Analysis of a Food-Borne Fungal Pathogen Outbreak: Virulence and Genome of a Mucor circinelloides Isolate from Yogurt

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ABSTRACT Food-borne pathogens are ongoing problems, and new pathogens are emerging. The impact of fungi, however, is largely underestimated. Recently, commercial yogurts contaminated with Mucor circinelloides were sold, and >200 consumers became ill with nausea, vomiting, and diarrhea. Mucoralean fungi cause the fatal fungal infection mucormycosis, whose incidence has been continuously increasing. In this study, we isolated an M. circinelloides strain from a yogurt container, and multilocus sequence typing identified the strain as Mucor circinelloides f. circinelloides. M. circinelloides f. circinelloides is the most virulent M. circinelloides subspecies and is commonly associated with human infections, whereas M. circinelloides f. lusitanicus and M. circinelloides f. griseocyanus are less common causes of infection. Whole-genome analysis of the yogurt isolate confirmed it as being close to the M. circinelloides f. circinelloides subgroup, with a higher percentage of divergence with the M. circinelloides f. lusitanicus subgroup. In mating assays, the yogurt isolate formed sexual zygospores with the (-) M. circinelloides f. circinelloides tester strain, which is congruent with its sex locus encoding SexP, the (+) mating type sex determinant. The yogurt isolate was virulent in murine and wax moth larva host systems. In a murine gastromucormycosis model, Mucor was recovered from fecal samples of infected mice for up to 10 days, indicating that Mucor can survive transit through the GI tract. In interactions with human immune cells, M. circinelloides f. lusitanicus induced proinflammatory cytokines but M. circinelloides f. circinelloides did not, which may explain the different levels of virulence in mammalian hosts. This study demonstrates that M. circinelloides can spoil food products and cause gastrointestinal illness in consumers and may pose a particular risk to immunocompromised patients.

IMPORTANCE The U.S. FDA reported that yogurt products were contaminated with M. circinelloides, a mucoralean fungal pathogen, and >200 consumers complained of symptoms, including vomiting, nausea, and diarrhea. The manufacturer voluntarily withdrew the affected yogurt products from the market. Compared to other food-borne pathogens, including bacteria, viruses, and parasites, less focus has been placed on the risk of fungal pathogens. This study evaluates the potential risk from the food-borne fungal pathogen M. circinelloides that was isolated from the contaminated commercial yogurt. We successfully cultured an M. circinelloides isolate and found that the isolate belongs to the species M. circinelloides f. circinelloides, which is often associated with human infections. In murine and insect host models, the isolate was virulent. While information disseminated in the popular press would suggest this fungal contaminant poses little or no risk to consumers, our results show instead that it is capable of causing significant infections in animals.

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D aily essentials, such as food, are exposed to contamination/infestation by pathogenic microbes and eventually provide a source of infections. Food-borne intestinal infectious disease is a continuous problem in the health care system and causes considerable social and economic burdens. Among others, bacteria are normally considered prominent pathogens, and three well-known food-borne bacteria include Salmonella spp., Campylobacter spp., and Escherichia coli (reviewed in reference 1). Listeria monocytogenes is an emerging bacterial pathogen associated with food (2). Viral pathogens also cause food-borne illness (3, 4). Noroviruses are one of the most common agents for gastroenteritis and cause especially severe symptoms in immunocompromised patients (4–6). Other examples of food-borne viral pathogens include hepatitis A virus, hepatitis E virus, and rotavirus (1). In addition, para-
sites can cause food-borne infectious diseases, and currently, ~300 parasitic worms and ~70 protozan species are known to infect humans and animals (reviewed in reference 1).

However, studies evaluating fungi as food-borne pathogens and assessing associated risks are limited. In September 2013, there was a food-borne illness outbreak after consumers ingested yogurts contaminated with mold. More than 200 individuals suffered from vomiting, nausea, and diarrhea after consumption of the yogurt (7, 8). The U.S. Food and Drug Administration (U.S. FDA) immediately analyzed the responsible mold and identified it as Mucor circinelloides. M. circinelloides belongs to the order Mucorales, among the lineages of early-diverging fungi known as zygomycetes, and is one of the causal agents of the fungal infection mucormycosis. The increase of immunocompromised cohorts in recent years caused by, for example, transplantation, malignancies, HIV/AIDS, steroids, diabetes, and neutropenia, has been mirrored by an increase in the incidence of mucormycosis. Significantly, ~15% of patients with severe neutropenia develop mucormycosis (9, 10). The mortality from this fungal infection is high, ranging from 68 to 100% (9, 11). The main infection sites are the lungs, the sinuses, soft tissues, skin, and the bloodstream (12, 13). Gastrointestinal (GI) mucormycosis causes symptoms that include nonspecific abdominal tenderness and distention with nausea and vomiting, and case reports (14) have been published (15–18). Transplant recipients are especially susceptible to gastrointestinal mucormycosis following ingestion of causative fungal species (17). One such case was reported in a bone marrow transplant recipient who ingested naturopathic medicine contaminated with Mucor, resulting in the development of gastrointestinal mucormycosis (16). The mortality rate of gastrointestinal mucormycosis is as high as 85%, and the infection is often disseminated, resulting in higher rates of mortality (15).

Mucor species are the second most common mucoralean fungus causing mucormycosis, surpassed only by Rhizopus species (19, 20). According to the U.S. FDA report, M. circinelloides was associated with the outbreak of food-borne illnesses after consumption of the contaminated yogurt. The M. circinelloides species complex consists of several distinct species or subspecies: M. circinelloides f. lusitanicus, M. circinelloides f. circinelloides, and M. circinelloides f. griseocyanus (21, 22). M. circinelloides f. circinelloides isolates are more often associated with patients and display higher virulence in the murine mucormycosis model (21).

In this study, we obtained a plain Chobani yogurt that was within the manufacturer’s voluntary date recall range and also in the production lot subject to recall. This sample was provided by a couple in Texas who both consumed the contaminated product. Both individuals developed nausea and diarrhea, and one also developed vomiting. From the container, we isolated an M. circinelloides strain, designated Mucho, and subsequently identified the isolate as belonging to the M. circinelloides f. circinelloides subgroup. Whole-genome analysis further supports that the yogurt isolate belongs to M. circinelloides f. circinelloides and is distinct from the M. circinelloides f. lusitanicus and M. circinelloides f. griseocyanus subgroups. In virulence tests with two mucormycosis virulence models, we found that the isolate infects animals and causes mortality. Our results demonstrate that M. circinelloides is a food-borne pathogen that can cause lethal mucormycosis and suggest that caution should be exercised with respect to fungal pathogens in food, particularly for individuals who are immunocompromised.

RESULTS
Isolation of a mold from a yogurt within the company recall range. The U.S. Food and Drug Administration (FDA) released a report that there was a recall of yogurt made by Chobani from a factory located in Twin Falls, ID. The recalled yogurts were labeled as those with a best by date between 11 September and 7 October 2013. The IMS code assigned was 16-012 (7). It was reported to the FDA that more than 300 individuals who consumed yogurts produced by the company experienced gastrointestinal discomfort, including nausea, cramps, vomiting, and diarrhea. An immediate investigation found that the yogurts were contaminated with M. circinelloides (23).

A couple in Corpus Christi, TX, experienced moderate to moderately severe illness following consumption of part of a plain yogurt from Chobani. One of them experienced repeated vomiting and diarrhea for two entire days with two days of missed work; the other was severely nauseated with diarrhea for a few days without vomiting (see Text S1 for the note from the couple). According to the couple, an apparent mold grew in the yogurt placed in the refrigerator. We obtained the yogurt container, and it was dated for expiration on 30 September 2013, which was within the recall date period, and had the IMS code 16-012 (Fig. S1). We isolated a mold from the yogurt container, and the mold displayed the typical growth features that are observed in Mucor species, i.e., formation of aseptate hyphae, aerial hyphae decorated with a ball-like sporangium, and a sporangium containing thousands of asexual spores (data not shown).

Identification and phylogenetic analyses of the yogurt isolate. For identification, a multilocus sequence typing (MLST) analysis was conducted with three genes used in the Fungal Tree of Life Project (24). Initially, seven isolates were cultured from seven different locations within the suspect container. An intragenic spacer region (ITS), a large subunit rRNA gene (LSU rRNA), and an RNA polymerase subunit gene (RPB1) were amplified and sequenced from the genomes of each isolate. All sequences for each of the three genes from the seven isolates were identical (data not shown), indicating that, at least at these loci, the seven isolates are indistinguishable. One of the seven isolates was designated Mucho and selected for further analysis.

We also tested 16 other yogurt products from Chobani with different “best by” dates and IMS codes (see Table S1 in the supplemental material). None of them were found to be contaminated with M. circinelloides. However, we isolated a Yarrowia lipolytica isolate from an opened yogurt product, which is not known as a pathogen and is associated with lipid production as an industrial microorganism (reviewed in references 25 and 26).

The M. circinelloides species complex includes at least three species or subspecies: M. circinelloides f. circinelloides, M. circinelloides f. lusitanicus, and M. circinelloides f. griseocyanus (21, 22). Maximum likelihood trees were constructed with the three genes obtained and known sequences for the same three genes from representative M. circinelloides f. circinelloides (NRRL3614, NRRL3615, and ATCC11010), M. circinelloides f. lusitanicus (CBS277.49, ATCC1216a, ATCC1216b, and NRRL3631), and M. circinelloides f. griseocyanus (ATCC1207a and ATCC1207b) isolates (see reference 21 and references therein) (Fig. 1). The three individual gene trees displayed similar patterns, indicating...
that there are three distinct groups represented by *M. circinelloides f. circinelloides*, *M. circinelloides f. lusitanicus*, and *M. circinelloides f. griseocyanus*. No examples of phylogenetic incongruence were observed, and it is therefore evident that Mucho belongs to the *M. circinelloides f. circinelloides* subgroup. We also included an *M. circinelloides f. circinelloides* isolate, 1006PhL, recently sequenced at the Broad Institute (www.broadinstitute.org/annotation/genome/rhizopus_oryzae/MultiHome.html) (27), in this analysis and conclude that this isolate also belongs to the *M. circinelloides f. circinelloides* subgroup. The *M. circinelloides f. circinelloides* 1006PhL isolate was obtained from the skin of a normal human volunteer during a skin mycobiome study (27).

**Whole-genome comparisons: Mucho versus *M. circinelloides f. lusitanicus* and Mucho versus 1006PhL (**M. circinelloides** f. **circinelloides**).** Whole-genome sequencing reveals that the genome of Mucho is much more similar to the *M. circinelloides f. circinelloides* 1006PhL isolate than to the *M. circinelloides f. lusitanicus* CBS277.49 isolate (http://jgi.doe.gov/Mucci2/Mucci2.home.html) (27). However, there are still substantial polymorphisms differentiating Mucho from 1006PhL (Fig. 2A). The genome of 1006PhL shares only 85.03% identity with CBS277.49 over the 14 megabases successfully aligned using Nucmer (part of the MUMmer package), while the genome of Mucho shares 85.11% identity with CBS277.49 over 11.2 megabases. The Mucho and 1006PhL isolates share substantially more of their sequences, with 65.1% of the genomes aligning with 96.5% identity using Nucmer. Using a reference-based assembly instead, the Mucho genome is still differentiated from the 1006PhL genome by approximately 630,000 single-nucleotide polymorphisms (SNPs) over the 72.2% of the genome that was callable (97.6% identity).

Interestingly, the *M. circinelloides f. lusitanicus* genome was not suitable as a reference for genome assembly of Mucho using the short-read component of the Burrows-Wheeler aligner (BWA)(12.2% coverage over 5×); *M. circinelloides f. circinelloides* was much more compatible (87.5% coverage over 5×). This appears to be the result of substantial rearrangement of the genomes between *M. circinelloides f. circinelloides* and *M. circinelloides f. lusitanicus*, as well as, likely, a result of low sequence identity. An alignment of the first contig from 1006PhL with the entire genome of CBS277.49 maps to three different *M. circinelloides f. lusitanicus* scaffolds and includes at least six large inversions and a number of regions that were not aligned between the two genomes (Fig. 2B).

Taken together, these findings suggest that *M. circinelloides f. circinelloides*, *M. circinelloides f. lusitanicus*, and *M. circinelloides f. griseocyanus* are different enough to be three distinct species rather than simply subspecies. Moreover, even with the *M. circinelloides f. circinelloides* subgroup, the finding that isolates differ by as much as 3.5% at the whole-genome level suggests substantial diversity compared to that of other well defined fungal species.

**Mucho is sexually fertile.** The mating ability of Mucho was tested with the *M. circinelloides f. lusitanicus* ([−] CBS277.49 and [+]) NRRL3631, *M. circinelloides f. griseocyanus* ([+]) ATCC1207a and (−) ATCC1207b, and *M. circinelloides f. circinelloides* ([−]) NRRL3614 and (+) NRRL3615 isolates. The formation of zygospores was monitored after 4 weeks of coculture in

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**FIG 1** Phylogenetic analyses of the yogurt isolate Mucho. Three genes (ITS, LSU rRNA, and RPB1) were used to construct the phylogenetic trees. In all cases, there are three distinct groups, represented by *M. circinelloides f. circinelloides* (Mcc), *M. circinelloides f. lusitanicus* (Mcl), and *M. circinelloides f. griseocyanus* (Mcg). No phylogenetic incongruence was found. The three trees show that Mucho and 1006PhL belong to *M. circinelloides f. circinelloides*. The bootstrap support is 1,000, and the scales indicate base substitutions per position.
the dark (Fig. 3). Mucho mated with the (−) M. circinelloides f. circinelloides strain NRRL3614 but not with the (+) NRRL3615 isolate, indicating that Mucho is a (+) mating type strain. No intersubspecies mating with either M. circinelloides f. lusitanicus or M. circinelloides f. griseocyanus isolates was observed (data not shown). M. circinelloides has a sex locus encoding a high mobility group (HMG) domain protein as the sex determinant, and each mating type carries an allelic HMG transcription factor gene, sexP for (+) and sexM for (−) mating type (28). The Mucho genome was found to contain the sexP gene from the sex locus, which is in full accord with its (+) mating specificity.

Mucho is virulent in systemic infections in murine and Galleria hosts. Among the three M. circinelloides species or subspecies, the M. circinelloides f. circinelloides subgroup is the most commonly associated with clinical infections (21). Our previous findings indicate that M. circinelloides f. circinelloides is the most virulent in the murine host model system, whereas the M. circinelloides f. lusitanicus and M. circinelloides f. griseocyanus isolates are less virulent/avirulent. However, different levels of virulence are also observed among M. circinelloides f. circinelloides isolates (21). To assess the virulence of Mucho and 1006PhL, we employed two host systems: larvae of the wax moth Galleria mellonella and mice (Fig. 4).
Four M. circinelloides f. circinelloides isolates (NRRL3614, NRRL3615, IP1873.89, and CNRMA03.154) were tested and compared with Mucho and 1006PhL. Male BALB/c mice (~20 g) were infected through tail vein injection with 10⁶ spores resuspended in 200 μl of sterile phosphate-buffered saline (PBS). In accord with our previous results (21), NRRL3614 was significantly less virulent than NRRL3615 (P = 0.0404). The trend of the killing curves may indicate that Mucho is more virulent than the least virulent NRRL3614 strain and less virulent than the most virulent NRRL3615 strain (Fig. 4A). A statistical analysis, however, did not support that the survival curve of mice infected with Mucho was significantly different from that of either isolate (P = 0.2062 for Mucho versus NRRL3615 and P = 0.2404 for Mucho versus NRRL3614). However, it is clearly apparent that Mucho is virulent in the murine tail vein injection model. Similar results were obtained with the human skin isolate 1006PhL: no significant difference in virulence was observed for 1006PhL versus NRRL3615 (P = 0.0788) or 1006PhL versus NRRL3614 (P = 0.2813). In the Galleria larva host model, all M. circinelloides f. circinelloides isolates (including Mucho and 1006PhL) displayed significant virulence compared to the results for the PBS control (for example, P < 0.0001 for Mucho versus PBS) (Fig. 4B). No significant difference in the virulence of the M. circinelloides f. circinelloides strains was observed (P = 0.7071).

We also tested the virulence of these strains with male CD1 (~30 g) mice as hosts (Fig. 4C). Spores (10⁶) were inoculated via tail vein injection, and all conditions and methods of monitoring were the same as described above for BALB/c mice (see also Materials and Methods). The mortality of CD1 mice after infection with the M. circinelloides f. circinelloides strains was lower than that of BALB/c mice. For example, only one mouse died after infection with Mucho, 1006PhL, or IP1873.89. We found in monitoring body weight postinfection (p.i.) that the cohort of mice infected with Mucho underwent significant weight loss by 2 days p.i.; for example, three mice lost ~30% of their body weight by 3, 4, or 6 days p.i., one of which progressed to imminent mortality and was sacrificed on day 3 p.i. Infections with the 1006PhL, NRRL3615, CNRMA03.154, and IP1873.89 isolates that were virulent in BALB/c mice (Fig. 4A) all resulted in obvious weight loss between 2 and 6 days p.i. On the other hand, the least virulent strain, NRRL3614, caused less apparent weight loss, which is in accord with the results of the virulence tests with BALB/c mice. In all cases, the infected mice eventually recovered their initial body weight, indicating that the weight difference between the two mouse strains tested (~20 g for BALB/c and ~30 g for CD1) might contribute to different levels of virulence or that the CD1 mouse background may be less susceptible to M. circinelloides infection than the BALB/c background.

Gastrointestinal colonization of Mucho in the murine host. We developed a murine gastrointestinal host model to assess the risk from ingestion of Mucor. Diabetic and nondiabetic murine host models were tested by using the BALB/c mouse strain. Groups of diabetic and nondiabetic mice were infected with Mucho via oral gavage. From each mouse, we collected feces daily (three mice tested from each diabetic or nondiabetic mouse host) and placed them on yeast extract-peptone-dextrose (YPD) medium. Interestingly, fecal samples up to day 10 p.i. displayed Mucor growth after 24 h of incubation at 30°C, which indicates that Mucor can survive passage through the mouse GI tract (Fig. 5A; see Table S2 in the supplemental material). Only one diabetic mouse was found to lose significant weight (~20% of body weight at day 2 p.i.); however, overall, we did not observe apparent weight loss from oral infection with Mucho (Fig. 5B). Interestingly, the colons from infected mice sacrificed at days 2, 5, and 10 p.i. for GI dissection and histopathology tended to be shorter than those from noninfected mice (Fig. 5C); however, this trend is not statistically supported due to the small sample size from each day. In histopathological analysis, we did not observe apparent inflammatory symptom development or fungal invasion in the colons or ceca (data not shown).

Mucho induces cytokine production from immune cells. The fact that M. circinelloides f. circinelloides isolates display greater virulence than M. circinelloides f. lusitanius isolates suggests that the two species or subspecies may interact differentially with immune cells. To test this hypothesis, we examined the cytokine responses of THP-1 human monocytes following exposure to M. circinelloides f. circinelloides (Mucho) and M. circinelloides f. lusitanius (R7B) strains. Of 41 cytokines examined (see Table S3 in the supplemental material), one cytokine, alpha interferon 2 (IFN-α2), appeared to increase moderately in response to all three strains (Fig. S2), indicating activation of the interferon response by this fungal species. Surprisingly, the proinflammatory chemokines interleukin-8 (IL-8), monocyte chemotactant protein 1 (MCP-1), and macrophage inflammatory protein 1α (MIP-1α), key factors in the recruitment of neutrophils and other immune cells, were only induced by the M. circinelloides f. lusitanius isolate (Fig. 6 and Fig. S2). Interestingly, another related Mucorales fungal pathogen, Rhizopus delemar (RA99-880), also did not induce the proinflammatory cytokines tested (Fig. 6 and Fig. S2). This suggests that the M. circinelloides f. circinelloides and R. delemar strains fail to trigger or inhibit proinflammatory chemokine production, which may contribute to the greater virulence of the M. circinelloides f. circinelloides subspecies. Two other pathogenic fungi, Aspergillus fumigatus and Candida albicans, were also tested; in these cases, A. fumigatus induced both IL-8 and MIP-1α, whereas C. albicans only induced MIP-1α.

DISCUSSION

Fungal infections have increased as the cohorts immunocompromised by infections and/or medical conditions mount. Fungal pathogens as food-borne pathogens, however, have been neglected compared to other well known food-borne pathogens, including bacteria, viruses, and parasites. Food-borne infectious diseases are a serious ongoing problem for the health care system.
FIG 4 Virulence of Mucho in two host models. Fungal spores were injected by tail vein infection into murine hosts and via pseudopods for wax moth larvae. (A) When male BALB/c mice (~20 g) were used as hosts, Mucho displayed moderate virulence in comparison to the results for the most virulent isolate, NRRL3615, and the least virulent isolate, NRRL3614. The difference in virulence between NRRL3615 and NRRL3614 is significant \( P = 0.0404 \); however, the differences in virulence of Mucho and NRRL3615 or NRRL3614 are not statistically significant \( P = 0.2064 \) and \( P = 0.2404 \), respectively. Similar results were observed for the virulence of the 1006PhL strain (see text for details). (B) All of the \( M. \) \( cirrhitus \) \( f. \) \( cirrhitus \) isolates tested were significantly virulent in the wax moth larva host model compared to the results for the PBS control \( (P < 0.0001) \); however, the difference in virulence between the isolates was not significant \( (P = 0.7071) \). (C) When male CD1 mice (~30 g) were used as hosts, mortality from the \( M. \) \( cirrhitus \) \( f. \) \( cirrhitus \) strain infection was lower; for example, under the given conditions, one mouse each died from infection with Mucho, 1006PhL, or IP1873.89. However, substantial weight loss was observed in all of the mice infected with the \( M. \) \( cirrhitus \) \( f. \) \( cirrhitus \) strains, with the exception of mice infected with NRRL3614 \( (x \) axis, days postinfection; \( y \) axis, percentage compared to initial weight). Between days 2 and 6 p.i., the infected mice underwent weight loss of up to ~30% of the initial weight at the time of infection. The infected mice that survived eventually regained their preinfection body weight. The NRRL3614 isolate that exhibited the least virulence in the BALB/c mouse host model also displayed lower virulence than the other \( M. \) \( cirrhitus \) \( f. \) \( cirrhitus \) isolates tested, and all of the infected mice exhibited only subtle weight loss between days 2 and 4 p.i.
and cause tremendous economic and social burdens (1). New pathogens are emerging unexpectedly. It is therefore reasonable to raise precautionary concerns with regard to fungi as food-borne infectious disease agents.

There was a recent outbreak of illnesses related to *M. circinelloides* contamination in commercial yogurt products. Consumers complained of discomfort after eating the yogurt products, according to a U.S. FDA report. As a result, the manufacturer voluntarily withdrew their products that had “best by” dates within a certain time range (best by dates of 11 September to 7 October 2013 with the IMS code 16-012). These events prompted us to investigate the mold responsible for this recall and potential health concerns related to it by investigating the causative agent in animal host models.

The mold isolated from the contaminated yogurt, designated Mucho, was identified as *M. circinelloides f. circinelloides* by MLST, whole-genome comparisons, and mating analyses (Fig. 1, 2, and 3). There are at least three species or subspecies in the *M. circinelloides* species complex, *M. circinelloides f. circinelloides*, *M. circinelloides f. lusitanicus*, and *M. circinelloides f. griseocyanus*. Comparison of the *M. circinelloides f. lusitanicus* and *M. circinelloides f. circinelloides* genomes reveals dramatic rearrangements, as well as significant differences in sequence identity. It is highly likely that, as a result, there is a substantial reproductive barrier separating these two lineages. The Mucho isolate mated only with a (*H11001*) *M. circinelloides f. circinelloides* strain and not with (*H11001*) *M. circinelloides f. lusitanicus* or (*H11001*) *M. circinelloides f. griseocyanus* isolates. Even if an *M. circinelloides f. circinelloides* strain may be able to mate with an *M. circinelloides f. lusitanicus* strain, the progeny are likely to be either less fit or inviable. This suggests that there may be a species barrier between the *M. circinelloides f. circinelloides* and *M. circinelloides f. lusitanicus* subtypes, consistent with their divergent and rearranged genome sequences.

Among the species or subspecies, *M. circinelloides f. circinelloides* is known to be more virulent in the murine host model and, in particular, is more frequently associated with human infections (21). Different outcomes during interactions with cultured immune cells may explain the differences in virulence (Fig. 6), where an *M. circinelloides f. lusitanicus* isolate induced proinflammatory cytokines but two *M. circinelloides f. circinelloides* isolates did not.
Our subsequent virulence tests found that the Mucho strain is virulent in animal hosts (Fig. 4), which may be explained by differential induction of proinflammatory cytokines by M. circinelloides f. lusitanicus and M. circinelloides f. circinelloides. However, in the oral gavage model experiment (Fig. 5), the Mucho isolate did not cause apparent disease symptoms based on weight monitoring postinfection (except in one mouse). Nevertheless, the Mucho strain survived passage through the mouse GI tract based on recovery of the fungus from fecal samples, indicating that M. circinelloides can survive passage through the GI tract. These results implicate Mucor species as potential causal agents for serious food-borne illness, especially for immunocompromised patients. A series of case reports of gastrointestinal mucormycosis further supports that Mucor can cause fatal fungal infections through ingestion of contaminated foods or medicines (15–18, 29).

It is as yet unclear whether Mucor infection is directly responsible for the illnesses reported in the M. circinelloides outbreak related to the yogurts. An alternative explanation is that intoxication with secondary metabolites, such as mycotoxins, could be responsible for this incident. Possibly the contaminated yogurts could contain harmful toxins produced by Mucor. Ergot fungi provide an example. Ergot toxin is an alkaloid fungal toxin produced by Claviceps species, and the consumption of rye and other cereals contaminated with ergot toxin causes ergotism (30, 31). The symptoms caused by the toxin include gangrene of the skin and are associated with the tragic Salem witch trials of 1692 that were held in Salem Village, MA. This food-borne ergotism was misinterpreted as a symptom of witchcraft, resulting in the persecution and execution of 20 Puritans (32, 33). Another example of a well known mycotoxin is aflatoxin, produced by Aspergillus species (30). The responsible fungal species colonize grains and peanuts and contaminate them with aflatoxin; the toxin was first known as the source of turkey X disease (34). Aflatoxin is a carcinogen posing a serious medical problem for the health care system (35). In addition, T-2 toxin produced by Fusarium spp. causes alimentary toxic aleukia, and consumption of bread contaminated with the toxin resulted in the deaths of ~100,000 people in the Soviet Union from 1942 to 1948 (36).

The genomes of M. circinelloides f. circinelloides Mucho and 1006PhL and M. circinelloides f. lusitanicus CBS277.49 contain genes predicted to be involved in the production of secondary metabolites, which indicates that Mucor might produce harmful toxins (Table 1; see Fig. S3 in the supplemental material). Each of the genes identified in these pathways from 1006PhL is present in the Mucho genome, and some of the proteins encoded are clearly more conserved than others. Genes predicted to encode proteins involved in the synthesis of terpenes and bacteriocins were particularly well conserved. Although the related species Rhizophus microsporus produces rhizoxin via the endosymbiotic bacterium Burkholderia rhizoxinica, it is not known whether any Mucor species harbor endosymbionts or produce mycotoxins (37). However, our whole-genome sequencing did not reveal sequences of endosymbiotic bacteria.

There are other reports that fungal contamination associated with other daily essentials or medications can cause serious medical threats. A fungal meningitis outbreak occurred after epidural injection of methylprednisolone contaminated with the ascomycete fungus Exserohilum rostratum (38–40). A survey study of 328 patients who had been administered the steroid contaminated...
with this fungus and who then developed infections revealed that 81% developed central nervous system (CNS) infections and the remaining 19% suffered from non-CNS infections (29). Multiple fungal keratitis outbreaks were reported between 2004 and 2006 among contact lens wearers in Hong Kong, Singapore, France, and the United States (41–44). The fungus Fusarium that contaminated contact lens solutions was the responsible pathogen, which resulted in a withdrawal of ReNu with MoistureLoc (Bausch & Lomb) from the world market on 15 May 2006 (42). Fungal keratitis is an inflammation of the cornea and can cause permanent loss of eyesight.

It is obvious that fungal pathogens pose a severe problem for the health care system, and the ensuing outcomes of infection often cause a serious loss of quality of life or even blindness or death. However, compared to other pathogens, less attention has been afforded to fungal pathogens as contaminants of our daily essentials, such as foods, medical devices, medications, and the facilities that manufacture them. Prevention of fungal contamination and careful examination should be practiced. Our results in this study provide evidence that understudied Mucor species can cause a potentially serious illness following ingestion with foods.

### MATERIALS AND METHODS

**Strains and culture conditions.** A plain Chobani yogurt dated best by 30 September 2013 and with the IMS code 16-012 was obtained from the batch that was subject to recall (see Fig. S1 in the supplemental material). Yogurt samples from seven different spots within the suspect container were transferred with sterile applicators onto potato dextrose agar (PDA) medium containing 50 μg/ml ampicillin and kanamycin to control residual live bacteria. After 3 days of incubation at 30°C, mold mycelia grew out on the plates. After two cycles of streak purification, spores from each strain were collected. For spore production from all spots on the plates. After two cycles of streak purification, spores from each strain were collected. For further virulence tests, the spore suspensions were washed with sterile distilled water was added to the plates and spores were collected. For further virulence tests, the spore suspensions were washed with sterile PBS.

For mating, V8 medium (pH 7) was inoculated with two strains approximately 1 in. apart from each other. After 4 weeks of coculture in the light, plates were collected. For spore production from all spots on the plates. After two cycles of streak purification, spores from each strain were collected. For further virulence tests, the spore suspensions were washed with sterile distilled water was added to the plates and spores were collected. For further virulence tests, the spore suspensions were washed with sterile PBS.

**TABLE 1 Secondary metabolite genes in the genomes of Mucho, 1006PhL, and CBS277.49 strains**

<table>
<thead>
<tr>
<th>Gene accession number</th>
<th>Category</th>
<th>1006PhL</th>
<th>Mucho</th>
<th>CBS277.49</th>
<th>Putative function</th>
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<tr>
<td>HMPREF 1544_00936</td>
<td>Terpene</td>
<td>J999696</td>
<td>Mucci1_e_gw1.6.146.1</td>
<td>3-Methylcrotonyl-coenzyme A (CoA) carboxylase biotin-containing subunit</td>
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<tr>
<td>HMPREF 1544_00937</td>
<td>Terpene</td>
<td>J999697</td>
<td>extGenewise1Plus.C_070865</td>
<td>Farnesyltransferase</td>
<td></td>
</tr>
<tr>
<td>HMPREF 1544_01407</td>
<td>Terpene</td>
<td>J999698</td>
<td>e_gw1.02.1791.1</td>
<td>Farnesyl-diphosphate farnesyltransferase</td>
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<tr>
<td>HMPREF 1544_07394</td>
<td>Terpene</td>
<td>J999705</td>
<td>e_gw1.05.994.1</td>
<td>Lanosterol synthase</td>
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<tr>
<td>HMPREF 1544_08078</td>
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<tr>
<td>HMPREF 1544_08719</td>
<td>Terpene</td>
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<td>e_gw1.01.1613.1</td>
<td>NADH dehydrogenase (ubiquinone) complex</td>
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<tr>
<td>HMPREF 1544_08721</td>
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<td>HMPREF 1544_08722</td>
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<tr>
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<td>fgenesh1_kg.01_g_373_1623</td>
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<tr>
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<td>Mucci1_e_gw1.244.1</td>
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<td>Homocitrate synthase</td>
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<tr>
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<td>J999716</td>
<td>extGenewise1Plus.C_010092</td>
<td>α-Aminoacidopate-semialdehyde dehydrogenase</td>
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<tr>
<td>HMPREF 1544_00134</td>
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<td>Mucci1_fgeneshMC_pg.5_g_71</td>
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<tr>
<td>HMPREF 1544_07114</td>
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<td>HMPREF 1544_07116</td>
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<td>Mucci1_fgeneshMC_pg.3_g_1109</td>
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<td>Genemark1.7267_g</td>
<td>Acyl-CoA synthetases (AMP-forming)/AMP- acid ligases II (lipid metabolism)</td>
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</tbody>
</table>

dark, the mating plates were observed by using a Nikon Eclipse E400 microscope equipped with a Nikon DXM1200F camera.

**Phylogenetic analysis.** ITS, LSU rRNA, and RPB1 used for the Fungal Tree of Life Project (45) were amplified from genomic DNA of the Mucho isolate. The primers used for ITS were ITS1, TCCGTAAGTGAACCTG CGG, and ITS4, TCTCCGCTTACTATGATGC; for LSU rRNA, the primers were D1/D2 LRDNA, GCATATCAATAAGGGAAGGAAAAG, and LR3, GGTCCGTGTTTCAAGACGG; and for RPB1, they were RPB1-Ac, GARTGYCCDGDCAYTYYGG, and RPB1-Cr, CCNGCDATNTCR TTTCACATRA. PCR products were obtained for each gene, sequenced, and aligned with CLUSTALW. The aligned DNA sequences were used to construct phylogenetic trees by using the PhyML 3.0 software (46). The three genes of the *Yarrowia lipolytica* isolate were also sequenced, and the sequences obtained were analyzed with Blast to identify the species. The GenBank nucleotide accession numbers for the Mucho ITS, LSU rRNA, and RPB1 sequences are KJ882046, KJ882055, and KJ882056, respectively.

**Whole-genome analysis** A reference genome was generated for the 1006PhL isolate of *M. circinelloides* and *M. circinelloides* RPB1 sequences obtained were analyzed with Blast to identify the species. The GenBank nucleotide accession numbers for the Mucho ITS, LSU rRNA, and RPB1 sequences are KJ882046, KJ882055, and KJ882056, respectively.

**Virulence tests in animal models.** Spores for animal infections were resuspended in sterile PBS, and the numbers of spores were counted using a hemocytometer. PBS containing 50,000 spores or PBS alone was injected into a cohort of wax moth larvae for each strain (10 larvae per strain), as described previously (21, 50, 51). For the murine systemic infection model, groups of male BALB/c and CD1 mice (5 per each strain) were used; for the diabetic mouse host model, the mice were rendered diabetic by injecting streptozocin (190 mg per kg of body weight) as previously described (52, 53). Three male BALB/c mice (4 to 6 weeks old) were infected with the Mucho strain, and two mice were mock infected with sterile water as a control. Body weight was measured daily. Fecal samples were collected daily from each mouse separately and placed onto YPD medium to examine the growth of *Mucor*. The fungus from the feces from day 1 was confirmed by sequencing the ITS, and later recovery of Mucho was confirmed by morphological analysis, including observation of aseptated hyphae and sporangium formation, which are hallmarks of the *M. corales* fungi. An absence of fungal burden was detected from uninfected mouse controls. The experiments were repeated in duplicate, and all mice were sacrificed approximately at day 10 p.i. or at day 2, 5, or 10 p.i. for GI dissection and histopathology.

**Interactions with immune cells.** THP-1 cells were purchased from the American Type Culture Collection (ATCC). Cells were maintained at 37°C in a 5% CO2 atmosphere. THP-1 cells were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum. For *Mucor* infection experiments, cells were plated at 1 × 10^5^ cells in 100 μl of medium and infected with spores at a multiplicity of infection of 1. Supernatants were collected for cytokine analysis at 24 h. Forty-one cytokines were measured using the Milliplex MAP (multi-analyte panel) human cytokine/chemokine magnetic bead panel (Millipore) by the Duke Immunology Reconstitution & Biomarker Analysis Shared Resource. IL-8 and MIP-1α enzyme-linked immunosorbent assays (R&D Systems) to verify changes seen with Luminex were conducted on supernatants according to the manufacturer’s instructions. Three other pathogenic fungus strains, RA99-880 (R. delemar), AF293 (A. fumigatus), and SC5314 (C. albicans), were included in addition to the R7B (*M. circinelloides* f. *l. tusianicus*), Mucho (*M. circinelloides* f. *circinelloides*), and 1006PhL (*M. circinelloides* f. *circinelloides*) strains.

The murine animal studies were conducted at the Duke University Medical Center in full compliance with all of the guidelines of the Duke University Medical Center Institutional Animal Care and Use Committee (IACUC) and in full compliance with the United States Animal Welfare Act (Public Law 98–198). The Duke University Medical Center IACUC approved all of the vertebrate animal studies under protocol number A061-12-03. The studies 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).

**SUPPLEMENTAL MATERIAL** Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01390-14/-/DCSupplemental.

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**REFERENCES**


