

**INVESTIGATION OF THE MICROBIOME
INVOLVED IN THE PROGRESSION OF ROSACEA**

PhD thesis outlines

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1 Introduction

Rosacea is a chronic inflammatory skin disease that predominantly affects individuals with a lighter skin phenotype in middle to later adulthood. The condition is characterized by the presence of erythema, flushing, telangiectasia, and inflammatory lesions. In some cases, there may be ocular involvement. Despite the high prevalence and significant impact on quality of life, the precise pathomechanism underlying rosacea remain incompletely understood.

Recent innovations and advances in diagnostic methodologies have resulted in a substantial improvement in the investigation of rosacea pathogenesis. Notably, the increasing utilisation of non-culture-based techniques has facilitated a more comprehensive analysis of host–microbe interactions. Next-generation sequencing (NGS) technologies have had a profound impact on the field of microbiome research, facilitating detailed characterisation of microbial composition and diversity across diverse body sites. The analysis of the bacterial 16S rRNA gene is a widely utilised method for the identification and classification of bacterial taxa. Meanwhile, the sequencing of the internal transcribed spacer (ITS) region serves as a pivotal technique for the investigation of the mycobiome.

These approaches have led to significant advances in our understanding of the functional and compositional characteristics of microbial communities that colonize the human body, including the skin, gut, and blood. The human microbiome is defined as the sum of all microorganisms present in and on the human body. It has been estimated that this comprises 100 trillion microorganisms, including

bacteria, archaea, viruses, fungi, and protozoa. Collectively, these microorganisms play a crucial role in maintaining immune homeostasis and modulating inflammatory processes.

A growing body of research has examined the role of the microbiome in rosacea. However, the findings from these studies have been inconsistent and, in some cases, controversial. Furthermore, the mycobiome has received comparatively little research attention. To the best of our knowledge, this is the first study to simultaneously assess differences in the skin, stool, and blood microbiome, as well as the mycobiome, in individuals with and without rosacea.

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. Analyse 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 Dermatocology 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 1.

Table 1. 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)	Type 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 ZymoBIOMICS 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 (53). 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 was 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 was 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 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 presents 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 finding 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 suggest 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.

5 Bibliography

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