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MAJOR ARTICLE

Low Diversity in Nasal Microbiome Associated With *Staphylococcus aureus* Colonization and Bloodstream Infections in Hospitalized Neonates

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Background. *Staphylococcus aureus* is a leading cause of infectious morbidity and mortality in neonates. Few data exist on the association of the nasal microbiome and susceptibility to neonatal *S. aureus* colonization and infection.

Methods. We performed 2 matched case–control studies (colonization cohort—neonates who did and did not acquire *S. aureus* colonization; bacteremia cohort—neonates who did [colonized neonates] and did not [controls] acquire *S. aureus* colonization and neonates with *S. aureus* bacteremia [bacteremic neonantes]). Neonates in 2 intensive care units were enrolled and matched on week of life at time of colonization or infection. Nasal samples were collected weekly until discharge and cultured for *S. aureus,* and the nasal microbiome was characterized using 16S rRNA gene sequencing.

Results. In the colonization cohort, 43 *S. aureus*–colonized neonates were matched to 82 controls. At 1 week of life, neonates who acquired *S. aureus* colonization had lower alpha diversity (Wilcoxon rank-sum test $P < .05$) and differed in beta diversity (omnibus MiRKAT $P = .002$) even after adjusting for birth weight ($P = .01$). The bacteremia cohort included 10 neonates, of whom 80% developed bacteremia within 4 weeks of birth and 70% had positive *S. aureus* cultures within a few days of bacteremia. Neonates with bacteremia had an increased relative abundance of *S. aureus* sequences and lower alpha diversity measures compared with colonized neonates and controls.

Conclusions. The association of increased *S. aureus* abundance and decrease of microbiome diversity suggest the need for interventions targeting the nasal microbiome to prevent *S. aureus* disease in vulnerable neonates.

Keywords. bloodstream infection; microbiome; neonates; *Staphylococcus aureus*.

Staphylococcus aureus remains one of the leading causes of infectious morbidity and mortality of hospitalized infants [1]. *S. aureus* is the primary cause of central line–associated bloodstream infections (CLABSIs), ventilator-associated pneumonia, and surgical site infections in neonates and children [2–11]. In addition, hospitalized infants experience higher rates of invasive disease due to *S. aureus* than most other age groups [1].

S. aureus can colonize the nasal cavity as well as other anatomic sites, and colonization predisposes to subsequent infection [12]. Recent research has explored nuances of the nasal microbiome that may contribute to *S. aureus* colonization and infection. High-throughput sequencing studies in adults suggest that the biodiversity of the nasal microbiome, the distribution of bacterial communities, and the abundance of specific bacteria are associated with *S. aureus* colonization [13–15].

However, the microbiota of neonates is distinct from that of older children and adults. The nasal microbiota of an adult is relatively diverse with an even distribution of bacterial taxa, making it less susceptible to changes provoked by environmental exposures [16–20]. By contrast, a newborn's nasal microbiota typically demonstrates lower biodiversity, higher bacterial burden, and greater variance in organism abundance, making it more dynamic and susceptible to the effects of environmental exposures [15, 21–23]. This dynamic state may predispose to dysbiosis and lead to an increased risk of colonization and domination of the neonatal microbiota by pathogenic species [21, 24, 25]. The role the nasal microbiota plays in susceptibility of neonates to the acquisition of *S. aureus* colonization and development of *S. aureus* bloodstream infections remains unknown.

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Our objective was to compare the nasal microbiomes of 3 groups of neonates in the neonatal intensive care unit (NICU) with known exposure to *S. aureus*: those who did not acquire *S. aureus* colonization, those who acquired *S. aureus* colonization, and those who developed an *S. aureus* bloodstream infection. The purpose of this comparison was to elucidate how bacterial communities may interact to provide resistance or susceptibility to *S. aureus* colonization and infection.

METHODS

Study Design, Setting, and Population

We performed 2 matched case–control studies to measure the association of clinical characteristics and the evolving nasal microbiota in hospitalized neonates. All neonates were admitted to the Johns Hopkins Hospital (JHH) NICU or the Johns Hopkins Bayview Medical Center (JHBMC) NICU. Eligible neonates were (1) enrolled in a clinical trial, the TREAT PARENTS Trial [26], and tested for *S. aureus* colonization at trial enrollment and weekly until NICU discharge or acquisition of *S. aureus* colonization (neonates were not decolonized in this trial; only parents were) or (2) admitted to the JHH and JHBMC NICUs and had weekly nasal swabs collected as part of a comprehensive *S. aureus* control program. Colonization was defined as growth of *S. aureus* using the method described below. All neonates were monitored for invasive *S. aureus* infections. We designed 2 matched case–control cohorts based on available stored samples: the Colonization Cohort—neonates within the TREAT PARENT Trial who acquired *S. aureus* colonization (colonized neonates) and did not acquire *S. aureus* colonization (control) were matched 1:2, respectively, based on the week of life (WOL) that a case acquired colonization; and the Bacteremia Cohort—neonates who developed *S. aureus* bacteremia (bacteremic neonates), neonates who acquired *S. aureus* colonization (colonized neonates), and neonates who did not acquire *S. aureus* colonization (controls) were matched 1:1:1, based on the WOL that the case developed bacteremia, such that a child with bacteremia during WOL 4 was matched to a colonized neonate who acquired *S. aureus* colonization during WOL 4 and a control who had not acquired *S. aureus* colonization by WOL 4.

Patient Consent

This study was approved by the Johns Hopkins University Institutional Review Board. Written informed consent was obtained for neonates in the TREAT PARENTS Trial, and waiver of consent was granted for use of remnant samples collected for clinical care.

Data Collection

Data from participants were collected from the electronic medical record, which included basic demographic and clinical information. Antibiotic use was defined as any antibiotic administered in the week before sample collection.

Sample Collection and 16S rRNA Gene Sequencing

To identify neonates with *S. aureus* colonization, flocked swabs were used to collect samples from nares, inoculated into broth-enriched culture, and plated on sheep blood agar and selective agar plates. Colonies were screened by coagulase testing and confirmed using the BD Phoenix Microbial identification system. Residual samples were stored at –80°C before undergoing DNA extraction, 16S rRNA gene sequencing, and bacterial community profiling. DNA were isolated as previously described [27–29], after which the V3V4 16S hypervariable regions of the 16S rRNA gene were PCR amplified using the universal primers 319F and 806R [28, 30]. 16S rRNA gene amplicon sequencing was then performed on Illumina HiSeq 2500 with 300-bp PE reads as previously described, such that a sampling depth of ~50 000 16S reads was obtained on average per sample [28, 31, 32]. This strategy allowed us to characterize bacterial community composition with high resolution, even for bacterial species with low abundance.

16S rRNA Gene Amplicon Sequence Analysis

Raw paired-end reads were merged into consensus fragments and cleaned, and high-quality passing 16S sequences were submitted for high-resolution taxonomic assignment using Resphera Insight, as previously described [33–36]. This approach is capable of providing species-level assignments and distinguishing *S. aureus* from other closely related species. Samples with less than 1040 total read counts were removed from analysis. All microbiome samples were subsampled (rarefied) to an even depth for all downstream analysis. Four alpha diversity metrics (number of observed species, Chao1, Shannon index, and inverse Simpson index) were calculated and considered continuous variables for all analyses. Beta-diversity analyses were performed using Bray-Curtis dissimilarity and weighted and unweighted UniFrac distances [37, 38].

Statistical Analysis

Baseline characteristics of participants were compared between cases and controls using 2-sample *t* tests and chi-square tests for continuous variables and categorical variables, respectively. Alpha diversity metrics at the first WOL were compared between cases and controls using nonparametric Wilcoxon rank-sum or Kruskal-Wallis tests and linear regression models adjusting for birth weight. Planned sensitivity analyses included adjusting for antibiotic use and subset analysis for treatment assignment in the TREAT PARENTS trial to include neonates whose parents were not decolonized. Principal coordinate analysis and MiRKAT were conducted to visualize and statistically evaluate differences in beta-diversity between cases and controls [39]. Differences in relative abundance of individual taxa were assessed at the genus and species levels. To reduce the number of multiple comparisons, we filtered out rare taxa, keeping only

taxa that were present in at least 10% of the samples or had a relative abundance of 5% in at least 1 sample. Wilcoxon rank-sum tests were used to compare the relative abundances between cases and controls at each WOL. Associations were considered significant at the false discovery level of .05 [40]. Alpha and beta diversity analyses were performed using the "phyloseq" package (version 1.32.0) [41]. All analyses were conducted in R (version 4.0.2).

RESULTS

Colonization Cohort

Forty-three neonates within the TREAT PARENTS Trial who acquired *S. aureus* colonization (colonized neonates) were identified and matched to 82 neonates who did not acquire *S. aureus* colonization (controls). A total of 610 nares samples were available for sequencing. After removing samples with less than 1040 read counts, 504 samples from 120 participants remained in the analysis (43 cases and 77 controls). Neonates who acquired *S. aureus* colonization had a lower gestational age and lower birth weight compared with the matched control neonates who did not acquire *S. aureus* colonization (Table 1). Delivery mode and sex were similar between the 2 groups.

Of the 120 neonates, 79 had microbiome data available at the first WOL. Compared with controls, colonized neonates had lower alpha diversity (number of species, Chao1, Shannon index, and inverse Simpson index; Wilcoxon rank-sum test, $P < .05$) (Figures 1 and 2). To account for differences between colonized neonates and controls in birth weight, adjusted models demonstrated that higher observed species, Shannon index, and inverse Simpson index at the first WOL were statistically associated with a lower risk of *S. aureus* colonization ($P < .05$), but not for Chao1 ($P = .184$). Further adjustment including both birth weight and antibiotic use found similar differences in alpha diversity comparing colonized neonates and controls. We also conducted a sensitivity analysis that included only neonates whose parents were not decolonized in the trial and found an association between lower observed species and Shannon index and *S. aureus* colonization ($P = .026$ and 0.039, respectively), while the association was not significant for Chao1 ($P = .71$) or Inverse Simpson Index ($P = .11$).

Differences in microbiome beta diversity at the first WOL were observed between colonized neonates and controls. Figure 2B shows the principal coordinate plots for microbiome beta-diversity assessed by Bray-Curtis, weighted UniFrac, and UniFrac distances for colonized neonates and controls. We then applied MiRKAT to assess the association between beta diversity and acquisition of *S. aureus* colonization. In the unadjusted analysis, strong associations existed between microbiome beta diversity and *S. aureus* colonization (all $P < .05$). After adjusting for birth weight, the observed distance in UniFrac distance was associated with acquisition of *S. aureus* colonization ($P = .004$). Using Bray-Curtis and weighted UniFrac distance, the adjusted association was not significant ($P = .084$ and $P = 0.208$, respectively). We further applied the omnibus MiRKAT, a robust version of MiRKAT that takes into consideration all 3 distance matrices, and found a significant association overall in beta diversity and acquisition of *S. aureus* colonization ($P = 0.002$) that remained after adjusting for birth weight ($P = 0.01$). Adjusting for antibiotic use produced similar results.

The median time to acquisition of *S. aureus* colonization in cases was the third WOL. We further explored the factors contributing to shifts in microbiome alpha diversity in the weeks before acquisition of *S. aureus* colonization in the colonized neonates and matched controls (Supplemental Figure 1). Week of colonization is defined as the week a case became colonized, and as the matching week of age for controls. Only colonized neonates who acquired colonization after the first WOL and their matched controls were included in this analysis. For example, neonates who acquired colonization during the second or third WOL contributed alpha diversity data for the first WOL and first and second WOL, respectively, representing 1 and 2 weeks before colonization (WBC). The Shannon diversity index was borderline lower in colonized neonates compared with controls 2 WBC ($P = .05$, linear regression adjusting for birth weight), and similar non–statistically significant trends were noted for the other 3 alpha diversity measures. At 1 WBC, there was a similar trend for all 4 alpha diversity measures even

Table 1. Patient Characteristics of 2 Matched Case–Control Cohort Studies to Assess Colonization[a] And Bacteremia[b]

	Colonization Cohort		Bacteremia Cohort		
Variable	Controls	Colonized Neonates	Controls	Colonized Neonates	Bacteremic Neonates
Neonates analyzed, No.[c]	77	43	9	8	10
Gestational age, mean (SD)	34.10 (4.28)	30.16 (4.16)	31.11 (3.74)	30.35 (4.40)	25.93 (1.93)
Birth weight, mean (SD)	2.21 (0.96)	1.40 (0.77)	1.45 (0.79)	1.42 (1.13)	0.96 (0.36)
Sex, male, No. (%)	39 (50.6)	27 (62.8)	4 (44.4)	6 (75.0)	5 (50.0)
Vaginal delivery, No. (%)	29 (37.7)	15 (34.9)	2 (22.2)	2 (25.0)	1 (10.0)

[a]Included neonates who did and did not acquire *S. aureus* colonization.

[b]Included neonates with *Staphylococcus aureus* bacteremia (bacteremic neonates, cases) and neonates who did (colonized neonates) and did not (controls) acquire *S. aureus* colonization.

[c]Analytic group included samples with >1040 read counts.

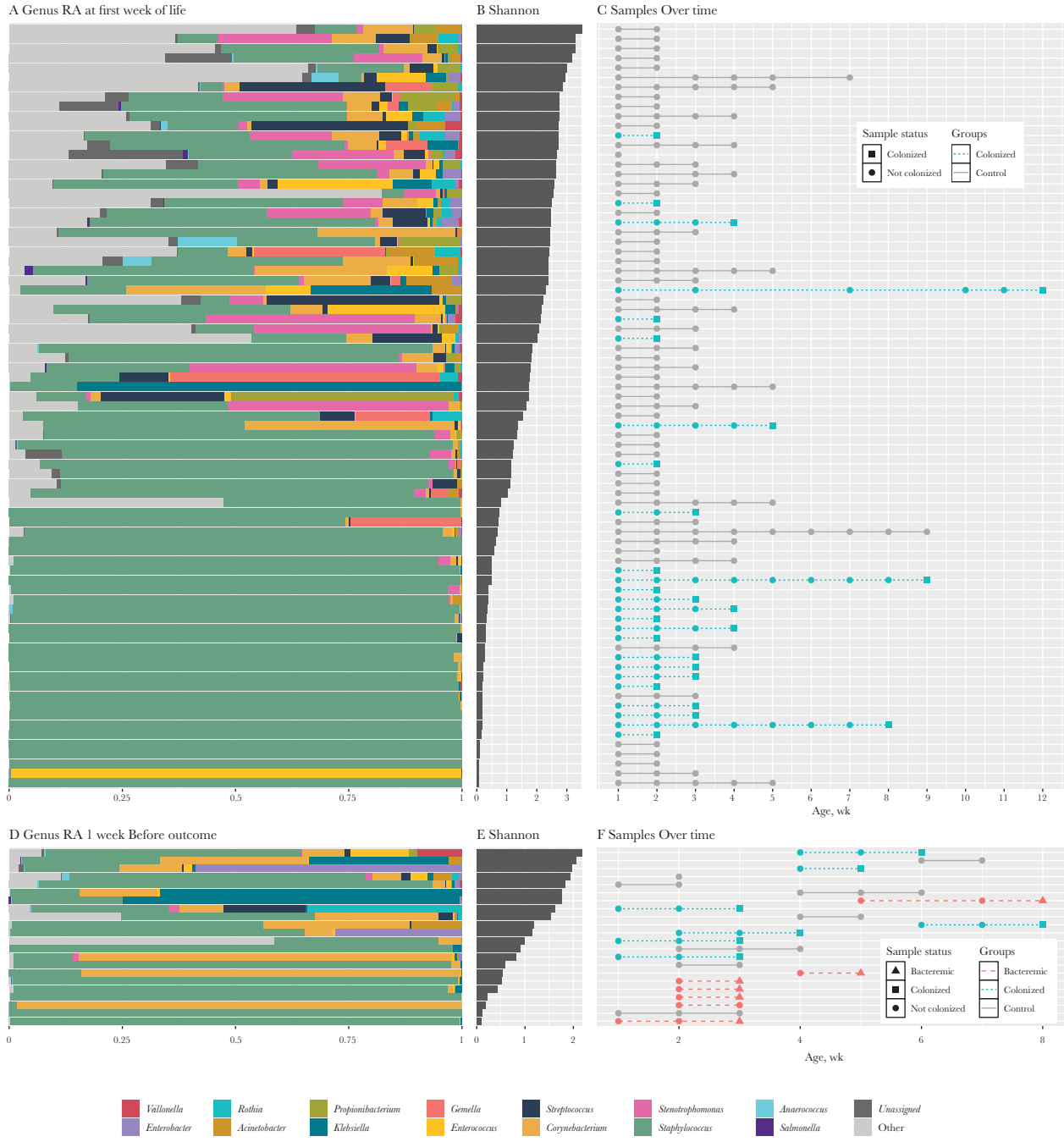

Figure 1. Association of nasal microbiome and *Staphylococcus aureus* colonization and bacteremia. A, Horizontal bars represent microbiome relative abundances (RAs) at the first week of life (WOL) in the colonization cohort (limited to neonates with an available sample at the first WOL, n = 79 of 120). These bars represent genus-level assignments, so some control neonates have predominately *Staphylococcus* due to other *Staphylococcus* species (not *S. aureus*). B, Bar plot showing the corresponding Shannon index at the first WOL in the colonization cohort. C, Available microbiome sequencing samples at each WOL in the colonization cohort. Blue line represents neonates who acquired *S. aureus* colonization as detected by culture. Shapes of points represent whether the individual microbiome samples grew *S. aureus* in culture (colonized, square) or did not grow *S. aureus* in culture (not colonized, circle). D, Horizontal bars represent microbiome relative abundances 1 week before outcome in the bacteremia cohort (n = 22 of 27 individuals). E, Bar plot showing the corresponding Shannon index 1 week before outcome in the bacteremia cohort. F, Available microbiome sequencing samples at each WOL in the bacteremia cohort for neonates who had a sample the week before outcome. Line color represents whether the neonate developed bacteremia (red) or acquired *S. aureus* colonization (blue) as measured by culture. Shapes of points represent whether the individual microbiome samples grew *S. aureus* in culture (colonized, square), did not grow *S. aureus* in culture (not colonized, circle), or were taken at time of *S. aureus* bacteremia (bacteremic, triangle).

though the difference was not significant. There was no difference in alpha diversity at the week of colonization adjusting for birth weight.

S. aureus sequences were detected in most samples, even when the conventional culture methods, including broth enrichment, did not detect *S. aureus*. Only 7 samples had no

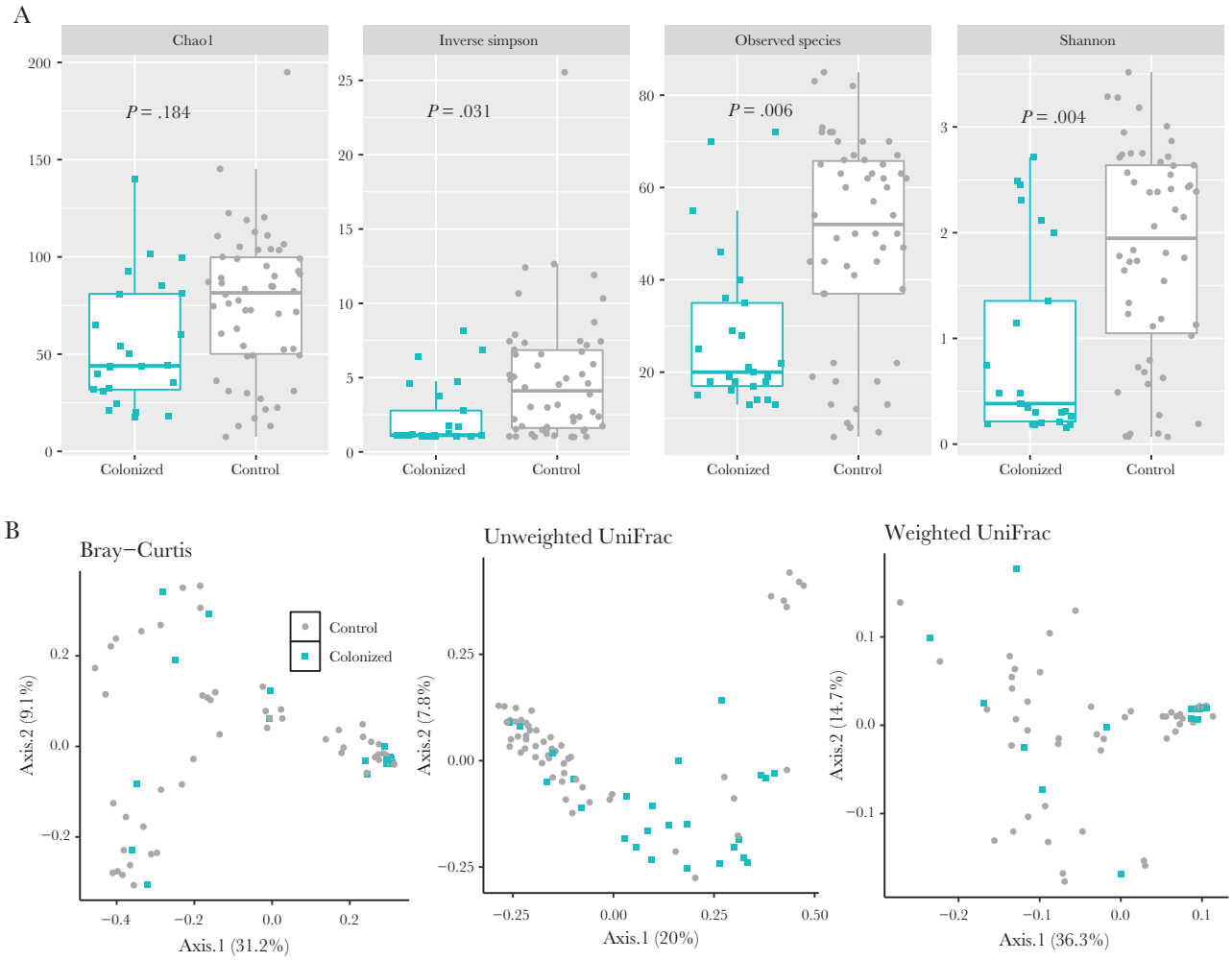

Figure 2. A, Alpha diversity indices at first week of life (WOL) comparing neonates who acquired *Staphylococcus aureus* colonization (colonized neonates, blue) with neonates who did not acquire *S. aureus* colonization (controls, gray). The *P* values were obtained from linear regression models associating microbiome alpha diversity and group status (colonized vs control), adjusting for birth weight. B, Principal coordinate plots of beta diversity analyses at first WOL comparing neonates who acquired *S. aureus* colonization during neonatal intensive care unit (NICU) admission (colonized neonates, blue) with neonates who did not acquire *S. aureus* colonization during NICU admission (controls, gray).

detected *S. aureus* sequences. There exists a clear correlation over time between relative abundance of *S. aureus* sequences in colonized neonates and controls (Supplemental Figure 2), such that colonized neonates have an increased abundance of *S. aureus* sequences at the time of colonization.

Bacteremia Cohort

Ten neonates who developed *S. aureus* bacteremia were identified (bacteremic neonates). Eight of 10 neonates developed bacteremia within 4 weeks of birth, and 7 of 10 had a first positive nares culture within a few days before or after the date of positive blood culture. The 10 bacteremic neonates were matched to 10 neonates who acquired *S. aureus* colonization (colonized neonates) and 11 neonates who did not acquire *S. aureus* colonization (controls). There were 103 nasal microbiome samples available for sequencing. After removing samples with fewer than 1040 total read counts, 98 samples remained, with 34, 33, and 31 samples from the colonized neonates (n = 8), controls (n = 9), and bacteremia neonates (n = 10), respectively (Table 1). Bacteremic neonates had a lower gestational age compared with the colonized and control neonates. The selected samples were collected at 3 time points: 2 weeks before bacteremia, 1 week before bacteremia, and the week of bacteremia. There were on average 8 samples at each time, limiting the statistical power of the analysis.

Despite the small sample size, there were large differences in all 4 alpha diversity measures comparing bacteremic neonates, colonized neonates, and controls in the week before bacteremia (Figure 3). Bacteremic neonates had lower alpha diversity than the colonized neonates, who in turn had lower alpha diversity than controls. At the week of bacteremia, bacteremic neonates had lower alpha diversity than colonized neonates; however,

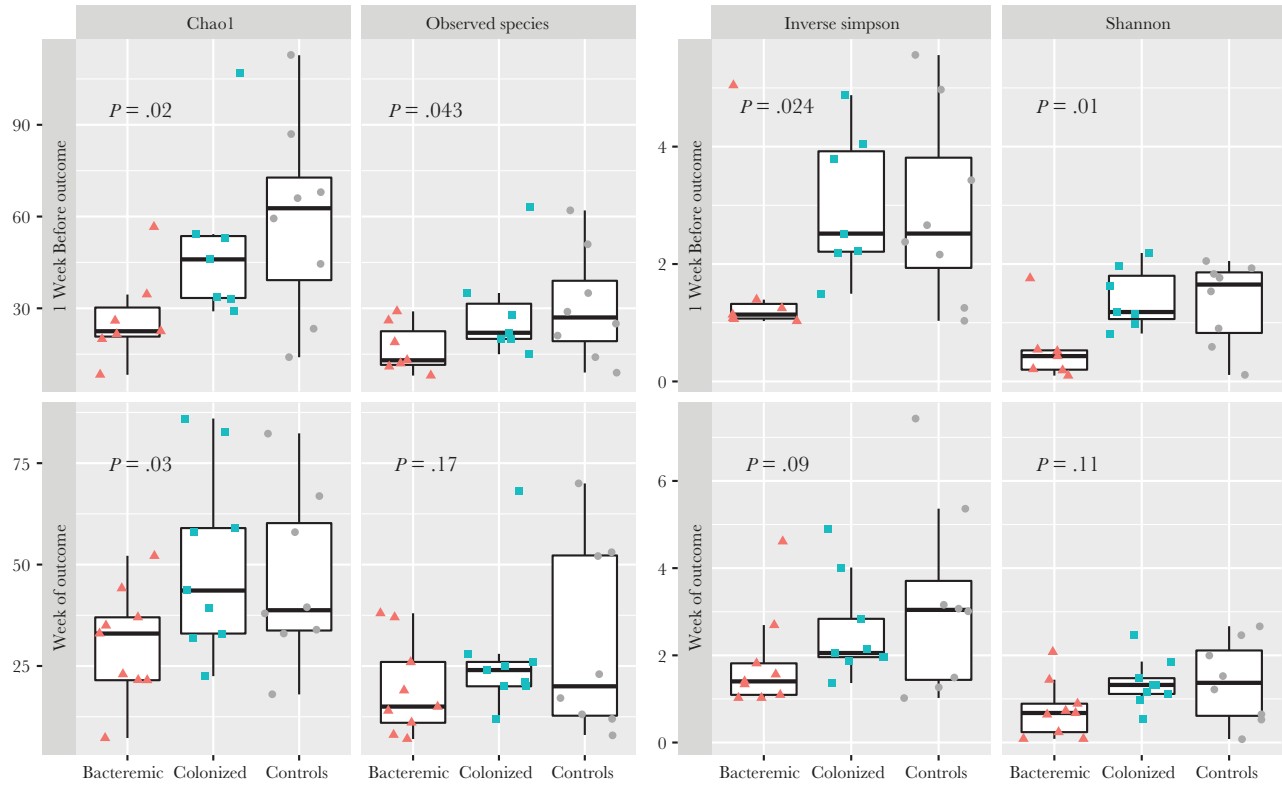

Figure 3. Alpha diversity for neonates with *Staphylococcus aureus* bacteremia (bacteremic neonates [red]), neonates who acquired *S. aureus* colonization (colonized neonates positive [blue]), and neonates who did not acquire *S. aureus* colonization (control [gray]). Upper panel represents 1 week before the outcome (bacteremia, colonization, or matched control), and the lower panel represents the week of the outcome. The *P* values shown are from the Kruskal-Wallis tests that compared the 3 groups.

there was no difference in alpha diversity measures between the colonized neonates and controls.

There exists a clear correlation over time between the relative abundance of *S. aureus* sequences in the 3 groups (Figure 4). Bacteremic neonates had an increased abundance of *S. aureus* sequences compared with colonized neonates, who in turn had a higher abundance than controls.

DISCUSSION

Few data exist to explain what predisposes some neonates in the NICU to acquire *S. aureus* colonization or develop invasive *S. aureus* infections. These case-controlled studies of neonates suggest that lower bacterial biodiversity, differences in bacterial community structures (beta diversity), and higher relative abundance of nasal *S. aureus* were associated with *S. aureus* colonization and bacteremia. This association of dysbiosis with invasive *S. aureus* disease presents an opportunity to promote greater nasal microbiome diversity as potentially protective against *S. aureus* colonization and bacteremia in neonates.

In neonates tested weekly from birth, the nasal microbiome in the first WOL demonstrated lower biodiversity and differences in bacterial community structure in neonates who subsequently acquired *S. aureus* colonization while in the NICU than those who did not acquire colonization. After adjusting for birth weight and antibiotic use, the association between alpha or beta diversity and *S. aureus* colonization remained significant. This cohort was unique given that all neonates had a known exposure to *S. aureus* as at least 1 parent was colonized with *S. aureus*. To elucidate when the nasal microbiome may predispose neonates to *S. aureus* colonization, we characterized the microbiome in the weeks leading up to detected *S. aureus* colonization. In addition to differences at week 1 of life, we also found lower biodiversity and differences in bacterial community structure 2 weeks before neonates had *S. aureus* colonization detected by culture compared with a similar WOL for those who did not acquire colonization. Our findings are supported by prior studies in adults [13, 14] and neonates [23, 36] linking bacterial communities containing lower biodiversity with *S. aureus* colonization, suggesting that increased nasal microbiome diversity may provide colonization resistance in neonates.

In addition to differences in nasal community composition, some studies suggest an association between specific organisms and the presence or absence of *S. aureus* colonization. A higher relative abundance of organisms such as *Dolosigranulum pigrum* and certain *Corynebacterium* species has been described in the nares of infants who do not have *S. aureus* colonization

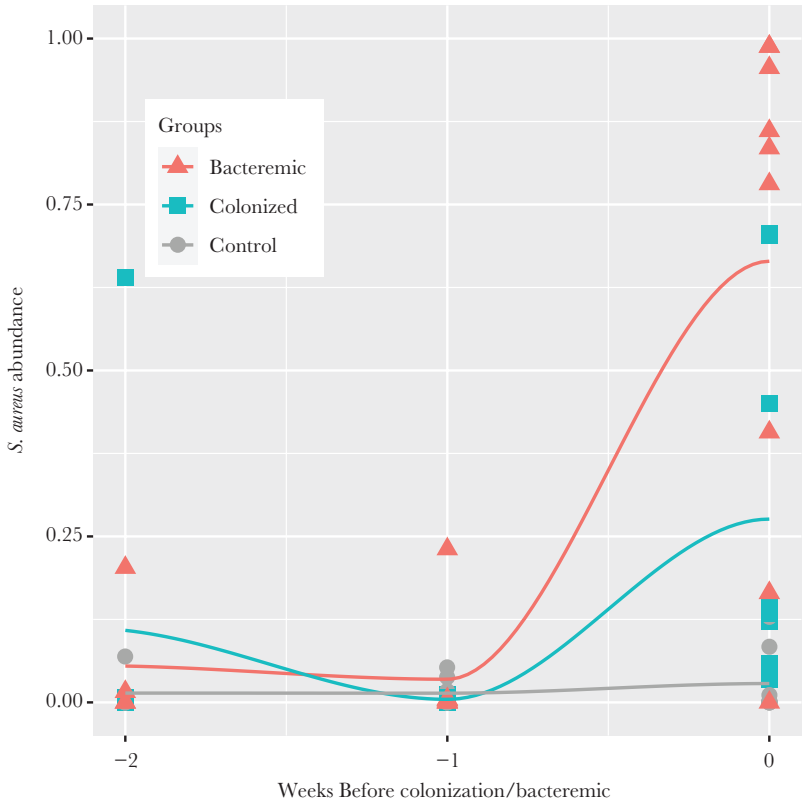

Figure 4. Relative abundance of *Staphylococcus aureus* comparing neonates who developed *S. aureus* bacteremia (bacteremic neonates), neonates who acquired *S. aureus* colonization (colonized neonates control), and neonates who did not acquire *S. aureus* colonization (controls). Time 0 is the week of a positive culture result (or matched week of life for controls), and negative times are the weeks before the culture results.

[36, 42]. In hospitalized neonates, a higher relative abundance of *Corynebacterium* species was found in neonates who did not acquire *S. aureus* colonization compared with those who did [36]. These studies have led to speculation that specific organisms may play a role in providing colonization resistance to *S. aureus*. In our current study, despite species-level assignments of sequencing results, we did not replicate this finding or identify these bacterial species in higher abundance in neonates without *S. aureus* colonization. One unique characteristic of this cohort that may explain this difference is that neonates were followed from birth and mostly became colonized with *S. aureus* in the first month of life, a time characterized by rapid evolution of the microbiome. Prior studies have compared older infants who may have a more mature microbiome. Future longitudinal studies should further explore taxa-level differences in the nasal microbiome between neonates, infants, and children, as the microbial composition and predisposing factors to *S. aureus* colonization may differ in these populations.

The question of greatest clinical importance is why some exposed neonates develop *S. aureus* bacteremia. *S. aureus* nasal colonization is a known predisposing factor to *S. aureus* infections [13, 14, 43]. In the studied neonates, individuals with bacteremia had lower alpha diversity indices before acquisition of colonization than neonates who acquired *S. aureus* colonization and those who were not colonized, supporting the hypothesis that dysbiosis may predispose to both colonization and bacteremia. Additionally, neonates who developed *S. aureus* bacteremia had a markedly higher abundance of *S. aureus* than those who acquired *S. aureus* colonization but did not develop subsequent bacteremia. An association between relative abundance of pathogenic organisms and bacteremia has been demonstrated across studies of the intestinal microbiome in the adult population. Increased relative abundance of *Klebsiella pneumoniae* carbapenemase–producing *Klebsiella pneumonia* (KPC-Kp) in the adult gut microbiome has been associated with KPC-Kp bacteremia [42]. In hematopoietic stem cell transplant patients, intestinal domination by a particular organism has been associated with a many-fold increase in risk of bacteremia with the dominant pathogen [44]. These observations, while addressing different populations and organisms, support our finding that higher relative abundance of *S. aureus* is associated with higher risk of bacteremia. Overall, a combination of lower biodiversity, an uneven distribution of organisms, and *S. aureus* domination may together contribute to risk of infection in neonates and may serve as a biomarker for predicting disease in this high-risk population.

While this study presents many significant findings, it also has some limitations. First, despite being the largest study to evaluate the nasal microbiome in neonates who acquire *S. aureus* colonization and infection, the sample size of bacteremic neonates was limited. This may limit the generalizability and power of the study to detect some differences between groups. Second, 17% of available samples did not yield 1040 reads after sequencing, possibly due to ultra-low biomass; these were excluded from the analysis. These excluded samples were equally distributed across groups. Third, sampling the nasal cavity in a newborn can be challenging due to the small vestibule and an often-moving target, but all samples were collected by a study team member using standardized collection methods to limit variability and sampling error. Fourth, standard challenges with 16S rRNA amplicon sequencing analysis, such as differential amplification of certain sequences due to long polymorphisms in the V3 region, can result in taxonomic bias toward clades with greater gene copy numbers (GCNs) [45]. Finally, conventional culture may misidentify some colonized neonates as controls, but the clinical significance of *S. aureus* operational taxonomic unit in a culture-negative sample is unknown. Any potential misclassification of colonized neonates as controls should bias results toward the null.

In conclusion, this study found significant differences in the neonatal nasal microbiome between neonates who did not acquire *S. aureus* colonization, those who acquired *S. aureus* colonization, and those who developed *S. aureus* bacteremia. Future studies should investigate whether increasing diversity and evenness within the neonatal nasal microbiome promote colonization resistance to *S. aureus* and reduce the risk of invasive *S. aureus* infection in critically ill neonates.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases online*. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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