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MIcrobiome profiles in breast milk from healthy women depend on mode of delivery, geographic location and interaction with bacteria

Running title: Breast milk healthy mycobiome in different geographic locations.

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Abstract

Recent studies report the presence of fungal species in breast milk of healthy mothers, suggesting a potential role on infant mycobiome development. In the present work, we aimed to determine whether the healthy human breast milk mycobiota is influenced by geographical location and mode of delivery, as well as investigate its interaction with bacterial profiles in the same samples. A total of 80 mature breast milk samples from 4 different countries were analysed by Illumina sequencing of the ITS1 region, joining the 18S and 5.8S regions of the fungal rRNA region. Basidiomycota and Ascomycota were found to be the dominant phyla, with *Malassezia* and *Davidiella* being the most prevalent genera across countries. A core formed by *Malassezia, Davidiella, Sistotrema* and *Penicillium* was shared in the milk samples from the different origins, although specific shifts in mycobiome composition were associated with geographic location and delivery mode. The presence of fungi in the breast milk samples was further confirmed by culture and isolates characterization, and fungal loads were estimated by qPCR targeting the fungal ITS1 region. Co-occurrence network analysis of bacteria and fungi showed complex interactions that were influenced by geographical location, mode of delivery, maternal age and pre-gestational Body Mass Index. The presence of a breast milk mycobiome was confirmed in all the samples analysed, regardless of the geographic origin.

Importance

During the last years, human breast milk has been documented as a potential source of bacteria for the newborn. Recently, we have reported the presence of fungi in breast milk from healthy mothers. It is well-known that environmental and perinatal factors could affect milk bacteria; however, the impact on milk fungi is still unknown. The current manuscript describes fungal communities (mycobiota) in breast milk samples across different geographic locations and the influence of mode of delivery. We also provide novel insights on bacteria-

48 fungi interactions taking into account environmental and perinatal factors. We identified a
49 core of four genera shared across locations, constituted by *Malassezia, Davidiella, Sistotrema*
50 and *Penicillium* which have been reported to be present in the infant gut. Our data confirm
51 the presence of fungi in breastmilk across continents and support the potential role of breast
52 milk on the initial seeding of fungal species to the infant gut.
53
54

Introduction

Early human microbial gut colonization is an essential step-wise process with an impact on the immunological and metabolic programming of later health (1–3). Fungi residing in the human gut have been recognized as an important part of the gut microbiota, and although research on the field is scarce, the mycobiome could have important roles on human health status (4–8). Although information about fungal communities in the infant is generally lacking, there is evidence that fungal species (mainly yeast-like) can be found in the gut since early life (9–11). Only a few reports have documented fungal transfer from mothers to infants , but little is known about how the mycobiome is shaped during this period (12–14). Recent prospective studies have revealed that altered gut mycobial patterns precede atopic wheeze and asthma development, and suggested fungal-bacterial interactions that would influence early-life patterns of microbial alpha diversity (15, 16).

Breast milk is an important source of bacteria to the infant, and together with oligosaccharides, contribute to the settlement of the gut microbiota characteristic to the healthy breast-fed child, with a strong impact on immune surveillance within the gastrointestinal environment, and thereby also other membranes of the body (17–19). A recent study suggested the presence of a diversity of fungal species in human breast milk of healthy mothers, including *Malassezia*, *Candida* and *Saccharomyces* as the most common genera, by means of high-throughput sequencing, microscopy and other culture-independent techniques (20). Moreover, viable yeasts, predominantly *Candida parapsilosis* and *Rhodotorula mucilaginosa* species, were isolated and characterized. This finding provides a new angle to the infant mycobiome development, and calls for further evaluation of the key determinants of their composition. Furthermore, complex interactions between bacteria and fungi have been reported in the human gut, oral cavity, skin and vagina (16, 21–24) and thus, such are also likely to occur in breast milk.

81 In addition, accumulating evidence suggests that some environmental factors could
82 influence breast milk composition(25–27). In particular, geographic location, delivery mode,
83 maternal Body Mass Index (BMI) and age have been suggested to have an impact on breast
84 milk bacterial composition (28–35), although their potential impact on the milk's fungal
85 fraction is still to be elucidated.

86 In the present study, we characterized the breast milk mycobiota of healthy breast-
87 feeding mothers from four different countries (Spain, Finland, South Africa and China), in order
88 to investigate the potential influence of geographic location and mode of delivery on its
89 composition. Fungal loads in the samples were estimated, and co-occurrence networks
90 between specific fungi and bacteria were analysed for potential interactions depending on
91 mode of delivery across the different countries.

92

93 ## Results

94 **Subject Description**

95 The characteristics of the subjects participating in the study are listed in **Table 1**.
96 The mean age of the mothers (n=80) was 33.52 (SD±4.87), with no statistical differences
97 between countries. Mean pre-gestational BMI was 24.06 (SD±3.85), normal weight.
98 Chinese mothers had significantly lower BMI= 21.71 (SD±1.97), considered as normal
99 weight. Differences in BMI between samples from mothers delivery vaginally or by C-
100 section were only observed in South African and Finish women, where mothers delivering
101 by C-section had higher BMI, 26.67 (SD±1.41) and 26.30 ± 2.57, respectively; although this
102 difference was only significant in the South African group (p<0.05).

103

104 **Fungal cells detection in breast milk**

105 80 milk samples were analysed by qPCR targeting the ITS1-5.8S rRNA region. Results
106 showed that 16/20 Spanish samples had detectable levels of fungi (80%; median values,
107 195,142 cells/ml); 9/20 of the Chinese samples (45%; median value= 170,732 cells/ml); 7/20 of
108 the Finish samples (35%; median value= 199,480 cells/ml), and 14/20 of South African samples
109 (70%; median value=371,119 cells/ml). No significant differences were observed between
110 geographic locations, nor by mode of delivery (**Figure S1**).

111 The presence of fungal cells in the milk was further confirmed by culture in fungal-
112 specific culture media and identification of the isolates by 18S rRNA sequencing, as well as by
113 microscopy after incubation of the milk samples with calcofluor-white fungal stain. A summary
114 of the results is available in the Supplemental Material (**Table S1 and Figure S2**).

115

116 **Fungal composition of breast milk: impact of geographical area and perinatal factors**

117 After sequencing the ITS1 fungal region, a mean of 107,765 taxonomically
118 assigned, clean and filtered sequences per sample (SD±45,493), with an average length of
119 301 bp were obtained. All breast milk samples contained fungal DNA and they were
120 dominated by two phyla: Basidiomycota (58.65%) and Ascomycota (41.03%). South African
121 samples had significantly higher levels of Ascomycota and lower levels of Basidiomycota
122 compared to the other countries ($p<0.05$).

123 Discriminant Analysis of Principal Components (DAPC), which transforms data using a
124 principal components analysis (PCA) and subsequently identifies clusters using discriminant
125 analysis (DA), showed that South African samples clustered in distance from the other
126 countries, mainly due to the increased levels of *Rhodotorula mucilaginosa* (**Figure 1**).

127 Taxonomic analysis at genus level showed that breast milk samples were dominated by
128 *Malassezia* (40.6% average abundance), followed by *Davidiella* (9.0%), that was prevalent
129 regardless of the location or the donor's type of delivery **(Figure 2a)**. The effects of country of

130 origin and mode of delivery on the breast milk fungal composition were analysed, and
131 reflected that milk mycobiota differed significantly across geographic location (PERMANOVA,
132 p=0.005) and mode of delivery (PERMANOVA, p=0.023). Redundant analysis (RDA) confirmed
133 the effect of geographic location on breast milk fungal composition (p=0.001), although that of
134 mode of delivery did not reach statistical significance. Kruskal-Wallis test was implemented to
135 compare phylotypes at genus level across samples. Results showed that *Malassezia* was
136 statistically less abundant in South African samples (p<0.05), and *Penicillum* and *Rhodotorula*
137 abundances were lower in Chinese samples (p<0.01); while *Saccharomyces* was more
138 abundant in Spanish and Finnish samples (p<0.01) compared to the rest of locations. No
139 statistical significant effect of maternal age, gestational BMI nor antibiotic intake during
140 delivery was detected on breast milk microbial composition by using MaAsLin analysis.

141 Despite the differences, a core of four genera shared across the four countries was
142 identified, including *Malassezia, Davidiella, Sistotrema* and *Penicillium*. *Wallemia* and
143 *Aspergillus* were only found in samples from Finland, *Botrytis* and an unidentified
144 Saccharomycetales in South African samples, and an unidentified Malasseziales in Spanish
145 samples. *Rhodotorula* was present in samples from other countries except China **(Figure 2b)**.

146 Comparisons between samples were further analysed at species level. LefSe results
147 showed differentially abundant fungi between countries. *Rhodotorula mucilaginosa* and
148 *Saccharomycetales sp.* were more abundant in South African samples, while *Malassezia furfur*
149 was more prevalent in Chinese samples, and an *Ascomycota sp*. was more abundant in Spanish
150 samples **(Figure 2c)**.

151 Taking into account mode of delivery, mycobiota composition was different across the
152 milk samples from different geographic origins. Kruskal- Wallis test reflected that *Cryptococcus*
153 was statistically significantly higher in milk samples of women delivering vaginally, as
154 compared to those who delivered by C-section (p=0.028). At species level, in Chinese breast
155 milk samples, *Candida smithsonii* was significantly more abundant in vaginal deliveries;

156 *Sistotrema sp.* in C-section Spanish samples; *Ascomycota sp* in Finnish vaginal delivery samples,
157 and *Malasezzia restricta* in C-section samples; and *Malassezia restricta* and *Davidiella tassiana*
158 in C-section South African samples (LefSe analysis, $p<0.05$) **(Figure 2d)**.
159 Indices of alpha diversity and richness across the samples were similar and no statistical
160 differences were observed between geographic locations nor delivery mode (Figure S3).
161
162 **Fungal and bacterial interactions: a network analysis**
163 Network analyses of the bacteria and fungi present in the breast milk samples showed
164 complex interactions intra- and inter-domain, with different associations among organisms
165 depending on the country of origin and delivery mode, some of which were also influenced by
166 maternal features. For example, a *Malassezia* OTU (Fungi_1) correlated positively with a
167 *Streptococcus* (Bact_6) from vaginal delivery samples, and with a *Streptococcus* (Bact_1) from
168 C-section deliveries among Finnish samples, and the abundances were dependent of maternal
169 age. The same *Malassezia* OTU correlated positively with several *Streptococcus* OTUs in
170 samples from C-section deliveries from Chinese mothers, and also positively with an
171 Unclassified Bacilli (Bact_2) from South African samples and vaginal deliveries. Significant
172 influence of maternal age and BMI on specific bacterial and fungal organisms were also
173 observed (**Figure 3**). However, given that the density of fungal cells is at least one order of
174 magnitude lower than that of bacteria, the influence of fungi on the breast milk ecosystem
175 needs to be elucidated.
176 In order to study the diversity of the most common yeast in our samples, a
177 phylogenetic tree of the most prevalent *Malassezia* OTUs detected in this work across
178 geographic locations was performed, including known members of the *Malassezia* genus as a
179 reference (Figure S4). The tree shows a large diversity of *Malassezia* isolates with similarity to
180 at least four known species, including OTUs which could potentially represent new species.
181 With the exception of one OTU (Fungi 37, which was found to be uniquely present in China), all

182 other sequences were found in all countries and appear to be therefore ubiquitous. In relation
183 to mode of delivery, all the OTUs were present in breasmilk from mothers with both delivery
184 types.
185

186 **Discussion**

187 Breast milk is a continuous source of microbes that are transmitted, together with
188 many nutrients and protective compounds, to the infant gut during a critical period when the
189 key regulatory systems of the body are immature (17, 18). Although bacteria inhabiting human
190 breast milk have been extensively studied, the presence of fungi in the fluid had not been
191 assessed until recently, when a diversity of fungal phylotypes in breast milk from healthy
192 Spanish mothers was reported by our group(20). The mycobiome, the fungal fraction of the
193 human microbiome, is present in lower abundances and has been much less explored than the
194 bacterial fraction. However, its potential importance for human health and disease has
195 stimulated an increased interest on this field (5–7, 10). In the infant, fungal species can be
196 detected since very early in life [10,11,13]. However, the infant mycobiome is almost
197 unexplored, and information about its development is scarce. To ascertain the presence of
198 fungi in breast milk is difficult because of the possibility of contamination in samples with low
199 microbial density, and therefore multiple approaches and strict negative controls are needed
200 (15).

201 A recent study reported higher gut fungal diversities during the first months of life,
202 that decreased over time, while the diversities of the bacterial fraction increased in reciprocal
203 correlation, suggesting that potential inter-kingdom associations may drive microbial gut
204 dynamics(36).

205 In the present study, we have confirmed the presence of diverse fungal communities
206 in breast milk samples from Spain, Finland, China and South Africa. Fungi were detected in all
207 breast milk samples through massive DNA sequencing, with the two phyla Basidomycota and

208 Ascomycota being the most prevalent and presenting reciprocal patterns of abundance in all
209 countries except for South Africa, where Ascomycota levels were significantly higher and
210 Basidiomycota lower compared to the other countries. At genus level, *Malassezia*
211 predominated in all countries, followed by *Davidiella.* In our previous work reporting the
212 presence of fungi in breast milk, *Malassezia* also represented the most abundant genus (20).
213 Other genera found in the current manuscript such as *Alternaria*, *Rhodotorula, Saccharomyces*,
214 and *Candida* were also found in the mentioned study.

215 Results yielded by qPCR showed that >70% of Spanish and South African samples had
216 detectable levels of fungal DNA; 45% of Chinese samples and only 35% of Finish samples.
217 Fungal median load in all the samples was $2,5x10^5$ cells/ml, in agreement with our previous
218 results on Spanish samples.

219 Our findings reinforce the potential influence of environmental factors, in particular
220 geographic location and delivery mode, on breast milk fungal composition. Samples from
221 South Africa clustered distanced from the other countries according to their fungal
222 composition influenced by the higher levels of *Rhodotorula mucilaginosa* in those samples
223 (Figure 1). Although differences among samples from different geographic locations were
224 observed, a core constituted by four genera, *Malassezia*, *Davidiella*, *Sistotrema* and *Penicillium*
225 was shared in all countries (Figure 2b).

226 Breast milk mycobiota also differed depending on the mode of delivery (vaginal or C-
227 section) across countries. Specific fungi, such as the genus *Cryptococcus,* appeared to be more
228 prevalent among samples from mothers delivering vaginally, and specific shifts at species level
229 were also observed within each country. No differences in fungal diversity nor richness were
230 observed in the present study. Previously, we identified changes in breast milk microbiota
231 between locations, as well as in the milk metabolite profile(31, 37) using the same samples
232 analysed in this study.

233 Although the origin of breast milk fungi is unknown, most of the organisms detected in
234 this study can be found in other human niches. *Malassezia* are yeasts whose primary niche is
235 the human body (and other animals). In healthy individuals they are part of the normal
236 microbiota where they predominantly colonize the seborrheic parts of the skin (38), and are
237 commonly found in infants (9, 39–41). *Malassezia* has also been detected in significant
238 abundance in adult (11, 38, 42) and infant fecal samples (43), and therefore may play a role at
239 the intestinal level, and has also been described as an oral commensal (44). Although
240 *Malassezia* DNA has been detected in high proportions in breast milk before, no viable cells
241 could be recovered by classic culture methods from breast milk, (20) and further efforts should
242 be made to culture this organism, which has also been shown to be able to penetrate the cell
243 and survive intracellularly.

244 *Davidiella*, the second most prevalent fungi found in the samples of this study, has
245 been detected in the only published study about the characterization of vaginal microbiota and
246 mycobiota of asymptomatic women (45). In the same study, *Candida* was found to be the
247 predominant genus. Therefore, they may play an important role in the early colonization of
248 vaginally born infants. In our previous study on breast milk fungi, *Davidiella* could not be
249 detected(20), which could be associated to the differences on sequencing platforms and genes
250 targeted in both studies, as has been previously shown(46, 47). In addition, *Davidiella*
251 represents the sexual form of the *Cladosporium* genus (48). Fungi can have an asexual
252 (anamorph) and sexual (teleomorph) form that may be classified into different genera. This
253 sexual dimorphism can be a significant problem when classifying fungal sequences and the use
254 of different databases and/or sequencing of different genes can lead to conflicting
255 classifications. In a study with paediatric inflammatory bowel disease (IBD) patients,
256 *Cladosporium cladosporiodes* abundance decreased in IBD, while *Pichia jadinii* and *Candida*
257 *parapsilosis* increased compared to controls (49).

258 *Candida* is probably the most ubiquitous genera of the human mycobiome. It is the
259 major fungal genera detected in the adult oral cavity (50, 51) , and has also been detected in
260 the infant mouth, including several species as common inhabitants (*C. parapsilosis , C.*
261 *tropicalis*, *C. orthopsilosis,* etc.*)* (9, 52, 53). Several *Candida* species are also commonly present
262 in the adult skin and fecal samples (7, 42), and in the infant anus and fecal samples (9, 54).
263 Despite *Candida* can be responsible of vaginal infections (55), it is the most prevalent fungi in
264 the vaginal mycobiome of healthy women (45). Transmission of *Candida* from mother to infant
265 likely occurs, as the same fingerprinting of the DNA has showed identity between maternal
266 *Candida* from vagina, rectum, oral cavity and skin, and infant oral cavity and rectum (14).

267 Other prevalent fungi detected in our samples are commonly found in several body
268 niches. *Saccharomyces* are among the most abundant fungi in the gut (7, 42), and
269 *Saccharomyces cerevisiae* has been reported to be highly prevalent and abundant in the infant
270 oral and anal mycobiome during the first month of life (9). In a recent study, bacteria and fungi
271 from fecal samples in children suffering atopic wheeze were analysed, and Saccharomycetales
272 taxa appeared to be decreased in the atopic wheeze group, while the species *Pichia*
273 *kudriavzevii* was increased compared to controls (24). Others such as *Penicillium* or *Aspergillus*
274 can also be detected in fecal samples, and *Debaromyces hansenii* represents one of the main
275 species present in breastfed infants' gut (12). In the present study, we have detected
276 *Debaromyces* although none of the sequences have been classified as *D. hansenii*. However,
277 DNA from this species was previously detected in breast milk (20).

278 The study of inter-species interactions within a population is necessary to better
279 understand the microbiota's role. It is known that microorganisms can interact by competition
280 and sometimes collaboration, thereby influencing microbiota composition and host's health. It
281 has been demonstrated that cross-talk between bacteria and fungi can exist, modulating host
282 defence mechanisms, protecting against infections or collaborating to cause them (56, 57). For
283 example, synergies between oral *S. oralis* and *C. albicans* enhanced *C. albicans* invasion

284 through activation of host enzymes that cleave epithelial junction proteins (58). On the
285 contrary, *S. mutans* showed ability to modulate biofilm formation and to reduce *C. albicans*
286 virulence in an animal model (59). Some vaginal isolates of *Lactobacillus* strains have shown
287 anti-fungal activity *in vitro* against *Candida spp* and probiotic *L. rhamnosus* and *L. reuterii*
288 strains showed *in vitro* efficacy against *C. albicans* responsible of vaginal infections (24). To
289 understand microbial relationships, microbial networks analyses are indispensable, allowing
290 the identification and representation of the most influential members in a bacterial
291 community and their interactions with other microorganisms (60). In a recent work, bacterial
292 interactions in colostrum and mature milk of Italian and Burundian mothers were analysed,
293 and showed different bacterial networks among the two populations. The identified networks
294 were complex and dynamic, changing from colostrum to mature milk (61). In the present
295 study, we have analysed co-occurrence relationships between bacteria and fungi in breast
296 milk, observing a complex network of interactions between fungi and bacteria, and within the
297 same domain. Microbial interactions were influenced by delivery characteristics (mode of
298 delivery and geographic location), and maternal features (maternal BMI and age) influenced
299 the prevalence of particular microorganisms. Interesting positive correlations were observed
300 between several *Malassezia,* the most prevalent fungi detected in breast milk by sequencing,
301 and different streptococci, the latter representing one of the most common bacterial genera in
302 breast milk (62). Interestingly, in our previous study we observed a significant positive
303 correlation between *Malassezia* and bacterial load (20), and further experimental research
304 should analyse potential synergistic relationships between these genera.

305 Our data confirmed the presence of fungal DNA and fungal cells (including viable cells)
306 in breast milk samples from healthy mothers from four different geographic locations, by using
307 different approaches. This supports the existence of a "breast milk mycobiota" under healthy
308 conditions. Differences in composition associated to mode of delivery and country of origin
309 were observed. In addition, we observed some inter-domain microbial interactions in breast

310 milk that could lead to further *in vitro* studies. The presence of viable fungal cells suggests a
311 potential influence of breast milk on the infant's mycobiota development. However, data from
312 infant's gut mycobiota is missing in the present study, and further studies should address the
313 potential fungal transference from breast milk to the infant gut mycobiome. Although we tried
314 to prevent the contamination of maternal skin mycobiota by cleaning the breast prior to
315 sample collection (which has been previously shown to reduce bacteria in breast milk samples
316 (63)), it should be taken into account that certain retrograde flux occurs during breastfeeding,
317 and fungal species present in maternal skin and infant's mouth could be translocated to breast
318 milk, and *vice versa*(64). A greater understanding of the environmental influence on the
319 bacterial and fungal communities and their metabolic functions is also needed.
320

321 ## Material and Methods

322 **Subjects and Sampling**

323 Breast milk samples at 1-month post-partum were obtained from 80 healthy lactating
324 women from 4 different geographical locations (20 in each location), including China (Beijing
325 area), South Africa (Cape Town), Finland (South-western area), and Spain (Valencia,
326 Mediterranean area).

327 All mothers were practising exclusive breastfeeding. Subjects were grouped according
328 to mode of delivery: vaginal (n= 10 per country) and Caesarean-section (C-section) (n= 10 per
329 country). Maternal characteristics such as age, weight and pre-gestational body mass index
330 (BMI) were collected at the time of enrolment. All women who delivered via C-section received
331 prophylactic antibiotics, except Finnish women, for whom no prophylaxis is routinely used as
332 per the hospital policy. All participants were given detailed oral and written information, and
333 written informed consent was obtained for participation. The study protocol was approved by
334 the Ethics Committees of the respective participating institutions: Spain (Bioethics Committee
335 of CSIC and the Regional Ethics Committee for Biomedical Research), Finland (Ethics

336 Committee, Hospital District of Southwest Finland), China (Medical Research Board of Peking
337 University) and South Africa (University of Cape Town, Human Research Ethics Committee).
338 Previous to sample collection, nipples and mammary areola were cleaned with soap
339 and sterile water and soaked in chlorhexidine to reduce sampling of microorganisms residing
340 on the skin. Milk samples were collected in a sterile tube manually, discarding the first
341 drops. All samples were frozen at -20°C until further processing. To avoid bias, samples were
342 collected using the same standardised protocol in the four countries, and were processed and
343 analysed in a single laboratory.

344

345 **Microbial DNA Extraction and Sequencing**

346 Breast milk samples (1.5 ml) were centrifuged at 14,000 rpm for 20 min at 4°C to
347 remove fat, and pellets were used for total DNA extraction that involved mechanical and
348 chemical cell lysis. Bead beating was carried out using FastPrep® (FP120-230, Bio 101
349 ThermoSavant, Holbrook, NY, USA), and the InviMag® Stool DNA kit (Stratec Molecular, Berlin,
350 Germany) was used with the King Fisher magnetic particle processor (Thermo Fisher Scientific
351 Oy, Vantaa, Finland). The DNA extraction protocol was also followed with water to use as
352 negative controls. Isolated DNA concentrations were measured using a Qubit® 2.0 Fluorometer
353 (Life Technology, Carlsbad, CA, USA).
354 Primers targeting the highly variable fungal internal transcriber spacer ITS1 of the
355 fungal 18S ribosomal rRNA gene (forward: TAGAGGAAGTAAAAGTCGTAA, reverse:
356 TTYRCTRCGTTCTTCATC) (65) with adaptors were used for sequencing on an Illumina Miseq
357 platform. Sequencing was carried out at the Foundation for the Promotion of Health and
358 Biomedical Research, FISABIO (Valencia, Spain). No-template controls (NTCs) and negative
359 controls during DNA extraction were included to rule out potential contaminations at the time
360 of DNA extraction or sequencing.

361

362 **Fungal load**

363 qPCR amplification and detection of the ITS1-5.8S rRNA conserved fungal region was
364 performed as previously described (17), using the primers ITS1F: 5'-TCCGTAGGTGAACCTGCGG;
365 and 5.8 R: 5'-CGCTGCGTTCTTCATCG. Each reaction mixture of 20 µl was composed of 10 µl of
366 KAPA Sybr Fast qPCR Kit (KAPA Biosistems), 0.4 µl of each primer (10 µM concentration) and 2
367 µl of template DNA; using an annealing temperature of 61°C in a Light Cycler 480 Real-Time
368 PCR System (Roche Technologies). All amplifications were performed in duplicates and a
369 negative control was included in each reaction plate. Samples with Ct values equal or higher
370 than the negative control were considered as negative for fungal DNA.

371

372 **Breast milk culture and identification of fungal colonies**

373 100 µl of selected breast milk samples were plated in four solid fungal-selective media:
374 Sabouraud (Conda-Pronadisa) supplemented with cloramphenicol 0.05 g/l (Roche); Rose
375 Bengal (Conda-Pronadisa) supplemented with chloramphenicol 0.5 g/l (Roche); YPD (40 g/l
376 dextrose, 40 g/l peptone, 20 g/l yeast extract and 40 g/l agar) supplemented with 25 µg/ml of
377 streptomycin/25 U/ml of penicillin (Biowest); and YNB (Sigma) with 8% ethanol and 25 µg/ml
378 of streptomycin/25 U/ml of penicillin (Biowest). All plates were incubated aerobically at 37°C,
379 as previously described (17). DNA from the isolated colonies was extracted and amplified by
380 PCR using primers targeting the 18S rRNA gene (forward: 5'-GTAGTCATATGCTTGTCTC; and
381 reverse: 5'-CCATTCCCCGTTACCCGTTG). PCR products were sequenced in an Applied
382 Biosystems® 3730/3730xl DNA Analyzer at University of Valencia (Spain) and isolates were
383 identified by using the BLAST algorithm in the NCBI database, with minimum 98% sequence
384 identity.

385

386 **Microscopic analyses of fungi in milk**

387 In order to identify fungal cells in breast milk, samples were incubated with calcofluor-
388 white stain that dye the fungal and yeast's cell walls. Samples were visualized with
389 fluorescence microscopy using a Nikon Eclipse E90i microscope (Nikon Corporation) with a
390 100× objective. Images processing was performed using the NIS-Elements BR v3.22 software
391 (Nikon).

392

393 **Data Analysis**

394 ITS1 reads were pair-end joined using FLASH program (66) applying default
395 parameters. Resulting sequences were end-trimmed in 20 bp sliding windows with average
396 quality value >30, and length >50 bp, using the Prinseq-lite program (67). Chimeric reads were
397 eliminated using UCHIME algorithm (68), resulting in a total of 9,797,578 reads. Taxonomy
398 assignment of the remaining sequences was performed using Ribosomal Database Project
399 classifier standalone tool (69) with the UNITE fungal ITS v 7.2 trainset (70), and an 80%
400 confidence threshold. Sequences where clustered into operational taxonomical units (OTUs)
401 based on 99% identity, and representative OTUs sequences were obtained using CD-hit
402 software (71). OTU tables were rarefied to 9200 sequences per sample to avoid variations in
403 sequencing depth, and Shannon and Chao1 indexes were calculated using the "plyr" and
404 "vegan" packages from R software (version 3.2.2) (72).

405

406 **Statistical Analysis**

407 Calypso software (version 8.2) (73) was used to obtain Venn diagram for shared
408 phylotypes; Discriminant Analysis of Principal Components (DAPC) was performed at OTU
409 level, using geographic location as factor; and PERMANOVA and redundancy analysis (RDA),
410 were applied to study the statistical effect of country and delivery mode on the breast milk

411 fungal composition. Kruskal-Wallis test was implemented to study genus-level taxonomical
412 differences between countries and delivery mode, using GraphPad PRISM^{R} 6 (GraphPad
413 Software). Linear discriminant analysis effect size (LefSe) (74) algorithm was used to detect the
414 most differentially abundant fungi between countries, and between vaginal and C-section
415 deliveries in each country, at species level. In order to control the potential effects of maternal
416 age, maternal BMI pre-delivery and antibiotic use at delivery, MaAsLin (multivariate analysis
417 with linear model)(75) was applied, which finds associations between metadata and microbial
418 abundances. Other statistical analysis and graphs were performed using GraphPad PRISM^{R} 6.
419
420 **Analysis of bacterial and fungal co-occurence**
421 Sequences from the 16S rRNA gene of the same samples, from Kumar *et al* (31) were
422 obtained from NCBI (SRA accession: SRP082263 and submission ID: SUB1772296). Quality
423 filtering, chimera checking and OTU clustering were as followed for the ITS1 reads.
424 RDP classifier was used to taxonomically assign the bacterial (against RDP's 16S rRNA training
425 set 16) (76) and fungal (against the UNITE v 07-04-2014 trainset (70)) representative OTU
426 sequences. Samples with less than 1500 sequences were excluded from the analysis.
427
428 For the bacterial datasets, OTUs with a higher relative abundance in any of the two
429 controls than in the breast milk samples were treated as putative contaminants and discarded.
430 This procedure could not be performed on the fungal datasets, since the sequencing of the
431 two controls yielded too few reads. Nevertheless, the low fraction of reads assigned to
432 putative contaminants in the bacterial datasets (2% on average) leads us to believe that the
433 samples were essentially contamination-free. Both the bacterial and fungal OTU tables were
434 rarefied to 1500 sequences per sample. OTUs from both the bacterial and fungal datasets
435 having an overall relative abundance higher than 1% of the total reads, or appearing in at least
436 one sample with a relative abundance higher than 5%, were combined into a single table.

437 Associations between pairs of bacterial and fungal OTUs were calculated using the Maximal
438 Information Coefficient, as implemented in MICtools (77). Pseudo p-values were obtained by
439 generating 200,000 null matrices, and further transformed to Storey's Q-values to correct for
440 multiple hypothesis testing with the Benjamini-Hochberg method. Correlations with a False
441 Discovery Rate lower than 0.01 were deemed significant. Further, we divided the samples into
442 8 groups according to the combination of the 4 countries and 2 delivery modes. We used linear
443 regression to calculate correlations between pairs of OTUs and factors (Age, BMI) in a given
444 group. For each group, only OTUs appearing in at least 4 samples and with a relative
445 abundance higher than 2% in at least one sample were included. Correlations with a p-value
446 lower than 0.05 were deemed significant. Network analysis was performed on Cytoscape (78).
447

448 **Phylogenetic relationships between *Malassezia* reads**

449 ITS sequences of the 20 most abundant OTUs assigned to the *Malassezia* genus by the
450 RDP classifier were combined with those of known *Malassezia* representatives from the UNITE
451 v07-04-2014 database (70). A multiple sequence alignment was constructed with MAFFT
452 v7.313 (79). *Cryptococcus neoformans* was selected as an outgroup, and its ITS sequence was
453 added to the alignment using the *add* option from MAFFT. The resulting alignment was
454 manually curated and further refined with MUSCLE v3.8.31 (80). Phylogenetic trees were
455 inferred with RaxML v8 (81) and MrBayes v3.2 (82), using 1000 replicates and 1,000,000
456 generations respectively. TreeGraph2 (83) was used to combine and visualize the maximum
457 likelihood and bayesian inference trees.
458

459 ## Data availability

460 All ITS1 sequences have been deposited in the European National Archive (ENA) server
461 under the study ID PRJEB25581. Samples accession IDs: ERS2311788-2311867.
462

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Competing interests

Conflict of interest. The authors declare that they have no conflict of interest.

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716 ## Figure Legends

717 **Figure 1. Breast milk samples cluster separately according to fungal composition.** DAPC
718 analysis showing relationships in fungal composition among samples from different locations.
719 Canonical loading plot show differentially abundant bacterial OTUs in the groups. The
720 individual peaks show the magnitude of the influence of each variable on the separation of the
721 groups (threshold level=0.05). n=80 (n per country=20). SA= South Africa.

722

723 **Figure 2. Effect of geographical location and mode of delivery on fungal composition in**
724 **breast milk samples. (a)** Fungal relative abundances at genus level across countries. Only
725 genera present in more than 1% abundance in at least 20% of the samples are represented. **(b)**
726 Shared phylotypes across countries at genus level. *, core of four fungal genera shared across
727 geographic locations. Venn's diagram cut-off: 0.5. **(c)** Differentially abundant species in breast
728 milk samples depending on geographic location, as inferred by the LEfSe algorithm. The
729 threshold for logarithmic discriminant analysis *(LDA)* score was 2, and $p < 0.05$. n= 20 per
730 country (total n=80). **(d)** Differentially abundant species in breast milk samples depending on
731 delivery mode and geographic location, as inferred by the LEfSe algorithm. The threshold for
732 logarithmic discriminant analysis *(LDA)* score was 2, and *p-value* < 0.05. n=80 (vaginal
733 deliveries n=40, C-section deliveries n=40). SA= South Africa.

734

735 **Figure 3. Co-occurrence network of bacteria and fungi in breastmilk samples depending on**
736 **maternal features and delivery mode.** Green nodes represent bacterial OTUs, blue nodes
737 represent fungal OTUs, and yellow nodes represent features. Nodes size indicates OTU
738 abundance. Pie chart colours represent the overall distribution of each OTU across countries.
739 Each link indicates a significant (p<0.05) interaction between OTUs or features in samples from
740 a given combination of country and delivery mode (Vaginal, C-section). Link colour denotes the
741 country, and line type indicates delivery mode. SA= South Africa.

742

743

744 **Table 1. Clinical characteristics of donors providing the human milk samples for the study.**

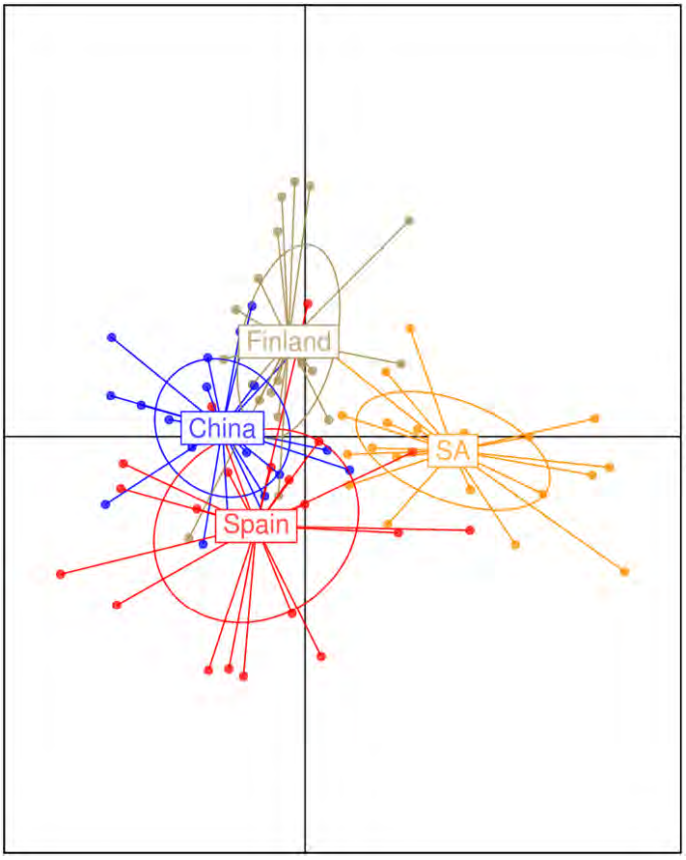
745

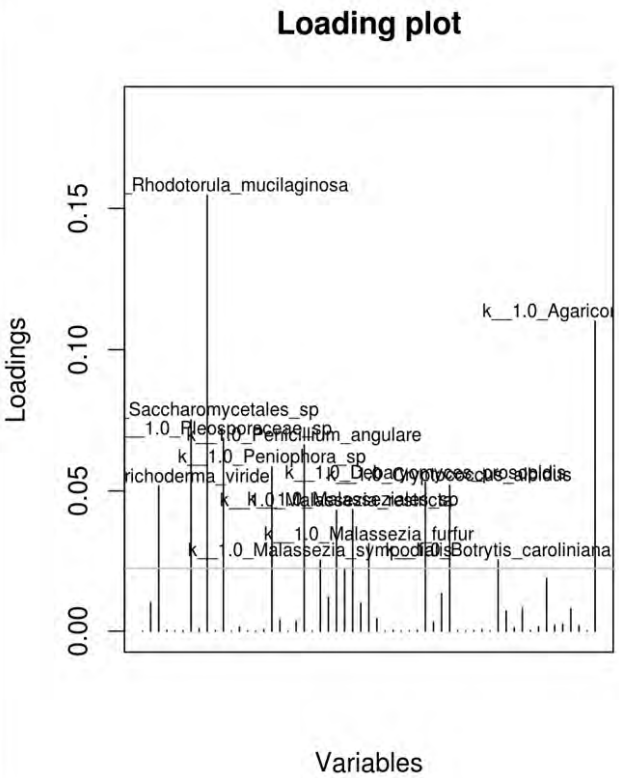
	Delivery mode	Age	*p* value	BMI ± SD	*p* value
Finland	C-section (10)	35.20 ± 4.07		26.30 ± 2.57	
	Vaginal (10)	33.70 ± 6.02	0.820	22.65 ± 8.60	0.185
	Total (20)	34.45 ± 5.06	ns	24.47 ± 6.46	ns
Spain	C-section (10)	34.50 ± 2.59		24.34 ± 1.47	
	Vaginal (10)	32.20 ± 5.16	0.288	24.25 ± 1.43	0.630
	Total (20)	33.35 ± 4.14	ns	24.30 ± 1.41	ns
South Africa	C-section (10)	36.60 ± 6.08		26.67 ± 1.41	
	Vaginal (10)	31.50 ± 5.76	0.944	24.81 ± 2.67	0.043
	Total (20)	34.05 ± 2.29	ns	25.75 ± 2.29	ns
China	C-section (10)	32.60 ± 2.95		21.49 ± 2.29	
	Vaginal (10)	31.90 ± 4.25	0.970	21.92 ± 1.54	0.449
	Total (20)	32.25 ± 3.58	ns	21.71 ± 1.97	0.004
All	C-section (10)	34.72 ± 4.25		24.70 ± 2.83	
	Vaginal (10)	32.32 ± 5.20	0.058	23.41 ± 2.11	0.072
	Total (20)	33.52 ± 4.87	ns	24.06 ± 3.85	ns

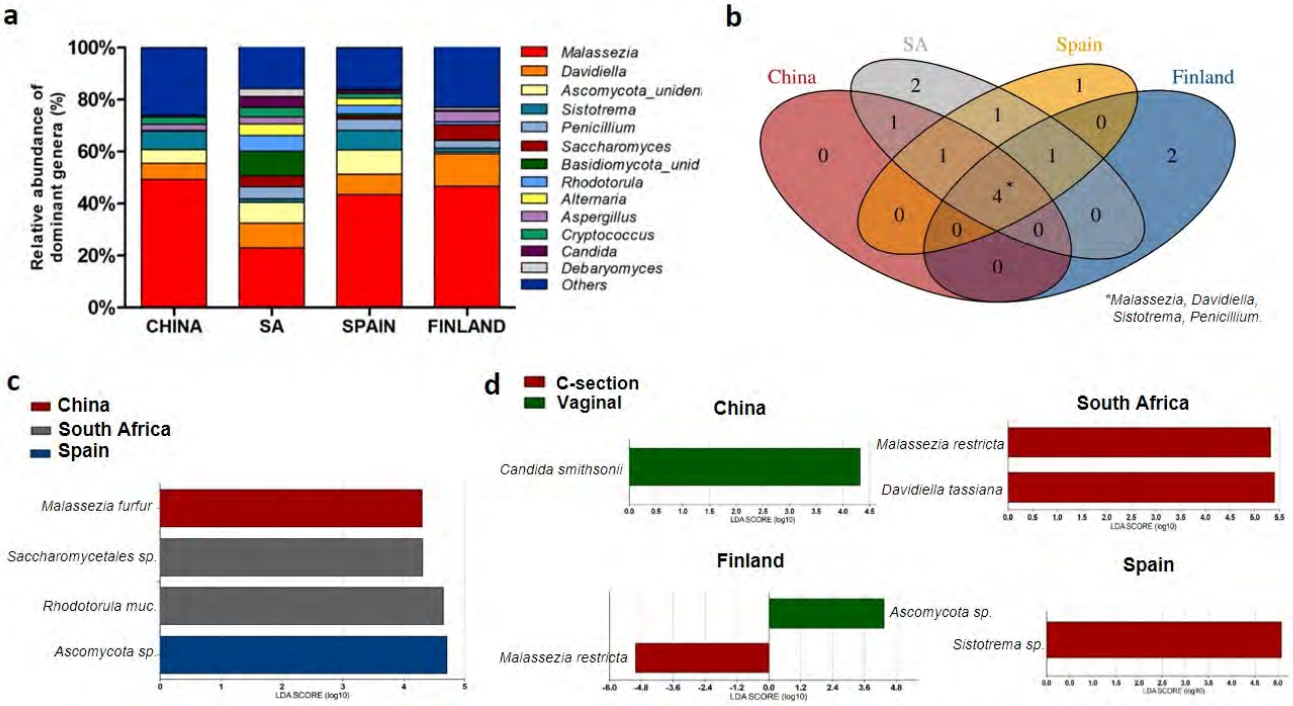
746

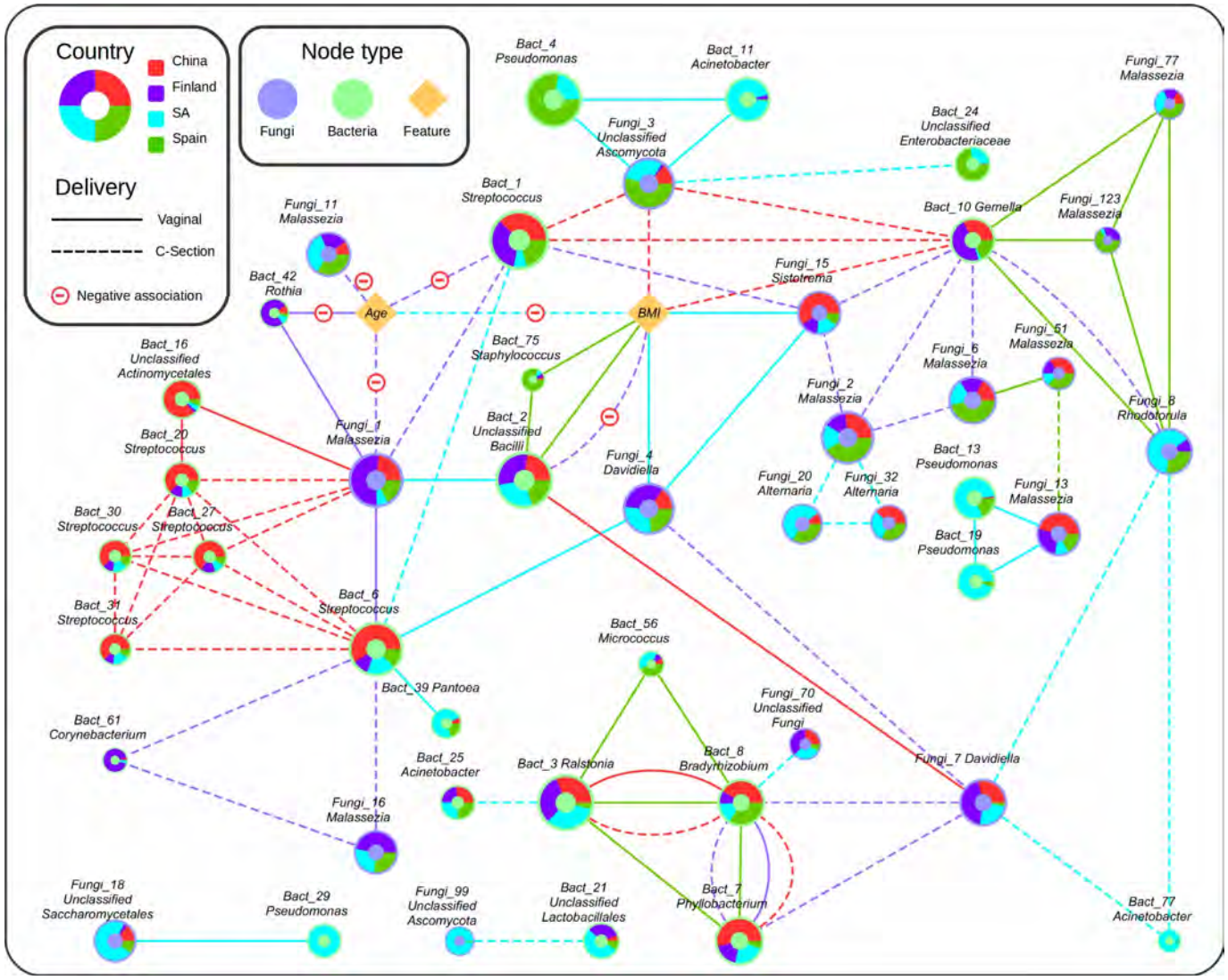
747 ns= not significant

748


Finland
China
SA
Spain


Loading plot
Loadings
Variables
0.00
0.05
0.10
0.15
Rhodotorula_mucilaginosa
k__1.0_Agaricom
Saccharomycetales_sp
__1.0_Pleosporaceae_sp
k__1.0_Penicillium_angulare
k__1.0_Peniophora_sp
richoderma_viride
k__1.0_Malassezia_furfur
k__1.0_Malassezia_symbodialis
Botrytis_caroliniana


a
100%
80%
60%
40%
20%
0%
Relative abundance of dominant genera (%)
CHINA
SA
SPAIN
FINLAND
Malassezia
Davidiella
Ascomycota_uniden
Sistotrema
Penicillium
Saccharomyces
Basidiomycota_unid
Rhodotorula
Alternaria
Aspergillus
Cryptococcus
Candida
Debaryomyces
Others
b
China
SA
Spain
Finland
*Malassezia, Davidiella, Sistotrema, Penicillium.
c
China
South Africa
Spain
Malassezia furfur
Saccharomycetales sp.
Rhodotorula muc.
Ascomycota sp.
LDA SCORE (log10)
d
C-section
Vaginal
China
Candida smithsonii
South Africa
Malassezia restricta
Davidiella tassiana
Finland
Ascomycota sp.
Malassezia restricta
Spain
Sistotrema sp.


Country
China
Finland
SA
Spain
Delivery
Vaginal
C-Section
Negative association
Node type
Fungi
Bacteria
Feature
Bact_4 Pseudomonas
Bact_11 Acinetobacter
Fungi_77 Malassezia
Fungi_3 Unclassified Ascomycota
Bact_24 Unclassified Enterobacteriaceae
Bact_1 Streptococcus
Fungi_11 Malassezia
Bact_10 Gemella
Fungi_123 Malassezia
Fungi_15 Sistotrema
Bact_42 Rothia
Age
BMI
Bact_75 Staphylococcus
Fungi_51 Malassezia
Fungi_6 Malassezia
Bact_16 Unclassified Actinomycetales
Fungi_2 Malassezia
Fungi_8 Rhodotorula
Bact_2 Unclassified Bacilli
Bact_20 Streptococcus
Fungi_1 Malassezia
Fungi_4 Davidiella
Bact_13 Pseudomonas
Fungi_20 Alternaria
Fungi_32 Alternaria
Fungi_13 Malassezia
Bact_30 Streptococcus
Bact_27 Streptococcus
Bact_19 Pseudomonas
Bact_31 Streptococcus
Bact_6 Streptococcus
Bact_56 Micrococcus
Bact_39 Pantoea
Fungi_70 Unclassified Fungi
Bact_61 Corynebacterium
Bact_25 Acinetobacter
Bact_3 Ralstonia
Bact_8 Bradyrhizobium
Fungi_7 Davidiella
Fungi_16 Malassezia
Fungi_18 Unclassified Saccharomycetales
Bact_29 Pseudomonas
Fungi_99 Unclassified Ascomycota
Bact_21 Unclassified Lactobacillales
Bact_7 Phyllobacterium
Bact_77 Acinetobacter