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Colonization with the commensal fungus *Candida albicans* perturbs the gut-brain axis through dysregulation of endocannabinoid signaling

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Abstract

Anxiety disorders are the most prevalent mental health disorder worldwide, with a lifetime prevalence of 5–7% of the human population. Although the etiology of anxiety disorders is incompletely understood, one aspect of host health that affects anxiety disorders is the gut-brain axis. Adolescence is a key developmental window in which stress and anxiety disorders are a major health concern. We used adolescent female mice in a gastrointestinal (GI) colonization model to demonstrate that the commensal fungus *Candida albicans* affects host health via the gut-brain axis. In mice, bacterial members of the gut microbiota can influence the host gut-brain axis, affecting anxiety-like behavior and the hypothalamus-pituitary-adrenal (HPA) axis which produces the stress hormone corticosterone (CORT). Here we showed that mice colonized with *C. albicans* demonstrated increased anxiety-like behavior and increased basal production of CORT as well as dysregulation of CORT production following acute stress. The HPA axis and anxiety-like behavior are negatively regulated by the endocannabinoid N-arachidonylethanolamide (AEA). We demonstrated that *C. albicans*-colonized mice exhibited changes in the endocannabinoidome. Further, increasing AEA levels using the well-characterized fatty acid amide hydrolase (FAAH) inhibitor URB597 was sufficient to reverse both neuroendocrine phenotypes in *C. albicans*-colonized mice. Thus, a commensal fungus that is a common colonizer of humans had widespread effects on the physiology of its host. To our knowledge, this is the first report of microbial

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5-Conflicts of Interest

MH is a member of the advisory board for Sophren Therapeutics and JM is a member of the advisory board for SAGE Therapeutics.

Declaration of interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

manipulation of the endocannabinoid (eCB) system that resulted in neuroendocrine changes contributing to anxiety-like behavior.

Graphical abstract

Keywords

Endocannabinoid; microbiota; *Candida albicans*; anxiety; corticosterone

1. Introduction

Anxiety disorders are the most prevalent mental health disorder worldwide, with a lifetime prevalence of 5–7% of the human population (Baxter et al., 2013). The etiology of anxiety disorders is incompletely understood, although many different genetic and environmental factors have been identified (Leonardo and Hen, 2008). The gut-brain axis, the bi-directional communication between the gut and the brain, has been shown to affect anxiety disorders (Luna and Foster, 2015).

One component of the gut-brain axis thought to play a role in anxiety disorders is the hypothalamus-pituitary-adrenal (HPA) axis. The endocrine output of the HPA axis, the stress hormone corticosterone (CORT), is a broad regulator of multiple physiological systems. Under basal conditions, CORT release is regulated by the circadian rhythm and helps to maintain homeostasis (Son et al., 2018). CORT is also a key part of the psychological stress response and a rapid increase in circulating CORT is essential for mounting an effective response to stress (Sapolsky et al., 2000). Dysregulation of the HPA axis is correlated with mental health disorders including anxiety and depression (Kallen et al., 2008). Multiple clinical studies found HPA axis hyperactivity in patients with depression (Stetler and Miller,

2011). Mice bred for high anxiety behavior display increased immobility in the Forced Swim Test and a blunted CORT response to stress, further supporting a role for dysregulation of the HPA axis in development of anxiety and depression-like behaviors (Sotnikov et al., 2014).

The gut microbiota, the diverse assemblage of microbes that colonizes the GI tract, has been shown to affect virtually every aspect of human health, including the gut-brain axis. Researchers using germ-free mouse models demonstrated that the gut microbiota plays an important role in the development and regulation of the HPA axis (Sudo et al., 2004). Studies have also shown that specific probiotic bacteria can affect the gut-brain axis, as treatment with organisms of the *Bifidobacterium* and *Lactobacillus* genera decrease anxiety-like behavior and normalize HPA axis function in mice (Messaoudi et al., 2011). In mice the HPA axis of adolescent but not adult mice is acutely sensitive to changes in the bacterial microbiota (Sudo et al., 2004). Additionally, studies of human populations show that the development of anxiety disorders during adolescence is a significant concern (Siegel and Dickstein, 2012) and that anxiety disorders are significantly more prevalent in the female population (McLean et al., 2011). In the research that follows, we used adolescent female mice in a gastrointestinal (GI) colonization model to investigate the commensal fungus *Candida albicans* and its interaction with the gut-brain axis.

Candida albicans colonizes the GI tract of ~60% of the human population (Raimondi et al., 2019) and has been shown to colonize the mouth, stomach, small and large intestine (Cannon and Chaffin, 2016; Cohen et al., 1969; Zwolska-Wciso et al., 1998). Although a major colonizer of humans, its role as a commensal microbe is largely uncharacterized. GI *C. albicans* colonization induces local and systemic immune changes which are protective against systemic *C. albicans* infection and disease (Shao et al., 2019), and against infection with bacterial pathogens such as *Clostridioides difficile* (Markey et al., 2018) and *Staphylococcus aureus* (Shao et al., 2019). In the context of recovery after antibiotic treatment, *C. albicans* colonization can alter the bacterial microbiota and is correlated with increased abundance of *Enterococcus faecalis* and decreased abundance of *Lactobacillus* spp. (Mason et al., 2012a, 2012b).

The mammalian endocannabinoid (eCB) system regulates both the HPA axis and anxiety-like behavior. It consists of two neuroactive lipids, N-arachidonylethanolamide (AEA) and 2-arachidonoylglycerol (2-AG) and their receptors CB1 (distributed throughout the nervous system) and CB2 (present primarily on immune cells) (Devane et al., 1992; Mechoulam et al., 1995). AEA and 2-AG are produced in response to activation in post-synaptic neurons, and act at CB1 on pre-synaptic neurons to limit neurotransmitter release. AEA and 2-AG signaling through CB1 has been shown to regulate the release of the classical neurotransmitters glutamate and gamma-aminobutyric acid as well as other neuromodulators including serotonin, norepinephrine, dopamine and acetylcholine (Schlicker and Kathmann, 2001). AEA and 2-AG levels are regulated by substrate availability and the activity of their synthetic and degradative enzymes (Placzek et al., 2008). In rodents, manipulation of CB1 signaling affects basal and stress-induced activation of the HPA axis as well as performance in tests for anxiety-like behavior (Barna et al., 2004; Hill et al., 2011).

In the work that follows, we characterized the neuroendocrine effect of colonization with the commensal fungus *C. albicans* on the murine host and investigated the molecular mechanism by which gastrointestinal colonization with *C. albicans* resulted in significant changes in host behavior and HPA axis regulation.

2. Results

2.1 A single oral inoculation with *Candida albicans* is sufficient to establish gastrointestinal colonization without inflammatory disease

The murine colonization model is described in Fig. 1A. Co-housing of up to 23 5-week-old mice in a large cage was used to standardize gut microbiota by coprophagy. The mice were acclimated and handled daily while co-housed (Fig. 1A). They were then either inoculated with a single dose of *C. albicans* or mock-inoculated with phosphate-buffered saline (PBS) and transferred to small cages in groups of 3–4. All mice were tested for colonization and *C. albicans* was measurable in fecal pellets of all inoculated mice after 24h and 3 days after inoculation (Fig. 1B) and was detected throughout the GI tract after sacrifice (Fig. S1). We assessed behavior two days post-inoculation and sacrificed mice three days after inoculation (Fig. 1A). *C. albicans*-colonized mice did not exhibit symptoms of illness or lose >5% bodyweight (Fig. S2). A multiplex ELISA was used to measure IL-1 β , IFN- γ , IL-10 and IL-6 in serum from mock-inoculated and *C. albicans*-colonized mice (Fig. 1C). Cytokine measurements were normally distributed. All cytokines detected were well below the levels typically observed in mice with *C. albicans* disease (Tuite et al., 2005) and there were no significant differences between mock-colonized and *C. albicans*-colonized mice (Fig. 1C, mixed effects analysis followed by Sidak's multiple comparisons test) indicating that the systemic inflammatory immune response was not activated by *C. albicans* GI colonization. This model of the initial stages of colonization with *C. albicans* was not associated with disease, indicating that our model represents the first stages of true commensal GI colonization.

Previous work demonstrated that short-term colonization (7 days) with *C. albicans* in the absence of antibiotic treatment does not significantly alter the bacterial microbiota although there are some small taxonomic shifts (Erb Downward et al., 2013). To determine whether the bacterial microbiota was significantly changed in our 3 day model of *C. albicans* colonization, we analyzed the bacterial microbiota of the cecum of mice sacrificed three days post-inoculation using 16S rRNA DNA sequencing and the QIIME analysis pipeline (Caporaso et al., 2010). The composition and diversity of bacterial taxa were not significantly altered by the introduction of *C. albicans* (Fig. 1D–F), consistent with previous results (Erb Downward et al., 2013). Mann-Whitney U test showed that no taxa were significantly different in relative abundance between the microbiota of mock-colonized and *C. albicans*-colonized mice (Fig. 1D). The overall diversity of the microbiota was also not significantly different (Fig. 1E). We analyzed beta diversity and summarized the results using principal coordinates analysis (PCoA) (Fig. 1F). PERMANOVA analysis of the PCoA found that there was not significant separation of populations based on *C. albicans* colonization (pseudo-F statistic=1.7, $p=0.12$, 999 permutations). To summarize, mice

inoculated with a single dose of *C. albicans* were stably colonized with neither disruption of the bacterial microbiota nor invasive disease.

2.2 *C. albicans* colonization increases anxiety-like behavior in the EPM

To assess the effect of GI *C. albicans* colonization on host emotional behavior, standard tests for anxiety-like and stress-coping behavior were used. Two days post-inoculation (Fig. 1A), all mice were subjected to a single behavioral test, either the elevated plus maze (EPM) test for anxiety-like behavior (Walf and Frye, 2007) or the forced swim test (FST) for stress-coping behavior (Hascoët and Bourin, 2009). Mice were allowed to explore the EPM for a 5-minute trial which was scored by a blinded observer for time spent and entries into the closed and open arms. Anxiety-like behavior is detected as avoidance of the open arms, expressed as percentage arm time (time spent in specified arms divided by the total arm time). Time in seconds in each zone is shown in Fig. S3.

C. albicans-colonized mice spent significantly less time in and made fewer entries into the open arms of the EPM compared to the mock-colonized mice (Fig. 2A–B, Mann-Whitney test, $p < 0.05$). There was a significant increase in the percentage of time spent in the closed arms in the *C. albicans*-colonized mice but no difference in the number of entries into the closed arms (Fig. 2C–D). There was no effect of *C. albicans* colonization on the duration of time spent in the center or the total number of entries into all arms (Fig. 2E–F), indicating that *C. albicans* colonization did not alter overall activity but specifically increased anxiety-like behavior in this test.

The forced swim test (FST) for stress-coping behavior was used to examine the effect of *C. albicans* colonization on a second aspect of emotional behavior, again two days post-inoculation. Mice were placed in a cylinder containing 22°C tap water for a six-minute trial which was video-recorded and later scored by a blinded observer for time spent immobile. A passive-coping response in this assay was defined as the amount of time spent floating immobile rather than the active coping response defined as swimming or actively struggling. There was no difference in the time spent immobile between the *C. albicans*-colonized mice and the mock-colonized mice (Fig. 2G), indicating that *C. albicans* colonization did not affect stress-coping behavior in this test.

2.3 *C. albicans* colonization alters basal production of the stress hormone CORT

The underlying biology of anxiety-like behavior is multifaceted (Nutt et al., 2002). One neuroendocrine pathway that has been shown to be dysregulated in human patients with anxiety is the hypothalamus-pituitary-adrenal (HPA) axis (Kallen et al., 2008). To determine whether *C. albicans* colonization affected the HPA axis, we sacrificed mice three days post-inoculation and measured circulating serum CORT under basal conditions and in response to acute psychological stress.

C. albicans-colonized and mock-colonized mice were sacrificed unstressed and CORT measured in trunk blood. *C. albicans*-colonized mice had significantly higher unstressed basal CORT (Fig. 3A Welch's t-test, $p = 0.0012$). When the effect of circadian rhythm on serum CORT was examined, basal CORT in both groups was shown to increase over time throughout the afternoon, as expected (Chung et al., 2017) (Fig. 3B). Basal CORT in *C.*

albicans-colonized mice, however, began to rise sooner than in mock-colonized mice and thus was significantly higher in *C. albicans*-colonized mice sacrificed two-to-five hours prior to lights out, consistent with a circadian advance of CORT production (Fig. 3B, ANOVA followed by Sidak's multiple comparison test, $p < 0.05$). These results indicate that *C. albicans* colonization dysregulates circadian control of the HPA axis. Further studies of basal CORT were conducted only with mice sacrificed between 14:00–17:00.

To determine whether the changes to basal CORT regulation affected the stress-responsive function of the HPA axis, mice were subjected to 30 minutes of restraint stress and then sacrificed immediately or after 30 minutes or 60 minutes of recovery. *C. albicans*-colonized and mock-colonized mice had comparable peak CORT immediately after stress and after 30 minutes of recovery from stress (Fig. 3C). After 60 minutes of recovery from stress, the mock-colonized mice had CORT comparable to the baseline CORT shown in Fig. 3A and CORT in *C. albicans*-colonized mice returned to an elevated baseline (Fig. 3C, Student's t-test, $p = 0.042$).

Activation of the HPA axis begins in the brain, through excitation of the CRH-producing neurons of the paraventricular nucleus (PVN) of the hypothalamus. We used immunofluorescence to quantify expression of c-FOS protein in the PVN as a marker for neuronal activation. Nuclear c-FOS protein was not detected in the PVN of unstressed mock-colonized or *C. albicans*-colonized mice (Fig. 3D, left). In mice subjected to 30 minutes of stress and 30 minutes of recovery, nuclear c-FOS protein was detected in both mock-colonized and *C. albicans*-colonized mice (Fig. 3D, right). The ratio of c-FOS positive to total nuclei in the PVN of stressed samples was comparable in the mock-colonized and *C. albicans*-colonized mice (Fig. 3E). We also used qRT-PCR to measure expression of *Fos* in the hypothalamus of mice sacrificed without stress or immediately after 30 minutes of stress. *Fos* was detectable in the hypothalamus of unstressed mice and was not significantly different between mock-colonized and *C. albicans*-colonized mice (Fig. S4). After stress, *Fos* expression increased significantly in both mock-colonized and *C. albicans*-colonized mice and was comparable between these groups (Fig. S4). Thus, the PVN remains stress-responsive in the *C. albicans*-colonized mice and is not sufficiently activated at baseline by *C. albicans* colonization to be detected with these methods.

2.4 Neuroendocrine changes observed in *C. albicans*-colonized mice are mediated by the endocannabinoid system

The neuroactive lipid endocannabinoid (eCB) AEA, through its interactions with the CB1 receptor, is a key regulator of anxiety-like behavior and both stress-induced and basal CORT production (Bluett et al., 2014; Hermanson et al., 2013; Hill et al., 2013). The circadian advance of basal CORT and increased anxiety-like behavior observed in the *C. albicans*-colonized mice resemble the phenotype of the CB1 knock-out mouse (Barna et al., 2004), suggesting a deficit of eCB-CB1 signaling as a result of *C. albicans* colonization. Therefore, we hypothesized that one way that *C. albicans* colonization interacted with the gut-brain axis was via the endocannabinoid system.

To test this hypothesis, we used the well-characterized drug URB597 to increase AEA levels and therefore amplify AEA-CB1 signaling through inhibition of the AEA degradative

enzyme FAAH (Fegley et al., 2005). Mice were given an intraperitoneal (IP) injection of vehicle or URB597 (1mg/kg) 4 hours prior to sacrifice under unstressed conditions, to determine whether elevating AEA levels affected basal CORT. Using a 2-way ANOVA we determined that there was a significant interaction between the drug and colonization conditions ($F(1, 64)=12.6$, $p=0.0007$). We used Sidak's multiple comparisons post-hoc test and found that *C. albicans*-colonized mice given vehicle injection had significantly higher basal CORT than mock-colonized mice given vehicle as expected (Fig. 4A, adjusted $p=0.0023$). *C. albicans*-colonized mice treated with URB597 showed significantly decreased basal CORT compared to *C. albicans*-colonized mice given vehicle (Fig. 4A, adjusted $p=0.0046$). This result is consistent with an AEA deficit in the *C. albicans*-colonized mice underlying the elevated basal CORT.

URB597 was administered 4–6 hours prior to testing in the EPM (0.1–0.15mg/kg) to determine whether increasing AEA-CB1 signaling was sufficient to alleviate anxiety-like behavior in the *C. albicans*-colonized mice. Due to variability in activity level between cohorts of IP-injected mice, data are shown as the percentage of time spent in the open arm normalized to the experimental mean of the mock-colonized vehicle group. We used a 2-way ANOVA to determine the effect of URB597 and found that both colonization with *C. albicans* and treatment with URB597 had a significant effect on relative open arm time (Fig. 4B, $F(1, 50)=7.86$, $p=0.0072$ and $F(1, 50)=9.88$, $p=0.0028$, respectively). There was no significant interaction between these factors. URB597 significantly increased open arm time in *C. albicans*-colonized mice (post-hoc t-test corrected for multiple comparisons, $p=0.00097$) and open arm time was comparable between URB597-treated PBS and *C. albicans*-colonized mice indicating that increasing AEA-CB1 signaling was sufficient to normalize anxiety-like behavior. There was no effect of URB597 treatment on overall activity as measured by total arm entries (Fig. 4C). Together, these results demonstrate that increasing AEA-CB1 signaling with the FAAH inhibitor URB597 reversed the effect of *C. albicans* colonization on basal CORT production and anxiety-like behavior, supporting the model that the neuroendocrine phenotypes observed in *C. albicans*-colonized mice result from decreased AEA-CB1 signaling.

2.5 Relationship between basal CORT and brain AEA levels in *C. albicans*-colonized mice

To test the hypothesis that AEA levels were reduced in colonized mice, AEA levels in the forebrain were measured. Mice were sacrificed on day 3 post-inoculation and forebrain samples analyzed by mass spectrometry. We did not observe a significant difference between AEA in the forebrains of *C. albicans*-colonized mice compared to mock-colonized mice (Fig. 4D, Student's t-test, $p=0.30$). However, a trend towards a negative correlation between basal serum CORT and AEA in the *C. albicans*-colonized mice (Fig. 4F, $R^2=0.44$, F-test, $p=0.0509$) but not in the mock-colonized mice (Fig. 4E, $R^2=0.119$, F-test, $p=0.45$) was observed. We used k-means clustering to analyze basal CORT in the mock-colonized and *C. albicans*-colonized mice and found that the *C. albicans*-colonized mice separated into a higher CORT cluster and a lower CORT cluster that included the majority of the mock-colonized mice (Fig. S5A). AEA levels were significantly lower in *C. albicans*-colonized mice that belonged to the higher CORT cluster than the lower CORT cluster (Fig. S5B). Taken together, these results indicate that *C. albicans* colonization had variable effects on

bulk AEA levels in the mouse forebrain and reduced forebrain AEA was observed in colonized mice with greater dysregulation of the HPA axis.

A second canonical eCB, 2-arachidonoylglycerol (2-AG), was also measured in the forebrain of mice. There was no difference between mock-colonized and *C. albicans*-colonized mice in bulk levels or degree of correlation between 2-AG and serum CORT (Fig. S6).

2.6 *C. albicans* colonization alters the gut endocannabinoidome

We performed untargeted metabolomic analysis of the cecum contents of mice to determine whether *C. albicans* colonization altered the metabolite composition of the GI tract and thereby altered eCB metabolism. Of the 735 compounds measured, 22 were significantly differentially abundant in mock-colonized versus *C. albicans*-colonized mice (Table 1, $p < 0.05$). This result is below the false discovery rate and therefore pathway enrichment analysis was performed to investigate the relevance of the 22 hits. Of the 5 pathways that contained more than one significant compound, two were significantly enriched for *C. albicans*-dependent changes: sterols and eCBs (Fisher's exact test, Holm's correction for multiple comparisons, $p < 0.0001$ and $p = 0.031$ respectively). The enrichment in eCB family compounds indicated that *C. albicans* colonization altered eCB metabolism in the gut.

Two such eCB compounds, N-acylethanolamides (NAEs), linoleoyl and linolenoyl ethanolamide, were significantly increased in the *C. albicans*-colonized mice (Fig. 4G–H, Student's t-test, $p = 0.0037$ and $p = 0.019$). An additional NAE, oleoyl ethanolamide, was increased in the *C. albicans*-colonized mice but not significantly (Fig. 4I, Student's t-test, $p = 0.078$). These compounds are structurally similar to AEA but have different acyl groups. AEA itself is present at low levels in the GI tract and was not detected in any of the samples. The increase in 18-C NAEs implies that *C. albicans* colonization altered NAE metabolism. Feeding studies have shown that dietary enrichment for a specific fatty acid (FA) results in overproduction of the NAE derived from the enriched FA and limits production of alternate NAEs (Sihag and Jones, 2019). Membrane phospholipid acyl chains can also act as substrates for the production of NAEs. We measured a trend towards an increase in the 18-C FA linolenate (Fig. 4J, Student's t-test, $p = 0.05$) as well as a significant increase in the lysophospholipid 1-linoleoyl-glycerophosphocholine (Fig. 4K, Student's t-test, $p = 0.017$) in the GI tract of *C. albicans*-colonized mice. Both of these compounds could be converted into their respective 18-C NAEs and increase production of 18C NAEs linoleoyl and linolenoyl ethanolamide while limiting production of AEA. Thus *C. albicans*-induced changes in precursor compound abundance in the GI tract could contribute to the alterations in AEA levels observed in the *C. albicans*-colonized mice.

2.7 Altered hepatic lipid metabolism in *C. albicans*-colonized mice reflects gut metabolite changes

In addition to the specific enrichment of two lipid subpathways, sterols and eCBs, and the trend towards increased linolenate described above, we observed an overall increase in lipid compounds containing long-chain polyunsaturated fatty acids (PUFAs) in the *C. albicans*-colonized mice (Fig. S7). Lipids are absorbed from the GI tract into the blood and pass

through the liver, a central organ for lipid metabolism which is highly regulated by dietary lipid levels (Xie et al., 2010). We measured liver gene expression to determine whether the changes in lipid levels we quantified in the cecum were sufficient to induce a physiological change in the host.

Mice were sacrificed after three days of *C. albicans* colonization and qRT-PCR was used to measure the expression of lipid-responsive genes in the liver (primer sequences in Table S1). Decreased expression of the mRNA (*Scd1*) encoding the enzyme stearoyl-coA desaturase (SCD1) was detected in the *C. albicans*-colonized mice compared to the mock-colonized mice (Fig. 5A, Mann-Whitney U-test, $p=0.0086$), consistent with a transcriptional response to increased PUFA-containing lipids from the GI tract (Lee et al., 1998). Gene expression of regulators of lipogenesis, *Fads1* (acyl-CoA 8–3 desaturase) and *Fads2* (acyl-CoA 6 desaturase), the transcription factor sterol regulatory element-binding protein 1 (SREBP1-c) encoded by *Srebp1* and fatty acid synthase (*Fasn*) (Oosterveer et al., 2009), were not significantly changed by *C. albicans* colonization (Fig. 5B), implying that the increased lipid abundance measured in the cecum was not a result of host lipogenesis but rather that the increased abundance of PUFAs resulted from *C. albicans* colonization and was sufficient to induce hepatic transcriptional changes.

Also, increased mRNA expression of two enzymes that regulate hepatic gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK-C) encoded by *Pck1* and glucose-6-phosphatase (G6Pase) encoded by *G6pc* was detected in the *C. albicans*-colonized mice compared to the mock-colonized mice (Fig. 5C, Student's t-test, $p=0.027$, Fig. 5D, Mann-Whitney U-test, $p=0.0134$). These enzymes can be regulated by both dietary lipid abundance (Massillon et al., 2003) and by basal serum CORT in accordance with the circadian rhythm (Reddy et al., 2007). Thus, the increased expression of gluconeogenic enzymes may reflect both circadian advance of CORT and increased abundance of dietary PUFAs present in *C. albicans*-colonized mice. Regardless, these changes in hepatic gene expression demonstrate that *C. albicans* alters host physiology beyond the GI tract and could have a significant impact on host metabolism.

3.1 Discussion

C. albicans is the most common fungal member of the human gut microbiota. We investigated the effect of *C. albicans* colonization on the gut-brain axis and observed specific changes in the endocannabinoidome related to a stress-like behavioral and neuroendocrine phenotype. Previous investigations of the gut-microbiota-brain axis have shown that colonization with commensal bacterial species normalized the HPA axis and behavioral phenotypes in germ-free animals. Sudo *et al* showed that monocolonization of germ-free mice with the probiotic *Bifidobacterium infantis* reversed the hyperactive CORT response to restraint stress (Sudo et al., 2004) and Heijtz *et al* showed that full reconstitution of the microbiota normalized behavior of germ-free mice in the EPM (Diaz Heijtz et al., 2011). Chronic administration of probiotic *Lactobacillus plantarum* to specific-pathogen-free (SPF) mice was sufficient to decrease anxiety-like behavior in the EPM (Liu et al., 2016) and a combination of *Lactobacillus helveticus* and *Bifidobacterium longum* decreased anxiety-like behavior in rats and psychological stress in healthy human volunteers (Messaoudi et al.,

2011). Additional studies of the bacterial microbiota-gut-brain axis have identified the immune system (Ait-Belgnaoui et al., 2012) as well as direct signaling through the vagus nerve (Bravo et al., 2011) as mechanisms by which changes to the gut microbiota result in alterations of the HPA axis and behavior.

In contrast to the anxiolytic effect of probiotic bacteria supplementation, the addition of *C. albicans* to the intact microbiota of mice increased basal CORT and anxiety-like behavior, resembling neuroendocrine phenotypes observed after infection with the parasite *Trichuris muris* (Bercik et al., 2010) or the mouse gastrointestinal pathogen *Citrobacter rodentium* (Lyte et al., 2006). *C. albicans* in contrast is a commensal and did not induce significant systemic inflammation. Although the neuroendocrine phenotypes observed in *C. albicans*-colonized mice were more limited in scope and scale than those observed in other microbiota manipulation models, it is worth noting that *C. albicans* is a non-pathogenic, common colonizer of the GI tract and thus would not be expected to induce widespread physiological changes. *C. albicans* colonization changed the gut endocannabinoidome and altered eCB signaling to produce an observed increase in basal CORT and anxiety-like behavior. To our knowledge, this is the first communication to report that a commensal fungus, *C. albicans*, affects neuroendocrine host phenotypes and that microbiota-induced changes to eCBs can affect the brain and behavior.

Treatment with the FAAH inhibitor URB597 alleviated both the elevated basal CORT and increased anxiety-like behavior observed in *C. albicans*-colonized mice, indicating a common mechanism of insufficient AEA. URB597 treatment had no effect on basal CORT in the mock-colonized control mice and significantly decreased basal CORT in the *C. albicans*-colonized mice. URB597 decreased anxiety-like behavior in both the mock-colonized control and *C. albicans*-colonized mice, indicating that behavior in the EPM is sensitive to URB597 treatment in both non-anxious controls and anxious *C. albicans*-colonized mice. The comparable behavior in the *C. albicans*-colonized and mock-colonized mice treated with URB597 demonstrated that increasing AEA levels was sufficient to eliminate the *C. albicans*-induced increase in anxiety-like behavior, supporting eCB signaling as a mechanism by which *C. albicans* colonization altered behavior.

Consistent with the results of the URB597 experiments, we found a trend towards a correlation in *C. albicans*-colonized mice between basal CORT and brain AEA, similar to what has been seen in chronic stress models, both in unstressed mice and those subjected to subsequent acute stress (Hill et al., 2010). K-means cluster analysis further supported the interpretation that mice with the greatest CORT response to *C. albicans*-colonization exhibited the greatest decrease in brain AEA content. This analysis supported the model that an AEA deficit was responsible for the neuroendocrine changes observed in *C. albicans*-colonized mice. Previous investigations found that AEA levels specifically within the prefrontal cortex (Hill et al., 2011) and the amygdala (Hill et al., 2010) were responsible for the downregulation of CORT during recovery from stress and for anxiety-like behavior, respectively. It is possible that within those discrete brain regions, *C. albicans*-colonized mice exhibited a larger decrease in AEA than the decrease we measured in the forebrain as a whole. Untargeted metabolomic analysis of cecum contents further supported the model that *C. albicans* colonization altered eCB metabolism, as two alternate NAEs, linoleoyl and

linolenoyl ethanolamide, were increased in abundance in the *C. albicans*-colonized mice as were compounds containing their FA precursors. We suggest that these changes in GI tract eCB levels could reflect systemic changes in lipid availability and NAE production and therefore disruption of normal eCB metabolism. Such disruption could contribute to the altered eCB-CB1 signaling and consequent neuroendocrine changes observed in the *C. albicans*-colonized mice. We did not detect changes in the levels of precursors or metabolic intermediates of 2-AG in the cecum contents and did not observe a difference in 2-AG in the brain. Thus we suggest that the altered eCB-CB1 signaling and neuroendocrine changes observed in the *C. albicans*-colonized mice are driven by changes to AEA metabolism.

Investigation into anxiety disorders specifically during adolescence is relevant to our understanding of human health (Siegel and Dickstein, 2012). The current study was performed with adolescent mice, which were sensitive to HPA axis dysregulation by GI tract colonization with *C. albicans*. Previous work investigating the gut microbiota and the HPA axis demonstrated a window during which mice are sensitive to microbiota manipulation, as Sudo *et al* (2004) found that hyperreactivity of the HPA axis in germ-free mice could be reversed through microbiota transplant in adolescent (6-week-old) mice but not in adult (8-week-old or 14-week-old) mice. Lee *et al* (2013) showed that AEA levels in the brain increased significantly between adolescence and adulthood, and thus the adolescent brain may be especially sensitive to the degree of eCB disruption observed in the *C. albicans*-colonized mice. Researchers have also demonstrated significant age-related differences in HPA axis regulation and response in both rodents (Romeo *et al.*, 2014) and humans (Gunnar *et al.*, 2009; Netherton *et al.*, 2004).

More broadly, these results highlight the sensitivity of the HPA axis and related anxiety-like behavior to changes in AEA levels, and provide evidence that the eCB system is a viable drug target for anxiety disorders. Indeed, a recent study in healthy human volunteers demonstrated that treatment with a different FAAH inhibitor, PF-04457845, raised AEA levels and protected against the development of stress-induced anxiety (Mayo *et al.*, 2019), demonstrating that FAAH inhibition and increased AEA can reverse activation of stress responses and modulate emotional behavior in humans as it does in mouse models. Altogether this work demonstrates the ability of the gut metabolome to have a significant impact on the brain and behavior, and illustrates the ability of the common gut commensal fungus *C. albicans* to affect the host beyond the GI tract.

3.2 Conclusions

We have shown that GI colonization with the human commensal fungus, *C. albicans*, can alter systemic host health through the gut-brain axis. Although *C. albicans* colonization was limited to the GI tract, mice so colonized exhibited endocannabinoidome changes that resulted in dysregulation of basal CORT production and increased anxiety-like behavior. We suggest that *C. albicans* is able to induce these changes through manipulation of lipid availability in the GI tract, in agreement with previous studies that have shown the importance of GI lipid pools for eCB production throughout the host (Sihag and Jones, 2019). This study illustrates a novel mechanism by which a member of the microbiota can

impact host health, by modulating the GI tract metabolome to change eCB levels throughout the host and alter the gut-brain axis.

4. Materials and Methods

Detailed methods are presented in the Supplemental Information

4.1 Animals

Up to 23 five-week-old female C57BL/6 mice (Jackson Laboratory) were cohoused in a large cage (24"×17") and given sterile food, water and bedding. After four days of acclimation, mice were transferred to standard cages: 3–4 mice inoculated with *C. albicans* by gentle pipetting into their mouths or 3–4 mice mock-inoculated. On day one post-inoculation, fresh fecal pellets were collected from all mice and plated on YPD-SA to confirm and measure colonization with *C. albicans* in all inoculated mice. No fungal growth was detected in mock-inoculated mice. Mice were put through a behavioral test on day two and sacrificed on day three post-inoculation without stress (Sarkar et al., 2011). After sacrifice cecum contents were collected and plated on YPD-SA to measure endpoint *C. albicans* colonization level.

The total duration of the acute colonization model and housing at Tufts University was one week. Animals were only used in one study: mice were acclimated, inoculated or mock-inoculated, used in a single behavioral test on day two post-inoculation and either sacrificed without stress on day three or subjected to restraint stress and then sacrificed on day three (Fig. S8).

All experiments were done in compliance with NIH Guide for the Care and Use of Laboratory Animals and Tufts University IACUC guidelines.

4.2 Strains and growth conditions

C. albicans strain CKY101 (Brown et al., 1999) was used for all experiments. For preparation of mouse inoculum, cells were grown at 37°C in standard yeast media for 24 hours. 25µl (5×10^7 cells) of cells in phosphate-buffered saline (PBS) with 2% sucrose was orally fed to mice by gently pipetting into their mouths with a pipettor. 25µl of 2% sucrose in PBS was fed in the same manner to mice for mock-inoculation.

4.3 Drug treatment

URB597 (Sigma Aldrich) and URB937 (Cayman Chemical) were dissolved in 18:1:1 normal saline:PEG400:Tween80 (Sigma Aldrich) and administered to mice via intraperitoneal injection at a dosage of 0.1–1mg/kg bodyweight four hours prior to behavioral testing or sacrifice.

4.4 Restraint stress

Mice were placed in a 50ml conical tube with two airholes enclosed with a rubber stopper for 30 minutes. After this restraint, mice were placed back in their home cage and

anesthetized and sacrificed or allowed to recover in the home cage for 30 or 60 minutes and then anesthetized and sacrificed.

4.5 Elevated Plus Maze

Behavioral testing in the Elevated Plus Maze (EPM) was performed as described in Walf *et al* (2007) under moderately bright indirect light from 12pm–5pm. After a 5m trial in the EPM, the mouse was placed in a fresh sterile cage. All trials were recorded with a video-camera from above and scored by a blinded observer.

4.6 Forced Swim Test

Behavioral testing in the forced swim test was performed as described in Can *et al* (2012). A mouse was removed from its home cage and placed in the beaker of non-sterile tap water for a six-minute trial which was recorded by video-camera from the side and scored after the fact by a blinded observer. After testing, mice were placed in a fresh sterile cage. Beaker was sterilized between trials.

4.7 Bacterial microbiota analysis

The cecum, including contents, was dissected from mice after sacrifice on day three and was immediately frozen on dry ice. Microbial DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturer's protocol. Libraries were prepared from each sample and sequenced as described (Caporaso *et al.*, 2012). The resulting fastq files were used as input for downstream analysis using QIIME (1.8.0)(Caporaso *et al.*, 2010). The resultant operational taxonomic unit (OTU) tables contained the relative abundance of bacterial taxa in each sample. Analysis of alpha and beta diversity were performed using standard QIIME scripts.

4.8 Measurement of hormones and cytokines in serum

Trunk blood was collected into serum separator blood collection tubes (BD) after sacrifice by decapitation. All CORT measurements shown are from terminal blood collection such that only one CORT measurement per mouse was performed. Tubes were then spun to separate serum. Serum was divided into aliquots and frozen at -80°C . Serum corticosterone was measured using a Corticosterone ELISA Kit (Enzo Life Sciences) following the manufacturer's small volume protocol. Manufacturer reported sensitivity down to 27pg/ml of CORT. Authors observed an average intra-assay coefficient of variance of 5.7% and an average inter-assay coefficient of variance of 8.9%. Serum cytokines were measured using a multiplex ELISA (Quanterix) following manufacturer's protocol. Manufacturer reported sensitivity for each cytokine individually: mIFN- γ 7.1pg/ml, mIL-1 β 1.1pg/ml, mIL-6 6.4pg/ml, mIL-10 3.0pg/ml, mIL-12p70 0.37pg/ml. Due to volume of mouse serum required for assay, authors did not have sufficient sample to calculate inter-assay coefficient of variance.

4.9 Immunohistochemistry for cFOS in hypothalamus slices

Whole brains were dissected and fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 24h at 4°C . They were then cryopreserved in sucrose gradient, rapidly

frozen in isopentane chilled on dry ice, and stored at -80°C . Free-floating sections were prepared using a cryostat. Sections of interest were incubated with 1:5000 dilution of rabbit anti-mouse cFos antibody (Sigma Aldrich F7799) for 72h, then stained using anti-rabbit IgG (VectaStain Elite ABC Kit) and streptavidin-Alexa488 (Molecular Probes). Sections were imaged using a Zeiss microscope with Apotome attachment. Quantification was performed using ImageJ. The PVN was identified using the DAPI image to define the outline of the PVN and cFOS positive nuclei were counted within this region. For each brain at least two sections containing the PVN were quantified and the ratio of cFOS/DAPI positive nuclei averaged.

4.10 Extraction and measurement of AEA in forebrain

Whole brain was dissected and the forebrain separated at approximately Bregma -5.5 . The forebrain sample taken in this manner should not include most of the midbrain, cerebellum, pons or medulla. Lipid extraction from forebrain samples was performed as described previously (Morena et al., 2015). AEA was measured using mass spectrometry as described previously (Qi et al., 2015).

4.11 Untargeted metabolomic analysis of cecum contents

The cecum was dissected after sacrifice on day three and contents were squeezed into a tube and immediately frozen in dry ice/ethanol bath and stored at -80°C . Extraction of metabolites and untargeted mass spectrometry analysis were performed by Metabolon. Statistical analysis of results was performed using Metabolon Metabolync tools.

4.12 Real-time quantitative PCR analysis

Tissues were frozen at -80°C in RNALater (Invitrogen). RNA was purified from tissues using QIAzol for lysis and extraction with a Qialyzer, followed by column purification using the Ambion Purelink Mini kit (Invitrogen). cDNA was synthesized using SuperScript III (Invitrogen) with oligo-dT priming and the manufacturer's protocol. qPCR reactions were performed using SYBR Green Master Mix (Applied Biosystems) and a LightCycler 480 II (Roche) instrument.

4.13 Statistical analysis

Statistical analysis was performed using GraphPad Prism8.4.0. The majority of the data was analyzed using a two-tailed t-test to compare the means of the two experimental groups in question. For data with two dimensions (four groups) 2-way ANOVA was used. When the standard deviation of the data was significantly different between groups, Welch's t-test was used; otherwise Student's t-test was used. Throughout $\alpha=0.05$ was used to assess significance. When multiple comparisons were required, the Bonferroni correction was used to adjust the value of α . Sidak's test for multiple comparisons was used in some instances as a post-hoc test. K-means clustering was performed in R. Data was checked for normality using the D'agostino-Pearson omnibus test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *C. albicans* GI colonization increases anxiety-like behavior and basal serum CORT
- Treatment with FAAH inhibitor URB597 reversed both behavior and CORT phenotypes
- *C. albicans* altered lipid and endocannabinoid levels in the brain and GI tract
- Changes in hepatic gene expression reflected increased lipids measured in GI tract

Figure 1: Gastrointestinal colonization with *Candida albicans* after a single inoculation without disruption of bacterial gut microbiota or invasive disease

A) Timeline for acute colonization model. 5-week-old female mice were cohoused for four days and handled daily. Mice were then moved into smaller groups in standard cages and orally inoculated with 5×10^7 CFU of *C. albicans* or given buffer. On day one post-inoculation, fecal pellets were sterilely collected from mice and plated on YPD-SA to measure colonization levels. On day two post-inoculation, all mice underwent a behavioral test. On day three post-inoculation mice were anesthetized with isoflurane and sacrificed by decapitation. Some mice were subjected to restraint stress prior to sacrifice. B) *C. albicans* CFU/g of fecal pellets was measured on day 1 post-inoculation and CFU/g of cecum contents was measured after sacrifice on day three post-inoculation. No culturable fungi

were measured on either day from mock-colonized mice. Day 1: N=56, Day 3: N=50. Figure includes data from 6 cohorts. Bars indicate geometric mean. C) A multiplex ELISA was used to measure inflammatory cytokines in the serum of mice. N.D, not detectable. Mixed effects analysis followed by Sidak's post-hoc test. Mock-colonized N=13, *C. albicans*-colonized N=14. Figure includes data from 3 cohorts. D–F) Microbiota analysis of the cecum contents of mice was performed using standard 16S rRNA DNA sequencing and QIIME analysis pipeline. Mock-colonized N=8 and *C. albicans*-colonized N=8. Figure includes data from 2 cohorts. D) The relative abundance of all bacterial families detected with a median relative abundance greater than 0 is shown. Bar shows geometric mean with SEM E) The average alpha diversity metric Chao1 was calculated to determine the diversity of the microbiota at different levels of sampling. The average Chao1 score of the experimental groups is shown. F) Beta diversity was calculated using weighted UniFrac scores and principal coordinates analysis was performed. For C–D, symbols indicate individual mice and bars indicate the average. Mock-colonized mice are represented as solid dots and bars; *C. albicans*-colonized mice are represented as open circles and bars.

Figure 2: *C. albicans* colonization increases anxiety-like behavior

On day two post-inoculation mice underwent either the Elevated Plus Maze (EPM) or the Forced Swim Test (FST). Trials in the behavioral tests were video-recorded and scored by a blinded observer after the fact. For the EPM data (A-F) that follows, mock-colonized N=22 and *C. albicans*-colonized N=19 and statistical analysis performed was Mann-Whitney U-test (A-C, G) or Student's t-test (D-F), $p < 0.05$. Figures include data from two cohorts. A) Percentage of time spent in the open arms of the EPM. B) Total number of entries into the open arms of the EPM. C) Percentage of time spent in the closed arms of the EPM. D) Total entries into the closed arms of the EPM. E) Percentage of time spent in the neutral central square of the EPM (neither open nor closed arms). F) Total entries during the trial (sum of the open and closed arm entries), a metric for locomotor activity. G) Total time spent immobile during the six-minute FST trial. Immobility was defined as no movements beyond those required to stay afloat. For FST data: mock-colonized N=33, *C. albicans*-colonized N=24. Figure includes data from three cohorts. Throughout, symbols represent individual mice and bars indicate average with the SEM. Mock-colonized mice are represented as solid dots and bars; *C. albicans*-colonized mice are represented as open circles and bars.

Figure 3: *C. albicans* colonization increases basal serum CORT production

A) Corticosterone (CORT) in trunk blood of mice sacrificed without stress was measured by ELISA. Mock-colonized N=15, *C. albicans*-colonized N=19. Figure includes data from four cohorts. Data was analyzed using Welch's t-test, $p<0.05$ B) CORT in trunk blood of mice sacrificed at different times of day. In addition to the data summarized in A (mice sacrificed from 13:00–17:00), B includes mice sacrificed without stress from 17:00–20:00. Data was binned in one-hour increments. Mock-colonized: 13:00 N=5, 14:00 N=9, 15:00 N=11, 16:00 N=6, 17:00 N=9, 18:00 N=8, 19:00 N=8. *C. albicans*-colonized: 13:00 N=6, 14:00 N=13, 15:00 N=10, 16:00 N=11, 17:00 N=10, 18:00 N=6, 19:00 N=14. C) Mice were subjected to 30 minutes of restraint stress and then sacrificed immediately (0m recovered) or after 30 minutes or 60 minutes of recovery as shown on the x-axis. Mock-colonized: 0m N=14, 30m N=15, 60m N=13. *C. albicans*-colonized: 0m N=15, 30m N=13, 60m N=12. D)

Immunohistochemistry was used to visualize c-FOS protein with Alexa488 in the paraventricular nucleus (PVN) of mice using the 20X objective. DAPI was used to visualize nuclei and Alexa488 to visualize c-FOS. The PVN of mice sacrificed unstressed (left) do not exhibit nuclear c-FOS staining, while PVN of mice sacrificed after 30 minutes of stress and 30 minutes of recovery (right) are positive for c-FOS. Representative images are shown. E) The ratio of c-FOS positive nuclei to total nuclei (DAPI) quantified the degree of PVN c-FOS staining measured in the mock-colonized and *C. albicans*-colonized mice after 30 minutes of stress and 30 minutes of recovery. Mock-colonized N=10, *C. albicans*-colonized N=9. For A and C, symbols indicate individual mice and bars indicate the average with the SEM. For B, symbols indicate average and error bars indicate SEM. For E, each symbol shows the average ratio of c-FOS/DAPI nuclei from 2–3 brain slices that contained the PVN from an individual mouse. Throughout, mock-colonized mice are solid dots and bars and *C. albicans*-colonized mice are open circles and bars.

Figure 4: Neuroendocrine phenotypes of the *C. albicans*-colonized mice are mediated through disruption of the endocannabinoid system

A-C) Mice were treated with the fatty acid amide hydrolase (FAAH) inhibitor URB597, a well-characterized drug which increases AEA levels by blocking degradation. Mice were given either URB597 or vehicle control by intraperitoneal injection 4–6 hours prior to sacrifice (A) or testing in the EPM (B-C). Prior to post-hoc testing data was analyzed by 2-way ANOVA. A) Mice were treated with 1mg/kg of URB597 or vehicle. ELISA was used to measure CORT in trunk blood of mice sacrificed without stress. Sidak's post-hoc test, $p < 0.05$. Mock-colonized: vehicle N=15, URB597 N=19. *C. albicans*-colonized: vehicle N=16, URB597 N=18. Figure includes data from four cohorts. B-C) Mice were treated with 0.1mg/kg (triangles) or 0.15mg/kg (circles) URB597 or vehicle, then tested in the EPM. Mock-colonized: vehicle N=17, 0.1mg/kg URB597 N=4, 0.15mg/kg URB597 N=8. C.

albicans-colonized: vehicle N=13, 0.1mg/kg URB597 N=4 0.15mg/kg URB597 N=8. B) Percentage time spent in the open arms, normalized to the average percent open time of the mock-colonized vehicle control, is shown. T-test corrected for multiple comparisons, $p<0.05$. C) Total arms entries, a metric for overall locomotor activity is shown. D) AEA (pmol/g tissue) was measured in forebrain samples by mass spectrometry. E-F) Linear regression of basal serum CORT and forebrain AEA (D) in individual mice are shown. (D-F) Mock-colonized N=7, *C. albicans*-colonized N=9. Untargeted mass spectrometry was used to measure relative abundance of 735 compounds in the cecum contents of mice. N-acylethanolamides (G-I), a free fatty acid (J) and a lysophospholipids (K) containing an 18-carbon chain with varying degrees of unsaturation are shown here. Raw data was log-transformed. Mock-colonized N=8, *C. albicans*-colonized N=8. Student's t-test, $p<0.05$. Symbols indicate individual mice and bars indicate average. Solid circles and bars are mock-colonized mice and open circles and bars are *C. albicans*-colonized mice.

Figure 5: *C. albicans* colonization affects hepatic lipid-responsive gene expression

RNA was extracted from the liver of mice sacrificed unstressed on day three. cDNA was synthesized and qRT-PCR was used to quantify liver gene transcription. RNA gene expression is normalized to GAPDH and expressed relative to the average expression of the mock-colonized mice. A) Stearoyl-CoA desaturase 1 (*Scd1*) RNA expression in the liver is shown. Mock-colonized N=24, *C. albicans*-colonized N=27. Figure includes data from four cohorts. B) Expression of multiple lipogenic genes is shown: acyl-CoA 8–3 desaturase (*Fads1*), acyl-CoA 6 desaturase (*Fads2*), sterol regulatory element-binding protein 1 (*Srebp1*) and fatty acid synthase (*Fasn*). Mock-colonized N=11, *C. albicans*-colonized N=10. Figure includes data from two cohorts. C) Expression of the gluconeogenesis enzymes phosphoenolpyruvate carboxykinase (*Pck1*) (mock-colonized N=24, *C. albicans*-colonized N=23) and D) glucose-6-phosphatase (*G6Pc*) is shown (mock-colonized N=20, *C. albicans*-colonized N=18). Figures include data from four cohorts. Symbols represent individual mice and bars indicate average with the SEM. Solid symbols and bars indicate mock-colonized mice and open symbols and bars indicate *C. albicans*-colonized mice. Mann-Whitney U-test was used for statistical analysis of A and D; Welch's t-test was used for analysis of B and C, $p < 0.05$.

Table 1:
22 metabolites significantly altered by *C. albicans* colonization

Mice were either mock-colonized or colonized with *C. albicans*. They were sacrificed without stress on day three post-inoculation and the cecum contents were squeezed into a tube and snap-frozen. Extraction of metabolites and untargeted mass spectrometry analysis were performed by Metabolon. Statistical analysis of results was performed using Metabolon Metabolync tools. Only compounds with a significant difference in abundance in the *C. albicans*-colonized versus mock-colonized mice are listed. Statistical analysis was not corrected for multiple comparisons, t-test, $p < 0.05$. Mock-colonized PBS N=8, *C. albicans*-colonized N=8.

Biochemical compound	Pathway	P-value (t-test)	Fold Change
indole-3-carboxylate	Amino Acid	0.001594	1.028581099
tartronate (hydroxymalonate)	Xenobiotics	0.00236	0.948908087
1-lignoceroyl-GPC (24:0)	Lipid	0.002615	1.056459907
linoleoyl ethanolamide	Lipid	0.00372	1.029836368
suberate (C8-DC)	Lipid	0.007619	0.987119582
1,2-dilinoleoyl-digalactosylglycerol (18:2/18:2)*	Lipid	0.011453	1.016990862
N-acetylglutamine	Amino Acid	0.012994	0.976312103
1-oleoyl-GPC (18:1)	Lipid	0.013467	1.034327387
beta-sitosterol	Lipid	0.01369	1.014459847
trans-4-hydroxyproline	Amino Acid	0.014716	0.987789428
gamma-tocopherol/beta-tocopherol	Cofactors and Vitamins	0.016379	1.010246153
1-linoleoyl-GPC (18:2)	Lipid	0.016602	1.035658474
heptadecatrienoate (17:3)*	Lipid	0.017057	1.024691248
linolenoyl ethanolamide	Lipid	0.0188	1.034021742
1,2-dilinoleoyl-galactosylglycerol (18:2/18:2)*	Lipid	0.023423	1.023983049
bilirubin (E,E)*	Cofactors and Vitamins	0.029299	1.061286204
stigmasterol	Lipid	0.030937	1.012926246
campesterol	Lipid	0.038772	1.017016776
alpha-tocotrienol	Cofactors and Vitamins	0.040974	1.016694223
3-methyl-2-oxobutyrate	Amino Acid	0.041846	0.979357347
cholesterol	Lipid	0.042509	1.02118387
2'-deoxyadenosine	Nucleotide	0.043641	0.923940457