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Intestinal mycobiome associated with diagnosis of inflammatory bowel disease based on tissue biopsies

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Abstract

Analysis of mycobiome from formalin-fixed, paraffin-embedded (FFPE) biopsies should preferentially detect only fungi which are actually present in the intestine wall, in contrast to stool samples, which are limited by the diet composition. Next generation sequencing provides the advantage of analyzing many species from a single sample. Consequently, canonical correspondence analysis divided fungal genera present in FFPE intestinal tissues into three well-defined experimental groups (negative controls - NC, Crohn's disease - CD, ulcerative colitis - UC). Simultaneously, the analysis showed that particular fungal genera are associated with these experimental groups and several fungal genera occurred in all experimental groups equally. Our results also showed a noticeable increase of Ascomycota proportion from NC, through CD to UC. Fungal genera *Malassezia*, *Cladosporium* and *Toninia* occurred in all experimental groups assuming that they are common components of the intestinal mycobiome. Other fungal genera found only in the NC experimental group were non-pathogenic and might bring some benefits. In contrast, CD and UC samples were characterized by an accumulation of genera with inhibitive effects on growth of other fungal genera and the presence of opportunistic pathogens. Furthermore, a decrease in the fungal genus *Malassezia* in inflammatory tissues was observed; Specifically, the UC experimental group showed a connection between the presence of *Candida* and seven time's lower amounts of *Malassezia* (compared to amounts found in NC). The CD experimental group was characterized by the simultaneous presence of *Engyodontium album* with *Lecanicillium*, and indicates a possible pathogenic effect of *Ramularia* in disease development.

Lay Summary

Mycobiome analysis of formalin-fixed and paraffin-embedded biopsies may highlight actual fungal genera composition in the intestinal wall. Interestingly, experimental groups of Crohn's disease and ulcerative colitis clearly differed by structure of their mycobiomes.

Key words: mycobiome, FFPE samples, ulcerative colitis, Crohn's disease, NGS.

Introduction

Fungal diagnostic is a difficult topic, mostly because of the high resistance of fungal cells and their widespread distribution in the environment, food and human body. Especially samples from the gastrointestinal tract contain multiple fungi species, and casual methods are not able to identify such a broad range of fungal species, which can lead to misidentification. An appropriate method for fungal microbiome diagnostics is next generation sequencing (NGS) and due to quick development, NGS is becoming cheap and available for diagnostics purposes.

Mycobiome (fungal microbiome) is another possible component of unknown pathogenesis of inflammatory bowel disease (IBD). Many studies have shown unbalanced bacterial and fungal microbiomes.[1–3] Since bacterial biodiversity is reduced in both broadest IBD subtypes, fungal biodiversity in Crohn's disease (CD) remains unchanged. Consequently, disruptions occur among the interactions of the bacterial and fungal microbiomes. On the other hand, the fungal biodiversity in ulcerative colitis (UC) is increased, creating new interactions among kingdoms that can also lead to an inflammation.[4] Moreover, the fungal microbiome in the same patient differs between healthy and inflammatory tissue. For example, *Saccharomyces cerevisiae* and *Filobasidium uniguttulatum* are associated with non-inflamed mucosa, whereas *Xylariales* order is associated with inflamed mucosa.[2] Thus, any changes in the fungal and bacterial biodiversity can lead to an amplification of pathogens and disease development.

Human gut fungal microbiome has been intensively studied since 2008. The most of these studies were performed on stool samples. Namely, the Human Microbiome Project cohort revealed that the dominated genera in healthy human feces were *Saccharomyces*, *Malassezia*, and *Candida*.[5] However, stool samples may often show a high prevalence of *Saccharomyces* due to the ingestion of yeast-containing foods such as bread and beer.[4,6]

Mycobiome analysis of (FFPE) biopsies should prevent this uncertainty. Biopsies are taken by colonoscopy, which requires a special diet for 4 days before and 1 day before only of liquids. Moreover, medication with laxative effects finishes the cleaning process. Therefore, only fungi embedded in the tissue could remain in the intestine. On the other hand, disadvantages of FFPE samples represent DNA degradation by formalin and possible contamination during the histological processing of tissue. According to Flury et al.,[7] the fungal DNA degradation by formalin is negligible. Thus, this study reveals results of NGS mycobiome analysis from FFPE samples of CD, UC patients and healthy controls, divides fungal genera into three experimental groups, determines their affiliation to health and disease, suggests fungal genera interactions, and shows specific fungal genera related to the diagnosis.

Methods

FFPE intestinal biopsies from the years 2017 to 2019 kept in the archive of Bioptická laboratoř s.r.o. were analyzed during the years 2018–2019. These samples were obtained from the small intestine, colon and rectum by colonoscopy or gastroscopy. Each excised biopsy tissue was fixed in 10% formalin and histologically processed. Ten samples with UC diagnosis, eight samples with CD diagnosis and nine samples with no proven inflammatory bowel disease (NC) were selected based on a histological diagnosis. Detailed sample's information is listed in Table 1.

Table 1. Patients information

Sample number	Sex	Age	Histological diagnosis	Disease state
1	Woman	64	Ulcerative colitis	Mild
2	Woman	36	Ulcerative colitis	Severe
3	Man	30	Ulcerative colitis	Severe
4	Man	23	Ulcerative colitis	Mild
5	Man	54	Ulcerative colitis	Mild
6	Man	68	Ulcerative colitis	Mild
7	Woman	22	Ulcerative colitis	Mild
8	Man	27	Ulcerative colitis	Mild
9	Man	27	Ulcerative colitis	Mild
10	Woman	42	Ulcerative colitis	Moderate
11	Woman	58	Crohn disease	Normal
12	Woman	31	Crohn disease	Stenosis
13	Woman	28	Crohn disease	Relapsis
14	Man	38	Crohn disease	Stenosis
15	Man	40	Crohn disease	Fistulizing
16	Man	71	Crohn disease	Segmental
17	Woman	46	Crohn disease	Normal
18	Man	46	Crohn disease	Normal
19	Woman	50	Without inflamation	None
20	Woman	66	Without inflamation	None
21	Woman	31	Without inflamation	None
22	Man	39	Without inflamation	None
23	Man	23	Without inflamation	None
24	Man	46	Without inflamation	None
25	Woman	46	Without inflamation	None
26	Woman	45	Without inflamation	None
27	Woman	69	Without inflamation	None

DNA extraction was provided by BiOstic® FFPE Tissue DNA Isolation Kit (MO BIO laboratories, Inc., USA) and performed according to the manufacturer´s instructions. To prevent possible contamination, the whole isolation process was performed in a laminar box (BIO 130A2, Alpina, Poland). The paraffin blocks were cut on a rotary microtome. Between each sample, the microtome was decontaminated with a Termi-DNAtor (Biotools B&M Labs S.A.) and the first section from each sample was discarded. Subsequently, five sections of 5 μm thickness were cut and transferred to a 2 ml tube. To the tube were added 180 μl of the Solution FP1 and 20 μl of the Solution FP2, whose combination form a high activity dissolving buffer that breaks down wax and enhances Proteinase K activity. Next, 20 μl of the Solution FP3 (Proteinase K, 20 mg/ml) were added a mixed by the vortex. The sample was incubated at 55°C for 1 h and then at 90°C for 1 h. Next, the sample was centrifuged at 13 000 × *g* for 1 min and digested lysate was transferred to a new 2 ml collection tube. Next, 200 μl of the Solution FP4 (a chaotropic salt buffer) were added and mixed by the vortex. Next, 200 μl of the Solution FP5 (100% ethanol) were added and mixed by the vortex. The entire lysate (620 μl) was loaded onto the Spin Filter and was centrifuged at 10 000 × *g* for 1 min. Next, 500 μl of the Solution FP6 (wash buffer for removal of protein

and other non-aqueous contaminants from the membrane) were added and centrifuged at 10 000 x g for 1 min. Next, 500 μl of Solution FP7 (ethanol based wash buffer for removal of salt from the membrane) were added and centrifuged at 10 000 × *g* for 1 min. Next, the sample was centrifuged at 13 000 × *g* for 2 min to dry the membrane. Finally, the purified DNA was eluted with 50 μl of Solution FP8 (10 mM Tris pH 8.0). DNA concentration was measured on Qubit 2.0 (Thermofisher Scientific, USA); samples with a concentration ≥2 ng/μl were used for NGS analysis. Extracted DNA was stored at −20°C.

PCR analyses

Nested PCR was designed to capture both ITS regions (ITS1, ITS2) with primers ITS 5 and ITS 4 in the first round and ITS2 region with primers ITS 3 and ITS 4 in the second round of the PCR analysis.[8] First, PCR reaction was performed in a volume of 25 μl consisting of 12.5 μl HotStarTaq Master mix (1000 U, Qiagen), 1.0 μl of 10 μM primer (Generi Biotech, Czech Republic), 2.0 μl of DNA in the first round and 1.0 μl of DNA in the second round. RNAse free water was added to the PCR premix in order to reach the total volume of 25 μl. Second, the first round PCR amplification program started with an initial denaturation at 95°C for 14 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 1 min and extension at 72°C for 1 min. Final elongation was done at 72°C for 10 min. In the second round, the same program was used with the exception in denaturation at 94°C for 1 min and annealing at 51°C for 1 min. PCR reactions ran in a thermocycler SymplyAmp (Thermofisher Scientific, USA). Third, PCR products were visualized by electrophoresis in 2% agarose gel with addition of 3.2 μl Ethidium Bromide (Top-Bio, Czech Republic). Consequently, they were purified with AMPure XP (Beckmancoulter, USA) according to the manufacturer´s protocol.

Next generation sequencing (NGS)

Purified PCR products were additionally processed by KAPA Hyper plus kit (KHP) according to the manufacturer´s instructions with a few customizations. Namely, to reach an optimal length of 270 bp,[9] fragmentation at 37°C for 15 min was used. Further, samples were tagged by specific adapters and amplified by the program: 98°C/45 s; 6× (98°C/15 s, 60°C/30 s, 72°C/30 s); 72°C/1 min. Finally, the 2 nM library was analyzed in NextSeq 550 with a sequencing chemistry for 300 cycles. The sequencing fulfilled the manufacturer´s validity conditions. In addition, sequencing data were uploaded into the Illumina basespace, analyzed by ITS Metagenomic application. The UNITE Fungal ITS Database v7.2 is based on FASTA from: UNITE Community (2017): UNITE general FASTA release. Version 01.12.2017. UNITE Community. https://doi.org/10.15156/BIO/587475 includes singletons set as RefS (in dynamic files). Finally, fungal species names were united by server UniProt.org. Only fungal genera with a percentage of reads higher than 2% of the total number of reads in the sample were included into analyses.

Statistical analyses

Multivariate data on the representation of each genus in samples were analyzed using canonical correspondence analysis (CCA) in Canoco 5.1 software.[10] Since the data were arranged similar to percentages (i.e., 100 reads for each genus), we used arcsine data transformation prior to analysis. Fungi genera represented response variables, the number of unclassified reads was used as a covariate, and sex, age and experimental group of patients (UC, CD and NC) were used as explanatory variables. Forward selection of independent variables was used, and statistical significance was obtained by Monte Carlo permutation tests under 499 permutations. We also performed power analysis for our CCA according to Skalski et al.[11] using noncentrality parameter, numerator (number of experimental groups -1) and denominator degrees of freedom (number of patients – number of experimental groups) with qf and pf functions from the base package in R 4.0.3. software.[12] Further we simulated the power of our CCA analysis if we used 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 patients by changing the denominator degrees of freedom. Power was expressed by the proportion (0-1) and fitted by a non-linear estimation in Statistica 13 software.[13]

The relationships between scores from the first and second ordination axis and proportion of Ascomycota/Basidiomycota were tested by linear regressions using Statistica 13. Scores from ordination axes were further used to find the position of each fungal genus within the ordination space and to order all genera along the first and second ordination axis.

Further, the effects of independent variables were tested (age, sex, experimental group and its interactions) on the proportion of Ascomycota/Basidiomycota using generalized linear mixed models (GLMM) in R 4.0.3. First, a null model was built using lmer function (package Lme4) without independent variables and with random variable proportion of unclassified genera. Next, six alternative models were built (independent variables and their interactions: age, sex, experimental group, experimental group*age, experimental group*sex, age*sex). Finally, each alternative model was compared with a null model using ANOVA function and Chi test.

Finally, we calculated alpha (genera richness, Shannon's diversity index) and beta diversity (Jaccard similarity index) for all samples and for each experimental group separately after Shannon (1948) and Jaccard (1908).[14,15]

Results

All samples were correctly sequenced and the generated fastq files reported an average value of 2.6 million reads passing the quality filtering per sample. The mean of the total reads

Table 2. Results of CCA analysis on the effect of independent variables on the distribution of fungal genera within our samples

Independent variable	% of explained variability	pseudo-F	*P*
Experimental group	29.0	2.3	0.089
Age	26.6	1.2	0.268
Sex	16.4	0.7	0.868

classified into the genus level was 70% and the average amplicon length was 250 bp. The raw sequencing data are available on request.

Overall, the most common was the presence of *Malassezia* (32.1% of all reads), followed by *Cladosporium* (12.5%), *Schizopora* (7.2%), *Candida* (6.6%) and *Engyodontium* (5.4%). Other genera formed less than 5% of all reads. Based on CCA analysis, each experimental group showed an indicative distinct composition of fungal community (Table 2, Fig. 1). First, the NC experimental group was mainly connected with the presence of genera *Microidium*, *Incertomyces*, *Saturnispora*, *Hygrophorus*, *Extremus*, *Periconia*, *Botrytis*, *Aspergillus*, *Debaryomyces* and *Coniosporium*. Second, the CD experimental group was connected with the presence of genera *Ramularia*, *Xylodon*, *Corticium*, *Descolea*, *Neokalmusia*, *Engyodontium*, *Paraconiothyrum* and *Lecanicillium*. Finally, the UC experimental group was often connected with the presence of genera *Pichia*, *Filobasidium*, *Clonostachys*, *Alternaria*, *Candida*, *Rhabdocline*, *Helicoma*, *Trametes*, *Strobilurus*, *Naevala*, *Neoascochyta* and *Lemonniera*. In addition, genera *Toninia*, *Cladosporium*, *Malassezia*, *Penicillium* and *Vishniacomyza* occurred rather independently on experimental groups. Genus *Schizopora* was common for both groups of ulcerative colitis and Crohn's disease.

Along the first ordination axis, a clear gradient from negative control through ulcerative colitis to Crohn's disease was found. In contrast, a similar gradient was found along the second ordination axis, but the border of Crohn's disease and ulcerative colitis exchanged (Fig. 1). The effect of age and sex was not significant (Table 2). According to power analysis, our CCA reached power 80.7%. Increase of power to 90% would be possible by adding ten further patients (Fig. 2). Order of genera along the axes also differed between the first and second ordination axis. Both these gradients started by genera associated with negative control, but they continued differently (Fig. 3). Along the first ordination axis, the highest scores were found for genera associated with Crohn's disease (descendent order: *Lecanicillium*, *Engyodontium*, *Paraconiothyrum*, *Neokalmusia*, *Descolea*, *Corticium*, *Xylodon*). For the second ordination axis, the highest scores were found for genera associated with ulcerative colitis (descendent order: *Lemonniera*, *Naevala*, *Neoascochyta*, *Strobilurus*, *Helicoma*, *Trametes*, *Rhabdocline*, *Candida*, *Alternaria*, *Clonostachys*, *Filobasidium*, *Pichia*). This distribution of fungal genera among experimental groups was also supported by their abundances (% of reads, Fig. 4). For taxonomical and ecological affiliation of fungal genera, see Supplementary Content 1.

Proportion of Ascomycota/Basidiomycota followed the pattern found within the ordination space. Proportion of Ascomycota/Basidiomycota was marginally, significantly positively correlated with scores from first ($R^2 = 0.18$, $F = 2.13$, $\beta = 0.28$, $P = 0.112$, S2a) and second ordination axis ($R^2 = 0.17$, $F = 2.15$, $\beta = 0.27$, $P = 0.104$, S2b). Again, we revealed the exchanged position of patients with ulcerative colitis and Crohn's disease along the ordination axes. Along the first ordination axis, patients with Crohn's disease positioned towards the highest score. Along the second ordination axis, this was valid for ulcerative colitis. Both these relationships together formed the main gradient between healthy and ill patients that was visible also in our CCA analysis (Fig. 1A). Therefore, the proportion of Ascomycota was increased in both groups of Crohn's disease and ulcerative colitis compared to the negative control group. Additionally, we tested the effect of age, sex, experimental group and its interactions on the proportion of Ascomycota/Basidiomycota, but none of the tested variable/interaction had significant effect despite high percentages of explained variability. The least statistical significance was reported for the independent variable experimental group (S3). The patients within each experimental group showed closed affinity to each other based on occurrence of particular fungi genera (Fig. 1B).

Alpha diversity expressed by both genera richness and Shannon's diversity index decreased from the experimental group for ulcerative colitis, through Crohn's disease to negative control (Table 3). Beta diversity expressed by Jaccard similarity index was the lowest among samples for negative control and increased among samples for the experimental groups of ulcerative colitis and Crohn's disease (Table 3).

Discussion

Since fresh tissue samples are often not available for diagnostic testing, FFPE biopsies are appropriate samples for clinical diagnostic, because they are stable at room temperature, easily storable and contain accurate information directly from the patient's tissue. However, formalin fixation could be perceived as disadvantage, because it causes degradation and base alterations.[16] Nevertheless, the degradation of human DNA may not always predict the degradation of fungal DNA, because fungal cell wall has protective effect against formalin.[7,17] Furthermore, DNA extraction is a key step for successful fungal identification. FFPE samples require special protocols in order to extract small amounts of DNA suitable for amplification. These protocols of available commercial FFPE kits appear to be sufficient for fungal DNA extraction too, even without the use of cell wall disrupting enzymes such as chitinase.[18–20] The fungal detection was aimed to capture a long section of resident fungal DNA about 700 bp

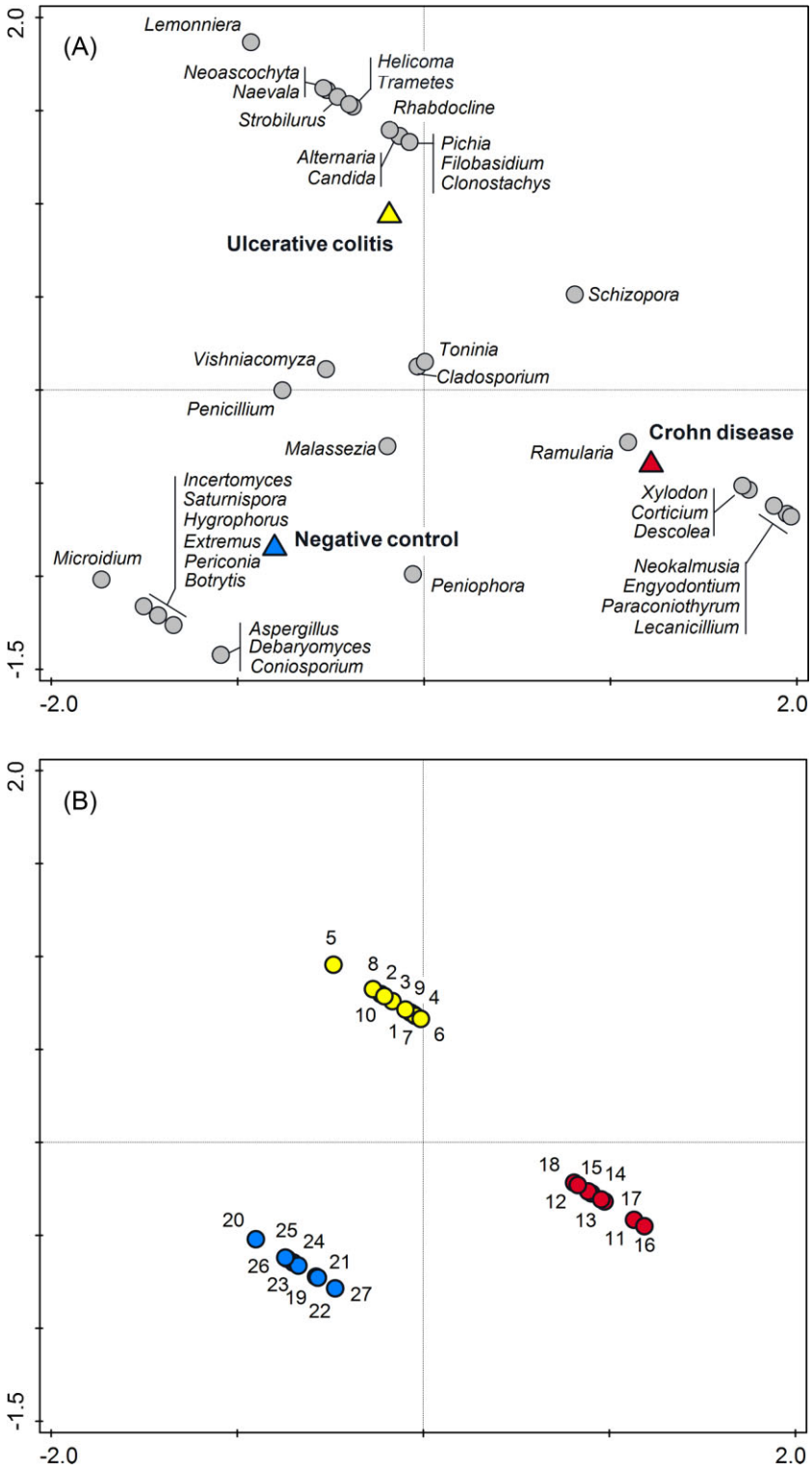

Figure 1. The projection scores for fungal genera regarding the experimental group (CCA analysis, first and second ordination axes together explained 63.2% of variability) and samples distribution.

(ITS 1 - 5.8S - ITS 2) to avoid capturing of non-resident fungal DNA, suggesting that non-resident fungi were breaks down by digestion.[21,22] Amplification of ITS 2 region in the second round of nested PCR should specify the input for sequencing analysis. Due to the amplification of the 700 bp DNA section there might be uncertainty about the failure to detect targeted DNA that may be fragmented by formalin. This may lead to a possible bias of the fungal population, which may be caused by the

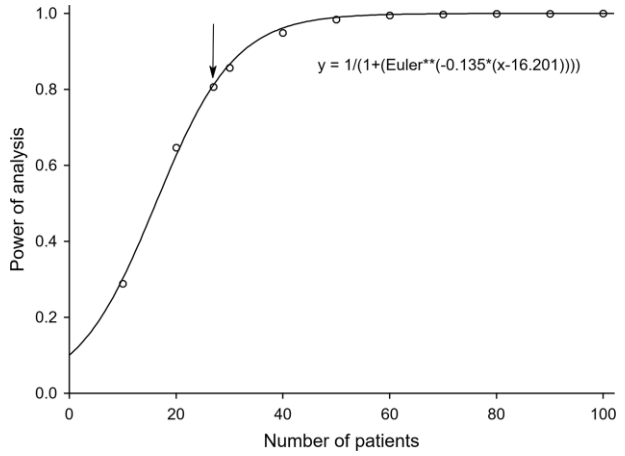

Figure 2. Simulation of power for CCA analysis for a different number of patients.

different cell wall abilities of various fungal genera. The melanin and other fungal pigments can inhibit PCR reaction via enzymatic inhibition of polymerase thus samples from cultured fungi must be diluted appropriately. In the case of bioptic samples, the quantity and quality of DNA for fungal species is unknown as well as the form in which are represented in sample e.c. hyphae, spore, blastic cell. Besides this, the morphological changes of fungi in the environment of the human gut are assumed via adaptive mechanisms that includes also melanin biosynthesis.[23] For example, yeasts are the easiest detectable fungi, filamentous fungi are more difficult to detect including Mucoromycetes which are the most difficult to detect.[16,18,20] Despite the prevailing circumstances, in our laboratory clinical experience, this methodology is able to detect routine clinical pathogens (e.g., *Alternaria spp*., *Cladosporium spp*., *Fusarium spp*., *Aspergillus spp*., *Cryptococcus spp*., *Trichophyton spp*., *Rhizopus spp*.).

FFPE sample analysis of three defined experimental groups (NC, CD, UC) showed several diagnosis-independent fungal genera. We suggest that three of them '*Malassezia*, *Cladosporium* and *Toninia*' represent the common part of the human gut mycobiome. Also, our data described a noticeable increase of Ascomycota proportion from negative controls, through Crohn's disease to ulcerative colitis, which is in agreement with available literature.[2,4,5] Further, the data showed specific fungal genera composition for each experimental group. Although their function is not sufficiently elucidated, we can estimate their purpose based on biochemical properties of proven enzymes. Finally, despite the relatively small groups of samples, which may not provide statistically robust results, the power analysis confirmed the correctness of our results.

The first independent fungal genus, *Malassezia*, is a mammalian skin commensal dependent on host lipids. This yeast is enriched for genes encoding lipases, phospholipases C, acid sphingomyelinases and aspartyl proteases, which all provide fatty acids from host lipids.[24] A potential beneficial effect of *Malassezia* in gut microbiome is its ability to ferment mono- and diacylglycerol to form short chain fatty acids (SCFA).[25] SCFA are formed from undigested food components, and they have many positive influences on the human body, such as energy for enterocytes and anti-inflammatory effects.[26,27] Furthermore, SCFA also inhibit filamentation and may prevent *Candida* sp. from causing disease.[28] For example, all our samples with proven *Candida* sp. have seven times lower amounts of *Malassezia* than negative controls. To sum up, by SCFA production *Malassezia* may positively affect the gut ecosystem, and prevent disease from fungal/*Candida* filamentation.

A second independent fungal genus, *Cladosporium*, is a filamentous fungus which often occurs as a plant pathogen in the outdoor environment but also as a black mold indoors. Commonly, it gets into the intestine with 'contaminated' food and its further impact depends on host immunity.[29,30] *Cladosporium* has several virulence factors. For example, peptidases are known to hydrolyze elastin and laminin, which could facilitate the penetration of young hyphae into the host connective tissue, or enolase and serine proteases causing extensive cross-reactivity and auto-reactivity.[31,32] Thus, *Cladosporium* is an opportunistic pathogen capable of penetrating barriers, especially in immunocompromised patients, irritate immune system and causing overreactions.

Third independent fungal genus, *Toninia*, is a fungal part of lichens occurring on the surface of soil, sand and rocks.[33] Lichen extracts, specifically norstictic and usnic acid show a relatively strong antimycobial activity but their antimicrobial activity is much stronger.[34] Therefore, we assume that *Toninia* might maintain a balance between fungal and bacterial kingdoms.

Negative controls were mostly composed of basic fungal genera (*Malassezia*, *Cladosporium* and *Toninia*) supplemented with additional specific genera. Only fungal genera highly correlated with the experimental group NC are discussed. First, *Debaryomyces* is often used in the food industry as cheese yeast,[35] and is one of the first fungi present in the human gut mycobiome in early life.[36] *Debaryomyces* is able to survive gastrointestinal conditions, adhere to the intestinal wall, and stimulate the immune system by its β-glucans from the cell wall.[37] Furthermore, *Debaryomyces hansenii* could be used as a probiotic for animals and even for humans.[37] Second, *Aspergillus* is ubiquitous in the natural environment and represents a dominant indoor pathogen and its spores are omnipresent. Therefore, *Aspergillus* is daily inhaled into the lungs, and it is also consumed with food.[32] Furthermore, *Aspergillus* can produce a wide spectrum of bioactive molecules. For example, musizin have antifungal and antioxidant effects,[38] and echinocandins are bioactive molecules with antifungal effect that inhibit the cell wall synthesis. The production of bioactive molecules depends on

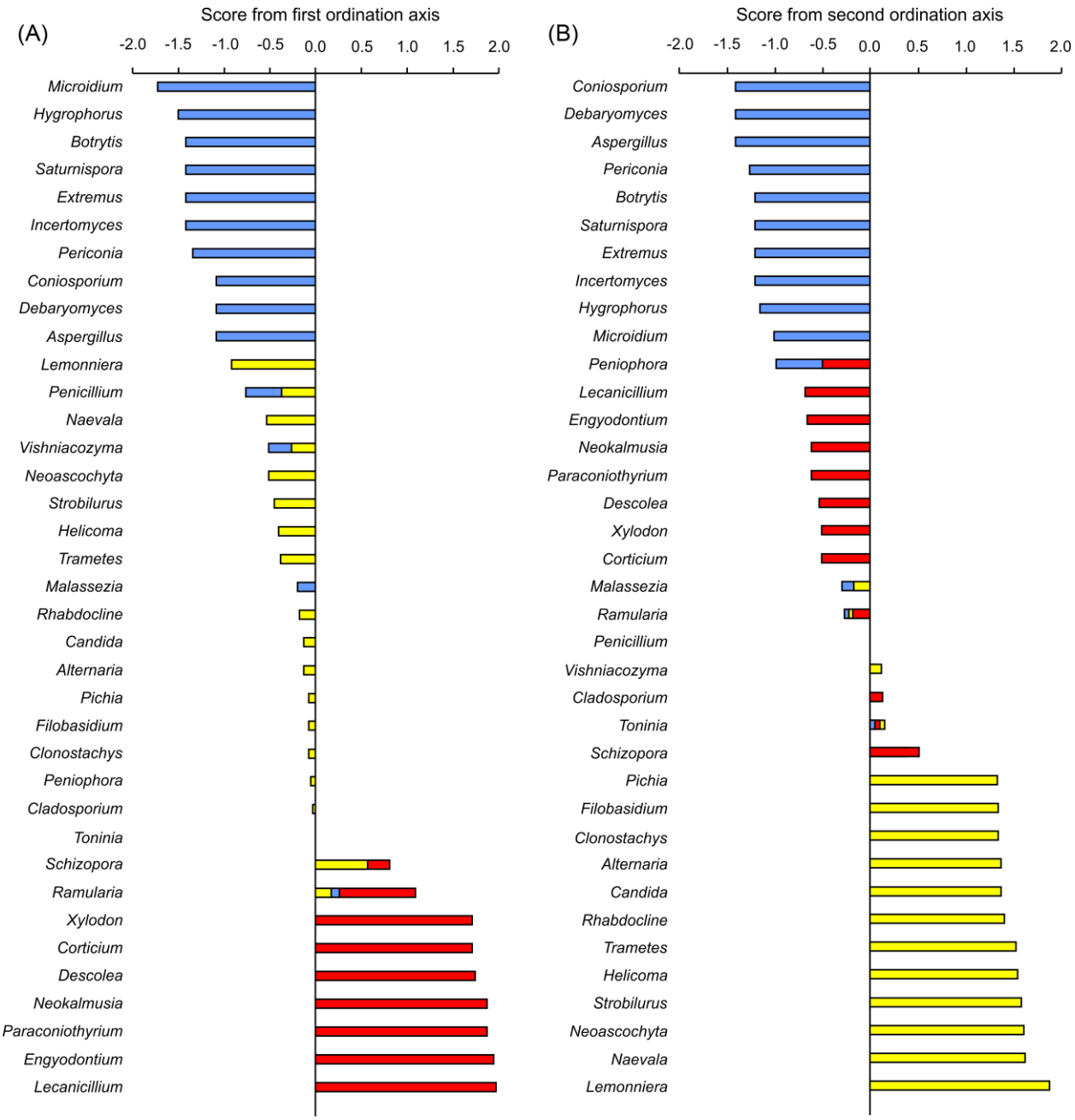

Figure 3. Detailed interpretation of fungal representation and their affiliation to diagnosis. Legend: blue – negative control, red – Crohn's disease, yellow – ulcerative colitis.

cultivation conditions including substrate, aeration and temperature. In respect to the bioptic samples, individuality of patient and medication, the real role of fungus in gastrointestinal environment is predicted only on the base of current environmental studies or partial microbiological studies *in vitro*. In addition, this filamentous fungus belongs in heathy gut mycobiome together with *Penicillium*, which is also commonly found in food and capable of growing in almost any environment.[39] Thirdly, *Coniosporium* is an environmental fungus forming black spots or patches on plant leaves, bamboo, rotten wood and rock surfaces.[40] In recent years, it was also classified as a human skin pathogen.[41] Thus, it may also come into the digestive system with contaminated food, but it has probably no pathogenic effects in the human gut. In conclusion, most fungal genera included in the negative control group are commonly found in the environment, their exact role in the intestinal mycobiome is yet unknown, but current knowledge suggests non-pathogenic activity and beneficial effects.

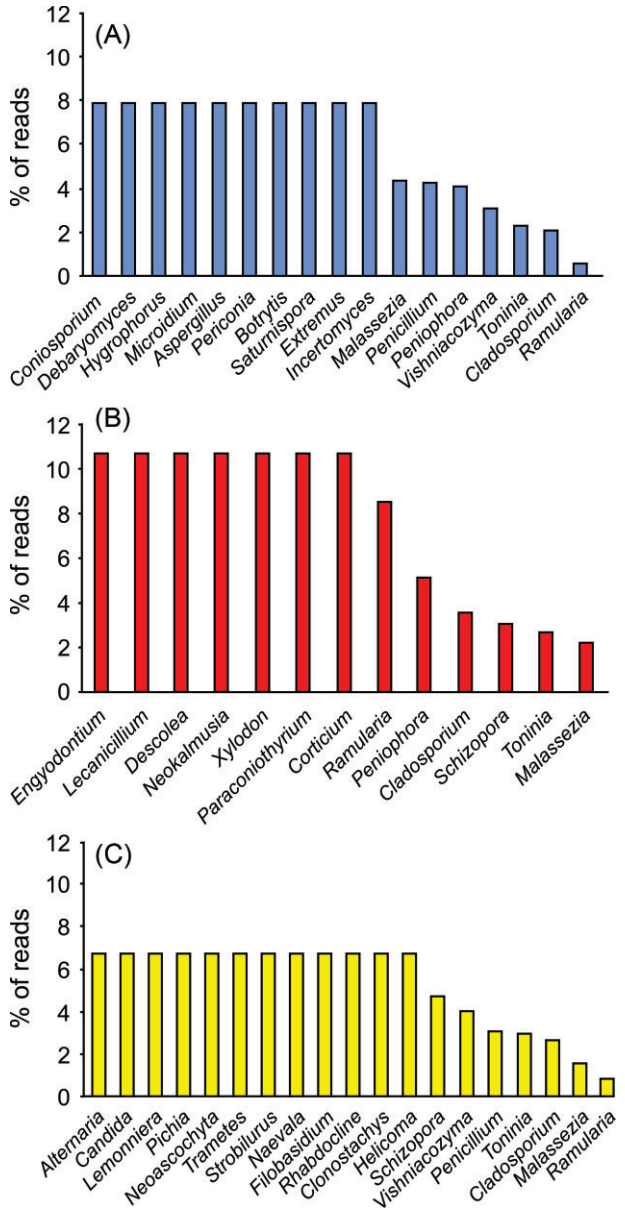

Figure 4. Percentages of reads for each fungal genus within (A) negative control, (B) Crohn's disease and (C) ulcerative colitis experimental group.

Basic fungal genera associated with CD mycobiome were similar to the NC group with lower amounts of *Malassezia* sp. and a noticeably higher incidence of *Ramularia*. Genus *Ramularia* is mostly a barley pathogen.[42] Also *Ramularia* produces a wide spectrum of secondary metabolites. Namely rubellin anthraquinones performed the antimicrobial activity against gram-negative bacteria.[43] Interestingly, *Ramularia* (together with *Fusarium* and *Aspergillus*) is also an endophytic fungus living in the plant *Rumex gmelini* Turcz which is used in Chinese medicine.[44] Despite producing beneficial secondary metabolite chrysophanol, which has antiviral and anti-inflammatory effects, *Ramularia* occurring without *Fusarium* and *Aspergillus* seems to be toxic.[44] Whereas we proved the genus *Ramularia* without them in all cases, it might be assumed that *Ramularia* plays a role in disease development. Another fungal genus found in CD samples was *Lecanicillium* which is pathogenic to insects and to other fungi, such as the commercially cultivated mushroom - *Agaricus bisporus*. Moreover, it was observed also as a human skin pathogen.[45–47] With its antifungal effects, *Lecanicillium* might also modulate the mycobiome composition and disturb the gut balance. Interestingly, in our samples *Lecanicillium* was always detected together with *Engyodontium album*. Some authors describe *Engyodontium album* as a causative agent of different kinds of dermatoses, respiratory tract diseases and endocarditis mostly in immunocompromised patients.[48,49] As a virulence factor may serve proteinase K that is commonly obtained from this species.[50] In conclusion, after the accumulation of species with fungal growth inhibitive effects, beneficial yeast *Malassezia* may decrease, and this process might release space for potential pathogens, such as *Ramularia* sp. or *Engyodontium album*.

UC experimental group was characteristic by an increased Ascomycota/Basidiomycota ratio than other groups and with specific fungal genera connections. The most interesting connection is a decrease of genus *Malassezia* and an increase of genus *Candida*. According to Zwolinska-Wcislo et al.,[51] genus *Candida* occurs in long-term ulcerative colitis and complicates ulcer healing. To improve the patient's condition either fluconazole antifungal therapy or probiotic therapy by *Lactobacillus acidophilus* is used, which inhibit *Candida* filamentation by producing SCFA.

In conclusion, our data showed variability in the presence of different fungal genera among experimental groups that can be also explained their possible cooperation/inhibition in the intestinal mycobiome. Future experiments may verify our results by functional tests of fungal interactions.

Table 3. Alpha and beta diversity for all samples and within each experimental group expressed as genera richness, Shannon's diversity index and Jaccard similarity index (mean ± s.d. [range])

Variable	All samples	Ulcerative colitis	Crohn's disease	Negative control
Genera richness	3.70 ± 1.68 (1–7)	4.20 ± 1.60 (1–7)	3.50 ± 1.58 (1–6)	3.33 ± 1.56 (1–6)
Shannon's diversity	1.36 ± 0.75 (0.25–0.74)	1.60 ± 0.72 (0.00–2.47)	1.29 ± 0.69 (0.00–2.30)	1.15 ± 0.75 (0.00–2.44)
Jaccard similarity	0.19 ± 0.20 (0.00–0.50)	0.23 ± 0.18 (0.00–0.50)	0.21 ± 0.26 (0.00–0.50)	0.17 ± 0.16 (0.00–0.50)

Supplementary material

Supplementary material is available at *MMYCOL* online.

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Author contributions

Data collection and analysis: JC. Statistical analysis: JR. Manuscript draft: JC and JR. Clinical experience and consultation: KC and MK. Final manuscript and approval: All authors.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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