Pediatric RESEARCH

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CLINICAL RESEARCH ARTICLE

Fungal cutaneous microbiome and host determinants in preterm and term neonates

Anshu A. Paul[1], Kristi L. Hoffman[2], Joseph L. Hagan[1], Venkatesh Sampath[3], Joseph F. Petrosino[2] and Mohan Pammi[1]

BACKGROUND: The neonatal cutaneous mycobiome has not been characterized in preterm infants. Invasive fungal infections in preterm neonates are associated with high mortality. The immaturity of the preterm skin predisposes neonates to invasive infection by skin colonizers. We report the clinical and host determinants that influence the skin mycobiome.

METHODS: Skin swabs from the antecubital fossa, forehead, and gluteal region of 15 preterm and 15 term neonates were obtained during the first 5 weeks of life. The mycobiome was sequenced using the conserved pan-fungal ITS2 region. Blood samples were used to genotype immune modulating genes. Clinical metadata was collected to determine the clinical predictors of the abundance and diversity of the skin mycobiome.

RESULTS: The neonatal mycobiome is characterized by few taxa. Alpha diversity of the mycobiome is influenced by antibiotic exposure, the forehead body site, and the neonatal intensive care unit (NICU) environment. Beta diversity varies with mode of delivery, diet, and body site. The host determinants of the cutaneous microbiome include single-nucleotide polymorphisms in *TLR4*, *NLRP3*, *CARD8*, and *NOD2*.

CONCLUSION: The neonatal cutaneous mycobiome is composed of few genera and is influenced by clinical factors and host genetics, the understanding of which will inform preventive strategies against invasive fungal infections.

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INTRODUCTION

The skin, which serves as a physical barrier to the outside environment, is underdeveloped and immature in preterm infants, including its microbial ecosystem.[1] The importance of the cutaneous microbiome in neonatal sepsis and neonatal immune development is gaining widespread acceptance. Colonization of the skin with pathogenic microbes is an important endogenous source of infection and its breach naturally or by medical procedures increases the risk for infection. The neonatal skin microbiome in preterm infants, especially the fungal microbiome has not been investigated adequately.

In contrast to the full-term infant, the preterm infant has a poorly developed epidermis that results in poor barrier function, leading to increased skin permeability. The unique physiochemical characteristics of the preterm skin may modify risk for colonization, infection, and neonatal sepsis.[2] The burden of neonatal sepsis is inversely correlated to gestational age with premature and low birth weight infants being at highest risk for sepsis, especially late-onset sepsis (LOS).[3] Invasive fungal infections (IFIs) account for 5 to 27% of LOS, with *Candida* being the third most common infectious organism of LOS in very and extremely low birth weight (VLBW and ELBW) infants.[4–6] Mortality associated with IFIs in neonates is estimated between 20 and 44% in different studies.[4] Culture-dependent studies have shown that the density of *Candida* colonization and multiple-site colonization have been associated with an increase in candidemia.[5,6] Culture-dependent methods provide an incomplete view of the mycobiome as many fungi cannot be cultured easily. With the availability of molecular identification by PCR and sequencing, we can better understand the mycobiome, its abundance, and diversity.

Host determinants that determine fungal colonization include components of innate immunity such as pattern recognition receptors (PRRs) that mediate immune responses to bacteria and fungi that colonize the newborn infant. The relationship between PRR genes and immunity to bacterial infections has been well described previously, but there are few studies that link these genes with anti-fungal immunity. In adults, single-nucleotide polymorphisms (SNPs) in PRRs like toll-like receptor protein 4 (TLR4) have been associated with increased susceptibility to *Aspergillus* and *Candida* infections[7] and defective TLR4 in mice has also been associated with increased susceptibility to *Candida* infections.[8] Other PRRs such as NOD-1 and NOD-like receptor protein (NLRP3) have also been implicated in anti-fungal immunity. *Malassezia* fungal presence on the skin has been shown to activate the NLRP3 inflammasome,[9] indicating that this innate immunity gene may have a role in moderating *Malassezia* presence. NOD-1-defective mice were found to have decreased *Aspergillus* outgrowth, indicating the absence of NOD-1 may be protective against *Aspergillus* infections.[10] We are not aware of any studies exploring the relationship between SNPs in these genes and the skin bacterial or fungal microbiome in neonates.

We undertook a prospective cohort study of term and preterm neonates in our NICU with longitudinal collection of skin swabs in the first weeks of life. The objectives for this study were to characterize the cutaneous mycobiome in preterm infants and compare it with that of term infants, to delineate clinical and host determinants that influence the diversity and composition of the

[1]Department of Pediatrics, Baylor College of Medicine, Houston, TX, USA; [2]Alkek Center for Microbiome and Metagenomics Research, Baylor College of Medicine, Houston, TX, USA and [3]Department of Pediatrics, Section: Neonatology, Children's Mercy Hospital, University of Missouri, Kansas City, MO, USA
Correspondence: Mohan Pammi (mohanv@bcm.edu)

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mycobiome, to find the relationship between the bacterial and fungal microbiomes, and to associate SNPs in PRRs to the cutaneous microbiome.

METHODS

Patient recruitment

The protocol was approved by the Institutional Review Board at Baylor College of Medicine. After informed parental consent, 15 term and 15 preterm infants were enrolled in the study after delivery. Seven of the 15 term infants were initially admitted to the newborn nursery, where the initial samples were collected. Follow-up sample collection occurred either at home (six infants) or at a Texas Children's Hospital outpatient clinic (one infant) by M.P. The remaining eight term infants and all 15 preterm infants were admitted and stayed in the NICU for the duration of the study for medical indications.

Sample collection and processing

A Cytopak cytosoft brush, CP-5B initially soaked in sterile 0.15 M NaCl with 0.1% Tween-20 (Fisher Scientific, Fair Lawn, NJ) was used for skin swab collection. Skin samples were obtained from ~4 cm^2 area on the forehead, antecubital fossa, and gluteal region. Samples were collected over a 5-week period: two samples from term infants (in the first 48 h and between 2–4 weeks of life) and five samples from preterm infants (in the first 48 h and weekly for 4 weeks). The samples were transported to the laboratory on the date of collection, where they were stored in −80 °C before analysis as a single batch.

DNA extraction and sequencing

The PowerLyzer PowerSoil DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) was used for DNA extraction from the skin samples. The internal transcribed spacer region 2 (ITS2) was sequenced. Primers used for amplification were ITS3 and ITS4, which contained adapters for MiSeq sequencing, linkers, and a 12mer Golay barcode (ITS4 only). The protocol followed for ITS2 sequencing is described in detail in a human microbiome project article by Nash et al.[11] Briefly, the PCRs contained Accuprine 10X PCR Buffer II, Acfcuprime Taq High Fidelity DNA Polymerase, the primers mentioned above, template DNA, and bovine serum albumin. The protocol for PCR included 2 min of 95 °C for denaturation, 35 cycles of 20 s of 95 °C for amplification, 45 s of 56 °C, 90 s of 72 °C, and finally 10 min of 72 °C.[11] DNA from samples were extracted within 2 weeks of arrival to the lab and all samples were processed as a single batch.

SNP genotyping

Genomic DNA was extracted from blood samples using the FlexiGene DNA kit (Qiagen Inc., Valencia, CA) and stored at 4 °C. To genotype the NOD-1 (rs6958571), NOD2 (rs2066844), ATG16L1 (rs2241880), CARD8 (rs2043211), NLRP3 (rs4353135, rs6672995, and rs35829419), TLR4 variants (rs4986790, rs4986791), and IRAK1 (rs1059703) variants, we performed a 5′ nuclease Taqman assay (Applied Biosystems, Foster City, CA) using custom or predesigned TaqMan SNP Genotyping Assay probes (ABI, Foster City, CA) as per the manufacturer's instructions.[12] The principle of the assay involves amplification of the genomic region of interest followed with ligation with allele-specific probes that emit a distinct fluorescent signal specific to the variant or reference allele. Samples were analyzed on ABI HT7900 with SDS 2.3 software package (probes available on request). Genotyping was done by personnel blinded to clinical outcomes.

Quality control

Ten percent of the samples were re-genotyped by an independent technician blinded to prior results. There was >99% concordance for all samples.

Data analysis

For visualization of the mycobiome communities, we used ATIMA (Agile Toolkit for Incisive Microbial Analysis), a software developed at the Alkek Center for Metagenomics and Microbiome Research at Baylor College of Medicine. ATIMA analyzes trends in alpha diversity, beta diversity, and taxa abundance comparing between different samples and their clinical correlates.[13] We used a rarefaction depth of >1000 reads for all samples.

For longitudinal analyses of alpha diversity, we used linear regression models using a base "lm" function, with p value indicating the probability that the slope of the line is zero. For analysis of alpha diversity, we used measures of observed operational taxonomic units (observed OTUs), which measures the richness of species found in a given sample, and Shannon diversity index (SDI), which accounts for how evenly the taxa are distributed in a given sample (the more evenly distributed, the higher SDI). Mixed-effects linear models with a first-order autoregressive covariance structure were used for hypothesis testing in order to accommodate the repeated measurements on the same patients over time and the clustering of sampling sites (regions) within the same patient.

We quantified the differences in microbial communities found in our different samples using beta diversity. For beta diversity, we used principal coordinate analysis (PCoA) with Jaccard distance metric to visualize between-sample differences. An unweighted Jaccard metric was used, which looks at the presence or absence of specific observed OTUs. We visualized the relative abundance of different taxa between samples using "stacked bar plots" and hierarchical clustering of taxa using "heatmap" through ATIMA. A $p < 0.05$ was considered to be significant.

Genome analysis

For SNP genotyping analysis, we assumed a genetic dominant model wherein the presence of one variant SNP allele would be dominant over the wild-type allele. In view of the small number of neonates for homozygous variants, which precluded any meaningful analysis, microbial taxonomical abundance and diversity were compared between infants without the SNP variant and infants who had one or more copies of the variant allele.

RESULTS

Clinical characteristics of the enrolled cohort

Fifteen preterm infants (birth weight 1015 ± 269 g, gestational age 27 ± 2.6 weeks) and 15 term infants (birth weight 3188 ± 538g , gestational age 38.6 ± 1.3 weeks) were enrolled (mean ± standard deviation). Patient characteristics are presented in Table 1.

Cutaneous mycobiome is characterized primarily by few taxa in both preterm and term neonates

The most abundant genera in both preterm and term neonates, in order of abundance, were *Malassezia*, *Candida*, *Cladosporium*, *Fusarium*, and *Cryptococcus* and the two most abundant species were *Malassezia restricta* and *Candida albicans* (Fig. 1). We found no significant difference of relative abundances of these genera or species between preterm and term infants. There were no statistical differences in each of the five most abundant genera in across chronologic age in either preterm or term infants (Supplemental Fig. S1). Each sample was composed of few observed taxonomic units (2.6 ± 3.1) (mean ± SD) with most samples having <15 units, and the highest number of units found in a sample was 23.

Increased fungal alpha diversity is associated with initial antibiotic exposure, NICU environment, and forehead body site

We defined "initial antibiotic exposure" as ≤48 h and "significant antibiotic exposure" as >48 h. When compared with infants exposed to no antibiotics, we found that fungal alpha diversity

Table 1. Clinical characteristics of the enrolled cohort.

	Preterm ($n = 15$)	Term ($n = 15$)
Mean gestational age (weeks) (SD)	27.29 (2.66)	38.52 (1.11)
Mean birth weight (g) (SD)	1010.40 (277.95)	3105.93 (569.76)
Gender (*n*)	Males (9) Females (6)	Males (9) Females (6)
Mode of delivery (*n*)	CS (10) Vaginal (5)	CS (7) Vaginal (8)
Sample collection location (*n*)	NICU (15)	NICU (8) Nursery ->Home (7)
Diet (*n*)	Maternal breast milk (8) Donor breast milk (2) Formula (1) TPN (5)	Maternal breast milk (12) Donor breast milk (0) Formula (2) TPN (1)
Antibiotic exposure (*n*)	Initial antibiotics (7) Significant antibiotics (5)	Initial antibiotics (5) Significant antibiotics (0)
Sepsis	6	0
Candidemia	1	0

CS C-section delivery, *NICU* neonatal intensive care unit, *TPN* total parenteral nutrition
We defined "initial antibiotics" if given for the first 48 h or less and "significant antibiotics" as more than 48 h of antibiotic exposure

measured by both observed OTUs and SDI were significantly higher for infants exposed to initial antibiotics (observed OTUs $p = 0.027$, SDI $p = 0.019$), but not for infants exposed to significant antibiotics (observed OTUs $p = 0.318$, SDI $p = 0.297$). We found no statistical difference between significant antibiotics and initial antibiotics (observed OTUs $p = 0.244$, SDI $p = 0.187$) (Fig. 2a). When we compared infants exposed to any antibiotics (initial antibiotics group + significant antibiotics group) to no antibiotics, we found significantly increased SDI ($p = 0.047$) and a trend of increased observed OTUs, although it did not reach significance ($p = 0.056$).

We also found that the NICU environment was associated with an increased SDI when compared with newborn nursery ($p = 0.037$) and the home environment ($p = 0.048$). However, fungal observed OTUs were not significantly different across the three locations ($p = 0.214$) (Fig. 2b).

All infants had samples collected from the antecubital fossa, the forehead, and the gluteal region. The forehead site was associated with increased observed OTUs ($p < 0.001$) and increased SDI ($p < 0.001$) when compared with the antecubital fossa and the gluteal region. There were no significant differences between gluteal region and antecubital fossa for either observed OTUs ($p = 0.282$) or SDI ($p = 0.079$) (Fig. 2c).

Prematurity, chronologic age, and corrected gestational age were not associated with changes in alpha diversity

We found no significant differences between preterm and term infants for observed OTUs ($p = 0.21$) or SDI ($p = 0.73$). No change in alpha diversity was observed throughout the 5-week period in preterm (observed OTUs $p = 0.22$, SDI $p = 0.65$) or term infants (observed OTUs $p = 0.47$, SDI $p = 0.47$) (Fig. 3). Neither gestational

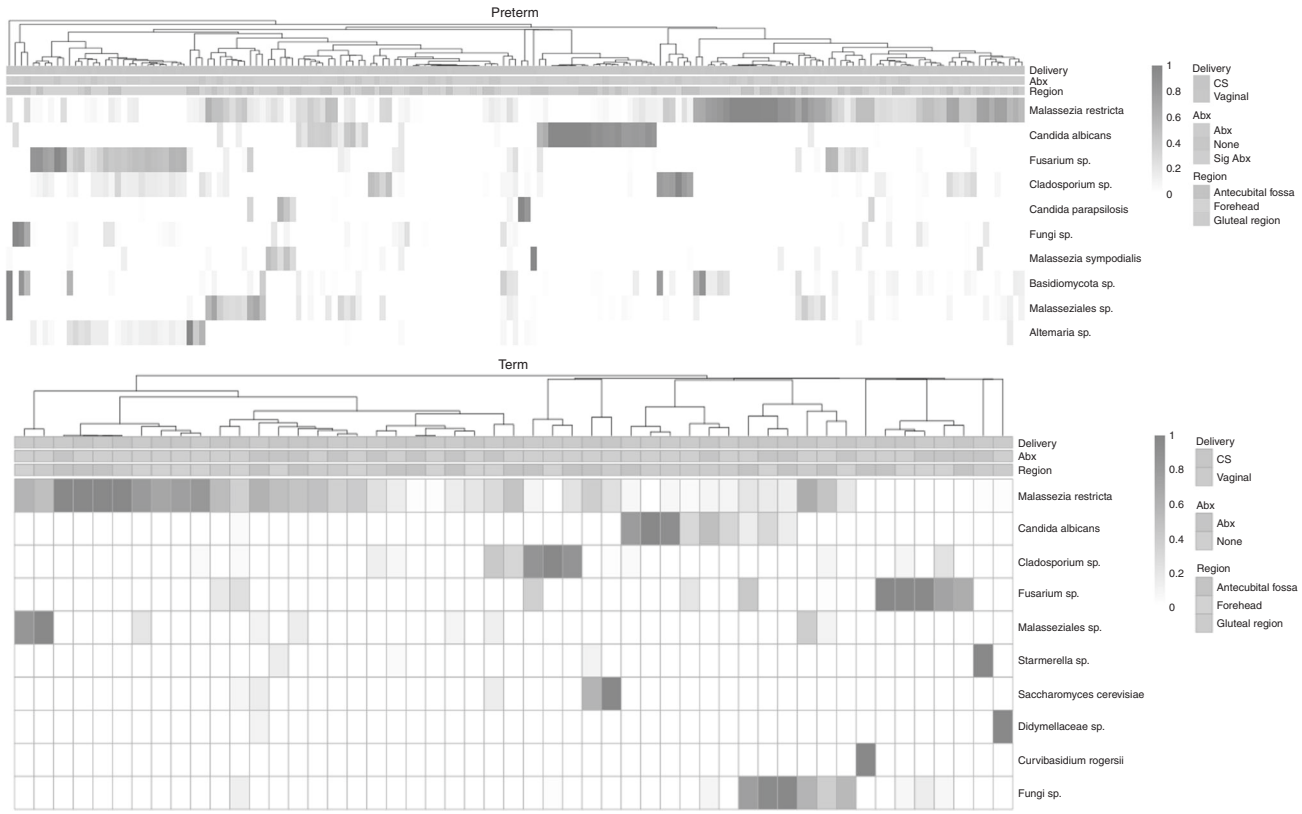

Fig. 1 Hierarchical clustering for preterm and term infants, demonstrating relative abundance at the species level. The two most abundant species in both preterm and term infants were *Malassezia restricta* and *Candida albicans*. The top three rows also show metadata for each sample: the mode of delivery ("CS" = C-section), antibiotic exposure ("Abx" = initial antibiotics, "Sig Abx" = significant antibiotics), and skin site.

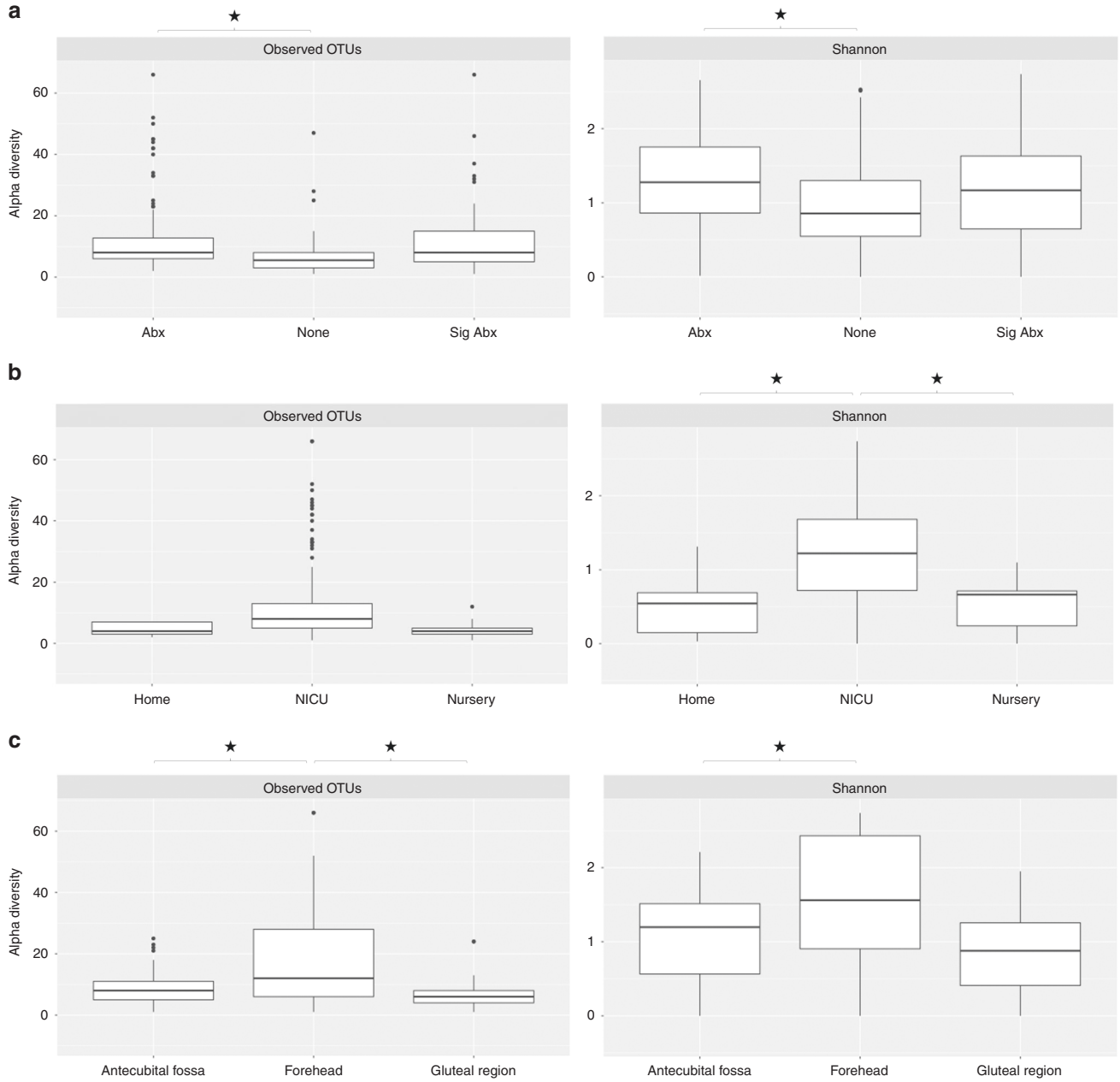

Fig. 2 Alpha diversity as measured by observed operational taxonomic units (OTUs, left panels) and Shannon diversity index (SDI, right panels). a Comparison between infants exposed to no antibiotics (None), initial antibiotics ≤48 h (Abx), and significant antibiotics (Sig Abx). The initial antibiotics group and no antibiotic group were found to be statistically different when comparing both observed OTUs ($p = 0.027$) and SDI ($p = 0.019$). **b** Comparison between infants located at home, in the NICU, and in the newborn nursery. Infants in the NICU had significantly increased SDI as compared with infants at home ($p = 0.048$) or in the newborn nursery ($p = 0.037$). No differences were found in observed OTUs. **c** Comparison between samples collected from the antecubital fossa, the forehead, and the gluteal region. The forehead was found to have statistically increased observed OTUs ($p < 0.001$) and SDI ($p < 0.001$) when compared with the other regions.

age (observed OTUs $p = 0.286$, SDI $p = 0.287$) nor corrected gestational age (observed OTUs $p = 0.73$, SDI $p = 0.73$) were associated with changes in fungal alpha diversity using linear regression methods.

We found no difference in alpha diversity with baths (before bath, after bath, and unknown) using observed OTUs ($p = 0.85$) or SDI ($p = 0.95$). There were no changes in alpha diversity with diet (comparing infants primarily fed total parenteral nutrition (TPN), donor expressed breast milk (dEBM), maternal expressed breast milk, or formula) in observed OTUs ($p = 0.61$) or SDI ($p = 0.53$).

Infant mycobiome beta diversity varied with mode of delivery, type of diet, and body site

Although we found no difference in fungal alpha diversity as measured by observed OTUs ($p = 0.889$), we did find a non-significant trend of increased SDI with C-section (CS) ($p = 0.068$). Using PCoA, there was variation in clustering of infants delivered vaginally versus via CS ($p = 0.001$, $R^2 = 0.036$) (Fig. 4a). Specifically, we noted an increased *Candida* relative abundance in vaginal delivery ($p \leq 0.001$, Fig. 5a). In addition, the relative abundance of *Candida* increased an estimated 6.6% per week for patients

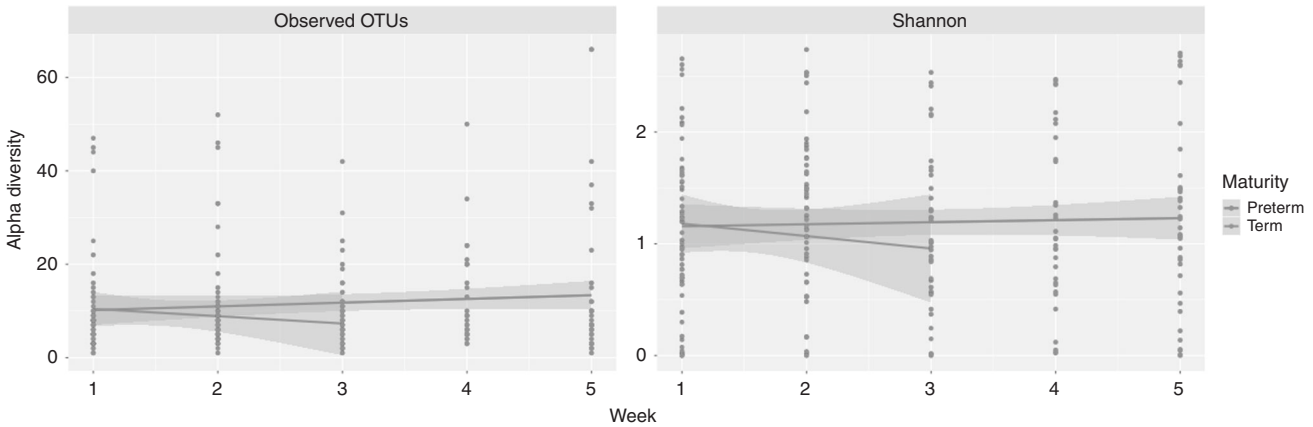

Fig. 3 Alpha diversity as measured by observed OTUs (left) and SDI (right) showing no significant difference across chronological age (from weeks 1 to 5) in either preterm (observed OTUs $p = 0.22$, SDI $p = 0.65$) or term infants (observed OTUs $p = 0.47$, SDI $p = 0.47$).

delivered vaginally compared to 2.6% per week for patients delivered via CS ($p = 0.005$, Fig. 5b).

PCoA analysis between the four different diet types: primarily fed maternal breast milk, donor breast milk, formula, or TPN, revealed significant variation in clustering of infants by type of diet ($p = 0.025$, Fig. 4b), with donor breast milk clustering separate from maternal breast milk, TPN, or formula.

When assessing by body site, PCoA revealed a significant difference in clustering of samples from the forehead ($p = 0.001$, Fig. 4c) as compared to the antecubital fossa and the gluteal region. We found no significant difference in beta diversity between preterm and term infants ($p = 0.188$). When only analyzing preterm infants, we see an increased relative abundance of the *Cladosporium* ($p = 0.002$), *Fusarium* ($p < 0.001$), and *Cryptococcus* ($p < 0.001$) genera in the forehead site when compared with antecubital fossa and gluteal region, which may account for the difference in clustering (Supplemental Fig. S2).

Only patient with culture-positive *Candidemia* noted to have an increased relative abundance of *Candida*

Of our 30 infants, six preterm infants (one infant had *Candida albicans* fungemia) and none of the term infants were diagnosed with late-onset sepsis. The patient with candidemia was noted to have the highest relative abundance of *Candida* across body sites, although it was not statistically significant (Supplemental Fig. S3).

Fungal alpha diversity is positively correlated with bacterial alpha diversity

We previously published on the cutaneous bacterial microbiome from the same cohort of infants.[14] In this study, we compared the alpha diversity of the fungal mycobiome with the bacterial microbiome. We found that bacterial observed OTUs were positively correlated with fungal observed OTUs ($p < 0.001$), but SDI of bacterial and fungal microbiomes were not significantly associated ($p = 0.314$).

Antibiotic exposure was found to be associated with a decrease in bacterial alpha diversity by Pammi et al.[14] In the current study, we observed any antibiotic exposure was associated with an increase in fungal alpha diversity: SDI ($p = 0.047$) and observed OTUs ($p = 0.056$). We report a significant positive correlation between bacterial and fungal OTUs for patients receiving no antibiotics ($p = 0.001$), initial antibiotics ($p = 0.005$) and significant antibiotics ($p < 0.001$) (Supplemental Fig. S4). The positive correlation between bacterial and fungal OTUs was significantly stronger for patients receiving initial antibiotics ($p < 0.001$) or significant antibiotics ($p < 0.001$) compared to no antibiotics.

TLR4 (*rs4986791)* SNP *and NLRP3 (rs6672995)* SNP are associated with variations in the cutaneous mycobiome

We genotyped ten SNPs genotyped to investigate their associations with the cutaneous bacterial microbiome and the mycobiome. Their distribution in preterm and term infants is noted in Table 2. Hardy–Weinberg equilibrium was confirmed for all the SNPs targeted except for the IRAK1 variant, which is X-linked.

The *TLR4 (rs4986791)* SNP was associated with a decrease in the fungal SDI ($p = 0.007$), but not observed OTUs ($p = 0.101$). The *NLRP3 (rs6672995)* SNP was associated with a decrease in the relative abundance of *Fusarium* genus ($p = 0.044$), but an increase in the relative abundance of *Malassezia* genus ($p < 0.001$). The *NLRP3 (rs4353135)* SNP was associated with increased relative abundance of the *Candida* genus ($p = 0.015$) as well as decreased relative abundance of *Malassezia* genus ($p = 0.002$). The *NOD1 (rs6958571)* SNP was associated with decreased relative abundance of the *Malassezia* genus ($p = 0.016$). The *CARD8 (rs2043211)* SNP was found to have a trend with increased relative abundance of the *Malassezia* genus, although it did not reach significance ($p = 0.051$). We did not find any interactions between the fungal mycobiome and the *NOD2 (rs2066844)*, *NLRP3 (rs35829419)*, *ATG16L1 (rs2241880)*, *IRAK1 (rs1059703),* or *TLR4 (rs4986790)* SNPs.

The *NOD2 (rs2066844)* SNP was associated with a decrease in the bacterial observed OTUs ($p = 0.004$). The remaining nine SNPs were not significantly associated with any changes in the bacterial microbiome.

DISCUSSION

The major findings of this study included characterization of the most common genera and species of fungi found on the neonatal skin, identification of the clinical determinants associated with changes in the mycobiome namely NICU environment, antibiotic exposure, forehead site, mode of delivery, and type of diet, and determination of host genetic factors that are associated with changes in the cutaneous microbiome: SNPs in *TLR4, NLRP3*, *CARD8*, and *NOD2*.

Infant mycobiome composition

We found that the skin mycobiome in infants is composed of few taxonomic units, with most infants having <15 taxa on their skin, similar to findings by Ward et al.[15] Although *Malassezia* is known to be the predominant taxa in adult skin, the relative abundance of *Malassezia* was previously found to be lower in the pediatric population[15] when looking at healthy term infants and children. In a study of preterm infants, however, Benjamin et al.[16] found *Malassezia* to be the most abundant colonizer followed by *Candida*, a finding similar to our study.

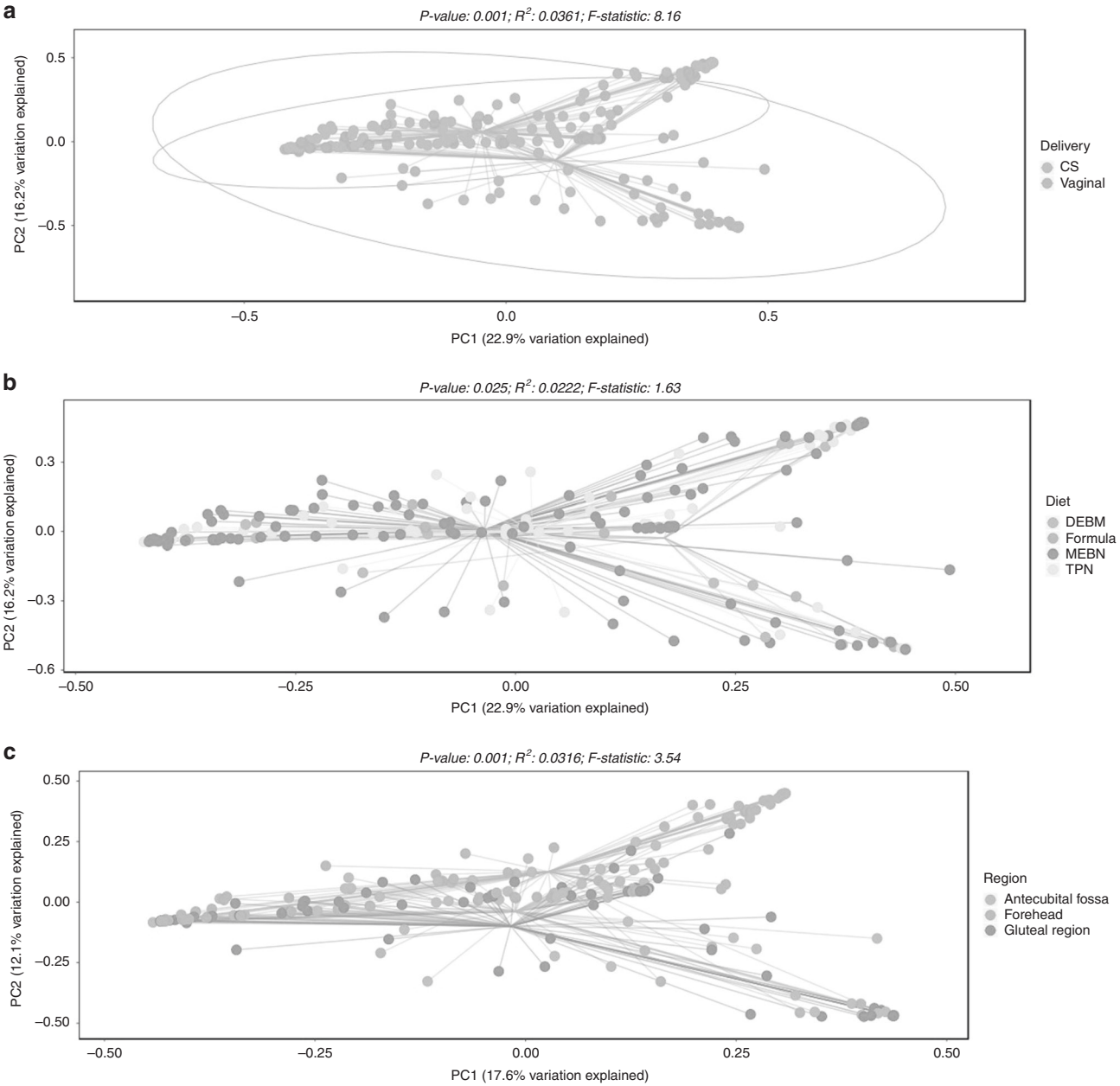

Fig. 4 Principal co-ordinates analysis (PCoA) derived from unweighted Jaccard distances. a Between samples from infants delivered via vaginal delivery (blue) and via C-section delivery ("CS," orange) demonstrating a statistically significant difference in beta diversity ($p = 0.001$) between the two sets. **b** Between samples from infants primarily fed maternal breast milk ("MEBM," green), donor breast milk ("DEBM," orange), formula (blue) and total parenteral nutrition ("TPN," yellow) ($p = 0.025$). Visually noted is a clustering of infants fed donor breast milk separate from the others. **c** Between samples taken from the antecubital fossa (orange), the forehead (blue), and the gluteal region (green), with clustering of samples from the forehead separate from the antecubital fossa and the gluteal region ($p = 0.001$).

Clinical determinants of fungal alpha diversity

The effects of antibiotic use on the bacterial microbiome of infants: in the reduction of diversity and in the emergence of antibiotic-resistant genes have been well established.[15,17] We found that antibiotic use was associated with increased fungal colonization, specifically *Candida albicans*, as reported in previous literature.[18,19] It is interesting to note that we found no significant difference in alpha diversity between the initial antibiotic group and the significant antibiotic group. Although the lack of significant difference between initial and significant antibiotic exposure could have been a result of a small sample size, this finding suggests that 48-h of antibiotics in neonates has a significant effect on the developing microbiome, which may have implications on sepsis and developing immunity. In our enrolled cohort, the most common antibiotics were ampicillin and gentamicin, followed by vancomycin and gentamicin. Only three infants received third-generation cephalosporins and all three were part of the "significant antibiotics" cohort. Due to the small number of infants exposed to cephalosporins, we did not separately analyze the effects of different antibiotic exposures on the mycobiome.

We found the NICU environment to be associated with an increase in fungal alpha diversity. Recent studies have shown a relationship between the bacterial microbiome of NICU surfaces and the infant gut.[20] Heisel et al.[21] found that many NICU surfaces serve as a reservoir for fungi and that *Candida albicans*, *Candida*

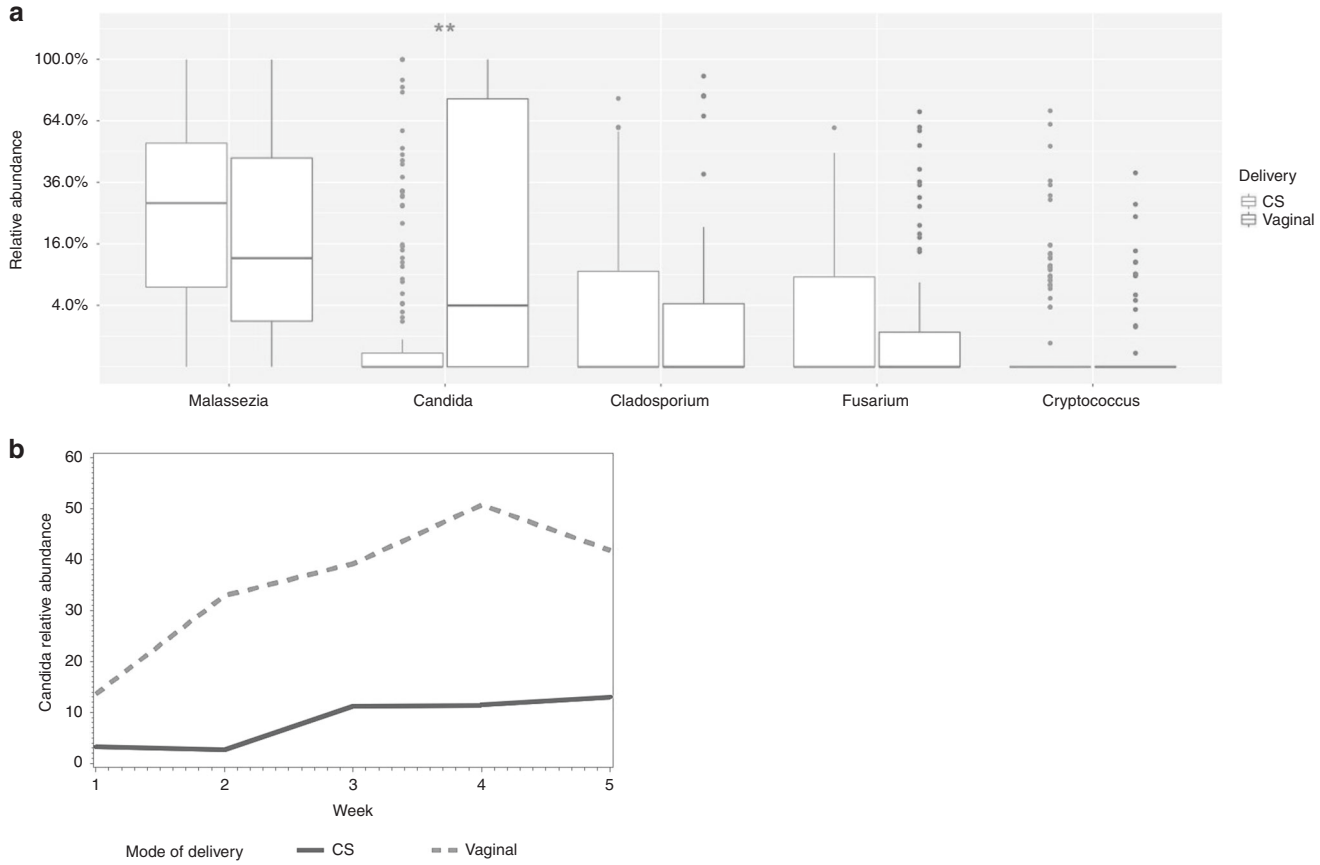

Fig. 5 **a** Relative abundance of the five most abundant genera in infant delivered via C-section ("CS," blue) and vaginally (red). *Candida* relative abundance is significantly increased in vaginally delivered infants ($p < 0.001$). **b** Relative abundance of *Candida* genus in C-section ("CS," blue) and vaginal delivered (red) infants across chronologic age (from weeks 1 to 5) showing persistently increased *Candida* abundance in vaginally delivered infants.

Table 2. Distribution of SNPs among preterm and term infants.

SNP	Alleles	Preterm infants ($n = 15$)	Term infants ($n = 15$)
NOD2 (rs2066844)	Heterozygous (CT)	4 (26.7%)	1 (6.7%)
	Homozygous (TT)	0	0
NOD1 (rs6958571)	Heterozygous (AC)	6 (40%)	6 (40%)
	Homozygous (CC)	1 (6.7%)	1 (6.7%)
NLRP3 (rs4353135)	Heterozygous (GT)	5 (33.3%)	4 (26.7%)
	Homozygous (GG)	1 (6.7%)	1 (6.7%)
NLRP3 (rs6672995)	Heterozygous (AG)	3 (20%)	6 (40%)
	Homozygous (AA)	0	0
NLRP3 (rs35829419)	Heterozygous (AC)	0	2 (13.3%)
	Homozygous (AA)	0	0
ATG16L1 (rs2241880)	Heterozygous (AG)	10 (66.7%)	7 (46.7%)
	Homozygous (GG)	2 (13.3%)	0
CARD8 (rs2043211)	Heterozygous (AT)	8 (53.3%)	4 (26.7%)
	Homozygous (TT)	0	2 (13.3%)
TLR4 (rs4986791)	Heterozygous (CT)	1 (6.7%)	1 (6.7%)
	Homozygous (TT)	0	0
TLR4 (rs4986790)	Heterozygous (AG)	4 (26.7%)	1 (6.7%)
	Homozygous (GG)	0	0
IRAK1 (rs1059703)	Heterozygous (TC)	1 (6.7%)	2 (13.3%)
	Homozygous (CC)	5 (33.3%)	3 (20%)

parapsilosis, and *Saccharomyces cerevisiae* were the most abundantly found fungi on these surfaces.[21] Our findings suggest that the NICU environment may host a more diverse mycobiome than the home or nursery environment, thus influencing the infant skin mycobiome and its diversity. Future studies involving identification of the mycobiome from different surfaces would be warranted to further understand this association. One limitation of the current study is the confounding effect of antibiotic exposure as none of the infants sampled at home or in the newborn nursery were exposed to antibiotics. Differences in other clinical and host determinants may have also confounded environment-related changes in the mycobiome.

The forehead has previously been associated with increased *Malassezia* colonization in both adults and children, hypothesized to be a result of the presence of sebaceous glands in the face.[22,23] In early infancy, there is androgen-driven sebaceous gland hyperplasia of the face in both preterm and term infants,[24] and adult studies have shown that sebum and hydration levels on the face affect both the diversity and the composition of the bacterial microbiome.[25] This may explain the increase in fungal alpha diversity on the forehead.

In addition, we found no increase in the fungal alpha diversity with chronologic age or corrected gestational age, indicating a lack of maturation or diversification of the mycobiome over time, supporting the findings of Ward et al.[15]

Mycobiome variations with mode of delivery, type of diet, and body site

Vertical transmission of *Candida* species from mother's oral or vaginal cavity to infant's oral cavity or gastrointestinal tract have

been demonstrated using DNA fingerprinting techniques,[26] culture,[27] API Candida Test,[27] and molecular sequencing techniques.[15] Our finding of increased *Candida* relative abundance in vaginally delivered infants as compared to CS delivered infants supports these studies. The only other study looking at beta diversity difference in skin mycobiome from Ward et al.[15] did not find a similar variability as our study. This difference may be due to small sample size or hospital-driven differences.

We also found diet-specific variations in mycobiome between infants fed primarily maternal breast milk, donor breast milk, formula, and TPN. Boix-Amorós et al.[28] has shown the presence of fungi in the breast milk from healthy lactating mothers, with the most abundant taxa being *Malassezia* and *Candida*. In contrast, pasteurization suppresses microbial growth in donor breast milk, although studies have primarily focused on bacterial and viral growth.[29–32] These differences may contribute to the variations observed here. The limitation of this analysis is that although we have classified infants into groups based on their primary diet, many infants were fed multiple diets during their stay in the NICU.

Skin studies in children have shown a diversity in fungal flora on the forehead,[23] similar to our finding that the forehead has a mycobiome that was significantly different from both the antecubital fossa and the gluteal region. When we look at all infants together, we found no difference in the relative abundance of the most abundant genera. However, when limiting the analysis to preterm infants, we found that the forehead has increased relative abundance of *Cladosporium, Fusarium*, and *Cryptococcus*. This has not been shown previously and investigation into the properties of these fungi that promote their growth on the forehead skin is warranted to understand this association.

Synergistic relationship between fungi and bacteria on the neonatal skin

Interestingly, we found a positive correlation between bacterial and fungal alpha diversity. Based on our findings of antibiotic exposure and its negative association with bacteria but positive association with fungi, we had hypothesized an inverse relationship between bacteria and fungi. However, even when limiting to antibiotic-exposed infants, our study found a positive correlation between bacteria and fungi. One possibility for this correlative relationship may be that our measures of observed OTUs only give us information on the presence or absence of a fungus and not on the richness or abundance.

Gram-positive organisms, such as *Staphylococcus*, *Streptococcus*, or *Enterococcus* have been shown to create a mixed bacteria–fungi biofilm with *Candida* that benefits the bacteria and fungi in the oral cavity, the gastrointestinal tract, and subcutaneous catheter models.[33–36] Given the predominant phylum found in the cutaneous bacterial microbiome of neonates, *Firmicutes*, is composed of Gram-positive species, there may be a similar interaction between the cutaneous mycobiome and bacterial microbiome.

SNPs associated with variations in the cutaneous fungal and bacterial microbiome

TLR4 is a receptor that recognizes pathogen-associated molecular patterns (PAMPs), which plays a role in activating the pro-inflammatory response. In humans, TLR4 SNPs have been associated with increased susceptibility to *Aspergillus* and *Candida* infections[7] and defective TLR4 in mice has also been associated with increased susceptibility to *Candida* infections.[8] Our study found that the *TLR4 (rs4986791)* SNP is associated with a decrease in fungal alpha diversity (SDI), but its clinical relevance needs more research.

Nod-like receptors, including NLRP3, are also PRRs that sense the presence of PAMPs. After sensing a pathogen, they can form into a large cytoplasmic complex known as an inflammasome, which eventually leads to cytokine activation that is part of our innate immune response. NLRP3 inflammasome activation has been linked to *Malassezia* fungal presence on the human skin.[9] In our study, we found the *NLRP3 (rs6672995)* SNP was associated with an increase in the relative abundance of *Malassezia* and decrease in the relative abundance of *Fusarium*. We also found the *NLRP3 (rs4353135)* SNP to be associated with an increase in relative abundance of *Candida*, but a decrease in relative abundance of *Malassezia*.

NOD proteins are PRRs that recognize bacterial cell wall ligands in the host cytosol and activate the immune response pathway. NOD-1 defects have been linked with decreased *Aspergillus* outgrowth.[10] We found that the *NOD1 (rs6958571)* SNP was associated with a decreased relative abundance of *Malassezia*.

CARD8 is thought to be a negative regulator of the *NLRP3* inflammasome.[37] Idosa et al.[38] found an association between the *CARD8 (rs2043211)* SNP and both Gram-negative and Gram-positive bacteremia.[38] We found a trend of increased relative abundance of *Malassezia* with the *CARD8 (rs2043211)* SNP.

Only the *NOD2 (rs2066844)* SNP was associated with changes in the bacterial microbiome (a decrease in the bacterial observed OTUs) in our cohort. This NOD2 SNP has previously been associated with changes in the abundance of certain bacteria: decrease in *F. prausnitzii* and *g_Roseburia*, and an increase in *Enterobacteriae* in patients with inflammatory bowel disease[39,40] Further studies looking at direct associations between NOD2 SNPs and cutaneous disease processes are warranted.

The strengths of this study include it being the first study looking at the cutaneous fungal microbiome in preterm infants, the longitudinal assessment over a 5-week period, and the analysis of both host and clinical determinants of the fungal and bacterial cutaneous microbiome. The limitations include the small sample size which limits our ability to detect significant differences in some clinical variables in preterm infants. In addition, there are clinical characteristics that were difficult to separate, like prematurity and NICU admission or samples being collected at home/clinic and term infants.

CONCLUSIONS

We report the first study of the cutaneous fungal microbiome in preterm infants. The clinical determinants that are associated with changes in the mycobiome are: NICU environment, antibiotic exposure, forehead body site, mode of delivery, and type of diet. Antibiotic exposure has been associated with IFIs in preterm infants and here we show that even <48 h of antibiotic exposure can lead to increased richness of the mycobiome. We found that vaginal delivery is associated with increased *Candida* richness in the cutaneous microbiome. We found several SNPs in immune-modulatory genes that were associated with changes in the cutaneous microbiome. Understanding the development and determinants of the cutaneous microbiome could lead to novel strategies for prevention of IFIs in vulnerable preterm infants.

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AUTHOR CONTRIBUTIONS

Substantial contribution to the conception and design: M.P. Acquisition of data: M.P., J.F.P., and V.S. Analysis and interpretation of data: A.A.P., K.L.H., J.L.H., V.S., and M.P. Drafting of manuscript: A.A.P. and M.P. All authors contributed to critical revisions of the manuscript and final approval of the version to be published.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41390-019-0719-7) contains supplementary material, which is available to authorized users.

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