moreover, although an increased abundance of *C. albicans* may result in opportunistic infections, the risk of candidiasis is low in patients with RA because they retain an effective immune response to *C. albicans* (Bishu et al., 2014).

The decreased abundance of *Aspergillus* and increased abundance of *Candida* in the feces of patients in our study suggest that such changes are specific to RA. Further studies regarding *Aspergillus* will provide insight into its role in the healthy fecal fungal community and its effect on human health. Our findings suggest that changes in the fungal community could be used as an indicator of fecal dysbiosis in patients with RA.

In conclusion, we investigated dysbiosis and fungal–bacterial interactions in the fecal microbial communities of patients with RA. Changes in fungal communities indicated significant dysbiosis between HC and patients with RA, whereas changes in bacterial communities did not. Future research should examine whether the

increased abundance of *C. albicans* is caused by immunosuppressive or immunomodulatory medications. Our results were limited in that they comprised bioinformatics-based predictions of the effects of RA-related changes on fecal microbial communities. *In vivo* experiments are required to confirm that RA alters the fungal community. Therefore, an experimental validation studies concerning the effects of *C. albicans* and *Aspergillus* on RA-related immune pathways are needed. *Aspergillus* was more abundant in the fecal fungal community of healthy Koreans, whereas *Saccharomyces* was comparable to patients with RA. Further research is necessary to clarify precisely our findings differ from the Western cohort.

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류마티스관절염 환자의 장내 미생물 군집 불균형

이 은 하

초 록

장내 미생물 군집 (Gut microbiota)은 장내에 살고 있는 다양한 박테리아, 바이러스, 곰팡이 및 기타 미생물들의 집합체를 의미한다. 인간의 경우 다른 신체 부위에 비해 장내에 가장 많은 미생물 군집을 가지고 있는데, 이들은 장내 환경과 상호작용을 통해 인간의 건강과 질병에 깊은 연관이 있다고 알려져 있다. 미생물 불균형(dysbiosis)은 장내 미생물 군집의 균형이 깨짐으로써 발생하며, 이는 다양한 질병과 관련이 있다고 보고되었다. 따라서 이 논문에서는 장내 박테리아와 곰팡이의 역할을 포함하여 대사성 질환, 신경성 질환, 면역조절 그리고 약물 대사에 미치는 영향에 대해 논의하였다. 장내 미생물 군집은 영양소 흡수 및 에너지 대사에 중요한 역할을 담당하고 있어서 미생물 군집의 불균형이 발생할 경우 심혈관 및 대사성 질환의 발생에 영향을 준다. 미생물 군집의 불균형은 염증성 반응을 유발하여 뇌-장간 축을 통해 뉴런의 손상을 유발하거나 면역조절에 문제를 일으키기도 한다.

장내 미생물 군집은 인체에서 면역 조절에 중요한 역할을 한다. 면역 조절에 문제가 발생하여 생기는 자가면역질환들은 장내 미생물 군집과의 상호작용 연구가 많이 이루어졌다. 이러한 연구들 중 대표적인 질환으로 류마티스관절염(RA)이 있으며, RA는 장내 미생물 군집과 밀접한 관련이 있다. 특히 곰팡이 세포벽이 병의 발생에 중요한 역할을 한다고 보고되었으나 이에 대한 자세한 정보는 부족하다. 따라서 본 연구에서는

RA 환자와 건강한 대조군으로부터 대변 미생물군집의 조성과 상관관계를 조사하였다. 결과적으로 RA 환자군에서는 박테리아보다 곰팡이 군집에서의 변화가 더 뚜렷하게 확인되었다. 정상인에 비해 *Aspergillus*는 감소하고, *Candida*가 크게 증가하는 특징을 보였으며, 미생물 군집 구조변화 분석 결과에서도 곰팡이가 핵심적인 역할을 하는 것으로 확인되었다. 이러한 결과는 곰팡이가 장내 미생물군집의 핵심 역할을 하며, RA의 발병 과정에 중요한 역할을 한다는 것을 시사한다.

장내 미생물 군집은 약물의 효과를 증가시키거나 반대로 부작용을 유발하기도 한다. 장내 박테리아가 약물 대사에 작용하는 것이 알려졌으며, 이를 활용한 개인 맞춤형 치료제 연구도 수행되고 있다. 최근에는 미생물 군집의 불균형을 회복시켜 질병을 예방하거나 치료에 적용하려는 연구가 진행되고 있다. 예를 들면 프리 또는 프로바이오틱스를 사용한 환자의 경우 장내 미생물군집의 불균형이 일부 회복되었고, 그 결과 면역조절 능력의 향상과 증상 완화의 효과를 보였다.

본 논문에서는 장내 미생물 군집의 불균형이 질병에 미치는 영향을 탐색하였고, 이를 활용한 질병 치료 연구에 단서를 제공하였다. 특히 류마티스관절염 질환에서 장내 곰팡이가 중요한 역할을 하는 것을 확인하였다. 이를 통해 장내 곰팡이 군집 연구의 중요성을 제안하였다. 이러한 장내 미생물군집체에 대한 다각적 검토는 질병의 치료 및 예방을 위한 연구에 새로운 접근법을 제시할 수 있다.

주요어: 장내 미생물 군집, *Candida*, *Aspergillus*, 미생물 군집 불균형, 류마티스관절염

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