

# Partial restoration of the microbiota of cesarean-born infants via vaginal microbial transfer

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**Exposure of newborns to the maternal vaginal microbiota is interrupted with cesarean birthing. Babies delivered by cesarean section (C-section) acquire a microbiota that differs from that of vaginally delivered infants, and C-section delivery has been associated with increased risk for immune and metabolic disorders. Here we conducted a pilot study in which infants delivered by C-section were exposed to maternal vaginal fluids at birth. Similarly to vaginally delivered babies, the gut, oral and skin bacterial communities of these newborns during the first 30 d of life was enriched in vaginal bacteria—which were underrepresented in unexposed C-section-delivered infants—and the microbiome similarity to those of vaginally delivered infants was greater in oral and skin samples than in anal samples. Although the long-term health consequences of restoring the microbiota of C-section-delivered infants remain unclear, our results demonstrate that vaginal microbes can be partially restored at birth in C-section-delivered babies.**

A major determinant of the microbiota composition of newborns is the mode of delivery. Vaginally delivered infants harbor bacterial communities resembling those of the maternal vagina, whereas C-section-delivered infants are enriched in skin microbiota<sup>1</sup>. The microbiome that colonizes the body of newborns can have a determinant role in educating the immune system<sup>2</sup>. Early interaction with commensal microbes is essential for healthy immune development and metabolic programming, and aberrant microbial colonization in newborns has been associated with long-term effects on host metabolism<sup>3</sup> or impaired immune development<sup>2</sup>. Epidemiological studies, although not showing causality, have reported associations between C-section delivery and an increased risk of obesity, asthma, allergies and immune deficiencies<sup>4–7</sup>. Rates of cesarean delivery are increasing worldwide and in some countries exceed 50% of total births<sup>8–10</sup>, a rate substantially greater than the

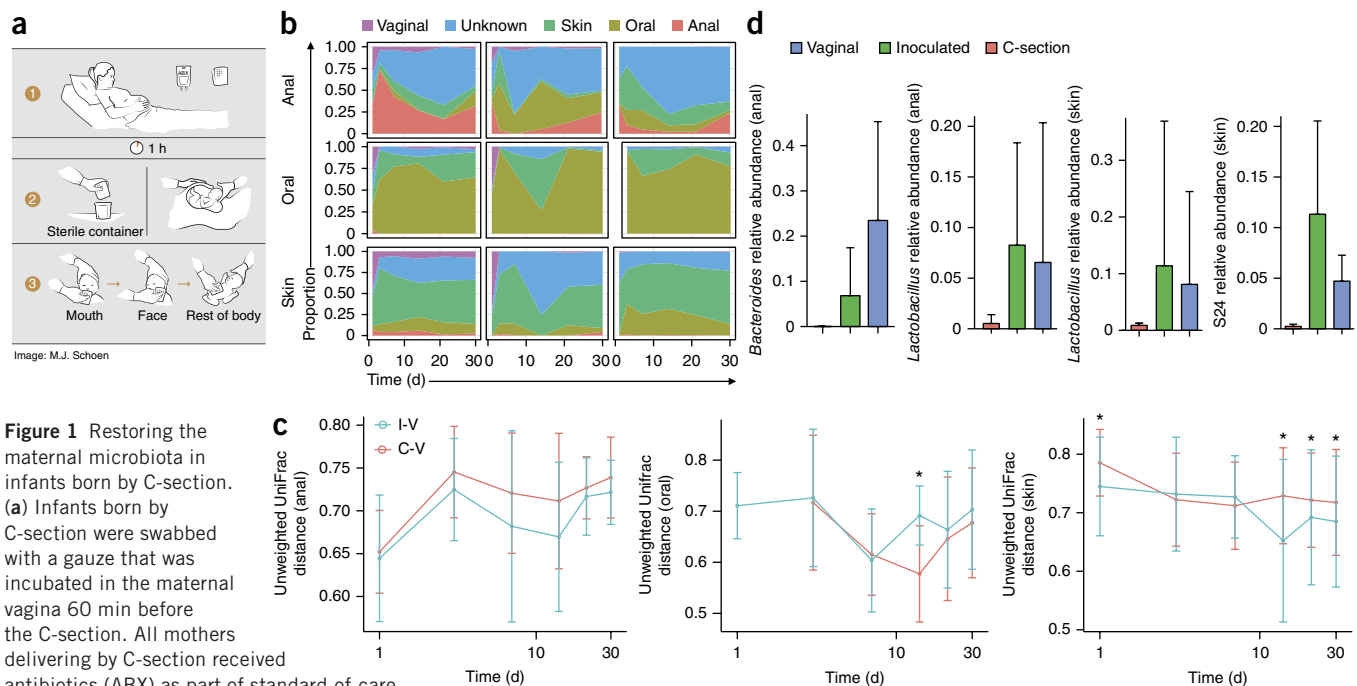
estimated 15% of births that require C-section delivery to protect the health of the mother or baby<sup>11</sup>.

Here we exposed C-section-delivered infants to their maternal vaginal fluids at birth and longitudinally determined the composition of their microbiota to assess whether it developed more similarly to vaginally born babies than to unexposed C-section-delivered infants. We collected samples from 18 infants and their mothers, including 7 born vaginally and 11 delivered by scheduled C-section, of which four were exposed to the maternal vaginal fluids at birth (**Supplementary Table 1**). Briefly, the microbial restoration procedure, or vaginal microbial transfer, consists of incubating sterile gauze in the vagina of mothers who were negative for group B *Streptococcus* (GBS), had no signs of vaginosis and had a vaginal pH < 4.5 during the hour preceding the C-section. Within the first 2 min of birth, babies were exposed to their maternal vaginal contents by being swabbed with the gauze, starting with the mouth, then the face and finally the rest of the body (**Fig. 1a**). A total of 1,519 samples were obtained from anal, oral and skin sites of infants and mothers at six time points during the first month of life (1, 3, 7, 14, 21 and 30 d after birth; **Supplementary Table 2**). Microbiome composition was characterized by sequencing the V4 region of 16S rRNA gene as previously described<sup>12</sup>, and 1,016 samples were used for analysis after quality filtering (see Online Methods). No adverse events were reported for any of the infants in this study.

Bacterial source-tracking<sup>13</sup> of the infant microbiome revealed that the microbiomes of the four C-section-delivered infants exposed to vaginal fluids resembled those of vaginally delivered infants, particularly so during the first week of life (**Fig. 1b**). The bacterial community distance between microbiome samples from exposed and vaginal newborns was lower in anal and skin samples (**Fig. 1c**). At day 1, regardless of body site, the microbiomes of babies that were delivered vaginally or by C-section but exposed to vaginal fluids was more similar to the maternal vaginal microbiomes than to those of C-section-delivered (but unexposed) infants (**Supplementary Fig. 1**). A progression toward a body-specific configuration was observed in all of the body sites, either gradually (anus) or quickly (oral and skin), but both vaginally delivered and exposed C-section-delivered newborns exhibited a vaginal microbiome-like signature that was absent in unexposed C-section-delivered babies (**Supplementary Fig. 2**). Although variations in microbiome composition between subjects within each of the groups exist (**Supplementary Figs. 3–7**), we confirmed the differences between unexposed and exposed C-section-delivered infants by building a random forest classifier for each body site<sup>14</sup>. Samples from unexposed C-section- and vaginally delivered infants could be classified with high accuracy, confirming that the mode of delivery shapes the microbial communities of the infants

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**Figure 1** Restoring the maternal microbiota in infants born by C-section. (a) Infants born by C-section were swabbed with a gauze that was incubated in the maternal vagina 60 min before the C-section. All mothers delivering by C-section received antibiotics (ABX) as part of standard-of-care treatment (top). The gauze was extracted before the procedure, kept in a sterile (middle) container and used to swab the newborn within the first 1–3 min after birth, starting with the mouth, then the face and the rest of the body (bottom). (b) Proportion of microbiota from anal (top), oral (middle) and skin (bottom) samples of infants delivered either vaginally (left;  $n = 7$  subjects sampled at six time points), by C-section and exposed to vaginal fluids (middle;  $n = 4$  subjects sampled at six time points) that are estimated to originate from different maternal sources (colored regions), using bacterial source-tracking. (c) Bacterial community distances (unweighted UniFrac distances) in anal (left), oral (middle) and skin (right) samples between vaginally delivered babies and C-section–delivered babies that were either exposed (I-V) or not exposed (C-V) to the vaginal gauze during the first month of life. Because the babies were sampled more frequently during the first week of life, the x axis is represented as a log scale. Error bars indicate mean  $\pm$  s.d. \* $P < 0.01$ ; analysis of variance (ANOVA) and Tukey's honest significant difference test. (d) Representative bacterial taxa enriched in infants with perinatal exposure to vaginal fluids during the first month of life. S24, *Bacteroidales* family S24-7 members. Error bars indicate mean  $\pm$  s.d.

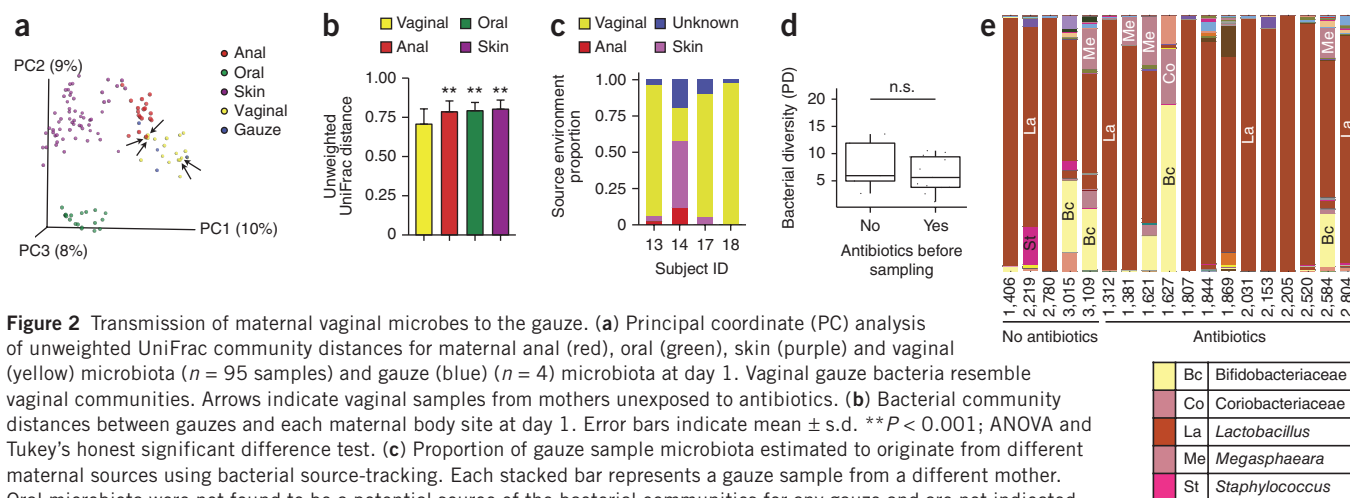
(Supplementary Table 3). In relation to C-section–delivered infants who were not exposed to vaginal fluids, anal, oral and skin samples from exposed C-section–delivered infants were classified less frequently as samples from C-section–delivered infants and more often as samples from vaginally delivered infants (Supplementary Table 3)—with oral and skin samples being more accurately classified than anal samples. A random forest classifier built from predicted metagenome content was equally able to distinguish vaginally delivered and C-section–delivered infants, with the exposed infants being classified as vaginally delivered using the oral and skin samples, and as C-section–delivered from the anal samples, but again less frequently than unexposed C-section–delivered infants (Supplementary Table 4).

Microbial colonization of body sites in the newborn occurs quickly, and changes proceed during the first month in all of the groups. In anal samples from exposed infants and vaginally delivered infants, there was an early enrichment of *Lactobacillus* followed by a bloom of *Bacteroides* from week 2, which was not observed in newborns that were not exposed to vaginal fluids (Fig. 1d and Supplementary Fig. 2). The anal microbiota of newborns here, as previously reported, remained distinct from that of adults even at 1 month of life<sup>15,16</sup>. The infant skin and oral microbiota, however, acquired a more adult-like configuration after the first week of life in all three groups. Unexposed C-section–delivered infants, however, lacked the vaginal bacteria that were restored by swabbing infants with gauze or that were present in vaginally delivered infants—particularly anal and skin *Lactobacillus* early in life, anal *Bacteroides* and members of the *Bacteroidales* family S24-7 in the skin (Fig. 1d and Supplementary Fig. 2). Maturation of

the infant gut microbiome occurs with the cessation of breastfeeding, and there are no differences until the fourth month of life between the microbiomes of infants who are exclusively breast-fed and those from infants whose diet is supplemented with formula<sup>15</sup>. Because all of the infants in our study received breast milk either exclusively or supplemented with formula during the first month of life (Supplementary Table 1), the microbiome composition profiles observed in each group do not appear to be due to the mode of feeding.

Neonatal bacterial diversity was highest at birth in the anal and oral sites, and this declined by the third day (Supplementary Fig. 8a,b). Although this phenomenon is not yet well understood, we have previously observed it in the gut microbiome of mice<sup>17</sup>. Although the post-natal decrease in digestive diversity might reflect the selective effect of milk on the gut and oral microbiomes, the initially higher diversity could be explained by *in utero* colonization of the neonate<sup>18,19</sup>. The bacterial diversity on the skin of newborns, in contrast, was lowest at birth and gradually increased during the first month of life (Supplementary Fig. 8c).

Because C-section–delivered infants were exposed to vaginal fluids through the use of sterile gauzes, we determined how similar the microbiomes of the gauzes were to those of samples obtained from the maternal body sites at day 1. We confirmed that the microbiota of the gauzes that were incubated in the maternal vagina were the most similar to those of the vaginal samples (Fig. 2a), with both being enriched for *Lactobacillus iners* (Supplementary Fig. 9). The bacterial community distance of each gauze sample to its own maternal vaginal sample was smaller than to those of other mothers, although



these differences were not significant (**Supplementary Fig. 10**). Power analysis estimated that it would require 35 samples per group to detect the observed effect size (Cohen's  $d = 0.68$ ) with a power of 80% and a significance level of 0.05. UniFrac distances from the gauzes to the vaginal samples were significantly smaller than those from the gauzes to the other body sites (analysis of variance (ANOVA),  $P < 0.01$ ; **Fig. 2b**); bacterial source-tracking further confirmed that the microbiota of the gauzes is mostly of vaginal origin (**Fig. 2c**).

Although our sample size was limited and sampling extended only through the first month after birth, our results suggest that by exposing the infant to the maternal vaginal microbiota, the bacterial communities of newborns delivered by C-section can be partially restored to resemble those of vaginally delivered babies. The partial microbiota restoration provided by the gauze might be due to the compounded effects of the antibiotic treatments that accompany the C-section procedure and the suboptimal bacterial transfer from the vagina to the gauze and then to the baby. However, there was no apparent clustering of the vaginal microbiota on the basis of exposure to antibiotics (**Fig. 2a**; arrows indicate mothers unexposed to antibiotics). Bacterial diversity was not lower in the vaginal microbiota of mothers who received antibiotics (**Fig. 2d**), and no clear differences were observed in taxonomic composition either (**Fig. 2e**). The abundance of *Lactobacillus* was not depleted in the vaginal microbiota of antibiotic-exposed mothers as compared to that of unexposed mothers (Student's  $t$ -test,  $P = 0.618$ ). Both exposed and unexposed C-section-delivered infants were comparable in terms of antibiotic exposure and feeding (**Supplementary Table 1**), suggesting that the differences observed in microbiome composition between these two groups can be most parsimoniously explained by the exposure to the vaginally swabbed gauze. A larger sample size will be needed to clarify the effect of perinatal antibiotics on the vaginal microbiome and, subsequently, on the microbiome of the gauze that was incubated in the maternal vagina and on the bacterial load received by the infant. Determining more effective approaches to transfer the maternal microbiota to newborns and, more importantly, establishing which keystone species newborn infants should acquire at birth, are important to replicating the beneficial effects provided by vaginal delivery in C-section-delivered infants.

The partial microbial restoration observed in our study could be due to the fact that infants are exposed a single time to topical application of vaginal fluids. Furthermore, particular body sites (such as mouth and skin) were more amenable to inoculation than others. A modified protocol that exposes newborns repeatedly to the gauze would more faithfully reproduce the extended exposure that vaginally delivered infants receive and might potentially improve microbial restoration, although this hypothesis remains to be tested. Enteral administration of key bacterial species could further supplement the method described here; however, extensive research would be required to ensure the safety and efficacy of such an approach. We stress that our work represents a proof of principle on a small cohort and with limited follow-up over time. Labor is a complex process that cannot be fully recaptured by our procedure, and which encompasses multiple factors beyond the mere transmission of microbes from mother to infant. Finally, extended longitudinal analysis of larger cohorts is needed to determine whether this procedure has any effects on diseases later in life.

## METHODS

Methods and any associated references are available in the [online version of the paper](#).

**Accession codes.** European Bioinformatics Institute (EBI): sequence data have been deposited under study accession number [PRJEB10914](#).

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

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## BRIEF COMMUNICATIONS

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### AUTHOR CONTRIBUTIONS

M.G.D.-B. designed the study. M.G.D.-B., K.M.D.J.-L., J.I.R.-V. and K.M. collected and processed specimens. M.G.D.-B. sequenced and generated data. N.S., L.M.C., A.A., A.G., N.A.B., S.J.S., M.H. and J.C.C. performed experiments. M.G.D.-B., N.S., L.M.C., A.A., A.G., N.A.B., S.J.S., M.H., R.K. and J.C.C. analyzed data. M.G.D.-B. and J.C.C. drafted the manuscript. All authors reviewed the final manuscript.

### COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the [online version of the paper](#).

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## ONLINE METHODS

**Study design.** The inclusion criteria for mothers participating in this study were healthy mothers, as assessed by their doctors who were delivering either vaginally or by scheduled C-section. Mothers scheduled to have a C-section were offered the opportunity to participate in the study and were divided into the two groups based on their willingness to have their newborns swabbed with the gauze. For the group of C-section–delivered infants exposed to maternal vaginal fluids, mothers had to have negative results for the standard-of-care tests of sexually transmitted diseases (STDs)—including HIV, *Chlamydia* and group B *Streptococcus* (GBS, standard test at 35 weeks by culturing)—no signs of vaginosis or viral infections as determined by their obstetrician and a vaginal pH < 4.5 at 1–2 h preceding the procedure. Of the 14 mothers whose infants were not exposed to the gauze (7 vaginal and 7 C-section deliveries), three were GBS positive, and of which two were delivered by C-section and one was by vaginal delivery (**Supplementary Table 1**). All mothers received standard-of-care treatment, including preventive perinatal antibiotics (Beta lactams: mostly cephalosporins or penicillin; see **Supplementary Table 1** for details) for mothers who underwent cesarean section or for vaginally delivering GBS-positive mothers. The study was approved by the Institutional Review Boards from the University of Puerto Rico, Medical Sciences Campus (A9710112) and from the Rio Piedras (1011-107) campus. All C-sections in this study were due to previous C-sections, and the procedure was conducted at the University Adult's Hospital, Puerto Rico Medical Center. Written informed consent was obtained from all participants.

**Microbial restoration procedure.** Within the hour prior to the procedure, maternal vaginal pH was measured using a sterile swab and a paper pH strip (Fisher). Once the pH was confirmed to be <4.5, an 8-cm × 8-cm four-layered gauze (Fisherbrand cat# 22028558) was folded like a fan, and then in half, wet with sterile saline solution and inserted in the vagina for 1 h. Right before the C-section surgery started, the gauze was extracted and placed in a sterile collector and kept at room temperature. As soon as the baby was brought to the neonate lamp and within 1 min after delivery, the infant was swabbed with the gauze, starting on the lips, followed by the face, thorax, arms, legs, genitals and anal region, and finally the back. The swabbing took approximately 15 s. The neonatologist then proceeded to perform the standard detailed examination of the newborn.

**Sample collection and processing.** Sampling with sterile swabs in different body sites took place within the first 5 min after birth in all babies (including the vaginal gauze–exposed C-section group, who were sampled after the gauze swabbing procedure), then at day 3 and then weekly for the first month. Sampled body sites included oral mucosa, forehead, right arm, right foot and anal region of the baby, and the same sites of the mother plus a vaginal swab (**Supplementary Table 2**). Gauze samples were obtained from a 1-cm<sup>2</sup> area from the center of the gauze. All samples were transported to the laboratory with ice packs within 2 h after collection and stored at –80 °C until further processing. DNA was extracted from samples using the MoBio Powersoil Kit according to the manufacturer's instructions, modified as described in the Earth Microbiome Project protocol (<http://www.earthmicrobiome.org/emp-standard-protocols/dna-extraction-protocol/>).

**Sequencing and data processing.** Sequencing of the swabs and gauzes was performed at the NYU Genome Technology Center using the Illumina MiSeq sequencing instrument, with v2 reagents and 2 × 250 cartridge. Raw reads were de-multiplexed and quality-filtered using QIIME v1.8.0 with default parameters<sup>20</sup>. Quality-filtered reads were clustered into operational taxonomic units (OTUs) using an open-reference algorithm<sup>21</sup> and Greengenes v13.8 as a reference set<sup>22</sup>. Samples that had at least 1,000 sequences ( $n = 1,016$ ) were further analyzed, resulting in a total of 6,515,724 sequences (mean 6,413 ± 4,593; median 5,360 sequences). Alpha diversity on rarefied tables was estimated by using Faith's phylogenetic diversity<sup>23</sup> and beta diversity by using unweighted UniFrac<sup>24</sup>.

**Bacterial source-tracking.** To estimate the sources of the microbial communities observed in each of the three infant groups at different body sites and time points, we used SourceTracker (v1.0), a Bayesian approach for bacterial source-tracking<sup>13</sup>. Samples from each body site in the infants were designated as sinks, and samples from all of the body sites of the corresponding mother were tagged as sources. Source-tracking of bacterial communities in the gauzes were performed similarly, designating gauzes as sinks and paired maternal body sites as potential sources.

**Supervised learning classification.** A random forest classifier was built for each body site using 500 trees and a leave-one-out error model as previously described<sup>14</sup>. To account for subsampling variability, the input tables were rarefied ten times and results averaged over the total. The confusion matrices represent the mean and s.d. percentage of samples from the true class assigned to each of the possible classes (vaginal, exposed, C-section) for each body site.

**Predicted metagenome.** OTU tables were filtered to remove *de novo* OTUs (i.e., not found in Greengenes). Metagenomic content was estimated using PICRUSt<sup>25</sup>, first normalizing each OTU in the filtered tables by its corresponding copy number and then predicting the abundance of functional traits from the normalized OTU counts.

**Deblurring.** To identify with higher resolution the *Lactobacillus* present in the samples we used deblurring, a novel denoising method for Illumina-based amplicon sequencing. By using the raw reads as input, deblurring processes each sample independently and tries to remove all sequences derived from sequencing-read errors or PCR errors, based on an upper bound on error probabilities. Briefly, the deblurring algorithm steps and parameters used for the current study are the following. (i) Trim the all reads to constant length (150 bp) and de-replicate, retaining the total number of reads per unique sequence. (ii) Subsample to 4,000 reads (per sample). (iii) Discard all singleton reads. (iv) Remove sequences containing known sequencing artifacts (PhiS or adaptor sequences). (v) Perform multiple-sequence alignment using MAFFT v7.130b<sup>26</sup>. (vi) Perform the actual deblurring: iterate on all sequences from highest frequency to lowest: (a) for each sequence, reduce the frequency of neighboring sequences (based on Hamming distance + indel (insertion and deletion)) according to the distance dependent maximal error profile—the error profile used ranges from 6% maximal read error for Hamming distance 1, 2% for distance 2, and down to 0.1% for Hamming distance 10; and (b) if the resulting frequency is lower than 0, then remove the sequence from the list. (vii) Remove chimeras from the resulting sequences using *de novo* chimera detection with usearch 5.2.236 and the parameter 'uchime\_denovo'.

The performance of deblurring has been validated in simulations and mock mixtures, and deblurring was shown to retain the exact actual sequences in the sample while removing most of the sequencing error- and PCR error-derived sequences, with the ability to detect sequences differing by as little as one nucleotide over the entire sequenced region. Additional details on deblurring, and the source code, can be found at <https://github.com/biocore/deblur>.

**Statistical analyses.** All technicians processing the samples were blinded to the group allocations. Computational analyses were not blinded owing to the use of supervised learning methods, which require knowledge of the groups. Because our study was designed as a proof of concept, sample size was not estimated *a priori*. Statistical tests were performed using QIIME 1.8.0 with default parameters and R 3.2.2.

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