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► **To cite this version:**

Alicia Moreno-Sabater, Delphine Sterlin, Lejla Imamovic, Fabienne Bon, Anne-Cecile Normand, et al.. Intestinal *Candida albicans* overgrowth in IgA deficiency. *Journal of Allergy and Clinical Immunology*, In press, 10.1016/j.jaci.2023.03.033 . hal-04099050

HAL Id: hal-04099050

<https://hal.science/hal-04099050v1>

Submitted on 16 May 2023

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1 Title: **Intestinal *Candida albicans* overgrowth in IgA deficiency**

2 Running title: **IgA impact on host-fungal mutualism**

3

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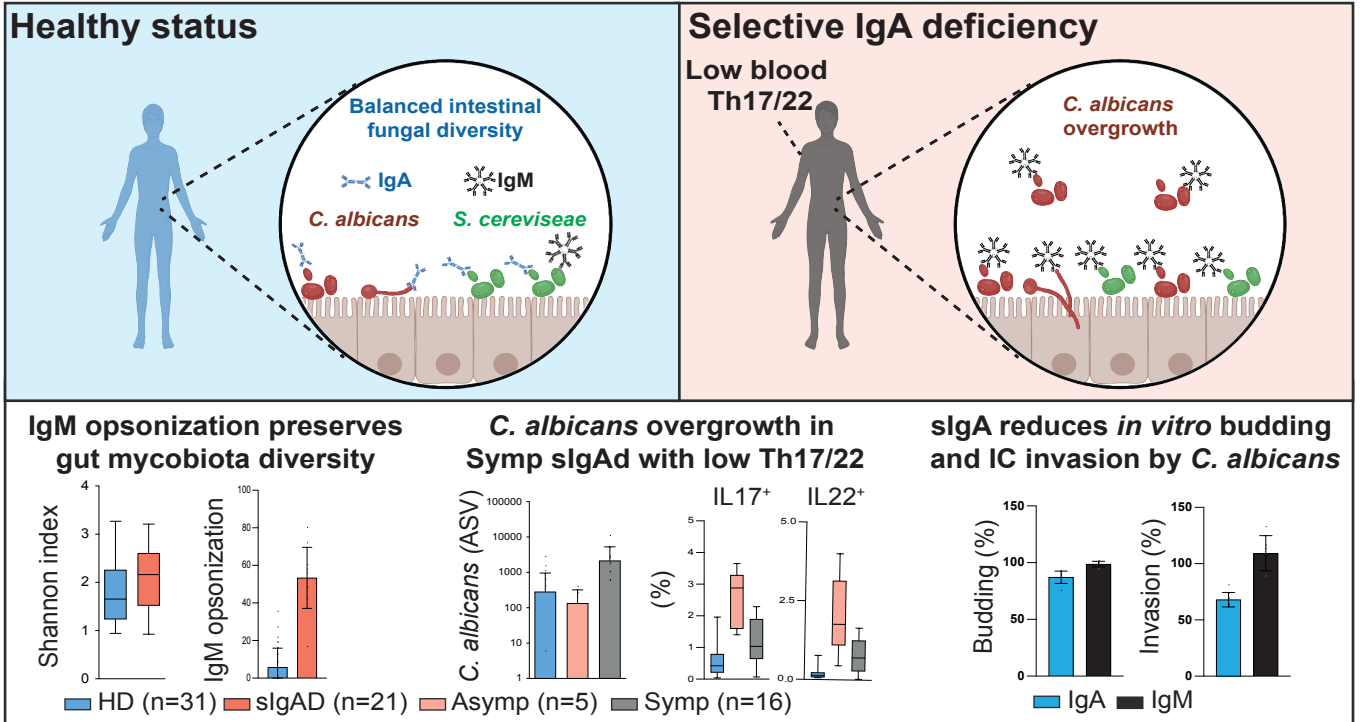
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Intestinal *Candida albicans* overgrowth in IgA deficiency



HD: healthy donor; slgAd: selective IgA deficient patient; Asymp: slgAd asymptomatic; Symp: slgAd symptomatic; ASV: amplicon sequence variant; IC: intestinal cell



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39

40 Word count : 4408

41 Abstract**42 Background**

43 Secretory IgA interacts with commensal bacteria, but its impact on human mycobiota ecology
44 has not been widely explored. In particular, it remains unknown whether human IgA-deficiency
45 is associated with gut fungal dysbiosis.

46 Objectives

47 Our goal was to study the impact of IgA on gut mycobiota ecology.

48 Methods

49 The Fungi-flow method was used to characterize fecal, systemic, and maternal IgA, IgM and
50 IgG responses against 14 representative fungal strains (yeast/spores or hyphae forms) in healthy
51 donors (HD, n= 34, 31 and 20, respectively), and also to compare gut mycobiota opsonization
52 by secretory antibodies in HD (n=28) and patients with selective IgA deficiency (SIgAd n=12).
53 Stool mycobiota composition was determined by ITS gene sequencing in HD (n= 23) and
54 SIgAd (n=17). Circulating CD4+ T cell cytokine secretion profiles were determined by
55 intracellular staining. Impact of secretory IgA, purified from breast milk (n=9),
56 on candida growth and intestinal Caco-2 cell invasion was tested *in vitro*.

57 Results

58 Homeostatic IgA binds commensal fungi with a body fluid-selective pattern of recognition. In
59 SIgAd patients, fungal gut ecology is preserved by compensatory IgM binding to commensal
60 fungi. Gut *Candida albicans* overgrowth nevertheless occurs in this condition, but only in
61 clinically symptomatic patients with decreased Th17/22 T cell responses. Indeed, secretory IgA
62 can reduce *in vitro* budding and invasion of intestinal cells by *C. albicans*, and therefore exert
63 control on this pathobiont.

64 Conclusion

65 IgA has a selective impact on *C. albicans* ecology to preserve fungal-host mutualism.

66 **Clinical implication**

67 Understanding of IgA-fungi interactions reveals the potential of secretory antibodies in
68 modulating mycobiota for therapeutic purposes.

69

70 **Capsule Summary**

71 IgA show distinct commensal fungi-specific recognition patterns across body fluids and
72 contribute to the prevention of intestinal *Candida albicans* overgrowth that cannot be
73 compensated by IgM in symptomatic IgA-deficient patients.

74

75

76 **Key words:**

77 Mycobiota, homeostatic IgA, IgA deficiency, IgM, *Candida albicans*

78

79 **Abbreviations**

80 ASV : Amplicon sequence variants

81 HD : Healthy donors

82 IL : Interleukin

83 MFI : Median fluorescence intensity

84 PCA: Principal component analysis

85 sIgA : Secretory immunoglobulin A

86 SIgAd : Selective IgA deficiency

87 Introduction

88 Mounting evidence of gut mycobiota involvement in human health and disease has increased
89 the interest in the immune mechanisms controlling host microbiota mutualism.¹ Commensal
90 fungi induce innate responses, implicating CX3CR1⁺ macrophages via CARD-9 signaling
91 pathway, as well as Th17 cell-mediated adaptive responses which are essential to maintain
92 mycobiota homeostasis.² Th17 cells also exert innate immune mechanisms, notably via the
93 production of interleukin (IL)-17A and IL-17F, both cytokines promoting local neutrophil
94 recruitment and playing an active role in the maintenance of integrity of epithelial barrier sites.³

95

96 The role of antibodies in intestinal host-fungi symbiosis remains in contrast poorly
97 characterized. Secretory immunoglobulin A (sIgA) is of particular interest, because of its
98 mucosal localization and its reported involvement in mediating microbial commensalism.⁴
99 Although the human mycobiota contains a large number of fungal genera,⁵ sIgA-fungal
100 interactions have focused on *Candida albicans*, a human commensal and a major fungal
101 pathogen.⁶ Hence, in a mouse model of oropharyngeal candidiasis, opsonization of this
102 commensal by sIgA prevented fungal epithelial adhesion and invasion, contributing to control
103 commensal *C. albicans* carriage.⁷ Central to the pathogenic potential of *C. albicans* is its ability
104 to convert from round yeast cells to a hyphenated form able to invade mucosal epithelial cells,
105 leading to blood infection. Importantly, sIgA mainly recognizes the latter morphotype,
106 suggesting that sIgA opsonization constitutes a regulatory mechanism of intestinal fungal
107 commensalism.^{8, 9} Indeed, in patients with Crohn's disease, reduced gut sIgA reactivity to
108 *Candida* hyphae-produced proteins was found to correlate with an increase in hyphal fungal
109 morphology.⁸

110

111 In addition to the regulatory role of intestinal sIgA, the involvement of systemic IgG in host-
112 mycobiota interactions has recently been highlighted. Systemic IgG responses targeting
113 *Saccharomyces cerevisiae* were initially considered secondary to fungal translocations due to
114 gut barrier defects in relation with intestinal bowel disease.¹⁰⁻¹³ We however recently reported
115 that systemic IgG responses against commensal fungi can also be detected in healthy subjects,¹⁴
116 while CARD9-deficient patients with systemic candidiasis present decreased anti-fungal
117 commensal IgG levels.¹⁵ Finally, in a CARD9-deficiency murine model, intestinal *C. albicans*
118 interactions with CX3CR1⁺macrophages failed to elicit IgG class switch recombination and
119 germinal center B cell expansion.⁸

120

121 The above-mentioned recent studies support the view that, besides cellular responses, anti-
122 fungal IgG responses are associated with a status of host-fungal mutualism. We postulated that
123 it is IgA that plays a key role in the establishment of this equilibrium by preserving gut
124 mycobiota diversity and intestinal barrier homeostasis. Here, we characterized fecal, systemic,
125 and maternal antifungal humoral responses in a cohort of healthy donors and evaluated
126 alterations of gut fungal diversity in a cohort of selective IgA deficient patients.

127

128 **Material and methods**

129 **Human samples**

130 Samples from patients with a selective IgA deficiency (SIgAd) and healthy donors (HD) used
131 in the present cross-sectional study are part of a previously published collection.¹⁶ The main
132 criterium for the inclusion of SIgAd patients were undetectable serum IgA (<0.07 mg/mL), but
133 normal IgG levels. Exclusion criteria were antibiotic therapy and laxative drug use during the
134 last 3 months before stool collection. Clinical and biological data were collected at inclusion
135 time. Fresh stool, fecal waters, purified microbiota, DNA from fresh stools, serum samples and
136 data from immunological status of HD and SIgAd patients were generated, as described
137 previously.¹⁶ Oral and written consent was obtained from patients before inclusion in the study.
138 Fresh breast milk of healthy lactating women was obtained from the Lactarium Regional d'Ile
139 de France (Hopital Necker Enfants Malades, Pôle Mère-Enfant). Breast milk sample use for
140 research was approved by the ethical committee (Avis CENEM 2020-VR
141 Biocol_lactothèque_v1.0_20190408). IgA was purified from breast milk and IgM from sera of
142 SIgAd patients using peptide M (InvivoGen).

143

144 **Fungal strains and culture conditions**

145 A fungal biobank, including 14 fungal strains, was generated from different genera commonly
146 described in human skin, lung, and intestinal mycobiota.¹⁴ Fungal reference strains were
147 obtained for *C. albicans* (ATCC90028) and *Aspergillus fumigatus* (ATCC204305), whereas
148 other fungal strains, isolated for clinical purpose, were obtained from *in vitro* culture of human
149 stool, skin and bronchoalveolar lavage samples. Fungi were cultured in Sabouraud agar medium
150 (Bio-Rad) between 30 and 35 °C, depending on the fungus studied. To generate budding forms,
151 yeast or spores from filamentous fungi were kept at 4 °C in RPMI-1640 medium (Gibco) for 2
152 h and then cultured at 30 °C under stirring for 9 h. At the end of the incubation period, cultures

153 were centrifuged at 21,000 g for 20 min. Supernatant was removed and fungi were resuspended
154 in cryopreservation medium.

155

156 **Quantification of humoral responses**

157 Systemic, as well as fecal water- and breast milk-derived, secretory IgA responses were
158 determined using the Fungi-flow method,¹⁴ as follows. Twenty-five microliters of fixed (2%
159 PFA/PBS) samples containing 2.5×10^5 fungi were distributed in a 96 well conic bottom plate.
160 Twenty-five microliters of normalized samples were then distributed in each well and incubated
161 for 20 min at 4 °C. Seventy microliters of PBS were added and samples were centrifuged at
162 3200g during 10 min. After removal of supernatant, fungi were incubated with a 25 µL mix (10
163 µg/mL) of goat anti-human IgG-Alexa Fluor 647 (Jackson Immunoresearch) or goat anti-
164 human IgA-FITC (Jackson Immunoresearch) or donkey anti-human IgM-PE (Jackson
165 Immunoresearch) for 20 min at 4 °C. After one washing step with PBS, fungi were resuspended
166 in 150 µL of PBS and analyzed on a Cytoflex flow cytometer (Beckman coulter). Unstained
167 spores or budding forms were used as negative control to determine background fluorescence.
168 Flow cytometry data were analyzed using the FlowJo program (version 10; Becton Dickinson)
169 and expressed as median fluorescence intensity (MFI).

170

171 **Quantification of *in vivo* antibody-binding levels to commensal fungi**

172 Whole microbiota was extracted by gradient purification in anaerobic conditions (Freter
173 chamber) as previously described.¹⁷ Microbiota samples were stored at -80°C and thawed
174 before use. Three microliters (10^7 microorganisms/µL) were fixed in 500 µL of 2% (w/v) PFA
175 in PBS, supplemented with 4 % of calcofluor white dye (Sigma) for 20 min at 4°C. After
176 washing with 1 mL PBS (9000 g for 10min at 4°C), bacteria and fungi were resuspended in
177 PBS at a concentration of 3×10^7 /mL with the appropriate secondary staining antibody, washed

178 and analyzed as above. An IgA isotype control antibody (FITC ChromPure Goat Serum,
179 Jackson ImmunoResearch) was used to correct for unspecific binding. In order to better define
180 mycobiota flow cytometry events among fecal microbiota, we determined the SSC/FSC
181 distribution of a fungal mock population containing most prevalent fungi found in human
182 mycobiota,⁵ to determine flow cytometry gates (Fig E1, A).

183

184 **DNA sequencing**

185 Sequencing libraries were constructed by amplifying the 18S rRNA gene ITS2 region using the
186 PCR amplification protocol previously described.¹⁴ DNA samples were subjected to PCR
187 amplification using the following primers: ITS2_FwTag
188 (5'CTTCCCTACACGACGCTCTTCCGATCTGTGARTCATCGAATCT TT-3') and ITS2_
189 RvTag (5'-GGAGTTCAGACGTGTGCTCTTCCGATCTGATATGCTTAAGTTCAGCGG
190 GT3'). Amplification reaction was performed with DNA MolTaq (Molzym, Bremen, Germany)
191 in a total volume of 50 µL containing 1 µM of each primer and 1 µL genomic DNA from
192 extracted stool samples. Cycling conditions were initial denaturation at 94 °C for 60 s, 35 cycles
193 of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and elongation at 72 °C for 45 s,
194 followed by a final elongation step at 72 °C for 7 min. PCR amplicon libraries were sequenced
195 on a MiSeq Illumina instrument (Genotoul, Toulouse, France) producing 2 X 300 bp paired-
196 end reads.

197

198 **ITS amplicon profiling**

199 We applied DADA2 (version 1.20.0) for further processing of demultiplexed sequence reads¹⁸
200 in R (version 4.1.0). The full amplicon DADA2 workflow: filtering, dereplication, sample
201 inference, chimaera identification, and merging of paired-end reads was employed by using a
202 default setting. We used standard filtering parameters: maxN=0, truncQ=2, rm.phix=TRUE and

203 maxEE=2. The maxEE parameter established the maximum number of “expected errors”
204 allowed in a read to 2, and cut off 210 bp for forward and 200 bp for reverse reads. Amplicon
205 sequence variants (ASV) generated by DADA2 analysis of the quality-filtered sequence data
206 were taxonomically classified against UNITE database
207 (sh_general_release_dynamic_10.05.2021_dev.fasta (release May 11th, 2021)).¹⁹ All ASVs
208 not matching taxa in the UNITE database were given the designation “NA” (unassigned). ASVs
209 without identified phylum were excluded from the analysis. To incorporate the ASVs that
210 included all taxonomically unassigned sequences, multiple sequencing comparisons were
211 performed using Blast.²⁰ The ASV sequences with > 97% similarity were identified as the same
212 species, > 95 % using as the same genus.

213

214 Mycobiome alpha and beta diversity were studied with the Phyloseq (version 1.36.0) and vegan
215 R package (2.5-7). Rarefaction was used to simulate an even number of reads per sample. The
216 rarefaction depth chosen was 90% of the minimum sample depth in the dataset (in this case
217 11725 reads per sample). DESeq2²¹ was used to produce a matrix containing differential
218 abundance values (e.g. log₂ fold changes) corresponding significance measures for species and
219 genus abundance (*p* and adjusted *p* values). An adjusted *p*-value < 0.01 was considered
220 statistically significant.

221

222 **Peripheral blood CD4+ T cell profiling**

223 Cytokines secreting T cells from frozen PBMC samples were detected after a 6-hour Phorbol
224 12-Myristate 13-Acetate (PMA) – Ionomycin calcium salt (Sigma Aldrich) stimulation and
225 Golgi blocking (Golgi stop, BD Biosciences) and stained with: CD4-APC-Cy7 (BD
226 Biosciences), CD3-PerCP (BD Biosciences), Aqua (LIVE/DEAD® Fixable Dead Cell Stain
227 Kit, Invitrogen), IL-22-A647 (e-Bioscience), IFN- γ -FITC (BD Biosciences), IL-17A-PE

228 (eBioscience) and analyzed on a FACSCanto II flow cytometer (BD Biosciences) and FACS
229 Diva (BD) software (BD Biosciences).

230 **Intestine enterocyte cell (IEC) line and growth conditions**

231 The cell line Caco-2, derived from a human colon adenocarcinoma, was purchased from the
232 American Type Culture Collection (ATCC n°HTB-37™). Cells were grown in Dulbecco's
233 modified Eagle's minimum essential medium (Gibco, Life Technologies) supplemented with
234 10% FBS (PAN Biotech) and 0.1mM non-essential amino acids (Dominique Dutscher), without
235 antibiotics or antifungal agents. Three hundred thousand cells were seeded onto 14 mm
236 diameter glass coverslips previously placed in 24-well plates. Cells were maintained in a
237 humidified incubator at 37°C with 5% CO₂ and were used for experiments 15 to 21 days after
238 seeding (Passages 10 to 20) after full differentiation.

239

240 ***C. albicans* filamentation adhesion and invasion assays**

241 For filamentation, adhesion and invasion assays, *C. albicans* (SC5314) were grown in liquid
242 YPD medium (yeast extract 1%, bacto-peptone 2%, and dextrose 2%, Fischer Bioreagent)
243 overnight at 37°C, in a shaking incubator. Fungal cells were then diluted in fresh liquid YPD
244 medium to a OD_{600nm} of 0.28 to 0.32 and then grown to log phase for another 2 hours in the
245 same conditions. A pellet of 5.10⁴ yeasts was resuspended with 100 µg/mL purified sIgA or
246 IgM. After an incubation for 20 min at 4°C, the yeast concentration was adjusted to 5.10⁴
247 yeasts/mL in cell culture media for further investigation. For filamentous assays, *C. albicans*,
248 either pre-treated or not with sIgA or IgM in RPMI-1640 medium were incubated into a 6
249 plastic dishes for 3 hours at 37°C, 5% CO₂. Fungal cells were then washed with PBS, scrapped
250 from the dishes and observed with a BX51 microscope using a X40 magnification (Olympus).
251 Adhesion and invasion assays were performed using differentiated Caco-2 cells. Briefly,

252 intestinal cells cultured on coverslips were infected with *C. albicans* pre-treated or not with
253 sIgA or IgM for 30 min or 2 hours at 37°C, respectively, as described elsewhere.²²

254

255 **Statistical analysis**

256 Statistical analysis was performed using GraphPad Prism (version 9) and JMP Pro (version 17).

257 Mann-Whitney test, Wilcoxon/Kruskal-Wallis test and Chi-square test were used whenever

258 necessary. Principal component Analysis (PCA) was performed using RStudio v1.3.1093

259 with FactoMineR PCA and Factoextra fviz_pca_biplot functions on MFI values.

260 **Results**

261 **Intestinal, systemic and maternal IgA differentially target commensal fungi**

262 Characterization of homeostatic IgA binding to 14 fungal strains from different genera of the
263 two main divisions *Ascomycota* and *Basidiomycota* was carried out by flow cytometry in fecal
264 waters, serum, and breast milk from a cohort of HD (Fig 1, A). Free sIgA fecal water binding
265 was observed against all fungal genera studied and showed high inter-individual variability (Fig
266 1, B). sIgA responses against members of the *Ascomycota* phylum, and *Saccharomycetales*
267 order, such as *Saccharomyces*, *Debaryomyces*, *Candida*, *Cyberlindnera* and *Yarrowia* were
268 lower than those of the *Hypocreales* order, *Fusarium* or *Acremonium*, or those of the
269 *Basidiomycota* phylum, *Rhodotorula*, *Malassezia* and *Trichosporon*. Analysis of Ig-opsonized
270 fungi in human purified gut microbiota (Fig 1, C and Fig E1, A) showed a preponderance of
271 sIgA⁺ as compared to sIgM⁺ fungal populations ($38.2 \pm 23\%$ vs $5.7 \pm 10\%$; $p < 0.0001$).
272 Systemic IgA responses exhibited also an inter-taxonomic variability, even against closely
273 related taxons of the *Saccharomycetales* order (Fig 1, D). The more intense responses were
274 observed against the *Candida* genus but also against *Aspergillus*, *Fusarium*, *Acremonium* and
275 *Penicillium*. Finally, as the maternal milk IgA repertoire largely mirrored the intestinal IgA
276 repertoire,²³ we characterized anti-fungal IgA activity in breast milk from healthy lactating
277 women (Fig 1, E). sIgA responses were mainly observed against *Debaryomyces*, *Candida* and
278 *Malassezia*. We also assessed IgA responses against the filamentous forms of *Candida*,
279 *Aspergillus*, and *Fusarium*. Interestingly, the more intense responses were observed against
280 *Candida* hyphae in serum and so less in breast milk as well, as compared to *Fusarium* and
281 *Aspergillus* (Fig 1, B-E). Principal component analysis (PCA) highlighted three distinct
282 patterns of IgA anti-mycobiota combined reactivity profiles (Fig 1, F) and further underline the
283 focalization of serum IgA responses against *Candida* hyphae as compared to *Aspergillus* and
284 *Fusarium* in healthy individuals (Fig 1, G). Together, these results show that homeostatic IgA

285 responses preferentially target specific members of the fungal community and suggest that
286 intestinal, systemic and maternal IgA repertoires are focused on different combinations of
287 genera, as underlined by combined PCA.

288

289 ***C. albicans* overgrowth in SIgAd patients**

290 To decipher the impact of IgA on homeostasis of gut mycobiota and its diversity, we
291 characterized the mycobiota composition in a cohort of IgA deficient patients (Fig 2, A and Fig
292 E1, B). Using the strategy described above, we first analyzed the percentage of fungal cells in
293 purified microbiota samples from HD and SIgAd patients (Fig 2, B). The results show that the
294 proportion of the fungal population in mycobiota was similar in HD and SIgAd patients (1.1%
295 ± 1.15 and $0.71\% \pm 0.64$, respectively). We then analyzed the percentage of IgA-opsonized
296 fungi. As expected, $38.2\% \pm 23.32$ of the gut fungal population was opsonized by intestinal
297 IgA in the HD population, whereas the IgA-opsonized population was absent in all but two
298 SIgAd patients.

299

300 To determine how the lack of IgA could alter the gut mycobiota ecology, we performed ITS-
301 gene sequencing of DNA obtained fecal samples (Fig 2, C-F). We observed a trend toward
302 increased diversity in SIgAd subjects for multiple alpha diversity metrics (observed ASVs,
303 Shannon Diversity Index and Chao1), although none of these differences reached statistical
304 significance ($p < 0.05$). We then analyzed whether IgA deficiency might affect the composition
305 of fungal populations from the phylum to the species level in terms of ASVs, relative abundancy
306 and prevalence. Interestingly, at the phylum level, significant differences were observed for the
307 *Ascomycota* phylum between the gut mycobiota of SIgAd subjects and that of HD ($p = 0.003$)
308 (Fig 2, D). We detected 72 fungal genera, with *Saccharomyces* being the more prevalent one,
309 followed by *Candida*, *Debaryomyces*, *Penicillium*, *Malassezia* and *Yaworria* genera (Fig E2).

310 Thirty-three out of 72 genera described were common to both populations whereas the other 39
311 genera, were either present in HD or SIgAd donors and belonged to the mycobiota rare
312 biosphere. Genera only present in the SIgAd population were yeasts such as *Nakaseomyces* or
313 *Metschnikowia* or filamentous fungi associated with diet or environment like *Aspergillus*,
314 *Podosphaera* or *Claviceps*.

315

316 Analysis at the class, order, family, genus and species level revealed important differences
317 between both populations with a significant increase in *C. albicans* overgrowth in SIgAd
318 mycobiota ($p=0.0046$) (Fig 2, E). We furthermore observed a loss of species diversity among
319 the *Candida* genus (Fig 2, F). Whereas five *Candida* species, in particular *C. sake*, were present
320 in HD, only three *Candida* species were identified in SIgAd patients with *C. albicans* being the
321 most abundant. This phenomenon was also observed for *Penicillium* species. In contrast, we
322 observed an increase in the species diversity of the *Kazarrstania* and *Yaworria* genera in fecal
323 samples of SIgAd patients. Therefore, although IgA deficiency does not induce a global
324 dysbiosis, it is associated with a promotion of *C. albicans* development.

325

326 **Mycobiota dysbiosis in symptomatic SIgAd patients**

327 Although many IgA-deficient individuals are asymptomatic, some are affected by recurrent
328 infections, have metabolic and gastrointestinal diseases and are at greater risk for developing
329 colorectal cancer.²⁴ To explore whether mycobiota composition could be related to clinical
330 phenotypes in the SIgAd population, we compared the intestinal mycobiota of HD and
331 asymptomatic SIgAd patients with that of symptomatic patients suffering from auto-immunity,
332 infections and/or intestinal disorders. The results show that alpha diversity, in particular the
333 Shannon, Simpson and invSimpson index, are significantly higher in symptomatic patients
334 when compared with HD and SIgAd patients (Fig 3, A). No significant differences were

335 observed in alpha diversity metrics between mycobiota of HD and asymptomatic SIgAd
336 patients. Comparison of relative abundance at class, order, genus and species level pointed out
337 that *C. albicans* and *S. cerevisiae* were overrepresented in symptomatic patients or
338 asymptomatic patients, respectively (Fig 3, B). Analysis of the observed ASV in both groups
339 illustrated a shift of mycobiota composition related to clinical phenotype, with a significant
340 increase of *C. albicans* and a decrease of *S. cerevisiae* in symptomatic patients (Fig 3, C).

341

342 **Symptomatic IgA-deficient individuals present with decreased Th17/22 counts**

343 As *C. albicans* is known to have an inflammatory effect,⁶ we then compared the frequency of
344 cytokine-secreting circulating CD4⁺ T cells in asymptomatic and symptomatic IgA-deficient
345 individuals (Fig 3, D-E). Proportions of interferon- γ (IFN- γ)⁺CD4⁺ T cells differed between HD
346 and asymptomatic but no differences were observed in SIgAd patients ($18.8 \pm 14\%$ vs $26.1 \pm$
347 6.4% , $p=0.516$). IL-17⁺- and IL-22⁺-secreting CD4⁺ T cells were increased in both populations
348 of SIgAd patients however, these cell populations were significantly lower in symptomatic
349 patients ($1.14 \pm 0.7\%$ vs $2.5 \pm 0.9\%$, $p=0.014$ and $0.75 \pm 0.5\%$ vs $2.0 \pm 1.3\%$, $p=0.019$
350 respectively). Double-positive IFN γ ⁺IL-17⁺ CD4⁺ T cells were also decreased in symptomatic
351 patients as compared with asymptomatic patients ($0.18 \pm 0.1\%$ vs $0.4 \pm 0.2\%$, $p=0.034$
352 respectively). Despite the limited number of individuals studied, our results underscore a
353 significant decrease of systemic Th17-type lymphocyte responses in symptomatic IgA-
354 deficient individuals.

355

356 **Secretory IgA controls *C. albicans* development and intestinal cell invasion**

357 The increase in *C. albicans* prevalence in the mycobiota of SIgAd donors prompted us to study
358 the impact of IgA responses in *C. albicans* yeast-to-hyphal transition, intestinal cell adhesion
359 and invasion. To this aim, we used an *in vitro* model of *C. albicans*-Caco-2 cells and IgA

360 purified from breast milk. The impact of purified systemic IgM from six SIgAd patients was
361 also evaluated. SIgA from nine different donors were found to significantly reduce *C. albicans*
362 hyphae development with reduction rates ranging from 10.27% to 23.8% ($p=0.002$; IgA versus
363 no-IgA conditions) (Fig 4, A). Breast-milk-derived sIgA also reduced yeast adhesion to Caco-
364 2 cells (reduction rates: 27.5% to 60%), although these differences did not reach statistical
365 significance (Fig 4B). Interestingly, yeast intestinal cell invasion was significantly reduced by
366 sIgA from eight of nine donors tested with a mean reduction rate of $33.68 \pm 4.3\%$ ($p=0.0012$;
367 IgA versus no IgA conditions) (Fig 4, C). In comparison, purified IgM did not significantly
368 impact hyphae development, nor intestinal cell adhesion or invasion. Thus, it appears that *in*
369 *vitro* antibody-mediated repression of *C. albicans* budding, intestinal cell adhesion and invasion
370 is mainly IgA-mediated.

371

372 **Compensatory secretory IgM binding to commensal fungi in SIgAd patients**

373 Although we did not *in vitro* measure a significant impact of IgM on *C. albicans* budding and
374 cell invasion, previous studies have nevertheless suggested that IgM might play *in vivo* a
375 compensatory role which might explain why IgA deficiency is not associated with massive
376 perturbations of gut microbial ecology.^{16, 25, 26} To explore whether sIgM could exert a similar
377 effect resulting in the preservation of gut mycobiota homeostasis in SIgAd patients, we
378 analyzed the sIgM-opsonized fungal community in purified microbiota. Indeed, sIgM was
379 detected at mycobiota surface in all SIgAd patients tested ($53.3\% \pm 16.2$) (Fig 5, A). We also
380 characterized sIgM responses in fecal waters (Fig 5, B). We observed a profile similar to that
381 observed for sIgA in HD population with a higher binding for those fungi that are less
382 represented in the gut, such as *Aspergillus*, *Malassezia* or *Trichosporon* as compared with that
383 observed for fungi from the *Saccharomycetales* order. This finding might suggest that

384 antibodies specific for abundant targets, such as fungi from the *Saccharomycetales* order, are
385 adsorbed at the surface of the latter and, are thus, less represented in fecal waters.

386

387 One classically described function of secretory immunoglobulins is immune exclusion of its
388 targets, in which antibody binding retains antigen in the intestinal lumen, thereby precluding
389 the priming of other immune responses, and lethal dissemination of commensal bacteria.²⁷ To
390 indirectly determine whether sIgM could play a role in the immune exclusion of fungal targets,
391 we compared antifungal systemic IgG responses in SIgAd and HD subjects. The results show
392 that IgG responses in both groups were similar with only significant differences in IgG
393 responses against two fungal genera (*Fusarium*, $p=0.0188$; *Trichosporon*, $p=0.0006$) (Fig 5, C).
394 We then compared systemic anti-fungal IgM responses in both populations (Fig E3). Systemic
395 IgM bound a large range of commensal fungi in HD as well as in SIgAd patients, with a
396 comparable inter-individual variability. Finally, the profile of systemic IgA responses in HD
397 was compared with that observed for IgM in SIgAd population in order to unveil a potential
398 compensatory role for IgM. Our results show that the IgA and IgM recognition patterns of the
399 sporulated forms did not differ between both groups (Fig 5, D). In contrast, comparison of the
400 patterns of filamentous form recognition showed that systemic IgA had a more selective
401 reactivity profile than IgM, with a preferential targeting of the *C. albicans* filament. Altogether,
402 these results confirm a partial compensatory impact of IgM on gut mycobiota in the absence of
403 IgA that does not fully extend to *C. albicans*.

404 **Discussion**

405 Here we show that homeostatic IgA binds to distinct strains of commensal fungi with a body
406 fluid-selective pattern of recognition in a cohort of healthy subjects. The profile of fungal
407 recognition by free sIgA in fecal waters was characterized by a reduced binding to the more
408 abundant genera, *Saccharomyces*, *Debaryomyces* and *Candida* and was related with a high rate
409 of opsonization of mycobiota by intestinal IgA. In contrast, systemic IgA was found to
410 preferentially target genera mainly implicated in human allergic responses or invasive fungal
411 infections, including *Penicillium*, *Candida*, *Fusarium* and *Aspergillus*,^{6, 28-30}. Finally, breast
412 milk-derived IgA responses were directed against *Candida*, *Debaryomyces* and *Malassezia*,
413 previously reported to colonize the mycobiota of healthy lactating mothers and their infants³¹
414 and, in case of fungal overgrowth, to be associated with the occurrence of inflammatory bowel
415 disease.³²⁻³⁴

416

417 To get further insight into the impact of IgA on host-mycobiota mutualism, we then compared
418 the gut mycobiota of IgA-deficient individuals and healthy subjects. Molecular characterization
419 of fungal commensal communities in both populations showed that IgA deficiency is not
420 associated with a significant perturbation of gut microbial ecology, given that, in SIgAd
421 patients, only an increase in intestinal diversity of fungal species belonging to the rare
422 mycobiota biosphere was observed. However, results from differential ASV analysis between
423 the mycobiota of HD and SIgAd patients assigned at order, class, family, genus and species
424 level showed an association of IgA deficiency with a significant proliferation of *C. albicans*.
425 This proliferation was associated with a reduction of other *Candida* species in SIgAd patients,
426 as compared with HD, suggesting an elective role of sIgA on species regulation inside the
427 *Candida* genus or revealing the preferential fitness of *C. albicans* over other species. Intestinal
428 mycobiota analysis within the population of the SIgAd patients also revealed a significantly

429 higher diversity with an overrepresentation of *C. albicans* in those presenting clinical
430 complications that contrasted with the dominant presence of *S. cerevisiae* in asymptomatic
431 sIgAd patients. The known anti-inflammatory and pro-inflammatory effects of *S. cerevisiae*³⁵
432 and *C. albicans*⁶ respectively, might contribute to the clinical phenotype observed in the sIgAd
433 population. While gut proliferation of *C. albicans* is associated with an increased CD4⁺Th17
434 systemic response,³⁶ *C. albicans* sIgAd-colonized patients showed lower IL-17⁺CD4⁺, IL-
435 22⁺CD4⁺ and IFN- γ ⁺IL17⁺ responses. These results corroborate and extend previous findings
436 showing that systematic sIgAd is associated with immune response defects³⁷ that may account
437 for their mycobiota altered ecological status and clinical manifestations observed in some
438 sIgAd patients. This interpretation is nevertheless limited by the fact that we did not have
439 access to gut biopsies in order to confirm that a global Th17 decrease is observed in
440 symptomatic sIgAd patients.

441

442 *C. albicans* is a commensal fungus of the human gut, but also causes life-threatening invasive
443 infections.³⁸ As reported previously in an experimental mouse model^{9, 15} and corroborated by
444 the present study, the pathogenic hyphal morphotype of *C. albicans*, once established, is
445 targeted by intestinal IgA. However, the *in vivo* study of IgA *C. albicans*-interaction on human
446 fungal-host mutualism is challenging given that *C. albicans* is not a mouse commensal. The
447 demonstration in the present study that sIgA significantly reduces hyphae development
448 corroborates a previous report showing that the lack of sIgA, directed against the *C. albicans*-
449 specific hypha antigen Sap6, in Crohn disease patients was associated with an increase in
450 hyphal fungal morphology.¹⁵ Moreover, sIgA was found to significantly reduce *C. albicans*
451 intestinal cell invasion, thereby implying that *C. albicans* opsonization by sIgA controls fungal
452 fitness and could reduce pathogenic fungal migration through the intestinal barrier.

453 It has been previously reported that IgA uniquely uncouples fungi colonization from
454 pathogenesis in humans.⁹ The clear *in vivo* and *in vitro* effects of IgA on *C. albicans*
455 development notwithstanding, it is important to stress that increased *C. albicans* fitness leading
456 to invasive or mucocutaneous candidiasis is never observed in human IgA deficiency.³⁹
457 Reminiscent to the compensatory effect of IgM specific for bacterial microbiota in human IgA
458 deficiency, we show that, in SIgAd patients, gut mycobiota is opsonized by IgM and that free
459 sIgM is able to recognize a diversity of fungal genera. The lack of gut fungal dysbiosis in these
460 patients therefore points to a compensatory role of IgM-fungal opsonization in order to maintain
461 mycobiota diversity. Our results also suggest that IgM might play an important role in
462 maintaining intestinal barrier integrity and controlling fungal antigen transfer because systemic
463 IgG responses against both sporulated and filamentous forms of commensal fungi are not
464 increased. Moreover, profiles of systemic IgA responses in HD against sporulated forms are
465 reminiscent of IgM responses in SIgAd patients, except for the hyphae forms of *C. albicans*,
466 for which IgA shows a higher selectivity. The later result is corroborated by the fact we did not
467 observe a significant impact of IgM on *C. albicans in vitro* budding and cell invasion and could
468 consequently explain the *C. albicans* overgrowth observed in SIgAd patients. IgM has nevertheless a
469 broad fungal reactivity that could be related to low affinity interactions of this isotype for the
470 β -(1,3)-glucan and chitin antigens which are conserved major fungal cell wall components.⁴⁰

471

472 Altogether, we show here that secretory and systemic IgA is able to recognize a large range of
473 fungal members of the human mycobiota and that it participates in fungal-host mutualism. At
474 the species level, intestinal *C. albicans* overgrowth in human IgA deficiency underscores the
475 pivotal role IgA in the intestinal fitness of this pathobiont fungus. Finally, the importance of
476 IgA-mediated fungal recognition in healthy subjects and the paradoxical lack of fungal
477 dysbiosis in human IgA deficiency could be explained by a partial compensatory role of IgM.

478 Author contributions

479 A. Moreno-Sabater, D. Sterlin, C. Fieschi, M. Malphettes, J. Fadlallah collected and prepared
480 the specimens from healthy donors and selective IgA deficient patients. V. Rigourd provided
481 breast milk samples. A. Moreno-Sabater, A-C. Normand, C. Bachmeyer, C. Hennequin
482 provided fungal strains. A. Moreno-Sabater, D. Sterlin, L. Imamovic, F. Bon, A-C. Normand,
483 C. Gonin, M. Bensalah, M. Gazzano, K. Dorgan, E. Ben Salah, A. Acherar, C. Parizot, F. Dalle,
484 J. Fadlallah designed and performed experiments. A. Moreno-Sabater, D. Sterlin, Lejla
485 Imamovic, A-C Normand, A. Acherar performed data analysis. A. Moreno-Sabater, D. Sterlin,
486 L. Imamovic, A-C. Normand and prepared figures. A. Moreno-Sabater, D. Sterlin, L.
487 Imamovic, Hans Yssel, J. Fadlallah, G. Gorochov designed the study and wrote the manuscript.

488

489 Acknowledgments

490 The study was financed by the Emergence program of Sorbonne Université (MycELIA- SU-
491 16-R-EMR-45; Principal investigator A. Moreno-Sabater) and by Fédération Hospitalo-
492 Universitaire (FHU) PaCeMM « Paris Center for Microbiome Médecine » (Grant to A. Moreno-
493 Sabater). Work in the G. Gorochov's team is supported by Institut National de la Santé et de la
494 Recherche Médicale (INSERM) and Sorbonne Université. L. Imamovic was partly supported
495 by Agence Nationale de la Recherche (ANR "BactRIA" R21219DD).

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- 596

597 **Figures Legends**

598 **Figure 1.** Selective homeostatic targeting of fungal community members by IgA shows body
 599 fluid-specific features. (A) Flow cytometry protocol used to probe IgA binding to fungal strains
 600 (yeast/spores or hyphae forms) following incubation with fecal water, serum or breast milk and
 601 secondary fluorophore-conjugated anti-IgA. (B) Analysis of human fecal free IgA binding
 602 (n=34) to a panel of cultivated strains covering 14 fungal genera. (C) Protocol used to probe
 603 immunoglobulins (Ig)-opsonized fungal populations of the human gut microbiota using flow
 604 cytometry. Calcofluor white (CFW) staining. Percentage of the gut mycobiota opsonized by
 605 secretory (s) IgA or IgM in healthy donors (n=28). (D-E) Analysis of systemic (n=31) and
 606 breast milk (n=20) IgA binding. (F-G) Principal component analysis of data from B, D, E
 607 figures and factorial analysis (variable relative contributions indicated by length and direction
 608 of respective arrow). Ellipses represent the 95% CI of patient distribution in each group. (B, D,
 609 E) Results are displayed as median fluorescence intensity (MFI) values. Bar plots show
 610 individual data with mean \pm s.d. Sac: *Saccharomyces*; Deb: *Debaryomyces*; Can: *Candida*;
 611 Cyb: *Cyberlindnera*; Yar: *Yarrowia*; Klu: *Kluyveromyces*; Asp: *Aspergillus*; Pen: *Penicillium*;
 612 Fus: *Fusarium*; Acr: *Acremonium*; Rho: *Rhodotorula*; Mal: *Malassezia*; Tri: *Trichosporon*;
 613 Cry: *Cryptococcus*. Images were created with BioRender.com.

614

615 **Figure 2.** Fungal gut ecology perturbation in selective IgA deficient (SIgAd) patients. (A)
 616 Strategy used to assess gut immunoglobulins (Ig) opsonized fungal population by flow
 617 cytometry and to characterize mycobiota composition by internal transcribed spacer (ITS) gene
 618 sequencing in healthy donors (HD) and SIgAd patients. (B) Proportion of CFW^{bright} population
 619 and double IgA⁺CFW^{bright} population among total gut microbiota in HD (n=28) and SIgAd
 620 (n=12). (C) Alpha diversity index in HD (n=23) and SIgAd (n=17). ASV: Amplicon sequence
 621 variant. (D) Observed ASV inside the Ascomycota and Basidiomycota phylum of the studied

622 populations. (E) Differential ASV between HD and SIgAd assigned at order, class, family,
623 genus and species level, ranked by statistical difference. Blue histograms represent fungal
624 population overrepresented in HD and red histograms in SIgAd patients. Significant differences
625 determined by Wilcoxon/Kruskal-Wallis test (* $p < 0.05$; ** $p < 0.01$). (F) Changes in the relative
626 abundance at species level in the *Candida*, *Penicillium*, *Kazachstania* and *Yarrowia* genera.
627 Patients with residual secretory IgA are colour-coded (black symbols). Bar plots show
628 individual data with mean \pm s.d. (B-D). Indicated p values were calculated using a Mann-
629 Whitney test. Images were created with BioRender.com

630

631 **Figure 3.** Clinical status of healthy donors (HD) and selective IgA deficient (SIgAd) patients
632 in relation with mycobiota composition and circulating CD4⁺ T cells. (A) Alpha diversity in
633 twenty-three HD and five asymptomatic (Asym) and eleven symptomatic (Sym) SIgAd
634 patients. Sym patients suffer from infections and/or autoimmune and/or intestinal
635 complications. ASV: amplicon sequence variants. (B) Differential ASV between asym and sym
636 patients assigned at order, class, family, genus and species level, ranked by statistical
637 difference. Red and black histograms represent fungal population overrepresented in asym and
638 in sym patients, respectively. F.i.s: fam_incertae_sedis; c.n.a.: class not assigned. Significant
639 differences determined by Wilcoxon/Kruskal-Wallis test (* $p < 0.05$; ** $p < 0.01$). (C) Number of
640 observed ASV for *C. albicans* and *S. cerevisiae* in gut mycobiota of HD, asym and sym SIgAd
641 patients. (D) Systemic inflammatory markers. Graphs show percentages of CD4⁺ T cells
642 expressing indicated cytokines determined by intracellular staining. (E) CD4⁺IL-22⁺, IL17⁺,
643 IFN γ ⁺IL17⁺ data from sym, and asym patients were grouped by hierarchical cluster analysis
644 according to Ward's method and plotted as a heat map. For all panels, bar plots show individual
645 data with mean \pm s.d, and p values were calculated with a Mann-Whitney test.

646

647 **Figure 4.** Impact of secretory IgA and IgM on *C. albicans* host mutualism. (A) *C. albicans*
648 (yeast form) was incubated in medium alone or in the presence of purified secretory IgA (sIgA)
649 or systemic IgM (B-C) in absence or presence of human intestinal Caco-2 cell monolayer. For
650 hyphae assay, *C. albicans* (yeast form) was incubated alone or in the presence of sIgA or IgM
651 for 3 hours at 37°C and observed with a BX51 microscope using X40 magnification (Olympus).
652 For adherence and invasiveness assays, *C. albicans* (yeast form) pre-incubated with or without
653 purified antibodies for 30 min or 2 hours, respectively, at 37°C. *C. albicans* assay concentration
654 were 5.10^4 cfu/mL. Purified sIgA from nine healthy lactating women and purified IgM from
655 six SIgAd patients were used at a final concentration of 100 µg/mL. Bar plots show the ratio
656 between IgA or IgM conditions and control conditions of three independent assays, *P* values
657 were calculated using a Mann-Whitney test. Images were created with BioRender.com.

658

659 **Figure 5.** Compensatory role of IgM in selective IgA deficiency (SIgAd). (A) Percentage of
660 the gut mycobiota fraction opsonized by secretory IgM in healthy donors (HD, n=28) and
661 SIgAd patients (n=12) determined by flow cytometry. (B) SIgAd fecal water IgM binding
662 (n=21) to yeast and hyphae forms of 12 commensal fungi. Plots show individual data with mean
663 \pm s.d. (C) Characterization of IgG systemic response in HD (n=31) and SIgAd (n= 21). Box
664 limits: upper and lower quartiles. MFI: Mean fluorescence intensity. (D) Radar plot comparison
665 of systemic IgM responses in SIgAd patients versus IgA responses in HD against spores/yeast
666 or hyphae forms of cultivated commensal fungi. Sac: *Saccharomyces*; Deb: *Debaryomyces*;
667 Can: *Candida*; Cyb: *Cyberlindnera*; Yar: *Yarrowia*; Klu: *Kluyveromyces*; Asp: *Aspergillus*;
668 Pen: *Penicillium*; Fus: *Fusarium*; Acr: *Acremonium*; Rho: *Rhodotorula*; Mal: *Malassezia*; Tri:
669 *Trichosporon*; Cry: *Cryptococcus*.

670

671 **Figure E1.** (A) Gating strategy to analyze intestinal IgA-opsonized mycobiota. A minimal
672 mock mycobiota corresponding to the most abundant fungal genera from the human gut
673 mycobiota (*Saccharomyces*, *Candida*, and *Debaryomyces*) was used to gate on fungal events
674 among the complex purified microbiota from a representative healthy donor (HD). Calcofluor
675 white (CFW) fungal-selective staining was used to probe fungal populations among gut
676 microbiota. The percentage of IgA-opsonized mycobiota among CFW^{bright} population is
677 indicated. (B-C) Systemic and fecal water immunoglobulin levels in HD (n=31) and selective
678 IgA-deficient (SIgAd) patients (n=21). Bar plots show individual data with mean \pm s.d.
679 Indicated *p* values were calculated using a Mann-Whitney test.

680

681 **Figure E2.** Comparative analysis of gut mycobiota diversity performed at genus level
682 highlights quantitative dominance of few genera from the Ascomycota phylum in both healthy
683 donors (HD) and in selective IgA deficient (SIgAd) patients. Analysis of fungal genera, relative
684 abundance and prevalence does not show significant differences between HD (n=23) and SIgAd
685 patients (n=17) suggesting that compensatory mechanisms occur in SIgAd to preserve gut
686 fungal diversity (Mann-Whitney and Chi-square tests). Bar plots show relative abundance
687 (RA), calculated as $\log_{10}(1/RA)$. In circles are indicated the prevalence of fungal genera in
688 HD (blue) as compared with SIgAd (red). The phylogenetic tree was constructed using Mega
689 X program and iTOL web application (<https://itol.embl.de/> last accessed: 11/2021).

690

691 **Figure E3.** Systemic IgM response against commensal fungi. Characterization by flow
692 cytometry of IgM systemic responses in healthy donors (HD, n=31) and selective IgA deficient
693 patients (SIgAd, n=21). Box limits: upper and lower quartiles. MFI, Mean fluorescence
694 intensity. Sac: *Saccharomyces*; Deb: *Debaryomyces*; Can: *Candida*; Cyb: *Cyberlindnera*; Yar:

- 695 *Yarrowia*; Klu: *Kluyveromyces*; Asp: *Aspergillus*; Pen: *Penicillium*; Fus: *Fusarium*; Acr:
696 *Acremonium*; Rho: *Rhodotorula*; Mal: *Malassezia*; Tri: *Trichosporon*; Cry: *Cryptococcus*.

Figure 1

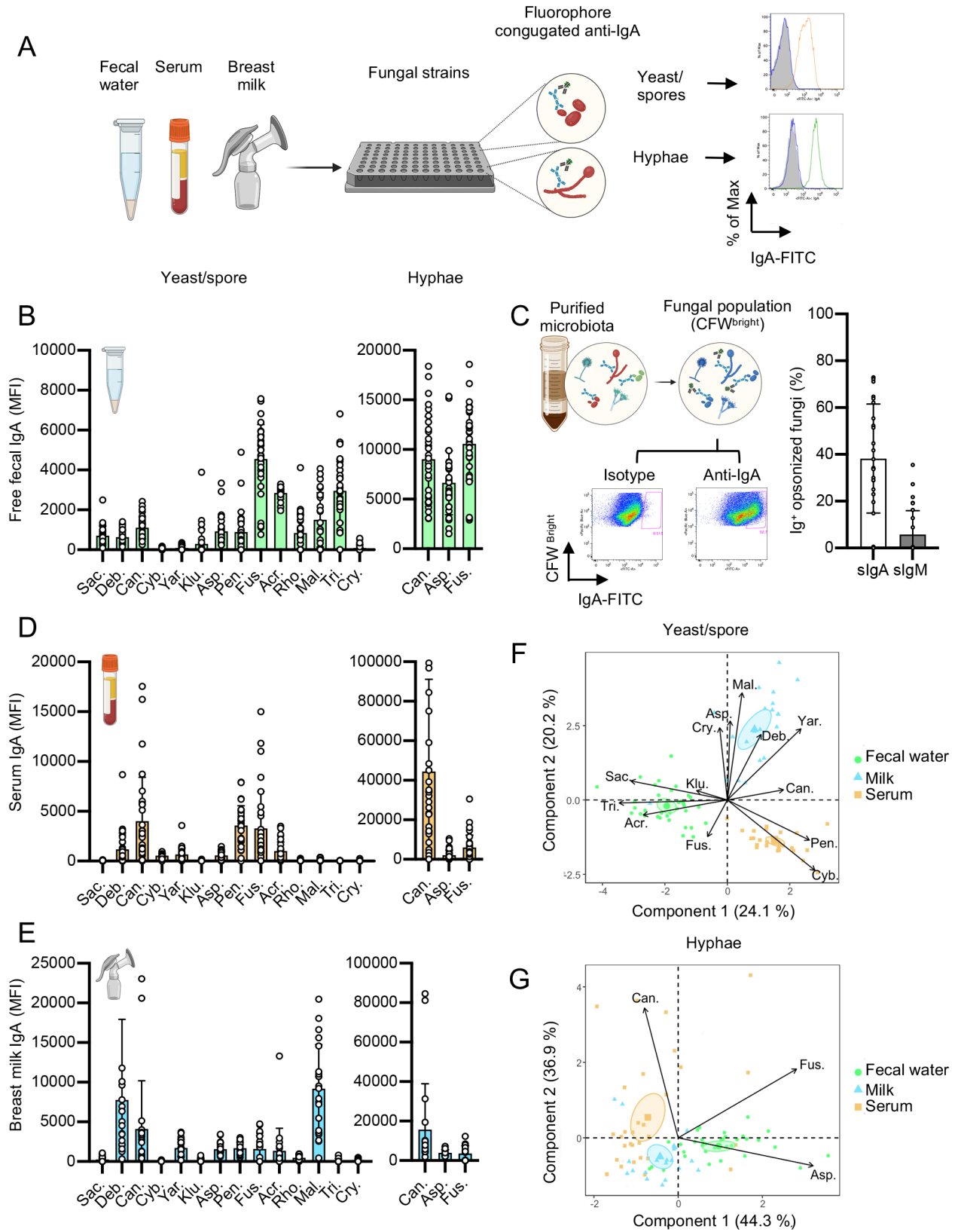


Figure 2

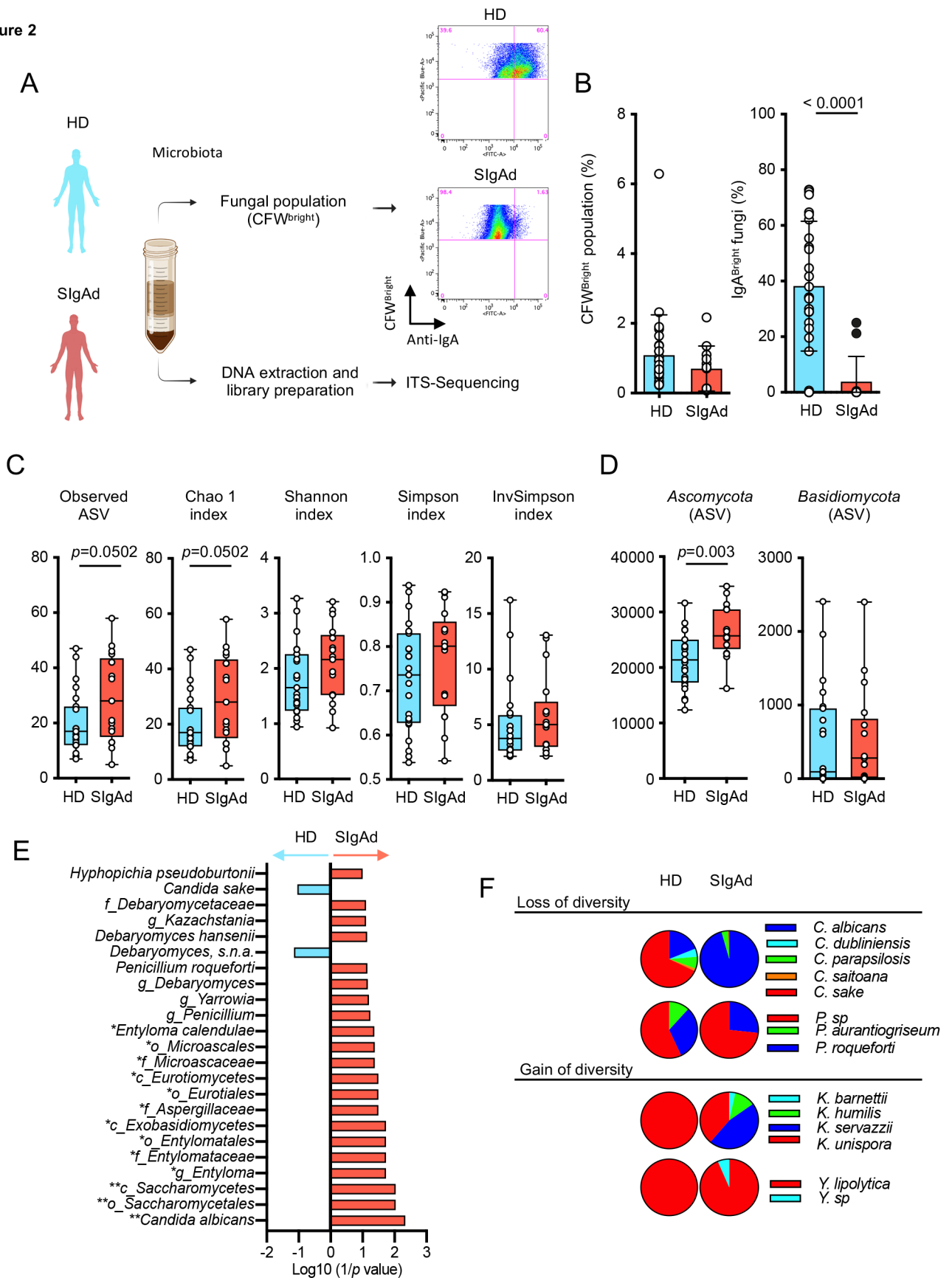
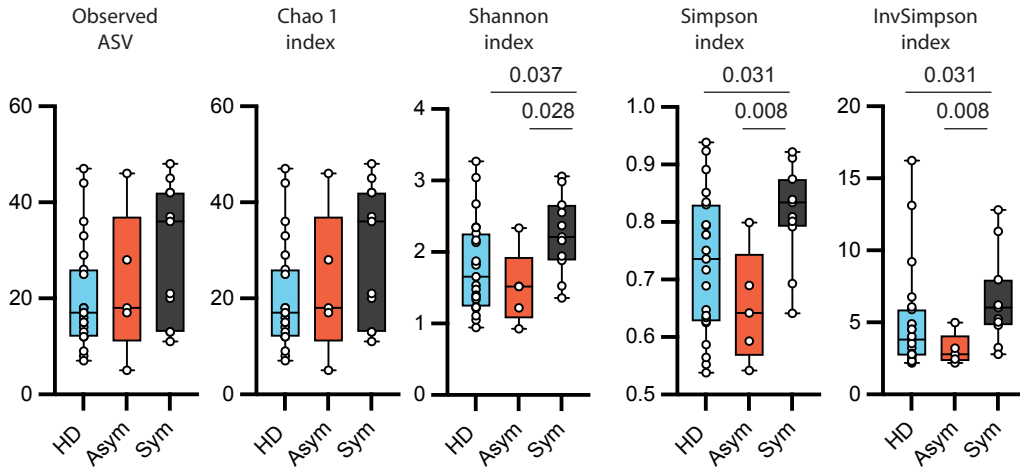
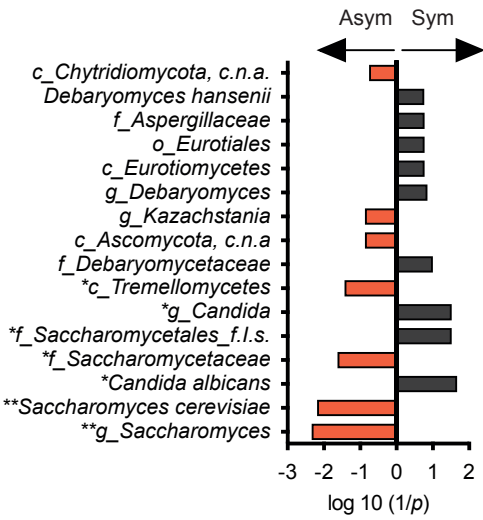


Figure 3

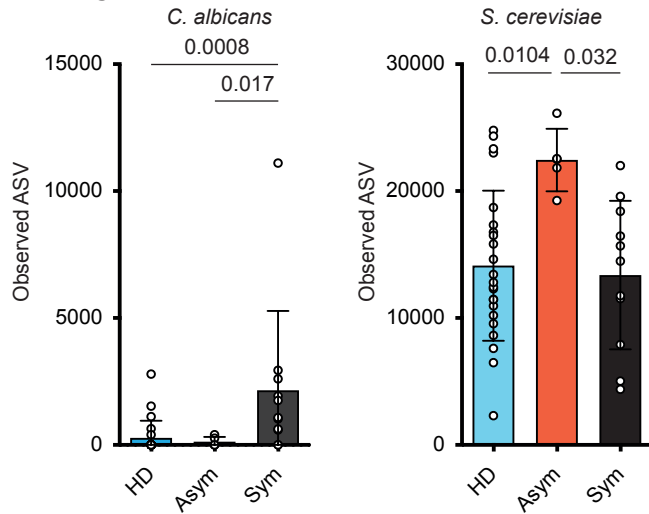
A



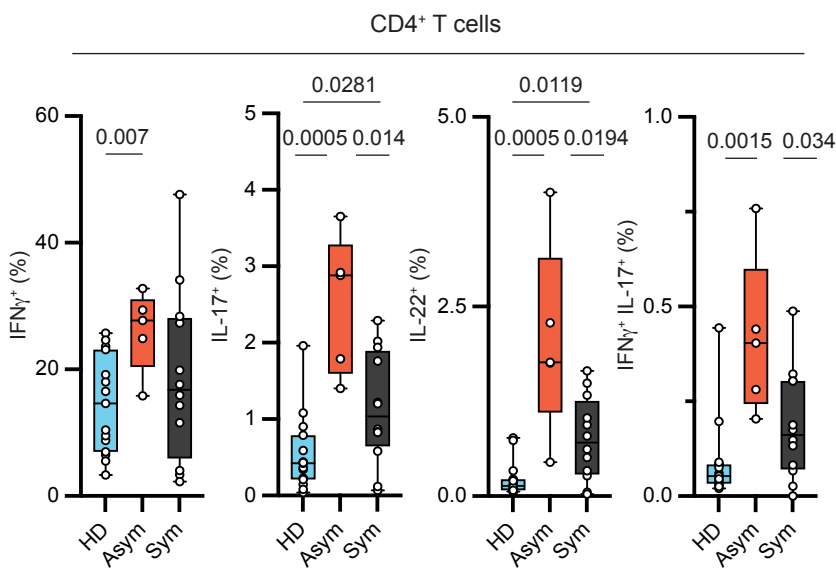
B



C



D



E

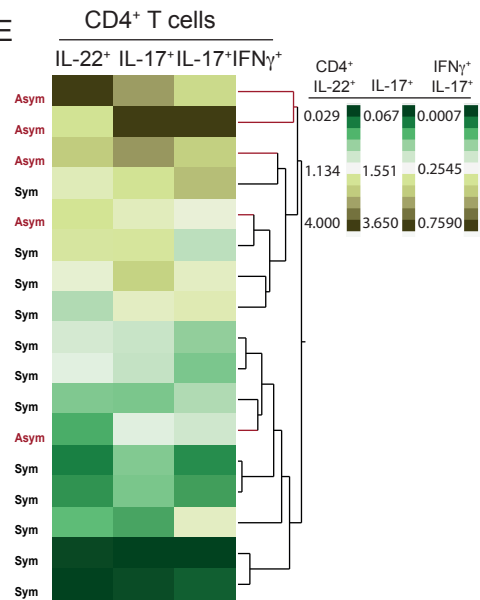


Figure 4

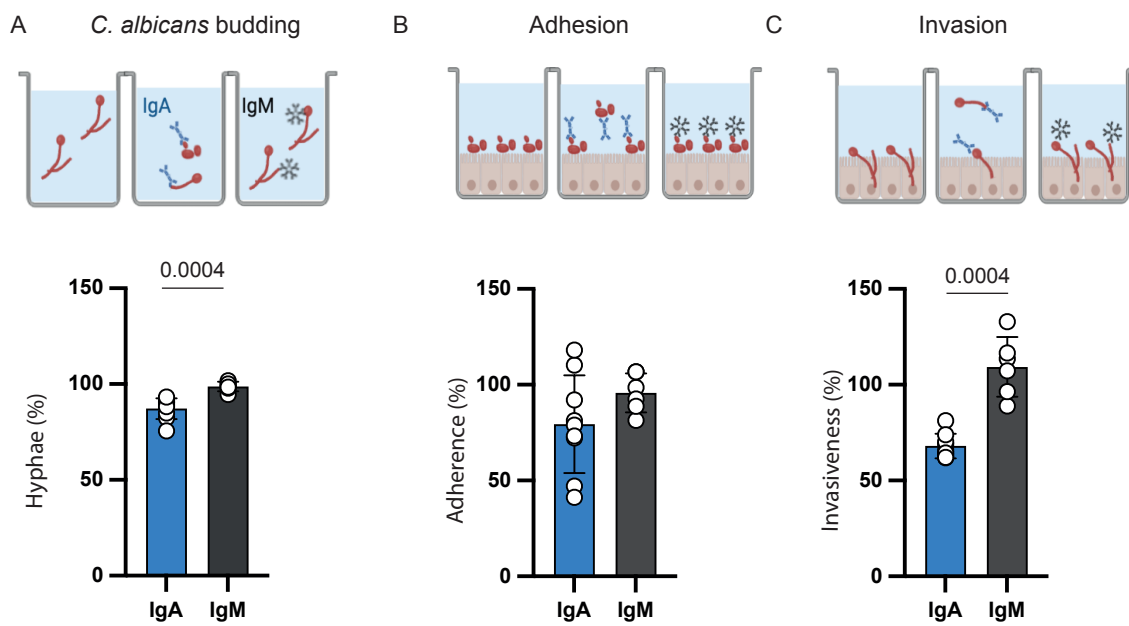
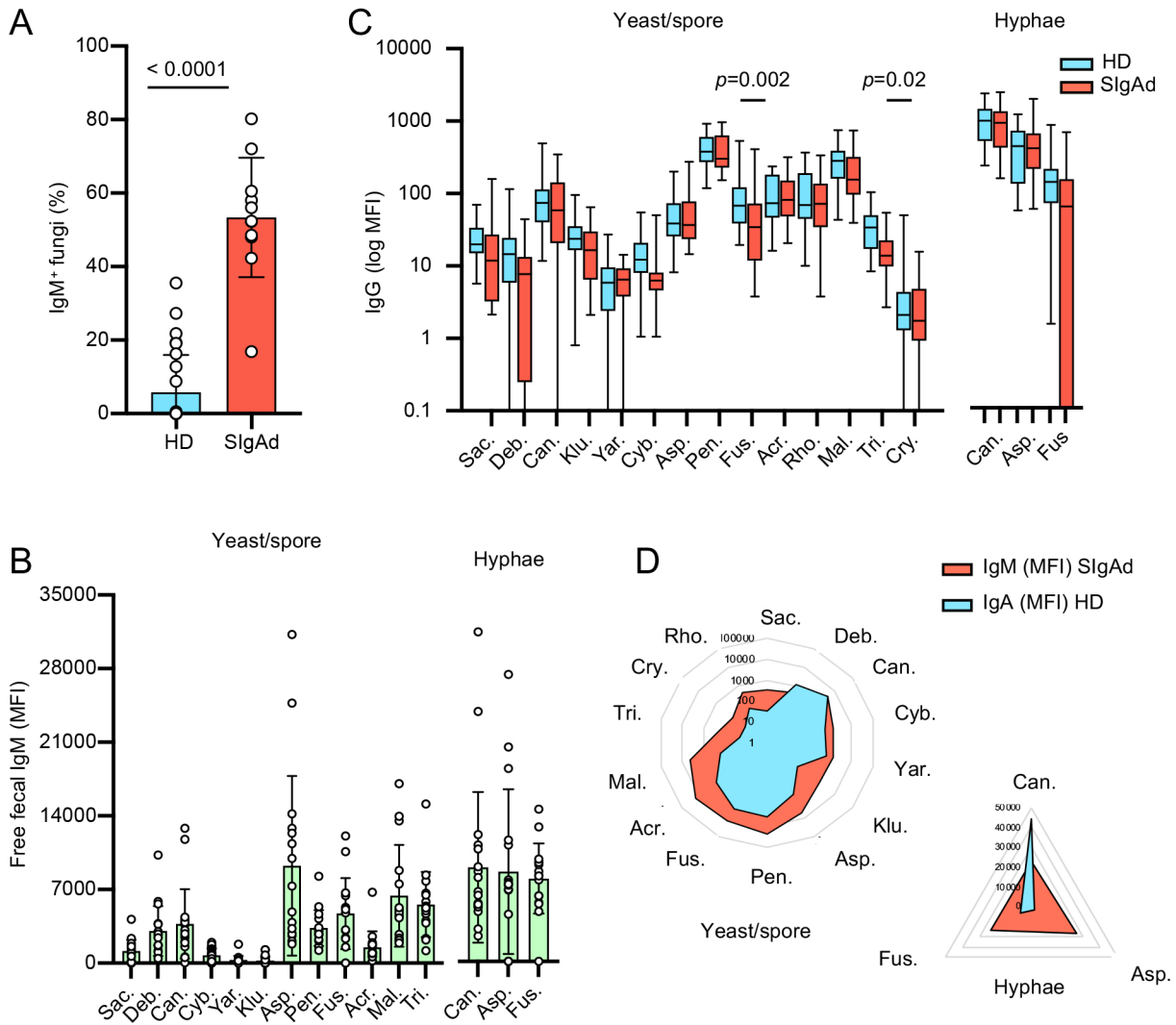


Figure 5



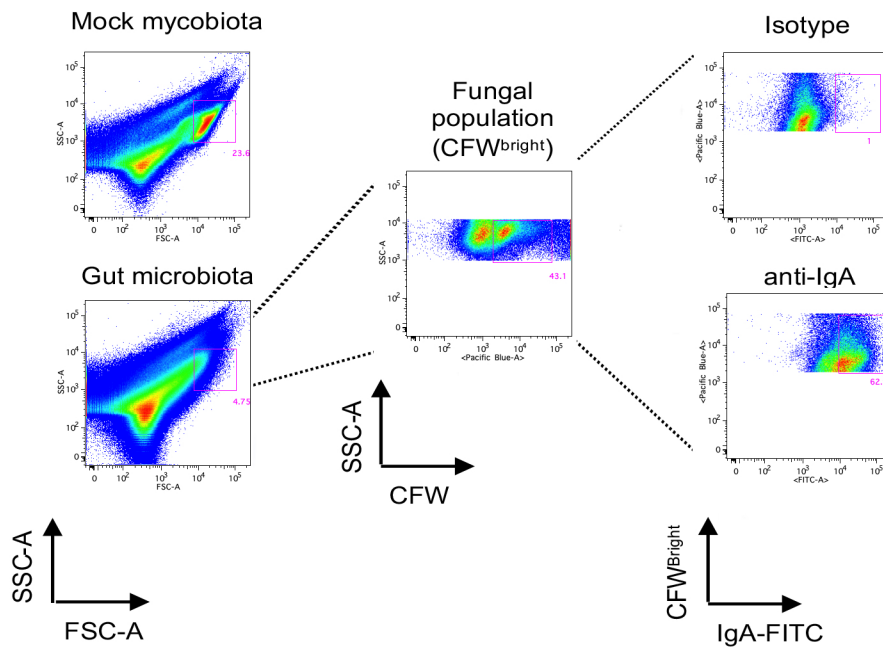
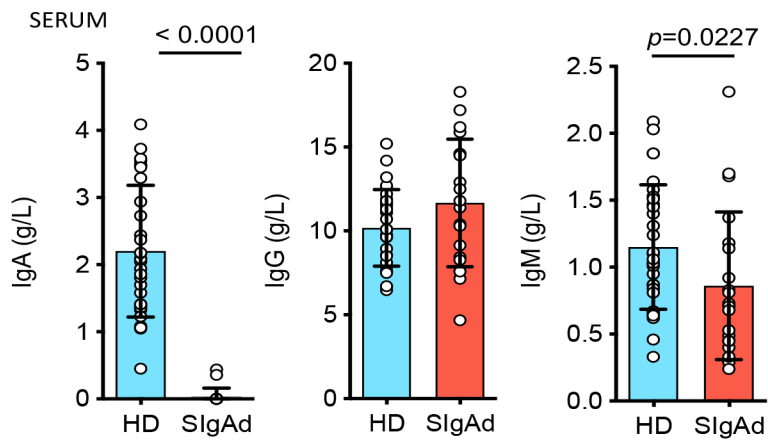
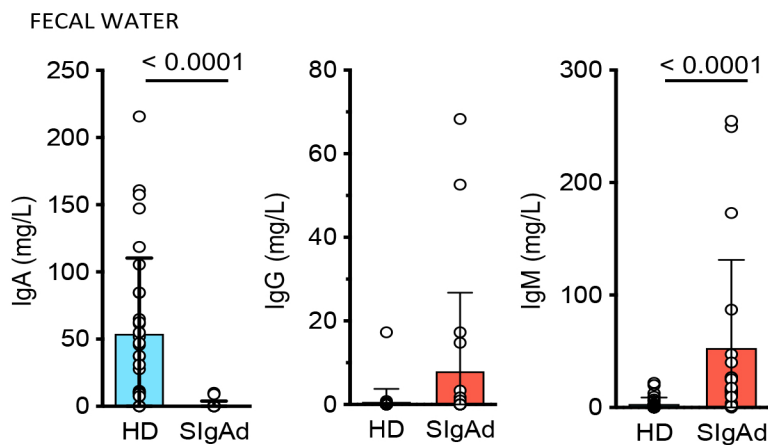
A**B****C**

Figure E2

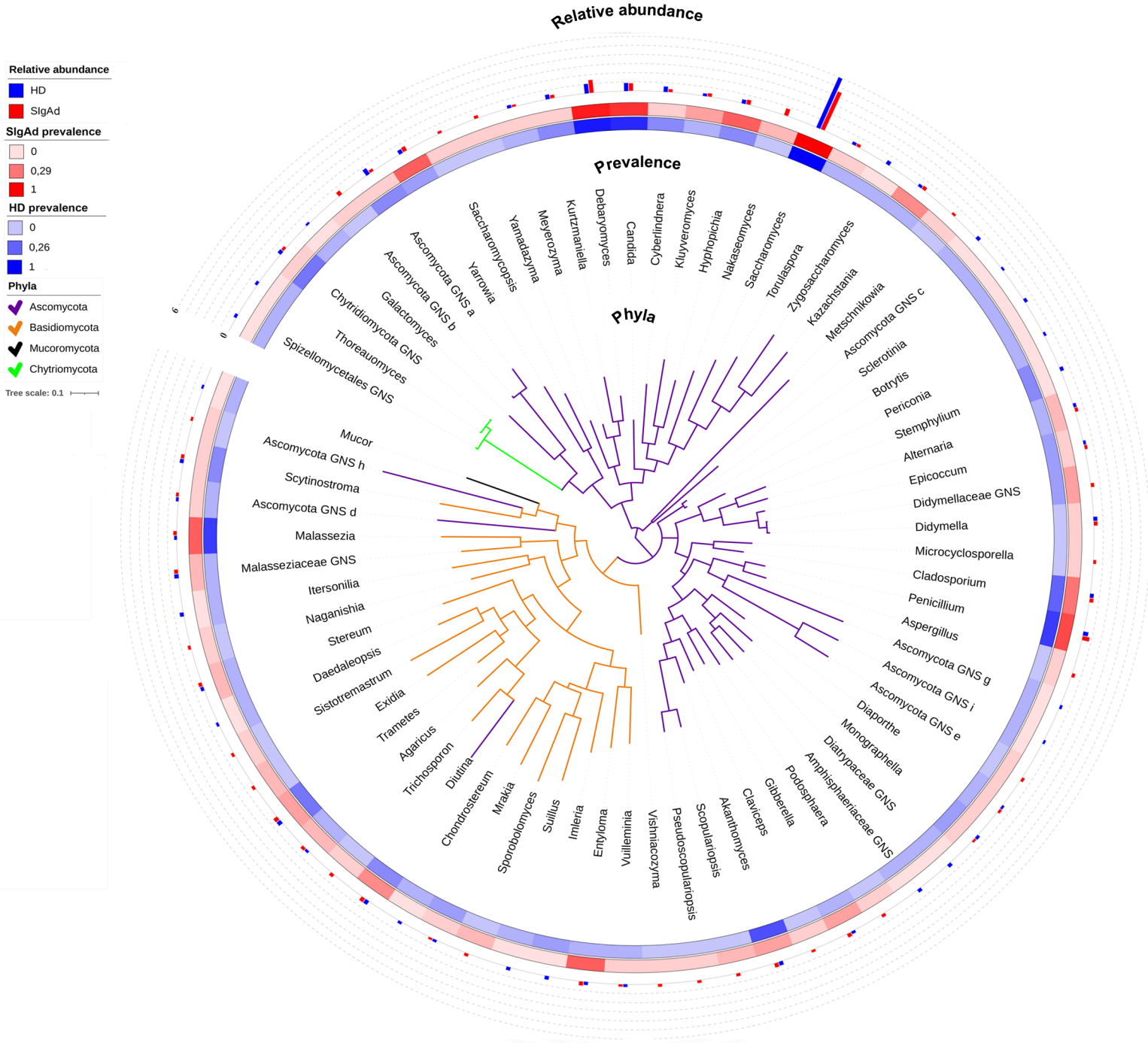


Figure E3

