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MICROBIAL ECOLOGY

Mycobiome Profiles in Breast Milk from Healthy Women Depend on Mode of Delivery, Geographic Location, and Interaction with Bacteria

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ABSTRACT Recent studies report the presence of fungal species in breast milk of healthy mothers, suggesting a potential role in 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 to investigate its interaction with bacterial profiles in the same samples. A total of 80 mature breast milk samples from 4 different countries were analyzed by Illumina sequencing of the internal transcribed spacer 1 (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 isolate characterization, and fungal loads were estimated by quantitative PCR (qPCR) targeting the fungal ITS1 region. Cooccurrence network analysis of bacteria and fungi showed complex interactions that were influenced by geographical location, mode of delivery, maternal age, and pregestational body mass index. The presence of a breast milk mycobiome was confirmed in all samples analyzed, regardless of the geographic origin.

IMPORTANCE During recent 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 can affect milk bacteria; however, the impact on milk fungi is still unknown. The current report describes fungal communities (mycobiota) in breast milk samples across different geographic locations and the influence of the mode of delivery. We also provide novel insights on bacterium-fungus interactions, taking into account environmental and perinatal factors. We identified a core of four genera shared across locations, consisting of *Malassezia, Davidiella, Sistotrema*, and *Penicillium*, which have been reported to be present in the infant gut. Our data confirm the presence of fungi in breast milk across continents and support the potential role of breast milk in the initial seeding of fungal species in the infant gut.

KEYWORDS fungi, Illumina sequencing, microbiota, mycobiota, breast milk

Citation Boix-Amorós A, Puente-Sánchez F, du Toit E, Linderborg KM, Zhang Y, Yang B, Salminen S, Isolauri E, Tamames J, Mira A, Collado MC. 2019. Mycobiome profiles in breast milk from healthy women depend on mode of delivery, geographic location, and interaction with bacteria. Appl Environ Microbiol 85:e02994-18. https://doi.org/10.1128/AEM.02994-18.

Editor Irina S. Druzhinina, Nanjing Agricultural University

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Received 13 December 2018
Accepted 13 February 2019
Accepted manuscript posted online 1 March 2019
Published 18 April 2019

Early human microbial gut colonization is an essential stepwise 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 in 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 species) can be found in the gut early in life (9–11). 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 have 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, contributes to the settlement of the gut microbiota characteristic of 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, therefore, such are also likely to occur in breast milk.

In addition, accumulating evidence suggests that some environmental factors might influence breast milk composition (25–27). In particular, geographic location, delivery mode, maternal body mass index (BMI), and age have been suggested to have an impact on breast milk bacterial composition (28–35), although their potential impact on the milk's fungal fraction is still to be elucidated.

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

RESULTS

Subject description. The characteristics of the subjects participating in the study are listed in Table 1. The mean age of the mothers ($n = 80$) was 33.52 years (standard deviation [SD], ±4.87 years), with no statistical differences between countries. The mean pregestational BMI was 24.06 (SD, ±3.85), normal weight. Chinese mothers had significantly lower BMI, 21.71 (SD, ±1.97), considered normal weight. Differences in BMI between mothers delivering vaginally or by Caesarean section (C-section) were observed only in South African and Finnish women, where mothers delivering by C-section had higher BMIs, 26.67 ± 1.41 and 26.30 ± 2.57, respectively, although this difference was significant only in the South African group ($P < 0.05$).

Fungal cell detection in breast milk. Eighty milk samples were analyzed by quantitative PCR (qPCR) targeting the ITS1–5.8S rRNA region. Results showed that 16 of 20 Spanish samples (80%; median value, 195,142 cells/ml), 9 of 20 Chinese samples (45%; median value, 170,732 cells/ml), 7 of 20 Finnish samples (35%; median value, 199,480 cells/ml), and 14 of 20 South African samples (70%; median value, 371,119 cells/ml) had detectable levels of fungi. No significant differences were observed between geographic locations or by mode of delivery (see Fig. S1 in the supplemental material).

TABLE 1 Clinical characteristics of donors providing human milk samples for the study[a]

Country	Delivery mode (no. of samples)	Age (yr) ± SD	*P* value	BMI ± SD	*P* value
Finland	C-section (10)	35.20 ± 4.07	0.820	26.30 ± 2.57	0.185
	Vaginal (10)	33.70 ± 6.02		22.65 ± 8.60	
	Total (20)	34.45 ± 5.06	ns	24.47 ± 6.46	ns
Spain	C-section (10)	34.50 ± 2.59	0.288	24.34 ± 1.47	0.630
	Vaginal (10)	32.20 ± 5.16		24.25 ± 1.43	
	Total (20)	33.35 ± 4.14	ns	24.30 ± 1.41	ns
South Africa	C-section (10)	36.60 ± 6.08	0.944	26.67 ± 1.41	0.043
	Vaginal (10)	31.50 ± 5.76		24.81 ± 2.67	
	Total (20)	34.05 ± 2.29	ns	25.75 ± 2.29	ns
China	C-section (10)	32.60 ± 2.95	0.970	21.49 ± 2.29	0.449
	Vaginal (10)	31.90 ± 4.25		21.92 ± 1.54	
	Total (20)	32.25 ± 3.58	ns	21.71 ± 1.97	0.004
All	C-section (40)	34.72 ± 4.25	0.058	24.70 ± 2.83	0.072
	Vaginal (40)	32.32 ± 5.20		23.41 ± 2.11	
	Total (80)	33.52 ± 4.87	ns	24.06 ± 3.85	ns

[a]ns, not significant.

The presence of fungal cells in the milk was further confirmed by culture in fungus-specific culture medium and identification of the isolates by 18S rRNA sequencing, as well as by microscopy after incubation of the milk samples with calcofluor white fungal stain. A summary of the results is available in Table S1 and Fig. S2 in the supplemental material.

Fungal composition of breast milk: impact of geographical area and perinatal factors. After sequencing of the ITS1 fungal region, a mean of 107,765 taxonomically assigned, clean, filtered sequences per sample (SD, ±45,493), with an average length of 301 bp, was obtained. All breast milk samples contained fungal DNA, and they were dominated by two phyla: Basidiomycota (58.65%) and Ascomycota (41.03%). South African samples had significantly higher levels of Ascomycota and lower levels of Basidiomycota than the other countries ($P < 0.05$).

Discriminant analysis of principal components (DAPC), which transforms data using a principal-component analysis (PCA) and subsequently identifies clusters using discriminant analysis (DA), showed that South African samples clustered at a distance from the other countries, mainly due to the increased levels of *Rhodotorula mucilaginosa* (Fig. 1).

Taxonomic analysis at the genus level showed that breast milk samples were dominated by *Malassezia* (40.6% average abundance), followed by *Davidiella* (9.0%), which was prevalent regardless of the location or the donor's type of delivery (Fig. 2a). The effects of country of origin and mode of delivery on breast milk fungal composition were analyzed and reflected that milk mycobiota differed significantly across geographic location (permutational multivariate analysis of variance [PERMANOVA], $P = 0.005$) and mode of delivery (PERMANOVA, $P = 0.023$). Redundant analysis (RDA) confirmed the effect of geographic location on breast milk fungal composition ($P = 0.001$), although that of mode of delivery did not reach statistical significance. The Kruskal-Wallis test was implemented to compare phylotypes at the genus level across samples. Results showed that *Malassezia* was statistically less abundant in South African samples ($P < 0.05$), and *Penicillum* and *Rhodotorula* abundances were lower in Chinese samples ($P < 0.01$), while *Saccharomyces* was more abundant in Spanish and Finnish samples ($P < 0.01$) than in samples from the rest of the locations. No statistically significant effect of maternal age, gestational BMI, or antibiotic intake during delivery was detected for breast milk microbial composition by using MaAsLin (multivariate analysis with linear model).

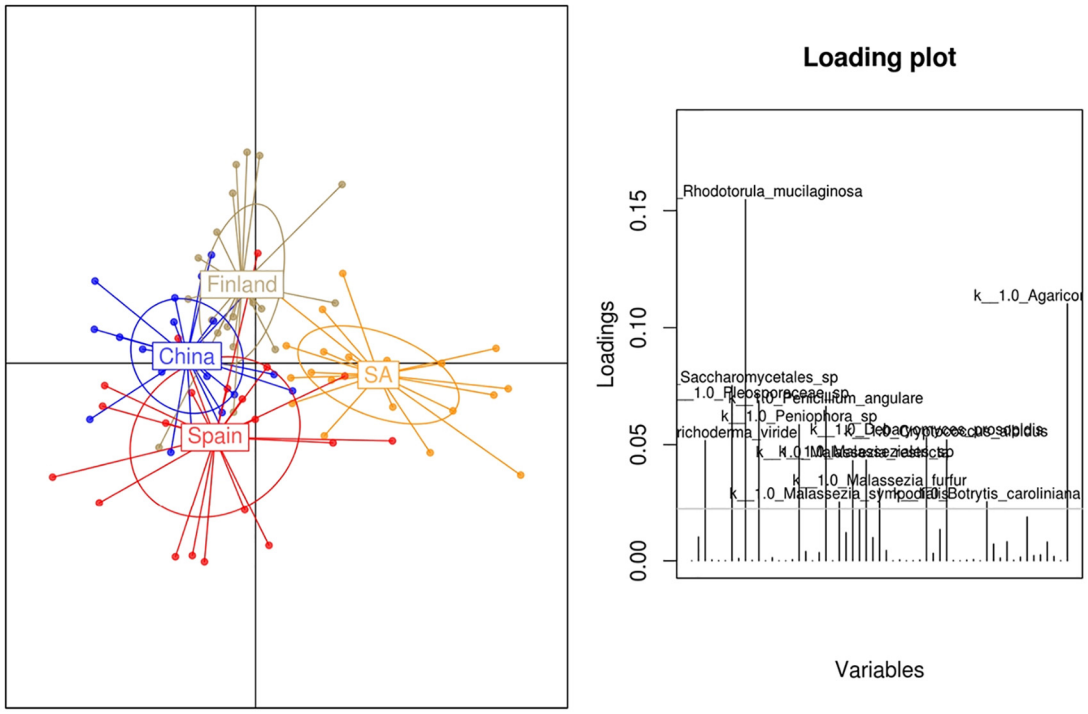

FIG 1 Breast milk samples cluster separately according to fungal composition. DAPC analysis shows relationships in fungal composition among samples from different locations. A canonical loading plot shows differentially abundant bacterial OTUs in the groups. The individual peaks show the magnitude of the influence of each variable on the separation of the groups (threshold level = 0.05). Total number of samples, 80 (number per country, 20). SA, South Africa.

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

Comparisons between samples were further analyzed at the species level. Linear discriminant analysis effect size (LefSe) results showed differentially abundant fungi between countries. *Rhodotorula mucilaginosa* and *Saccharomycetales* species were more abundant in South African samples, while *Malassezia furfur* was more prevalent in Chinese samples, and an *Ascomycota* sp. was more abundant in Spanish samples (Fig. 2c).

Taking into account mode of delivery, mycobiota compositions were different across the milk samples from different geographic origins. The Kruskal-Wallis test reflected that the occurrence of *Cryptococcus* was statistically significantly higher in milk samples of women delivering vaginally than in those who delivered by C-section ($P = 0.028$). At the species level, in Chinese breast milk samples, *Candida smithsonii* was significantly more abundant in samples of women with vaginal deliveries, *Sistotrema* species in samples from Spanish women with C-sections, *Ascomycota* species in samples from Finnish women with vaginal deliveries, *Malasezzia restricta* in samples from Finnish women with C-sections, and *Malassezia restricta* and *Davidiella tassiana* in samples from South African women with C-sections (LefSe analysis, $P < 0.05$) (Fig. 2d).

Indices of alpha diversity and richness across the samples were similar, and no statistical differences were observed between geographic locations or delivery modes (Fig. S3).

Fungal and bacterial interactions: a network analysis. Network analyses of the bacteria and fungi present in the breast milk samples showed complex intra- and interdomain interactions, with different associations among organisms depending on

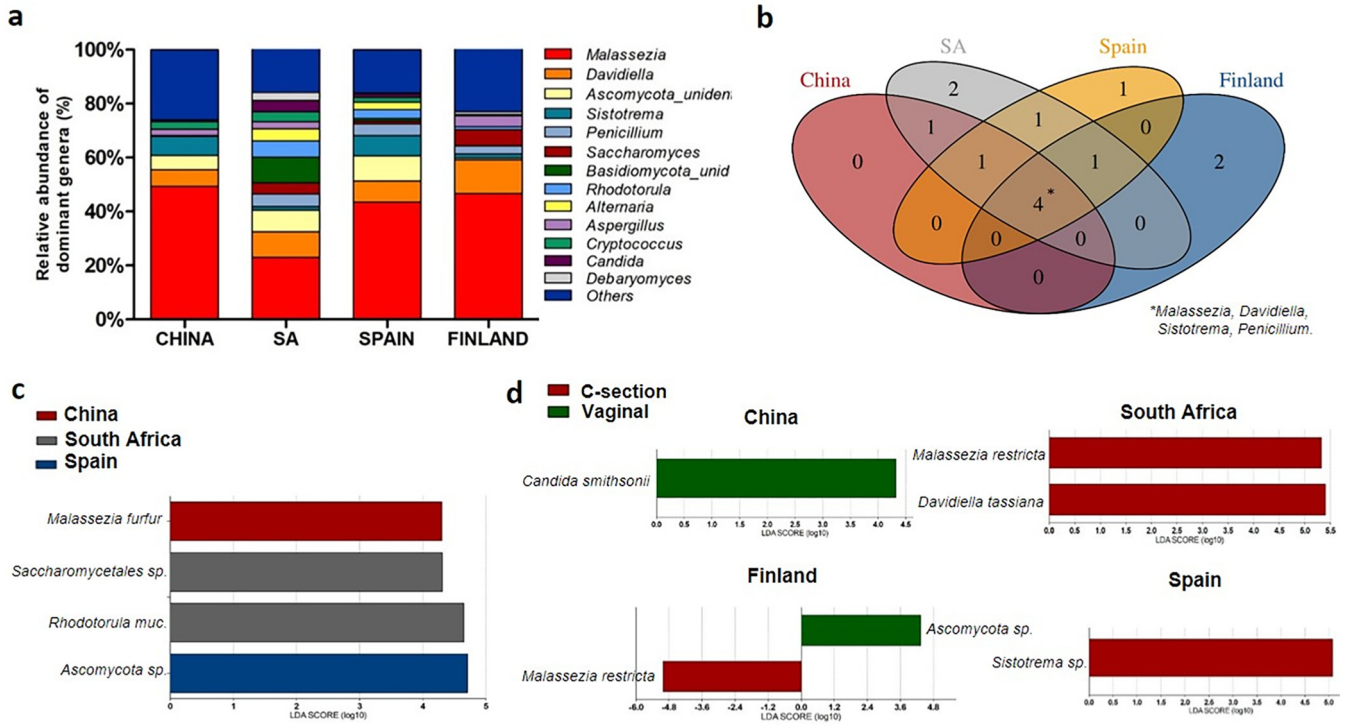

FIG 2 Effect of geographical location and mode of delivery on fungal composition in breast milk samples. (a) Fungal relative abundances at the genus level across countries. Only genera present in more than 1% abundance in at least 20% of the samples are represented. (b) Shared phylotypes across countries at the genus level. *, core of four fungal genera shared across geographic locations. Venn diagram cutoff, 0.5. (c) Differentially abundant species in breast milk samples depending on geographic location, as inferred by the LefSe algorithm. The threshold for the logarithmic discriminant analysis (LDA) score was 2, with a P of <0.05. Number of samples per country, 20 (total number, 80). (d) Differentially abundant species in breast milk samples depending on delivery mode and geographic location, as inferred by the LefSe algorithm. The threshold for the LDA score was 2, with a P value of <0.05. Total number of samples, 80 (samples from vaginal deliveries, 40; samples from C-section deliveries, 40). SA, South Africa.

the country of origin and delivery mode, some of which were also influenced by maternal features. For example, a *Malassezia* operational taxonomic unit (OTU) (Fungi_1) correlated positively with a *Streptococcus* (Bact_6) from vaginal delivery samples and with a *Streptococcus* (Bact_1) from C-section deliveries among Finnish samples, and the abundances were dependent on maternal age. The same *Malassezia* OTU correlated positively with several *Streptococcus* OTUs in samples from C-section deliveries from Chinese mothers and also positively with an unclassified member of *Bacilli* (Bact_2) from South African samples and vaginal deliveries. Significant influences of maternal age and BMI on specific bacterial and fungal organisms were also observed (Fig. 3). However, given that the density of fungal cells is at least 1 order of magnitude lower than that of bacteria, the influence of fungi on the breast milk ecosystem needs to be elucidated.

In order to study the diversity of the most common yeast in our samples, a phylogenetic tree of the most prevalent *Malassezia* OTUs detected in this work across geographic locations was determined, including known members of the *Malassezia* genus as a reference (Fig. S4). The tree shows a large diversity of *Malassezia* isolates with similarity to at least four known species, including OTUs which could potentially represent new species. With the exception of one OTU (Fungi 37, which was found to be uniquely present in China), all other sequences were found in all countries and appear to be therefore ubiquitous. In relation to mode of delivery, all the OTUs were present in breast milk from mothers with both delivery types.

DISCUSSION

Breast milk is a continuous source of microbes that are transmitted, together with many nutrients and protective compounds, to the infant gut during a critical period

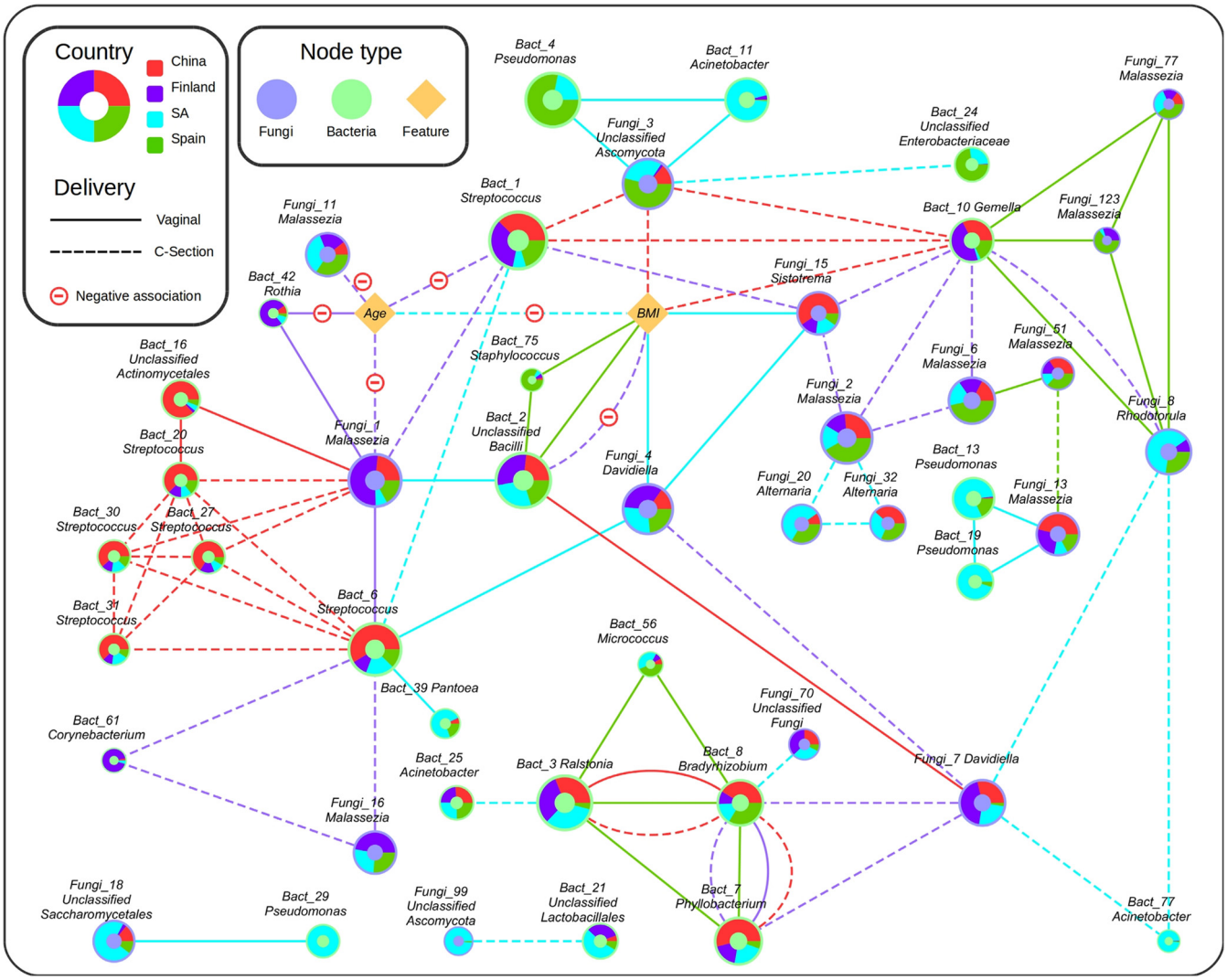

FIG 3 Cooccurrence network of bacteria and fungi in breast milk samples depending on maternal features and delivery mode. Green nodes represent bacterial OTUs, blue nodes represent fungal OTUs, and yellow nodes represent features. Node size indicates OTU abundance. Pie chart colors represent the overall distribution of each OTU across country. Each link indicates a significant ($P < 0.05$) interaction between OTUs or features in samples from a given combination of country and delivery mode (vaginal, C-section). Link color denotes the country, and line type indicates delivery mode. SA, South Africa.

when the key regulatory systems of the body are immature (17, 18). Although bacteria inhabiting human breast milk have been extensively studied, the presence of fungi in the fluid had not been assessed until recently, when a diversity of fungal phylotypes in breast milk from healthy Spanish mothers was reported by our group (20). The mycobiome, the fungal fraction of the human microbiome, is present in lower abundances and has been much less explored than the bacterial fraction. However, its potential importance for human health and disease has stimulated an increased interest in this field (5–7, 10). In the infant, fungal species can be detected very early in life (10, 11, 13). However, the infant mycobiome is almost unexplored, and information about its development is scarce. To ascertain the presence of fungi in breast milk is difficult because of the possibility of contamination in samples with low microbial density, and therefore multiple approaches and strict negative controls are needed (15).

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

In the present study, we have confirmed the presence of diverse fungal communi-

ties in breast milk samples from Spain, Finland, China, and South Africa. Fungi were detected in all breast milk samples through massive DNA sequencing, with the two phyla Basidomycota and Ascomycota being the most prevalent and presenting reciprocal patterns of abundance in all countries except for South Africa, where Ascomycota levels were significantly higher, and Basidiomycota levels lower, than those of the other countries. At the genus level, *Malassezia* predominated in all countries, followed by *Davidiella*. In our previous work reporting the presence of fungi in breast milk, *Malassezia* also represented the most abundant genus (20). Other genera found in the current study, such as *Alternaria*, *Rhodotorula, Saccharomyces*, and *Candida*, were also found in the previous study.

Results yielded by qPCR showed that >70% of Spanish and South African samples, 45% of Chinese samples, and only 35% of Finish samples had detectable levels of fungal DNA. The median fungal load in all the samples was 2.5×10^5 cells/ml, in agreement with our previous results for Spanish samples.

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

Breast milk mycobiota also differed depending on the mode of delivery (vaginal or C-section) across countries. Specific fungi, such as the genus *Cryptococcus,* appeared to be more prevalent among samples from mothers delivering vaginally, and specific shifts at species level were also observed within each country. No differences in fungal diversity or richness were observed in the present study. Previously, we identified changes in breast milk microbiota between locations, as well as in the milk metabolite profile (31, 35), using the same samples analyzed in this study.

Although the origin of breast milk fungi is unknown, most of the organisms detected in this study can be found in other human niches. *Malassezia* species are yeasts whose primary niche is the human body (and other animals). In healthy individuals, they are part of the normal microbiota, where they predominantly colonize the seborrheic parts of the skin (37), and are commonly found in infants (9, 38–40). *Malassezia* has also been detected in significant abundance in adult (11, 37, 41) and infant (42) fecal samples and therefore may play a role at the intestinal level; it has also been described as an oral commensal (43). Although *Malassezia* DNA has been detected in high proportions in breast milk before, no viable cells could be recovered by classic culture methods from breast milk, (20), and further efforts should be made to culture this organism, which has also been shown to be able to penetrate the cell and survive intracellularly.

Davidiella, the second most prevalent fungus found in the samples of this study, was detected in the only published study about the characterization of vaginal microbiota and mycobiota of asymptomatic women (44). In the same study, *Candida* was found to be the predominant genus. Therefore, these fungi may play an important role in the early colonization of vaginally born infants. In our previous study on breast milk fungi, *Davidiella* could not be detected (20), which could be associated with the differences on sequencing platforms and genes targeted in both studies, as has been previously shown (45, 46). In addition, *Davidiella* represents the sexual form of the *Cladosporium* genus (47). Fungi can have an asexual form (anamorph) and a sexual form (teleomorph) that may be classified into different genera. This sexual dimorphism can be a significant problem when classifying fungal sequences, and the use of different databases and/or sequencing of different genes can lead to conflicting classifications. In a study with pediatric inflammatory bowel disease (IBD) patients, *Cladosporium cladosporiodes* abundance decreased in patients with IBD, while *Pichia jadinii* and *Candida parapsilosis* increased, in comparison to controls (48).

Candida is probably the most ubiquitous genus of the human mycobiome. It is the

major fungal genus detected in the adult oral cavity (49, 50) and has also been detected in the infant mouth, including several species as common inhabitants (*C. parapsilosis*, *C. tropicalis*, *C. orthopsilosis*, etc.) (9, 51, 52). Several *Candida* species are also commonly present in adult skin and fecal samples (7, 41) and in infant anal and fecal samples (9, 53). Although *Candida* can be responsible for vaginal infections (54), it is the most prevalent fungus in the vaginal mycobiome of healthy women (44). Transmission of *Candida* from mother to infant likely occurs, as the same fingerprinting of the DNA has shown identity between maternal *Candida* from vagina, rectum, oral cavity, and skin and infant *Candida* from oral cavity and rectum (14).

Other prevalent fungi detected in our samples are commonly found in several body niches. *Saccharomyces* is among the most abundant fungus in the gut (7, 41), and *Saccharomyces cerevisiae* has been reported to be highly prevalent and abundant in the infant oral and anal mycobiome during the first month of life (9). In a recent study, bacteria and fungi from fecal samples in children suffering atopic wheeze were analyzed, and Saccharomycetales taxa appeared to be decreased in the atopic wheeze group, while the species *Pichia kudriavzevii* was increased, compared to controls (16). Others, such as *Penicillium* or *Aspergillus,* can also be detected in fecal samples, and *Debaromyces hansenii* represents one of the main species present in the gut of breastfed infants (12). In the present study, we detected *Debaromyces*, although none of the sequences have been classified as *D. hansenii*. However, DNA from this species was previously detected in breast milk (20).

The study of interspecies interactions within a population is necessary to better understand the microbiota's role. It is known that microorganisms can interact by competition and sometimes collaboration, thereby influencing microbiota composition and the host's health. It has been demonstrated that cross talk between bacteria and fungi can exist, modulating host defense mechanisms, protecting against infections, or collaborating to cause them (55, 56). For example, synergies between oral *Streptococcus oralis* and *C. albicans* enhanced *C. albicans* invasion through the activation of host enzymes that cleave epithelial junction proteins (57). On the contrary, *Streptococcus mutans* showed the ability to modulate biofilm formation and to reduce *C. albicans* virulence in an animal model (58). Some vaginal isolates of *Lactobacillus* strains have shown antifungal activity *in vitro* against *Candida* spp., and probiotic *Lactobacillus rhamnosus* and *Lactobacillus reuterii* strains have shown *in vitro* efficacy against *C. albicans* responsible for vaginal infections (24). To understand microbial relationships, microbial network analyses are indispensable, allowing the identification and representation of the most influential members in a bacterial community and their interactions with other microorganisms (59). In a recent work, bacterial interactions in the colostrum and mature milk of Italian and Burundian mothers were analyzed and showed different bacterial networks among the two populations. The identified networks were complex and dynamic, changing from colostrum to mature milk (60). In the present study, we have analyzed cooccurrence relationships between bacteria and fungi in breast milk, observing a complex network of interactions between fungi and bacteria and within the same domain. Microbial interactions were influenced by delivery characteristics (mode of delivery and geographic location), and maternal features (maternal BMI and age) influenced the prevalence of particular microorganisms. Interesting positive correlations were observed between several *Malassezia* spp., the most prevalent fungi detected in breast milk by sequencing, and different streptococci, the latter representing one of the most common bacterial genera in breast milk (61). Interestingly, in our previous study, we observed a significant positive correlation between *Malassezia* and bacterial load (20), and further experimental research should analyze potential synergistic relationships between these genera.

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

interdomain microbial interactions in breast milk that could lead to further *in vitro* studies. The presence of viable fungal cells suggests a potential influence of breast milk on the infant's mycobiota development. However, data from the infant gut mycobiota is missing in the present study, and further studies should address the potential fungal transference from breast milk to the infant gut mycobiome. Although we tried to prevent the contamination of maternal skin mycobiota by cleaning the breast prior to sample collection (which has been previously shown to reduce bacteria in breast milk samples [62]), it should be taken into account that certain retrograde flux occurs during breastfeeding, and fungal species present in maternal skin and the infant's mouth could be translocated to breast milk and *vice versa* (63). A greater understanding of the environmental influence on the bacterial and fungal communities and their metabolic functions is also needed.

MATERIALS AND METHODS

Subjects and sampling. Breast milk samples at 1-month postpartum were obtained from 80 healthy, lactating women from 4 different geographical locations (20 in each location), including China (Beijing area), South Africa (Cape Town), Finland (southwestern area), and Spain (Valencia, Mediterranean area).

All mothers were practicing exclusive breastfeeding. Subjects were grouped according to mode of delivery: vaginal ($n = 10$ per country) and Caesarean section (C-section) ($n = 10$ per country). Maternal characteristics such as age, weight, and pregestational body mass index (BMI) were collected at the time of enrollment. All women who delivered via C-section received prophylactic antibiotics, except Finnish women, for whom no prophylaxis is routinely used per the hospital policy. All participants were given detailed oral and written information, and written informed consent was obtained for participation. The study protocol was approved by the ethics committees of the respective participating institutions: Bioethics Committee of CSIC and the Regional Ethics Committee for Biomedical Research (Spain), Ethics Committee, Hospital District of Southwest Finland (Finland), Medical Research Board of Peking University (China), and University of Cape Town, Human Research Ethics Committee (South Africa).

Before sample collection, nipples and mammary areolas were cleaned with soap and sterile water and soaked in chlorhexidine to reduce sampling of microorganisms residing on the skin. Milk samples were collected in a sterile tube manually, with the first drops discarded. All samples were frozen at −20°C until further processing. To avoid bias, samples were collected using the same standardized protocol in the four countries and were processed and analyzed in a single laboratory.

Microbial DNA extraction and sequencing. Breast milk samples (1.5 ml) were centrifuged at 14,000 rpm for 20 min at 4°C to remove fat, and pellets were used for total DNA extraction, which involved mechanical and chemical cell lysis. Bead beating was carried out using FastPrep (FP120-230, Bio 101; ThermoSavant, Holbrook, NY, USA), and the InviMag stool DNA kit (Stratec Molecular, Berlin, Germany) was used with the King Fisher magnetic particle processor (Thermo Fisher Scientific Oy, Vantaa, Finland). The DNA extraction protocol was also used with water to serve as negative controls. Isolated DNA concentrations were measured using a Qubit 2.0 fluorometer (Life Technology, Carlsbad, CA, USA).

Primers targeting the highly variable fungal internal transcriber spacer ITS1 of the fungal 18S rRNA gene (forward, TAGAGGAAGTAAAAGTCGTAA; reverse, TTYRCTRCGTTCTTCATC) (64) with adaptors were used for sequencing on an Illumina MiSeq platform. Sequencing was carried out at the Foundation for the Promotion of Health and Biomedical Research, FISABIO (Valencia, Spain). No-template controls (NTCs) and negative controls during DNA extraction were included to rule out potential contaminations at the time of DNA extraction or sequencing.

Fungal load. qPCR amplification and detection of the ITS1–5.8S rRNA conserved fungal region was performed as previously described (17), using the primers ITS1F (5′-TCCGTAGGTGAACCTGCGG) and 5.8 R (5′-CGCTGCGTTCTTCATCG). Each reaction mixture of 20 μl was composed of 10 μl of KAPA Sybr Fast qPCR master mix (KAPA Biosistems), 0.4 μl of each primer (10 μM concentration), and 2 μl of template DNA, with an annealing temperature of 61°C in a LightCycler 480 real-time PCR system (Roche Technologies). All amplifications were performed in duplicate, and a negative control was included in each reaction plate. Samples with threshold cycle (C_T) values equal to or higher than the negative control were considered negative for fungal DNA.

Breast milk culture and identification of fungal colonies. One-hundred-microliter volumes of selected breast milk samples were plated in four solid fungus-selective media: Sabouraud (Conda-Pronadisa) supplemented with chloramphenicol, 0.05 g/liter (Roche); Rose Bengal (Conda-Pronadisa) supplemented with chloramphenicol, 0.5 g/liter (Roche); YPD (40 g/liter dextrose, 40 g/liter peptone, 20 g/liter yeast extract, and 40 g/liter agar) supplemented with 25 μg/ml of streptomycin–25 U/ml of penicillin (Biowest); and YNB (Sigma) with 8% ethanol and 25 μg/ml of streptomycin–25 U/ml of penicillin (Biowest). All plates were incubated aerobically at 37°C, as previously described (17). DNA from the isolated colonies was extracted and amplified by PCR using primers targeting the 18S rRNA gene (forward, 5′-GTAGTCATATGCTTGTCTC; reverse, 5′-CCATTCCCCGTTACCCGTTG). PCR products were sequenced in an Applied Biosystems 3730/3730xl DNA analyzer at the University of Valencia (Spain), and isolates were identified by using the BLAST algorithm in the NCBI database, with a minimum of 98% sequence identity.

Microscopic analyses of fungi in milk. In order to identify fungal cells in breast milk, samples were incubated with calcofluor white stain that dyes the cell walls of the fungi and yeasts. Samples were

visualized with fluorescence microscopy using a Nikon Eclipse E90i microscope (Nikon Corporation) with a 100× objective. Image processing was performed using the NIS-Elements BR v3.22 software (Nikon).

Data analysis. ITS1 reads were pair-end joined using the FLASH program (65), with default parameters applied. The resulting sequences were end-trimmed in 20-bp sliding windows with an average quality value of >30 and a length of >50 bp, using the Prinseq-lite program (66). Chimeric reads were eliminated using the UCHIME algorithm (67), resulting in a total of 9,797,578 reads. Taxonomy assignment of the remaining sequences was performed using the Ribosomal Database Project Classifier stand-alone tool (68) with the UNITE fungal ITS v7.2 trainset (69) and an 80% confidence threshold. Sequences were clustered into operational taxonomical units (OTUs) based on 99% identity, and representative OTU sequences were obtained using CD-hit software (70). OTU tables were rarefied to 9,200 sequences per sample to avoid variations in sequencing depth, and Shannon and Chao1 indexes were calculated using the "plyr" and "vegan" packages from R software (version 3.2.2) (71).

Statistical analysis. Calypso software (version 8.2) (72) was used to obtain a Venn diagram for shared phylotypes, discriminant analysis of principal components (DAPC) was performed at the OTU level, using geographic location as a factor, and PERMANOVA and redundancy analysis (RDA) were applied to study the statistical effect of country and delivery mode on breast milk fungal composition. The Kruskal-Wallis test was implemented to study genus-level taxonomical differences between countries and delivery modes, using GraphPad PRISM 6 (GraphPad Software). The linear discriminant analysis effect size (LefSe) (73) algorithm was used to detect the most differentially abundant fungi between countries, and between vaginal and C-section deliveries in each country, at the species level. In order to control the potential effects of maternal age, maternal BMI predelivery, and antibiotic use at delivery, MaAsLin (multivariate analysis with linear model) (74), which finds associations between metadata and microbial abundances, was applied. Other statistical analysis and graphing were performed using GraphPad PRISM 6.

Analysis of bacterial and fungal cooccurence. Sequences from the 16S rRNA gene of the same samples, from a report by Kumar et al. (31), were obtained from NCBI (SRA accession no. SRP082263 and submission ID SUB1772296). Quality filtering, chimera checking, and OTU clustering were done the same way as for the ITS1 reads.

RDP Classifier was used to taxonomically assign the bacterial (against the RDP 16S rRNA training set 16) (75) and fungal (against the UNITE v 07-04-2014 trainset) (70) representative OTU sequences. Samples with fewer than 1,500 sequences were excluded from the analysis.

For the bacterial data sets, OTUs with a higher relative abundance in any of the two controls than in the breast milk samples were treated as putative contaminants and discarded. This procedure could not be performed on the fungal data sets, since the sequencing of the two controls yielded too few reads. Nevertheless, the low fraction of reads assigned to putative contaminants in the bacterial data sets (2% on average) leads us to believe that the samples were essentially contamination-free. Both the bacterial and fungal OTU tables were rarefied to 1,500 sequences per sample. OTUs from both the bacterial and fungal data sets having an overall relative abundance higher than 1% of the total reads, or appearing in at least one sample with a relative abundance higher than 5%, were combined into a single table. Associations between pairs of bacterial and fungal OTUs were calculated using the maximal information coefficient, as implemented in MICtools (76). Pseudo *P* values were obtained by generating 200,000 null matrices and further transformed to Storey's Q-values to correct for multiple hypothesis testing with the Benjamini-Hochberg method. Correlations with a false discovery rate lower than 0.01 were deemed significant. Further, we divided the samples into eight groups according to the combination of the four countries and two delivery modes. We used linear regression to calculate correlations between pairs of OTUs and factors (age, BMI) in a given group. For each group, only OTUs appearing in at least four samples and with a relative abundance higher than 2% in at least one sample were included. Correlations with a *P* value lower than 0.05 were deemed significant. Network analysis was performed on Cytoscape (77).

Phylogenetic relationships between *Malassezia* reads. ITS sequences of the 20 most abundant OTUs assigned to the *Malassezia* genus by the RDP Classifier were combined with those of known *Malassezia* representatives from the UNITE v07-04-2014 database (69). A multiple sequence alignment was constructed with MAFFT v7.313 (78). *Cryptococcus neoformans* was selected as an outgroup, and its ITS sequence was added to the alignment using the "add" option from MAFFT. The resulting alignment was manually curated and further refined with MUSCLE v3.8.31 (79). Phylogenetic trees were inferred with RaxML v8 (80) and MrBayes v3.2 (81), using 1,000 replicates and 1,000,000 generations, respectively. TreeGraph2 (82) was used to combine and visualize the maximum likelihood and Bayesian inference trees.

Accession number(s). All ITS1 sequences have been deposited in the European Nucleotide Archive (ENA) server under study ID PRJEB25581. Samples were deposited under accession numbers ERS2312706 to ERS2312785.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02994-18.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

ACKNOWLEDGMENTS

We are grateful to all the participant families that provided biological samples for this study.

A.B.-A. and M.C.C. acknowledge the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (ERC Starting Grant project no. 639226). F.P.-S. was supported by Ministerio de Economía y Competitividad (MINECO) grant no. CTM2016-80095-C2-1-R (NOVAMAR). The Chinese group acknowledges support from Key Projects of Beijing Science and Technology (D141100004814002) and the Natural Scientific Foundation of Beijing (Z140001).

The authors declare that they have no conflicts of interest.

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