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## **Intestinal *Candida albicans* overgrowth in IgA deficiency**

Alicia Moreno-Sabater, Delphine Sterlin, Lejla Imamovic, Fabienne Bon, Anne-Cecile Normand, Cecile Gonnin, Marianne Gazzano, Merieme Bensalah, Karim Dorgham, Elyes Ben Salah, et al.

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

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

3

4 Alicia Moreno-Sabater PharmD, PhD<sup>a</sup>, Delphine Sterlin PharmD, PhD<sup>b\*</sup>, Lejla Imamovic  
5 PhD<sup>c\*</sup>, Fabienne Bon PhD<sup>d</sup>, Anne-Cecile Normand PhD<sup>e</sup>, Cecile Gonnin MSc<sup>f</sup>, Marianne  
6 Gazzano MSc<sup>f</sup>, Merieme Bensalah MSc<sup>f</sup>, Karim Dorgham PhD<sup>c</sup>, Elyes Ben Salah MSc<sup>f</sup>, Aniss  
7 Acherar MSc<sup>g</sup>, Christophe Parizot PhD<sup>f</sup>, Virginie Rigourd MD, MSc<sup>h</sup>, Hervé Begue PhD<sup>d</sup>,  
8 Frederic Dalle PharmD, PhD<sup>d,i</sup>, Claude Bachmeyer MD, MSc<sup>j</sup>, Christophe Hennequin MD,  
9 PhD<sup>k</sup>, Hans Yssel PhD<sup>c</sup>, Marion Malphettes MD, MSc<sup>l</sup>, Claire Fieschi MD, PhD<sup>l</sup>, Jehane  
10 Fadlallah MD, PhD<sup>l</sup>, Guy Gorochov MD, PhD<sup>b</sup>.

11 \*D. Sterlin, and L. Imamovic contributed equally to this work

12

13 <sup>a</sup> Sorbonne Université, Institut national de la santé et de la recherche médicale (INSERM),  
14 Centre d'Immunologie et des Maladies Infectieuses, Assistance Publique-Hôpitaux de Paris  
15 (AP-HP), Hôpital Saint Antoine, Paris, France.

16 <sup>b</sup> Sorbonne Université, INSERM, Centre d'Immunologie et des Maladies Infectieuses, AP-HP,  
17 Hôpital Pitié-Salpêtrière, Paris, France.

18 <sup>c</sup> Sorbonne Université, INSERM, Centre d'Immunologie et des Maladies Infectieuses, Paris,  
19 France.

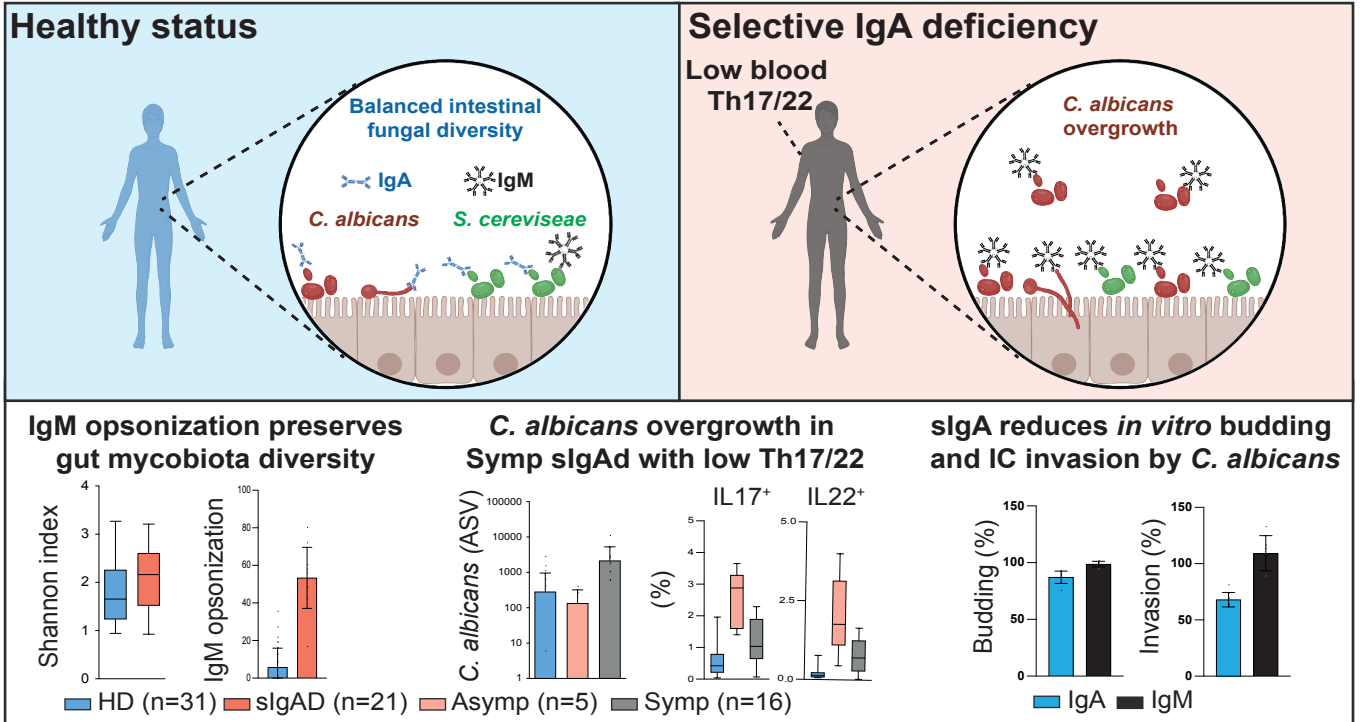
20 <sup>d</sup>UMR PAM Université de Bourgogne Franche-Comté (UBFC), AgroSup Dijon, Équipe Vin,  
21 Aliment, Microbiologie, Stress, Groupe Interactions *Candida*-muqueuses, Dijon, France.

22 <sup>e</sup> Service de Parasitologie-Mycologie, AP-HP, Groupement Hospitalier Pitié-Salpêtrière, Paris,  
23 France.

24 <sup>f</sup>Département d'Immunologie, AP-HP, Groupement Hospitalier Pitié-Salpêtrière, Paris, France.



## 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



25 <sup>g</sup> Sorbonne Université, INSERM, Institut Pierre Louis d'Épidémiologie et de Santé Publique,  
26 AP-HP, Hôpital Pitié-Salpêtrière, Paris, France.

27 <sup>h</sup> Lactarium régional d'Ile de France. AP-HP, Hôpital Necker-Enfants Malades, Paris, France.

28 <sup>i</sup> Department of Parasitology/Mycology, Dijon Bourgogne University Hospital, Dijon, France.

29 <sup>j</sup> Service de Médecine Interne, AP-HP, Hôpital Tenon, Paris, France.

30 <sup>k</sup> Sorbonne Université, INSERM, Centre de Recherche Saint-Antoine, AP-HP, Hôpital Saint  
31 Antoine, Paris, France.

32 <sup>l</sup> Université Paris Cité, Department of Clinical Immunology, Hôpital Saint-Louis, Paris, AP-  
33 HP, France.

34

35 Correspondence to G. Gorochov: [guy.gorochov@sorbonne-universite.fr](mailto:guy.gorochov@sorbonne-universite.fr)

36 Département d'Immunologie ; Hôpitaux Universitaires Pitié Salpêtrière - Charles Foix, 47/83

37 boulevard de l'Hôpital - 75013 Paris

38 Tel : + 33 1 42 17 79 30 Fax : + 33 1 42 17 74 90

39

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**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.<sup>1</sup> Commensal  
90 fungi induce innate responses, implicating CX3CR1<sup>+</sup> macrophages via CARD-9 signaling  
91 pathway, as well as Th17 cell-mediated adaptive responses which are essential to maintain  
92 mycobiota homeostasis.<sup>2</sup> 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.<sup>3</sup>

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.<sup>4</sup>  
99 Although the human mycobiota contains a large number of fungal genera,<sup>5</sup> sIgA-fungal  
100 interactions have focused on *Candida albicans*, a human commensal and a major fungal  
101 pathogen.<sup>6</sup> 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.<sup>7</sup> 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.<sup>8, 9</sup> 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.<sup>8</sup>

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.<sup>10-13</sup> We however recently reported  
115 that systemic IgG responses against commensal fungi can also be detected in healthy subjects,<sup>14</sup>  
116 while CARD9-deficient patients with systemic candidiasis present decreased anti-fungal  
117 commensal IgG levels.<sup>15</sup> Finally, in a CARD9-deficiency murine model, intestinal *C. albicans*  
118 interactions with CX3CR1<sup>+</sup>macrophages failed to elicit IgG class switch recombination and  
119 germinal center B cell expansion.<sup>8</sup>

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.<sup>16</sup> 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.<sup>16</sup> 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.<sup>14</sup> 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,<sup>14</sup> as follows. Twenty-five microliters of fixed (2%  
159 PFA/PBS) samples containing  $2.5 \times 10^5$  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.<sup>17</sup> 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  $3 \times 10^7$ /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,<sup>5</sup> 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.<sup>14</sup> 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  $\mu$ L containing 1  $\mu$ M of each primer and 1  $\mu$ L 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 reads<sup>18</sup>  
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)).<sup>19</sup> 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.<sup>20</sup> 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). DESeq<sup>21</sup> was used to produce a matrix containing differential  
218 abundance values (e.g. log<sub>2</sub> 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- $\gamma$ -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% CO<sub>2</sub> 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<sub>600nm</sub> of 0.28 to 0.32 and then grown to log phase for another 2 hours in the  
245 same conditions. A pellet of 5.10<sup>4</sup> 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 5.10<sup>4</sup>  
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% CO<sub>2</sub>. 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.<sup>22</sup>

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<sup>+</sup> as compared to sIgM<sup>+</sup> 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,<sup>23</sup> 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  $\pm 1.15$  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 *Yarrowia* 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.<sup>24</sup> 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,<sup>6</sup> we then compared the frequency of  
344 cytokine-secreting circulating CD4<sup>+</sup> T cells in asymptomatic and symptomatic IgA-deficient  
345 individuals (Fig 3, D-E). Proportions of interferon- $\gamma$  (IFN- $\gamma$ )<sup>+</sup>CD4<sup>+</sup> 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<sup>+</sup>- and IL-22<sup>+</sup>-secreting CD4<sup>+</sup> 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 $\gamma$ <sup>+</sup>IL-17<sup>+</sup> CD4<sup>+</sup> 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.<sup>16, 25, 26</sup> 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.<sup>27</sup> 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*,<sup>6, 28-30</sup>. 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 infants<sup>31</sup>  
414 and, in case of fungal overgrowth, to be associated with the occurrence of inflammatory bowel  
415 disease.<sup>32-34</sup>

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*<sup>35</sup>  
432 and *C. albicans*<sup>6</sup> 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<sup>+</sup>Th17  
434 systemic response,<sup>36</sup> *C. albicans* sIgAd-colonized patients showed lower IL-17<sup>+</sup>CD4<sup>+</sup>, IL-  
435 22<sup>+</sup>CD4<sup>+</sup> and IFN- $\gamma$ <sup>+</sup>IL17<sup>+</sup> responses. These results corroborate and extend previous findings  
436 showing that systematic sIgAd is associated with immune response defects<sup>37</sup> 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.<sup>38</sup> As reported previously in an experimental mouse model<sup>9, 15</sup> 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.<sup>15</sup> 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.<sup>9</sup> 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.<sup>39</sup>  
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  $\beta$ -(1,3)-glucan and chitin antigens which are conserved major fungal cell wall components.<sup>40</sup>

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

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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<sup>bright</sup> population  
 619 and double IgA<sup>+</sup>CFW<sup>bright</sup> 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<sup>+</sup> 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<sup>+</sup> T cells  
642 expressing indicated cytokines determined by intracellular staining. (E) CD4<sup>+</sup>IL-22<sup>+</sup>, IL17<sup>+</sup>,  
643 IFN $\gamma$ <sup>+</sup>IL17<sup>+</sup> 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<sup>bright</sup> 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**

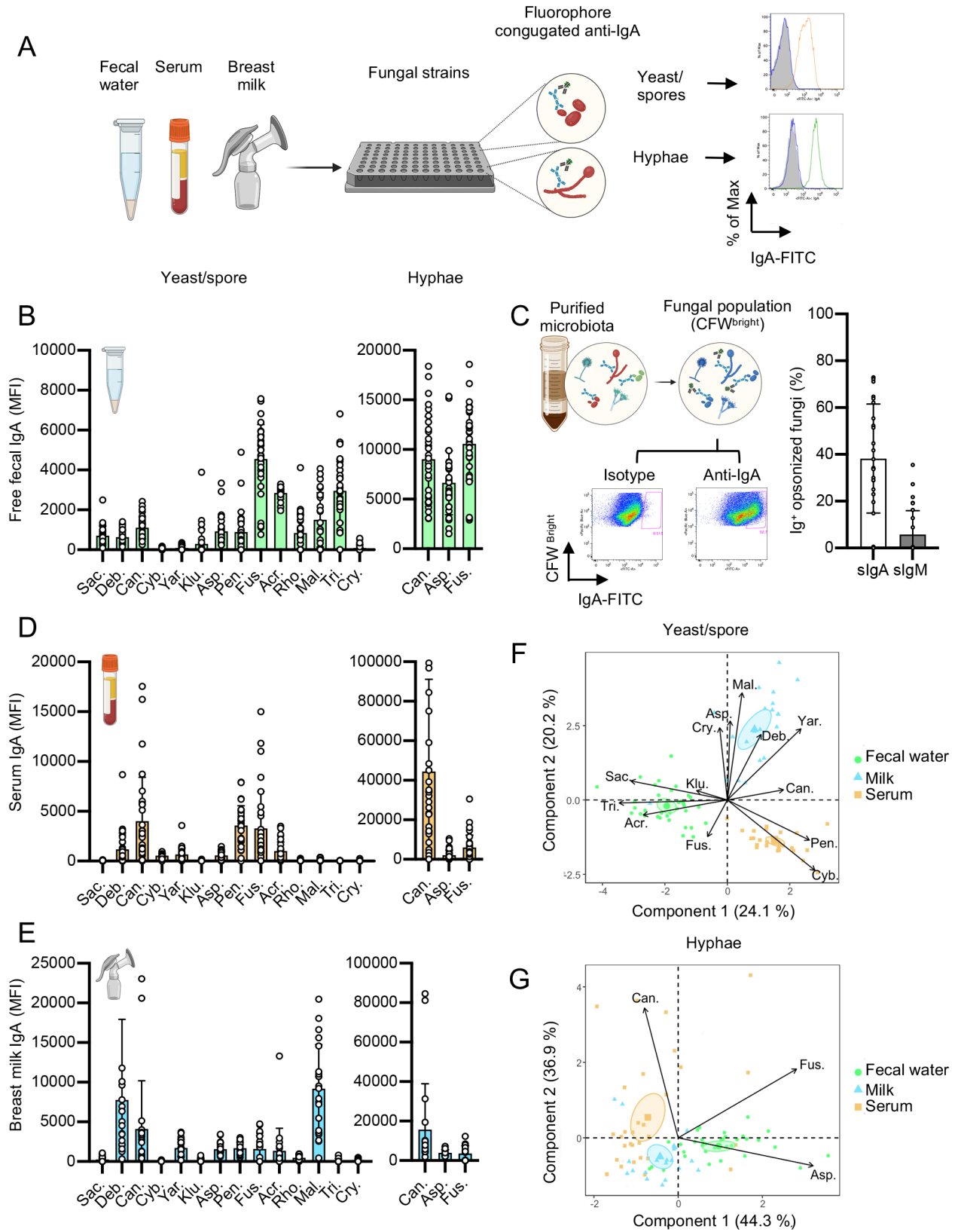


Figure 2

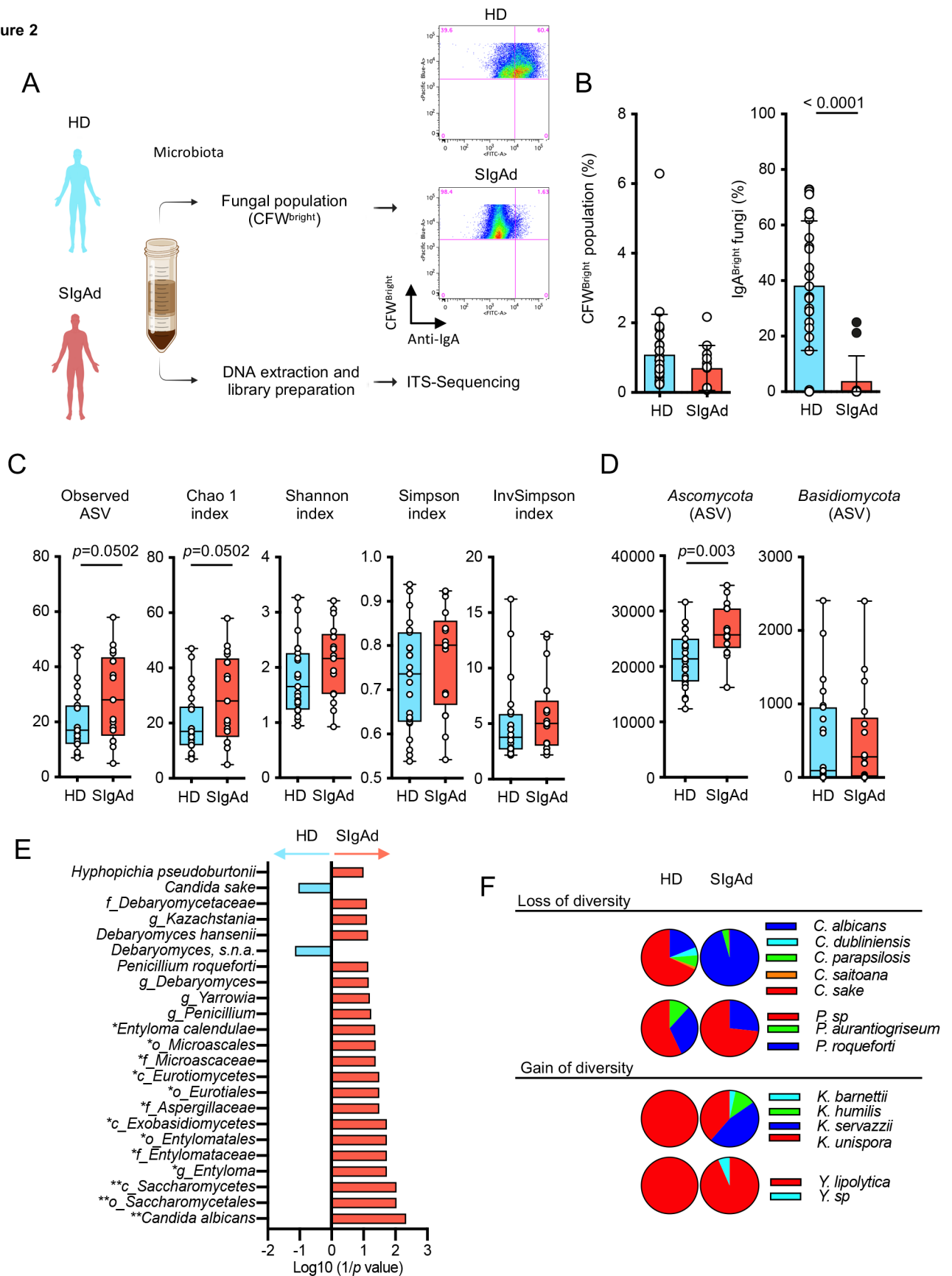


Figure 3

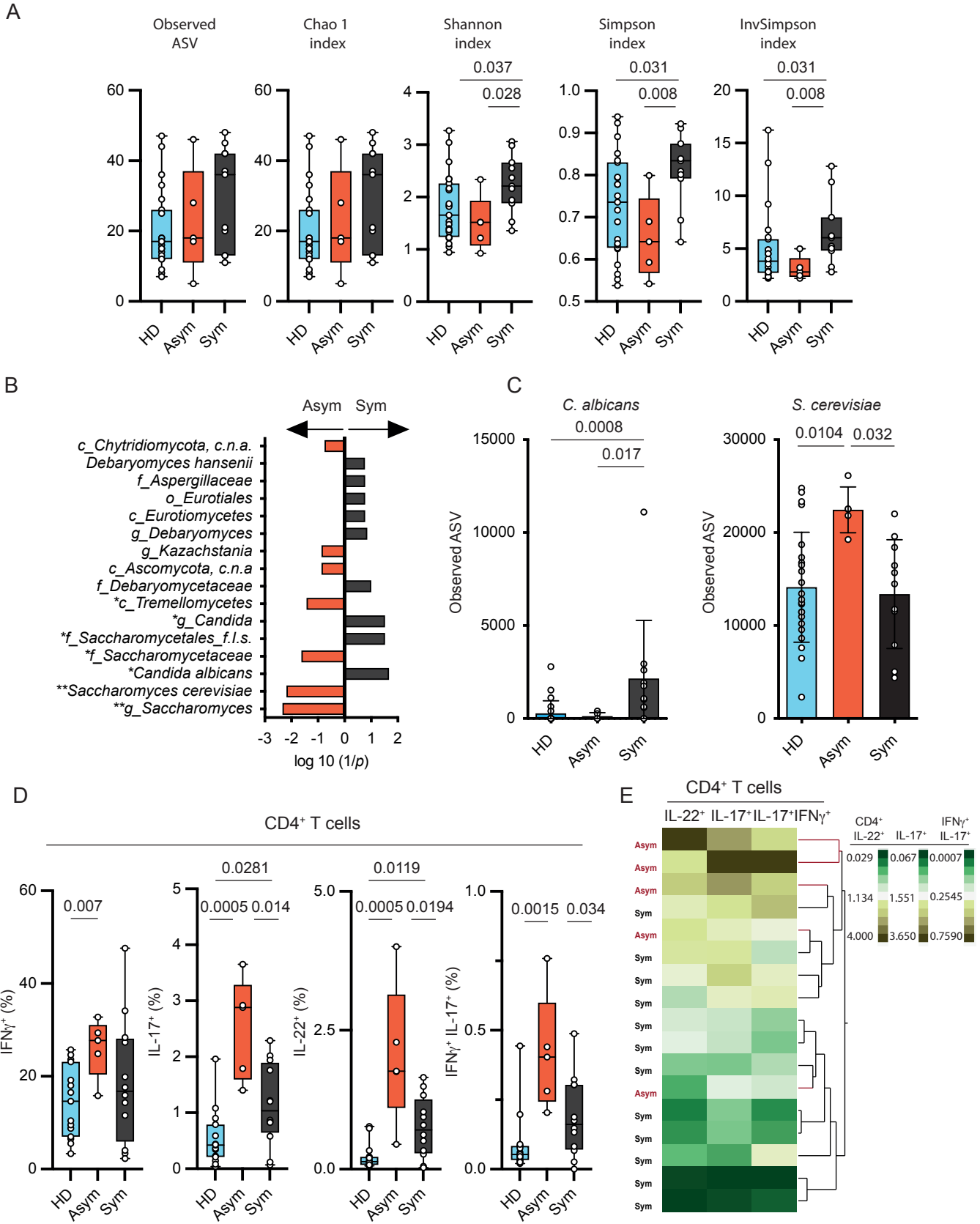


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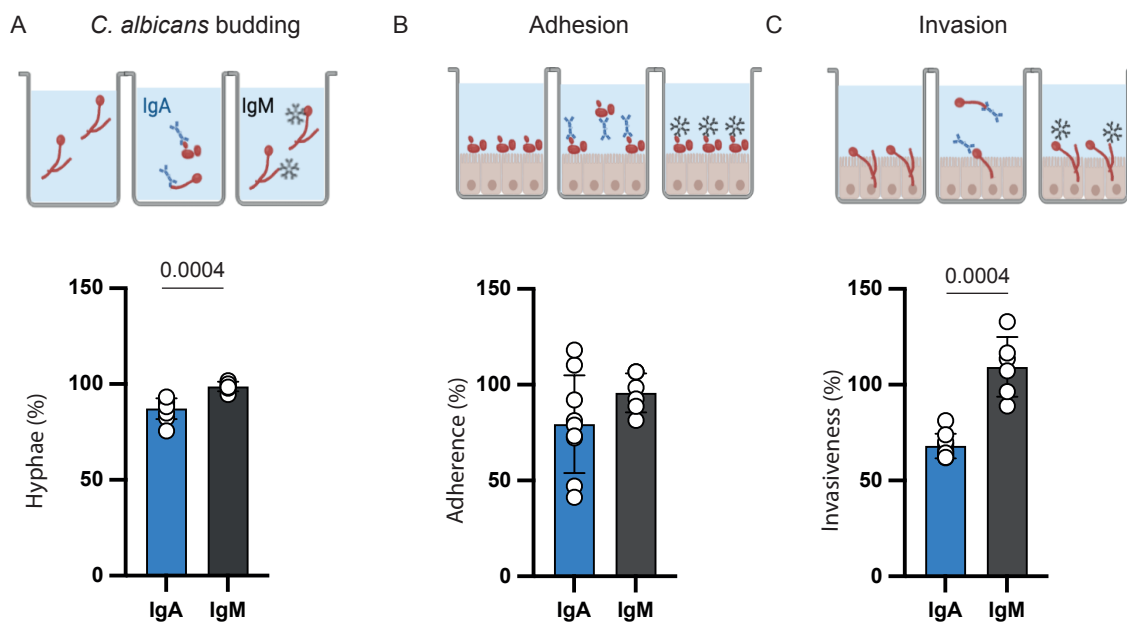
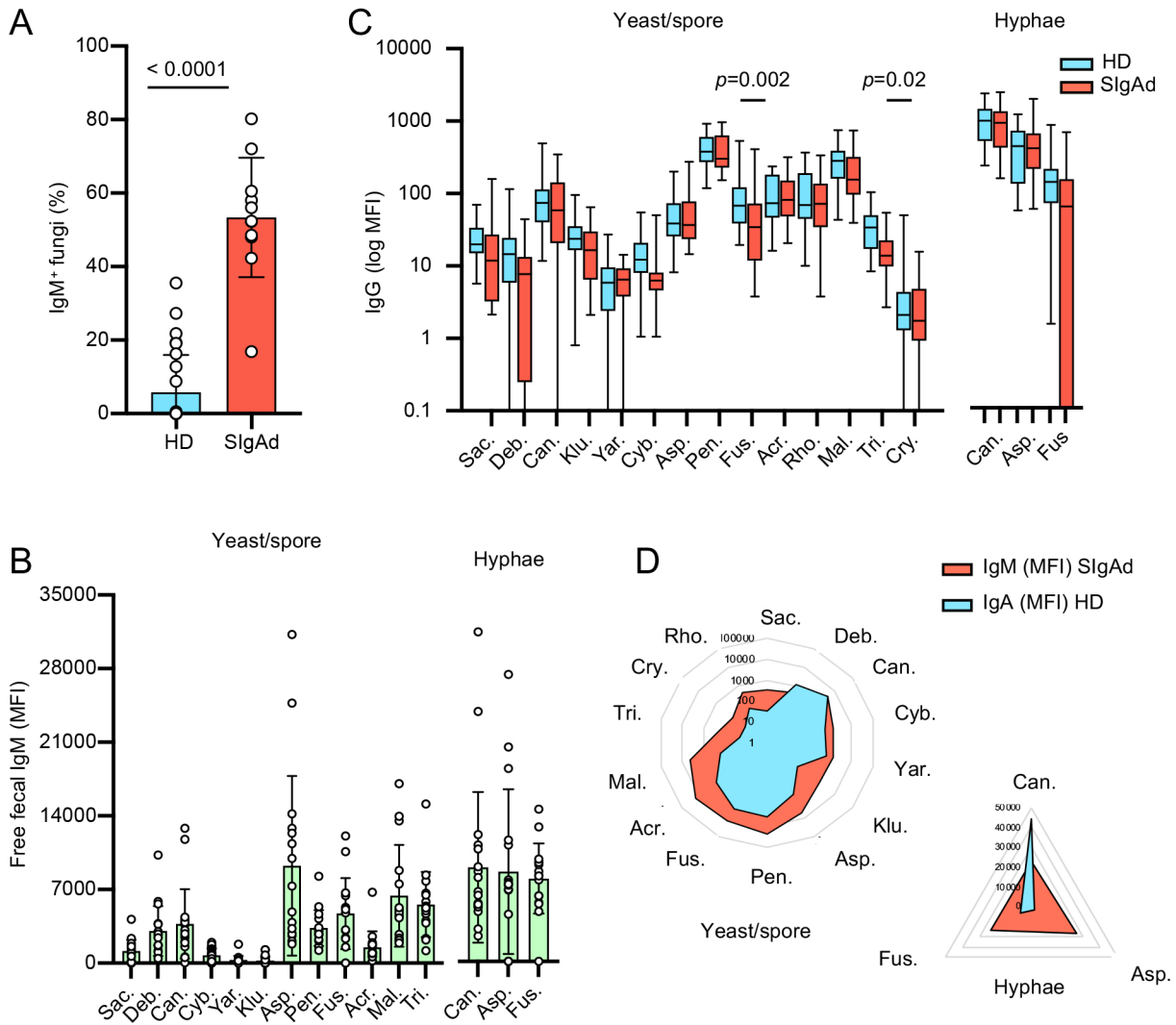


Figure 5



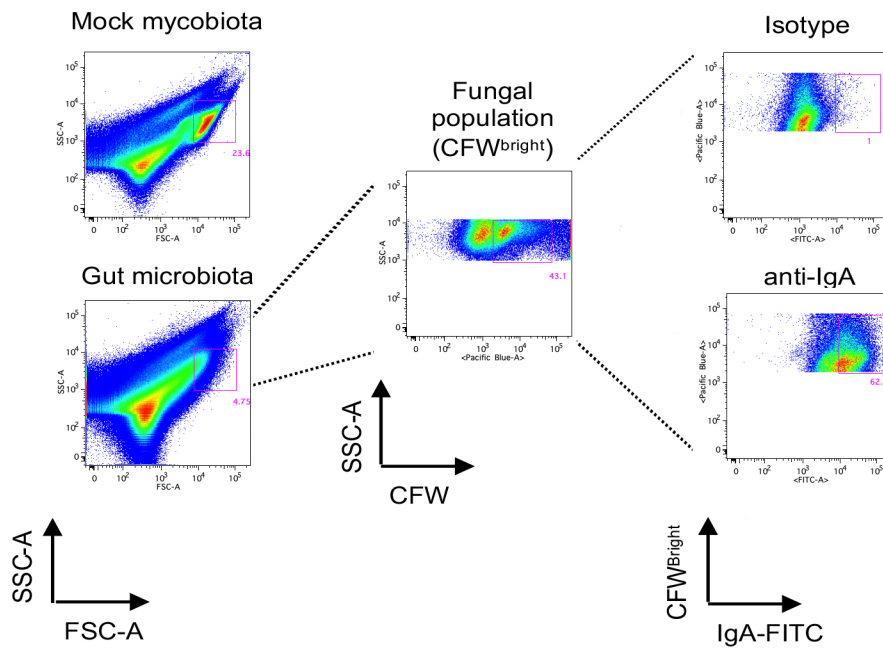
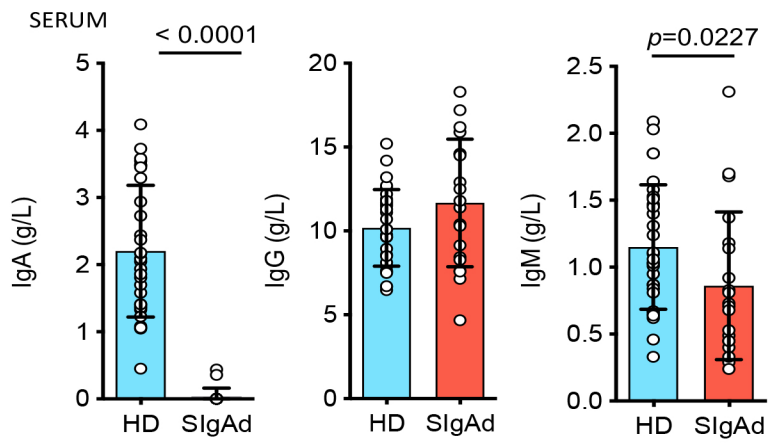
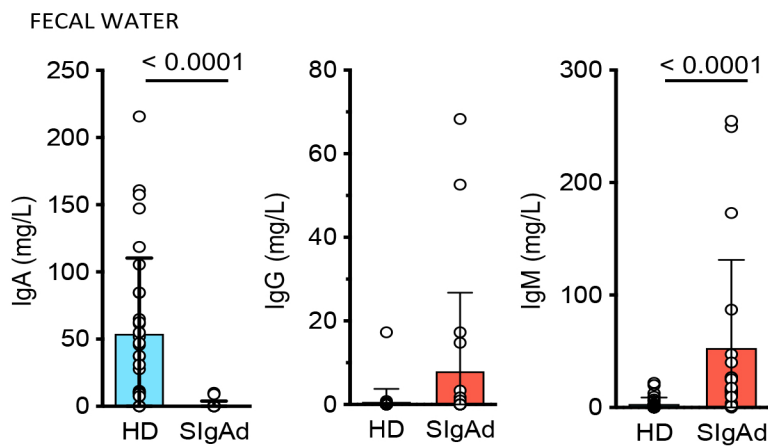
**A****B****C**



Figure E2

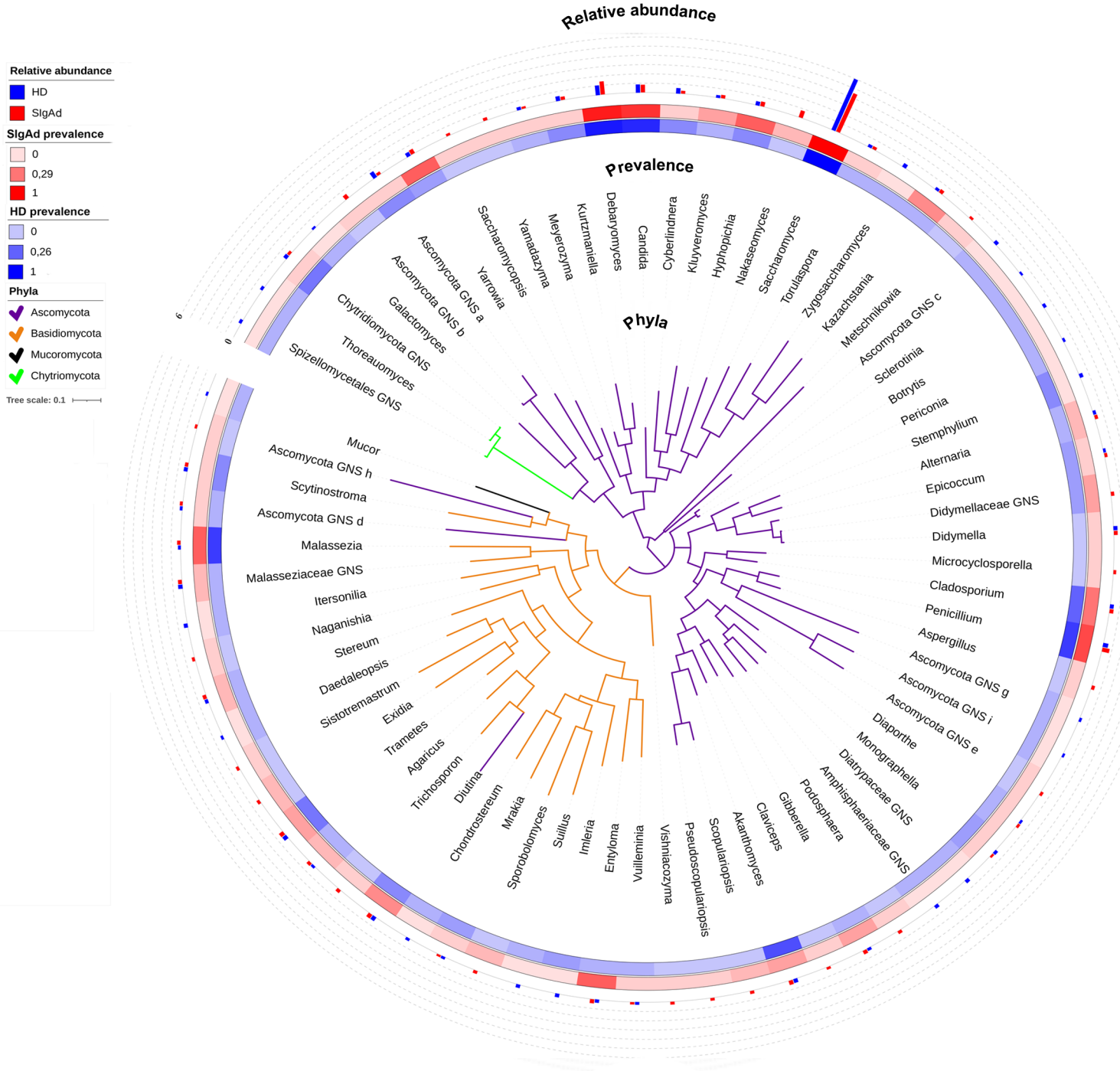


Figure E3

