Fungi and Fluoxetine: Effect of Fluoxetine on the Human Mycobiome

Original Research

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ABSTRACT

Background: The gut mycobiome and the role it plays in the gut-brain axis is an area of study in its infancy. Little is understood on how drugs might affect the mycobiome, and therefore the host. The Australian Bureau of Statistics reported that in 2018 around 13% of Australians suffer from anxiety, and around 10% from depression. The efficacy of treatment for these disorders is unpredictable, with just 30–40% of patients achieving remission. The gut microbiome may have a significant role in how treatment drugs are metabolised and the effect on neurotransmitters within the host. Interplay between the mycobiome and the microbiome may also affect treatment of these conditions.

Aim: The aims of this project are to provide a Minimum Inhibitory Concentration (MIC) of fluoxetine for two model strains of fungi, and to find the MIC of fluoxetine for endogenous fungal strains from human faecal samples.

Method: A serial dilution assay of fluoxetine in a micropore plate was inoculated with *Saccharomyces cerevisiae* and *Candida albicans* and incubated overnight. This assay was repeated using two human faecal sample cultures.

Results: Results demonstrate that fluoxetine inhibits *S. cerevisiae* at ~993 mg/L, and *C. albicans* at ~1100 mg/L. These MICs are greater than the physiological concentrations of fluoxetine, which is between 63 and 270 mg/L.

Conclusion: Endogenous fungal strains are inhibited by greater concentrations of fluoxetine than physiological concentrations found in the human gut with standard dosing.

INTRODUCTION

The human microbiome has been implicated as a vital component in homeostasis for several decades. The human gut microbiome is considered a 'virtual organ' composed of microorganisms and their genomes in the gastrointestinal environment.[1] These microorganisms include bacteria, viruses, archaea, protozoa, fungi, and helminths, which form a complex community that mutually interacts with the host organism to perform metabolic functions, which are unable to be achieved by the host alone.[1–3] The gut microbiome has been shown to have a critical role in the development and maturation of the immune system, protective immune responses, and regulating homeostasis.[2,4] Disturbance of the microbiome has been implicated in diseases such as inflammatory bowel disease, Alzheimer's disease, autism spectrum disorder, and major depressive disorder among others.[2]

The complex system of communication between the gastrointestinal tract, its microbiome, and the brain, has been encompassed by the term 'Microbiome-Gut-Brain Axis.'[5] This is a bidirectional network of neuro-immuno-endocrine

mediators and pathways between the gut and the central nervous system (CNS), the enteric nervous system (ENS), the autonomic nervous system (ANS) and the hypothalamic pituitary adrenal axis (HPAA).[2,5,6] While the pathways between the gut and the CNS may not be well understood, it has been shown that changes in the gut microbiome (dysbiosis) affect the normal function of the CNS and may have a negative impact on mood, and that mood disorders such as depression and anxiety, may potentiate dysbiosis in the gut.[7,8]

Antidepressant drugs such as fluoxetine have been shown to have antimicrobial activity and cause dysbiosis, which has implications in treatment efficacy.[9] Selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine, a class of drug commonly used to treat anxiety and depression, inhibits the neuronal reuptake of 5-hydroxytryptamine (5-HT), resulting in increased levels of serotonin and its prolonged availability at the synapse, which alleviates the symptoms of depression.[10–12] Fluoxetine is metabolised by cytochrome P450 (CYP450) isozymes, but unlike other SSRIs has an active metabolite, norfluoxetine, which is just as potent.[10,13] Fluoxetine has a half-life of 1–3 days, and norfluoxetine a half-life of 7–14 days, due to a large volume of distribution and being highly bound to plasma proteins, meaning high concentrations of both can reach the brain.[10,13]

The effect of antidepressants such as fluoxetine on the intestinal fungi of the gut microbiome is not yet well understood.[9] It is thought that fungal strains in the gut, or the mycobiome, are a small but essential part of the gut microbiome as they can, among other things, synthesise and secrete neurotransmitters similar to the bacterial component of the microbiome.[6] It has been estimated that 0.1% of genes found in faecal samples are fungal in origin.[6,14] However, culturing fungal isolates from faecal samples is difficult, and for some general isolation by culture is impossible.[6] While fungal diversity in the gut is low, variability between individuals, as well as within an individual over time, is high.[15] It has been demonstrated that faecal samples from one individual at two different time points will have a level of variability in their mycobiome much higher than the level of variability found in their bacteriome.[6,15,16] Establishing a core mycobiome has been difficult because of this time-point variability in the same individual, as well as the mycobiome variability between individuals.[15,16]

The most prevalent fungal species found so far are yeasts, as demonstrated in the gut mycobiome of the Human Microbiome Project (HMP) healthy cohort,[15] with *Saccharomyces cerevisiae*, *Malassezia restricta*, and *Candida albicans* being present in 96.8%, 88.3%, and 80.8% of samples respectively. Other yeast species from the genera *Candida*, *Galactomyces* and *Debaryomyces* have been reported, along with filamentous fungal species from the genera *Cladosporium*, *Aspergillus* and *Penicillium*.[6,15,16] While some of these fungal species are found as natural residents of the human body, most fungal species are thought to become part of the mycobiome from environmental origins and diet.[15,16]

It has been shown through animal studies that mycobiome dysbiosis may be involved in the development and progression of inflammatory diseases such as colitis and allergic airway disease, where it was found that prolonged treatment with antifungal drugs in wild-type mice increased the severity of these experimentally induced diseases.[4] Mycobiome dysbiosis linked to immune response has also been observed in patients with inflammatory bowel disease (IBD), which is persistent intestinal inflammation due to an inappropriate immune reaction.[6,14] There appears to be little known about the communication and relationships that must exist between the components of the gut microbiome.[6] There also appears to be little known about how different drugs affect the gut mycobiome. SSRIs seem to have some antifungal activity, however the species and strains tested were not comparable to those more commonly found in the gut mycobiome.[9,15]

An interesting case study characterising the bacteriome, mycobiome and virome of a teenager suffering from graft-versus-host disease after a stem cell transplant adds weight to these findings. This patient received a course of faecal microbiota transplants (FMT) in an attempt to replace the dysbiotic gut microbiota with microbes from a healthy donor.[17] Faecal samples from the patient after the FMT treatments demonstrated an expansion of multiple fungal strains, but a decrease in fungal diversity.[17] The results of this study also showed an inverse relationship between the mycobiome diversity and the bacteriome diversity after FMT treatments.[17] This shows that there is a link between the bacteria and fungi found within the gut microbiome, and changes to the balance held between them can have long lasting effects.

The purpose of this study is to establish how antidepressant medications might affect the fungi present in the human gut microbiome. This will be done by quantifying the minimum inhibitory concentration (MIC) of fluoxetine for two model strains of fungi, by attempting to isolate endogenous fungal strains from human faecal samples, and by finding the MIC of fluoxetine for these fungal strains.

METHOD

Frozen faecal samples were provided from a previous study, where permission was given to use the samples for future projects. Ethics approval number for this project is HE18-017. Three 200 mL aliquots each of potato dextrose agar (PDA) and 1x yeast extract peptone dextrose (YPD) solid media, and three 200 mL aliquots of 2xYPD liquid media were prepared and sterilised. YPD is a general medium used for the cultivation of fungal species.[18] PDA solid media was made using 7.8 g of potato dextrose agar (Oxoid) and 200 mL of reverse osmosis (RO) water. 1xYPD solid media was made using 4 g of peptone (Oxoid), 2 g of yeast extract (Oxoid), 4 g/L of glucose (Oxoid), and 4 g of agar (Oxoid), all accurately weighed and added to 200 mL of RO water. 2xYPD liquid media was made using 8 g peptone, 4 g yeast extract, and 8 g/L of glucose, all accurately weighed and added to 200 mL of RO water. The media was autoclaved to sterilise, then stored for later use.

Overnight cultures of *S. cerevisiae* and *C. albicans* (University of New England Culture Collection) were prepared. A 1:100 dilution for each model strain was prepared to use as inoculums using 60 µL of overnight culture in a 6 mL aliquot of 2xYPD liquid media added to a sterile tube.

2xYPD liquid media with antibiotics was prepared at a dilution of 100 µg/mL for ampicillin (Sigma-Aldrich), and 50 µg/mL for chloramphenicol (Sigma-Aldrich), to inhibit bacterial growth and select for fungal growth. Of the twelve 5 mL 2xYPD aliquots prepared, four were treated with ampicillin, four with chloramphenicol, and four untreated as positive controls. Faecal samples 109FS5 and 101FS5 were defrosted and vortexed. Approximately 200 µL of each sample was added separately to each different treated media and incubated overnight.

PDA and 1xYPD solid media were supplemented with ampicillin, chloramphenicol, or untreated, and poured into sterile plates. The faecal sample overnight cultures were diluted 1:100, 10 µL into 1 mL of 2xYPD liquid media. 100 µL of each diluted sample was spread onto two PDA plates and two 1xYPD plates treated with respect to the samples (ampicillin, chloramphenicol, or untreated) and incubated for 48 hours.

A 50 mL aliquot of 2x fluoxetine (Sigma-Aldrich) stock solution was prepared. 100 mg of fluoxetine was accurately weighed and added to a sterile tube with 50 mL of sterile RO water.

Two 96-well microplates were prepared, one for the model species and one for the faecal samples. A 1:2 serial dilution assay was prepared over five series with the starting concentration of 2000 mg/L, and a final concentration of 62.5 mg/L. Half the plate was inoculated with 100 µL of *S. cerevisiae* and the other half with *C. albicans*. 100 µL of sterile 2xYPD media was added to each well in Row H as a negative control. The plate was covered with protective film and incubated overnight at 35 °C in the plate reader (BMG Labtech Spectrostar Nano) with the optical density (OD), and therefore fungal growth, detected every 2 hours during incubation.

A 1:2 serial dilution assay was prepared over seven series with the starting concentration of 2000 mg/L, and a final concentration of 15.6 mg/L, for the faecal samples. Half the plate was designated to both faecal samples treated with ampicillin, and half the plate was designated to both faecal samples treated with chloramphenicol. Each sample was designated 3 columns. The faecal sample overnight cultures were diluted 1:100 in 2xYPD sterile media treated with ampicillin or chloramphenicol at a dilution of 1:1000 with respect to the samples. The plate was covered with protective film and incubated overnight at 37 °C in the plate reader with the optical density (OD) detected every 2 hours.

Data was collected from the plate reader and saved in Microsoft® Excel. Statistical analysis was performed using MS Excel, and GraphPad Prism software using the Lambert and Pearson MIC template.[19]

RESULTS

The data collected for the model fungal strains from the final cycle at 21 hours was used to determine the mean optical density and standard deviation in Microsoft® Excel. This was compared to the log concentration of fluoxetine using GraphPad and a curve fit in accordance with the MIC template used.[19] The results showed that model strain *S. cerevisiae* has a MIC for fluoxetine at approximately 993 mg/L (Figure 1), and model strain *C. albicans* is seen to have a MIC for fluoxetine at approximately 1100 mg/L (Figure 2).

FIGURE 1 - The log concentration of fluoxetine and the mean OD of *S. cerevisiae* after incubating for 21 hours. The MIC of fluoxetine for *S. cerevisiae* is ~993 mg/L

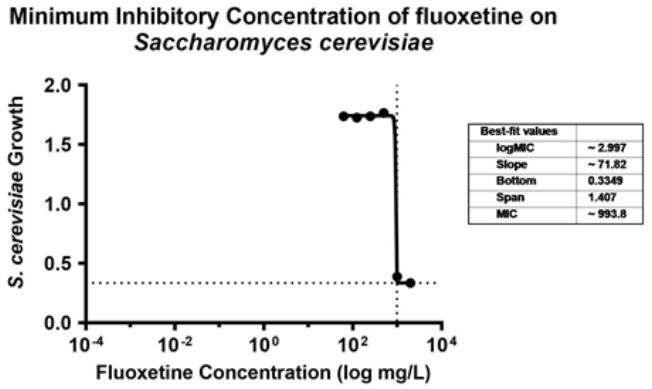
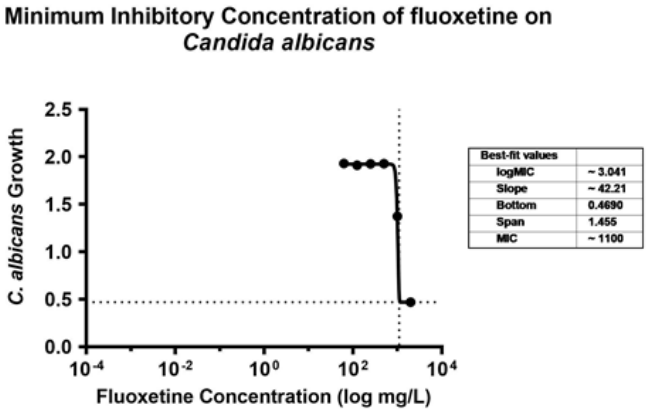


FIGURE 2 - The log concentration of Fluoxetine and the mean OD of *C. albicans* after incubating for 21 hours. The MIC of fluoxetine for *C. albicans* is ~1100 mg/L.



According to the literature, the MIC of fluoxetine for *C. albicans* species has been reported to be within a range of 156–625 mg/L and within a range of 256–512 mg/L.[20,21] Despite the MIC range in the literature being lower than the results of this project, there is consistency in the fact that *C. albicans* has a MIC for fluoxetine beyond the expected physiological concentrations. Unfortunately, there is a lack of literature showing the MIC of fluoxetine for *S. cerevisiae*, so no comparison was able to be made for this fungal strain. The results, however, also show a MIC of fluoxetine for *S. cerevisiae* to be greater than the expected physiological concentrations.

The results for the model strains show a distinct curve. The faecal samples, however, contain a multitude of unknown microbes, and therefore the curves are not as uniform. This is likely because the resulting colonies contain different species of fungi within a fungal community.

While some of the faecal samples in the fluoxetine assay yielded no significant growth, others produced a MIC curve similar to those seen in Figure 1 and Figure 2. As seen in Table 1, sample 101FS5 treated with ampicillin did not show significant amounts of growth. Sample 109FS5 treated with ampicillin showed some growth and a MIC for fluoxetine was able to be determined. Both samples 101FS5 and 109FS5 treated with chloramphenicol showed growth and a MIC for fluoxetine was determined for both.

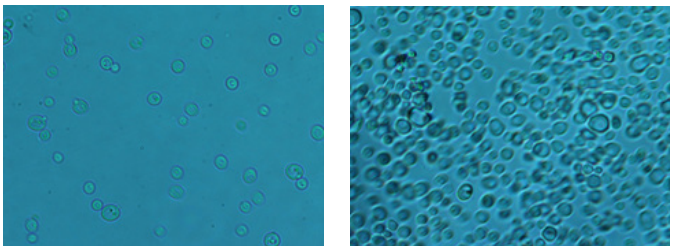
TABLE 1 - The MIC of fluoxetine for each faecal sample (101FS5, 109FS5) treated with two antibiotics (ampicillin and chloramphenicol).

FAECAL SAMPLE + ANTIBIOTIC	MIC OF FLUOXETINE (mg/L)
101FS5 + AMPICILLIN	None Determined
109FS5 + AMPICILLIN	~263.7
101FS5 + CHLORAMPHENICOL	1316
109FS5 + CHLORAMPHENICOL	584.3

Following the incubation period in the plate reader, the faecal samples treated with chloramphenicol showed more of an indication of microbial growth. To ascertain which class of microbes had been isolated, the contents of some of the wells were plated onto chloramphenicol-treated YPD agar plates and incubated for four days at 28 °C. Uniform colonies that seemed to be a yeast were observed on the agar plates for both 101FS5 and 109FS5, and microscope images taken (Figure 3). These will be stored in the University of New England (UNE) culture collection for possible future identification.

A second approach was used to isolate enteric fungi from the faecal sample overnight cultures on 1xYPD and PDA plates. For each overnight culture, two each of 1xYPD and PDA plates, treated with an antibiotic or left untreated, respective to the sample, were prepared and incubated. After 24 hours, colonies were observed that were likely to be bacterial. After 48 hours there was still no growth that could be described as fungal.

FIGURE 3 - Yeast colonies isolated from faecal samples 101FS5 (left) and 109FS5 (right). Microscopy images courtesy of Dr Gal Winter-Ziv.



The observed bacterial growth was most prevalent on the untreated plates for both faecal samples. The plates treated with ampicillin had more colonies present than those treated with chloramphenicol (Table 2). This suggests there could be an inverse relationship between the presence of bacterial growth on these spread plates, and the presence of fungal growth in the 96-well microplate.

TABLE 2 - The presence of bacterial colonies (+) or absence of growth (0) after incubation at 28 °C for 48 hours. No fungal growth was observed.

SAMPLE	CONTROL	AMPICILLIN	CHLORAMPHENICOL
109FS5 1XYPD	+ +	+ +	0 0
109FS5 PDA	+ +	+ +	0 0
101FS5 1XYPD	+ +	0 0	0 0
101FS5 PDA	+ +	0 0	0 0

DISCUSSION

The results of this project show that the fluoxetine MIC of the model fungal strains *S. cerevisiae* and *C. albicans* are both higher than the physiological concentrations of fluoxetine found in the large intestine with standard dosing. While the data does not support the hypothesis that fluoxetine has a direct inhibitory effect on the mycobiome, there is some evidence that there is an indirect inhibition.

The model strains show the effect of fluoxetine on a single fungal strain. The faecal samples, however, contain a multitude of unknown microbes. It was attempted to narrow the isolates to fungal species by adding antibiotics to the overnight cultures and to the plates. The resulting colonies most likely contained different species of fungi. How the faecal sample cultures performed in the fluoxetine assay is therefore more indicative of a fungal community, rather than the effect on a single strain.

The initial dose of fluoxetine for most indications is 20 mg daily, and titrated up by 10 mg every two weeks until the drug is effective, with a maximum dose of 60 mg daily.[22,23] The physiological concentrations of fluoxetine in the large intestine following a 20–40 mg dose are between 63–270 mg/L.[9] However, the MIC of fluoxetine for the model fungi, and the faecal sample cultures, is shown to be much higher than the physiological concentration range.

The literature shows that the MIC of fluoxetine on various strains of bacteria is much lower than that reported for fungal strains, and well within the physiological concentration range. As part of a broader study, the MIC of fluoxetine for 13 bacterial species was determined using a micro-well dilution assay, along with several other SSRIs.[24] The fluoxetine MIC range for these species was within the range of 4–128 mg/L.[24]

The antimicrobial effect of fluoxetine has been demonstrated on two resident bacterial strains in the human gut.[25] Fluoxetine was shown to completely inhibit growth of *Escherichia coli*, and to inhibit growth of *Lactobacillus rhamnosus* at concentrations above 400 mg/L.[25] Important to note is that *L. rhamnosus* is a probiotic that has been shown to improve social behaviours and stress-induced anxiety in male mice exposed to social defeat.[26,27] Another study showed a significant decrease in *Lactobacilli* species following fluoxetine treatment in male mice, also citing the regulation of body weight as a function of *Lactobacilli*.[28] Antimicrobial effects were also found using the SSRI citalopram, among other different classes of antidepressant medications, on commensal gut microbes including *C. albicans*.[29]

Recent studies have also uncovered a synergistic effect between SSRIs and antibiotics.[30] This synergistic effect has also been found to occur between SSRIs and antifungal agents against drug-resistant *Candida* species.[20,21,31] Using the antifungal agent fluconazole in combination with fluoxetine, the MIC of both drugs was decreased by at least half, and in some cases up to 97%, depending on the *Candida* strains tested with antifungal agents to treat drug-resistant fungal infections.[20,21,31]

Aside from drugs working synergistically to prevent bacterial and fungal growth, there is the effect that bacteria and fungi have on each other within the microbial communities of the gut. The faecal sample isolate results of this study suggest that the presence of bacterial growth may inhibit the growth of enteric fungi, and vice versa. Changes in the relationship between bacteria and fungi within the gut microbiome following antibacterial treatment or antifungal treatment has been reported in the literature. Broad spectrum antibiotics have been shown to decrease anaerobic bacteria within the gut, and have in turn promoted the growth of opportunistic pathogenic fungi such as *C. albicans*.[32] Another recent study revealed that three months after being administered a 6-day course of antibiotics, the bacteriome had mostly recovered, but the mycobiome was now in competition with the bacteriome rather than the mutualistic relationship seen in the baseline sample.[33] Additionally, antibiotic treatment resulted in an

increase in fungal species diversity, although temporarily. Samples taken early post antibiotic treatment showed that less commonly found fungal species had increased in abundance, while fungi commonly associated with the mycobiome had decreased in abundance.[33] However, this niche of less common fungal species had not managed to successfully colonise the gut microbiome three months post treatment.[33]

This study was limited to two model yeast species and two faecal samples. Further research to obtain the MIC of fluoxetine on other common enteric fungal species could be a future direction. Similarly, the MIC of other SSRIs and other classes of antidepressant medication on these fungal strains could also be a valuable future direction for research. Isolating and identifying enteric fungi from faecal samples, as well as observing enteric microbial communities treated with antidepressant medication could be another direction for future research.

Fluoxetine has been shown to have an inhibitory effect on fungal growth, but not at physiological concentrations found in the gut. However, the literature demonstrates that the MIC for fluoxetine on bacterial strains is well within these physiological concentrations. In conclusion, the effect fluoxetine may have on the gut mycobiome could be secondary to, and dependent on the bacterial component of the gut microbiome. This is supported by the literature, but further studies are required. There is scope for continuing research into how fungi and bacteria behave within microbial communities treated with fluoxetine, and how other enteric fungal species behave when treated with antidepressant medications.

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