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

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Classification

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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. We used a mouse model of gastrointestinal (GI) colonization 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 anandamide (AEA). *C. albicans*-colonized mice exhibited changes in endocannabinoid levels within the GI tract and a negative correlation between brain AEA levels and serum CORT. 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 manipulation of the endocannabinoid (eCB) system that resulted in neuroendocrine changes contributing to anxiety-like behavior.

Keywords

Endocannabinoid, microbiota, *Candida albicans*, anxiety, corticosterone

42

43 **Significance Statement**

44 Anxiety disorders are the most prevalent mental health disorder worldwide yet
45 their etiology is incompletely understood and treatment options are limited. The gut-
46 brain axis has been shown to influence the development of anxiety disorders. Recent
47 work has established that the bacterial gut microbiota can influence the gut-brain axis and
48 result in changes to neuroendocrine health including anxiety-like behavior in mice. We
49 describe a novel role for a fungal member of the gut microbiota, *Candida albicans*, that
50 influences the neuroendocrine system through manipulation of the endocannabinoid
51 system. Additionally, this work demonstrates that acute, limited manipulation of
52 endocannabinoids can have a significant impact on host health, emphasizing the potential
53 of this system as a target for the treatment of anxiety disorders.

54 **Introduction**

55 Anxiety disorders are the most prevalent mental health disorder worldwide, with a
56 lifetime prevalence of 5-7% of the human population[1]. The etiology of anxiety disorders
57 is complex and incompletely understood, although many different genetic and
58 environmental factors have been identified[2]. The gut-brain axis, the bi-directional
59 communication between the gut and the brain through multiple host systems, has been
60 shown to affect anxiety disorders[3]. Constant communication between the periphery and
61 the brain is required for normal homeostasis and overall health.

62 One component of the gut-brain axis thought to play a role in anxiety disorders is
63 the hypothalamus-pituitary-adrenal (HPA) axis. The endocrine output of the HPA axis,

64 the stress hormone corticosterone (CORT), is a broad regulator of host health including
65 the immune, neuroendocrine, metabolic and cardiovascular systems. Under basal
66 conditions, CORT release is regulated by the circadian rhythm and helps to maintain
67 homeostasis[4]. CORT is also a key part of the acute psychological stress response and a
68 rapid increase in circulating CORT is essential for mounting an effective response to
69 stress[5]. Dysregulation of the HPA axis and CORT production is correlated with mental
70 health disorders including anxiety and depression[6].

71 The gut microbiota, the diverse assemblage of microbes that colonizes the GI tract
72 of humans, has been shown to affect virtually every aspect of human health, including the
73 gut-brain axis. Researchers using germ-free mouse models demonstrated that the gut
74 microbiota plays an important role in the development and regulation of the HPA axis[7,8].
75 Studies have also shown that specific probiotic bacterial species can affect the gut-brain
76 axis, as treatment with *Bifidobacterium sp*. and *Lactobacillus sp*. decreases anxiety-like
77 behavior and normalizes HPA axis function in mice[9,10]. Investigation into the
78 contribution of the gut-microbiota-brain axis to mental health could reveal novel
79 regulators of anxiety-like behavior and offer mechanistic insight into the etiology of the
80 disease.

81 *Candida albicans* is a commensal fungus that colonizes the GI tract of ~60% of
82 the human population[11]. Although a major colonizer of humans, its role as a commensal
83 microbe is largely uncharacterized. Previous researchers have shown that GI *C. albicans*
84 colonization induces local and systemic immune changes which are protective against
85 systemic *C. albicans* infection and disease[12], and against infection with bacterial

86 pathogens such as *C. difficile*[13] and *S. aureus*[12]. The research that follows demonstrates
87 that *C. albicans* affects host health beyond the GI tract. We show that *C. albicans*
88 dysregulated HPA axis activity and increased anxiety-like behavior in a mouse model by
89 altering endocannabinoid signaling.

90 The mammalian endocannabinoid (eCB) system regulates both the HPA axis and
91 anxiety-like behavior. It consists of two major neuroactive lipids, N-
92 arachidonoylethanolamide (AEA) and 2-arachidonoylglycerol (2-AG) and their receptors
93 CB1 (distributed throughout the nervous system) and CB2 (present primarily on immune
94 cells)[14–16]. AEA and 2-AG are produced in response to activation in post-synaptic
95 neurons, and act at CB1 on pre-synaptic neurons to limit neurotransmitter release. AEA
96 and 2-AG levels are regulated by substrate availability and the activity of their synthetic
97 and degradative enzymes[17]. Using genetic and pharmacological tools in rodents,
98 researchers have demonstrated that manipulation of CB1 signaling affects basal and
99 stress-induced activation of the HPA axis as well as performance in tests for anxiety-like
100 behavior[18,19]. Here we show that treatment with the FAAH inhibitor URB597, which
101 increases AEA levels and thus CB1 signaling, is sufficient to reverse the effect of *C.*
102 *albicans* colonization on CORT and anxiety-like behavior. We also show that
103 colonization with *C. albicans* altered levels of eCBs in the gut and that brain AEA levels
104 correlated with the increase in serum CORT observed in the *C. albicans*-colonized mice.
105 These results establish a mechanism by which a specific member of the gut microbiota,
106 *C. albicans*, affects activity in the brain.

107 **Results**

108 **A single oral inoculation with *Candida albicans* is sufficient to establish**

109 **gastrointestinal colonization without inflammatory disease**

110 We used a murine colonization model to investigate the effects of *C. albicans*

111 inoculation on the mammalian host. Co-housing of up to 23 5-week-old mice in a large

112 cage was used to standardize gut microbiota by coprophagy and therefore only female

113 mice were used in these studies. The mice were acclimated to our facility and handled

114 daily while co-housed. They were then either inoculated with a single dose of *C.*

115 *albicans* or mock-inoculated with buffer and transferred to small cages in groups of 3-4.

116 *C. albicans* was measurable in fecal pellets after 24h and 3 days after inoculation (Fig.

117 1B) and was detected throughout the GI tract (Fig. S1). We assessed behavior two days

118 post-inoculation and mice were sacrificed after three days of colonization (Fig. 1A). *C.*

119 *albicans*-colonized mice did not exhibit symptoms of illness or lose >5% bodyweight

120 (Fig. S2). A multiplex ELISA was used to measure inflammatory cytokines in the serum

121 after sacrifice and low levels of circulating IL-1β, IFN-γ, IL-10 and IL-6 were measured

122 in mock-inoculated and *C. albicans*-colonized mice (Fig. 1C). Although IL-6 was

123 significantly increased in *C. albicans*-colonized mice (Student's t-test, $p=0.04$), IL-6 as

124 well as all other cytokines were well below the levels typically observed in mice with *C.*

125 *albicans* disease[21]. Thus, our model resulted in stable colonization of mice with *C.*

126 *albicans* in the GI tract that was not associated with disease, modeling commensal GI

127 colonization.

128 Previous work demonstrated that colonization with *C. albicans* in the absence of
129 antibiotic treatment had a small effect on the composition of the bacterial microbiota[22].
130 To determine whether the bacterial microbiota was significantly changed upon *C.*
131 *albicans* colonization, we analyzed the bacterial microbiota of the cecum of mice
132 sacrificed three days post-inoculation using 16s rRNA DNA sequencing and the QIIME
133 analysis pipeline[23]. The composition and diversity of bacterial taxa were not significantly
134 altered by the introduction of *C. albicans* (Fig. 1D-F), consistent with previous results[22].
135 Mann-Whitney U test and linear discriminant analysis effect size ((LEFSE) analysis[24]
136 showed that no taxa were significantly different in relative abundance between the
137 microbiota of mock-colonized and *C. albicans*-colonized mice (Fig. 1D). The overall
138 diversity of the microbiota was also not significantly different (Fig. 1E). We analyzed
139 beta diversity and summarized the results using principal coordinates analysis (Fig. 1F).
140 PERMANOVA analysis of the complete principal coordinates analysis found that there
141 was not significant separation of populations based on *C. albicans* colonization (pseudo-F
142 statistic=1.7306, p=0.117, 999 permutations). To summarize, mice inoculated with a
143 single dose of *C. albicans* were stably colonized with neither disruption of the bacterial
144 microbiota nor invasive disease.

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

146 To assess the effect of GI *C. albicans* colonization on host emotional behavior,
147 standard tests for anxiety-like and stress-coping behavior were used. Two days post-
148 inoculation (Fig. 1A), all mice were subjected to a single behavioral test, either the
149 elevated plus maze (EPM) test for anxiety-like behavior[25] or the forced swim test (FST)

150 for stress-coping behavior[26]. The EPM consists of two open, aversive arms and two
151 closed arms. Mice were allowed to explore the EPM for a 5-minute trial which was
152 scored by a blinded observer for time spent and entries into the closed and open arms.
153 Anxiety-like behavior is detected as avoidance of the open arms, expressed as percentage
154 arm time (time spent in specified arms divided by the total time spent all arms).

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

164 The forced swim test (FST) for stress-coping behavior was used to examine the
165 effect of *C. albicans* colonization on a second aspect of emotional behavior, again two
166 days post-inoculation. Mice were placed in a cylinder containing 22°C water for a six-
167 minute trial which was video-recorded and later scored by a blinded observer for time
168 spent immobile. A passive-coping response in this assay was defined as an increase in
169 the amount of time spent floating immobile rather than the active coping response
170 defined as swimming or actively struggling. There was no difference in the time spent
171 immobile between the *C. albicans*-colonized mice and the mock-colonized mice (Fig.

172 2G), indicating that *C. albicans* colonization did not affect stress-coping behavior in this
173 test.

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

175 The underlying biology of anxiety-like behavior is multifaceted[27,28]. One
176 neuroendocrine pathway that has been shown to be dysregulated in human patients with
177 anxiety is the hypothalamus-pituitary-adrenal (HPA) axis[6]. To determine whether *C.*
178 *albicans* colonization affected the HPA axis, we sacrificed mice three days post-
179 inoculation and measured circulating serum CORT under basal conditions and in
180 response to acute psychological stress.

181 *C. albicans*-colonized and mock-colonized mice were sacrificed unstressed. *C.*
182 *albicans*-colonized mice had significantly higher unstressed basal CORT (Fig. 3A
183 Welch's t-test, p=0.0012). When the effect of circadian rhythm on serum CORT was
184 examined, basal CORT in both groups was shown to increase over time throughout the
185 afternoon, as expected[29] (Fig. 3B). Basal CORT in *C. albicans*-colonized mice, however,
186 began to rise sooner than in mock-colonized mice and thus was significantly higher in *C.*
187 *albicans*-colonized mice sacrificed three-to-five hours prior to lights out, consistent with
188 a circadian advance of CORT production. These results indicate that *C. albicans*
189 colonization dysregulates circadian control of the HPA axis. Further studies of basal
190 CORT were conducted only with mice sacrificed between 14:00-16:00.

191 To determine whether the changes to basal CORT regulation affected the stress-
192 responsive function of the HPA axis, mice were subjected to 30 minutes of restraint stress
193 and then sacrificed immediately or after 30 minutes or 60 minutes of recovery. *C.*

194 *albicans*-colonized and mock-colonized mice had comparable peak CORT immediately
195 after stress (Fig. 3C) but *C. albicans*-colonized mice failed to recover from stress to the
196 same extent as the uncolonized mice after 60 minutes of recovery time (Fig. 3C,
197 Student's t-test, p=0.04). These results indicated that *C. albicans* colonization advanced
198 circadian basal CORT production and impaired negative feedback of the HPA axis after
199 stress and recovery.

200 Activation of the HPA axis begins in the brain, through excitation of the CRH-
201 producing neurons of the paraventricular nucleus (PVN) of the hypothalamus. We used
202 immunofluorescence to quantify expression of cFos protein in the PVN as a marker for
203 neuronal activation. Nuclear cFos protein was not detected in the PVN of unstressed
204 mock-colonized or *C. albicans*-colonized mice (Fig. 3D, left). In mice subjected to 30
205 minutes of stress and 30 minutes of recovery, nuclear cFos protein was comparably
206 detected in both mock-colonized and *C. albicans*-colonized mice (Fig. 3D, right). The
207 PVN remains stress-responsive in the *C. albicans*-colonized mice, and was not
208 sufficiently stimulated at baseline to be detected by this method. These results
209 demonstrate that *C. albicans* colonization significantly impacts the gut-brain axis through
210 altered basal and stress-induced HPA axis function and increased anxiety-like behavior.

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

213 The neuroactive lipid endocannabinoid (eCB) N-arachidonoylethanolamide
214 (anandamide; AEA), through its interactions with the CB1 receptor, is a key regulator of
215 anxiety-like behavior and both stress-induced and basal CORT production[30–32]. The

216 circadian advance of basal CORT, reduced feedback inhibition of stress-induced CORT
217 and increased anxiety-like behavior observed in the *C. albicans*-colonized mice resemble
218 the phenotype of the CB1 knock-out mouse[19], suggesting a deficit of eCB-CB1 signaling
219 as a result of *C. albicans* colonization. Therefore, we hypothesized that *C. albicans*
220 colonization was interacting with the gut-brain axis via the endocannabinoid system.

221 To test this hypothesis, we used the well-characterized drug URB597 to increase
222 AEA levels and therefore amplify AEA-CB1 signaling through inhibition of the AEA
223 degradative enzyme, fatty acid amide hydrolase (FAAH)[33,34]. Mice were given an
224 intraperitoneal injection of vehicle or URB597 (1mg/kg) 4 hours prior to sacrifice under
225 unstressed conditions, to determine whether elevating AEA levels was sufficient to
226 alleviate the high basal CORT of the *C. albicans*-colonized mice. *C. albicans*-colonized
227 mice given vehicle injection had significantly higher basal CORT than mock-colonized
228 mice as expected (Fig. 4A, Welch's t-test, p=0.0043). Administration of URB597 was
229 sufficient to decrease basal CORT in the *C. albicans*-colonized mice (Fig. 4A, Welch's t-
230 test, p=0.0062). This result is consistent with an AEA deficit in the *C. albicans*-
231 colonized mice underlying the elevated basal CORT. URB597 did not significantly
232 change basal CORT in the mock-colonized mice (Welch's t-test, p=0.0971).

233 URB597 was administered 4-6 hours prior to testing in the EPM (0.1-0.15mg/kg)
234 to determine whether increasing AEA-CB1 signaling was sufficient to alleviate anxiety-
235 like behavior in the *C. albicans*-colonized mice. Due to variability in activity level of
236 mice, data are shown as the percentage of time spent in the open arm normalized to the
237 experimental mean of the mock-colonized vehicle control. As expected, *C. albicans*-

238 colonized mice treated with vehicle had increased anxiety-like behavior compared to the
239 mock-colonized mice (Fig. 4B, Welch's t-test, p=0.0033). Treatment with URB597
240 significantly increased open arm time of the *C. albicans*-colonized mice (Fig. 4B,
241 Welch's t-test, p=0.0024), which indicates that increasing AEA-CB1 signaling was
242 sufficient to normalize anxiety-like behavior in the *C. albicans*-colonized mice. There
243 was no effect of URB597 treatment on overall activity as measured by total arm entries
244 (Fig. 4C). Together, these results demonstrate that increasing AEA-CB1 signaling with
245 the FAAH inhibitor URB597 reversed the effect of *C. albicans* colonization on basal
246 CORT production and anxiety-like behavior, suggesting that the neuroendocrine
247 phenotypes observed in *C. albicans*-colonized mice result from decreased AEA-CB1
248 signaling.

249 **Basal CORT and brain AEA levels are negatively correlated in *C. albicans*-colonized**
250 **mice**

251 To test the hypothesis that AEA levels were reduced in colonized mice, AEA
252 levels in the forebrain were measured. Mice were sacrificed on day 3 post-inoculation
253 and forebrain samples analyzed by mass spectrometry. We observed only a trend
254 towards decreased AEA in the forebrains of *C. albicans*-colonized mice compared to
255 mock-colonized mice (Fig. 4D, Student's t-test, p=0.3). However, a negative correlation
256 between basal serum CORT and AEA in the *C. albicans*-colonized mice (Fig. 4F,
257 R^2=0.4416, F-test, p=0.05) but not in the mock-colonized mice (Fig. 4E, R^2=0.119, F-
258 test, p=0.4486) was observed. Thus mice that responded to *C. albicans* colonization with
259 increased basal CORT did indeed have lower levels of AEA in their forebrain. Previous

260 results have demonstrated a similar negative correlation between basal CORT and AEA
261 levels in the prefrontal cortex of mice[35]. Taken together, these results indicate that *C.*
262 *albicans* colonization had variable effects on bulk AEA levels in the mouse forebrain and
263 reduced forebrain AEA was observed in colonized mice with greater dysregulation of the
264 HPA axis.

265 A second canonical eCB, 2-arachidonoylglycerol (2-AG), was also measured in
266 the forebrain of mice. There was no difference between mock-colonized and *C. albicans*-
267 colonized mice in bulk levels or degree of correlation between 2-AG and serum CORT
268 (Fig. S4).

269 ***C. albicans* colonization alters the gut endocannabinoidome**

270 We performed untargeted metabolomic analysis of the cecum contents of mice to
271 determine whether *C. albicans* colonization altered the metabolite composition of the GI
272 tract and thereby altered eCB metabolism. Of the 735 compounds measured, 21 were
273 significantly differentially abundant in mock-colonized versus *C. albicans*-colonized
274 mice (Table S2, Student's t-test, $p<0.05$, uncorrected). This result is below the false
275 discovery rate and therefore pathway enrichment analysis was performed to investigate
276 the relevance of the 21 hits. Of the 5 pathways that contained more than one significant
277 compound, two were significantly enriched for *C. albicans*-dependent changes: sterols
278 and eCBs (Fisher's exact test, Holm's correction for multiple comparisons, $p<0.0001$ and
279 $p=0.0305$ respectively). The enrichment in eCB family compounds indicated that *C.*
280 *albicans* colonization altered eCB metabolism in the gut, consistent with the eCB
281 changes observed in the brain.

282 Two such eCB compounds, N-acylethanolamides (NAEs), linoleoyl and
283 linolenoyl ethanolamide, were significantly increased in the *C. albicans*-colonized mice
284 (Fig. 4G-H, Student's t-test, p=0.0037 and p=0.0188). An additional NAE, oleoyl
285 ethanolamide, was increased in the *C. albicans*-colonized mice but not significantly (Fig.
286 4I, Student's t-test, p=0.08). These compounds are structurally similar to anandamide
287 (AEA) but have different acyl groups. AEA itself is present at low levels in the GI tract
288 and was not detected in any of the samples. The increase in 18-C NAEs implies that *C.*
289 *albicans* colonization altered NAE metabolism. Feeding studies have shown that dietary
290 enrichment for a specific fatty acid (FA) results in overproduction of the NAE derived
291 from the enriched FA and limits production of alternate NAEs[37,38]. Membrane
292 phospholipid acyl chains can also act as substrates for the production of NAEs. We
293 measured a trend towards an increase in the 18-C FAs linolenate and linoleate (Fig. 4J-K,
294 Student's t-test, p=0.05 and p=0.06) as well as a significant increase in the
295 lysophospholipid 1-linoleoyl-glycerophosphocholine (Fig. 4L, Student's t-test, p=0.02) in
296 the GI tract of *C. albicans*-colonized mice. All of these compounds could be converted
297 into their respective 18-C NAEs and increase production of 18C NAEs linoleoyl and
298 linolenoyl ethanolamide while limiting production of AEA, contributing to the alterations
299 in AEA levels observed in the *C. albicans*-colonized mice.

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

302 In addition to the specific enrichment of two lipid subpathways, sterols and eCBs
303 and the trends towards increased 18-C FAs described above, we observed an overall

304 increase in lipid compounds containing long-chain polyunsaturated fatty acids (PUFAs)
305 in the *C. albicans*-colonized mice (Fig. S3). Lipids are absorbed from the GI tract into
306 the blood and pass through the liver, a central organ for lipid metabolism which is highly
307 regulated by dietary lipid levels[39]. We measured liver gene expression to determine
308 whether the changes in lipid levels we quantified in the cecum were sufficient to induce a
309 physiological change in the host.

310 Mice were sacrificed after three days of *C. albicans* colonization and qRT-PCR
311 was used to measure the expression of lipid-responsive genes in the liver. Decreased
312 expression of the mRNA (*Scd1*) encoding the enzyme stearoyl-coA desaturase (SCD1)
313 was detected in the *C. albicans*-colonized mice compared to the mock-colonized mice
314 (Fig. 5A, Student's t-test, $p=0.006$), consistent with a transcriptional response to
315 increased PUFA-containing lipids from the GI tract[40]. Gene expression of regulators of
316 lipogenesis, *Fads1* (acyl-CoA 8-3 desaturase) and *Fads2* (acyl-CoA 6 desaturase), the
317 transcription factor sterol regulatory element-binding protein 1 (SREBP1-c) encoded by
318 *Srebf1* and fatty acid synthase (*Fasn*)[41], were not significantly changed by *C. albicans*
319 colonization (Fig. 5B), implying that the increased lipid abundance measured in the
320 cecum was not a result of host lipogenesis. Also, increased mRNA expression of two
321 enzymes that regulate rate-limiting steps of hepatic gluconeogenesis,
322 phosphoenoylpyruvate carboxykinase (PEPCK-C) encoded by *Pck1* and glucose-6-
323 phosphatase (G6Pase) encoded by *G6pc* was detected in the *C. albicans*-colonized mice
324 compared to the mock-colonized mice (Fig. 5C-D, Student's t-test, $p<0.05$), consistent
325 with a liver response to increased dietary lipids[42]. These genes are positively regulated

326 by endocrine mediators CORT and glucagon, as well as by dietary lipids[42,43] and so may
327 also be upregulated by the elevated basal CORT present in the *C. albicans*-colonized
328 mice. These results imply that the increased abundance of PUFAs measured in the GI
329 tract was sufficient to induce hepatic transcriptional changes.

330 To summarize, we demonstrated that colonization of mice with the commensal
331 fungus *C. albicans* resulted in significant changes to the gut-brain axis including
332 increased anxiety-like behavior and altered activity of the HPA axis. We identified
333 altered eCB levels in the brain and GI tract of the *C. albicans*-colonized mice and
334 demonstrated that increasing levels of AEA through administration of URB597 was
335 sufficient to reverse the *C. albicans*-induced increase in CORT production and decrease
336 anxiety-like behavior. These results demonstrate that the gut microbiota can alter activity
337 in the brain through modulation of eCB signaling.

338 **Discussion**

339 *C. albicans* is the most common fungal member of the human gut microbiota. We
340 investigated the effect of *C. albicans* colonization on the gut-brain axis and observed
341 specific changes in the endocannabinoidome related to a stress-like behavioral and
342 neuroendocrine phenotype. Previous investigations of the gut-microbiota-brain axis have
343 shown that colonization with commensal bacterial species decreased CORT produced in
344 response to stress and decreased anxiety-like behaviors[8,9]. In contrast to these bacteria,
345 commensal *C. albicans* increased basal CORT and increased anxiety-like behavior,
346 resembling the neuroendocrine phenotypes observed after infection with the parasite
347 *Trichuris muris*[44] or the mouse gastrointestinal pathogen *Citrobacter rodentium*[45].

348 Researchers demonstrated that infection with these pathogens increased anxiety-like
349 behavior through increased inflammation. *C. albicans* in contrast is a commensal and did
350 not induce significant systemic inflammation. Rather, *C. albicans* colonization changed
351 the gut metabolome and altered eCB signaling to produce the observed increase in basal
352 CORT and anxiety-like behavior. To our knowledge, this is the first communication to
353 report that a commensal fungus, *C. albicans,* affects neuroendocrine host phenotypes and
354 that microbiota-induced changes to eCBs can affect the brain and behavior.

355 Treatment with the FAAH inhibitor URB597 alleviated both the elevated basal
356 CORT and increased anxiety-like behavior observed in *C. albicans*-colonized mice,
357 indicating a common mechanism of insufficient AEA. Consistent with the results of the
358 URB597 experiments, we measured a trend towards decreased AEA in the forebrain of
359 *C. albicans*-colonized mice, and found a correlation in *C. albicans*-colonized mice such
360 that those with the largest increase in basal CORT also had the lowest AEA, similar to
361 what has been seen in chronic stress models[35]. This negative correlation supported the
362 model that an AEA deficit was responsible for the neuroendocrine changes observed in
363 *C. albicans*-colonized mice. Previous investigations found that AEA levels specifically
364 within the prefrontal cortex[18] and the amygdala[35,46] were responsible for the
365 downregulation of CORT during recovery from stress and for anxiety-like behavior,
366 respectively. It is possible that within those discrete brain regions, colonized mice
367 exhibited a larger decrease in AEA than the decrease we measured in the forebrain as a
368 whole. Untargeted metabolomic analysis of cecum contents further supported the model
369 that *C. albicans* colonization altered eCB metabolism, as two alternate NAEs, linoleoyl

370 and linolenoyl ethanolamide, were increased in abundance in the *C. albicans*-colonized
371 mice as were compounds containing their FA precursors. We suggest that these changes
372 in GI tract eCB levels could reflect systemic changes in lipid availability and NAE
373 production and therefore disruption of normal eCB metabolism. Such disruption could
374 contribute to the altered eCB-CB1 signaling and consequent neuroendocrine changes
375 observed in the *C. albicans*-colonized mice.

376 Previous work investigating the gut microbiota and the HPA axis demonstrated a
377 window during which mice are sensitive to microbiota manipulation, as Sudo *et al*[8] found
378 that hyperreactivity of the HPA axis in germ-free mice could be reversed through
379 microbiota transplant in adolescent (6-week-old) mice but not in adult (8-week-old or 14-
380 week-old) mice. The current study was performed with mid-adolescent (5-week-old)
381 mice, which were sensitive to HPA axis dysregulation by GI tract colonization with *C.*
382 *albicans*. Lee *et al* showed that AEA levels in the brain increased significantly between
383 adolescence and adulthood[47], and thus the adolescent brain may be sensitive to the degree
384 of eCB disruption observed in the *C. albicans*-colonized mice. Previous researchers have
385 also demonstrated significant developmental differences in HPA axis regulation and
386 response in both rodents[48] and humans[49,50]. Adolescence in humans is a dynamic period
387 of development during which stress and anxiety disorders present a significant health
388 concern[51]; therefore ongoing research into neuroendocrine health using adolescent
389 models is relevant to human health. Women are significantly more likely to experience
390 anxiety disorders than men[52], and therefore this study using a female mouse model

391 provides valuable insight into dysregulation of the HPA axis and anxiety-like behavior in
392 a female population.

393 More broadly, these results highlight the sensitivity of the HPA axis and related
394 anxiety-like behavior to changes in AEA levels, and provide additional evidence that the
395 eCB system is a viable drug target for anxiety disorders. Indeed, a recent study in healthy
396 human volunteers demonstrated that treatment with a different FAAH inhibitor, PF-
397 04457845, raised AEA levels and protected against the development of stress-induced
398 anxiety[53], demonstrating that FAAH inhibition and increased AEA can reverse activation
399 of stress responses and modulate emotional behavior in humans as it does in mouse
400 models. Altogether this work demonstrates the ability of the gut metabolome to have a
401 significant impact on the brain and behavior, and illustrates the ability of the common gut
402 commensal fungus *C. albicans* to affect the host beyond the GI tract.

403 **Materials and Methods**

404 Detailed methods are presented in the Supplemental Information

405 **Animals**

406 Up to 23 five-week-old female C57BL/6 mice (Jackson Laboratory) were
407 cohoused in a large cage (24”x17”) and given sterile food, water and bedding. After four
408 days of acclimation, mice were transferred to standard cages with 3-4 mice inoculated
409 with *Candida albicans* or 3-4 mice given a mock-inoculum in each cage. Mice were put
410 through a behavioral test on day two and sacrificed on day three post-inoculation without
411 stress[54].

412 All experiments were done in compliance with Tufts University IACUC guidelines.

413 **Strains and growth conditions**

414 *C. albicans* strain CKY101[55] was used for all experiments. For preparation of
415 mouse inoculum, cells were grown at 37°C in standard yeast media for 24 hours. 25µl
416 ($5x10^7$ cells) of cells in PBS with 2% sucrose was fed to mice for gastrointestinal
417 colonization. 25µl of 2% sucrose in PBS was fed to mice for mock-inoculation.

418 **Drug treatment**

419 URB597 (Sigma Aldrich) and URB937 (Cayman Chemical) were dissolved in
420 18:1:1 normal saline:PEG400:Tween80 (Sigma Aldrich) and administered to mice via
421 intraperitoneal injection at a dosage of 0.1-1mg/kg bodyweight four hours prior to
422 behavioral testing or sacrifice.

423 **Restraint stress**

424 Mice were placed in a 50ml conical tube with two airholes enclosed with a rubber
425 stopper for 30 minutes. After this restraint, mice were placed back in their home cage
426 and anesthetized and sacrificed or allowed to recover in the home cage for 30 or 60
427 minutes and then anesthetized and sacrificed.

428 **Elevated Plus Maze**

429 Behavioral testing in the Elevated Plus Maze (EPM) was performed as described
430 in Walf *et al* 2013[25]. After a 5m trial in the EPM, the mouse was placed in a fresh sterile
431 cage. All trials were recorded with a video-camera from above and scored by a blinded
432 observer.

433 **Forced Swim Test**

434 Behavioral testing in the forced swim test was performed as described in Can *et al*
435 2012 [56]. A mouse was removed from its home cage and placed in the beaker of water for
436 a six-minute trial which was recorded by video-camera from the side and scored after the
437 fact by a blinded observer. After testing, mice were placed in a fresh sterile cage.

438 **Bacterial microbiota analysis**

439 The cecum, including contents, was dissected from mice after sacrifice on day
440 three and was immediately frozen on dry ice. Microbial DNA was extracted using the
441 QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturer's protocol. Libraries
442 were prepared from each sample and sequenced as described[57]. The resulting fastq files
443 were used as input for downstream analysis using QIIME (1.8.0)[23]. The resultant OTU
444 tables contained the relative abundance of bacterial taxa in each sample. Analysis of
445 alpha and beta diversity were performed using standard QIIME scripts.

446 **Measurement of hormones and cytokines in serum**

447 Trunk blood was collected into serum separator blood collection tubes (BD) after
448 sacrifice by decapitation. Tubes were then spun to separate serum. Serum was divided
449 into aliquots and frozen at -80°C.

450 Serum corticosterone was measured using a Corticosterone ELISA Kit (Enzo Life
451 Sciences) following the manufacturer's small volume protocol. Serum cytokines were
452 measured using a multiplex ELISA (Quanterix) following manufacturer's protocol.

453 **Immunohistochemistry for cFOS in hypothalamus slices**

454 Brains were dissected and fixed in 4% (w/v) paraformaldehyde in PBS for 24h at
455 4°C. They were then cryopreserved in sucrose gradient, rapidly frozen in isopentane
456 chilled on dry ice, and stored at -80°C. Free-floating sections were prepared using a
457 cryostat. Sections of interest were incubated with 1:5000 dilution of rabbit anti-mouse
458 cFos antibody (Sigma Aldrich F7799) for 72h, then stained using anti-rabbit IgG
459 (VectaStain Elite ABC Kit) and streptadvidin-Alexa488 (Molecular Probes). Sections
460 were imaged using a Zeiss microscope with Apotome attachment.

461 **Extraction and measurement of AEA in forebrain**

462 Lipid extraction from forebrain samples was performed as described previously[58].
463 AEA was measured using mass spectrometry as described previously[59].

464 **Untargeted metabolomic analysis of cecum contents**

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

470 **Real-time quantitative PCR analysis**

471 Tissues were frozen at -80°C in RNALater (Invitrogen). RNA was purified from
472 tissues using QIAzol for lysis and extraction with a Qialyzer, followed by column
473 purification using the Ambion Purelink Mini kit (Invitrogen). cDNA was synthesized
474 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.

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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.

References

1. Baxter, A. J., Scott, K. M., Vos, T. & Whiteford, H. A. Global prevalence of anxiety disorders: a systematic review and meta-regression. *Psychological Medicine* **43**, 897–910 (2013).
2. Leonardo, E. D. & Hen, R. Anxiety as a Developmental Disorder. *Neuropsychopharmacol* **33**, 134–140 (2008).
3. Luna, R. A. & Foster, J. A. Gut brain axis: diet microbiota interactions and implications for modulation of anxiety and depression. *Curr. Opin. Biotechnol.* **32**, 35–41 (2015).
4. Dallman, M. F. *et al.* Feast and Famine: Critical Role of Glucocorticoids with Insulin in Daily Energy Flow. *Frontiers in Neuroendocrinology* **14**, 303–347 (1993).
5. Sapolsky, R. M., Romero, L. M. & Munck, A. U. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr. Rev.* **21**, 55–89 (2000).
6. Kallen, V. L. *et al.* Associations between HPA axis functioning and level of anxiety in children and adolescents with an anxiety disorder. *Depression and Anxiety* **25**, 131–141 (2008).

7. Neufeld, K. M., Kang, N., Bienenstock, J. & Foster, J. A. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterol. Motil.* **23**, 255–264, e119 (2011).
8. Sudo, N. *et al.* Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *J. Physiol. (Lond.)* **558**, 263–275 (2004).
9. Messaoudi, M. *et al.* Assessment of psychotropic-like properties of a probiotic formulation (Lactobacillus helveticus R0052 and Bifidobacterium longum R0175) in rats and human subjects. *Br. J. Nutr.* **105**, 755–764 (2011).
10. Ait-Belgnaoui, A. *et al.* Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. *Psychoneuroendocrinology* **37**, 1885–1895 (2012).
11. Raimondi, S. *et al.* Longitudinal Survey of Fungi in the Human Gut: ITS Profiling, Phenotyping, and Colonization. *Front Microbiol* **10**, 1575 (2019).
12. Shao, T.-Y. *et al.* Commensal Candida albicans Positively Calibrates Systemic Th17 Immunological Responses. *Cell Host & Microbe* **25**, 404-417.e6 (2019).
13. Markey, L. *et al.* Pre-colonization with the commensal fungus Candida albicans reduces murine susceptibility to Clostridium difficile infection. *Gut Microbes* **9**, 497–509 (2018).
14. Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C. & Bonner, T. I. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* **346**, 561–564 (1990).
15. Devane, W. A. *et al.* Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* **258**, 1946–1949 (1992).
16. Mechoulam, R. *et al.* Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem. Pharmacol.* **50**, 83–90 (1995).
17. Placzek, E. A., Okamoto, Y., Ueda, N. & Barker, E. L. Mechanisms for recycling and biosynthesis of endogenous cannabinoids anandamide and 2-arachidonylglycerol. *Journal of Neurochemistry* **107**, 987–1000 (2008).
18. Hill, M. N. *et al.* Recruitment of prefrontal cortical endocannabinoid signaling by glucocorticoids contributes to termination of the stress response. *J. Neurosci.* **31**, 10506–10515 (2011).
19. Barna, I., Zelena, D., Arszovszki, A. C. & Ledent, C. The role of endogenous cannabinoids in the hypothalamo-pituitary-adrenal axis regulation: in vivo and in vitro studies in CB1 receptor knockout mice. *Life Sciences* **75**, 2959–2970 (2004).
20. Tiraboschi, I. C. N. *et al.* Congenital candidiasis: confirmation of mother-neonate transmission using molecular analysis techniques. *Med Mycol* **48**, 177–181 (2010).
21. Tuite, A., Elias, M., Picard, S., Mullick, A. & Gros, P. Genetic control of suceptibility to Candida albicans in susceptible A/J and resistant C57BL/6J mice. *Genes & Immunity* **6**, 672–682 (2005).
22. Erb Downward, J. R., Falkowski, N. R., Mason, K. L., Muraglia, R. & Huffnagle, G. B. Modulation of post-antibiotic bacterial community reassembly and host response by Candida albicans. *Scientific reports* **3**, 2191 (2013).
23. Caporaso, J. G. *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **7**, 335–336 (2010).

549 24. Segata, N. *et al.* Metagenomic biomarker discovery and explanation. *Genome Biol*
550 **12**, R60 (2011).
551 25. Walf, A. A. & Frye, C. A. The use of the elevated plus maze as an assay of
552 anxiety-related behavior in rodents. *Nature Protocols* **2**, 322–328 (2007).
553 26. Hascoët, M. & Bourin, M. The Forced Swimming Test in Mice: A Suitable Model
554 to Study Antidepressants. in *Mood and Anxiety Related Phenotypes in Mice* 85–118
555 (Humana Press, Totowa, NJ, 2009). doi:10.1007/978-1-60761-303-9_6.
556 27. Kalueff, A. V., Wheaton, M. & Murphy, D. L. What's wrong with my mouse
557 model?: Advances and strategies in animal modeling of anxiety and depression.
558 *Behavioural Brain Research* **179**, 1–18 (2007).
559 28. Nutt, D. J., Ballenger, J. C., Sheehan, D. & Wittchen, H.-U. Generalized anxiety
560 disorder: comorbidity, comparative biology and treatment. *Int J Neuropsychopharmacol*
561 **5**, 315–325 (2002).
562 29. Chung, S. *et al.* Cooperative roles of the suprachiasmatic nucleus central clock
563 and the adrenal clock in controlling circadian glucocorticoid rhythm. *Scientific Reports* **7**,
564 46404 (2017).
565 30. Patel, S., Roelke, C. T., Rademacher, D. J., Cullinan, W. E. & Hillard, C. J.
566 Endocannabinoid Signaling Negatively Modulates Stress-Induced Activation of the
567 Hypothalamic-Pituitary-Adrenal Axis. *Endocrinology* **145**, 5431–5438 (2004).
568 31. Moreira, F. A., Kaiser, N., Monory, K. & Lutz, B. Reduced anxiety-like
569 behaviour induced by genetic and pharmacological inhibition of the endocannabinoid-
570 degrading enzyme fatty acid amide hydrolase (FAAH) is mediated by CB1 receptors.
571 *Neuropharmacology* **54**, 141–150 (2008).
572 32. Hill, M. N. *et al.* Suppression of amygdalar endocannabinoid signaling by stress
573 contributes to activation of the hypothalamic-pituitary-adrenal axis.
574 *Neuropsychopharmacology* **34**, 2733–2745 (2009).
575 33. Fegley, D. *et al.* Characterization of the fatty acid amide hydrolase inhibitor
576 cyclohexyl carbamic acid 3'-carbamoyl-biphenyl-3-yl ester (URB597): effects on
577 anandamide and oleoylethanolamide deactivation. *J. Pharmacol. Exp. Ther.* **313**, 352–
578 358 (2005).
579 34. Piomelli, D. *et al.* Pharmacological profile of the selective FAAH inhibitor KDS-
580 4103 (URB597). *CNS Drug Rev* **12**, 21–38 (2006).
581 35. Hill, M. N. *et al.* Endogenous cannabinoid signaling is essential for stress
582 adaptation. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 9406–9411 (2010).
583 36. Imperatore, R. *et al.* Genetic deletion of monoacylglycerol lipase leads to
584 impaired cannabinoid receptor CB1R signaling and anxiety-like behavior. *Journal of*
585 *Neurochemistry* **135**, 799–813 (2015).
586 37. Sihag, J. & Jones, P. J. H. Dietary fatty acid profile influences circulating and
587 tissue fatty acid ethanolamide concentrations in a tissue-specific manner in male Syrian
588 hamsters. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*
589 **1864**, 1563–1579 (2019).
590 38. Artmann, A. *et al.* Influence of dietary fatty acids on endocannabinoid and N-
591 acylethanolamine levels in rat brain, liver and small intestine. *Biochimica et Biophysica*
592 *Acta (BBA) - Molecular and Cell Biology of Lipids* **1781**, 200–212 (2008).

593 39. Xie, Z. *et al.* Analysis of transcriptome and metabolome profiles alterations in
594 fatty liver induced by high-fat diet in rat. *Metabolism* **59**, 554–560 (2010).
595 40. Lee, K. N., Pariza, M. W. & Ntambi, J. M. Conjugated Linoleic Acid Decreases
596 Hepatic Stearoyl-CoA Desaturase mRNA Expression. *Biochemical and Biophysical*
597 *Research Communications* **248**, 817–821 (1998).
598 41. Oosterveer, M. H. *et al.* High Fat Feeding Induces Hepatic Fatty Acid Elongation
599 in Mice. *PLOS ONE* **4**, e6066 (2009).
600 42. Massillon, D., Arinze, I. J., Xu, C. & Bone, F. Regulation of Glucose-6-
601 phosphatase Gene Expression in Cultured Hepatocytes and H4IIE Cells by Short-chain
602 Fatty Acids ROLE OF HEPATIC NUCLEAR FACTOR-4α. *J. Biol. Chem.* **278**, 40694–
603 40701 (2003).
604 43. Chen, G. Liver lipid molecules induce PEPCK-C gene transcription and attenuate
605 insulin action. *Biochemical and Biophysical Research Communications* **361**, 805–810
606 (2007).
607 44. Bercik, P. *et al.* Chronic gastrointestinal inflammation induces anxiety-like
608 behavior and alters central nervous system biochemistry in mice. *Gastroenterology* **139**,
609 2102-2112.e1 (2010).
610 45. Lyte, M., Li, W., Opitz, N., Gaykema, R. P. A. & Goehler, L. E. Induction of
611 anxiety-like behavior in mice during the initial stages of infection with the agent of
612 murine colonic hyperplasia Citrobacter rodentium. *Physiol. Behav.* **89**, 350–357 (2006).
613 46. Gray, J. M. *et al.* Corticotropin-Releasing Hormone Drives Anandamide
614 Hydrolysis in the Amygdala to Promote Anxiety. *J. Neurosci.* **35**, 3879–3892 (2015).
615 47. Lee, T. T.-Y., Hill, M. N., Hillard, C. J. & Gorzalka, B. B. Temporal changes in
616 N-acylethanolamine content and metabolism throughout the peri-adolescent period.
617 *Synapse* **67**, 4–10 (2013).
618 48. Romeo, R. D. *et al.* Pubertal shifts in adrenal responsiveness to stress and
619 adrenocorticotropic hormone in male rats. *Psychoneuroendocrinology* **42**, 146–152
620 (2014).
621 49. Netherton, C., Goodyer, I., Tamplin, A. & Herbert, J. Salivary cortisol and
622 dehydroepiandrosterone in relation to puberty and gender. *Psychoneuroendocrinology* **29**,
623 125–140 (2004).
624 50. Gunnar, M. R., Wewerka, S., Frenn, K., Long, J. D. & Griggs, C. Developmental
625 changes in hypothalamus–pituitary–adrenal activity over the transition to adolescence:
626 Normative changes and associations with puberty. *Dev Psychopathol* **21**, 69–85 (2009).
627 51. Siegel, R. S. & Dickstein, D. P. Anxiety in adolescents: Update on its diagnosis
628 and treatment for primary care providers. *Adolescent Health, Medicine and Therapeutics*
629 **3**, 1 (2012).
630 52. McLean, C. P., Asnaani, A., Litz, B. T. & Hofmann, S. G. Gender differences in
631 anxiety disorders: Prevalence, course of illness, comorbidity and burden of illness.
632 *Journal of Psychiatric Research* **45**, 1027–1035 (2011).
633 53. Mayo, L. M. *et al.* Elevated anandamide, enhanced recall of fear extinction, and
634 attenuated stress responses following inhibition of fatty acid amide hydrolase (FAAH): a
635 randomized, controlled experimental medicine trial. *Biological Psychiatry* (2019)
636 doi:10.1016/j.biopsych.2019.07.034.

637 54. Sarkar, J., Wakefield, S., MacKenzie, G., Moss, S. J. & Maguire, J.
638 Neurosteroidogenesis Is Required for the Physiological Response to Stress: Role of
639 Neurosteroid-Sensitive GABAA Receptors. *J. Neurosci.* **31**, 18198–18210 (2011).
640 55. Brown, D. H., Jr., Giusani, A. D., Chen, X. & Kumamoto, C. A. Filamentous
641 growth of Candida albicans in response to physical environmental cues and its regulation
642 by the unique CZF1 gene. *Molecular microbiology* **34**, 651–62 (1999).
643 56. Can, A. *et al.* The Mouse Forced Swim Test. *J Vis Exp* (2012) doi:10.3791/3638.
644 57. Caporaso, J. G. *et al.* Ultra-high-throughput microbial community analysis on the
645 Illumina HiSeq and MiSeq platforms. *ISME J* **6**, 1621–1624 (2012).
646 58. Morena, M. *et al.* Training-Associated Emotional Arousal Shapes
647 Endocannabinoid Modulation of Spatial Memory Retrieval in Rats. *J. Neurosci.* **35**,
648 13962–13974 (2015).
649 59. Qi, M., Morena, M., Vecchiarelli, H. A., Hill, M. N. & Schriemer, D. C. A robust
650 capillary liquid chromatography/tandem mass spectrometry method for quantitation of
651 neuromodulatory endocannabinoids. *Rapid Commun. Mass Spectrom.* **29**, 1889–1897
652 (2015).
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657 **Figure Legends**

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

660 A) Timeline for acute colonization model. 5-week-old female mice were
661 cohoused for four days and handled daily. Mice were then moved into smaller groups in
662 standard cages and orally inoculated with $5x10^7$CFU of *C. albicans* or given buffer. On
663 day one post-inoculation, fecal pellets were sterilely collected from mice and plated on
664 YPD-SA to measure colonization levels. On day two post-inoculation, all mice
665 underwent a behavioral test. On day three post-inoculation mice were anesthetized with
666 isoflurane and sacrificed by decapitation. Some mice were subjected to restraint stress
667 prior to sacrifice. B) *C. albicans* CFU/g of fecal pellets was measured on day 1 post-
668 inoculation and CFU/g of cecum contents was measured after sacrifice on day three post-
669 inoculation. No culturable fungi were measured on either day from mock-colonized
670 mice. Day 1: N=56, Day 3: N=50. Figure includes data from 6 cohorts. Bars indicate
671 geometric mean. C) A multiplex ELISA was used to measure inflammatory cytokines in
672 the serum of mice. N.D, not detectable. Student's t-test, $p<0.05$. Mock-colonized N=13,
673 *C. albicans*-colonized N=14. Figure includes data from 3 cohorts. D-F) Microbiota
674 analysis of the cecum contents of mice was performed using standard 16s rRNA DNA
675 sequencing and QIIME analysis pipeline. Mock-colonized N=8 and *C. albicans*-
676 colonized N=8. Figure includes data from 2 cohorts. D) The relative abundance of all
677 bacterial families detected with a median relative abundance greater than 0 is shown. Bar
678 shows geometric mean with SEM E) The average alpha diversity metric Chao1 was
679 calculated to determine the diversity of the microbiota at different levels of sampling.
680 The average Chao1 score of the experimental groups is shown. F) Beta diversity was
681 calculated using weighted UniFrac scores and principal coordinates analysis was
682 performed. For C-D, symbols indicate individual mice and bars indicate the average.
683 Mock-colonized mice are represented as solid dots and bars; *C. albicans*-colonized mice
684 are represented as open circles and bars.

685

686 **Figure 2: *C. albicans* colonization increases anxiety-like behavior**
687 On day two post-inoculation mice underwent either the Elevated Plus Maze
688 (EPM) or the Forced Swim Test (FST). Trials in the behavioral tests were video-
689 recorded and scored by a blinded observer after the fact. For the EPM data (A-F) that
690 follows, mock-colonized N=22 and *C. albicans*-colonized N=19 and statistical analysis
691 performed was Welch's t-test, $p<0.05$. Figures include data from two cohorts. A)
692 Percentage of time spent in the open arms of the EPM. B) Total number of entries into
693 the open arms of the EPM. C) Percentage of time spent in the closed arms of the EPM.
694 D) Total entries into the closed arms of the EPM. E) Percentage of time spent in the
695 neutral central square of the EPM (neither open nor closed arms). F) Total entries during
696 the trial (sum of the open and closed arm entries), a metric for locomotor activity. G)
697 Total time spent immobile during the six-minute FST trial. Immobility was defined as no
698 movements beyond those required to stay afloat. For FST data: mock-colonized N=33,
699 *C. albicans*-colonized N=24. Figure includes data from three cohorts. Throughout,
700 symbols represent individual mice and bars indicate average. Mock-colonized mice are
701 represented as solid dots and bars; *C. albicans*-colonized mice are represented as open
702 circles and bars.

703

704

705 **Figure 3: *C. albicans* colonization increases basal CORT and alters feedback**
706 **inhibition after acute stress**
707 A) Corticosterone (CORT) in trunk blood of mice sacrificed without stress
708 was measured by ELISA. Mock-colonized N=15, *C. albicans*-colonized N=19. Figure
709 includes data from four cohorts. Data was analyzed using Welch's t-test, $p<0.05$ B)
710 CORT in trunk blood of mice sacrificed at different times of day. In addition to the data
711 summarized in A (mice sacrificed from 13:00-16:00), B includes mice sacrificed without
712 stress from 17:00-18:00. Data was binned in one-hour increments. Mock-colonized:
713 13:00 N=5, 14:00 N=9, 15:00 N=11, 16:00 N=8, 17:00 N=9, 18:00 N=5. *C. albicans*-
714 colonized: 13:00 N=6, 14:00 N=13, 15:00 N=10, 16:00 N=11, 17:00 N=8, 18:00 N=9.
715 C) Mice were subjected to 30 minutes of restraint stress and then sacrificed immediately
716 (0m recovered) or after 30 minutes or 60 minutes of recovery as shown on the x-axis.
717 Mock-colonized: 0m N=14, 30m N=15, 60m N=13. *C. albicans*-colonized: 0m N=15,
718 30m N=13, 60m N=12. D) Immunohistochemistry was used to visualize cFos protein
719 with Alexa488 in the paraventricular nucleus (PVN) of mice using the 20X objective.
720 DAPI was used to visualize nuclei and Alexa488 to visualize cFOS. The PVN of mice
721 sacrificed unstressed (left) do not exhibit cFOS staining, while PVN of mice sacrificed
722 after 30 minutes of stress and 30 minutes of recovery (right) are positive for cFOS.
723 Representative images are shown. For all figures, symbols indicate individual mice and
724 bars indicate the average. Mock-colonized mice are solid dots and bars and *C. albicans*-
725 colonized mice are open circles and bars.

726

727 **Figure 4: Neuroendocrine phenotypes of the *C. albicans*-colonized mice are**
728 **mediated through disruption of the endocannabinoid system**
729 A-C) Mice were treated with the fatty acid amide hydrolase (FAAH) inhibitor
730 URB597, a well-characterized drug which increases AEA levels by blocking degradation.
731 Mice were given either URB597 or vehicle control by intraperitoneal injection 4-6 hours
732 prior to sacrifice (A) or testing in the EPM (B-C). A) Mice were treated with 1mg/kg of
733 URB597 or vehicle. ELISA was used to measure CORT in trunk blood of mice
734 sacrificed without stress. Welch's t-test, $p<0.05$. Mock-colonized: vehicle N=15,
735 URB597 N=19. *C. albicans*-colonized: vehicle N=16, URB597 N=18. Figure includes
736 data from four cohorts. B-C) Mice were treated with 0.1mg/kg (triangles) or 0.15mg/kg
737 (circles) URB597 or vehicle, then tested in the EPM. Mock-colonized: vehicle N=17,
738 0.1mg/kg URB597 N=4, 0.15mg/kg URB597 N=8. *C. albicans*-colonized: vehicle
739 N=13, 0.1mg/kg URB597 N=4 0.15mg/kg URB597 N=8. B) Percentage time spent in
740 the open arms, normalized to the average percent open time of the mock-colonized
741 vehicle control, is shown. Welch's t-test, $p<0.05$. C) Total arms entries, a metric for
742 overall locomotor activity is shown. D) AEA (pmol/g tissue) was measured in forebrain
743 samples by mass spectrometry. E-F) Linear regression of basal serum CORT and
744 forebrain AEA (D) in individual mice are shown. D-F Mock-colonized N=7, *C.*
745 *albicans*-colonized N=9. Untargeted mass spectrometry was used to measure relative
746 abundance of 735 compounds in the cecum contents of mice. N-acylethanolamides (E-
747 G), free fatty acids (H-I) and lysophospholipids (J) containing an 18-carbon chain with
748 varying degrees of unsaturation are shown here. Raw data was log-transformed. Mock-
749 colonized N=8, *C. albicans*-colonized N=8. Student's t-test, $p<0.05$. Symbols indicate
750 individual mice and bars indicate average. Solid circles and bars are mock-colonized
751 mice and open circles and bars are *C. albicans*-colonized mice.

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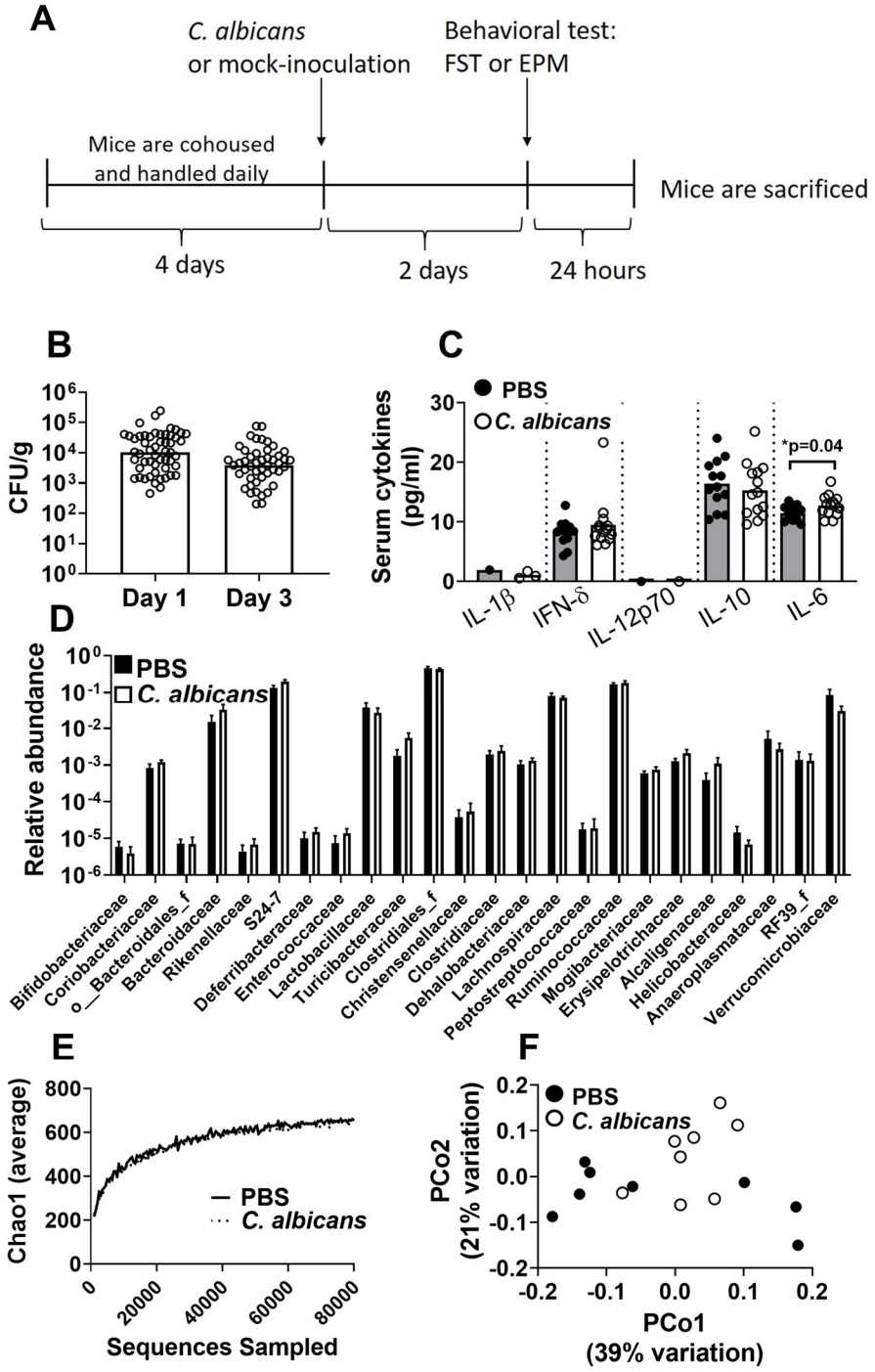
755 **Figure 5: *C. albicans* colonization affects hepatic lipid-responsive gene expression**
756 RNA was extracted from the liver of mice sacrificed unstressed on day three.
757 cDNA was synthesized and qRT-PCR was used to quantify liver gene transcription.
758 RNA gene expression is normalized to GAPDH and expressed relative to the average
759 expression of the mock-colonized mice. A) Stearoyl-CoA desaturase 1 (*Scd1*) RNA
760 expression in the liver is shown. Mock-colonized N=24, *C. albicans*-colonized N=27.
761 Figure includes data from four cohorts. B) Expression of multiple lipogenic genes is
762 shown: acyl-CoA 8-3 desaturase (*Fads1*), acyl-CoA 6 desaturase (*Fads2*), sterol
763 regulatory element-binding protein 1 (*Srepbf1*) and fatty acid synthase (*Fasn*). Mock-
764 colonized N=11, *C. albicans*-colonized N= 10. Figure includes data from two cohorts.
765 C) Expression of the gluconeogenesis enzymes phosphoenolpyruvate carboxykinase
766 (*Pck1*) (mock-colonized N=24, *C. albicans*-colonized N=23) and D) glucose-6-
767 phosphatase (*G6Pc*) is shown (mock-colonized N=20, *C. albicans*-colonized N=18).
768 Figures include data from four cohorts. Symbols represent individual mice and bars
769 indicate average. Solid symbols and bars indicate mock-colonized mice and open
770 symbols and bars indicate *C. albicans*-colonized mice.

771

772 **Figures**

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



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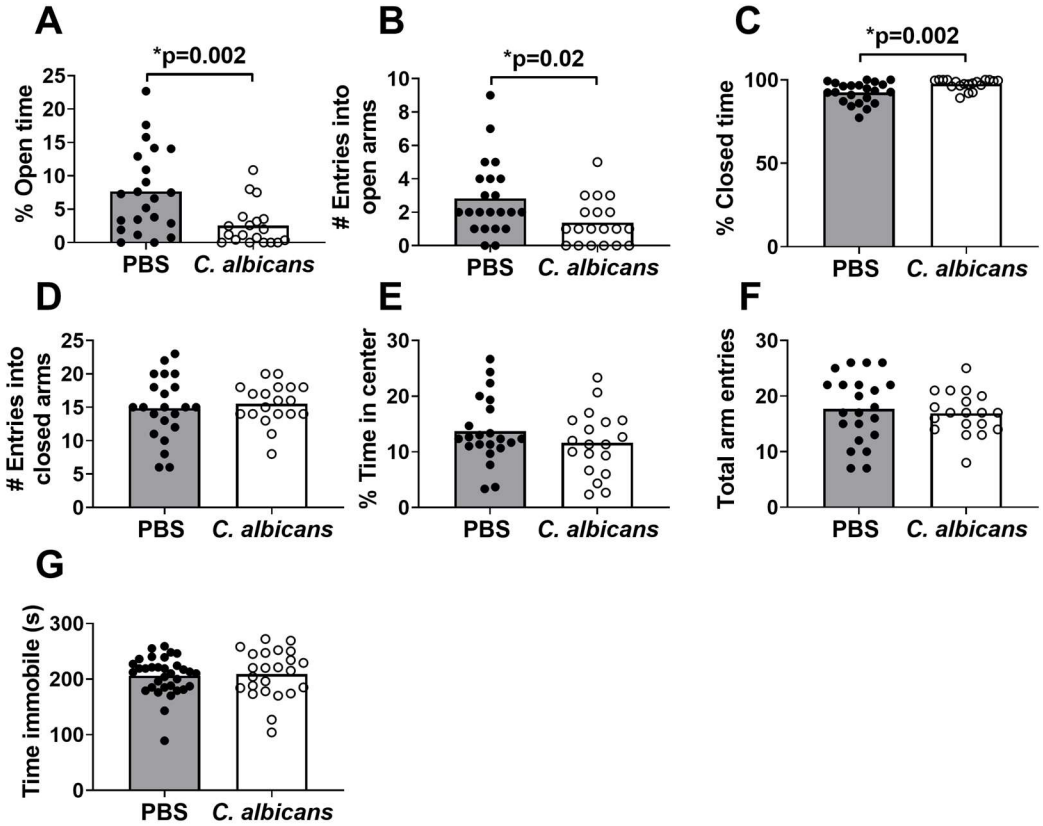
777 **Figure 2: *C. albicans* colonization increases anxiety-like behavior**



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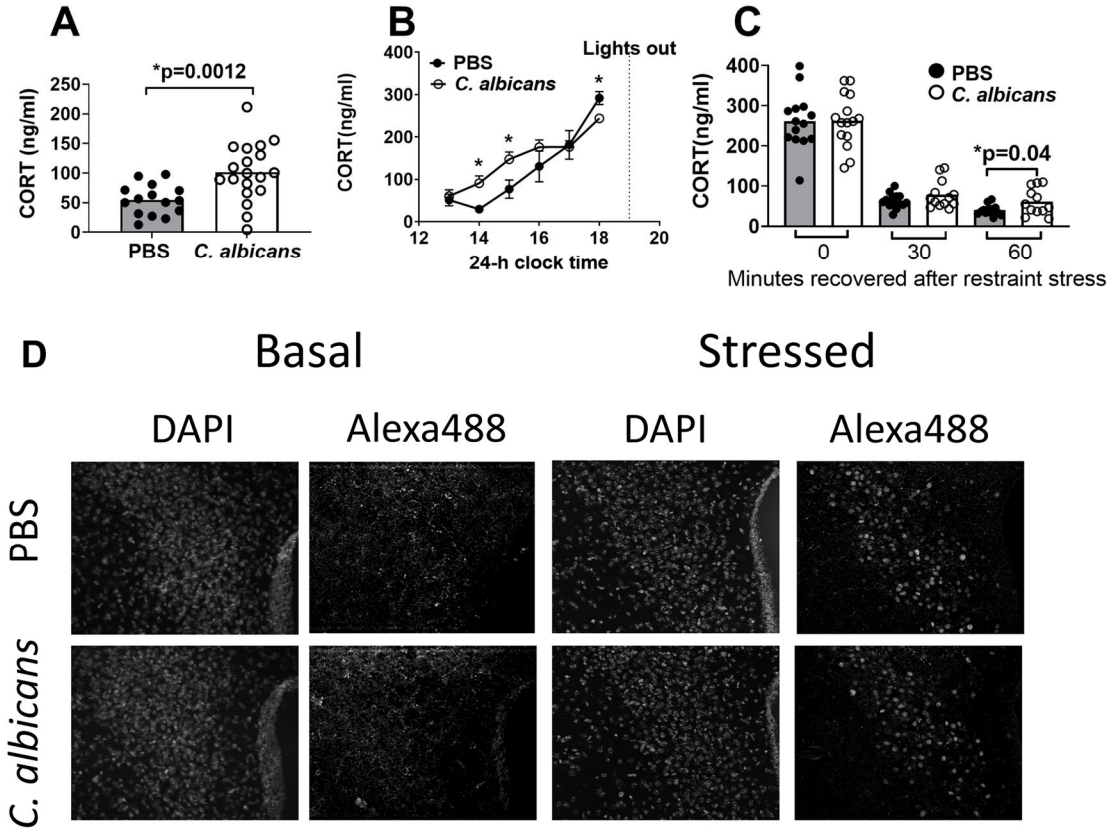
780 **Figure 3: *C. albicans* colonization increases basal CORT and alters feedback**
781 **inhibition after acute stress**



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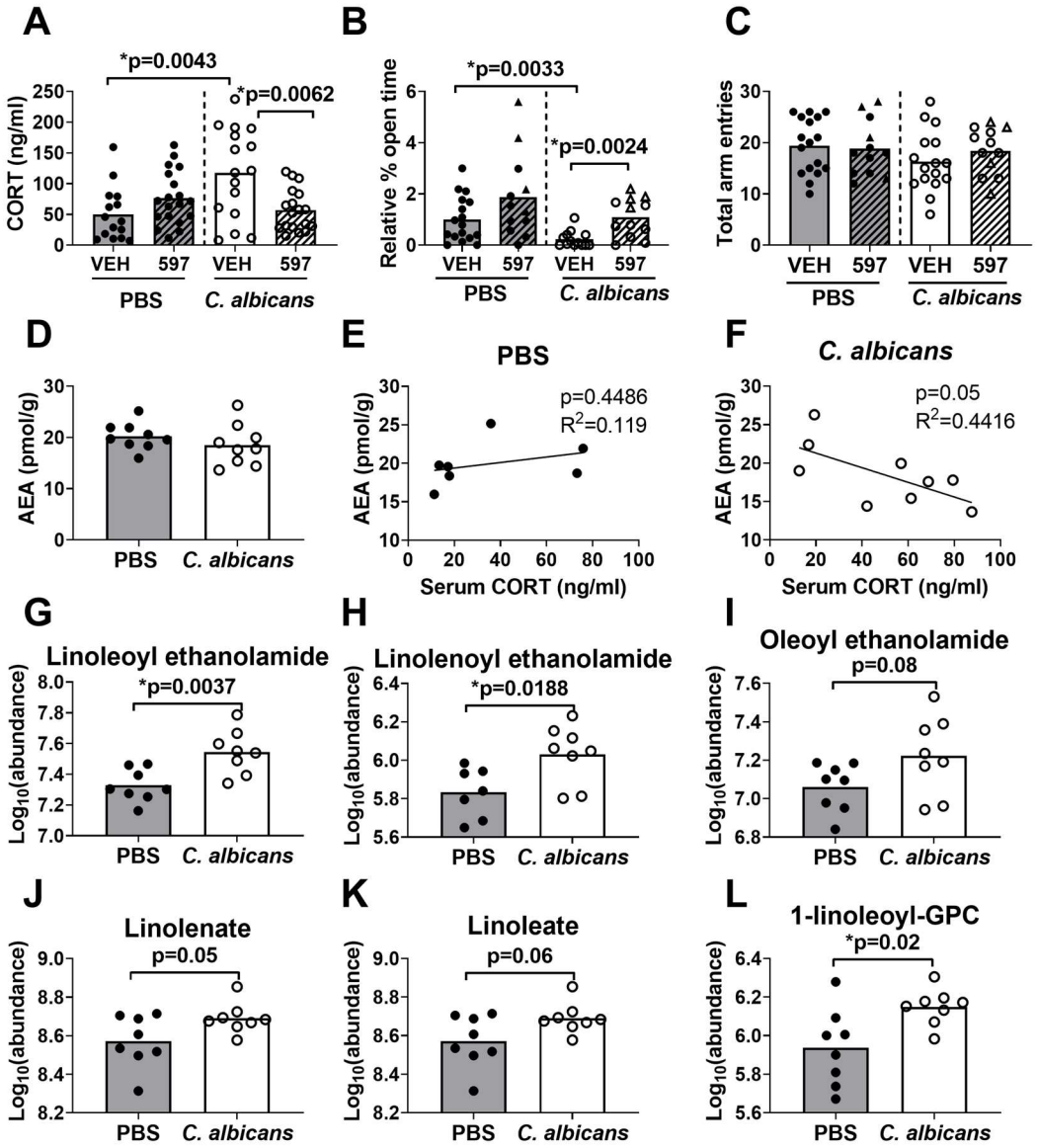
784 **Figure 4: Neuroendocrine phenotypes of the *C. albicans*-colonized mice are**
785 **mediated through disruption of the endocannabinoid system**



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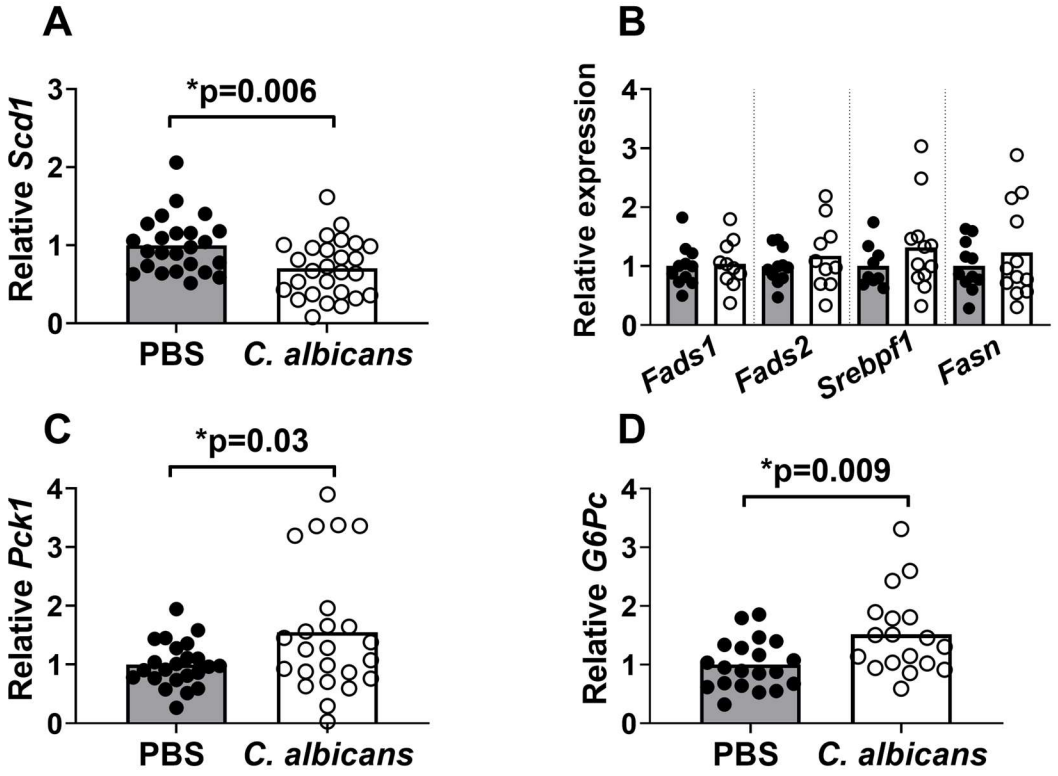
789 **Figure 5: *C. albicans* colonization affects hepatic lipid-responsive gene expression**



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