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Breastfeeding-associated microbiota in human milk following supplementation with *Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus* La-5, and *Bifidobacterium animalis* ssp. *lactis* Bb-12

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ABSTRACT

Breastfeeding is one of the major factors affecting the early development of the infant gut microbiota, and weaning is associated with a shift in the gut microbiota toward a more adult composition. Through breastfeeding, infants receive bioactive components that shape their microbiota while also being exposed to the breast milk and breast surface microbial communities. Recent studies have suggested the possibility of an entero-mammary route of microbial transfer, opening the possibility of infant gut microbiota modulation through maternal probiotic supplementation. In this study, we have analyzed breast milk samples collected at 10 d and 3 mo postpartum from women participating in the Probiotics in the Prevention of Allergy among Children in Trondheim placebo controlled trial. Women who were randomized to the probiotic arm of the Probiotics in the Prevention of Allergy among Children in Trondheim trial received a fermented milk supplemented with *Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus* La-5, and *Bifidobacterium animalis* ssp. *lactis* Bb-12, consuming this daily from 4 wk before their expected due date until 3 mo after birth. In total, 472 breast milk samples were assessed for the administered bacteria using quantitative real-time PCR and the microbiota transferred during breastfeeding was analyzed using 16S ribosomal RNA gene sequencing of 142 samples. We found that breastfeeding is unlikely to be a significant source of *L. rhamnosus* GG, *L. acidophilus* La-5, and *B. animalis* ssp. *lactis* Bb-12 for infants in the probiotic arm of the trial. Furthermore, maternal supplementation did not significantly affect the overall composition of the breast milk microbiota transferred during breastfeeding. We also present a descriptive analysis of this microbiota, which was largely dominated by *Streptococcus* and *Staphylococcus* genera at both 10 d and 3 mo postpartum. Samples collected at 3 mo postpartum had a statistically significant lower presence and relative abundance of the *Staphylococcus* genus. These samples also had a greater number of observed species and diversity, including more operational taxonomic units from the *Rothia*, *Veillonella*, *Granulicatella*, and *Methylbacterium* genera.
Key words: human milk, probiotics, atopic dermatitis, microbiota

INTRODUCTION

Breastfeeding is one of the major factors affecting the early development of the infant gut microbiota and weaning is associated with a shift in the gut microbiota toward a more adult-like composition (Wopereis et al., 2014; Bäckhed et al., 2015; Rodríguez et al., 2015). Multiple components of breast milk contribute to these effects, including human milk oligosaccharides, which promote the growth of some microbes, and lysozymes, lactoferrin, and antimicrobial peptides, which inhibit the growth of others (Cacho and Lawrence, 2017). Additionally, breastfeeding is a source of a diverse range of microbes that are found both on the breast surface and within the mammary glands of lactating women (Fitzstevens et al., 2017). Culture-dependent and -independent techniques have demonstrated a dominance of bacteria belonging to the *Staphylococcus*, *Streptococcus*, and *Propionibacterium* genera, as well as the presence of lactic acid bacteria and bifidobacteria in breast milk (Fitzstevens et al., 2017). The origin of these bacteria is thought to be a combination of the microbiotas associated with the mother's skin flora, the infant's oral mucosa, and the maternal gut. Recent studies have suggested the possibility of an entero-mammary route with selective trafficking of commensal bacteria from the maternal gut to the mammary glands via dendritic cells and macrophages (Rodríguez, 2014; Treven et al., 2015).

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In the Probiotics in the Prevention of Allergy among Children in Trondheim (**ProPACT**) placebo controlled trial, we found that maternal ingestion of 3 probiotic strains, while pregnant and breastfeeding, resulted in an almost 40% reduction in the cumulative incidence of atopic dermatitis among offspring at 2 yr of age (Dotterud et al., 2010). Women in the probiotic arm of the trial consumed fermented milk supplemented with *Lactobacillus rhamnosus* GG (**LGG**), *Lactobacillus acidophilus* La-5 (**La-5**), and *Bifidobacterium animalis* ssp. *lactis* Bb-12 (**Bb-12**), whereas women in the placebo group consumed heat-treated fermented milk without supplemented bacteria. Analysis of stool samples from the ProPACT study revealed that women in the probiotic arm had a higher prevalence and relative abundance of all 3 probiotic bacteria strains at 3 mo postpartum in their stool samples. A similar result was observed for the children of these women at 10 d and 3 mo of age, although only for the LGG (Dotterud et al., 2015). Breastfeeding may therefore be an ongoing source of LGG for these infants. Previous studies suggest that maternal supplementation with *L. rhamnosus* LC705 (Nasiraii et al., 2011), *Lactobacillus reuteri* (Abrahamsson et al., 2009), and *Lactobacillus fermentum* CECT5716 and *Lactobacillus salivarius* CECT5713 (Arroyo et al., 2010) may result in an increased presence of the administered bacteria in the breast milk of some, but not all, women. To our knowledge, no studies have investigated the transfer of LGG, *L. acidophilus,* or *Bifidobacterium* species through breastfeeding after maternal supplementation. *Lactobacillus rhamnosus* GG is of particular interest because it has been the most commonly administered bacteria in atopic dermatitis prevention studies and was observed to be transferred to infants in the ProPACT study.

In the present study, we investigated the bacteria transferred through breastfeeding using breast milk samples taken at 10 d and 3 mo postpartum from women participating in the ProPACT trial. The samples were collected without sterilization of the breast areola and are considered to give a more representative analysis of the bacteria ingested by suckling infants. We have therefore adopted the term "breastfeeding-associated microbiota" suggested by Sakwinska et al. (2016) to describe this bacterial community, which involves the breast milk and breast surface microbiotas in human milk ingested by the suckling infant. Our aim was to investigate whether maternal probiotic supplementation with LGG, La-5, and Bb-12 affected the presence of these strains among the bacteria transferred during breastfeeding at 10 d and 3 mo postpartum, and their association with the later development of atopic dermatitis. We also assessed the general microbiota associated with breastfeeding, considered temporal trends, and the relationships between the composition of the microbiota transferred during breastfeeding, probiotic supplementation, and atopic dermatitis.

MATERIALS AND METHODS

Participant Recruitment and Sample Collection

This study analyzed 472 breast milk samples collected from 252 women participating in the ProPACT trial. The design and clinical results from this randomized, placebo controlled trial have been described in detail elsewhere (Dotterud et al., 2010; Simpson et al., 2015). Briefly, 415 women, who intended to breastfeed, were randomized to receive a commercially available fermented milk (Biola, Tine AS, Oslo, Norway) containing 5×10^{10} cfu of LGG and Bb-12 and 5×10^{9} cfu of La-5 per 250 mL or a placebo fermented milk that contained no probiotic bacteria and was heat treated after fermentation. Participating women were to consume 250 mL per day of their allocated study milk from 36 wk gestation until 3 mo postpartum. Their infants did not receive any probiotic supplementation. The children were assessed for signs and symptoms of allergy-related diseases through questionnaires and clinical examination at 2 and 6 yr of age. Atopic dermatitis was diagnosed according to the UK Working Party diagnostic criteria (Williams et al., 1994) at the clinical examinations.

Participating women were provided with sterile sample tubes and were requested to collect breast milk at 10 d and 3 mo postpartum. The timing, with respect to time of day or whether fore- or hindmilk was collected, was not standardized. The women did not receive explicit instructions regarding washing or sterilization of the breast surface before sample collection. Samples were frozen in their home freezer until transportation to the laboratory where they were subsequently stored at −80°C. All available breast milk samples were included in the current study, provided that the child attended the 2-yr clinical follow-up (Figure 1).

Analysis of Microbiota

Breast milk samples (2 mL) were centrifuged at $21{,}500 \times g$ for 30 min. The resulting pellet was resuspended in 100 μL of stool transport and recovery buffer and DNA was isolated using LGC Mag DNA extraction kit (LGC Genomics, Middlesex, UK) on a KingFisher FLEX magnetic particle processor (Thermo Fisher Scientific, Waltham, MA) according to the manufacturers' instructions. Samples were analyzed for total bacteria

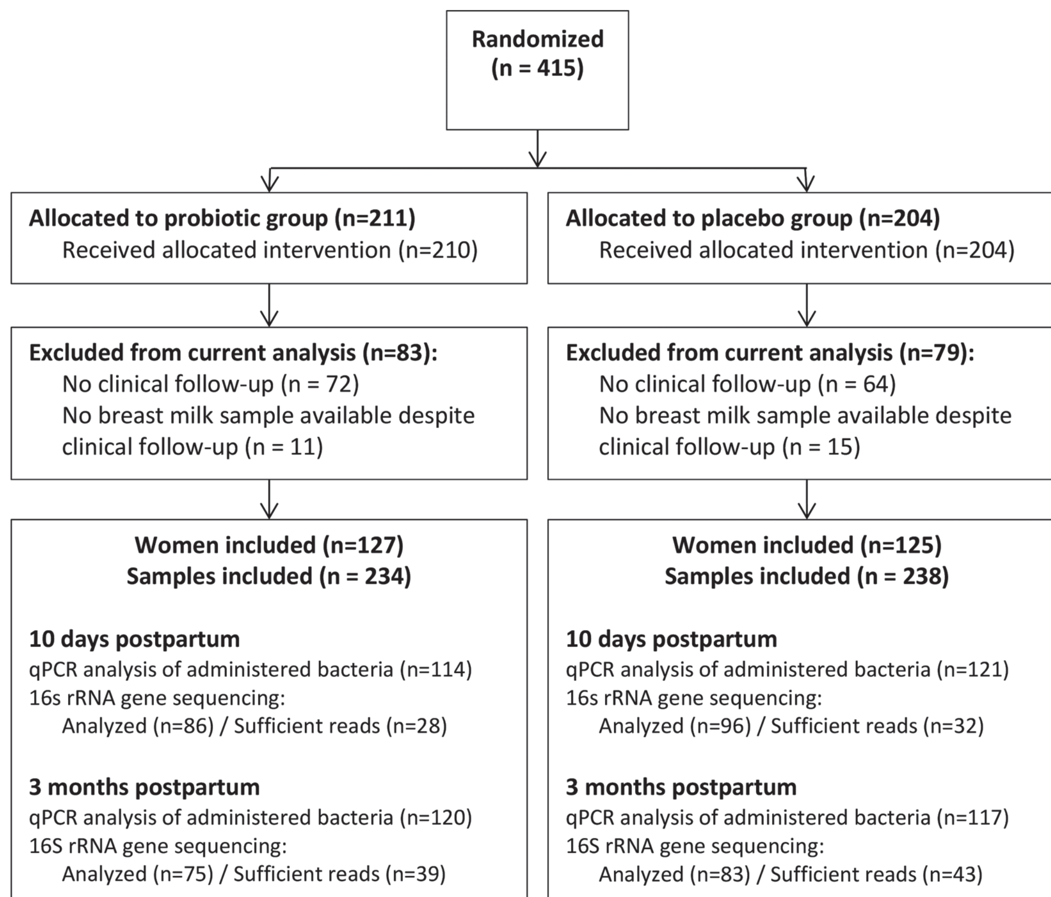

Figure 1. Flow diagram of participants in the Probiotics in the Prevention of Allergy among Children in Trondheim study and analysis of the bacterial content of breast milk samples is given. The number of eligible women who had provided at least one breast milk sample. The precise number of samples analyzed at each time point with each method varies as indicated. Samples were considered to have a sufficient number of reads if the 16S rRNA gene sequencing analysis returned ≥1,000 reads. qPCR = quantitative PCR.

and the 3 administered bacteria using quantitative real-time PCR (**qPCR**) and using 16S rRNA gene sequencing for the general microbial composition.

The qPCR was performed using a double-stranded DNA binding dye-based EvaGreen assay (Biotium Inc., Fremont, CA) for assessing LGG and La5, and a probe-based TaqMan assay (Thermo Fisher Scientific) for Bb12 and total bacteria. The primers and probes for these assays are detailed in Table 1. For the EvaGreen assays, the reaction volumes of 20 μL contained 1 × HOT FIREpol EvaGreen (Solis BioDyne, Tartu, Estonia), forward and reverse primers with a final concentration of 0.2 μ*M* and 1 μL of template DNA. A LightCycler-480 machine (Roche, Basel, Switzerland) was used for the qPCR reaction using the following settings: heating to 95°C for 15 min, followed by 40 cycles of denaturing at 95°C for 15 s, annealing and extension at 60°C for 1 min. Melting point curves were obtained by heating the samples from 60 to 95°C at 0.1°C per second and measuring fluorescence at each 0.2°C temperature increment. For the TaqMan assay, the reaction volume of 20 μL contained 4 μL of 1 × HOT FIREpol, 0.4 μL of forward and reverse primers with a final concentration of 0.2 μ*M*, the probe with a final concentration of 250 n*M* and 1 μL of template DNA. The LightCycler-480 settings for this assay were heating to 95°C for 12 min, followed by 50 cycles of denaturing at 95°C for 30 s, annealing and extension at 60°C for 1 min. For both the EvaGreen and TaqMan assays, the negative control was 1 μL of nuclease-free water and the positive control was DNA isolated from Biola or Bb12 cultures supplied by Tine AS (the producer of Biola) for the measurement of the administered bacteria and 1 μL of *Escherichia coli* DNA for the analysis of total bacteria.

The general microbial composition of the samples was assessed using 16S rRNA gene sequencing of 300-bp paired end reads on the Illumina MiSeq platform (Illumina Inc., San Diego, CA) with v3 sequencing chemistry at The Centre for Integrative Genetics (CIGENE, Ås, Norway). Following DNA extraction, the

Table 1. Primers and probes

Bacteria[1]	Assay[2]	Forward/reverse primer and probe name	DNA sequence (5′–3′)
Total	TaqMan	Forward primer: Univ_F	TCCTACGGGAGGCAGCAGT
		Reverse primer: Univ_R	GGACTACCAGGGTATCTAATCCTGTT
		Probe: universal probe	FAM-GTATTACCGCGGCTGCTGGCAC-TAMRA
LGG	EvaGreen	Forward: L.rhamn1	CAATCTGAATGAACAGTTGTC
		Reverse: L.rhamn2	TATCTTGACCAAACTTGACG
La-5	EvaGreen	Forward: La-5–5L	TTACGCCAGTCCAAGGGTAG
		Reverse: La-5–5R	CAGAATGCCCGCAAGTTATC
Bb-12	TaqMan	Forward primer	AGA ACC ACG GCG GCG TC
		Reverse primer	CGC GGT CTT CTC GAG CAC T
		Probe	FAM-TGC GCT CGC CGA CG-MGB

[1]LGG = *Lactobacillus rhamnosus* GG; La-5 = *Lactobacillus acidophilus* La-5; Bb-12 = *Bifidobacterium animalis* ssp. *lactis* Bb-12.
[2]TaqMan (Thermo Fisher Scientific, Waltham, MA); EvaGreen (Biotium Inc., Fremont, CA).

V3 to V4 region of the 16S rRNA gene was amplified using PRK341F/PRK806R primers adapted for Illumina sequencing. The results were analyzed with the Quantitative Insights Into Microbial Ecology (QIIME) pipeline, utilizing AmpliconNoise to denoise the data and remove chimeras, uclust for operational taxonomic unit (**OTU**) clustering at 97% similarity and the Ribosomal Database Project classifier for assigning taxonomic identity to the identified OTU. A total of 1,712,182 sequences were generated, with a median of 4,517.6 reads per sample.

Statistical Analysis

Statistical analyses and graphics were conducted using Stata IC 13.1 (StataCorp, College Station, TX) and MATLAB 2016b (The MathWorks Inc., Natick, MA). The effect of probiotic supplementation on the presence of the administered bacteria in breast milk at each time point was estimated using the qPCR data and is presented as a risk ratio (**RR**) and 95% confidence interval, accompanied with a Fisher exact test. Samples were considered positive if the EvaGreen assays had a threshold cycle (**Ct**) value below 35 and a melting point of 86°C for LGG or 80°C for La-5. For Bb-12 and total bacteria, samples were considered positive if they had a Ct value below 35 on the TaqMan assay.

The 16S rRNA gene sequencing data was rarefied to 1,000 reads before descriptive and comparative analysis of the breastfeeding-associated microbiota. Samples with less than 1,000 reads were not included in these analyses. The effect of probiotic supplementation on the presence and relative abundance of individual genera at each time point was assessed using a χ^2 test (or a Fisher exact test as indicated) and a Wilcoxon rank-sum test, respectively. An OTU was considered "present" if it accounted for at least 1% of reads (10 reads) in a given sample in the rarefied data set. To avoid excessive multiple comparisons of uncommon or very low abundance OTU, comparisons were only estimated for OTU and genera that were present in at least 10% of the samples (i.e., $\geq$ 10 reads in $\geq$6 samples from 10 d or $\geq$9 samples from 3 mo). For each set of comparisons, a raw *P*-value of $\leq$0.05 was considered of potential interest. False discovery rates (**FDR**) were obtained using the Benjamini-Hochberg method in the multiproc Stata command and an FDR $<$0.1 was considered acceptable. Associations between the presence or relative abundance of the common genera and the following factors were also considered: stage of lactation, the development of atopic dermatitis, presence of older siblings, mode of delivery, maternal atopy, and sex of the child. The association between the presence of common genera and the development of atopic dermatitis was also assessed using multivariable logistic regression models including treatment allocation, presence of older siblings, maternal atopy, and sex of the child as additional covariates.

The effect of probiotic supplementation and stage of lactation on the α diversity was assessed using the Shannon and Simpson diversity indices, phylogenetic diversity, and species richness (observed species). Beta diversity was investigated using principal coordinates analysis (**PCoA**) derived from unweighted and weighted UniFrac, distance matrices, and Bray Curtis dissimilarity matrix. Each PCoA plot was reviewed with respect to stage of lactation, treatment allocation, and the previously mentioned mother-infant characteristics.

RESULTS

Participants

A total of 472 breast milk samples from 252 women were available for analysis (Figure 1). Due to low quantities of bacterial DNA, fewer samples could be assessed using 16S rRNA gene sequencing (Figure 1). The baseline family characteristics and allergy-related disease outcomes among children in the current study are representative of the original ProPACT study population

Table 2. Baseline characteristics of families and allergy-related disease in the children at 2 yr

Item	Treatment allocation				Risk ratio (95% CI)
	Probiotic		Placebo		
	n	Value	n	Value	
Characteristics					
Age, mother, yr mean (SD)	127	30.5 (3.9)	125	30.4 (4.1)	
Sex (male), child, no. (%)	127	66 (52.0)	125	51 (40.8)	
Siblings, no. (%)	127	60 (47.2)	125	53 (42.4)	
Atopy in family, no. (%)	127	90 (70.9)	125	92 (73.6)	
Maternal atopy, no. (%)	127	58 (45.7)	124	65 (52.4)	
Maternal smoking,[1] no. (%)	127	8 (6.3)	125	11 (8.8)	
Pet,[2] no. (%)	127	33 (26.0)	125	35 (28.0)	
Compliant,[3] no. (%)	123	111 (90.2)	123	110 (89.4)	
Mode of delivery, vaginal, no. (%)	123	108 (87.8)	124	108 (87.1)	
Allergy-related disease at 2 yr					
Atopic dermatitis,[4] no. (%)	127	29 (22.8)	125	43 (34.4)	0.66 (0.44–0.99)
Current asthma, no. (%)	127	7 (5.5)	125	12 (9.6)	0.57 (0.23–1.41)
ARC,[5] no. (%)	123	1 (0.8)	124	0 (0.0)	NA
Current sensitization,[6] no. (%)	120	18 (15.0)	119	14 (11.7)	1.28 (0.67–2.44)
Skin prick test positive, no. (%)	109	6 (5.5)	106	6 (5.7)	0.97 (0.32–2.92)
sIgE positive, no. (%)	80	17 (21.3)	89	12 (13.5)	1.58 (0.80–3.09)

[1]Maternal smoking reported during pregnancy, or 6 wk or 12 mo postpartum.

[2]Reported a household pet during pregnancy or the child's first year of life.

[3]Compliance with the study protocol was defined as consumption of the study milk on at least 50% of days from 36 wk gestation to 12 postpartum, no consumption of other products with probiotics and at least partial breastfeeding until 3 mo postpartum.

[4]Cumulative incidence of atopic dermatitis.

[5]ARC = allergic rhinoconjunctivitis (cumulative incidence).

[6]Allergic sensitization defined as positive skin prick test (wheal ≥3 mm) and or positive sIgE (≥0.35 kUL^{-1}). Not all children had both measured.

(Table 2 and Supplemental Table S1; https://doi.org/10.3168/jds.2017-13411). The baseline characteristics of the families with adequate reads on 16S rRNA gene sequencing analysis were also comparable; however, the preventative effect of probiotic supplementation on atopic dermatitis was not observed in this subgroup (Supplemental Table S1; https://doi.org/10.3168/jds.2017-13411).

Transfer of Administered Probiotic Bacteria Strains Via Breast Milk

Based on results from the qPCR assays, the majority of the breast milk samples had measurable quantities of bacterial DNA with 392 of 472 samples positive for total bacteria. Samples without measurable bacteria included 39 samples collected at 10 d postpartum (20 and 19 from the probiotic and placebo groups, respectively) and 41 samples from 3 mo postpartum (26 and 15 from the probiotic and placebo group, respectively). However, only 8 women from the probiotic group and 1 from the placebo group had detectable levels of the any of the administered bacteria. At 10 d postpartum, LGG was present in 2 of 114 samples collected from women in the probiotic group and 1 of 121 samples from the placebo group (RR 4.2, 95% CI 0.5–36.8, Fisher's exact $P = 0.21$). The La-5 was detected in 2 samples from the probiotic group only at 10 d postpartum (RR not applicable, $P = 0.49$). Neither LGG nor La-5 were found in breast milk samples collected 3 mo postpartum (n = 120 and 117 for the probiotic and placebo groups, respectively). The Bb-12 was present in breast milk samples from women in the probiotic group only, including 5 samples at 10 d postpartum (RR not applicable, $P = 0.03$) and 2 samples at 3 mo postpartum (RR not applicable, $P = 0.50$). Two women with Bb-12 in breast milk at 10 d also had one of the other administered bacteria in their milk sample, one with LGG and one with La-5. Additionally, the woman with both LGG and Bb12 at 10 d was also found to have Bb12 in her breast milk at 3 mo. Given that so few samples were positive for the administered probiotic bacteria, comparison of the relative abundance of these bacteria between the probiotic and placebo groups was not feasible. Similarly, it was not feasible to conduct any formal statistical assessment of the association between the presence of the administered bacteria in breast milk samples and previously analyzed mother and infant stool samples (Dotterud et al., 2015) or the development of atopic dermatitis in infants. A summary of these results for

mother-infant pairs with positive breast milk samples are presented in Supplemental Table S2 (https://doi.org/10.3168/jds.2017-13411).

Breastfeeding-Associated Microbiota at Different Stages of Lactation

Due to low quantity and quality of bacterial DNA in the breast milk samples, not all samples were sequenced and fewer samples had sufficient reads to be included in further analysis. Samples that were removed before sequencing had a median Ct value for total bacteria of 33.4 [interquartile range (**IQR**) 31.9–36.1, n = 128] and samples that were sequenced, but had insufficient reads, had a median Ct value of 31.7 (IQR 29.7–33.8, n = 199). Ultimately, sufficient reads were obtained for 142 samples from 125 women (median Ct value 30.2, IQR 27.9–32.0). These reads were attributed to a total of 307 OTU belonging to 147 genera. Considering the composition of the milk microbiota at the genus level, 45 and 69 genera were present (≥10 reads) in samples collected at 10 d and 3 mo, respectively. However, only 11 genera were present in at least 10% of samples at either time point and no genus was present in all samples (Table 3 and Supplemental Table S3 for OTU level summary; https://doi.org/10.3168/jds.2017-13411). Even at the phyla level, no phylum was present in all breast milk samples, although *Firmicutes* bacteria were present in all but one 3-mo sample (data not shown). The general breastfeeding-associated microbiota at 10 d and 3 mo postpartum was dominated by *Streptococcus* and *Staphylococcus* genera in most women (Supplemental Figure S1; https://doi.org/10.3168/jds.2017-13411). At both time points, a subgroup of women had a milk microbiota dominated by other genera, such as *Acinetobacter* and genera from the *Bacillaceae* family (Supplemental Figure S1). Additionally, some individual women had moderate to high relative abundances of uncommon genera, such as *Klebsiella*, *Lactobacillus zeae*, *Alkanindeges*, *Stenotrophononas*, and genera from the *Caulobacteraceae* family (data not shown, these genera fall into the category of "other" in Supplemental Figure S1).

When assessing the influence of the stage of lactation on specific genera, we found a higher presence and relative abundance of *Staphylococcus* in 10-d compared with 3-mo samples (Table 3 and Figure 2a). The samples from 10 d postpartum also had a lower prevalence and relative abundance of *Rothia* and *Veillonella* genera and a higher relative abundance of unassigned bacteria in 10-d samples. The *Granulicatella* and *Methylbacterium* had higher prevalence in 3-mo samples and *Granulicatella* had a higher relative abundance. The number of observed species, phylogenetic diversity, and Shannon's

Table 3. Presence and relative abundance of the 11 genera present in at least 10% of samples at either 10 d or 3 mo postpartum[1]

Taxonomic classification	10 d (n = 60)				3 mo (n = 82)				Prevalence: 10 d vs. 3 mo		RA: 10 d vs. 3 mo	
	Pos.	Median	IQR	Range	Pos.	Median	IQR	Range	*P*-value[2]	FDR[2]	*P*-value[3]	FDR[3]
Streptococcus	60	547	287–779	11–964	79	726	406–843	2–936	0.134	0.229	0.061	0.112
Staphylococcus	52	151	45–320	0–908	58	30	8–102	0–817	0.012	0.027	<0.001	<0.001
Gemellaceae family	36	18	3–48	0–555	52	15	2–44	0–179	0.679	0.679	0.598	0.598
Rothia	10	0	0–6	0–327	41	10	2–22	0–97	<0.001	<0.001	<0.001	<0.001
Veillonella	10	1	0–6	0–58	33	6	1–19	0–73	0.003	0.009	<0.001	<0.001
Unassigned	23	6	2–23	0–188	22	3	0–10	0–75	0.146	0.229	0.006	0.015
Acinetobacter	11	0	0–5	0–898	21	1	0–11	0–966	0.305	0.343	0.074	0.116
Hemophilus	6	0	0–1	0–211	13	1	0–5	0–244	0.311	0.343	0.112	0.137
Bacillaceae family	6	0	0–2	0–414	14	1	0–5	0–651	0.231	0.318	0.102	0.137
Granulicatella	1	0	0–0	0–82	21	1	0–10	0–75	<0.001	<0.001	<0.001	<0.001
Methylbacterium	1	0	0–2	0–13	12	1	0–4	0–195	0.008	0.022	0.162	0.178

[1]Pos. = number of samples with at least 10 reads for given genera; IQR = interquartile range; RA = relative abundance; FDR = false discovery rate.

[2]*P*-value and FDR for χ^2 test.

[3]*P*-value and FDR for Wilcoxon rank sum test.

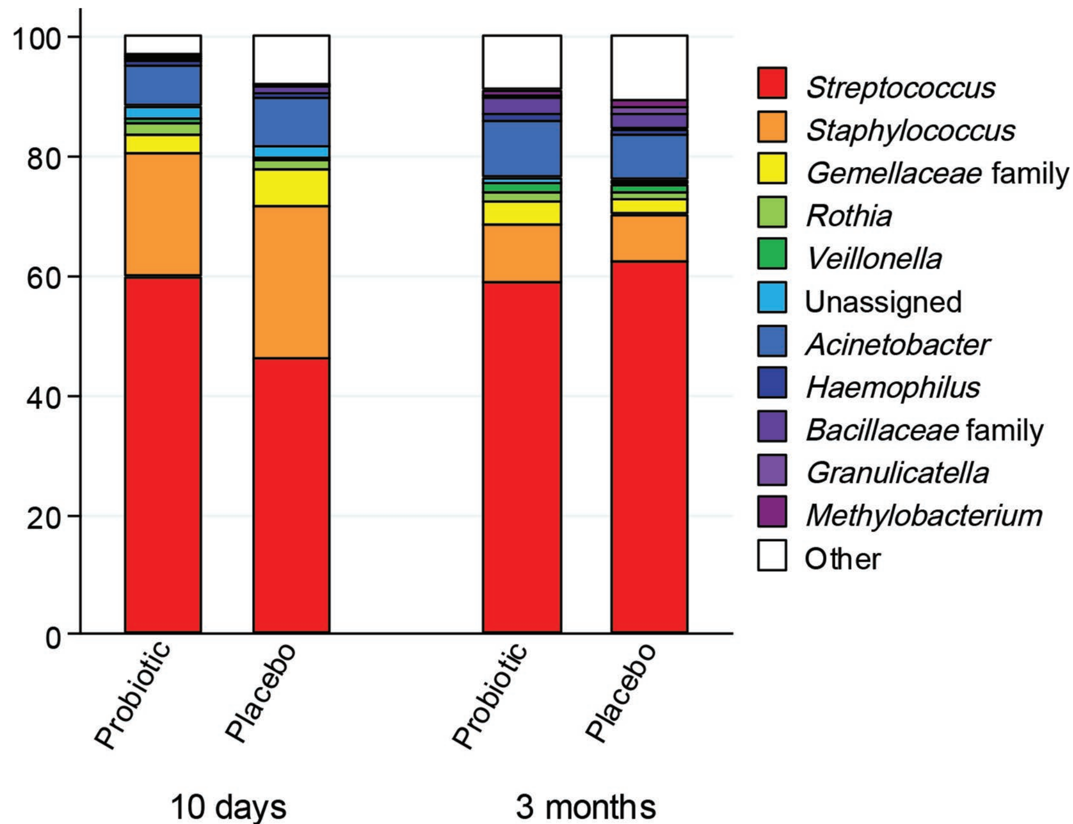

Figure 2. Overview of proportion of reads attributable to the 11 genera present in at least 10% of samples at 10 d or 3 mo in the probiotic and placebo groups.

diversity index of the breastfeeding-associated microbiota increased from 10 d to 3 mo postpartum (Shannon's index median (IQR): 1.70 (1.08–2.42) vs 2.15 (1.63–2.47), $P = 0.016$, Supplemental Figure S2; https://doi.org/10.3168/jds.2017-13411). The PCoA plots based on weighted and unweighted UniFrac distance matrices and Bray Curtis dissimilarity matrix did not indicate a clear separation in the breastfeeding-associated microbiota by stage of lactation (Supplemental Figure S3; https://doi.org/10.3168/jds.2017-13411), although 10-d samples were statistically more likely to be associated divergent microbiotas in the unweighted UniFrac and Bray Curtis analyses (data not shown).

An adequate number of reads was achieved in both 10-d and 3-mo samples from 17 women (Supplemental Figure S4; https://doi.org/10.3168/jds.2017-13411). Individually, these women had between 5 and 17 OTU (median 10, IQR 7–11) represented at either time point, with between 0 and 5 OTU present in both samples (median 3, IQR 2–4). These persistent OTU accounted for a large proportion of total reads in most women: median of 80.0% (IQR 36.7–93.2%, range 0.0–99.4%) of the total reads at 10 d and 72.5% (31.8–85.0, range 0.0–92.8) of the reads at 3 mo. However, 6 of 17 women had substantial changes in the OTU-level composition of their breast milk microbiota, with the persistent OTU accounting for less than 40% of reads at either or both time points. One of these women had no persistent OTU, suggesting a total replacement of the breastfeeding-associated microbial community between 10 d and 3 mo (M10 in Figure S4; https://doi.org/10.3168/jds.2017-13411).

At each time point, probiotics had no statistically significant effect on the presence or relative abundance of any of the individual genera that were present in at least 10% of the samples (Supplemental Table S4, https://doi.org/10.3168/jds.2017-13411, and Figure 2). Furthermore, probiotic supplementation was not found to have a statistically significant effect on the α or β diversity of the breast milk microbiota at either time point (Supplemental Figures S2 and S3; https://doi.org/10.3168/jds.2017-13411). In the subgroup of 17 women with 16S rRNA gene sequencing data, probiotic supplementation may have a positive influence on the stability of the breastfeeding-associated microbiota (RR of stable breastfeeding-associated microbiota after probiotic supplementation: 2.37, 95% CI 0.94–5.97, Fisher's exact $P = 0.050$).

Breastfeeding-Associated Microbiota and Atopic Dermatitis in Offspring

Finally, we considered the association between the general breastfeeding-associated microbiota and the development of atopic dermatitis. The presence of the *Staphylococcus* and *Veillonella* genera at 3 mo postpartum tended to be associated with a borderline greater risk of developing atopic dermatitis, although the FDR for these comparisons was unacceptably high (RR 1.93, 95% CI 0.92–4.06, $P = 0.052$ for *Staphylococcus*; RR 1.67, 95% CI 1.00–2.78, $P = 0.048$ for *Veillonella*; and FDR = 0.285 for both). Similarly, a higher relative abundance of *Staphylococcus* at 3 mo appeared to increase the risk of atopic dermatitis before considering the FDR [median number of reads per 1,000 (IQR): 40 (18–128) vs. 14 (5–63) for children with and without atopic dermatitis, respectively, $P = 0.045$, FDR: 0.50]. No other associations were found between the development of atopic dermatitis and the presence or relative abundance of the common genera in 10-d or 3-mo breast milk samples (data not shown). The relationship between the breastfeeding-associated microbiota and the development of atopic dermatitis is potentially confounded by other maternal-infant characteristics, such as the presence of older siblings, mode of delivery, maternal atopy, and the sex of the child. The *Gemellaceae* family was more common in 10-d samples from mothers with a personal history of allergy (23/31 vs. 13/29, $P = 0.020$, FDR = 0.081), whereas the *Rothia* genera was more commonly observed in 10-d samples from mothers with no allergic history (9/29 vs. 1/31, $P = 0.004$, FDR = 0.031). Samples collected at 3 mo from mothers of female infants also had a higher presence and relative abundance of the *Rothia* genera compared with mothers of male infants (present in 30/48 vs. 11/34 for female and male infants, respectively, $P = 0.007$, FDR = 0.079, and median (IQR): 14 (5–27) vs. 4 (0–12), respectively, $P = 0.005$, FDR = 0.055). Also at 3 mo postpartum, *Streptococcus* was more commonly present in breast milk samples after vaginal delivery, although the FDR was unacceptably high (65/66 vs. 23/14 in vaginal vs. cesarean delivery, $P = 0.022$, FDR = 0.246). No other statistically significant associations between mother-infant characteristics and the presence or relative abundance of individual bacterial genera were observed at either time point (data not shown). The presence and relative abundance of the *Staphylococcus* and *Veillonella* genera, which were highlighted for their potential association with the development of atopic dermatitis, do not appear to be related to any of these maternal-infant characteristics. Further, the strength of association between the presence of individual bacterial genera and the development of atopic dermatitis was not significantly altered by the addition of these maternal-infant characteristics as covariates in a multivariable logistic regression models (data not shown). Neither the development of atopic dermatitis nor these mother-infant characteristics were associated with a clear separation of samples when reviewing the PCoA plots, suggesting that the β-diversity of these samples was not defined by these characteristics (Supplemental Figure S3; https://doi.org/10.3168/jds.2017-13411).

DISCUSSION

Our findings indicate that pre- and postnatal maternal probiotic supplementation with LGG, La-5, and Bb-12 leads to the presence of these bacteria in breast milk samples for only a small subgroup of women. Thus, breast milk was not a meaningful source of LGG, La-5, or Bb-12 for the infants in the ProPACT trial, and the observed preventative effect of the ProPACT regimen on atopic dermatitis cannot be attributed to the transfer of these bacteria through breastfeeding. Using 16S rRNA gene sequencing, we also demonstrate that the general breastfeeding-associated microbiota of human milk is largely dominated by *Streptococcus* and *Staphylococcus* genera at both 10 d and 3 mo postpartum and that maternal probiotic supplementation does not have a substantial effect on this microbiota or its diversity.

One of the major strengths of this study is the use of samples collected in a randomized, placebo controlled trial with clinical follow-up. This allows us to conduct an unbiased assessment of the effect of probiotic supplementation on the presence of administered bacteria and the breastfeeding-associated microbiota. Furthermore, the qPCR analysis has a high sensitivity and specificity for the administered bacteria. Therefore, with only 9 of 252 women found to have any administered bacteria in their breast milk at either time point, breast milk is unlikely to be a substantial source of LGG, La-5, or Bb-12 for infants after maternal supplementation. Although we found a statistically significant increase in the presence of Bb-12 at 10 d postpartum in the probiotic group, over 95% of the samples had no detectable Bb-12 and we do not consider this statistical significance to imply a clinically meaningful transfer of Bb-12 during breastfeeding. In a previous study, we found that as many as 50 out of 129 (39%) infants in the probiotic group had LGG present in their stool samples at 10 d of age and 56 of 122 (46%) at 3 mo of age. This represented a statistically significant increase in the presence of LGG, which was found in only 7 of 133 (5%) and 23 of 121 (19%) stool samples from infants in the placebo group at 10 d and 3 mo, respectively. In contrast, the current study identified LGG in only 3 breast milk samples collected at 10 d postpartum

and none at 3 mo, suggesting that breastfeeding cannot have been the source of LGG for the majority of infants who had this bacteria present in their stool samples.

In previous randomized studies of maternal supplementation, *Lactobacillus* species have been recovered from breast milk in 12 to 53% of women after supplementation (Abrahamsson et al., 2009; Arroyo et al., 2010; Nasiraii et al., 2011; Hurtado et al., 2017). In a small trial, 4 of 10 women had viable cultures of *L. rhamnosus* LC705 isolated in breast milk samples after supplementation with that probiotic (Nasiraii et al., 2011). Also using culture-based techniques, Abrahamsson et al. (2009) demonstrated that 12% of women in their probiotic group had viable *L. reuteri* in colostrum samples, compared with 2% in the placebo group. Similarly, in a 3-arm trial, Arroyo et al. (2010) investigated *L. fermentum* CECT5716, *L. salivarius* CECT5713 or antibiotics in the treatment of mastitis and found that just over 50% of women in both probiotic arms, and none in the antibiotic arm, had lactobacilli cultured in breast milk samples after 21 d of supplementation. Results from subsequent trials suggest that maternal supplementation with *L. fermentum* CECT5716 may increase levels of that bacteria (Hurtado et al., 2017), but not the overall abundance of lactobacilli (Maldonado-Lobon et al., 2015; Hurtado et al., 2017). It is worth noting that both *L. fermentum* CECT5716, and *L. salivarius* CECT5713 were initially isolated from human milk samples, suggesting that these bacteria have a natural affinity for the breast milk microbiota. The low proportion of milk samples with either LGG, La-5, or Bb-12 in the current study may reflect strain-specific differences in their ability to be transferred to the milk microbiota. We also found that maternal probiotic supplementation did not significantly affect the general breastfeeding-associated microbiota, which was investigated using 16S rRNA gene sequencing. Furthermore, the development of atopic dermatitis was not convincingly associated with individual genera or the overall composition of this microbiota.

Over the past 5 yr, several studies have employed sequencing methods to characterize the breast milk or breastfeeding-associated microbiota and its association with mother-infant characteristics such as stage of lactation (Hunt et al., 2011; Cabrera-Rubio et al., 2012; Jost et al., 2013; Sakwinska et al., 2016; Urbaniak et al., 2016; Murphy et al., 2017), prematurity (Urbaniak et al., 2016), mode of delivery and elective versus nonelective caesarean section deliveries (Cabrera-Rubio et al., 2012, 2016; Kumar et al., 2016; Sakwinska et al., 2016; Urbaniak et al., 2016), infant sex (Urbaniak et al., 2016), maternal weight (Cabrera-Rubio et al., 2012; Collado et al., 2012), geography (Kumar et al., 2016; Li et al., 2017), and mastitis (Jiménez et al., 2015). Each of these mother-infant characteristics has been described to influence the microbiota composition, particularly in the smaller studies (n = 7–20). Three of the larger studies (n = 133, n = 90, and n = 39) report no clear distinction in the breast milk microbiota based on stage of lactation, prematurity, or mode of delivery (Sakwinska et al., 2016; Urbaniak et al., 2016; Li et al., 2017). Our results suggest that stage of lactation, sex, maternal atopy, and mode of delivery may influence the presence or relative abundance of specific genera. However, neither these characteristics, nor the presence of siblings, defined the overall composition of the breastfeeding-associated microbiota. Due to lack of information, we were unable to assess the effect of maternal antibiotic use and signs or symptoms of mastitis. Information regarding antibiotic use and the presence of mastitis would have been particularly useful with respect to the observation that probiotic supplementation may promote stability of the breastfeeding-associated microbiota.

Another recurrent theme among some of the previous smaller studies is the presentation of a "core" breast milk microbiota of between 6 and 12 OTU or genera, which are found in 90 to 100% of samples (Hunt et al., 2011; Jiménez et al., 2015; Murphy et al., 2017). In the present study we did not observe a "core" breastfeeding-associated microbiota, despite participants coming from a small geographic area. This is likely due to the high degree of individual variability and relatively large number of women included in our study. Indeed, the lack of a "core" microbiota is consistent with the conclusion of a recent systematic review, which identified *Staphylococcus* and *Streptococcus* as the only genera to be repeatedly reported as dominant breast milk-related genera (Fitzstevens et al., 2017). Methodological differences in sample collection and laboratory analysis are likely to influence the observed microbiota in our study and other published studies.

Our 16S rRNA gene sequencing data probably represent a combination of the breast milk and breast surface microbiotas because women were not provided with instructions for sterile collection. This has presumably resulted in contamination from the skin microbiota and possibly the infant oral microbiota, and may account for the higher relative abundance of *Staphylococcus* and *Streptococcus* genera in our results compared with previous sequencing studies that have used sterile collection protocols. Indeed, Sakwinska et al. (2016) demonstrate that the method of collection significantly influences the microbiota composition, although it was the higher presence and relative abundance of *Acinetobacter* species that appeared to characterize the nonsterile samples in their study. Due to the nonsterile sample collection method, we consider our results more rep-

resentative of the microbiota transferred while breastfeeding, rather than what infants receive from breast milk alone. We note here that we presume the relative abundance, but not the presence, of the administered bacteria would have been affected by the nonsterile collection. We therefore believe that the collection method is unlikely to have masked a significant transfer of the administered bacteria via breast milk and we consider the qPCR results to be a clear indication that breastfeeding was not a substantial route of probiotic transfer in the ProPACT study. These findings would also suggest that, even if probiotic supplementation altered the breast milk microbiota, the overall composition of the microbiota transferred during breastfeeding was not statistically significantly altered. The results of the 16S rRNA gene sequencing are also limited because only 30% of samples had enough reads to be included in further analysis and only 17 individuals had adequate reads at both time points. The baseline characteristics of the women with successful sequencing was representative of the original ProPACT population, and with 142 successfully sequenced samples, this currently represents the largest sequencing study of the breastfeeding-associated microbiota. Nonetheless, due to the high degree of individual variation, large studies are required to further investigate this microbiota and its association with mother-infant characteristics and infant health outcomes.

This study was motivated by the findings of the ProPACT trial, namely, that maternal probiotic supplementation reduced the cumulative incidence of atopic dermatitis in offspring at 2 yr (Dotterud et al., 2010) and led to a higher prevalence and relative abundance of LGG in infant stool samples up to 3 mo of age (Dotterud et al., 2015). In adults, continuous ingestion of LGG is required to maintain measurable quantities in stool samples (Alander et al., 1999). Given that ongoing transfer does not appear to come from breastfeeding, the observation of LGG in infant stool samples at 3 mo of age suggests that either early transfer is sufficient to establish a stable colonization with LGG in the relatively microbe-free newborn gut, or that children are receiving continued transfer of LGG via some other route from their mother. On a technical note, due to the low quantities of bacterial DNA in breast milk samples, future studies of the breast milk or breastfeeding-associated microbiota should consider starting with a greater volume of milk and establishing methods to maximize DNA extraction from milk.

In conclusion, we find that maternal supplementation with LGG, La5, and Bb-12 does not result in substantial transfer of these bacteria via breastfeeding. The breastfeeding-associated microbiota is largely dominated by *Streptococcus* and *Staphylococcus* genera at both 10 d and 3 mo postpartum.

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