SEMMELWEIS EGYETEM

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Ph.D. értekezések

3400.

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INVESTIGATION OF THE MICROBIOME INVOLVED IN THE PROGRESSION OF ROSACEA

PhD thesis

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Budapest
2026

Table of Contents

List of Abbreviations

^{14}C	Carbon 14
B. oleronius	*Bacillus oleronius*
BRNL2	Butyrophilin-like 2
CARD15	Caspase recruitment domain-containing protein 15
CGRP	Calcitonin gene-related peptide
CP	Control patient
D. folliculorum	*Demodex folliculorum*
DNA	Deoxyribonucleic acid
ETR	Erythematotelangiectatic rosacea
GST	Glutathione S-transferase
H_2S	Hydrogen sulfide
HLA	Human leukocyte antigen
HLA-DRA	Human leukocyte antigen DR isotype alpha chain
HMP	Human Microbiome Project
H. pylori	*Helicobacter pylori*
INF-γ	Interferon gamma
IL-1β	Interleukin-1 beta
IL-8	Interleukin-8
IL-17	Interleukin-17
IPL	Intense pulsed light
ITS	Internal transcribed spacer
IQR	Interquartile range
KLK-5	Kallikrein-5
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
LL-37	Active form of cathelicidins antimicrobial peptide
NGS	Next-Generation Sequencing
NO	Nitric oxide
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
PACAP	Pituitary adenylate cyclase-activating peptide

PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational Multivariate Analysis of Variance
PPR	Papulopustular rosacea
PRR	Pattern Recognition Receptor
ROS	Reactive oxygen species
ROSCO	Rosacea Consensus
rRNA	Ribosomal ribonucleic acid
RP	Patient with rosacea
SCFAs	Short-chain fatty acids
S. epidermidis	*Staphylococcus epidermidis*
SIBO	Small intestine bacterial overgrowth
TACR3	Tachykinin receptor 3
Th1	T helper cell 1
Th17	T helper cell 17
TLRs	Toll-like receptors
TLR 2	Toll-like receptor 2
TNF-α	Tumor necrosis factor alpha
TRP channels	Transient receptor potential channels
UV	Ultraviolet

1 Introduction

1.1 Rosacea

Rosacea is a chronic inflammatory skin disease that affects the face, most often observed in individuals of a lighter skin phenotype, typically in middle age. The condition is characterized by the presence of skin manifestations, including flushing or persistent erythema, telangiectasia, papules, pustules, and hyperplasia of the connective tissue and sebaceous glands. In some cases, there may be ocular involvement. Rosacea is a significant source of discomfort on a global scale, often exerting a substantial impact on their quality of life (1). The precise pathogenesis of this condition remains to be fully understood. The potential pathological mechanisms encompass a dysregulation of the innate and possibly also of the acquired immune system (2), a disruption of neurovascular signaling (3), connective tissue degeneration, nutritional and chemical factors, ultraviolet (UV) and sun exposure, functional abnormalities of the pilosebaceous unit and microbial infections (4).

1.1.1 Epidemiology

The global prevalence of rosacea is estimated to be approximately 5%. The extant literature indicates that women are affected more frequently than men (5, 6). In women, the onset of the disease typically occurs at the age of 35, with a peak prevalence between the ages of 61 and 65. Conversely, men typically present with the disease at a later stage, often after the age of 50, with the highest incidence observed between the ages of 76 and 80 (7). It is noteworthy that children can also be affected by rosacea. However, its prevalence in this age group is significantly lower (8).

1.1.2 Pathogenesis

The precise pathogenesis of rosacea remains to be definitively elucidated. It is hypothesized that a variety of endogenous and exogenous factors contribute to the development and maintenance of the disease.

There exists a multitude of potential triggers for rosacea flare-ups or exacerbation of existing symptoms. These include, but are not limited to, ultraviolet (UV) exposure, psychological stress, the consumption of alcoholic beverages, spicy foods, and dairy products (9).

In addition to these environmental factors, studies also point to a genetic predisposition. For instance, a twin study by Aldrich et al. (10) demonstrated that the genetic factor accounts for approximately 50% of the risk of developing the disease, while the remaining 50% appears to be attributable to environmental influences. The molecular basis of genetic involvement remains to be fully elucidated. Potentially relevant genes include glutathione S-transferase (GST), butyrophilin-like 2 (BRNL2), various human leukocyte antigen DR isotype alpha chain (HLA-DRA) loci, nucleotide-binding oligomerization domain-containing protein 2 / caspase recruitment domain-containing protein 15 (NOD2/CARD15), and tachykinin receptor 3 (TACR3) (11).

Additionally, neurovascular activity has been identified as a contributing factor in the pathophysiology of rosacea (3). A significant number of patients have reported experiencing recurrent flushing, as well as persistent erythema on the face. The phenomenon of transient erythema, clinically referred to as “flushing”, is likely attributable to the presence of various bioactive substances, including pituitary adenylate cyclase-activating peptide (PACAP), substance P, calcitonin gene-related peptide (CGRP), adrenomedullin, transient receptor potential (TRP) channels, and nitric oxide (NO) (11). In addition to the sensory nervous system, the autonomic nervous system is also considered to be a cause of neurovascular inflammation. Conversely, persistent erythema has been shown to be a consequence of cytokine-induced inflammation. The primary actors in this process are T cells, macrophages, and mast cells (12, 13).

The integrity of the skin surface is paramount as a physical barrier that enables the functionality of the innate immune system. However, individuals afflicted with rosacea exhibit heightened sensitivity of the skin, augmented transepidermal water loss, and the presence of *Demodex* mites, which disrupt the integrity of the epidermis (14). Research has demonstrated that, in addition to pattern recognition receptors (PRRs), the concentration of toll-like receptor 2 (TLR-2) is elevated in rosacea patients compared to healthy individuals (15). As demonstrated in Figure 1, these are activated by microbes, which in

turn trigger keratinocytes to produce proinflammatory cytokines and chemokines, including interleukin-8 (IL-8), interleukin-1 beta (IL-1β), and tumor necrosis factor alpha (TNF-α). Vascular hyperreactivity is initiated by chemotaxis of neutrophils, which, in turn, is triggered by increased concentrations of cytokines and chemokines (16). Furthermore, TLR-2 has been demonstrated to enhance the expression of the serine protease kallikrein-5 (KLK-5). In conjunction with protease-3, these enzymes subsequently cleave cathelicidins into their active form, LL-37 (17). Research has demonstrated that skin affected by rosacea exhibits augmented expression of cathelicidin and KLK-5 in comparison to that of healthy individuals (18, 19). LL-37 exerts an antimicrobial effect, modulates neutrophil chemotaxis, and stimulates the release of cytokines and chemokines from mast cells (20). These effects are reflected in telangiectasia, erythema, and inflammation (21). LL-37 is also involved in angiogenesis by enhancing the proliferation of endothelial cells and promoting the expression of extracellular matrix components (22).

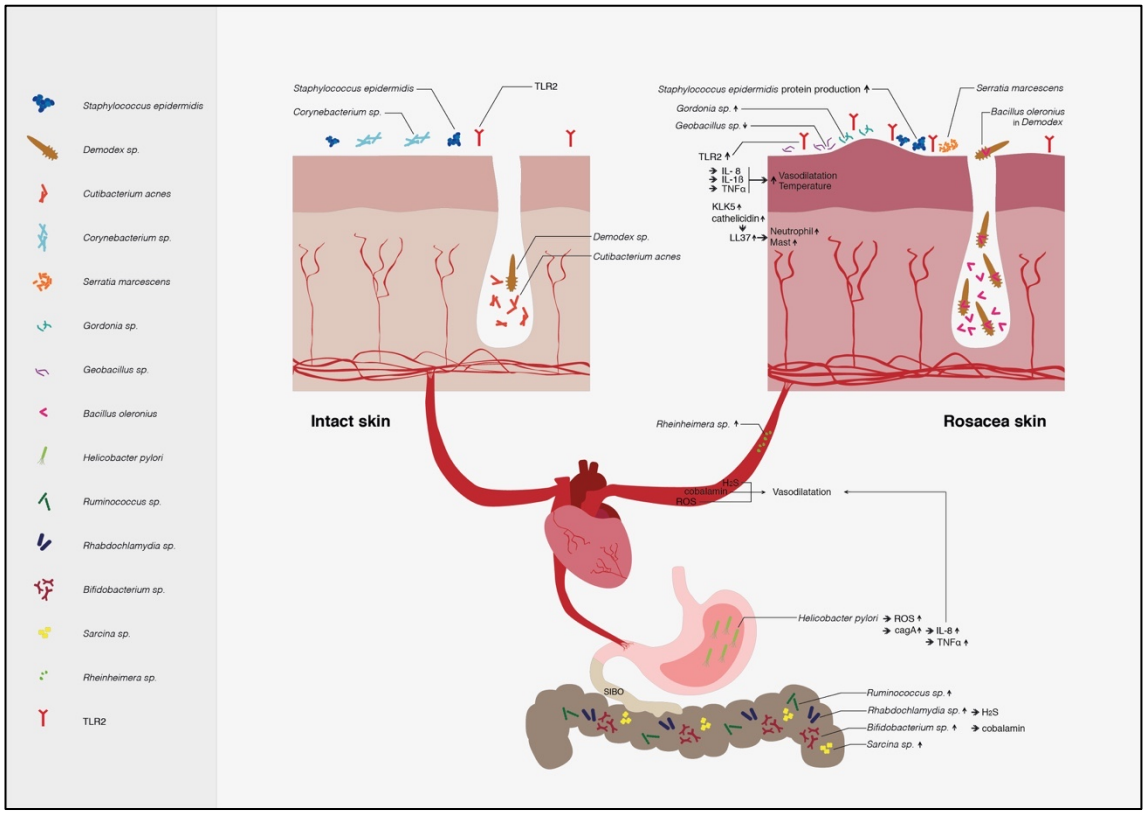

Figure 1. Interaction between microorganisms and immune response in rosacea-affected skin (23)

In addition to the innate immune response, the adaptive immune response also plays an important role in the pathogenesis of rosacea. Buhl et al. demonstrated that the inflammatory infiltration, which is observed in all rosacea phenotypes, is associated with an increased presence of T helper cell 1 (Th1) and T helper cell 17 (Th17) cells. Consequently, this results in the upregulation of proinflammatory cytokines, including TNF-α, interferon gamma (INF-γ) and interleukin 17 (IL-17) (2).

The hypothesis that there is a correlation between microorganisms and the pathogenesis of rosacea has been postulated for some time. These include *Demodex* mites, *Staphylococcus epidermidis* (*S. epidermidis*), *Bacillus oleronius* (*B. oleronius*), and *Helicobacter pylori* (*H. pylori*).

Demodex folliculorum (*D. folliculorum*), a species of mite, has been observed to inhabit the hair follicles of human skin (24). Research has demonstrated that the population density of *D. folliculorum* is elevated in patients diagnosed with rosacea compared to individuals considered to be healthy (25).

S. epidermidis is the most prevalent commensal bacterium on healthy human skin. Consequently, the pathogenic role of these bacteria must be reevaluated. A substantial body of research has demonstrated that these bacteria are predominantly present in pustules and exhibit an altered secretion profile. Research has demonstrated that rosacea patients exhibit increased skin temperature in comparison with healthy controls. Furthermore, *S. epidermidis* strains have been observed to manifest an altered protein expression pattern under conditions of elevated temperature. These findings suggest the potential involvement of certain temperature-dependent proteins in the pathogenesis of the disease (26).

The hypothesis that *D. folliculorum* facilitates the transfer of additional microorganisms to the skin has been postulated. This hypothesis is supported by the successful isolation of *B. oleronius*, a Gram-negative rod-shaped bacterium, from mites found on patients with papulopustular rosacea (27). Patients diagnosed with erythematotelangiectatic rosacea exhibit serum reactivity to the 62- and 83-kDa protein of *B. oleronius*, suggesting a potential role for these highly immunogenic proteins in the etiology of the condition (28). When exposed to *B. oleronius* proteins, neutrophils have been observed to

exhibit degranulation and produce inflammatory cytokines (29, 30). In addition, it has been demonstrated that the elevated temperature of inflamed rosacea skin facilitates the proliferation of *B. oleronius* and increases the production of its 62-kDa protein (31), which is likely to perpetuate the inflammatory response.

The role of *H. pylori* in the pathogenesis of rosacea remains a subject of debate, with opinions ranging from coincidental to plausible or inconclusive, depending on the diagnostic methods and patient populations under consideration. A significant body of research utilizing the ^{14}C-urea breath test has identified a markedly elevated rate of *H. pylori* colonization in patients diagnosed with rosacea, in comparison to the control group (32, 33). A number of reports have indicated the presence of seropositivity for *H. pylori* in individuals diagnosed with rosacea, although this finding is not universally accepted (34-38). *H. pylori* has been demonstrated to produce reactive oxygen species (ROS), including nitric oxide (NO), which has been shown to be elevated in the plasma of rosacea patients (39, 40). NO plays a pivotal role in vasodilation, inflammation, and immune modulation in the skin. It has been hypothesized that NO from *H. pylori* could contribute to the flushing and erythema seen in rosacea, or even play a role in the disease's inflammation (41). Furthermore, *H. pylori* has been observed to express the cagA cytotoxin, which has been demonstrated to induce the release of pro-inflammatory cytokines such as TNF-α and IL-8, thereby further promoting inflammation (42).

1.1.3 Clinical forms of Rosacea and diagnostic

Rosacea was originally divided into four subtypes: erythematotelangiectatic (ETR), inflammatory papulopustular (PPR), phymatous and ocular. However, the ROSCO (Rosacea Consensus) classification introduced in 2017 abounded this rigid system in favour of phenotypic classification (43). The aim was to enable a more flexible and individualized diagnosis, given that rosacea is usually associated with variable objective skin changes that are often accompanied by subjective symptoms.

Accordingly, the diagnosis of cutaneous rosacea is made on the basis of two diagnostic features as well as major and minor features (Tab. 1).

Table 1. Diagnostic criteria of cutaneous rosacea according to Schaller et al. (44)

Diagnostic features	**Major features**	**Minor features**
Phymatous changes	Flushing / transient erythema	Burning sensation of the skin
Persistent erythema	Papules and pustules	Stinging sensation of the skin
	Telangiectasias	Dry sensation of the skin
		Oedema

In approximately 20-50% of cases, cutaneous rosacea is accompanied by ocular rosacea. In approximately 90% of cases, the ocular rosacea manifests as an isolated condition (45). The condition typically manifests in both eyes and is not contingent on the intensity of the cutaneous manifestations. The diagnosis is made clinically in collaboration with the ophthalmologists (46). The presence of clinical signs indicative of ocular rosacea has been observed to include posterior blepharitis, Meibomian gland dysfunction, and secondary inflammatory changes to the ocular surface (45).

1.1.4 Treatment

The general measures for treating rosacea are tailored to the respective phenotypes. A comprehensive approach to prevention involves the identification and avoidance of potential triggers, including but not limited to elevated temperatures, direct exposure to sunlight, the consumption of alcohol and spicy foods, and psychological distress. This strategy is further supported by the implementation of ultraviolet (UV) protection measures, the regular application of appropriate skin care regimes, and the dissemination of educational material to patients. In cases where psychological counselling is deemed necessary, it can be pursued as a form of support (45).

Persistent erythema can be treated topically with brimonidine or, considering off-label use, systemically with beta-blockers. It has been demonstrated that physical procedures, including intense pulsed light (IPL) and laser therapy, can be an effective treatment option for telangiectasias (45).

The papulopustular phenotype of rosacea can be treated topically with metronidazole 7.5 mg/g, azelaic acid 150 mg/g, or ivermectin 10 mg/g. A combination of different topical agents is also a possibility. In addition to the aforementioned agents, permethrin, benzyl benzoate, retinoids, and calcineurin inhibitors have been utilized off-label. In cases where a patient exhibits an inadequate response to purely topical treatment, or in instances where the symptoms are deemed severe, the administration of systemic treatment with low-dose doxycycline (40mg) is recommended. This recommendation is made with the understanding that the decision to pursue systemic treatment should be made in conjunction with the decision to administer topical treatment, if deemed necessary. Low-dose isotretinoin (47), tetracyclines, and azithromycin can also be used off-label. In cases of severe rosacea, a combination of systemic and topical therapy should be used concurrently (45).

Systemic treatment with low-dose isotretinoin has been considered for cases of connective tissue and sebaceous gland hyperplasia (off-label). Alternatively, surgical procedure or laser therapy is available (45).

The ocular phenotype of rosacea necessitates interdisciplinary care in collaboration with ophthalmologists. In addition to maintaining consistent eyelid margin care and the use of tear substitutes, topical treatment with ciclosporin can be employed. Alternatively, azithromycin, tetracyclines, or topical steroids can be used (off-label). Doxycycline (in low or conventional doses) and azithromycin can be administered systemically (both off-label). Another treatment that can be considered is IPL (45, 48). Figure 2 provides an overview of the current guidelines for the recommended treatment of rosacea, which were compiled by experts in the field. The guidelines were developed through a structured consensus process (45).

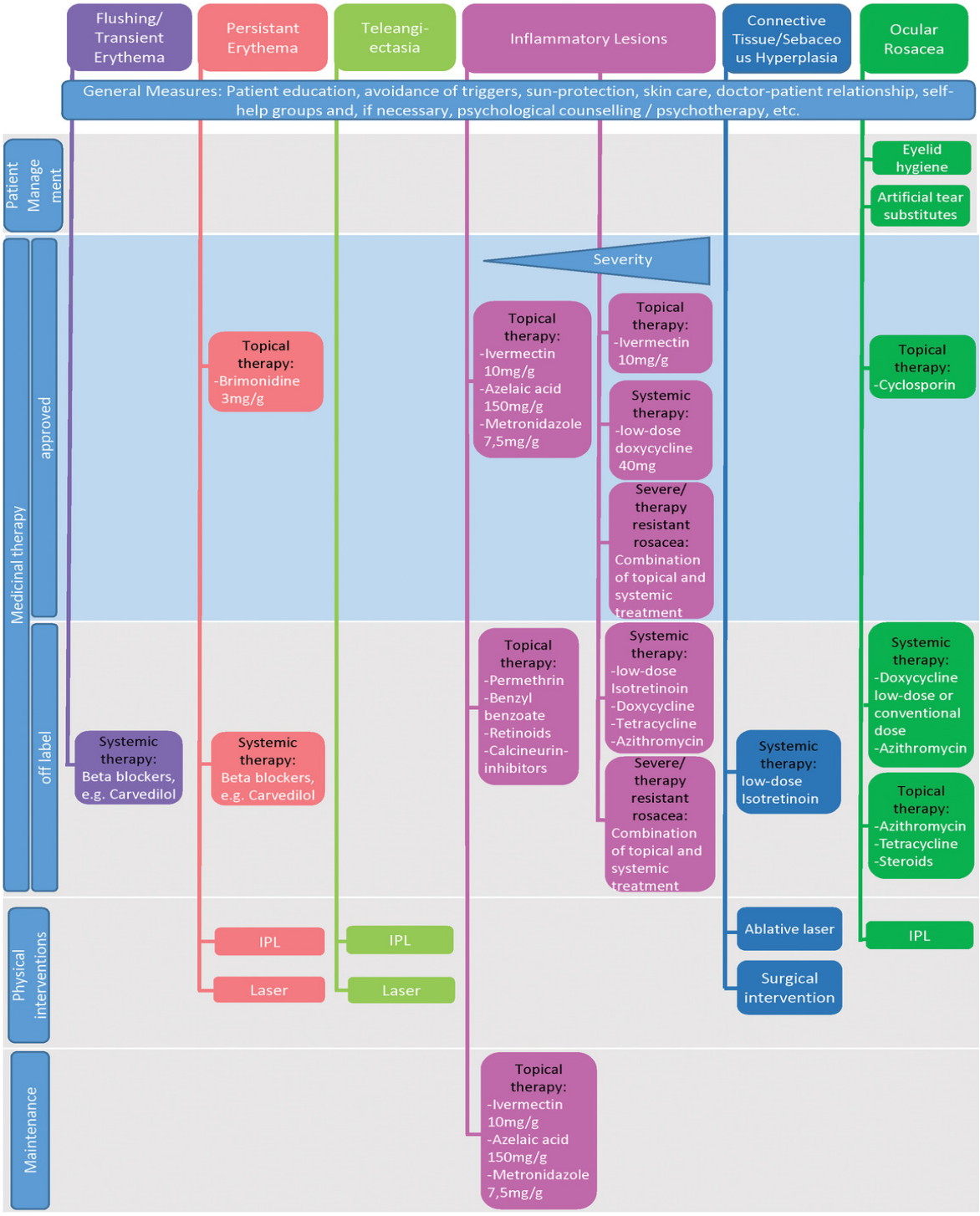

Figure 2. Overview of treatment options in rosacea by Clanner-Engelshofen et al. (45)

1.1.5 Psychosocial aspects

A considerable number of patients diagnosed with rosacea experience significant discomfort due to their persistent cutaneous condition. Patients frequently report a diminished quality of life, as well as symptoms of depression and anxiety, particularly in relation to stigmatization and social exclusion (49). The psychological burden is closely related to the severity of the symptoms (50). However, when treatment is targeted and appropriate, it can significantly improve the quality of life of those affected (51).

1.2 Microbiome

The microbiome is defined as the sum of all microorganisms that colonize and influence a macroorganism. These include bacteria (microbiome), archaea, viruses (virome), fungi (mycobiome), protozoa, and parasites. The vast majority of microorganisms are non-pathogenic and coexist in a state of symbiosis with host cells. The Human Microbiome Project (HMP) sought to identify and characterize all the microorganisms residing on human epithelial surfaces, with the objective of enhancing our comprehension of how the human microbiome affects health and disease (52). The advent of molecular genetic testing in recent years has enabled the analysis of the composition of the microbiome in a specific anatomical region, as well as the inference of biochemical activities from the microbial composition (53).

2 Objectives

1. Characterize and compare the bacterial and fungal microbiome compositions of skin, blood, and stool in individuals with rosacea and in healthy controls.

2. Investigate whether alterations in the gut microbiome (bacterial and fungal), potentially translocated via the bloodstream, contribute to the observed changes in the skin microbiome of rosacea patients.

3. Identify specific bacterial and fungal genera in stool and skin samples that are significantly associated with rosacea and explore their potential correlation with disease pathology.

4. Analyze the predicted biochemical activity of bacterial communities to establish mechanistic links between microbiome alterations and rosacea pathogenesis.

5. Assess the co-occurrence and potential synergistic interactions between bacterial and fungal species in the gut and skin and their role in rosacea development and progression.

3 Methods

3.1 Ethical Considerations

Samples were collected in accordance with the guidelines approved by the Ethics Committee of Semmelweis University (SE RKEB: 282/2020). The study was conducted in strict compliance with the ethical principles of the Declaration of Helsinki, which ensure the protection and dignity of all study participants. All participants gave their written consent to participate in the study and to the publication of anonymised data from their study results. The data presented in the thesis are completely anonymised and do not allow any conclusions to be drawn about individual persons.

3.2 Sample collection

As part of this microbiome study, skin, blood and stool samples from a total of 18 patients with newly diagnosed rosacea and healthy controls were analysed. The data was collected between February and August 2021 at the outpatient clinic of the Department of Dermatology, Venerology and Dermatooncology at Semmelweis University in Budapest, Hungary.

Only untreated patients with a first clinical diagnosis of rosacea were included. Exclusion criteria were pregnancy, the use of pro- or antibiotics in the last six months, gastrointestinal diseases or complaints in the last four weeks and any topical or systemic therapy for a skin disease in the same period. Nine healthy volunteers with a comparable age and gender distribution were recruited as a control group. The characteristics of the study participants are shown in Table 2.

Table 2. Characteristics of the study participants in terms of number, gender distribution, median age (in years) with interquartile range (IQR) and clinical rosacea phenotypes.

	Number of participants, gender (m/f)	**Age, year (Median + IQR)**	**Types of rosacea**
Rosacea patients	18, (4/14)	42, IQR: 15	14 PPR, 1 ETR, 3 PPR + ETR
Control patients	9, (2/7)	39, IQR: 13.5	NA

For each study participant, a minimum of 3 mL of whole blood was collected in citrate-filled VACUETTE® tubes (Greiner Bio-One, Stonehouse, UK). Skin swabs and stool samples were collected in Zymo DNA/RNA Shield (Zymo Research Corp., Irvine, CA, USA). The skin samples were obtained using sterile foam-tipped swabs from both cheeks. The swabs were swabbed vigorously for 30 seconds in each area to ensure adequate sample acquisition, with the swabs being rotated during this process. The study participants were instructed to refrain from washing their faces for a period of 24 hours prior to sample collection. All samples were stored at -80°C immediately after collection and kept until deoxyribonucleic acid (DNA) extraction.

3.3 DNA isolation

Genomic DNA was isolated from skin and stool samples using the Zymo-BIOMICS DNA Miniprep Kit (Zymo Research Corp., Irvine, CA, USA). For the extraction of DNA from blood samples, the NucleoSpin Blood Mini Kit (Macherey-Nagel, Allentown, PA, USA) was used according to the manufacturer's instructions.

3.4 16S rRNA Gene Library Preparation

To analyse the bacterial communities, the V3-V4 region of the 16S ribosomal ribonucleic acid (rRNA) gene was amplified using specifically tagged primers. Polymerase chain reaction (PCR) amplification and subsequent purification of the products were performed according to the standard Illumina protocol. The quality and fragment size of the amplified products were analysed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) with the DNA 1000 Kit.

Sequencing was performed on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) using the MiSeq Reagent Kit v3 (600 cycles, paired-end). Sequencing was performed with equimolar concentrations of pooled PCR products. Negative controls for DNA extraction and PCR were included in each analysis run to detect possible contamination.

All analyses were performed in technical duplication from two independently isolated DNA samples per patient. The raw sequencing data were obtained via Illumina BaseSpace and subsequently processed using the CosmosID bioinformatics platform (54). Taxonomic and species-specific assignment was performed using the 16S workflow of CosmosID-HUB Microbiome, based on the naïve Bayes classifier from the DADA2 package using the SILVA database (version 138).

3.5 ITS Gene Library Preparation

The conventional Illumina protocol for analysing fungal metagenomes was adapted to enable the investigation of internal transcript spacer (ITS) mycobiota. PCR conditions were optimised by increasing the volume of purified DNA used to 6.25 µL per reaction and reducing the primer volume to 3 µL to minimise primer dimer formation. The number of amplification cycles was increased to 30 to ensure sufficient enrichment of the target region. To remove non-specific amplification products and primer-dimers, a two-step purification procedure was performed, consisting of two consecutive bead clean-ups with 25 µL and 10 µL QuantaBio SparQ PureMag beads (QIAGEN, Germantown, MD, USA). This allowed the desired amplicons to be effectively enriched.

3.6 Illumina MiSeq Sequencing

All experimental steps were performed in duplicates to minimise contamination and increase reproducibility. Negative controls for both DNA extraction and PCR amplification were included in each batch to detect possible contamination-related artefacts from reagents. The quality and size distribution of the PCR-libraries were checked using the Agilent 2100 Bioanalyzer and the DNA 1000 Kit (Agilent Technologies, Waldbronn, Germany). Libraries were then normalised to equimolar concentrations, pooled and sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) using the MiSeq Reagent Kit v3 (600cycles, paired-end). The raw sequence data were retrieved via Illumina BaseSpace and analysed using the CosmosID bioinformatics platform (CosmosID Inc., Germantown, MD, USA).

3.7 Statistical data analysis

Comparative statistical analyses were conducted on sample cohorts to evaluate microbial diversity and taxonomic differences. Alpha diversity (Chao1 index) was assessed using the Wilcoxon Rank Sum test, while beta diversity was evaluated via Principal Coordinates Analysis (PCoA) based on Bray-Curtis and Jaccard distance matrices, followed by PERMANOVA (Permutational Multivariate Analysis of Variance). The statistical significance variations in the relative abundance of bacterial taxa among cohorts were determined by implementing the Mann-Whitney U-test. All statistical analyses were performed using integrated tools within the CosmosID bioinformatics platform (CosmosID Inc., Germantown, MD, USA), with statistical significance set at a two-tailed p-value ≤ 0.05. Furthermore, LefSe (Linear Discriminant Analysis Effect Size) analysis was conducted via CosmosID to identify significant features – including taxonomic groups and metabolic pathways – that distinguish the two cohorts.

4 Results

A subsequent investigation into four blood samples obtained from patients suffering from rosacea and one control blood sample revealed only low levels of bacterial 16S rRNA reads. All samples from these individuals were excluded from the comparative studies. A sufficient amount of read numbers in stool, blood and skin samples from the fourteen rosacea patients and eight control patients were collected for the purpose of comparison and evaluation of the data. The median number of reads within a single sample, irrespective of the sample type, was 186,792 (IQR: 16,548) after 16S rRNA sequencing, and 20,067 (IQR: 18,558) after ITS sequencing.

4.1 Composition of the bacterial microbiome of the skin, blood, and stool of rosacea patients and healthy controls

Irrespective of whether patients suffering from rosacea or samples from healthy subjects were analyzed, differing results pertaining to the composition of the microbiome were obtained, depending on the type of sample. The principal component analysis (PCoA) diagram illustrates that the stool, blood and skin microbiome results have created three distinct groups (Figure 3A). The heatmap diagram of bacterial genera revealed that bacteria occurred in stool, blood and skin samples, with different abundances (Figure 3B). The genera *Cutibacterium*, *Corynebacterium* and *Neisseria* were found to be prevalent in the skin microbiome, yet were scarcely detected or undetectable in the stool and blood microbiome. The genus *Staphylococcus* was detected in some blood microbiomes, appeared in high abundance in the skin microbiomes and was absent in the stool microbiomes.

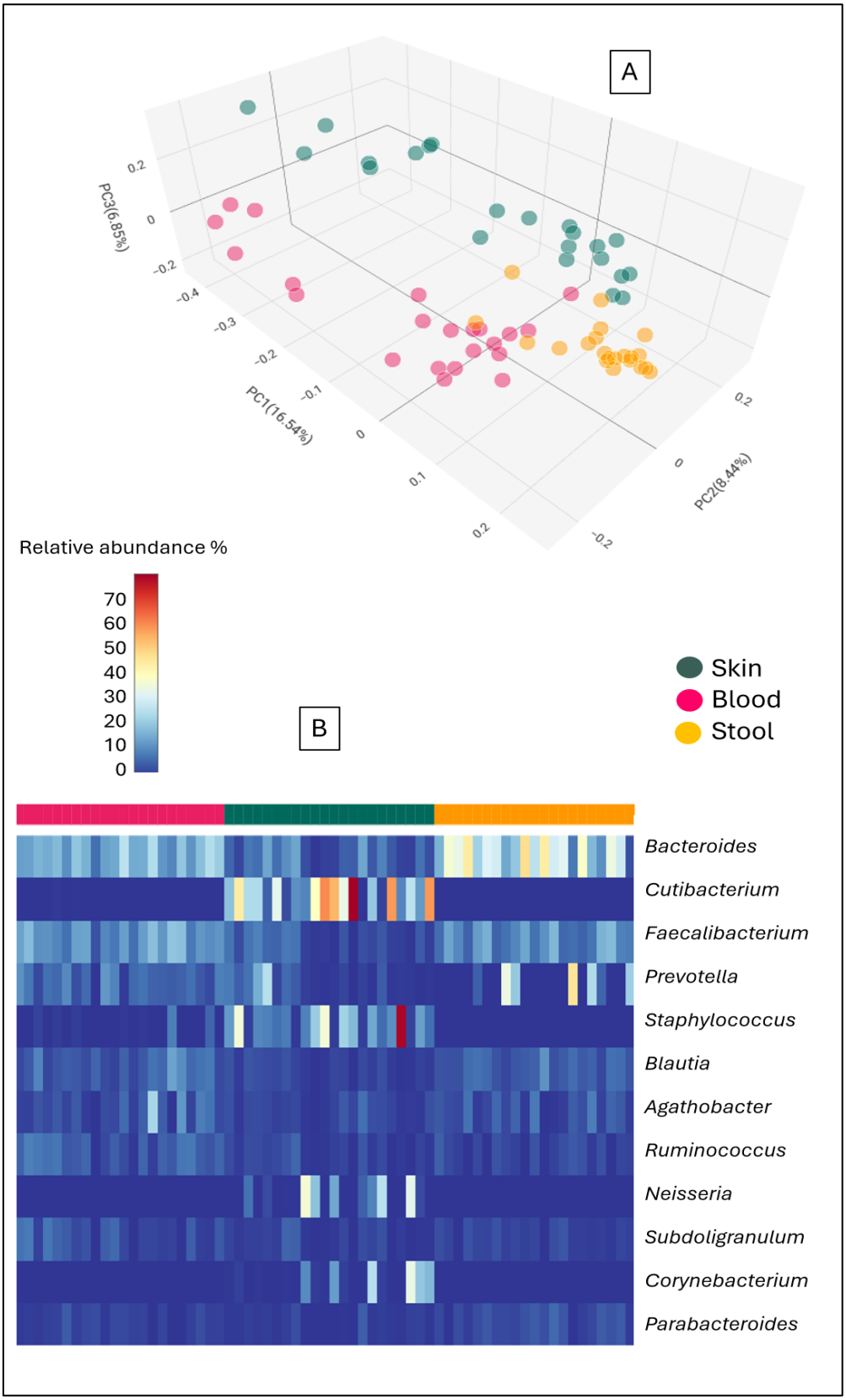

Figure 3. Principal component analysis **(A)** and heatmap **(B)** of bacterial abundance of stool, blood and skin samples of rosacea patients and control healthy volunteers (55)

A comparison of the Chao1 alpha diversity results of rosacea patients and control patients revealed no significant differences among the stool or blood samples. No significant disparities in beta diversity were identified when comparing the samples of rosacea patients and healthy controls, irrespective of whether they were obtained from stool or blood samples. In the Bray–Curtis principal coordinate beta diversity representation, the blood data of rosacea patients and control subjects appeared to be different, but the difference was not statistically significant ($p = 0.074$). The Chao1 alpha diversity of the skin microbiome in patients suffering from rosacea was found to be significantly lower than that observed in healthy controls ($p \leq 0.001$). As demonstrated in the Bray–Curtis beta diversity principal coordinate analysis figure below, the samples of rosacea patients and the control samples are organized into two significantly different sets ($p \leq 0.0001$) (Figure 4A, B).

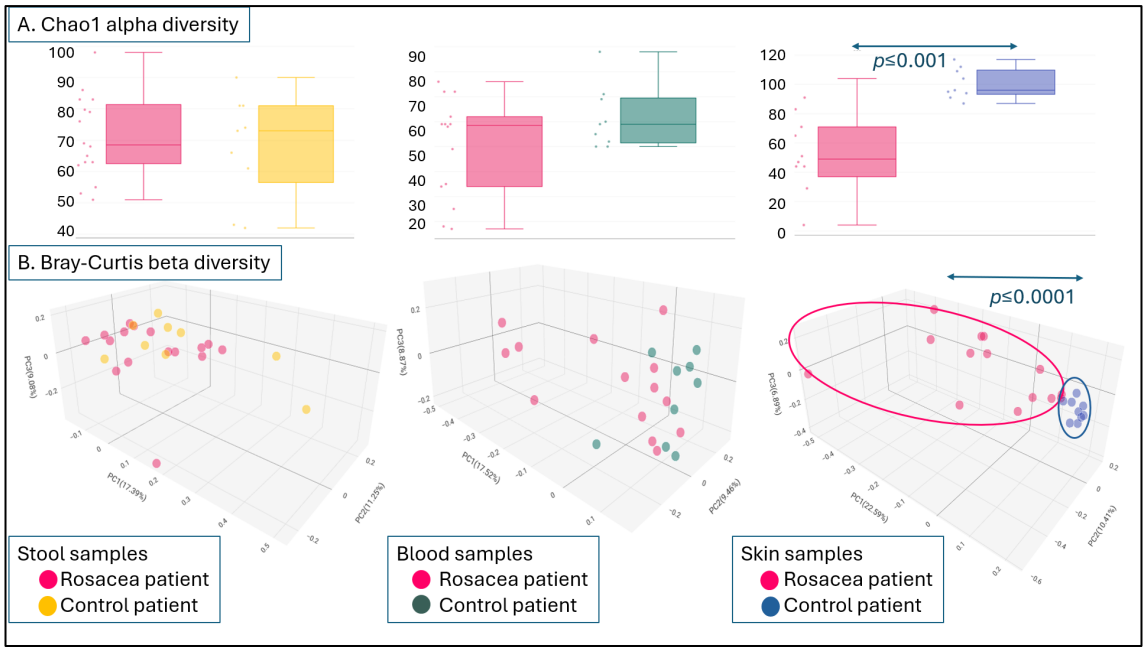

Figure 4. Comparison of Chao1 alpha diversity **(A)** or Bray-Curtis beta diversity **(B)** of stool, blood, and skin samples from the rosacea patients and control patients (55)

On the one hand, the skin microbiome of rosacea patients differs from that of healthy controls by comprising a greater number of taxa, resulting in higher alpha diversity. Conversely, the healthy microbiome exhibits a more diverse composition, resulting

in lower relative abundances of its most dominant taxa compared to those observed in the rosacea microbiome.

It has been observed that certain microbial taxa appear to be specific either to healthy skin or to skin affected by rosacea. The disparities in key microbiome components between the two cohorts (Figure 5A) are reflected in their median abundance values. However, due to the presence of high inter-individual variability (Figure 5B), statistically significant differences were found in only a few genera.

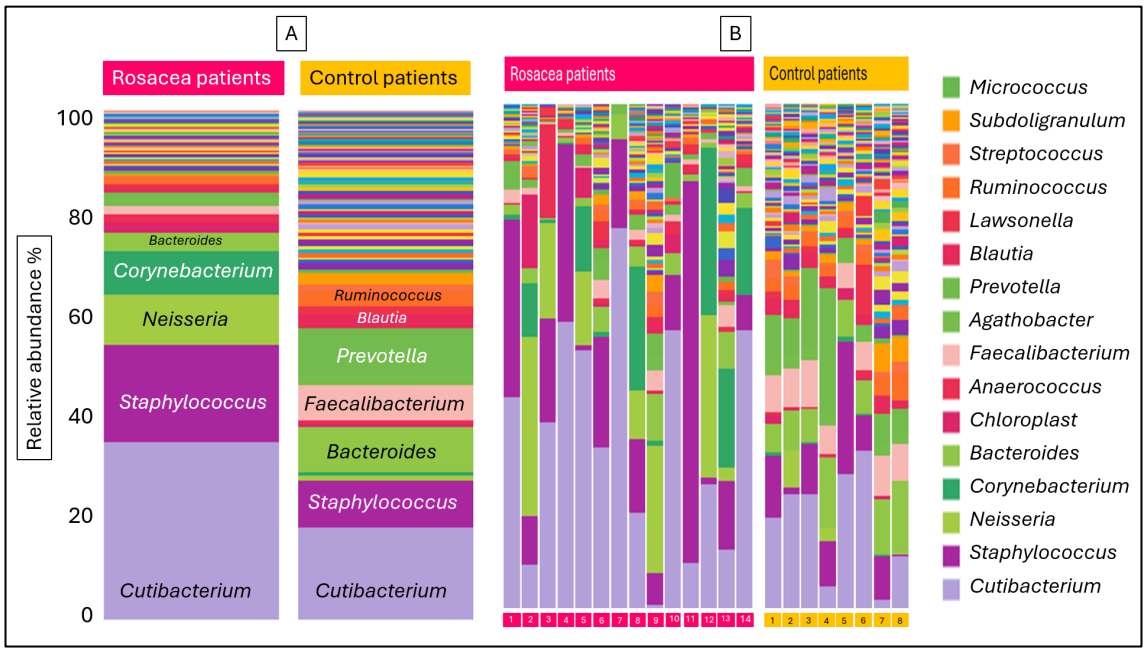

Figure 5. The relative abundance of the genus in the composition of the skin microbiome of rosacea patients and control individuals is illustrated in stacked bar representation, with the data aggregated by cohort **(A)** and individual data **(B)** (55)

Of the genus abundances that are characteristic of rosacea patients, *Neisseria* and *Corynebacterium* were found to be significantly higher, while *Cutibacterium* and *Staphylococcus* were not found to be significantly higher than in healthy patients.

In contrast, genera such as *Bacteroides*, *Faecalibacterium*, *Prevotella*, *Blautia*, *Ruminococcus*, and *Subdoligranulum* were found to be significantly more abundant in the skin microbiome of healthy individuals (Table 3). The six genera that demonstrated sig-

nificant disparities in the skin microbiome (favouring healthy controls) manifested comparable abundances in both the negative control and rosacea stool samples, with no statistically significant variations observed.

It is noteworthy that *Cutibacterium*, *Neisseria*, *Staphylococcus*, and *Corynebacterium* were absent from stool samples and present at low levels in only a few blood samples.

A significant discrepancy in the abundance of *Ruminococcus* and *Subdoligranulum* was identified in blood samples between rosacea patients and healthy controls (Table 3).

Table 3. Median relative abundances of bacterial genera in skin samples from rosacea patients and healthy controls. Genera with significantly higher abundances and corresponding *p*-values are shown in bold. (55)

Sample type	Genus	Rosacea patients	Control patients	*p*-value
Skin	*Cutibacterium*	35.14	20.93	0.088
	Neisseria	**18.65**	**1.35**	**0.023**
	Staphylococcus	13.97	8.82	0.152
	Corynebacterium	**10.56**	**0.21**	**0.015**
	Bacteroides	**2,94**	**7.49**	**0.001**
	Faecalibacterium	**1.28**	**7.42**	**<0.001**
	Prevotella	**0,47**	**6.75**	**0.001**
	Blautia	**0,83**	**2.81**	**0.001**
	Ruminococcus	**0.69**	**2.48**	**0.001**
	Subdoligranulum	**0.28**	**1.71**	**0.017**
Blood	***Ruminococcus***	**4.7**	**7.13**	**0.032**
	Subdoligranulum	**1.95**	**4.65**	**0.033**

No significant differences were observed in the abundance distributions of *Bacteroides*, *Faecalibacterium*, *Blautia*, and *Prevotella* between blood and stool samples.

However, the abundance of all four genera was significantly lower in skin samples compared to both blood and stool. Furthermore, the abundance of *Ruminococcus* and *Subdoligranulum* was found to be higher in blood samples than in stool samples. Both genera exhibited significantly greater abundance in blood than in skin samples (Figure 6).

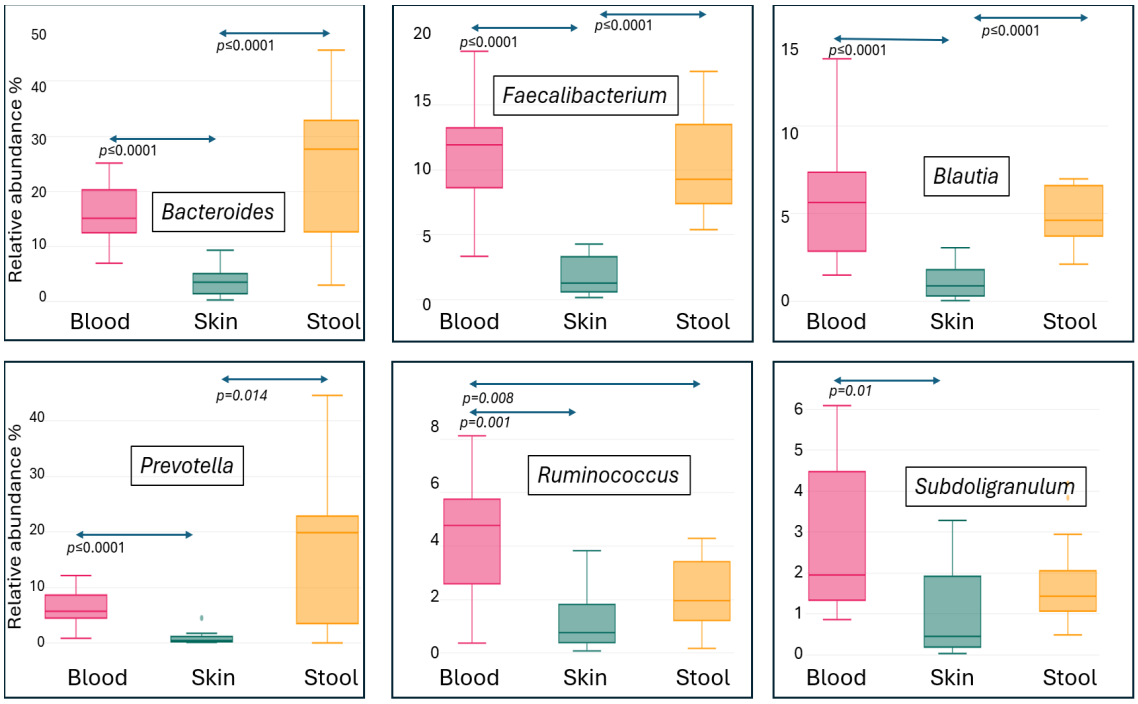

Figure 6. Abundance distribution of *Bacteroides*, *Faecalibacterium*, *Blautia*, *Prevotella*, *Ruminococcus* and *Subdoligranulum* genera among the different sample types (55)

While no substantial disparities were observed in the overall prevalence of predominant microbial components between the rosacea and control groups, irrespective of whether samples were from stool or blood, certain less prevalent genera exhibited statistically significant variations. In the blood of control patients, the relative abundance of *Subdoligranulum*, *Ruminococcus*, *Lachnospira*, and *Bifidobacterium* was significantly higher, whereas *Eubacterium* and *Odoribacter* were significantly less prevalent. In control stool samples, the relative abundance of *Lachnospira*, *Anaerostipes*, *Bifidobacterium*, and *Romboutsia* was found to be significantly higher, while the abundance of *Coriobacteriales* was found to be significantly lower.

4.2 Correlation between the altered bacterial composition and the pathomechanism of rosacea

Linear Discriminant analysis Effect Size (LEfSe), which identifies distinguishing features significantly associated with a specific group, showed that only the stool samples from the control group met the required linear discriminant analysis (LDA) and statistical significance thresholds. The investigation revealed that the gut microbiome of the control patients exhibited elevated activity in polysaccharide degradation pathways. Specifically, metabolic activities involving fructuronate, galacturonate, and related pathways were more prominent, contributing to increased production of short-chain fatty acids (SCFAs) such as acetate and butyrate. These compounds are known for their anti-inflammatory effects (Figure 7).

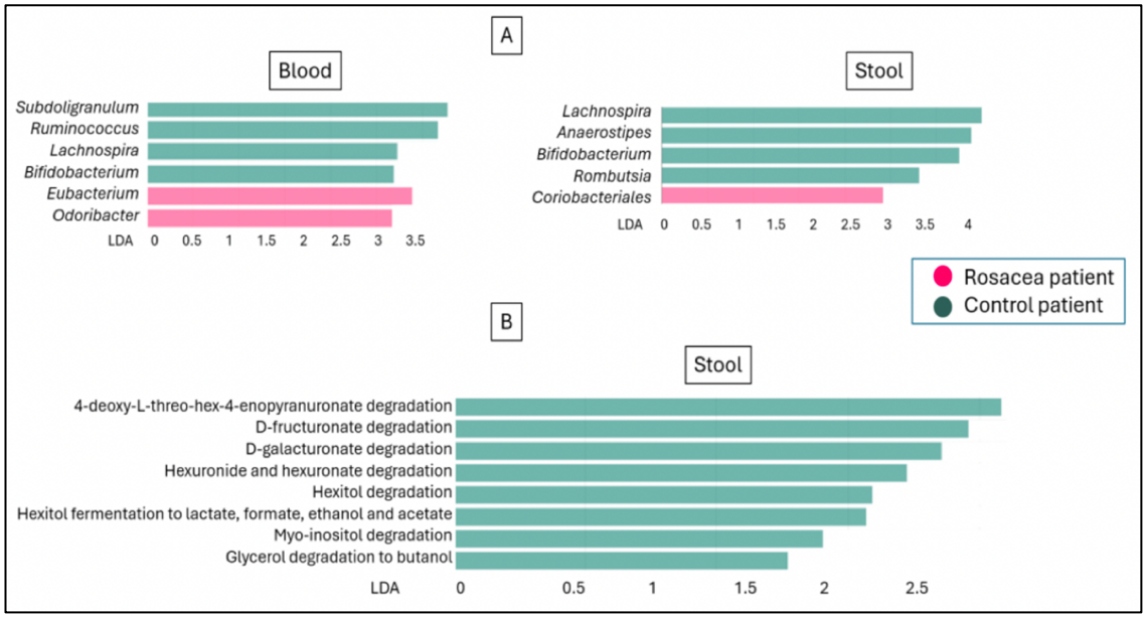

Figure 7. LEfSe bar chart; visual representation of discriminative features of genera abundances among the control and rosacea blood and stool samples **(A)**, and discriminative biochemical pathways in stool samples **(B)**. (55)

A comparison of the biochemical activity in the skin microbiomes of healthy individuals and rosacea patients revealed significant differences: A total of 46 distinct pathways were found to be more prevalent in patients suffering from rosacea, while 72 were

more common in healthy subjects. As illustrated in Figure 8, the figure exclusively highlights pathways that demonstrate significance levels ranging from $p \leq 0.001$ to $p = 0.01$.

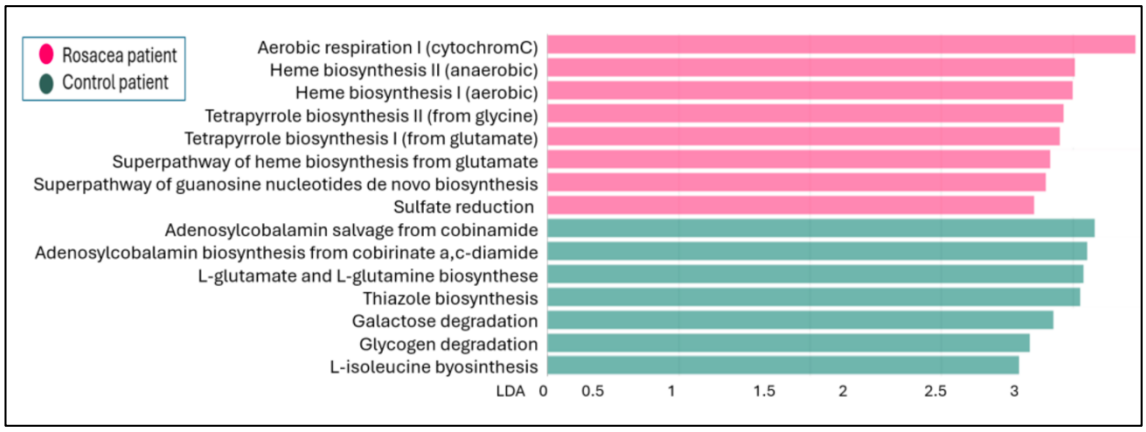

Figure 8. LefSe analysis of metabolic pathways of the rosacea and control skin microbiome. (55)

The skin microbiome associated with rosacea exhibited elevated activity in multiple pathways associated with heme biosynthesis. In contrast, the healthy skin microbiome exhibited heightened activity in pathways such as adenosylcobalamin (a derivative of vitamin B12) biosynthesis. The microbiome of the control group has also been demonstrated to produce elevated levels of L-isoleucine.

4.3 Composition of the fungal microbiome of the skin, blood, and stool of rosacea patients and healthy controls

Distinct mycobiome profiles were identified across various sample types, irrespective of whether the samples originated from rosacea patients or healthy controls. In the Jaccard beta diversity Principal Coordinate Analysis (PCoA) plot, fungal communities from stool, blood, and skin samples formed three clearly separated clusters (Figure 9A), indicating significant differences in community composition. This separation was further supported by a stacked bar plot, which demonstrated marked differences in fungal

abundance across the three sample types (Figure 9B). It is noteworthy that all fungal genera detected in blood samples were also present in stool and skin samples, albeit typically at intermediate abundance levels in blood.

A comparison of the skin and stool samples obtained from patients suffering from rosacea revealed that several genera were found to be significantly more abundant in the skin samples. These genera included *Alternaria* ($p \leq 0.001$), *Aspergillus* ($p \leq 0.0001$), *Malassezia* ($p \leq 0.0001$), *Ochrolechia* ($p \leq 0.01$), and *Venturiales* ($p \leq 0.0001$). Conversely, stool samples demonstrated significantly elevated levels of *Ascomycota* ($p \leq 0.0001$), *Candida* ($p \leq 0.0001$), and *Chaetothyrales* ($p \leq 0.0001$), *Didymosphaeria* ($p \leq 0.0001$), *Penicillium* ($p \leq 0.0001$), *Rinodina* ($p \leq 0.01$), *Russula* ($p \leq 0.001$), and *Saccharomyces* ($p \leq 0.001$), as well as *Tomentella* ($p \leq 0.01$). Nevertheless, the median relative abundance of *Chaetothyrales*, *Rinodina*, and *Russula* in stool samples remained below 5%.

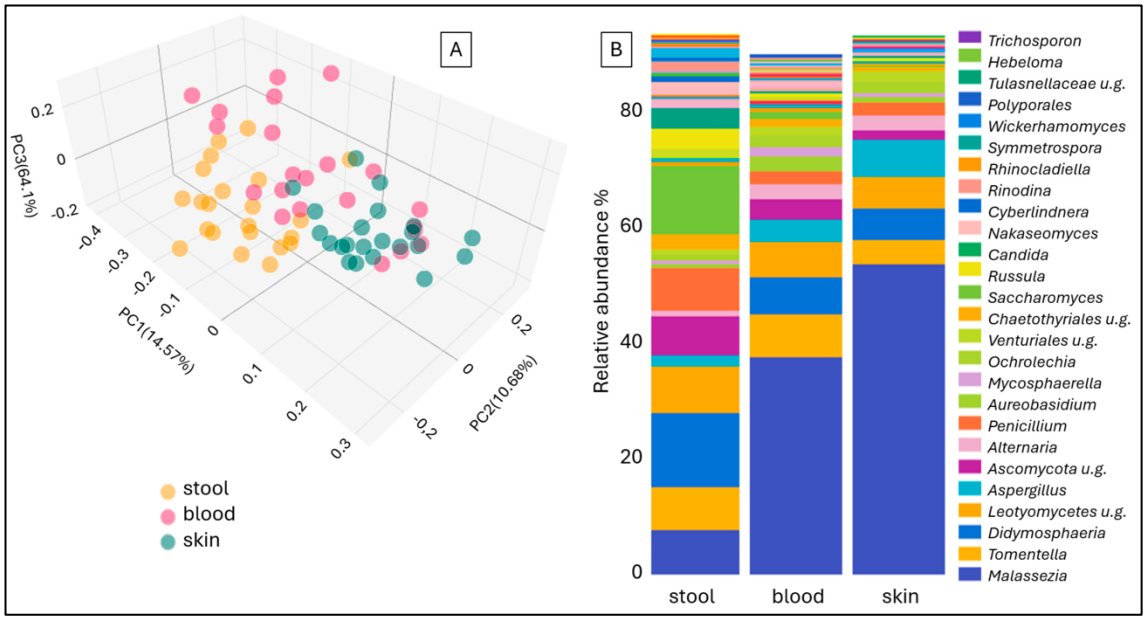

Figure 9. Jaccard beta diversity Principal Coordinate analysis (PCoA) **(A)** and aggregated stacked bar taxon abundance at genus level **(B)** of stool, blood and skin samples of study participants (56)

No significant differences were observed in alpha diversity between stool, blood, and skin samples from rosacea patients and healthy controls, indicating that the number

of fungal taxa detected was similar across groups within each sample type (Figure 10A). Similarly, the analysis of beta diversity revealed no significant differences in the composition or relative abundance of the fungal community between rosacea and control samples across all sample types (Figure 10B). A comparative analysis was also conducted, which revealed no significant disparities in the abundance of fungal genera across the skin, blood, or stool mycobiomes of the two study groups.

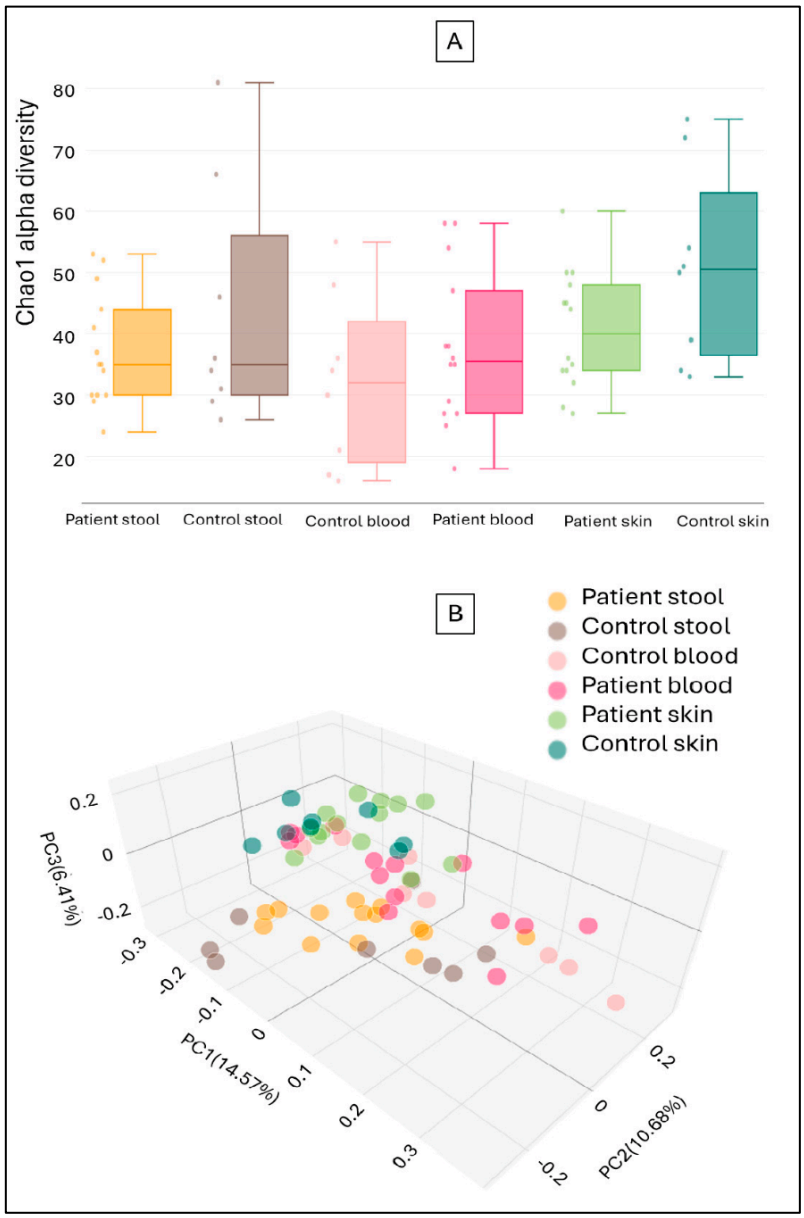

Figure 10. Box plot representation of Chao1 alpha diversity **(A)** and Principal Coordinate Analysis (PCoA) of Jaccard beta diversity **(B)** in stool, blood, and skin samples from rosacea patients and controls. (56)

4.4 Connection between the simultaneous presence of bacteria and fungi

Fungal DNA has been shown to enter the bloodstream via various body surfaces, including the oral mucosa, genital tract, urinary tract, skin, and gastrointestinal tract. The present study focused on comparing the blood mycobiome with those of skin and stool. As demonstrated in Figure 11, no fungal taxa were identified in the blood that could be definitively attributed to the stool alone. Of particular interest was the inverse correlation observed between *Saccharomyces* and *Candida* in stool samples, suggesting a potential dynamic relationship between these two fungal species within the gastrointestinal environment. Furthermore, bacterial taxa found in the blood may originate from multiple anatomical sites. As demonstrated in Figure 12, the presence of bacterial genera commonly associated with the gut microbiome has been observed in the blood at significant levels. Conversely, skin-associated genera such as *Corynebacterium*, *Cutibacterium*, *Neisseria*, and *Staphylococcus* manifest in the blood at low abundances and typically do not exceed background levels, along with other skin-derived bacterial taxa.

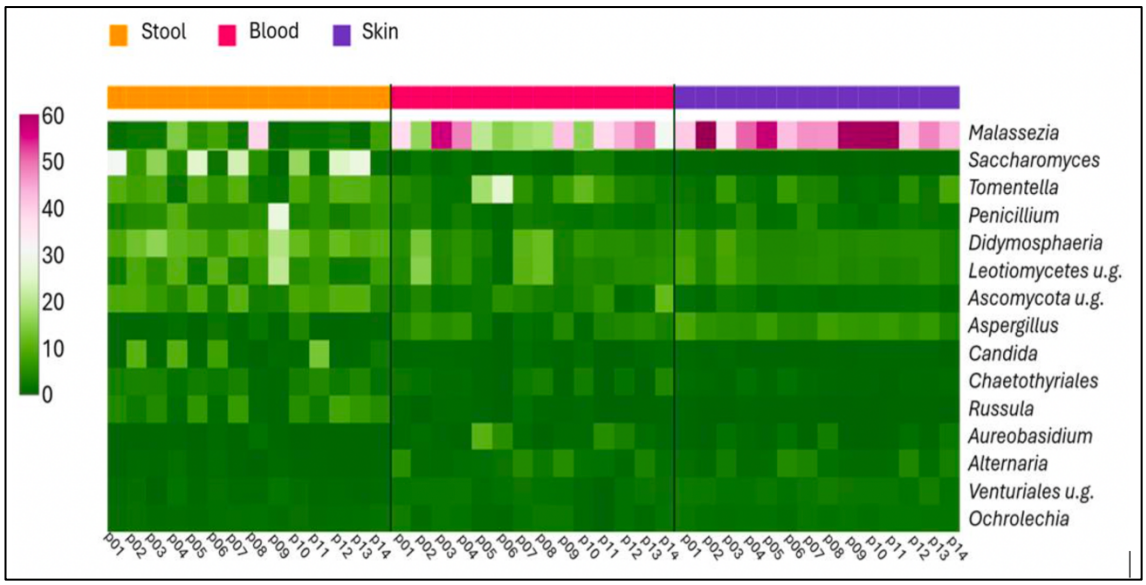

Figure 11. Heatmap presentation of the 15 most prevalent fungal genera detected in stool, blood, and skin samples from 14 rosacea patients (p1-p14) (56)

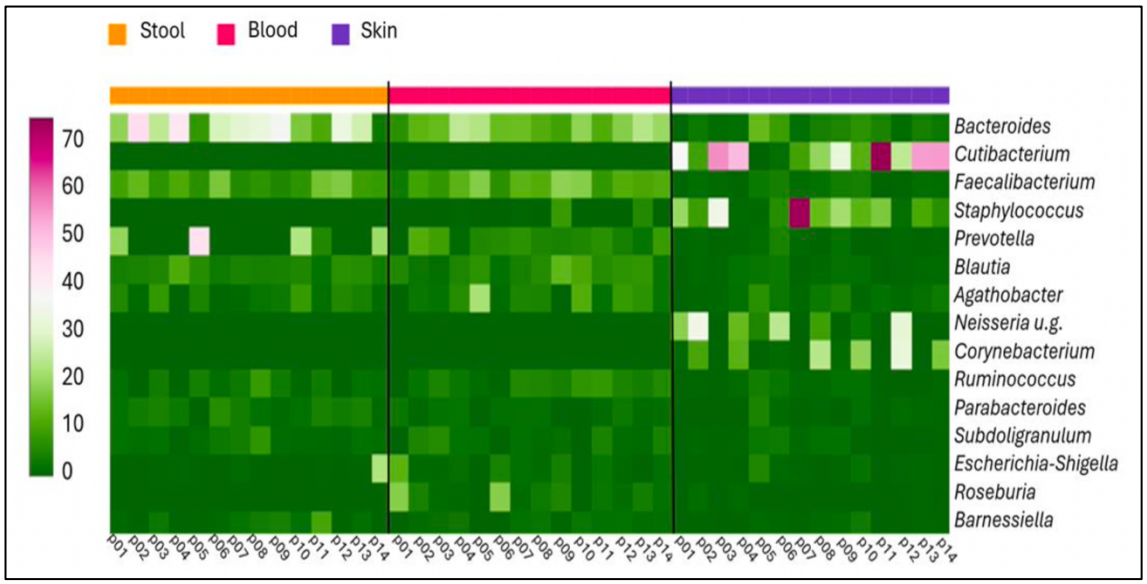

Figure 12. Heatmap presentation of the 15 most prevalent bacterial genera detected in stool, blood, and skin samples from 14 rosacea patients (p1-p14) (56)

The heatmap in Figure 11 demonstrates that the genus *Malassezia* accounted for over 40% of the fungal community in all skin samples. However, the presence of the genus was only detected at significant levels in a limited number of corresponding blood samples. This observation gives rise to the question of whether the composition of the skin bacterial microbiome influences the translocation of fungal DNA into the blood-stream, with the potential to affect skin integrity and permeability.

As demonstrated in Figure 13, the grouping of skin samples was based on the presence or absence of *Malassezia* in the blood, categorized as high or low abundance. A significant difference in beta diversity between the two groups ($p = 0.001$) reflects notable variations in skin bacterial community composition. Specifically, subjects with elevated levels of *Malassezia* in the blood exhibited significantly higher levels of *Cutibacterium* on the skin ($p = 0.002$). Conversely, subjects exhibiting low levels of *Malassezia* in the blood demonstrated significantly higher levels of *Faecalibacterium* ($p = 0.02$) and *Prevotella* ($p = 0.01$) on the skin.

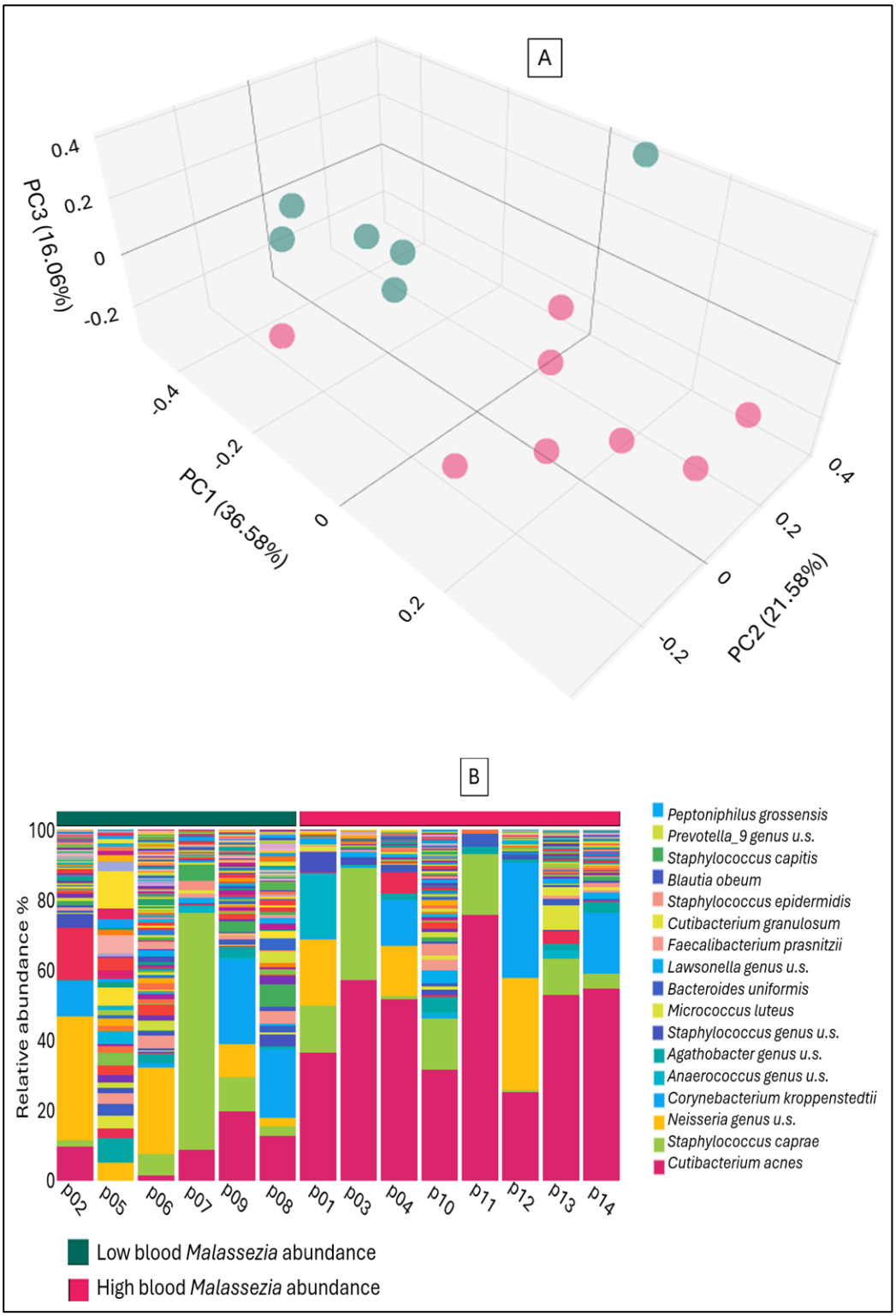

Figure 13. Principal Coordinate Analysis (PCoA) of Jaccard beta diversity **(A)** and stacked bar bacterial abundance **(B)** in skin samples of rosacea patients according to blood *Malassezia* abundance (56)

Stool samples from individuals diagnosed with rosacea exhibited a significantly higher median relative abundance of several fungal genera compared to skin samples, including *Ascomycota*, *Candida*, *Chaetothyrales*, *Didymosphaeria*, *Penicillium*, *Rinodina*, *Russula*, *Saccharomyces*, and *Tomentella*. It is noteworthy that *Ascomycota*, *Didymosphaeria*, and *Tomentella* exhibited a higher relative abundance in blood samples compared to stool samples.

The median relative abundances of *Chaetothyrales*, *Rinodina*, and *Russula* in stool samples remained below 5%, indicating a relatively minor role in the gut mycobiome. In the course of the research, particular attention was paid to the most prominent genera, *Saccharomyces* and *Candida*, which were selected for further analysis on the basis of their potential clinical relevance in the context of rosacea pathophysiology.

In stool samples from patients suffering from rosacea, *Candida* exhibited a higher median relative abundance, whereas *Saccharomyces* appeared at lower levels compared to healthy controls. However, these differences did not reach statistical significance. As demonstrated in Figure 14, the relative abundances of bacterial genera associated with these two fungal taxa exhibit inverse trends in the gut. In individuals with elevated *Saccharomyces* levels, the bacterial genera *Prevotella* ($p = 0.01$) and *Agathobacter* ($p = 0.006$) were found to be significantly more abundant. Conversely, elevated levels of these bacterial taxa were also observed in samples exhibiting reduced *Candida* abundance (Figure 15).

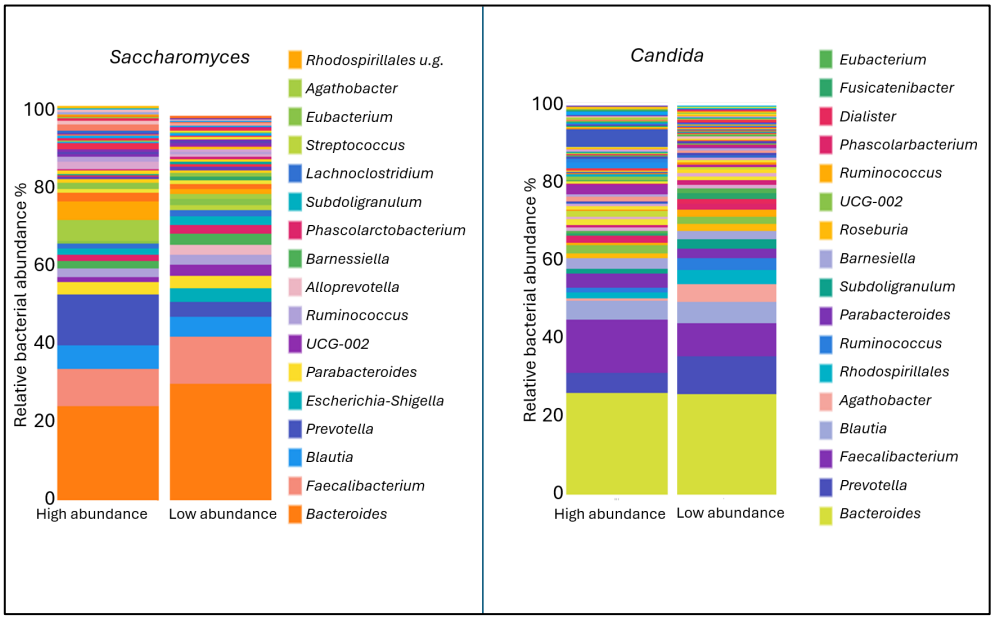

Figure 14. The composition of the bacterial microbiome in groups with high and low *Saccharomyces* or *Candida* abundance shown in stacked bar charts (56)

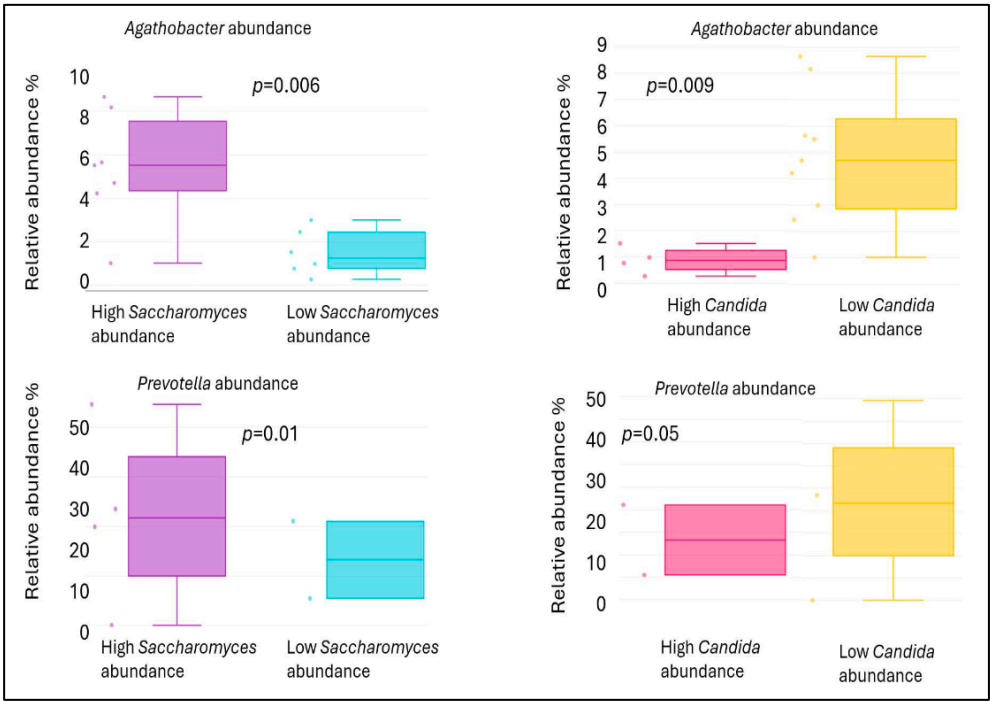

Figure 15. The abundance of *Agathobacter* and *Prevotella* in the stool of rosacea patients depends on the abundance of *Saccharomyces* and *Candida.* (56)

5 Discussion

The advent of novel innovations and advances in diagnostic methodologies has rendered it increasingly feasible to investigate the pathogenesis of rosacea. Despite this, the precise pathomechanism of rosacea remains to be fully elucidated.

In the contemporary era, a considerable portion of knowledge is derived from non-culture-based methodologies. The advent of next-generation sequencing (NGS) technology has led to substantial progress in the realm of detailed research concerning the composition of the human microbiome. In particular, analysis of the bacterial 16S rRNA gene region is a key method for investigating the structure and diversity of microbial communities. This region contains both conserved and hypervariable segments, which enable the construction of universal primers for the identification of a wide range of bacterial species (57). Additionally, the ITS region is frequently employed in the analysis of the mycobiome, functioning as a specific target for the identification of fungi (58). The results of numerous DNA sequencing studies have contributed to a significant increase in knowledge about the functional properties of microbial populations that colonize various parts of the human body, such as the skin, intestines, and blood. The total number of microorganisms in the human body is estimated at approximately 100 trillion, including representatives from various domains, such as bacteria, archaea, viruses, fungi, and protozoa (59).

A plethora of studies have previously investigated the microbiome in patients diagnosed with rosacea. However, the findings reported have been inconsistent and at times contentious. The objective of these studies was to further research into the microbiome and the as yet under-researched mycobiome in rosacea, with the aim of gaining new insights. To the best of our knowledge, this was the first study to simultaneously detect differences in the stool, blood and skin microbiome as well as mycobiome in individuals with and without rosacea.

Research has indicated that individuals diagnosed with rosacea exhibit a higher incidence of celiac disease, Crohn's disease, *H. pylori* infections, ulcerative colitis, irritable bowel syndrome, and small intestinal bacterial overgrowth (SIBO) when compared

to healthy control individuals (60-63). The underlying mechanism by which these associations occur is believed to be related to the composition of the gut microbiome. For instance, Agnoletti et al. (64) observed that SIBO predominantly presents in papulopustular phenotypes, while *H. pylori* infections are predominantly observed in erythrosis. This observation has been corroborated in subsequent studies, which demonstrated that the symptoms of rosacea improve following antibacterial treatment of SIBO (63, 65). A number of research groups have investigated the gut microbiome. However, the results of these investigations have been inconsistent.

Our study revealed no statistically significant disparities in alpha or beta diversity within the bacterial stool microbiome between patients diagnosed with rosacea and the healthy control group. The literature on alpha diversity is contradictory. Similar to our study, Nam et al. (66) (12 RP, 251 CP) found no significant difference in diversity. In contrast, Chen et al. (67) (11 RP, 110 CP) and Guertler et al. (68) (54 RP, 50 CP) found significantly reduced alpha diversity, while Moreno-Arrones et al. (69) (15 RP, 15 CP) found significantly increased alpha diversity. These differing results may be due to the small number of rosacea patients in each study (between 11 and 54 patients with rosacea). Alternatively, differences in diet due to the different locations of the studies could also play a role.

Consistent with prior research of Chen et al. (67), our analysis indicated elevated levels of carbohydrate degradation via fructuronate and galacturonate metabolic pathways in the stool microbiome of the control group. This feature was significantly diminished in individuals diagnosed with rosacea. These predicted metabolic differences suggest a diminished production of anti-inflammatory short-chain fatty acids (SCFAs) in the gut microbiome of rosacea patients compared to that of healthy controls.

A substantial presence of genera was identified in the stool samples, though these varied among the study cohorts. In patients diagnosed with rosacea, the genus *Coriobacteriales* was predominantly identified, while in the control group, a higher abundance of the genera *Lachnospira*, *Anaerostipes*, *Bifidobacterium*, and *Robustia* was observed. According to the extant literature, the prevalence of other bacterial taxa is of considerable significance.

To date, only a few studies have investigated the blood microbiome in connection with dermatological diseases like psoriasis (70) or hidradenitis suppurativa (71). In the context of our microbiome study, we observed no statistically significant disparities in alpha or beta diversity metrics in the blood samples between patients diagnosed with rosacea and a healthy control group. To date, only one research group has investigated the blood microbiome of rosacea. The research group Yun et al. (72) (10 RP, 30 CP) also found no significant difference in alpha diversity, but did find a difference in beta diversity. The relative frequency of *Chromatiaceae* and *Fusobacteriaceae* families was found to be significantly higher in the blood of patients diagnosed with rosacea. At the genus level, 14 bacterial taxa exhibited differential frequencies between rosacea patients and healthy controls. A particularly noteworthy finding was the elevated prevalence of the genus *Rheinheimera* among rosacea patients. *Rheinheimera* is classified as a gram-negative, aerobic bacterium that contains lipopolysaccharides in its outer membrane. These lipopolysaccharides may play a role in the pathogenesis of chronic inflammation associated with rosacea by activating TLRs.

The presence of microbial products has been identified as a contributing factor to the escalation of inflammatory responses within the gastrointestinal tract. It is also possible for the bacteria to reach the skin via the blood. In a similar manner, the blood might serve as a conduit between the bacteria that comprise the gut and skin microbiome.

A connection between the cutaneous microbiome and rosacea has long been suspected. The correlation between the presence of *Demodex* mites and rosacea was first demonstrated in 1932 (73). At that time, rudimentary methods such as a methylene blue staining and a microscopic observation were employed to demonstrate the involvement of various bacteria, including *S. epidermidis*, *B. oleronius*, and *C. acnes*, in the pathogenesis of rosacea (74). As demonstrated in previous studies, a dysbiotic condition of the cutaneous microbiome has been associated with various dermatological conditions, including acne vulgaris (75), atopic dermatitis (76), seborrheic dermatitis (77), psoriasis or alopecia areata (78).

The present study revealed statistically significant disparities in both alpha and beta diversity between patients diagnosed with rosacea and the healthy control group. The

analysis of the patient group diagnosed with rosacea revealed a decrease in alpha diversity. This finding is consistent with a study by Thompson et al. (79) (19 RP, 19 CP). However, Wang et al. (80) (36 RP, 22 CP) found increased alpha diversity in patients with rosacea. Studies by Zaidi et al. (81) (10 RP, 20 CP) and Rainer et al. (82) (19 RP, 19 CP) demonstrated no statistically significant difference in bacterial alpha diversity. The present study is singular in its demonstration of a substantial discrepancy in beta diversity. The results of our study demonstrated a substantial increase in the prevalence of the genera *Neisseria*, which is consistent with the findings of Xiong et al. (83) (17 RP, 27 CP), and *Corynebacterium*, as also reported by Rainer et al. (82) (19 RP, 19 CP). Furthermore, an increased frequency of *Cutibacterium* was observed. Studies by Thompson et al. (79) (19 RP, 19 CP) and Xiong et al. (83) (17 RP, 27 CP) support the increased frequency of *Cutibacterium* on the skin of rosacea patients, whereas studies by Rainer et al. (82) (19 RP, 19 CP) and Wang et al. (80) (36 RP, 22 CP) do not. The observed variability in the frequency of *Cutibacterium* can be attributed to the distinct biological activities exhibited by various strains of *Cutibacterium acnes*. These strains have been shown to exert either a protective effect or a potentially deleterious effect, depending on the specific strain and its interaction with the host. However, *Cutibacterium* is generally regarded as beneficial due to its role in occupying ecological niches on the skin, thereby preventing colonization by potentially harmful microorganisms. Furthermore, propionic acid and other metabolic products have been shown to assist in maintaining the skin's pH balance and impeding the proliferation of harmful bacterial strains. However, certain *Cutibacterium acnes* strains possess virulence factors that enhance their disease-causing properties. In particular, their ability to form biofilms and produce proinflammatory mediators can trigger immune responses in the host that ultimately lead to tissue damage (84).

In addition, as in Wang et al. (80) (36 RP, 22 CP), Murillo et al. (85) (30 RP, 17 CP), and Xiong et al. (83) (17 RP, 27 CP), an increased frequency of *Staphylococcus* was observed. With regard to the genus *Staphylococcus*, its pathogenic role in rosacea remains uncertain, as it is a natural component of the healthy skin microbiome (86). The elevated skin temperatures observed in patients with rosacea relative to those in healthy individuals, in conjunction with the documented secretion of diverse proteins by *Staphylococcus epidermidis* strains cultivated at elevated temperatures, suggest a plausible pathogenic role for these proteins (26).

In regard to biochemical pathways, the skin microbiome of the study's cohort of patients afflicted with rosacea exhibited an elevated concentration of tetrapyrroles and heme. An increase in heme concentrations has been demonstrated to activate proinflammatory cytokines and free radicals in immune cells, including macrophages and endothelial cells (87). The activation of this process is facilitated by the engagement of TLRs. This process results in the manifestation of inflammation, a hallmark feature of rosacea.

Furthermore, the results of this study demonstrated that elevated hydrogen sulfide (H_2S) production appeared to be associated with a predominance of sulfate-reducing bacteria within the subject's skin microbiome. H_2S exerts a multi-faceted role in the inflammatory response, functioning as both a pro- and anti-inflammatory mediator, depending on the specific context. In a similar manner, Chen et al. (67) observed an increase in H_2S production in the gut microbiome of rosacea patients. This, in turn, resulted in impaired colonic butyrate oxidation and disrupted epithelial permeability, ultimately compromising epithelial barrier function (88).

Furthermore, our study revealed increased adenosylcobalamin production in the skin microbiome of the healthy control group. In contrast, Chen et al. (67) found increased cobalamin transport in the stool microbiome of rosacea patients. Huang et al. (89) found that hydroxycobalamin, a vitamin B12 derivative like adenosylcobalamin, can reduce flushing and persistent erythema in rosacea patients. According to the current literature, it is unclear whether vitamin B complex preparations can worsen or improve rosacea symptoms (90).

Our study also revealed that the healthy control group exhibited increased production of thiazoles and L-isoleucines. Thiazole derivatives and L-isoleucine are known to modulate proinflammatory cytokines and pathways, thereby having an anti-inflammatory effect (91, 92).

Bacteria such as *Faecalibacterium* and *Prevotella* were detected in our healthy control group. These bacteria produce many anti-inflammatory metabolites. However, these bacteria are anaerobic and cannot, therefore, be used for local skin therapy. Nevertheless, other studies have demonstrated the positive effects of probiotic bacteria on the skin due to their anti-inflammatory properties (93, 94).

In addition to bacteria (microbiome), archaea, viruses (virome) and protozoans, fungi (mycobiome) are also part of the healthy skin flora. In contrast to the extensive research conducted on the bacterial microbiome, the human mycobiome has received comparatively less attention from the research community. However, recent studies have demonstrated that a dysbiosis of the mycobiome also plays a significant role in skin health (95). For instance, the mycobiome has been implicated in various dermatological diseases, including tinea versicolor (96), seborrheic dermatitis (97), and atopic dermatitis (98, 99). These alterations have been shown to promote immune system dysfunction and inflammation, which can compromise the integrity of the skin barrier through fungal allergens and their enzymatic activities (97, 99). In contrast, a study by Wang et al. (80) (36 RP, 22 CP) demonstrated that there was no significant difference either in the diversity or abundances of fungal genera in the cutaneous mycobiome. This is so far the only study about rosacea and its mycobiome.

In the present study, substantial disparities in both beta diversity and fungal abundance were identified in the stool, blood, and skin mycobiomes of both the rosacea patient group and the control group. Among patients diagnosed with rosacea, the most pronounced differences were detected in the stool and skin mycobiomes. Furthermore, the stool mycobiome exhibited higher relative abundances of *Saccharomyces* and *Candida*. Concurrently, data from the Human Microbiome Project demonstrated that the fungal genera *Saccharomyces*, *Malassezia*, *Candida*, and *Cyberlindnera* were among the most prevalent in the stool mycobiome (100).

The intestinal mycobiome demonstrates considerable variability over time, both between individuals (interindividual) and within the same individual (intraindividual), suggesting a relatively low degree of stability in comparison to the bacterial microbiome. The instability is partly attributable to fungal taxa that are predominantly of exogenous origin, mainly derived from environmental sources and diet. A significant proportion of the fungal genera identified in stool samples appear to be transient, likely unable to persist under the physiological conditions of the gut, such as elevated temperature, variable pH, and hypoxic environment. The extant body of supporting evidence indicates a significant correlation between the frequency of *Saccharomyces* detection and dietary intake. In contrast, the presence of *Candida* species has been associated with oral hygiene practices (101).

Conversely, our study identified *Malassezia* as the most prevalent genus within the skin mycobiome. This finding aligns with the conclusions of previous studies (102). Furthermore, *Malassezia* has been implicated in the pathogenesis of various other dermatological conditions, including pityriasis versicolor, seborrheic dermatitis, dandruff, atopic dermatitis, psoriasis, folliculitis, and onychomycosis, where associations between this genus and disease symptoms have been suggested (103).

In contrast to the findings observed in beta diversity, no significant differences in alpha diversity were detected in stool, blood, and skin samples, neither in the rosacea group nor in the control group. Alpha diversity exhibited no substantial disparities between the groups. Nevertheless, incongruence with Wang et al. (80) (36 RP, 22 CP), we identified substantial disparities in the prevalence of fungal genera among rosacea patients and healthy control subjects in skin samples. Our research group was the first to also investigate the stool and blood mycobiome. However, no significant differences in the frequency of fungi were found in this area of study as well.

The findings indicate that the relative abundance of fungal genera in the blood mycobiome does not directly reflect the composition of the skin or gut mycobiomes, likely due to the additional influence of fungal DNA originating from sites such as the oral cavity, respiratory tract, and urogenital tract. It is noteworthy that *Malassezia* consistently prevailed as the predominant fungal genus in blood samples where the relative abundance of any other fungal genus exceeded 30%. While *Malassezia* was present at over 40% relative abundance on the skin of rosacea patients, its DNA was only detected at significant levels in a subset of corresponding blood samples. Patients with high *Malassezia* abundance in both skin and blood also exhibited elevated levels of *Cutibacterium* on the skin, suggesting a potential relationship between skin microbial composition and fungal translocation into the bloodstream.

The role of *Cutibacterium* in rosacea appears to be complex and partially contradictory. It has been demonstrated that certain strains, including *Cutibacterium acnes subsp. defendens*, possess the capacity to manifest anti-inflammatory properties (83). Conversely, others, particularly in conjunction with *Staphylococcus*, have been hypothesized to contribute to proinflammatory processes (74). The present findings suggest that *Cutibacterium*, in conjunction with *Malassezia*, may contribute to the disruption of skin integrity in patients suffering from rosacea.

A comparative analysis of stool samples revealed significantly higher median relative abundances of specific fungal genera in rosacea patients than in controls, including *Ascomycota*, *Candida*, *Chaetothyrales*, *Didymosphaeria*, *Penicillium*, *Rinodina*, *Russula*, *Saccharomyces*, and *Tomentella*. Of these, *Ascomycota*, *Didymosphaeria*, and *Tomentella* exhibited higher relative abundance in blood than in stool, suggesting that fungal DNA may enter the bloodstream via routes other than the gut. While *Saccharomyces* and *Candida* were found to be more prevalent in stool samples from patients suffering from rosacea, the differences observed when compared to healthy individuals did not reach statistical significance.

An analysis of the relationship between fungal and bacterial communities in the gut revealed that stools with high *Saccharomyces* abundance were significantly enriched in *Prevotella* and *Agathobacter*, both genera associated with anti-inflammatory activity. Conversely, stools exhibiting a predominance of *Candida* exhibited significantly lower levels of these bacteria. This pattern lends support to the hypothesis that the composition of the fungal microbiota in the gut may modulate the presence of beneficial bacterial taxa and, in turn, influence inflammatory pathways relevant to the pathophysiology of rosacea.

While not all group differences attained statistical significance, the observed associations imply that fungal-bacterial interaction within the gut may contribute to systemic immune modulation and inflammation in rosacea. Further studies focusing on the functional dynamics of these interactions will be critical for clarifying their roles.

Taken together, these findings highlight the potential systemic relevance of the bacterial and fungal microbiome in rosacea, extending beyond the local skin environment. The precision of a foundation for integrative microbiome research across multiple microbial kingdoms and body compartments is a key objective, with the ultimate aim of informing the development of more targeted diagnostic and therapeutic approaches.

Notwithstanding the encouraging results of these studies, it is imperative to take into account the inherent limitations when interpreting the findings. The cross-sectional study design imposes limitations on the capacity to derive causal inferences. Moreover, the restricted sample size may impede the generalizability of the findings. Consequently, the data could not be grouped according to the rosacea phenotypes. It is also noteworthy

that the microbiome demonstrates considerable variations among diverse populations. The present studies incorporated a limited number of rosacea patients from a single nation, thereby limiting the generalizability of its findings. This geographical and demographic restriction represents a significant limitation. Additionally, the intricacies inherent in fungal taxonomy and the challenges associated with accurately profiling samples with minimal biomass, such as blood, necessitate meticulous interpretation. A notable limitation of the study is its exclusive focus on the relationship between bacterial and fungal microbiome composition and rosacea. A potential research approach for future studies would be to investigate the presence and frequency of *Demodex* mites in relation to these factors. To validate and expand upon these findings, further research is necessary. This research should include long-term studies with larger cohorts and functional analyses.

6 Conclusions

1. The skin microbiome of rosacea patients shows clear differences compared to that of healthy individuals – both in alpha and beta diversity and in the increased frequency of certain bacterial genera such as *Staphylococcus*, *Corynebacterium*, *Cutibacterium* and *Neisseria*. Conversely, no substantial disparities in genus composition were detected in the stool or blood microbiomes. Furthermore, patients diagnosed with rosacea do not demonstrate significant disparities in fungal diversity or genus abundance in skin, blood, or stool samples when compared to healthy controls.

2. A direct transfer of changes in the gut microbiome via the blood to the skin cannot be confirmed at the present time.

3. The analysis revealed that the skin microbiome of rosacea patients is characterized by the prevalence of bacterial genera such as *Cutibacterium*, *Corynebacterium* and *Neisseria*, as well as fungal genera including *Alternaria*, *Aspergillus*, *Malassezia*, *Ochrolechia*, and *Venturiales*. In stool samples, the presence of bacterial genera such as *Bacteroides*, *Prevotella* and *Faecalibacterium*, along with elevated levels of fungal genera including *Ascomycota*, *Candida*, and *Chaetothyrales*, *Didymosphaeria*, *Penicillium*, *Rinodina*, *Russula*, and *Sacharomyces*, as well as *Tomentella*, was observed. These findings suggest a potential correlation between the altered microbial composition and the pathological condition of rosacea, highlighting specific taxa that may contribute to or result from disease-related dysbiosis.

4. It is striking that the metabolic products predicted from the composition of the skin and gut microbiome of rosacea patients have pro-inflammatory properties. In contrast, anti-inflammatory metabolites are in the foreground in rosacea-free individuals. These results suggest that microbiome-based therapeutic approaches could offer promising potential for the treatment of rosacea.

5. The observed relationship between fungal and bacterial communities in the gut suggests that the concurrent existence of certain microbes may influence the pathogenesis of rosacea. A high abundance of *Saccharomyces* was associated with an increase in anti-inflammatory genera, such as *Prevotella* and *Agathobacter*. Conversely, *Candida* dominance was associated with lower levels of these beneficial bacteria. These findings lend support to the hypothesis that the composition of the fungal microbiota in the gut could modulate bacterial populations, potentially influencing inflammatory pathways relevant to rosacea. While not all observed differences attained statistical significance, the results suggest that fungal-bacterial interactions within the gastrointestinal tract may potentially contribute to systemic immune modulation and inflammation.

7 Summary

Rosacea is a chronic inflammatory skin disorder that affects approximately 5% (5) of the population and is frequently encountered in clinical practice. Despite its prevalence, the precise pathogenesis remains to be elucidated. Recent research suggests that dysbiosis of the microbiome may play a role. Our research team was the first to simultaneously analyse both the bacterial microbiome and the fungal mycobiome of the skin, stool, and blood in rosacea patients and healthy controls.

Our analysis revealed that the skin microbiome of rosacea patients differs significantly from that of healthy individuals, as indicated by alpha and beta diversity metrics. Conversely, no substantial disparities in bacterial or fungal composition were identified in stool or blood samples. Furthermore, the fungal diversity and abundance exhibited no significant disparities between patients and controls across the sampled tissues.

The hypothesis that alterations in the gut microbiome are directly transferred to the skin via the bloodstream was not confirmed. However, the skin of patients with rosacea was found to be characterized by distinct bacterial (*Cutibacterium*, *Neisseria*) and fungal (*Malassezia*, *Aspergillus*, *Alternaria*, and others) genera. Stool samples revealed increased levels of *Bacteroides*, *Prevotella*, *Faecalibacterium*, and fungal taxa such as *Candida*, *Saccharomyces*, and *Penicillium*. These findings suggest a potential link between altered microbial profiles and rosacea pathophysiology.

Notably, the predicted microbial metabolites in rosacea patients exhibited a predominantly pro-inflammatory profile, while anti-inflammatory metabolites predominated in healthy individuals. Furthermore, the data suggest the presence of interaction between fungal and bacterial populations within the gastrointestinal tract. Specifically, elevated levels of *Saccharomyces* were observed to be associated with the presence of anti-inflammatory bacteria, such as *Prevotella* and *Agathobacter*. In contrast, *Candida* dominance was found to be correlated with a decrease in these anti-inflammatory bacteria. These observations lend support the hypothesis that the fungal mycobiome has the capacity to modulate bacterial communities and potentially influence inflammation relevant to rosacea. Further studies involving larger cohorts are necessary to enhance our comprehension of these intricate interactions and to cultivate microbiome-based therapeutic strategies.

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9 Bibliography of the candidate's publications

9.1 Publications directly related to this thesis

Joura MI, Brunner A, Nemes-Nikodém É, Sárdy M, Ostorházi E. Interactions between immune system and the microbiome of skin, blood and gut in pathogenesis of rosacea. **Acta Microbiol Immunol Hungarica**. 2021;68(1):1-6. **IF: 2,298**

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9.3 Conference presentations and posters related to this thesis

Joura MI, Brunner A, Nemes-Nikodém É, Sárdy M & Ostorházi E. Interactions between immune system and the microbiome of skin, blood and gut in pathogenesis of rosacea (poster), PhD Scientific Days of Semmelweis University Budapest 2021.

Joura MI, Jobbágy A, Dunai ZA, Makra N, Bánvölgyi A, Kiss N, Sárdy M, Sándor SE, Holló P, Ostorházi E. Microbiome Alterations in Rosacea: A Multi-Site Analysis of Stool, Blood, and Skin Dysbiosis (presentation), Annual conference of ESDR Antwerpen 2025.

Joura MI, Jobbágy A, Dunai ZA, Makra N, Bánvölgyi A, Kiss N, Sárdy M, Sándor SE, Holló P, Ostorházi E. Microbiome Alterations in Rosacea: A Multi-Site Analysis of Stool, Blood, and Skin Dysbiosis (poster), Annual conference of EADV Paris 2025.

Joura MI, Jobbágy A, Dunai ZA, Makra N, Bánvölgyi A, Kiss N, Sárdy M, Sándor SE, Holló P, Ostorházi E. Investigation of the microbiome involved in the progression of rosacea (presentation), Annual conference of the Hungarian Dermatological Society, Budapest 2025.

9.4 Conference presentations and posters not directly related to this thesis

Joura MI, Jäger K. Case Report: Refreshment or danger? - Exanthema after a swimming trip (presentation), Annual conference of Austrian Society of Dermatology and Venereology, Graz 2024.

Joura MI, Jäger K. Case Report: Diagnosis and Management of a West Nile Virus Infection Presenting with Exanthema (poster), EADV Symposium Prague 2025.

Joura MI, Roka F. Rare, aggressive, therapeutically challenging – a
case report on cutaneous angiosarcoma (presentation), Annual Meeting of the Austrian Society for Dermatosurgery, Vienna 2025.

Joura MI, Roka F, Loewe R. Rare, aggressive, therapeutically challenging – a case report on cutaneous angiosarcoma (presentation), Annual conference of the Austrian Society of Dermatology and Venereology, Vienna 2025.

10 Acknowledgements

First and foremost, I would like to express my sincere gratitude to my supervisor, Assoc. Prof. Dr. Eszter Ostorházi, for her invaluable guidance, mentorship, and unwavering support throughout the course of this project. Her optimism and positive attitude have deeply influenced and inspired me.

I am also very grateful to the entire Microbiome Laboratory at the University Clinic of Microbiology. My heartfelt thanks go to Nóra Makra, Dr. Zsuzsanna Dunai, and Éva Nemes-Nikodém for their generous support and for teaching me the practical skills required for laboratory work.

I wish to extend my sincere thanks to Prof. Dr. Miklós Sárdy, who supported me from the very beginning - not only in my scientific endeavors but also in clinical practice. His passion for dermatology, his unique approach to color theory, and his empathetic care for patients have left a lasting impression on me.

Furthermore, I would like to thank Prof. Dr. Péter Holló, current Head of the University Clinic of Dermatology in Budapest, for giving me the opportunity to continue my research at the clinic.

I am also thankful to Dr. András Bánvölgyi, Dr. Norbert Kiss, Dr. Sarolta Eszter Sándor, and Dr. Antal Jobbágy for their tremendous support throughout my work.

My sincere appreciation also goes to the entire team at the University Clinic of Dermatology for warmly welcoming me and supporting me throughout the completion of my PhD.

I would also like to express my sincere gratitude to all patients who participated in this study. Their willingness to contribute, time commitment, and trust made this research possible.

Finally, I would like to express my deepest gratitude to my family and friends. Their constant encouragement, patience, and support during challenging times have been a source of strength and have played a significant role in the success of this dissertation.