Impact of *Streptococcus salivarius* K12 on Nasopharyngeal and Saliva Microbiome: A Randomized Controlled Trial

Suvi Sarlin, MD,*† Mysore V. Tejesvi, PhD,‡ Jenni Turunen, MSc,‡ Petri Vänni, MSc,‡ Tytti Pokka, MSc,*† Marjo Renko, MD, PhD,†‡§ and Terhi Tapiainen, MD, PhD*†‡§

**Background:** Probiotic lactobacilli have been ineffective in preventing acute otitis media. In contrast to lactobacilli, alpha-hemolytic streptococci belong to the core microbiome of nasopharynx.

**Methods:** We investigated the effects of *Streptococcus salivarius* K12 probiotic on the saliva and nasopharyngeal microbiome in 121 children attending daycare. Children were randomly allocated to receive oral K12 product for 1 month or no treatment. We obtained saliva and nasopharyngeal samples at study entry, at 1 and 2 months. The next-generation sequencing of the bacterial 16S gene was performed.

**Results:** After the intervention, the diversity of saliva or nasopharyngeal microbiome did not differ between groups. The proportion of children with any otopathogen did not differ between the groups. At 1 month, the abundance of otopathogens in nasopharynx was lower in K12 group compared with that in control children (34% vs. 55%, *P* = 0.037). When we compared each otopathogen separately, *Moraxella* was the only group lower in the treatment group. We could not verify the reduction of *Moraxella* when an alternative Human Oral Microbiome Database taxonomy database was used. In children receiving K12 product, the mean abundance of *S. salivarius* was greater in saliva after the intervention (0.9% vs. 2.0%, *P* = 0.009).

**Conclusions:** The use of *S. salivarius* K12 probiotic appeared to be safe because it did not disrupt the normal microbiome in young children. Even though a short-term colonization of *S. salivarius* was observed in the saliva, the impact of *S. salivarius* K12 probiotic on the otopathogens in nasopharyngeal microbiome remained uncertain.

**Key Words:** acute otitis media, probiotics, nasopharyngeal microbiome, saliva, next generation sequencing

(Pediatr Infect Dis J 2021;40:394–402)
the microbiologic effects of oral *S. salivarius* K12 probiotic, an over-the-counter probiotic product, on nasopharyngeal and saliva microbiome in a randomized controlled trial. In addition, we compared the relative abundance of otopathogens in nasopharyngeal microbiome and *S. salivarius* in nasopharyngeal and saliva microbiome during the study.

**METHODS**

**Study Design and Study Population**

The study design of this research was a randomized controlled clinical trial that was open to the families but blinded for the microbiologic analyses. The children were randomly allocated to a treatment or control group (2:1); those in the treatment group received oral *S. salivarius* K12 product, while those in the control group did not receive treatment but did undergo an otherwise identical clinical and microbiologic follow-up. Children ≤3 years old were randomly allocated to receive either *S. salivarius* K12 soluble powder or no treatment and older children were randomly allocated to receive *S. salivarius* K12 chewable tablets or no treatment. We first sent information letters about the clinical trial to all the parents of children 1–6 years of age who attended 15 daycare centers in the city of Oulu, Finland. In addition, study physicians attended parents’ meetings held in the evening at the daycare centers and gave presentations about the clinical trial before the study.

We offered participation to all children attending the daycare centers, as attending daycare is a known risk factor for AOM in children. Only children whose parents gave their written informed consent were enrolled. A clinical ear examination with otoscope was performed upon study entry to reveal currently present otorhoea or middle ear effusion. We excluded children with present chronic secretory otitis media or other middle ear effusion at study entry, continuous antibiotic prophylaxis or an immune system disorder. Children with a history of recurrent otitis media or otorhoea and children with present or earlier tympanostomy tubes were included. During the study period, altogether 92% of the children in the city of Oulu had received at least 1 dose of PCV10 vaccine. In total, 27% of children ≤3 years and 14% of 3–6 years old children received the influenza vaccination in 2017–2018 influenza season.

Children were recruited between October 5, 2017, and November 25, 2017. The study physician collected background data about the patients, including previous antibiotic history from electronic medical records and the national electronic prescription registry.

The study plan was found acceptable by the Ethical Committee of Oulu University Hospital, Oulu, Finland, and by the Finnish Medicines Agency. The study was registered at clinicaltrialsregister.eu, with EudraCT registration code 2017-000820-83, before the study. The study design and reporting were performed according to CONSORT guidelines.

**Randomization and Sample Size**

The randomization lists and sheets were created by a bio-statistician with computerized block randomization using permuted blocks of 6 according to age. One to three years old children were not randomized to receive chewing tablet products. Parents were not involved in the randomization process. The individual randomization sheets were then inserted into opaque envelopes with ascending numbers on the cover. The study physician opened each sealed randomization envelope after receiving the written informed consent. The interindividual variation in the nasopharyngeal microbiome was still largely unknown during the planning phase of the study. However, a sample size of 40 children had yielded clinically different outcomes in gut microbiome studies. Thus, we decided to recruit at least 40 children per group.

**Study Products and Feasibility Questionnaire**

Study products containing *S. salivarius* K12 were used daily for 30 days after the study entry. A *S. salivarius* chewable tablet is available in Finland as a commercial over-the-counter probiotic product (ToothGuide, GutGuide Ltd., Halikko, Finland). A daily dose was 1 chewable tablet containing 1 × 10^9^ colony-forming units (CFUs) of *S. salivarius* K12, a quantity that has previously been successful in the nasopharynx of adults. A daily dose of oral soluble powder, produced by Bluestone Pharma GmbH, Switzerland, for GutGuide Ltd., contained *S. salivarius* K12 5 × 10^7^ CFU per sachet.

Parents were instructed to administer the daily dose after the children had brushed their teeth every evening before going to sleep. The products were given to the parents with written instructions at recruitment. Children in the control group did not receive any products but received the same information about the follow-up. The consumption of other probiotic products during this trial was discouraged verbally. After the study, the parents were sent a questionnaire about the feasibility of the products. We estimated the ease of use with a corresponding Likert score (1 = strongly disagree, 2 = disagree, 3 = no opinion, 4 = agree, 5 = strongly agree).

**Study Visits and Microbiologic Samples**

The study physician obtained nasopharyngeal samples and saliva samples at study entry, at 1 and 2 months after the study entry. A nasopharyngeal bacterial swab sample was obtained using a sterile flocked swab (Copan, Brescia, Italy) and transferred immediately into a sterile tube containing 1 mL liquid Ames solution (eSwab, Copan, Brescia, Italy). Saliva samples were collected using saliva collection sponge swabs (Visitec, Beaver-Visitec). All samples were cooled and delivered on wet ice for processing and storage. The saliva tubes were centrifuged for 17 minutes at 1500 rpm. The saliva collection sponge swabs were then discarded, and the saliva was stored as such. All samples were stored at −20°C until DNA extraction and next-generation sequencing.

**Microbiome Analysis of Nasopharyngeal and Saliva Samples**

DNA extraction for both nasopharyngeal and saliva samples was performed using the QIAamp PowerFecal DNA Kit, according to the protocol provided by the manufacturer with a few modifications. Before DNA extraction, the nasopharyngeal samples were centrifuged at 10,000 rpm for 10 minutes, and the supernatant was discarded to increase the yield of DNA. After this, extraction started from the beginning. For both sample types, the incubation temperature in step 4 of the manufacturer’s instructions was increased to 95°C. The C2 and C3 solutions, as well as their respective incubations and centrifuging, were combined. The final product was eluted to 50 μL to further increase the DNA yield. The concentrations and purities were measured using a Nanodrop spectrometer.

The bacterial hypervariable regions V4–V5 of the 16S rRNA gene were amplified using primers F519 and 926tfPr1. The F519 primer contained Ion Torrent pyrosequencing adapter sequence A (Lifescience Technologies), a 9-bp unique barcode sequence, and 1 nucleotide linker. The 926tfPr1 primer contained an Ion Torrent adapter trPr1 sequence. Polymerase chain reaction (PCR) was performed on a Veriti 96-Well Thermal Cycler by Applied Biosystems in triplicates, each containing 1 × Phusion GC buffer, 0.4 μM of forward and reverse primers, 200 μM of deoxyribonucleotide triphosphates (dNTPs), 0.5 μM of Phusion enzyme (Thermo Scientific, Vantaa, Finland), and approximately 30–50 ng of genomic community DNA as the template and molecular grade water, for a total reaction
volume of 50 μL. Triplicate reactions were made for all samples, and after PCR, the triplicates were combined into 1 sample. The following cycling conditions were used for bacteria: 35 cycles of 98°C, 10 seconds; 65°C, 15 seconds; and 72°C, 30 seconds after an initial denaturation of 98°C, 3 minutes. The PCR products were purified with the AMPure XP PCR Clean-up Kit by Agencourt Bioscience. The DNA concentration was measured with a bioanalyzer DNA chip by Agilent Technologies.

For the forward primer, a common 926trP1 primer was used (Table, Supplemental Digital Content 1, http://links.lww.com/INF/E255). For reverse primers, 192 unique, barcoded primers were prepared. PCR was performed on the Veriti 96-Well Thermal Cycler by Applied Biosystems. Triplicate reactions were made of each sample, and after PCR, the triplicates were combined into 1 sample. The gel for the agarose gel electrophoresis was made of 1 × TE buffer and 1.5% agarose. Ethidium bromide was used as a fluorescent tag. A total of 15 μL of the DNA sample was mixed with 5 μL of loading dye. The first well was filled with 10 μL using a MassRuler or GeneRuler 100 bp DNA ladder, and the rest were filled with 20 μL of the DNA sample and loading dye mix. The agarose gel electrophoresis was left running for approximately 75 minutes, with 90 V and 400 mA. The gels were imaged using a VersaDoc instrument. The PCR products were purified using the AMPure XP PCR Clean-up Kit by Agencourt Bioscience. DNA concentration was measured with a bioanalyzer DNA chip by Agilent Technologies.

The samples were pooled in equimolar amounts, and the pooled sample was purified again using the AMPure XP Kit. The DNA concentration for the final product was measured using the Quant-iT PicoGreen dsDNA Assay Kit by Thermo Fisher Scientific. For sequencing, the Ion PGM Hi-Q View OT2 Kit for 400 bp protocol, Ion PGM Hi-Q View Sequencing Kit, and Ion 316 v2 chips were used. The sequencing was performed with an Ion Torrent PGM instrument.

Raw reads from the sequencing runs were obtained from the Ion Torrent PGM instrument in multiplexed FASTQ-formatted text files. Sequences shorter than 200 base pairs were removed with Cutadapt and imported into QIIME 2 (qiime2-2018.8). In QIIME 2, the reads were demultiplexed using barcode metadata, and primers were trimmed using the QIIME 2 Cutadapt plugin. Sequences were denoised using the QIIME 2 DADA2 plugin, with the trimming length from the left set at 30 and truncated to a total length of 360. After DADA2, data from each run were combined into a single feature table and representative sequence files. Features that were found only in 1 sample and those that had less than 100 reads in all samples were removed using the QIIME 2 feature table plugin. We performed all analyses in duplicate because we used 2 different reference databases for taxonomy classification of sequencing results: SILVA and Human Oral Microbiome Database (HOMD). Taxonomy was assigned using the QIIME 2 feature-classifier plugin, with a Naïve Bayes classifier trained on the SILVA and HOMD, trimmed to the 16S primers used in PCR. Every feature not classified into the kingdom “bacteria” was removed from both the feature table and representative sequence files. Chimeric reads were removed from sequences with the QIIME 2 vsearch plugin. The feature table and representative sequence files. Chimeric reads were removed from sequences with the QIIME 2 vsearch plugin. All microbiome data were uploaded to NCBI BioProject database with accession number PRJNA603433.

Outcomes and Statistical Analysis

We first compared the change in the nasopharyngeal and saliva microbiome composition, including microbiome diversity features and the relative abundance of phyla and amplicon sequence variants, in children receiving S. salivarius K12 treatment and control children. Second, we compared the proportion of children with any otopathogen in the nasopharyngeal microbiome, and the mean relative abundance of otopathogens (S. pneumoniae, H. influenzae, Streptococcus pyogenes and Moraxella), calculated for each sample separately, in both the groups. In addition, in children receiving S. salivarius K12 probiotic, we compared the relative abundance of S. salivarius, not specific for K12 strain, in saliva and nasopharyngeal microbiomes before and after the 1-month intervention. Of note, the relative abundance variables used indicated the proportion of sequences compatible with the taxonomic sequences in the microbiome analysis and were not a measure of the absolute bacterial counts in the samples.

When analyzing the relative abundances, there were differences in the results obtained by using SILVA and HOMD reference databases used for taxonomic classification. For primary outcome analysis, diversity measures and abundance of main phyla and genera, both databases were used separately to investigate the reproducibility of the analysis. For otopathogen analysis, SILVA database was used because HOMD database did not recognize S. pneumoniae. HOMD database was used for S. salivarius analysis because SILVA database did not recognize S. salivarius. Neither database recognized Moraxella catarrhalis directly. For the outcome variable labeled as “Moraxella,” we produced an estimate for M. catarrhalis by extracting other specifically identified Moraxella species from all Moraxella features (Moraxella spp.) using both databases.

Statistical analyses were performed using IBM SPSS Statistics for Windows (Version 26.0, IBM Corp., Armonk, NY). Independent samples t-tests, with the 95% confidence interval (CI) of the differences, were used to determine differences in the relative abundances of otopathogens and of microbial features between the S. salivarius K12 and control groups.

RESULTS

Study Population

We enrolled 121 children in this study. Altogether, 81 children were randomly allocated to receive S. salivarius K12 product: 40 children in the S. salivarius K12 powder group and 41 in the S. salivarius K12 tablet group (Fig. 1). Altogether, 40 children were randomly allocated to the control group (Table 1). During the study, a total of 293 nasopharyngeal swabs and 296 saliva samples were collected. None of the children had chronic perforation or chronic otitis media. Two children had tympanostomy tubes in place at recruitment visit.

Microbiome Composition in Nasopharyngeal and Saliva Microbiomes

Microbiome composition of nasopharyngeal samples differed clearly from that of saliva samples in principal coordinate analysis (Figure, Supplemental Digital Content 2, http://links.lww.com/INF/E256). When comparing the microbiome composition between the treatment groups, there were no statistically significant differences in the diversity, or relative abundances of phyla between the S. salivarius K12 product group and the control group during the study in the nasopharyngeal or saliva microbiome [Table 2 according to SILVA, Supplemental Digital Content 3, http://links.lww.com/INF/E257 (Table) according to HOMD with amplicon sequence variants]. When comparing the nasopharyngeal
microbiomes of treatment groups with principal coordinate analysis after intervention, there was no clear clustering according to the treatment group (Figure, Supplemental Digital Content 4, http://links.lww.com/INF/E258). No statistical significance was found between the treatment groups using beta diversity distances (naso-pharynx $P = 0.23$ and saliva $P = 0.45$).

**Streptococcus salivarius** and Otopathogens

In children receiving *S. salivarius* K12 product, the mean relative abundance of *S. salivarius* increased during the 1-month study intervention from 0.9% (SD 1.6%) to 2.0% (SD 2.7%) in saliva (difference: 1.4%, 95% CI: 0.3%–2.0%, $P = 0.009$) (Fig. 2). After the intervention was stopped, the mean relative abundance of *S. salivarius* in saliva microbiome decreased to 0.8% (SD 1.3%) at 2 months (95% CI of the difference 0.4%–2.1%, $P = 0.004$). In the nasopharyngeal microbiome, the mean relative abundance of *S. salivarius* did not increase statistically significantly during the intervention (1.9% (SD 5.0%) at 0 month vs. 3.4% (SD 5.3%) at 1 month, 95% CI of the difference −3.6% to 0.6%, $P = 0.162$) (Fig. 2).

The proportion of children with any otopathogen in the nasopharyngeal microbiome did not differ between the groups after the intervention at one month (Table 3).

At study entry, otopathogens (*S. pneumoniae*, *H. influenzae*, *S. pyogenes* and *Moraxella*) consisted of 36% of the total nasopharyngeal microbiome in the *S. salivarius* K12 group and 40% in the control group, with no statistically significant difference between the groups (difference: 4%; 95% CI: −12% to 20%; $P = 0.62$) (Figure, Supplemental Digital Content 5, http://links.
TABLE 1. Baseline Characteristics in Control Children and in Children Receiving *S. salivarius* K12 Product

| Characteristic | S. salivarius K12 Group | Control Group |
|---------------|-------------------------|---------------|
| Total, N      | 81                      | 40            |
| Boys, %       | 41 (51)                 | 22 (55)       |
| Girls, %      | 40 (49)                 | 18 (45)       |
| Age, yrs (mean, SD) | 4.2 (1.5)         | 3.5 (1.5)     |
| No. AOM (mean, SD) | 3.0 (2.7)               | 3.7 (4.4)     |
| Parental smoking (yes, %) | 13 (19)               | 4 (10)        |
| Pacifier Ever, % | 57 (80)               | 25 (66)       |
| Months (mean, SD) | 16 (12)               | 11 (11)       |
| Number of siblings (mean, SD) | 2.0 (2.7) | 1.2 (1.2) |
| Siblings’ AOM (mean, SD) | 4.2 (6.7)               | 4.0 (7.1)     |
| Total previous courses of systemic antibiotics (mean, SD) | 2.8 (2.9) | 4.1 (5.6) |
| Duration of daycare, mo (mean, SD) | 22 (14)               | 18 (15)       |
| Breast-feeding Ever (%) | 68 (96)               | 38 (100)      |
| Duration of, months mean (SD) | 10 (6.9)               | 9.6 (5.1)     |
| Tympanostomy Ever (yes, %) | 12 (15)               | 6 (15)        |
| At recruitment (mean, SD) | 1.1 (2.2)               | 2 (5.0)       |

*The mean age of children receiving oral powder was 3.6 years (SD: 1.6), and the mean age of those receiving chewable tablets was 4.8 years (SD: 1.2).*

In total, 88% of the children in the oral powder group and 81% in the tablet group consumed the product daily. Altogether, 82% of the children in the oral powder group and 84% in the tablet group liked the products, with Likert score ≥4 upon asking the child if they found the study products good overall. In total, 94% of the children in the oral powder group and 77% in the tablet group were satisfied with the product’s taste. In total, 61% of the parents in the oral powder group and 87% in the tablet group found the product easy to use.

**DISCUSSION**

In this randomized trial, the biodiversity of the nasopharyngeal or saliva microbiomes did not change in children receiving *S. salivarius* K12 probiotic as compared with those receiving no treatment. Thus, the probiotics did not alter the overall composition of the microbiome or eradicate the normal nasopharyngeal or saliva microbiome in children. This is a relevant finding, as the physiologic functions of a normal microbiome are only partly understood.34 It is known that the increase in otopathogen abundance and loss of beneficial microbes precede recurrent upper respiratory tract infections and AOM.39,40 Thus, any profound changes in the microbiome are not desired because we currently lack thorough understanding of host–microbiome interactions and its impacts in healthy individuals. In this respect, the present study shows that *S. salivarius* K12 probiotic is likely safe to use in young children because it does not disrupt the normal microbiome.

In the present study, in children treated with oral *S. salivarius* K12 product, the relative abundance of *S. salivarius* in saliva microbiome was higher after than before intervention. Even though the relative abundance of bacteria, used in the study, do not measure the absolute bacterial counts in the samples, our finding is line with earlier reports showing that the local colonization is achieved after the consumption of *S. salivarius* probiotics.32–34 The observed effect appeared to be short-term and vanished within 1 month in the present study.

After the 1-month intervention, in the present study, the relative abundance of otopathogens in the nasopharyngeal microbiome was lower in children receiving K12 product than in those receiving no treatment. The relative abundance, commonly used in microbiome studies, does not measure absolute bacterial counts. Furthermore, *M. catarrhalis* was not directly detected by neither taxonomy reference database used in the study. Thus, the observed effect may be explained by the change in the abundance of commensals with close taxonomic proximity with *M. catarrhalis*. Finally, the proportion of children with at least 1 otopathogen in nasopharyngeal microbiome did not change between the groups. Thus, the impact of oral *S. salivarius* K12 probiotic on the otopathogens in the nasopharyngeal microbiome of children remains uncertain.

The strength of the present study is the high-quality randomized study design with an interesting microbiologic primary outcome, microbiome composition formed using modern 16S rRNA next-generation sequencing. It is meaningful to characterize the overall microbiome effects before starting any clinical trials investigating probiotics in children. Earlier, the impact of most probiotic products on microbiome in children has seldom been reported even though probiotics might interfere with normal microbiome and result in unexpected results.39 Furthermore, a prospective study...
**TABLE 2.** The Biodiversity of Nasopharyngeal and Saliva Microbiomes During the Study and the Changes in the Mean Relative Abundances (%) in the Microbiome at the Phylum Level, According to SILVA Taxonomy Classification

| Outcome | S. salivarius K12 | Control | 95% CI of the Difference |
|---------|------------------|---------|-------------------------|
| **Nasopharyngeal microbiome**<sup>*</sup> | | | |
| **At study entry** | | | |
| Relative abundance of main phyla, mean % (SD) | | | |
| Actinobacteria | 8.3 (16) | 14 (23) | -4.1 to 16 |
| Bacteroidetes | 9.3 (15) | 3.6 (4.1) | -9.9 to 1.6 |
| Firmicutes | 56 (29) | 58 (27) | -11 to 15 |
| Fusobacteria | 0.3 (1.1) | 0.4 (0.8) | -0.5 to 0.4 |
| Proteobacteria | 25 (30) | 24 (22) | -14 to 12 |
| Diversity, mean (SD) | | | |
| Observed OTUs | 21 (9.2) | 25 (13) | -9.5 to 1.4 |
| Shannon index | 2.6 (1.2) | 2.4 (1.0) | -0.7 to 0.3 |
| Faith index | 2.6 (1.1) | 2.4 (0.8) | -0.7 to 0.2 |
| **At 1 mo (after intervention)** | | | |
| Relative abundance of main phyla, mean % (SD) | | | |
| Actinobacteria | 8.3 (16) | 10 (18) | -7.4 to 11 |
| Bacteroidetes | 8.2 (12) | 2 (3.3) | -9.9 to 1.7 |
| Firmicutes | 56 (28) | 51 (35) | -22 to 11 |
| Fusobacteria | 0.5 (1.2) | 0.2 (0.4) | -0.8 to 0.1 |
| Proteobacteria | 25 (30) | 35 (38) | -7.5 to 28 |
| Diversity, mean (SD) | | | |
| Observed OTUs | 23 (12) | 20 (10) | -9.7 to 3.1 |
| Shannon index | 2.7 (1.2) | 2.1 (1.2) | -1.2 to 0.1 |
| Faith index | 2.5 (1.1) | 2.2 (1.1) | -0.8 to 0.4 |
| **At 2 mo (1 mo after stopping intervention)** | | | |
| Relative abundance of main phyla, mean % (SD) | | | |
| Actinobacteria | 9.5 (14) | 3.0 (3.7) | -12 to -1.4 |
| Bacteroidetes | 6.4 (10) | 7.9 (15) | -5.9 to 9.0 |
| Firmicutes | 54 (29) | 44 (35) | -29 to 9.9 |
| Fusobacteria | 0.3 (0.6) | 0.7 (1.2) | -0.4 to 1.1 |
| Proteobacteria | 29 (30) | 44 (37) | -6.1 to 35 |
| Diversity, mean (SD) | | | |
| Observed OTUs | 25 (11) | 28 (12) | -3.8 to 10 |
| Shannon index | 2.6 (1.1) | 2.4 (1.3) | -0.9 to 0.6 |
| Faith index | 2.7 (1.0) | 2.7 (0.9) | -0.6 to 0.7 |
| **Saliva microbiome**<sup>†</sup> | | | |
| **At study entry** | | | |
| Relative abundance of main phyla, mean % (SD) | | | |
| Bacteroidetes | 28 (12) | 26 (13) | -6.9 to 3.3 |
| Firmicutes | 52 (15) | 54 (15) | -1.0 to 8.5 |
| Fusobacteria | 4.2 (3.7) | 3.8 (2.7) | -1.8 to 1.0 |
| Proteobacteria | 14 (9.9) | 14 (8.5) | -4.1 to 3.7 |
| Diversity, mean (SD) | | | |
| Observed OTUs | 44 (13) | 48 (15) | -1.2 to 9.9 |
| Shannon index | 4.0 (0.6) | 4.2 (0.6) | -11 to 36 |
| Faith index | 4.0 (0.8) | 4.2 (0.8) | -12 to 52 |
| **At 1 mo (after intervention)** | | | |
| Relative abundance of main phyla, mean % (SD) | | | |
| Bacteroidetes | 20 (12) | 24 (12) | -0.02 to 0.09 |
| Firmicutes | 61 (18) | 56 (16) | -11 to 4.7 |
| Fusobacteria | 2.8 (4.8) | 2.8 (2.5) | -1.8 to 1.8 |
| Proteobacteria | 13 (11) | 14 (8.8) | -4.1 to 5.2 |
| Diversity, mean (SD) | | | |
| Observed OTUs | 37 (14) | 39 (14) | -3.7 to 8.9 |
| Shannon index | 3.8 (0.6) | 3.9 (0.5) | -9.6 to 43 |
| Faith index | 3.6 (0.9) | 3.7 (0.9) | -12 to 52 |
| **At 2 mo (1 mo after stopping intervention)** | | | |
| Relative abundance of main phyla, mean % (SD) | | | |
| Bacteroidetes | 23 (12) | 27 (14) | -2.1 to 10 |
| Firmicutes | 57 (15) | 54 (15) | -11 to 3.8 |
| Fusobacteria | 2.8 (2.5) | 3.7 (3.2) | -0.4 to 2.2 |
| Proteobacteria | 18 (14) | 14 (7.3) | -7.7 to 4.1 |
| Diversity, mean (SD) | | | |
| Observed OTUs | 39 | 43 | -2.0 to 10 |
| Shannon index | 3.8 | 3.9 | -19 to 46 |
| Faith index | 3.7 | 3.8 | -19 to 51 |

*The relative abundances do not indicate the absolute bacterial counts. None of the differences was statistically significant with a Bonferroni correction significance level adjusted for the multiple comparisons.

*The relative abundance of Acidobacteria, Cyanobacteria, Epsilonproteobacteria and Tenericutes was <2%, with no statistically significant differences between the groups.

†The relative abundance of Acidobacteria, Actinobacteria, Cyanobacteria, Epsilonproteobacteria, Moraxella, Haemophilus influenzae, Porphyromonas and Tenericutes was <2%, with no statistically significant differences between the groups.

OTU indicates operational taxonomic unit.
design with both nasopharyngeal and saliva samples from young children before and after the intervention was a demanding effort in practice and was successfully conducted.

There are several limitations in the present study. The relative abundances of bacteria are commonly reported in microbiome research as in the present study. The relative abundances do not measure the absolute bacterial counts, however, and may produce variable results depending on the chosen reference database and laboratory conditions. Furthermore, *Moraxella* variable, used in the present study, may partly consist of commensal bacteria. Finally, the sample size was limited for subgroup comparisons of different otopathogens. The use of other probiotics was discouraged during this trial. Probiotics and probiotic containing foods, however, were frequently used in the study population, which modify the treatment effects. Furthermore, the study participants had received pneumococcal conjugate vaccine (PCV10) as a part of their childhood immunization program. Recently, Salgado et al. 47 concluded that PCV10 vaccination status does not significantly alter the nasopharyngeal microbiome. Yet, PCV has markedly reduced the incidence of invasive pneumococcal diseases 48,49 and the incidence of AOM and the implied cost and disease burden from pneumococcal diseases.50–52 Even though the clinical effectiveness of *S. salivarius* K12 product may be useful to study in AOM prevention in the future, the impact of such intervention might have been greater before the pneumococcal conjugate vaccine era.

For the specific interpretation of the next-generation sequencing results, it is noteworthy that PCR-based amplification bias is well documented in the mixed-template PCRs.53 PCR-based methods may increase the low-concentrated DNA from the samples and lead to differences in the relative abundances of species. 54,55 DNA from different species amplify at different rates depending on the number of PCR cycles and primer affinity to the template.60 To overcome this limitation in the present study, we used a maximum of 35 PCR cycles during amplification and used strict quality measures in DADA2 analysis to remove chimeric sequences.57 The 16S rRNA sequencing is the most widely used tool to identify bacterial

### TABLE 3. The Proportion of Children With Otopathogens in the Nasopharyngeal Microbiome (SILVA Database) at Recruitment, After Intervention at 1 Month, and 1 Month After Stopping Intervention, According to the Treatment Group

|                      | S. salivarius K12 (%) | Control (%) | 95% CI of Difference |
|----------------------|-----------------------|-------------|----------------------|
| Recruitment          | N = 81                | N = 40      |                      |
| Streptococcus pnuemo-niae | 50                    | 48          | −24% to 20%          |
| Streptococcus pyogenes | 17                    | 19          | −15% to 21%          |
| Moraxella*           | 90                    | 100         | −2.6% to 21%         |
| Haemophilus influenzae | 22                    | 19          | −21% to 17%          |
| Any otopathogen†     | 91                    | 100         | −4.3% to 19%         |
| At 1 mo (after intervention) |                     |             |                      |
| S. pneumoniae        | 37                    | 36          | −24% to 25%          |
| S. pyogenes          | 21                    | 14          | −26% to 15%          |
| Moraxella*           | 82                    | 86          | −17% to 24%          |
| H. influenzae        | 32                    | 27          | −27% to 21%          |
| Any otopathogen†     | 95                    | 96          | −17% to 14%          |
| At 2 mo (1 mo after stopping intervention) |             |             |                      |
| S. pneumoniae        | 57                    | 39          | −45% to 13%          |
| S. pyogenes          | 11                    | 31          | −3.0% to 49%         |
| Moraxella*           | 87                    | 100         | −11% to 28%          |
| H. influenzae        | 19                    | 15          | −24% to 28%          |
| Any otopathogen†     | 89                    | 100         | −14% to 25%          |

*Likely contains commensals in addition to *M. catarrhalis* due to the characteristics of taxonomy reference database.
†Any otopathogen includes the proportion of children with *S. pneumoniae, S. pyogenes, Moraxella* or *H. influenzae.*

**FIGURE 2.** *Streptococcus salivarius* in nasopharyngeal microbiome and saliva during the study. The impact of *S. salivarius* K12 on the relative abundance of *S. salivarius* (not specific for K12 strain) in the nasopharyngeal microbiome (A) and in the saliva (B) in children receiving K12 product for 1 month after study entry.
communities and most of the next-generation sequencing platforms use short-length reads. The short-read sequencing has a limitation in identification at the species level. We have used Ion Torrent sequencing platform, with a read length of 400 bp to overcome the limitation. Ion Torrent platforms have a high error rate because of the nature of the semiconductor sequencing technology. However, it is well suited for targeted amplicon sequencing such as bacterial 16S and antimicrobial resistance gene sequencing.

In summary, in a randomized trial, oral S. salivarius K12 probiotic did not disturb the diversity of saliva or nasopharyngeal microbiomes in children attending daycare. Even though there was a short-term increase in S. salivarius in the saliva microbiome of children receiving oral K12 product, the impact on the otopathogens remained uncertain.

REFERENCES

1. Var LE, Kleinman KP, Raebel MA, et al. Recent trends in outpatient antibiotic use in children. Pediatrics. 2014;133:375–385.
2. Kilpi T, Herva E, Kajalainen T, et al. Bacteriology of acute otitis media in a cohort of Finnish children followed for the first two years of life. Pediatr Infect Dis J 2001;20:654–662.
3. Faden H, Duffy L, Wasielewski R, et al. Relationship between nasopharyngeal colonization and the development of otitis media in children. Towanda/Williams Pediatrics. J Infect Develop. 1997;175:1440–1445.
4. Smith-Vaughan H, Byun R, Halpin S, et al. Interventions for prevention of otitis media may be most effective if implemented in the first weeks of life. Int J Pediatr Otorhinolaryngol. 2008;72:57–61.
5. Chonmaitree T, Jennings K, Golovko G, et al. Nasopharyngeal microbiota in infants and changes during viral upper respiratory tract infection and acute otitis media. PLoS One. 2017;12:e0180630.
6. Faden H, Stanevich J, Brodsky L, et al. Changes in nasopharyngeal flora during otitis media of childhood. Pediatr Infect Dis J. 1990;9:623–626.
7. Reviak K, Mannidi D, Chonmaitree T. Association of nasopharyngeal bacte rial colonization during upper respiratory tract infection and the development of acute otitis media. Clin Infect Dis. 2008;46:e34–e37.
8. Jokinen J, Palmu AA, Kilpi T. Acute otitis media replacement and recurrence in the Finnish otitis media vaccine trial. Clin Infect Dis. 2012;55:1673–1676.
9. Naziat H, Saha S, Islam M, et al. Epidemiology of otitis media with otorrhoea among Bangladeshi children: baseline study for future assessment of pneumococcal conjugate vaccine impact. Pediatr Infect Dis J. 2018;37:715–721.
10. Gisselsson-Solen M. Trends in otitis media incidence after conjugate pneumococcal vaccination: a national observational study. Pediatr Infect Dis J. 2017;36:1027–1031.
11. Fireman B, Black SB, Shinefield HR, et al. Impact of the pneumococcal conjugate vaccine on otitis media. Pediatr Infect Dis J. 2003;22:10–16.
12. Palmu AA, Jokinen J, Nieminen H, et al. Vaccine-preventable disease incidence of pneumococcal conjugate vaccine in the Finnish invasive pneumococcal disease vaccine trial. Vaccine. 2018;36:1816–1822.
13. Heikkinnen T, Block SL, Toback SL, et al. Effectiveness of intranasal live attenuated influenza vaccine against all-cause acute otitis media in children. Pediatr Infect Dis J. 2013;32:669–674.
14. Norhayati MN, Ho JJ, Azman MY. Influenza vaccines for preventing acute otitis media in infants and children. Cochrane Database Syst Rev. 2017;10:CD10089.
15. Uhari M, Tapiainen T, Kontioikari T. Xylitol in preventing acute otitis media. Vaccine. 2000;19(suppl 1):S144–S147.
16. Hautalahti O, Renko M, Tapiainen T, et al. Failure of xylitol given three times a day for preventing acute otitis media. Pediatr Infect Dis J. 2007;26:423–427.
17. Tapiainen T, Luotonen L, Kontioikari T, et al. Xylitol administered only during respiratory infections failed to prevent acute otitis media. Pediatrics. 2002;109:E19.
18. Hatakka K, Blomgren K, Pohjavoisto S, et al. Treatment of acute otitis media with probiotics in otitis-prone children—a double-blind, placebo-controlled randomized study. Clin Nutr. 2007;26:314–321.
19. Hatakka K, Savilaitė E, Pönnikä A, et al. Effect of long term consumption of probiotic milk on infections in children attending day care centres: double blind, randomised trial. BMJ 2001;322:1327.
20. Lehtoranta L, Pitkäranta A, Korpela R. Probiotics in respiratory virus infections. Eur J Clin Microbiol Infect Dis. 2014;33:1289–1302.
21. Igartua C, Davenport ER, Gilad Y, et al. Host genetic variation in mucosal immunity pathways influences the upper airway microbiome. Microbiome. 2017;5:16.
22. Kumpu M, Kekkonen RA, Kautiainen H, et al. Milk containing probiotic did not disturb the diversity of saliva or nasopharyngeal microbiomes in children attending daycare. Even though there was a short-term increase in S. salivarius in the saliva microbiome of children receiving oral K12 product, the impact on the otopathogens remained uncertain.
44. de Weerth C, Fuentes S, Puylaert P, et al. Intestinal microbiota of infants with colic: development and specific signatures. Pediatrics. 2013;131:e550–e558.
45. Bolyen E, Rideout JR, Dillon MR, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. 2019;37:852–857.
46. Man WH, Clerc M, de Steenhuijsen Pitera WAA, et al. Loss of microbial topography between oral and nasopharyngeal microbiota and development of respiratory infections early in life. Am J Respir Crit Care Med. 2019;200:760–770.
47. Salgado VR, Fukutani KF, Fukutani E, et al. Effects of 10-valent pneumococcal conjugate (PCV10) vaccination on the nasopharyngeal microbiome. Vaccine. 2020;38:1436–1443.
48. Whitney CG, Farley MM, Hadler J, et al; Active Bacterial Core Surveillance of the Emerging Infections Program Network. Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. N Engl J Med. 2003;348:1737–1746.
49. Palmu AA, Kilpi TM, Rinta-Kokko H, et al. Pneumococcal conjugate vaccine and clinically suspected invasive pneumococcal disease. Pediatrics. 2015;136:e22–e27.
50. Ben-Shimol S, Givon-Lavi N, Leibovitz E, et al. Studying PCV impact on clinical presentation of otitis media helps to understand its pathogenesis. Vaccine. 2019;37:1–6.
51. Eythorsson E, Sigurdsson S, Hrafnikelsson B, et al. Impact of the 10-valent pneumococcal conjugate vaccine on antimicrobial prescriptions in young children: a whole population study. BMC Infect Dis. 2018;18:505.
52. Palmu AA, Rinta-Kokko H, Nohynek H, et al. Impact of national ten-valent pneumococcal conjugate vaccine program on reducing antimicrobial use and tympanostomy tube placements in Finland. Pediatr Infect Dis J. 2018;37:97–102.
53. Deagle BE, Thomas AC, Shaffer AK, et al. Quantifying sequence proportions in a DNA-based diet study using Ion Torrent amplicon sequencing: which counts count? Mol Ecol Resour. 2013;13:620–633.
54. Pawluczyk M, Weiss J, Links MG, et al. Quantitative evaluation of bias in PCR amplification and next-generation sequencing derived from metabarcoding samples. Anal Bioanal Chem. 2015;407:1841–1848.
55. O’Donnell JL, Kelly RP, Lowell NC, et al. Indexed PCR primers induce template-specific bias in large-scale DNA sequencing studies. PLoS One. 2016;11:e0148698.
56. Kelly RP, Shelton AO, Gallego R. Understanding PCR processes to draw meaningful conclusions from environmental DNA studies. Sci Rep. 2019;9:12133.
57. Nearing JT, Douglas GM, Comeau AM, et al. Denoising the denoisers: an independent evaluation of microbiome sequence error-correction approaches. Peerj. 2018;6:e5364.
58. Johnson JS, Spakowicz DJ, Hong BY, et al. Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. Nat Commun. 2019;10:5029.
59. Salipante SJ, Kawashima T, Rosenthal C, et al. Performance comparison of Illumina and Ion torrent next-generation sequencing platforms for 16S rRNA-based bacterial community profiling. Appl Environ Microbiol. 2014;80:7583–7591.
60. Besser J, Carleton HA, Gerner-Smidt P, et al. Next-generation sequencing technologies and their application to the study and control of bacterial infections. Clin Microbiol Infect. 2018;24:335–341.