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# DISTINCT COMPOSITION AND DISTRIBUTION OF THE GASTRIC MYCOBIOTA OBSERVED BETWEEN DYSPEPTIC AND GASTRIC CANCER PATIENTS EVALUATED FROM GASTRIC BIOPSIES

A. B. R. Hansen[1], T. B. Johannesen[2], M. R. Spiegelhauer[1], J. Kupcinskas[3,4], M. Urba[3,4], J. Skieceviciene[4], L. Jonaitis[3], T. H. Frandsen[1], L. Kupcinskas[3,4], K. Fuursted[2], L. P. Andersen[1]

[1]Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark
[2]Department of Clinical Microbiology and Infection Control, Statens Serum Institute, Copenhagen, Denmark
[3]Department of Gastroenterology, Lithuanian University of Health Sciences, Kaunas, Lithuania
[4]Institute for Digestive Research, Lithuanian University of Health Sciences, Kaunas, Lithuania

*Corresponding Author:* Amalie B. R. Hansen, MSc; email: amalie.boege.rud.hansen@regionh.dk

**Abstract – *Objectives:*** In recent years, studies have proved that the stomach is not sterile as previously believed and thereby harbors a unique gastric microbiota. Since most studies have investigated the bacterial composition of the gastric microbiota, the investigation of other microorganisms is still in its infancy. To date, the fungal composition of the stomach (the gastric mycobiota) has gained more attention in microbiota studies. Nevertheless, there is a lack of studies investigating the gastric mycobiota and the association to the pathogenesis of gastric diseases.
We aim to investigate the composition of the gastric mycobiota of patients diagnosed with dyspepsia or gastric cancer and define the persistent and transient fungal colonizers of the stomach.
***Patients and methods:*** Gastric biopsies from twenty-two patients diagnosed with dyspepsia and twelve patients diagnosed with gastric cancer were analyzed by 18S rDNA sequencing to compare the gastric mycobiota. The gastric biopsies were either unwashed or washed to distinguish fungal adherence. To compare the mycobiota from cancer tissue and normal tissue, the gastric biopsies from gastric cancer patients were taken from two sites; antrum (AN) and corpus from cancer area (CA).
***Results:*** The distribution and composition of the gastric mycobiota in gastric cancer and dyspeptic patients were significantly distinct. The most prominent difference was observed in the relative abundance of the fungal genus *Malassezia* as it was significantly increased in gastric cancer patients. *Malassezia* is an opportunistic pathogen, which has been shown to promote the formation of several cancer types. Thereby the results in this study indicate that *Malassezia* may play a role in the formation of gastric cancer, however, further investigation is needed.
***Conclusions:*** The results from this study show that the gastric mycobiota might has an important role for the pathogeneses of human gastric diseases, as significant changes in the gastric mycobiota are observed in gastric cancer patients compared to dyspeptic patients. This advocates more research within the role of gastric mycobiota as more knowledge can lead to new therapeutics.

**Keywords:** Fungi, Mycobiota, Stomach, Gastric microbiota, Gastric biopsies, Gastric cancer, Dyspepsia.

## INTRODUCTION

### The Gastric Microbiota

The human gastrointestinal (GI) tract harbors a complex and diverse microbial ecosystem called the GI microbiota that includes microorganisms from all three domains of life; Bacteria, Archaea and Eukarya[1-5]. The GI microbiota has a huge effect on human development and health involving several physiological functions and pathogenesis of diseases[1,6].

The stomach is an organ of the GI tract, which functions as a defense mechanism against ingested microorganisms and thereby shapes the entire microbial ecology of the GI tract[4,7,8]. It has initially been thought that the inhospitable ecological environment of the stomach is not suitable for microbial colonization and survival[2,9-12]. This is due to the protective defense barriers like low pH, gastric peristalsis, mucus thickness, and secretion of bile and acid[9,13]. However, in the 1980s the Gram-negative bacterium *Helicobacter pylori* (*H. pylori*) was discovered to colonize the stomach, which changed the view of considering the stomach as a sterile organ[7,9,10,13-15]. In the time after the discovery of *H. pylori,* it was thought that *H. pylori* was the only bacterium with the ability to colonize the stomach[10,14,16,17]. However, along with the advances in molecular-based methods, several studies[10,12-14,16-18] uncovered that the stomach harbors a diverse non-*H. pylori* microbiota called the gastric microbiota. To date, the investigation of the gastric microbiota in health and disease is still in its infancy and thereby not well understood[13].

### Fungal Composition of the Gastric Microbiota

Since bacteria are the most dominant domain of the gastric microbiota, they have been the major focus in most studies[1,4,6,13,19-24]. Despite that, recent studies have become aware of the importance of the less explored microorganisms of the gastric microbiota, such as Archaea and Eukarya, and how they may influence humans in both health and disease[2,8,13]. It is known that fungi and especially yeast can be isolated from gastric samples but to date, the fungal composition in the stomach, better known as the gastric mycobiota, is primarily investigated by culture-dependent methods[8,11,13,25]. In general, fungi represent a small proportion of the human microbiota but are supposed to have a huge influence on the human health and disease[1,3,20,26,27]. For example, a study[11] observed that patients with gastric ulcer showed high concentrations of fungi and that fungal colonization impaired the healing of the gastric ulcer.

The potential role of the gastric mycobiota in health and disease advocates the importance of establishing a baseline for the fungal composition in the stomach[3]. The progress in culture-independent methods like next-generation sequencing gives the researchers new research opportunities to study the human microbiota[1,6,11,13,20,26]. However, this approach presents some limitations regarding the analysis of the mycobiota, since it is particularly developed for bacteria[1,6,28]. To date, there are no optimal detection and analysis methods of the mycobiota; nevertheless, it requires development of standardized techniques and bioinformatics besides well-updated and curated databases[6,12,26,29]. The current method to choose is metabarcoding, where the fungal ribosomal locus is the preferable target for the barcode[1,29]. The ribosomal locus (same for all eukaryotes) contains three subunits; 18S, 5.8S and 28S, which are separated by two internal transcribed spacers (ITS1 and ITS2)[4,6,29].

### Previous studies of the gastric mycobiota

Since the culture-independent investigation of the gastric mycobiota is in its infancy, only few studies have attempted to characterize the fungal composition in the stomach[2,8,13]. A study[8], which investigated the mycobiota of gastric fluid from 25 patients, observed between 19-81 genus-level operational taxonomic units in the samples where *Candida* spp. were observed in all samples. Most studies[3,20,29-31] have focused on the mycobiota of the GI tract by basing their research on stool samples. These studies[3,6,20] demonstrate that *Ascomycota* and the *Basidiomycota* are the two dominant phyla in the GI tract. Furthermore, they show that the two major fungal genera in the GI tract are *Saccharomyces* and *Candida*[20,29,30].

A study[20] showed that the fungal diversity in the GI tract was significantly lower compared to the bacterial diversity and the intra- and inter-volunteer variability of the fungal community was high. Furthermore, recent studies[22,27,32-34] have shown that the fungal composition and diversity in patients diagnosed with diseases like intestinal bowel disease (IBD) are distinct from healthy individuals.

Based on the fact that fungi are observed in the stomach and probably influence our health, we need more knowledge to get a clear picture of the fungal composition and contribution of the gastric microbiota in both health and disease[12,35].

## Definition of the Persistent and Transient Mycobiota

To date, *H. pylori* is the only microorganism that has been shown to contain mechanisms for colonization of the human stomach[17]. Studies[9,36] claim that more than 65% of the bacterial phylotypes discovered in the stomach are also detected in the human oral cavity. This puts on a question mark on the assumption that the microorganisms found in the stomach are persistent colonizers[16,17]. Previous studies[37,38] that investigate the gastric microbiota have different views of this question. Some studies assume that a proportion of the microorganisms detected in the stomach are contaminants from the oral cavity or upper airways. Other studies[14,39-41] assume that the microbial community detected in the stomach illustrates a unique microbiota that is distinct from the oral microbiota.

The aim of this study was to investigate how the fungal composition in the human stomach differs in different disease states by comparing the gastric mycobiota from patients with dyspepsia and gastric cancer. Furthermore, the aim was to define the transient and persistent gastric mycobiota, which was performed by comparing washed or unwashed gastric biopsies.

## MATERIALS AND METHODS

### Sampling of Gastric Biopsies

This study includes twenty-two patients with dyspepsia and twelve patients with gastric cancer. The exclusion criteria for participation in the study were age below 18 years, use of proton-pump inhibitors (PPIs), and/or antibiotics within the last 3 months and previous treatment of gastric cancer. The gastric biopsies were sampled by gastroscopy between November 2017 and June 2019 at Kaunas Medical University, Lithuania. From each dyspeptic patient, three antral biopsies were sampled about four centimeters from the pylorus. From gastric cancer patients, three antrum biopsies were sampled about four centimeters from the pylorus (AN), and three biopsies were sampled from the cancer area in the corpus (CA). Out of the three biopsies from each sampling site, one biopsy was washed twice in PBS (washed), the second remained native (unwashed) and the third was immediately fixed in formalin for histology to examine the presence of *H. pylori* (used in a previous publication[42]). The washed and unwashed biopsies were placed in Portagerm pylori transport medium (bioMérieux, Marcy L'Etoile, France) and stored at -80°C.

### Microbiota Analysis (18S rDNA Gene Sequencing)

The frozen biopsies were transported to Statens Serum Institute (SSI) for microbiota analysis (18S rDNA gene sequencing). The microbiota analysis, including DNA extraction, library preparation and sequencing, was performed at SSI.

### *DNA extraction*

A QIAamp DNA mini Kit (Qiagen, Hilden, Germany) was used to extract DNA from the biopsies according to the manufacturer's instruction for tissues. A negative control with no material from samples was included for downstream analysis for each batch of DNA extraction.

## Primer design

To amplify the extracted DNA a two-step PCR was used by applying three different primer sets targeting the 18S rDNA gene. The 18S rDNA gene is assumed to be the most inter-species conserved gene between eukaryotes and enables identification of a broad spectrum of eukaryotes[43-45]. The sequences of the three primer sets are G3F1/G3R1 (GCCAGCAGCCGCGGTAATTC/ACATTCTTGGCAAATGCTTTCGCAG), G4F3/G4R3 (CAGCCGCGGTAATTCCAGCTC/GGTGGTGCCCTTCCGTCAAT) and G6F1/G6R1 (TGGAGGGCAAGTCTGGTGCC/ACGGTATCTGATCGTCTTCGATCCC). The G3 and G6 primer both target the hyper-variable region V3-V4 where G4 targets the hypervariable region V3-V5 of the 18S rDNA gene. Each primer set was aligned to the NCBI database, using NCBI's Primer-Blast, with standard settings (excluding predicted Refseq transcripts and uncultured/environmental samples) to test for unintended amplification.

## Library preparation and sequencing

The purified 18S rDNA was initially amplified by the same procedure as in the previous publicatio[42]. However, in this study, we used the 18S PCR setup and ran with an initial denaturation at 95°C for 3 min, 20 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 30 sec, and a final elongation at 72°C for 4 min.

## Bioinformatics

This study used BION-META (http://box.com/bion), for analyzing the sequence data from the 18S rDNA gene sequencing. BION-META is a newly developed analytical semi-commercial open-source package for 16S rDNA gene and other reference gene analysis[46]. The pipeline accepts raw sequences, allows non-overlapping paired reads for analysis, and is often accurate to the species level. The pipeline was used for de-multiplexing, sequence- and quality-based trimming, filtering, de-replication, clustering, chimera-checking, reference data similarities and taxonomic mapping and formatting. The sequence data was processed by following automated steps that are described in the supplementary methods and materials of the article[46].

## Statistics

Analysis of microbiota composition was performed in R version 3.5.0[47] using the packages phyloseq v. 1.24.2[48] and vegan v. 2.5-2[49]. The figures were created using ggplot2 v. 3.2.0[50]. Alpha diversity of samples, as well as relative abundances of individual genera, were compared between the groups with Wilcoxon rank sum tests and adjusted for multiple testing using Bonferroni correction. The analysis of similarities (ANOSIM) test was used to test statistically whether there was a significant difference between groups of sampling units.

## RESULTS

The number of reads assigned to fungal taxa ranged from 0 to 146968 with a median of 2808. Ten samples were excluded due to read counts below the chosen rarefaction threshold of 414. No significant differences were observed in the read count distribution when comparing biopsy treatment, sample area and diagnosis. 98.4% of fungal reads were classified to the genus level while 0.16% of reads were unclassified to the phylum level.

## No Significant Difference in Fungal Diversity Between Washed and Unwashed Gastric Biopsies

To distinguish the persistent and transient fungi in the stomach, the gastric biopsies were for each patient separated into two groups (washed or unwashed). Figure 1 shows that there is no significant difference (p=.60) in fungal diversity between washed and unwashed gastric biopsies. In further analyzes, the sequence data from washed biopsies is used.

**Figure 1.** The alpha diversity compared between unwashed and washed gastric biopsies. The alpha diversity is shown as Shannon diversity index (richness and evenness). Wilcoxon rank sum test ($p=.60$).
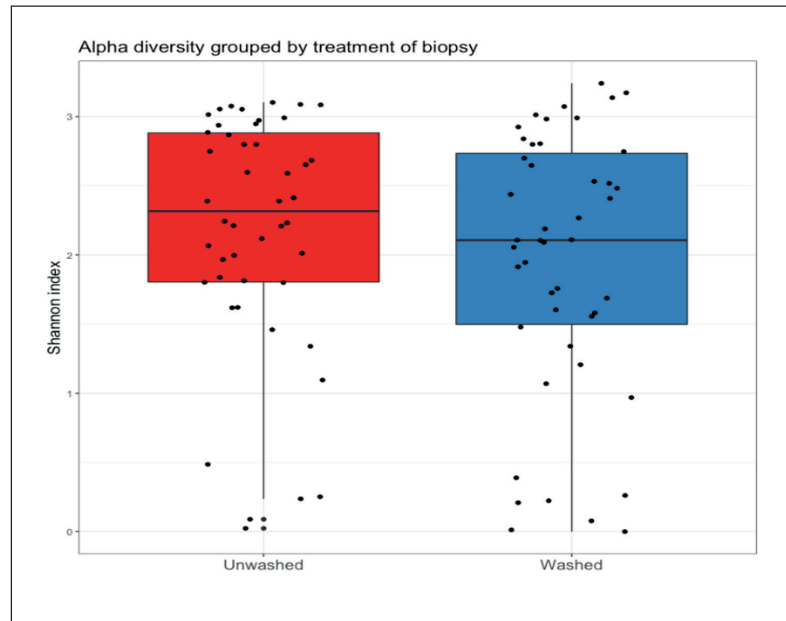


## A Stable Predictable Mycobiota of Gastric Biopsies

According to the data, it was relevant to investigate the 10 most abundant genera since they account for ~60% of the total fungal genera. When comparing the fungal distribution of the 10 most abundant genera in unwashed or washed gastric biopsies from both dyspeptic patients and gastric cancer patients, the overall distribution is stable but small differences are observed (Figure 2). For example, the average proportion of the fungal genus *Malassezia* in biopsies from dyspeptic patients decreases from 9.78% in unwashed biopsies to 5.78% in washed biopsies. On the other hand, when comparing the washed and unwashed gastric biopsies from gastric cancer patients, the average proportion of the fungal genus *Malassezia* increases from 17.33% in unwashed biopsies to 24.88% in washed biopsies (Figure 2).
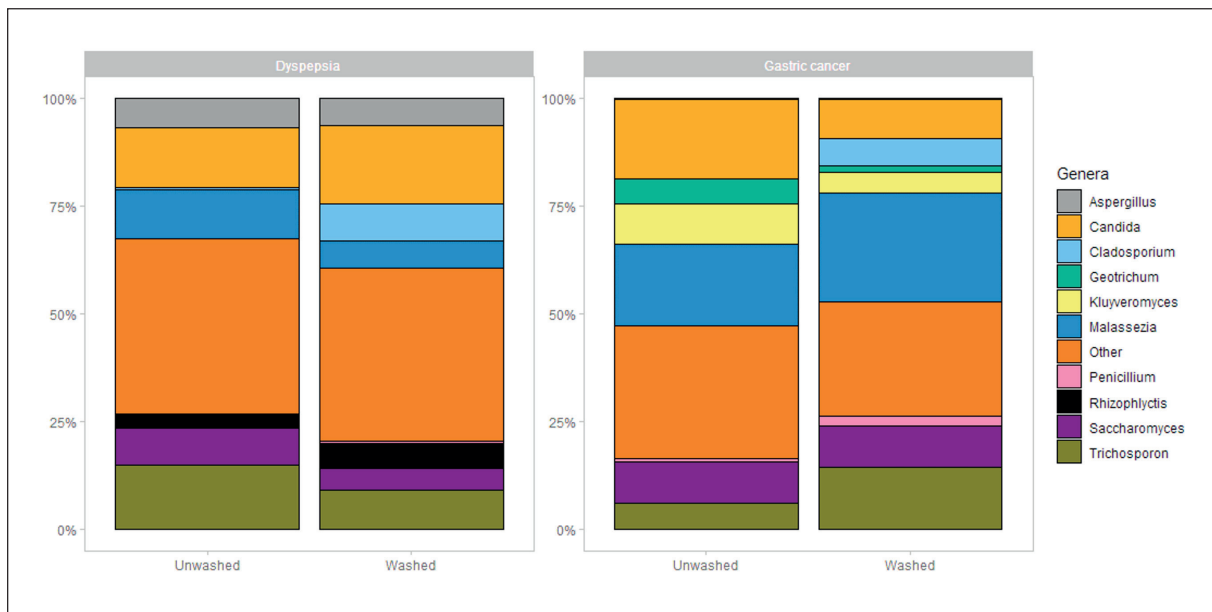


**Figure 2.** The average relative abundance of the 10 most abundant fungal genera in unwashed and washed gastric biopsies compared between dyspeptic and gastric cancer patients. The average relative abundance is shown as a percentage of total fungal reads.

The opposite tendency is observed for the genus *Candida*, which average proportion in dyspeptic patients increases from 11.83% in unwashed biopsies to 16.50% in washed biopsies (Figure 2). On the other hand, the average proportion of the genus *Candida,* in gastric cancer patients, decreases from 17.59% in unwashed biopsies to 9.14% in washed biopsies. Another difference is that the average proportion of the fungal genus *Cladosporium* is increased in washed biopsies compared to unwashed biopsies in both dyspeptic and gastric cancer patients (Figure 2).

### Dyspeptic and Gastric Cancer Patients Show No Significant Difference in Fungal Diversity

In this study, the diversity of the gastric mycobiota was investigated between two groups of patients that were diagnosed with either dyspepsia or gastric cancer. In figure 3A it is shown that there is no significant difference in fungal diversity compared between dyspeptic and gastric cancer patients ($p$=.54).

### Significantly Different Composition and Distribution of the 10 Most Abundant Fungal Genera Observed Between Dyspeptic and Gastric Cancer Patients

Figure 3B shows that dyspeptic and gastric cancer patients have different composition and distribution of the 10 most abundant fungal genera. This is confirmed by an ANOSIM test, which shows that the fungal abundances in gastric biopsies from dyspeptic and gastric cancer patients are significantly different ($p$=.009). In figure 3B it is shown that some genera are mainly observed in either dyspeptic patients or gastric cancer patients. For example, the genus *Aspergillus* is mainly observed in dyspeptic patients (5.77%) compared to gastric cancer patients (0.15%) (Figure 3B). On the other hand, the genus *Kluyveromyces* is mainly observed in gastric cancer patients (4.82%) compared to dyspeptic patients (0.01%) (Figure 3B). Moreover, the genus *Geotrichum* is only observed in gastric cancer patients (1.63%) (Figure 3B). In figure 3B it is shown that the average relative abundance of the genus *Candida* is higher in dyspeptic patients (16.50%) compared to gastric cancer patients (9.14%). A remarkable observation is that the genus *Malassezia* constitutes a larger percentage of the average distribution of the 10 most abundant genera in gastric cancer patients (24.88%) compared to dyspeptic patients (5.78%) (Figure 3B). In figure 3C it is shown that the difference in the relative abundance of the genus *Malassezia* between dyspeptic and gastric patients is significant ($p$=.004).

### Different Composition and Distribution of the 10 Most Abundant Fungal Genera Observed Between Two Sampling Sites From Gastric Cancer Patients

In this study, the gastric biopsies from the gastric cancer patients were taken either from AN or CA. This was done to distinguish the composition and distribution of mycobiota from the two areas. An ANOSIM test showed that the fungal abundances between AN and CA are not significantly different ($p$=.96). However, in figure 4 it is shown that the composition and distribution of the 10 most abundant fungal genera are different when comparing the sampling sites AN and CA. For example, figure 4 shows that the genus *Cladosporium* is only observed in the sampling site AN. Furthermore, the genus *Cladosporium* is mainly found in washed gastric biopsies comparing with unwashed gastric biopsies (Figure 4). For both sampling sites, it is observed that the average relative abundance of genus *Candida* is decreased in washed gastric biopsies compared with unwashed gastric biopsies (Figure 4). On the other hand, the average proportion of genus *Trichosporon* is increased in washed gastric biopsies compared with unwashed gastric biopsies for both sampling sites (Figure 4). However, when comparing the average proportion of genus *Trichosporon* in washed gastric biopsies between the two sampling sites, it is observed that the genus *Trichosporon* makes up a larger proportion in the AN sampling site (Figure 4). Conversely, it is observed that the genus *Saccharomyces* makes up a larger proportion in the CA sampling site (Figure 4).
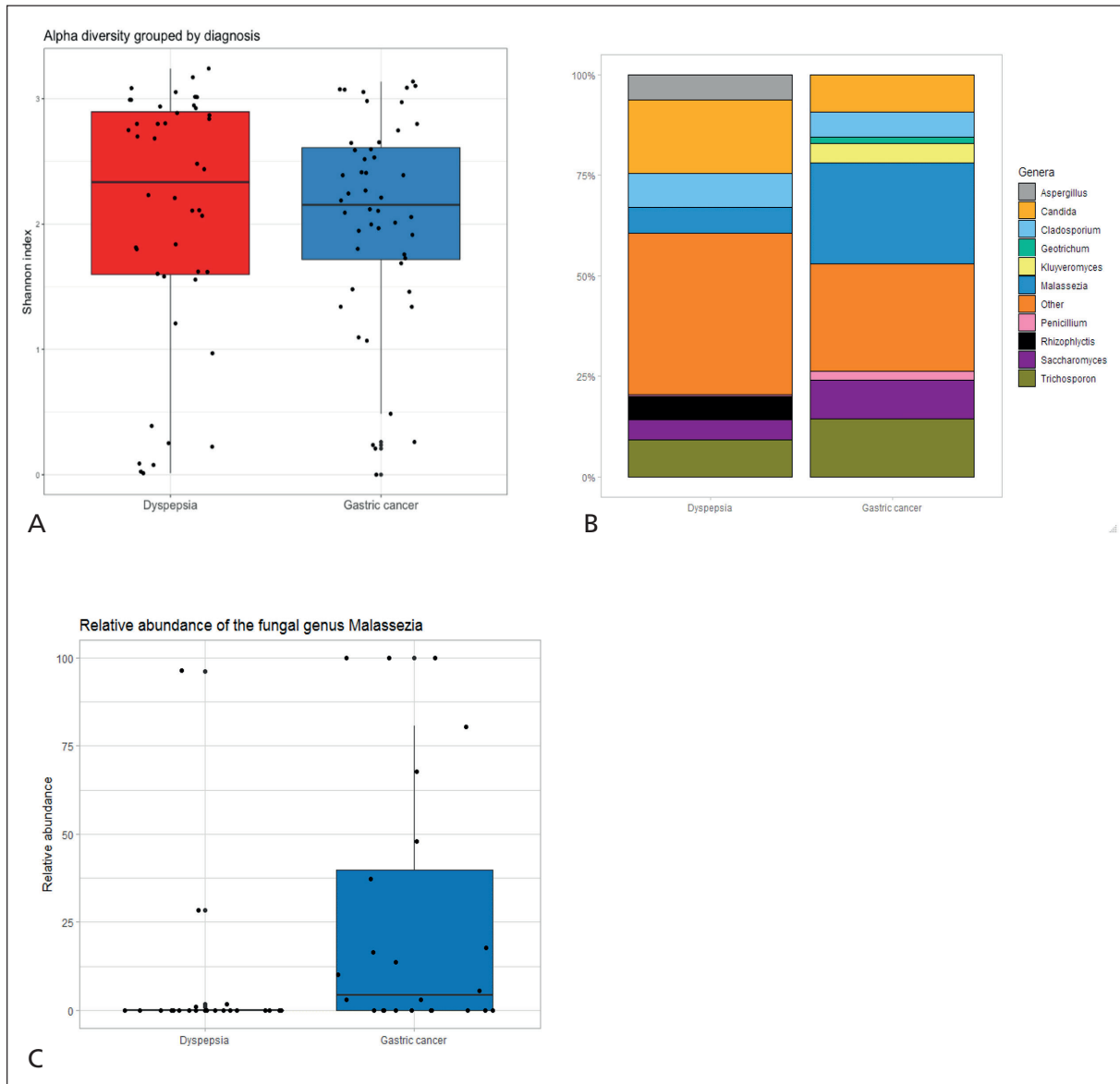
**Figure 3.** Comparison of fungal diversity and relative abundance of the 10 most abundant fungal genera between dyspeptic and gastric cancer patients. ***A***, The alpha diversity shown as Shannon diversity index (richness and evenness). ***B***, The average relative abundances of the 10 most abundant fungal genera. The average relative abundance is shown as a percentage of total fungal reads. ***C***, The relative abundance of the fungal genus Malassezia shown as a percentage of total fungal reads. Wilcoxon rank sum test ($p$=.004).

## Higher Basidiomycota:
## Ascomycota Abundance Ratio in Gastric Cancer Patients

As shown in figure 5A the gastric mycobiota of both dyspeptic and gastric cancer patients are dominated by the fungal phylum *Ascomycota* and *Basidiomycota*. Furthermore, gastric cancer patients show higher *Basidiomycota:Ascomycota* abundance ratio compared to dyspeptic patients (Figure 5A). In figure 5B it is shown that the relative abundance of the fungal phylum *Basidiomycota* is increased in gastric cancer patients, however not significantly ($p$=.12). In figure 5C it is shown that dyspeptic patients (red dots) are accumulated in the low right corner, which indicates that they on average have a lower *Basidiomycota:Ascomycota* abundance ratio compared to gastric cancer patients that are more equally distributed.
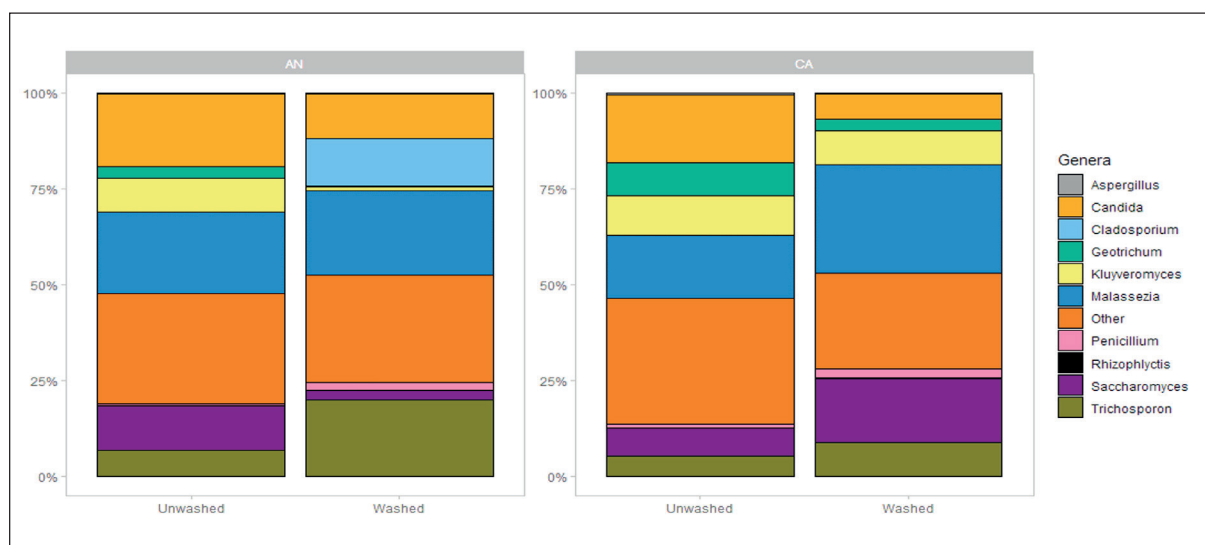
**Figure 4.** The average relative abundance of the 10 most abundant fungal genera compared between the two sampling sites of the gastric cancer patients; AN (antrum) and CA (corpus around the cancer area), which are further divided into groups of unwashed or washed gastric biopsies. The average relative abundance is shown as a percentage of total fungal reads.

## DISCUSSION

### Strong Adherence of Fungi to Gastric Biopsies

In this study, no significant difference in fungal diversity between unwashed and washed gastric biopsies was observed (Figure 1). It indicates that fungi are good adherers since the washing step does not influence fungal diversity. It correlates with our knowledge that fungi have remarkable adhesion and aggregation properties that are considered as an important virulence factor[51-53]. In general, adhesion of cells is managed by a class of specialized cell wall proteins called adhesin[51,53]. Fungi are known for their great phenotypical plasticity like internal tandem repeats in their adhesin genes, which trigger recombination and thereby formation of new versions of adhesins[51,54]. These properties make them able to adapt quickly to stressful environments like the stomach[51,55,56].

When comparing the differences in the average relative abundance of the 10 most abundant fungal genera in dyspeptic and gastric cancer patients between unwashed and washed gastric biopsies, it was observed that the washing step does not alter the overall proportions of the genera (Figure 2). It is consistent with the results in figure 1, which together indicate that the fungi perform a strong adhesion to the gastric biopsies and thereby promote a stable mycobiota in the stomach. However, most studies[57,58] that investigate the stability of the mycobiota indicate that the mycobiota shows high inter- and intra-variability over time. Nevertheless, only a few studies have investigated the stability of the mycobiota in the stomach over time, which advocates more research within this area. We chose to use sequence data from washed biopsies in further analyzes as we think it represents the persistent gastric mycobiota even though no differences were observed between unwashed and washed gastric biopsies.

In a previous publication[42], our laboratory used 16S rDNA sequencing to look at the bacterial composition of the gastric microbiota in dyspeptic and gastric cancer patients. When comparing the results of bacterial and fungal diversity it is clear that bacteria are the most abundant microorganism in the stomach. This distribution is also found in a study that investigated the microbiome of the piglet GI tract[28]. Moreover, the study showed that the diversity of fungi was differently distributed compared to bacteria along the GI tract[28]. As shown by other scholars[28], the diversity of bacteria increased along the GI tract with the lowest diversity in the stomach and the highest in the colon. On the other hand, the fungi showed a distinct distribution as the diversity was highest in the stomach and the colon but lower in the other parts of the GI tract[28]. These observations indicate that fungi may be more adapted to colonize the stomach
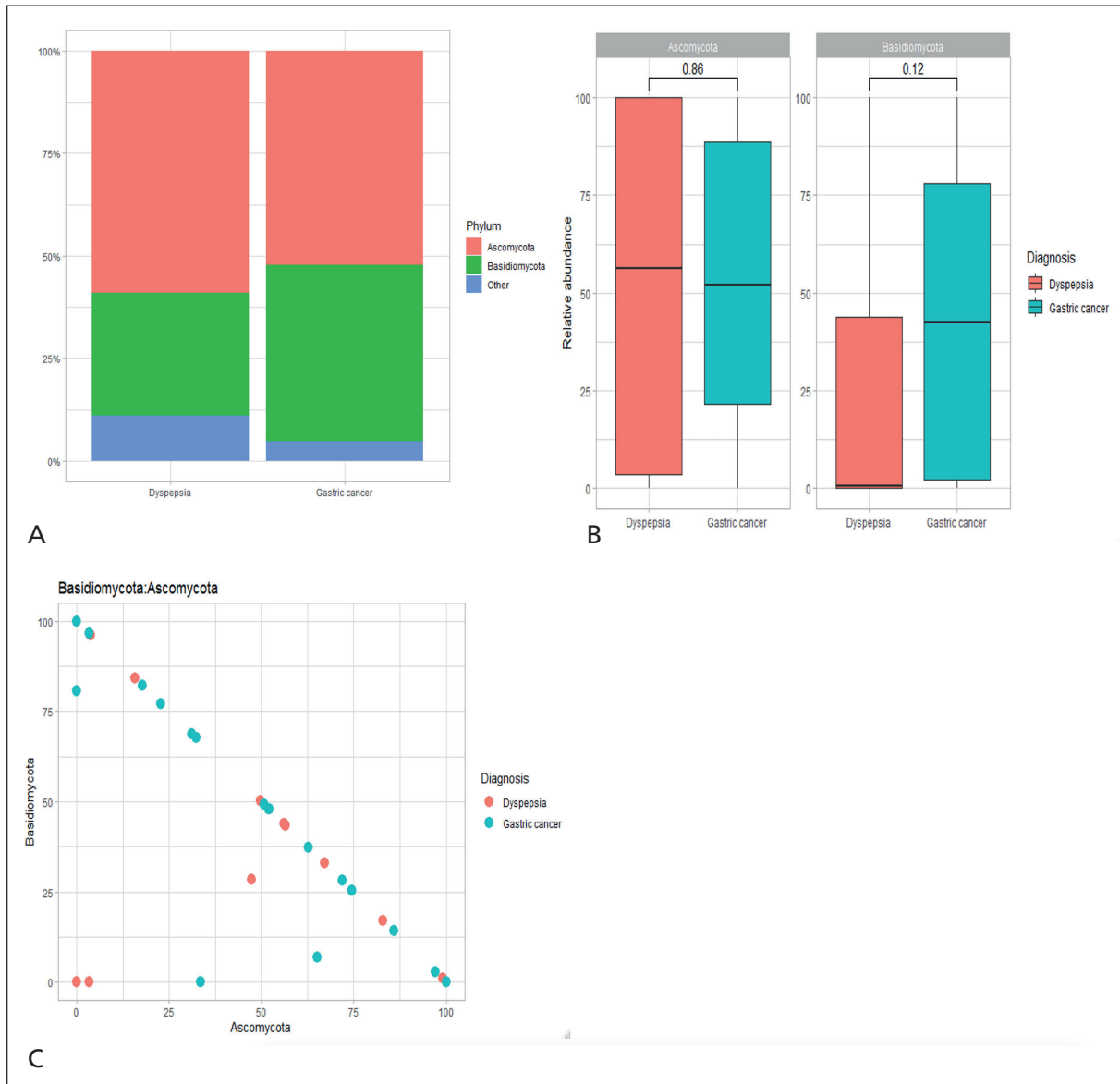
**Figure 5.** The relative abundance of the two main fungal phyla Basidiomycota and Ascomycota compared between dyspeptic and gastric cancer patients. **A**, The average relative abundance shown as a percentage of total fungal reads. **B**, Boxplot showing the relative abundance of the fungal phylum Ascomycota and Basidiomycota. **C**, Scatterplot showing the *Basidiomycota:Ascomycota* ratio. Each dot represents one sample.

compared to bacteria. A reason could be that fungi are more acid-tolerant because of specialized mechanisms to adapt to pH variations[28,59]. Another reason could be that there is less competition of resources from bacteria compared to the other parts of the GI tract[28].

## Distinct Gastric Mycobiota Observed Between Dyspeptic and Gastric Cancer Patients

In this study, we observed that the gastric mycobiota is distinct between dyspeptic and gastric cancer patients (Figure 3B). This has also been observed in other studies that investigate the composition of the mycobiota in humans diagnosed with different diseases[33,60,61]. However, in most of these studies[33,60,61], they compare the mycobiota of both sick and healthy individuals. In this study, samples from two patient groups (dyspepsia and gastric cancer) were compared. Since only 1-2% of dyspeptic patients are associated with the development of gastric cancer,

they are more identical to healthy individuals than gastric cancer patients[62]. Although, to get a clear answer if the mycobiota is distinct in the diseased patients, we should have included samples from healthy individuals. However, it is difficult since we analyzed gastric biopsies that are primarily obtained from diseased individuals.

A striking observation in this study is that some of the 10 most abundant genera were mainly observed in either dyspetic or gastric cancer patients (Figure 3B). The genera *Aspergillus* and *Rhizophlyctis* were mostly observed in dyspeptic patients whereas *Kluyveromyces* and *Penicillium* were mostly observed, and the genus *Geotricum* was only observed in gastric cancer patients. Besides *Aspergillus* and *Geotricum,* which are both normally found in humans[63,64], the other fungal genera (*Kluyveromyces, Penicillium* and *Rhizophlyctis)* are mainly found in soil or food products[65-67]. This could indicate that the differences in the gastric mycobiota might come from the individual patient's diet and environment. In other studies[5,30,57], the diet and environment have been mentioned as prominent drivers of the mycobiota.

### The Fungal Genus Malassezia May Have An Important Role in the Formation of Cancer Cells

In this study, we observed that the relative abundance of the fungal genus *Malassezia* was increased in gastric cancer patients compared to dyspeptic patients (Figure 3C). *Malassezia* is a fungal genus commonly found on the human skin but is also able to colonize the gut[68-72]. Moreover, *Malassezia* is known as an opportunistic pathogen since it is associated with several skin diseases like pityriasis versicolor, seborrheic dermatitis and Malassezia folliculitis[71,73,74]. The contribution of *Malassezia* in tumorigenesis has been shown in studies[60,69] that investigated colorectal cancer (CRC) and pancreatic ductal adenocarcinoma (PDA). In both studies, an increase in the abundance of *Malassezia* was observed in samples from cancer patients compared to controls[60,69]. Since our study shows the same correlation, it indicates that *Malassezia* might also contribute to the formation of gastric cancer, however, this needs further investigation.

Some mechanisms of *Malassezia* have been suggested to promote cancer formation. It is known that *Malassezia* synthesizes aryl hydrocarbon receptor (AhR) ligands, which some studies[69,73,75] suggest could promote basal cell carcinoma by activating the AhR. Ahr is a ligand-activated transcription factor that possesses numerous biological functions like detoxification, Nuclear Factor kappa B (NF-κB) regulation and immune regulation[73,76,77]. Notably, the bacterium *H. pylori*, which is the most important risk factor for the development of gastric cancer, is known to modulate several cellular components by its virulence factors like cytotoxin-associated gene A (CagA)[35,78,79]. For example, *H. pylori* can activate NF-κB signaling, which regulates several cellular processes that are important for both immune and inflammation responses and carcinogenesis[78-81]. Since *Malassezia* also might influence these cellular processes by AhR signaling, a synergistic effect with *H. pylori* might be present in gastric cancer patients. Another study[60], which investigated PDA, showed that activation of the complement immune system by ligation of fungal cell wall glycans to mannose-binding lectin (MBL) promotes the oncogenic progression. Together these findings suggest that *Malassezia* might manipulate the immune system and thereby lead to the progression of cancer formation. In future studies of gastric cancer, it could be interesting to investigate the influence of Malassezia in the progression of the cancer formation and the possible immune manipulation.

### Higher Basidiomycota:Ascomycota in Gastric Cancer Patients Is a Sign of Fungal Dysbiosis

In this study a higher *Basidiomycota:Ascomycota* ratio in gastric cancer patients was observed, however, it was not significant (Figure 5). Recent studies[33,69] suggest that the *Basidiomycota:Ascomycota* abundance ratio could be an indicator for fungal dysbiosis since a higher ratio is correlated with several gastrointestinal diseases. Our result indicates that gastric cancer patients are having fungal dysbiosis in the stomach, however, more investigation is needed. It is known that microbial dysbiosis can occur due to systemic immunosuppression, which is a consequence of cancer[35]. As we see a potential fungal dysbiosis in gastric cancer patients, it might have occurred due to their immunosuppressive state.

### Different Mycobiota in CA and AN Sample Sites

As seen in figure 4, the genus *Cladosporium* was only observed in the AN samples compared to the CA samples. The genus *Cladosporium* is a cosmopolitan and thereby found on most surfaces and some species are the most common fungal component isolated from air[82]. In several studies[58,83], *Cladosporium* is found as a common fungus in the intestines of humans and mice. However, little is known about its role in the GI microbiota[57].

Our results indicate that *Cladosporium* might not be able to colonize the area of the cancer tissue, which could be due to several factors. It is well known that the attachment of microorganisms to host cells is important for their colonization[84]. The attachment involves interaction between the microorganism's specific surface-bound adhesion molecules and their respective receptors on the host cell[85]. This feature is specific and selective and thereby discriminative to which microbes that can attach to the specific tissue[85]. Cancer cells are known to possess changed surface molecules which thereby alters the attachment site for the normal microbiota[85]. This results in reduced or inhibited attachment of certain microorganisms and promotes attachment of other microorganisms[85]. It indicates that the cancer tissue harbors a distinct microbiota compared to normal tissue[85]. Since we did not observe the genus *Cladosporium* in CA samples, it could thereby be caused by the altered surface structures of the cancer tissue, however, it needs more investigation.

Another factor that might inhibits the colonization of the genus *Cladosporium* on the cancer tissue is the fact that some areas of cancer cells are hypoxic due to poor supply of oxygen, also called tumor hypoxia[85,86]. Tumor hypoxia is caused by an altered cell metabolism in cancer cells, which for example leads to elevated reactive oxygen species (ROS) levels and thereby depleted oxygen levels[87,88]. In normal cells, low levels of ROS is an important factor for regulation of cell division, immune responses and inflammation[79,87-90]. However, high levels of ROS can cause oxidative damage, especially in DNA, which leads to mutations and thereby cancer[79,87-89]. *H. pylori* is known to induce ROS production, which is associated with the pathogenesis of *H. pylori*-related gastric diseases like gastric cancer[79,91]. It has been shown that high levels of ROS is important for every stage of cancer development, however, cancer cells need to counteract the high levels of ROS to avoid cell death, by elevating their antioxidant capacity[87,89,90]. A study[92,93] has shown that increased levels of hydrogen peroxide ($H_2O_2$), which is a member of the ROS family, inhibits the germination of the fungal plant pathogen *Cladosporium fulvum* (*C. fulvum*). This could indicate that the high levels of ROS in the cancer area make it impossible for the genus *Cladosporium* to colonize.

### Prokaryotic-Eukaryotic Interactions May Play a Role in Driving Diversity Fluctuations and Disease Pathogenesis

Several studies[27,28,34,58,61,94] have shown that interactions between fungi and bacteria have important influences on the microbial ecosystem. For example, a supposed endosymbiotic relationship between *H. pylori* and *Candida* spp. has been assumed to have an influence on the protection of *H. pylori* in the stomach[55]. This assumption is drawn from the fact that *H. pylori* has been isolated from *Candida* spp. yeasts vacuoles that are isolated from oral and gastric samples[12,55,95,96]. It is supposed that the vacuoles of *Candida spp*. function as a reservoir of *H. pylori* where it is protected against environmental stresses, provided with nutrients and vertically transmitted from human to human[55]. *H. pylori* has been designated as a class 1 carcinogen by the World Health Organization (WHO) since a chronic infection with *H. pylori* is the strongest risk factor for gastric adenocarcinoma[35]. Due to this fact, it would be interesting to study if *H. pylori* could be isolated from *Candida* spp. in the stomach of gastric cancer patients.

On the other hand, researchers have shown that fungi can positively modify the severity of infectious diseases by altering the microbiota[28,97]. For example, a study[97] has shown that oral administration of the dietary fungi *Candida kefyr* (*C. kefyr*) ameliorated the severity of experimental autoimmune encephalomyelitis (EAE), which is an animal model of brain inflammation. It was due to an alteration of the microbiota, which probably led to a positive shift in the immune response[97]. This indicates that beneficial fungi like *C. kefyr* could be a potential treatment of autoimmune diseases like multiple sclerosis[97].

In this regard, it would be interesting to investigate the inter-kingdom relationships in the gastric microbiota in further studies. This could give a broader picture of the dynamics in the microbial ecosystem of the stomach and might lead to new therapeutics.

## CONCLUSIONS

It is well known that dysbiosis in the bacterial composition of the gastric microbiota can promote disease, however, the role of the fungal composition it is still unknown. In this study, it was observed that gastric cancer patients harbored a changed dysbiotic mycobiota compared to dyspeptic patients. For example, the relative abundance of the fungal genus *Malassezia* was increased in gastric cancer patients, which indicates a potential role in cancer pathogenesis. Moreover, the mycobiota of the cancer tissue area (CA) was different from the mycobiota of normal tissue area (AN). This observation indicates a distinct surface structure and ecological environment of the cancer tissue, which thereby discriminate the microbial composition.

This study indicates that the gastric mycobiota probably has an important influence on gastric health. Since it is one of the first studies investigating the mycobiota in different gastric disease types, there is a big lack of knowledge. It advocates for more studies within this area, which can clarify how the mycobiota contributes to the dynamics of the gastric microbiota.

### Availability of Data and Materials:

The datasets used during the current study are available from the corresponding author on reasonable request.

### Statement of Ethics:

All patients participating in the study have signed an informed consent form. Kaunas Regional Bioethics Committee has approved the study protocol (Protocol No: BE-2-10; P1-BE-2-31).

### Conflicts of Interest:

The authors declare no conflict of interest.

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