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Gastrointestinal microbiota alteration induced by *Mucor circinelloides* in a murine model

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Abstract

Mucor circinelloides is a pathogenic fungus and etiologic agent of mucormycosis. In 2013, cases of gastrointestinal illness after yogurt consumption were reported to the US FDA, and the producer found that its products were contaminated with *Mucor*. A previous study found that the *Mucor* strain isolated from an open contaminated yogurt exhibited virulence in a murine systemic infection model and showed that this strain is capable of surviving passage through the gastrointestinal tract of mice. In this study, we isolated another *Mucor* strain from an unopened yogurt that is closely related but distinct from the first *Mucor* strain and subsequently examined if *Mucor* alters the gut microbiota in a murine host model. DNA extracted from a ten-day course of stool samples was used to analyze the microbiota in the gastrointestinal tracts of mice exposed via ingestion of *Mucor* spores. The bacterial 16S rRNA gene and fungal ITS1 sequences obtained were used to identify taxa of each kingdom. Linear regressions revealed that there are changes in bacterial and fungal abundance in the gastrointestinal tracts of mice which ingested *Mucor*. Furthermore, we found an increased abundance of the bacterial genus *Bacteroides* and a decreased abundance of the bacteria *Akkermansia muciniphila* in the gastrointestinal tracts of exposed mice. Measurements of abundances show shifts in relative levels of multiple bacterial and fungal taxa between mouse groups. These findings suggest that exposure of the gastrointestinal tract to *Mucor* can alter the microbiota and, more importantly, illustrate an interaction between the intestinal mycobiota and bacteriota. In addition, *Mucor* was able to induce increased permeability in epithelial cell monolayers *in vitro*, which might be indicative of unstable intestinal barriers. Understanding how the gut microbiota is shaped is important to understand the basis of potential methods of treatment for gastrointestinal illness. How the gut microbiota changes in response to exposure, even by pathogens not considered to be causative agents of food-borne illness, may be important to how commercial food producers prevent and respond to contamination of products

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aimed at the public. This study provides evidence that the fungal microbiota, though understudied, may play an important role in diseases of the human gut.

Keywords

microbiota; mycobiota; mycobiome; *Mucor*; fungi in the guts

Introduction

Mucor circinelloides is a fungus that belongs to the Mucorales family in the early diverging Mucoromycota phylum (Spata-fora *et al.*, 2016). Among other members of the Mucorales family, exposure to *M. circinelloides* causes the disease mucormycosis (Morin-Sardin *et al.*, 2017). Mucormycosis is a disease that primarily affects immunocompromised individuals, such as those with neutropenia, as well as diabetes mellitus and burn patients (Chayakulkeeree *et al.*, 2006). Despite the increased prevalence of mucormycosis that has resulted from an increase in immunocompromised patients, fatality rates reach as high as 90% in disseminated cases (Roden *et al.*, 2005), and current treatment options remain limited.

M. circinelloides has also been implicated in food product spoilage and development of gastrointestinal mucormycosis and fungemia following consumption in both immunocompromised and immunocompetent individuals (Aboltins *et al.*, 2006; Lazar *et al.*, 2014), the cause of which has been identified as post-pasteurization with hyphal phase *Mucor* (Snyder *et al.*, 2016). Additionally, *M. circinelloides* can produce the mycotoxin 3-nitropropionic acid (3NP) (Hollmann *et al.*, 2008). 3NP is an irreversible inhibitor of succinate dehydrogenase (Alston *et al.*, 1977), and ingestion of 3NP is known to result in neurodegenerative changes that have been studied in animal models of Huntington's disease (Brouillet *et al.*, 2005). 3NP has also been involved with food poisoning caused by contaminated sugarcane, in which the 3NP produced by *Arthrrium* species caused the onset of acute encephalopathy in humans during outbreaks of sugarcane mildew in China from 1972 to 1989 (He *et al.*, 1995). Therefore, *M. circinelloides* serves as a potential health threat regarding food-borne illness in addition to its more traditional clinical role in mucormycosis.

In September 2013, a recall was issued for yogurt products produced by a company after complaints of spoiled product and gastrointestinal illness following consumption of the yogurt (USAToday, 2013). The causative agent of this spoilage was determined by both the U.S. Food and Drug Administration (FDA) and the company to be *M. circinelloides* (FDA, 2013). In our previous study, a *M. circinelloides* strain was isolated from a contaminated yogurt sample that a consumer had provided (Lee *et al.*, 2014). This strain, named Mucho, was shown to be virulent in systemic exposure of mice in a manner comparable to that of strains isolated from clinical cases of mucormycosis. The study also noted that the colons of mice that ingested the strain tended to be shorter than those of mice that had not ingested it (Lee *et al.*, 2014). This is of interest, as shortening of the colon is associated with the intestinal inflammation seen in ulcerative colitis, one of the inflammatory bowel diseases

(IBDs) (Kim *et al.*, 2012). It is then important to consider how ingestion of this fungus may affect the gut.

Recently, emphasis has been placed on the role of the microbiota in the pathology of various human health conditions. Studies examining the microbiota in IBDs, including Crohn's disease, have also noted changes of composition in the disease state (Ott *et al.*, 2008; Li *et al.*, 2014; Hoarau *et al.*, 2016; Liguori *et al.*, 2016; Sokol *et al.*, 2017; Miyoshi *et al.*, 2018). These studies noted an overall decrease in beneficial bacteria, increase in the bacteria *Serratia marcescens* and *Escherichia coli*, decrease in the fungus *Saccharomyces cerevisiae*, and increase in the fungus *Candida albicans*. Inter-kingdom interactions were also observed through the positive correlation between *Candida tropicalis* and *S. marcescens* and *E. coli* (Hoarau *et al.*, 2016; Sokol *et al.*, 2017). Anorexia nervosa, characterized in part by severe malnourishment, has been associated with decreased gut diversity and alterations in microbial composition (Kleiman *et al.*, 2015). Additionally, altered diversity was shown to be associated with psychiatric measures, such as an increased risk of depression (Kleiman *et al.*, 2015). Obesity is associated with gastrointestinal illness and has also been associated with changes in the microbiota. Studies examining the bacteria associated with obesity have often found the condition to be negatively associated with relative abundance of Firmicutes members and positively associated with Bacteroides members (Devaraj *et al.*, 2013). More recently, *Mucor* species abundance was shown to parallel the degree of weight loss in obese individuals, being more abundant in non-obese individuals (Rodriguez *et al.*, 2015). This study also concluded that obese individuals could be distinguished from non-obese individuals through fungal composition, specifically utilizing the genera *Aspergillus*, *Mucor*, *Penicillium*, *Saccharomyces*, and *Eupenicillium*. From our current knowledge of the association between the microbiota and gastrointestinal illness, a reasonable hypothesis is that food-borne illness caused by *M. circinelloides* may also be associated with changes in the gut microbiota.

In this study, we isolated another *M. circinelloides* strain from a previously unopened yogurt that was within the company's 2013 recall range. Interestingly, the newly isolated *Mucor* strain is closely related but distinct from Mucho, the first strain isolated in our previous study. Following ingestion of this newly isolated *Mucor* strain, the murine gut microbiota was characterized by analyzing the bacterial microbiota and mycobiota in stool samples over the course of ten days. Analysis of gut alpha diversity revealed changes in bacterial and fungal diversity after ingestion of *Mucor*, suggesting a shift in the gut environment. This finding was supported by taxa compositional analysis, which suggested changes in the abundance of specific bacterial species related to gut health. These results demonstrate an example of fungal-driven alteration of the gut microbiota, in which ingestion of *Mucor* led to a shift in the gut microbiota to a potentially unhealthy state. Going forward, more emphasis should be placed on the role of fungi in relation to food-borne illness, the role of fungi in the gut, and interactions between intestinal bacteria and fungi.

Materials and Methods

Strains and culture conditions

An unopened black cherry flavor yogurt within the recall range was obtained before being stored in a freezer for approximately one year (Supplementary data Text S1). Yogurt samples were transferred on PDAm (Potato Dextrose Agar media, containing 4 g of potato starch, 20 g of dextrose, and 15 g of agar per L) media containing ampicillin (100 µg/ml) and kanamycin (50 µg/ml) by using sterile wooden applicators. The plates were incubated at 30°C for three days, when mycelial growth was observed. Three mycelial colonies were then subject to two vegetative rounds of streak purification, and spores from each colony were collected as described previously (Lee *et al.*, 2014). All three strains had identical DNA sequences at the *ITS*, *LSU rDNA*, and *RPB1* loci (see below). One of them was selected and designated AzMucho for further study. The first *M. circinelloides* isolate, Mucho (Lee *et al.*, 2014), and the human skin isolate 1006PhL (Findley *et al.*, 2013) were also used for this study.

For spore production, the strains were grown on PDAm for 3 to 4 days in the light. Deionized distilled water (2 ml) was added to the plates, which were gently rubbed with a sterile spreader to release spores from the mycelia. The spore suspension was collected and transferred into sterile micro-centrifuge tubes. The number of spores was calculated by using a hemocytometer. The whole genome analysis was performed as previously described (Lee *et al.*, 2014).

Phylogenetic analysis

The three loci including *ITS*, *LSU rDNA*, and *RPB1* were used for analysis as previously described (Lee *et al.*, 2014). Primers used for *ITS* are: ITS1, TCCGTAGGTGAACCTG CGG and ITS4, TCCTCCGCTTATTGATATGC; for *LSU rRNA*: D1/D2 LRDNA, GCATATCAATAAGCGGAGGAA AAG and LR3, GGTCCGTGTTTCAAGACGG; and for *RPB1*: RPB1-Ac, GARTGYCCDGGDCAYTTYGG and RPB1-Cr, CCNGCDATNTCRRTRTCCATRTA. The PCR amplicons were sequenced and analyzed. The whole genome analysis was performed as previously described for the first Mucho strain (Lee *et al.*, 2014). The AzMucho assembly was submitted to NCBI under the project accession number PRJNA-431470.

Virulence tests and microbiota analysis after *Mucor* ingestion in a murine model

Four to six-week-old male BALB/C mice (18–20 g body weight, Charles River Laboratories) were used for the virulence test. The animals were randomly distributed into two cages before the spore challenge. Spores for exposures were washed and resuspended in sterile PBS. For virulence tests, PBS (200 ml) containing 1×10^6 spores of *Mucor* strains was administered via tail vein injection. Survival of the mice was examined twice daily and body weight was monitored daily. Animals that appeared moribund or in pain were euthanized appropriately. Survival curve was generated and analyzed by using Kaplan-Meier survival curves with PRISM (GraphPad Software, Inc.).

Two independent microbiota analyses were performed with mice housed at the animal facility at the Duke university or The University of Texas at San Antonio (UTSA). Four- to six-week old male BALB/C mice, purchased from Charles River Laboratories, were used. Upon arrival, the animals were randomly distributed into different cages (5 mice per cage) and established for 10 days in a regular room at each facility before *Mucor* spore challenge. After ingestion, the animals were placed in a BSL2 room and maintained under 12 h day-and-night cycles throughout the experiments. Cohorts of the animals were ingested, by oral gavage, either spores (1×10^6) of the AzMucho strain, spores (1×10^6) of the 1006PhL strain, or PBS alone at day 0 before the night cycle. Body weight of the exposed and control mice was monitored daily until termination of the experiment. Fresh stool from mice was collected daily before the night cycle for up to 10 days post infection (or 9 days post infection at UTSA). The collected stool samples were immediately frozen at -80°C for later analysis. Duplicate stool samples from the *Mucor* exposed mice at day 2 were placed on PDAm to ensure *Mucor* spores had reached the GI tract. *Mucor* colonies formed from the stool samples collected from all 5 exposed mice (data not shown).

All murine experiments were conducted at Duke University and UTSA in full compliance with all of the guidelines of the both Institutional Animal Care and Use Committees (IACUC) and in full compliance with the United States Animal Welfare Act (Public Law 98–198). The UTSA IACUC approved all the murine studies under protocol number MU-104–2/20, and the Duke University IACUC approved all of the murine studies under protocol number A061–12-03. The experiments were conducted in the Division of Laboratory Animal Resources (DLAR) facilities that are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

3-nitropropionic acid analysis

For 3-nitropropionic acid (3NP) analysis, commercially available yogurt of the same sort as the originally contaminated one were inoculated with 1×10^6 spores of AzMucho in triplicates. Spores were injected through the foil yogurt lid which was subsequently sealed with tape and the whole container stored at 4°C until bloating of the lids were visible (2 weeks). Uninfected control yogurts of the same variety were incubated simultaneously. Analysis of 3NP was performed as initially described by Muir and Majak (Muir and Majak, 1984). Briefly, each yogurt was homogenized and 2 ml of each sample was mixed with 1 ml of 0.6 N perchloric acid and incubated on ice for 30 min. From the uninfected yogurt containers, 2 ml were additionally spiked with 3NP (Sigma Aldrich) to a final concentration of 20 μM and processed as described above. All perchloric acid treated samples were centrifuged at 3,000 g for 15 min and 500 μl of supernatant was filtered through 0.2 μm polyvinylidene fluoride filters before high performance liquid chromatography (HPLC) photodiode array (PDA) analysis. The samples were separated on a ZORBAX Eclipse XDB-C18 column (Agilent, 4.6 mm by 150 mm with a 5 μm particle size) isocratically with 0.15% (v/v) orthophosphoric acid (pH 2) using a Flexar Binary Liquid Chromatography (LC) Pump (PerkinElmer) coupled to a Flexar LC Autosampler (Perkin Elmer) and a Flexar PDA Plus Detector (PerkinElmer). The PDA was set to 210 nm. Each sample was run for 15 min and after each sample the column was washed with 95% methanol for 5 min and equilibrated with 0.15% (v/v) orthophosphoric acid for 5 min before injection of the next

sample. Identification of 3NP was performed using Chromera Manager (PerkinElmer) by comparison to an authentic standard (Sigma Aldrich).

Stool DNA isolation and sequencing for microbiota analysis

DNA was isolated from collected mouse stool samples by using the ZymoBiomix DNA Miniprep kit (Zymo Research Co.) with slight modifications made to the manufacturer's protocol. Before bead beating, samples were incubated at 70°C for 5 min, then at 37°C for 30 min. Bead beating was broken up into 5 processes of 1 min each with 1 min in between each process. As described in Kleiman *et al.* (2017), bacterial sequences in the isolated DNA samples were identified by amplification of the V4 variable region of the 16S rRNA gene by PCR (forward primer 5'-GAGTGCCAGCMGCCGCGG TAA-3', reverse primer 5'-ACGGACTACHVGGGTWTC TAAT-3'). Forward and reverse primers incorporated single nucleotide shifts in six different lengths. The first amplification used 200 ng of isolated DNA as a template, a mix of the forward and reverse 16S primers each at a concentration of 10 mM, and the KAPA2G Robust PCR Kit (KAPA Biosystems Inc.) (95°C for 3 min then 10 cycles of 95°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec, ending with 72°C for 5 min). A bacterial 16S V4 library for sequencing was generated via a second amplification using 5 ml of purified product from the first amplification as a template, 10 mM of forward and reverse primers containing Illumina MiSeq adapter sequences, 12 base pair barcodes (Caporaso *et al.*, 2012) specific for each sample, and the KAPA HiFi HotStart ReadyMix (95°C for 3 min, then 22 cycles of 95°C for 30 sec, ending with 72°C for 5 min). Fungal sequences in the isolated DNA samples were identified by amplification of the ITS1 region (forward primer 5'-CTTGGTCATTTAGAGGAAGTAA-3', reverse primer 5'-GCTGCGTTCTTCATCGATGC-3'). Forward and reverse primers incorporated single nucleotide shifts in six different lengths. First amplification used 200 ng of isolated DNA as a template, a mix of the forward and reverse ITS primers at a final concentration of 0.25 mM, and the TaKaRa Ex Taq PCR (Takara Bio USA Inc.) system (98°C for 10 sec, then 35 cycles of 98°C for 10 sec, 56.1°C for 30 sec, and 72°C for 2 min, ending with 72°C for 5 min). A fungal ITS1 library was constructed by a second amplification using 5 ml of purified product from the first amplification as a template, 10 mM of forward and reverse primers containing Illumina MiSeq adapter sequences, 12 base pair barcodes (Caporaso *et al.*, 2012) specific for each sample, and the KAPA HiFi HotStart ReadyMix (95°C for 3 min, then 22 cycles of 95°C for 30 sec, ending with 72°C for 5 min). PCR products from each amplification were cleaned utilizing the HighPrep PCR clean-up kit. Final cleaned PCR products were then diluted to be made equal in concentrations, pooled, and sequenced by the High-Throughput Sequencing Facility at the University of North Carolina at Chapel Hill School of Medicine or by the Genome Core at The University of Texas at San Antonio.

Analysis of 16S and ITS1 sequences

Demultiplexing and classification of 16S rRNA gene sequence data were performed utilizing the Quantitative Insights into Microbial Ecology (QIIME) pipeline using default settings (Walters *et al.*, 2010). Open reference operational taxonomic units (OTUs) were clustered utilizing the Uclust algorithm based on a 97% threshold for sequence similarity (Edgar, 2010). Taxonomic classification was determined using the Greengenes database. Classification of ITS1 data were performed utilizing the PIPITS pipeline (Gweon *et al.*,

2015), which clusters OTUs at a 97% sequencing similarity threshold by utilizing the RDP classifier. Taxonomic classification was determined using the UNITE database (Kõljalg *et al.*, 2005). The resulting OTU tables were filtered in QiIME to remove OTUs present in less than 25% of all samples and with an overall abundance less than 0.005% as a conservative cutoff (Bokulich *et al.*, 2013) for alpha diversity analysis and a more relaxed cutoff of 0.05% for beta diversity and taxonomic composition analysis due to the small sample size used.

Statistical analysis

Bacterial and fungal species richness (alpha diversity) were characterized by the number of species and the Chao 1 estimator of diversity for each sample using QiIME (Chao, 1984). Differences in alpha diversity between exposed and unexposed mice were tested with a multiple linear regression, which accounted for the possible interaction between exposure status and time since exposure using R version 3.3.3 and R packages vegan version 2.4–3 and phyloseq 1.19.1 (RCore-Team, 2017). Factors considered to calculate overall model for alpha diversity included day, *Mucor* spore or PBS ingestion, and the interaction between day and ingestion. Beta diversity analysis was performed with principle coordinate analysis of Bray-Curtis distances and permutational analysis of variance using adonis implementation in R. Taxa abundance of bacterial and fungal groups were calculated at the phylum, class, order, family, and genus levels using QiIME, and graphs depicting these levels were generated using R. Statistical significance of groups was determined using the Kruskal-Wallis test together with false discovery rate (FDR) correction (Benjamini and Hochberg, 1995). Taxa analysis was confirmed and visualized using the Linear Discriminant Analysis (LDA) Effect Size (LEfSe) web application (Segata *et al.*, 2011). An alpha level of 0.05 was used for all tests, but after FDR-correction, a level of 0.1 was used due to the small sample and exploratory nature of this study.

In vitro monolayer permeability assay after exposure of epithelial cells to *Mucor*

Caco-2 human colonic epithelial cell lines were cultured in MEM (Gibco) media supplemented with 10% heat-inactivated FBS (Gibco) in the presence of 1% penicillin/streptomycin (Gibco), 1% MEM Non-Essential Amino Acid (Gibco) and were grown at 37°C in a 5% CO₂ humidified incubator. For epithelial monolayer development, 2.5×10^5 Caco-2 cells were seeded into 24-well trans-well plates with a pore size of 0.4 mm and were maintained under standard conditions. Monolayers were cultured until tight junctions formed between cells for three weeks. Caco-2 cell monolayers then were exposed to *Mucor* (AzMucho and 1006PhL). After 20 h of exposure, fluorescein isothiocyanate (FITC)-labeled dextran (Sigma) (50 ng/ml) was added to the upper chamber of the trans-wells, and monolayers were incubated for additional 4 h. Fluorescence was measured in the upper and lower chambers of the trans-well plates using a fluorometer at an excitation wavelength of 485 nm and an emission wavelength of 530 nm to determine the percent flux of FITC-dextran across the monolayer (Maharshak *et al.*, 2015). Trypsin was used as a positive control for enhanced permeability. Three biological repeats were included for a statistic analysis (*t*-test with Welch's correction) and the result was visualized by using PRISM (GraphPad).

Results

Isolation of a *Mucor circinelloides* strain from an unopened yogurt within the recall range

In 2013, a yogurt company voluntarily withdrew its yogurt products produced from a factory located in Twin Falls, ID after more than 300 consumers had reported gastrointestinal illnesses to the US FDA. In a previous study, we isolated a *M. circinelloides* strain from an opened yogurt container within the recall range and analyzed the virulence, whole genome sequences, and interactions of the strain with mammalian hosts (Lee *et al.*, 2014).

For this study, a consumer in Phoenix, AZ, who was affected by the outbreak with associated illness, provided an unopened yogurt sample that was within the recall range and had been stored in a household freezer for about a year since the incident (Supplementary data Text S1). Subsequently, we isolated a *M. circinelloides* strain from this yogurt sample and named it AzMucho (see Materials and Methods). AzMucho was as virulent as the previous strain, Mucho (also isolated from a contaminated yogurt) in a murine systemic mucormycosis model (AzMucho vs Mucho, $P = 0.8342$) (Supplementary data Fig. S1). For phylogenetic identification of AzMucho, a multi-locus sequence typing (MLST) analysis was performed with three of the genomic loci used for the fungal tree of life project (James *et al.*, 2006). The loci include an intergenic spacer region (*ITS*) between two ribosomal sub-unit genes, a large ribosomal subunit gene (LSU *rDNA*), and an RNA polymerase subunit gene (*RPB1*). The sequences of the three loci amplified from the AzMucho genomic DNA were indistinguishable from those from Mucho (data not shown; Genbank accession numbers are KJ588204, KJ588205, and KJ588206, respectively) (Lee *et al.*, 2014). Mucho and AzMucho were *Mucor circinelloides* f. *circinelloides* and not distinguishable based on this MLST analysis. Based on an analysis of the whole genome, the genome of AzMucho differed by 6899 variants compared to that of Mucho. Comparisons with the whole genome sequences of ATCC1207a (*M. circinelloides* f. *griseocyanus*), ATCC1207b (*M. circinelloides* f. *griseocyanus*), CBS277.49 (*M. circinelloides* f. *lu-sitanicus*), and 1006PhL (*M. circinelloides* f. *circinelloides*) showed 69433 shared variants separated Mucho and AzMucho from the rest of the sequenced population (Supplementary data Fig. S2). This suggests indicating that Mucho and AzMucho are closely related but distinct isolates and it is likely that at least two isolates, and possibly more, were responsible for the yogurt spoilage. Alternatively, both could be the same isolate after accumulation of different SNPs during vegetative growth.

Ingestion of AzMucho influences bacterial species richness (alpha diversity)

Our initial examination found that AzMucho did not produce the toxin 3NP (Supplementary data Fig. S3), which is known to be produced by some *M. circinelloides* isolates and other fungi (He *et al.*, 1995; Hollmann *et al.*, 2008). It is therefore possible that the GI discomfort that consumers experienced was associated with an alteration of the microbiota. We investigated the possible effect of ingestion of *Mucor* on the intestinal microbiota. Alpha diversity of gut microbiota after AzMucho ingestion by male BALB/C mice housed in two independent animal facilities, UTSA and Duke university, were analyzed (Supplementary data Fig. S4). In our previous study, *Mucor* can survive in the mouse GI tract at least for 10 days and our *Mucor* colony forming unit (CFU) analysis from the stool also supports it

(Supplementary data Fig. S4) (Lee *et al.*, 2014). The stool samples therefore were collected up to day 10 post infection from animals at Duke or day 9 from animals at UTSA. Mice housed at the UTSA facility also ingested heat killed AzMucho or another *Mucor* strain, 1006PhL.

Cohorts of five mice ingested, by oral gavage, either AzMucho spores (exposed group) or PBS alone (unexposed group) at the Duke facility. *Mucor* ingestion was not likely affecting overall health of exposed mice based on the body weight monitoring, in which only one mouse experienced weight loss and eventually recovered (Supplementary data Fig. S1B). Fresh stool was collected from the mice daily for ten days, and DNA was extracted from the samples. Bacterial and fungal sequences in the isolated DNA were identified as OTUs utilizing 16S V4 and ITS1 primers, respectively, and Illumina MiSeq sequencing (see Materials and Methods for details). Bacterial and fungal OTU diversity were estimated, through OTU counts and Chao1 indices, and were tested for outliers using Cook's distance (Cook, 1977; Chao, 1984). The single sample outlier in bacteria and the two outliers in fungi with greatest Cook's distances were identified for removal prior to alpha diversity analysis.

In analysis of the microbiota of the mice housed at Duke, the overall model for bacterial Chao1 index (Fig. 1A left and Supplementary data Table S1) was significant ($F_{3,94} = 26.12$, $P < 0.05$), exhibiting higher bacterial diversity after ingestion of *Mucor* and over the course of the 10 day-period. The overall model denotes a summary of the entire linear regression with all factors including day, strain ingested, and the interaction between day and strain. Both day and exposure status were significant predictors of the Chao1 index. However, there was no significant interaction between day and exposure status. The overall model for number of bacterial OTUs (Fig 1A right and Supplementary data Table S1) was significant ($F_{3,94} = 19.76$, $P < 0.05$). Both day and exposure status were significant predictors of number of OTUs, as seen in the Chao1 index. There was also a significant interaction between day and exposure status. These results show that exposed mice have a higher overall bacterial diversity than their unexposed counterparts. It is important to note that both groups of mice experienced an increase in diversity with time. This increase, even in unexposed mice, may be because the mice used were juveniles (four to six weeks old upon arrival at the Duke animal facility), and thus their microbiota may still have been developing. However, it appears that exposure to *Mucor* altered the natural growth of the developing microbiota by lowering the rate at which bacterial alpha diversity increased in these mice.

An independent experiment was performed in the UTSA facility. At day 0, mice in all cohorts exhibited similar alpha diversity, indicating that the changes presented here are reflective of the fungal spore ingestions (data not shown). Interestingly, the overall model for bacterial Chao1 index when comparing live AzMucho with the PBS control (Fig. 1B) was significant ($F_{3,94} = 12.05$, $P < 0.05$), exhibiting lower bacterial diversity after ingestion of *Mucor* and over the course of the 10 day-period. Both day and the interaction term between day and strain were significant predictors of the Chao 1 index. Similarly, the overall model for bacterial OTU count when comparing live AzMucho with the PBS control was significant ($F_{3,94} = 7.801$, $P < 0.05$), exhibiting lower bacterial diversity after ingestion of *Mucor* (Fig. 1B). Both day and the interaction term between day and strain were significant predictors of the number of OTUs. These results indicate that *Mucor* ingestion can cause a

shift of bacterial abundance in either direction, dependent on the facility and/or animal cohorts. It is noteworthy that bacterial alpha diversity was increased in the microbiota of the mice housed at Duke but was decreased in mice housed at UTSA. Similar alpha diversity change variations have been observed in patients with Crohn's disease, in which fungal alpha diversity was increased, decreased, or unaltered when compared to healthy control groups, depending on the cohorts in the studies (Ott *et al.*, 2008; Li *et al.*, 2014; Hoarau *et al.*, 2016; Liguori *et al.*, 2016; Sokol *et al.*, 2017; Miyoshi *et al.*, 2018). In this study, it is possible that the differences in diet (different food for animals) and environment may have affected the direction of bacterial alpha diversity change. However, this study at least shows that ingestion of AzMucho resulted in changes to bacterial alpha diversity.

We then examined if *Mucor* needs to be alive to cause bacterial abundance change. The overall model for bacterial Chao1 index when comparing live AzMucho to heat killed AzMucho was significant ($F_{3,95} = 16.14$, $P < 0.05$), exhibiting lower bacterial diversity after ingestion of live *Mucor* than after ingestion of killed *Mucor* over the course of the 10-day period (Fig. 1B). The interaction term between day and strain was a significant predictor of the Chao1 index. Similarly, the overall model for bacterial OTU count when comparing live AzMucho with heat killed AzMucho was significant ($F_{3,95} = 12.3$, $P < 0.05$), exhibiting lower bacterial diversity after ingestion of live *Mucor*. The interaction term between day and strain was a significant predictor of the number of OTUs. Interestingly, the ingestion of heat killed AzMucho, although lower than live AzMucho, resulted in a change in alpha diversity compared to that of PBS, where the overall models for bacterial Chao1 and number of bacterial OTUs were significant ($F_{9,95} = 4.775$, $P < 0.05$ and $F_{3,95} = 4.06$, $P < 0.05$, respectively). This indicates that fungal spore component(s) can affect the bacterial microbiota (see Discussion).

Next, two different *Mucor* strains were compared. The overall model for bacterial Chao1 index when comparing live AzMucho to the 1006PhL strain was significant ($F_{3,95} = 8.271$, $P < 0.05$), exhibiting lower bacterial diversity after ingestion of AzMucho than after the alternative *Mucor* strain (Fig. 1B left). The interaction term between day and strain was a significant predictor of the Chao1 index. Similarly, the over-all model for bacterial OTU count when comparing live AzMucho with 1006PhL was significant ($F_{3,95} = 7.19$, $P < 0.05$), exhibiting lower bacterial diversity after ingestion of AzMucho. The interaction between day and strain was a significant predictor of the number of OTUs. Every one of the above described bacterial alpha diversity models remained significant after Bonferroni correction for multiple testing (data not shown). It appears that the bacterial alpha diversity changes after *Mucor* ingestion are likely strain-dependent. Considering all bacterial alpha diversity analyses together, ingestion of live AzMucho resulted in changes in bacterial abundance in two separate animal facilities and in a strain dependent manner. In addition, live *Mucor* is required to alter the bacterial abundance.

AzMucho ingestion effects on alpha diversity and abundance of fungal species

Considering animals housed at the Duke facility, the overall model for fungal Chao1 index (Fig. 2A) was non-significant ($F_{3,94} = 2.55$, $P = 0.06$), as was the overall model for number

of fungal OTUs (Fig. 2B) ($F_{3,94} = 1.32$, $P = 0.27$). As with bacterial diversity, unexposed mice experienced an increase in fungal alpha diversity over time.

However, in a test with animals housed at the UTSA facility, the overall model for fungal Chao1 index when comparing live AzMucho with the PBS control was significant ($F_{3,94} = 3.698$, $P < 0.05$), exhibiting higher fungal diversity after ingestion of *Mucor* spores over the course of the 10-day period (Fig. 2B). This remained significant after Bonferroni correction for multiple testing. Similarly, the overall model for fungal OTU count when comparing live AzMucho with the PBS control was significant ($F_{3,94} = 2.964$, $P < 0.05$), exhibiting higher fungal diversity (Fig. 2B) after ingestion of *Mucor*, although this did not remain significant after Bon-ferroni correction.

Interestingly, the overall model for fungal Chao1 index when comparing live AzMucho to heat killed AzMucho was not significant ($F_{3,94} = 1.7$, $P = 0.1723$). Similarly, the overall model for fungal OTU count when comparing live AzMucho with heat killed AzMucho was not significant ($F_{3,95} = 1.213$, $P = 0.3082$). These results indicate that dead AzMucho can also alter mycobiota, though to a lesser extent than live spores. It is therefore possible that cell wall associated fungal components may be associated with the ability to induce fungal alpha diversity changes. Unlike bacterial alpha diversity, the heat killed AzMucho spores did not alter fungal alpha diversity, where the overall model for fungal Chao1 was non-significant ($F_{3,95} = 2.162$, $P = 0.098$), as was the overall model for fungal OTUs ($F_{3,95} = 1.956$, $P = 0.126$).

The overall model for fungal Chao1 index when comparing live AzMucho to the 1006PhL strain was significant ($F_{3,95} = 3.257$, $P < 0.05$), exhibiting higher fungal diversity after ingestion of AzMucho than after the alternative *Mucor* strain. Day was a significant predictor of the Chao1 index. This did not remain significant after Bonferroni correction. The over-all model for fungal OTU count when comparing live AzMucho with 1006PhL was marginally different ($F_{3,95} = 2.67$, $P = 0.05196$). This result further support that the shift in microbiota after *Mucor* ingestion can be strain-dependent. However, changes in fungal alpha diversity and abundance after AzMucho ingestion are seen to a lesser extent when compared to bacterial diversity. The patterns of these alterations in fungal alpha diversity in different *Mucor* spore conditions are congruent with the changes in bacterial alpha diversity, in which fungal alpha diversity changes are strain-dependent and require live *Mucor* spores.

***Mucor* ingestion alters beta diversity and abundance of certain taxa**

Dissimilarity of intestinal microbiota of the animals housed at the Duke facility for all samples was visualized by principle coordinate analysis (PCoA) of Bray-Curtis distances (Bray and Curtis, 1957) and statistical significance was determined with the Adonis implementation of permutational analysis of variance (PERMANOVA) (Oksanen *et al.*, 2012). Analysis of bacterial microbiota (Fig. 3A and Supplementary data Fig. S5), when accounting for both live AzMucho ingestion and day, revealed a significant effect of both factors, where bacteria beta diversity was increased upon exposure and through the course of 10 days ($P = 0.016$ for AzMucho ingestion and $P = 0.001$ for day). Analysis of fungal microbiota (Fig. 3B and Supplementary data Fig. S6), when accounting for both AzMucho ingestion and day, also revealed a significant effect of both factors, where similar to the

bacterial beta diversity, the fungal beta diversity was also increased upon exposure and through the course of 10 days ($P=0.029$ for AzMucho ingestion and $P=0.001$ for day).

PERMANOVA analyses were then restricted to each individual day to explore on which specific days there was a significant effect of live AzMucho ingestion on beta diversity (Supplementary data Table S2 and Fig. 3C). There was a significant effect for bacterial samples on days 5, 6, and 10, and there was a significant effect for fungal samples on days 1, 3, 5, 6, and 10. These results have some overlap with the days of interest found during alpha diversity analysis and support the hypothesis that ingestion of AzMucho induced an alteration in the gastrointestinal microbiota of these mice. For ease of viewing, beta diversity plots were limited to individual days. Day 6 for both bacteria and fungi is shown for the purposes of this report (Fig. 3). Visualization of all days simultaneously and individually are available in supplemental data (Supplementary data Figs. S5 and S6).

In tests with animals housed at the UTSA facility, there was a significant effect of live AzMucho ingestion for the bacterial population on days 6 and 7, and on the fungal population on day 4 and 5 (Supplementary data Figs. S7 and S8). Interestingly, the ingestion of heat-killed AzMucho also showed a significant effect on bacterial population on days 3 and 4, and on fungal population on days 1, 4, and 7. Although heat-killed AzMucho did not significantly alter bacterial and fungal alpha diversities compared to live AzMucho, the dead *Mucor* spores can shift the bacterial and fungal populations (Supplementary data Fig. S7). This result further supports that fungal cell wall component(s) may contribute to the change in microbiota. Another strain 1006PhL, however, only had a significant effect on fungal population on day 1 (Supplementary data Fig. S7), which is in accordance with the bacterial and fungal alpha diversity analyses after AzMucho or 1006PhL ingestion. This further suggests that there is a *Mucor* strain dependent alteration of microbiota. For example, the 1006PhL strain may not encode gene(s) responsible for the microbiota change, or it may express the gene(s) at a lower level compared to AzMucho. Thus, the effect of 1006PhL ingestion resulted in subtle shift in microbiota.

Relative bacterial taxa composition of the intestinal microbiota of the animals housed at the Duke facility was compared between unexposed and exposed (live AzMucho compared to PBS ingestion) mice on day 8, based on the comparison of alpha diversity on individual days. Samples from days 5, 6, and 9, which also showed significant differences in alpha diversity, and all other time points were also subjected to compositional analysis (Supplementary data Table S3). By day 8, mice which ingested live AzMucho had significantly higher levels of the genus *Bacteroides* ($P=0.016$) and significantly lower levels of the genus *Akkermansia*, particularly the species *A. muciniphila* ($P=0.016$), than their unexposed counterparts (Fig. 4). Interestingly, *Bacteroides* species are known to be involved in inflammation in the gut (Wu *et al.*, 1998; Saitoh *et al.*, 2002; Bamias *et al.*, 2007) and *A. muciniphila* is associated with healthy guts (Everard *et al.*, 2013; Reunanen *et al.*, 2015). Therefore, the shift in bacterial species suggests an unhealthy shift in the guts of exposed mice. In a separate experiment performed with animals housed in the UTSA facility, the genus *Bacteriodes* ($P=0.028$) was also significantly higher by day 7 in the animals which ingested AzMucho. However, this change was not observed in animals which ingested heat-

killed AzMucho or 1006PhL. The bacterial taxa changes in each group are listed in Supplementary data Tables S4, S5, and S6.

Because fungal alpha diversity differed only on day 1 in animals housed at the Duke facility, we compared the relative fungal taxa composition of the intestinal microbiota between unexposed and exposed (live AzMucho and PBS ingestion) mice on day 10, which served as the endpoint of data collection. Samples from day 1 and all other time points (Supplementary data Table S7) were also subjected to compositional analysis. By day 10, exposed mice showed significantly lower levels of the genera *Acrostalagmus* ($P = 0.026$) and *Sarocladium* ($P = 0.047$) than their unexposed counterparts (Fig. 5). The fungal taxa changes seen in the animals housed in the UTSA facility are listed in Supplementary data Tables S8, S9, and S10. Interestingly, these analyses did not reveal a significant difference in *Mucor* between the two groups. This may be due to *Mucor* spores having undergone lysis in the stomach, although some are capable of passage through the GI tract (Lee *et al.*, 2014). Alternatively, *Mucor* spores are spread throughout the GI tract, so a single gavage of *Mucor* spores might not produce an amount of DNA sufficient to reach statistical significance in this taxonomic analysis.

Exposure to AzMucho increases monolayer permeability in epithelial cells

To investigate if AzMucho can permeabilize the epithelial monolayer barrier, CaCo-2 cell monolayers were exposed to AzMucho (10^5 spores) and 1006PhL (10^5 spores) along with PBS as a control (Fig. 6). Trypsin was also used as a positive cell permeabilizer. Upon exposure to AzMucho, the permeability of the CaCo-2 monolayers increased by approximately 3.3-fold compared to PBS control, which is significantly higher ($P = 0.0217$). These results suggest that AzMucho may have an ability to cause disruption of the enteric epithelial barrier. Interestingly, exposure to the 1006PhL strain did not result in an increase in the permeability at the given condition (1006PhL vs PBS, $P = 0.4369$; 1006PhL vs AzMucho, $P = 0.0187$). These results are congruent with the relatively lower shift in microbiota by ingestion of 1006PhL compared to that by ingestion of AzMucho.

Discussion

Our results demonstrate a significant change in bacterial alpha diversity in the intestinal microbiota of mice that ingested *Mucor*. Our results also show shifts in the composition of the microbiota, specifically through changes in the bacteria *A. muciniphila* and *Bacteroides species*. Interestingly, both bacterial taxa that differed between groups are known to be associated with gut health. Specifically, *Bacteroides* species have been implicated as a cause behind the inflammatory antibody response towards the gut microbiota seen in inflammatory bowel disease (IBD) (Saitoh *et al.*, 2002). *Bacteroides* species have also been shown to be involved in the exacerbation of inflammation in the gut, which is associated with Crohn's disease, as well as an increase in permeability of intestinal epithelial cells in patients with IBD (Wu *et al.*, 1998; Bamias *et al.*, 2007). *Bacteroides* species are present at higher levels in exposed mice, implying a shift in the microbial composition towards a potentially more pro-inflammatory environment. *A. muciniphila*, however, is known to be positively associated with gut health in that this species has been shown to control inflammation and

strengthen epithelial barrier integrity within the gut (Everard *et al.*, 2013; Reunanen *et al.*, 2015). *A. muciniphila* is present at lower levels in exposed mice, thus bolstering the idea that the microbial composition has experienced a shift towards a potentially unhealthy environment. However, shifts in bacterial taxa were variably significant after FDR-correction, and no shift at any fungal taxa level maintained significance after FDR-correction (see Supplementary data Tables for FDR-corrections). This may be due in part to the small sample size ($n = 5$ per group), and thus limited statistical power, used. In addition, it is also possible that a single gavage of *Mucor* would not be sufficient to cause a serious dysbiosis, rather than the moderate alteration in the microbiota (bacteria and fungi) compositions seen in our experiments. The lack of significant alteration in abundance of *Mucor* species between exposed and unexposed animals indicates this as a possibility.

Interestingly, ingestion of heat killed AzMucho also resulted in changes in beta diversity. This may suggest that fungal cell wall associated factor(s) can affect the intestinal microbiota. The genome of Mucho also encodes gene clusters involved in production of potentially harmful toxins (Lee *et al.*, 2014), and it is therefore possible that *Mucor* can produce cell wall associated toxin(s) that is detrimental to GI tract health. The overall lack of microbiota shift seen when using another *Mucor* strain, 1006PhL, can be explained by the possibility that the isolate may not produce the causal toxin(s), unlike Mucho or AzMucho. This observation is also in accordance with the findings that AzMucho may cause a leaky gut phenotype by testing the permeability assay using CaCo-2 cell tight junctions. On the other hand, the 1006PhL strain did not cause increased permeability (Fig. 6). Leaky gut or enhanced permeability of intestinal epithelial barrier is a common symptom in IBD and IBS (Odenwald and Turner, 2013). It is therefore possible that *Mucor* may play a role in the development of symptoms associated with GI discomfort. However, it is not clear that *Mucor* produces toxin(s) that would result in microbiota dysbiosis and/or disruption of enteric epithelial cell barrier in animals. Nevertheless, this strain dependent microbiota shift will provide a platform to identify how *Mucor* causes the microbiota shift.

Despite the small sample size used, overall, the examination of the composition of the gut microbiota of these mice supports the hypothesis that ingestion of AzMucho induces an alteration in the bacterial component of the gut in the days following ingestion. Our findings therefore suggest that consuming *Mucor* can alter the microbiota of the gut. It is possible that the alteration of the microbiota caused by *Mucor* might not be enough to be translated into a dysbiosis in the GI tracts of humans. Alternatively, *Mucor* might have affected processes in the stomach and/or small intestine that may not be apparent from the stool DNA analysis. Interestingly, in our separate study to analyze the bacterial and fungal microbiota in South Korean patients with Crohn's disease ($n = 35$) compared to healthy individuals ($n = 52$), abundance of *Mucor* was found to be significantly higher in the patient group (Serrano, Mueller, Huh, Choi, and Lee, unpublished data). Interestingly however, *Mucor* was not found to be significantly increased in studies of patients with CD from the United States and European countries (Hoarau *et al.*, 2016; Sokol *et al.*, 2017).

The change in bacterial alpha diversity in our experiments is consistent with previous studies relating to diseases, such as Crohn's disease, ulcerative colitis (UC), irritable bowel syndrome (IBS), and anorexia nervosa, in which the disease state is associated with altered

alpha diversity and shifts in microbiota composition of the gut (Kleiman *et al.*, 2015; Hoarau *et al.*, 2016; Sokol *et al.*, 2017). Specifically, these studies have demonstrated alterations in the prevalence of bacteria and fungi, such as Ruminococcaceae and Enterobacteriaceae genera, *C. tropicalis*, and *Saccharomyces cerevisiae*, which are linked to intestinal disorders (Kleiman *et al.*, 2015; Hoarau *et al.*, 2016; Sokol *et al.*, 2017) (Mueller, Serrano, and Lee, unpublished data). This then suggests that ingestion of AzMucho alters the stability of the gut microbiota, possibly pushing towards a less healthy state by inducing an alteration of the resident bacteria. It may be, if this alteration is translatable to humans, that the gastrointestinal illness experienced by consumers of *Mucor*-contaminated yogurt might have been, in part, due to a disruption of their gut microbiota. This would be consistent with previously established associations between gut dysbiosis and gastrointestinal discomfort (Chichlowski and Rudolph, 2015; Botschuijver *et al.*, 2017).

It is well known that induced alterations of the gut bacteria both affect gut health and often result in gastrointestinal discomfort, such as in the case of fungal overgrowth during the course of antibiotic use (Erdogan and Rao, 2015) and antibiotic-associated diarrhea caused by bacterial pathogens such as *Clostridium difficile* (Young and Schmidt, 2004). Intestinal fungi have the ability to cause inflammation in the gut. For example, improper recognition of fungi in the GI tract due to mutations in the gene encoding Dectin-1, a C-type lectin receptor, results in exacerbation of colitis (Iliev *et al.*, 2012). A dysbiosis of the enteric mycobiota is linked to inflammation in Crohn's disease (Li *et al.*, 2014). In particular, modulation of purine metabolism by *S. cerevisiae* has been shown to be associated with worsening colitis and increased gut barrier permeability in a murine model (Chiaro *et al.*, 2017). *Candida* colonization increases pro-inflammatory cytokine production that is associated with IBD (Kumamoto, 2011). Previous studies have further demonstrated an interaction between fungi and bacteria in the gut, and specifically, how that interaction relates to diseases of the gut (Hoarau *et al.*, 2016; Sokol *et al.*, 2017). Antifungal drug derived microbiota dysbiosis results in enhanced colitis and exacerbated allergy airway disease in a murine model (Wheeler *et al.*, 2016; Leonardi *et al.*, 2018). Our study, shown in Supplementary data Fig. S9, also supports fungal dysbiosis driven inflammation, in which after fluconazole and fluocytosine treatment the guts of mice secreted higher amounts of inflammatory cytokines, including IL-1b, IL-6, and INFg (see Supplementary data Fig. S9 and legend). Some fungi are also known, but to a lesser extent than bacteria, for their usefulness in promoting gut health. For example, *Saccharomyces boulardii*, a fungus which is commercially available as a probiotic supplement, is utilized in part to reduce the risk of development of antibiotic-associated diarrhea (Szajewska and Mrukowicz, 2005).

It is clear that both fungi and bacteria play important roles in the gut, and that maintaining a harmony between the two should be kept in mind when considering the prevention and treatment of gastrointestinal illness. Despite this, the role of fungi in the gut and in food-borne illness has been vastly understudied. It is evident then that more consideration should be given, and more studies conducted, regarding the role of the mycobiota in gut health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Bacterial alpha diversity in stool samples from mice exposed to *M. circinelloides* through ingestion and unexposed mice during ten days of observation.

Alpha diversity was measured using Chao-1 index (left) and number of bacterial OTUs (right) present. The bacterial alpha diversity was measured from mice housed at the Duke facility (A) and the UTSA facility (B). Live AzMucho ingested mice showed a higher diversity than the unexposed mice at the Duke; on the other hand, live AzMucho ingested mice showed a lower diversity than the unexposed mice at the UTSA facility. Ingestion of heat-killed AzMucho and the 1006PhL strain did not alter alpha diversity compared to the unexposed mice. Standard error bars are shown. Significant days (identified in Supplementary data Table S1, $P < 0.05$) with the data from the mice at Duke are denoted with stars.

Fig. 2. Fungal alpha diversity in stool samples from mice exposed to *M. circinelloides* through ingestion and unexposed mice during ten days of observation.

Alpha diversity was measured using Chao-1 index (left) and number of fungal OTUs (right) present. The fungal alpha diversity was measured from mice housed at the Duke facility (A) and the UTSA facility (B). The overall linear model was non-significant in mice at the Duke facility but significant in mice at the UTSA facility. Standard error bars are shown. A significant day (identified in Supplementary data Table S1, $P < 0.05$) with the data from the mice at Duke is denoted with stars.

Fig. 3. Beta diversity between exposure groups at all time points. Bray-Curtis dissimilarity was visualized by PCoA for (A) bacterial and (B) fungi on day 6 with the data obtained from mice at Duke. (C) Effect size of *Mucor* exposure by live AzMucho for individual time points was calculated using PERMANOVA. Significant days (identified in Supplementary data Table S2, $P < 0.05$) are denoted with stars.

Fig. 4. Shift in bacterial microbiota composition in exposed mice on day 8 and in fungal microbiota composition in exposed mice day 10.

(A) LEfSe analysis was used to determine which bacterial taxonomic units showed a significant difference between groups before FDR-correction. The data were obtained from the mice housed at Duke. Further bacteria taxa analysis data are available in Supplementary data. Bacterial taxa enriched in the exposed group were indicated with a negative LDA score (red bars) and bacterial taxa abundant in unexposed group were indicated with a negative LDA score (green bars). (B) LEfSe analysis was used to determine which fungal taxonomic units showed a significant difference between groups before FDR-correction. The data were obtained from the mice housed at Duke. Further fungal taxa analysis data are available in Supplementary data. Fungal taxa enriched in the exposed group were indicated with a negative LDA score (red bars) and fungal taxa abundant in unexposed group were indicated with a negative LDA score (green bars). The LDA scores indicates the effect size of each differently enriched taxon. LDA stands for Linear Discriminant Analysis.

Fig. 5. Exposure to AzMucho increases the permeability of epithelial cell monolayers.

CaCo-2 cell monolayers were co-cultured with spores of AzMucho or 1006PhL or PBS. The flux of FITC-dextran across the monolayers was significantly higher when they were exposed to AzMucho compared to PBS (* $P = 0.0217$). However, when the monolayers were exposed to 1006PhL, the difference is not significant compared to PBS ($P = 0.4369$). AzMucho caused greater permeability compared to 1006PhL (** $P = 0.0187$). Trypsin treatment groups were served as a positive control. Each group was tested at least 3 times and two independent experiments exhibited the similar results. Data were visualized as mean $\pm$ SD. Statistical significance was measured using Welch's t test.