1 **TITLE**

2 Gastrointestinal, Vaginal, Nasopharyngeal, and Breast Milk Microbiota profiles and Breast Milk
3 Metabolomic changes in Gambian infants over the first two months of lactation: a prospective
4 cohort study

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List of abbreviations

BM: breast milk

GBS: group B streptococcus

GC-MS: gas chromatography-mass spectrometry

1H-NMR: 1-H-nuclear magnetic resonance

HMOs: human milk oligosaccharides

nMDS: non-metric multidimensional scaling analysis

OTUs: operational taxonomic units

PCR: polymerase chain reaction

RM-MCCV-PLS: repeated measures Monte Carlo cross-validated partial least squares analysis

STGG: skim-milk tryptone, glucose glycerol medium

ABSTRACT

Background: Microbiota composition in breast milk affects intestinal and respiratory microbiota colonization and the mucosal immune system's development in infants. The metabolomic content of breast milk is thought to interact with the microbiota and may influence developing infant immunity.

Methods: 107 Gambian mothers and their healthy, vaginally delivered, exclusively breastfed infants were included in our study. We analyzed 32 breast milk samples, 51 maternal rectovaginal swabs and 30 infants' rectal swabs at birth. We also analyzed 9 breast milk samples and 18 infants' nasopharyngeal swabs 60 days post-delivery. We used 16S rRNA gene sequencing to determine the microbiota composition. Metabolomic profiling analysis was performed on colostrum and mature breast milk samples using a multiplatform approach combining 1-H Nuclear Magnetic Resonance Spectroscopy and Gas Chromatography-Mass Spectrometry.

Results: Bacterial communities were distinct in composition and diversity across different sample types. Breast milk composition changed over the first 60 days of lactation. α-1,4- and α-1,3-fucosylated human milk oligosaccharides, and other 33 key metabolites in breast milk (monosaccharides, sugar alcohols and fatty acids) increased between birth and day 60 of life.

Conclusions: This study's results indicate that infant gut and respiratory microbiota are unique bacterial communities, distinct from maternal gut and breast milk, respectively. Breast milk microbiota composition and metabolomic profile change throughout lactation. These changes may contribute to the infant's immunological, metabolic, and neurological development and could consist the basis for future interventions to correct disrupted early life microbial colonization.

INTRODUCTION

It has been proposed that human gut microbiota play a significant role in maintaining lifelong health.[1] Perturbations in the infant gut microbiota composition have been associated with increased susceptibility to various diseases early in life.[1,2] Less is known about the respiratory microbiota, but studies have identified disruptions in the respiratory microbiota in the first months of life to predict future respiratory health.[3]

The initial colonization and establishment of microbiota in infancy constitute a complex and dynamic process, influenced by multiple factors, such as mode of delivery,[4] gestational age at birth,[5] type of feeding,[6] antibiotic treatment,[7] maternal diet,[8] environmental exposures,[9] and host genetics.[10]

Human milk is considered a significant contributory factor to the development of the infant gut microbiota either by directly seeding the infant gut, [11,12] or through the role of human milk oligosaccharides (HMOs) in promoting the growth of *Bifidobacterium* species in the human gut.[13] The same may be true of the respiratory microbiome with breastfeeding potentially affecting patterns of colonization.[14,15] Also, it has been recently recognized that the breast milk metabolomic profile changes over time in the transition from colostrum to mature milk to promote the growing infant's immunological maturation and neurological development.[16] However, the interplay between the breast milk metabolome and the infant microbiome is, as yet, unknown.

In this study, we used 16S rRNA gene sequencing to characterize the gut and nasopharyngeal microbiome in healthy, vaginally delivered infants from the Gambia, together with the breast milk and rectovaginal microbiome of their mothers. In addition, we assessed the metabolomic changes of breast milk over the first two months of life in the same cohort.

MATERIALS AND METHODS

Study Population

Our Gambian samples were nested within a large longitudinal cohort study to examine risk factors for group B streptococcus (GBS) colonization in Gambian mothers and their infants.[17]

The study was conducted between January 15, 2014, and January 31, 2015, in Faji Kunda Hospital and Jammeh Foundation for Peace Hospital, two public health centers in Gambia's urban coastal Banjul region with 12,500 births each year. These two centers were chosen because they represent the antenatal services that Gambian women typically have. The inclusion criteria were age 18-45 years, negative HIV test during pregnancy, written informed consent obtained from the infant's mother, ability to comply with the study procedures as judged by a member of the research team, low-risk pregnancy defined as no evidence of pre-eclampsia, cardiomyopathy, maternal gestational diabetes, placental previa, twin pregnancy or any other condition or situation that substantially increased the risk of pregnancy based on the investigator's clinical judgement, meaning that participation would not be in the best interests of the infants. Women were not recruited if they were planning to move outside the study area for at least the first 12 weeks following delivery (preventing follow-up visits), were enrolled in other studies requiring blood/breast milk sampling or swabs, were not planning to breastfeed, developed complications of delivery (pre-eclampsia, antepartum haemorrhage, caesarian section), or if the infant was born below 32 weeks' gestation as assessed by Ballard score, had a birth weight less than 2.5 kg, diagnosed with congenital abnormalities requiring prolonged hospital stay (>48 hours), showed symptoms or signs of significant illness or infection at birth, required resuscitation and intensive care, or for any other reason that would prevent the study endpoints being assessed in the infant effectively as judged by the investigator. From the 750 mother-infant pairs recruited into the main study, 107 mothers and their infants were randomly included in the microbiota study.

Sample collection

We collected breast milk samples, maternal rectovaginal swabs and infants' rectal swabs at birth (D0). We also collected breast milk samples and infants' nasopharyngeal swabs 60 days post-delivery (D60). Swabs (Copan, UK) were collected in skim-milk tryptone, glucose glycerol (STGG) medium, refrigerated at 4 °C and transported to the laboratory within 6 h, to be vortexed and stored at -70°C. For the breast milk collection, mothers were requested to wash their hands with soap and wipe their breasts with sterile cotton wool and 0.02% chlorhexidine before hand-expressing a milk sample from each breast into separate sterile containers. After collection, milk

samples were refrigerated at 4 °C and transported to the laboratory within 4 h. Then the samples were spun at 3000 g for 30 min to remove lipids and were frozen at -70 °C.

Microbiota Analysis

DNA extraction, library preparation, and sequencing

Samples were transferred to Imperial College London for analysis. DNA was purified using Fast DNA™ SPIN Kit for Soil (MP BIOMEDICALS, CA, USA) according to the manufacturer's instructions.[18] The microbiota composition was established by conducting a nested polymerase chain reaction (PCR) to amplify first V3-V5 [19] and then V4 hypervariable regions of the 16S rRNA gene.[20]

Sequence processing and microbial species abundance estimates

Amplicon sequence data were analyzed using Mothur v1.43.0 [21] according to the Mothur MiSeq SOP.[22] Overlapping sequencing reads were merged into contigs, cleaned and aligned to a V4 restricted version of the SILVA reference database (version 138).[23] Sequences were clustered into operational taxonomic units (OTUs) at 97% similarity using the OptiClust algorithm and classified using both the SILVA and GreenGenes (version 13_8_99) reference databases.[24] Any contaminating taxa identified at significant levels within the negative controls were filtered out of the sample set; these included members of the *Rhizobiales* order and *Sericytochromatia*. Samples were "normalized" by subsampling at 3000 sequences to balance sample inclusion with sufficient coverage (mean Good's coverage = 98.1%, standard deviation 0.008%).

Metabolomics

Metabolic profiling analyses of breast milk samples were conducted using established 1-H-Nuclear Magnetic Resonance (1H-NMR) and Gas chromatography-mass spectrometry (GC-MS) metabolic profiling analysis methods. These methods have been described in detail in previous publications.[25–27]

Statistical Analyses

Differences in alpha diversity metrics were compared using the Shannon's diversity index measure of community richness, observed species and the Chao1 index, and differences in beta diversity using the Bray-Curtis distance measure of community dissimilarity. Alpha diversity was explored using the Shapiro test for normality, with significant differences calculated by the non-normal distribution Wilcox test. P-values were adjusted for multiple comparisons using the Benjamini-Hochberg method. When comparing the relative abundance of genera between sample groups, we applied an arbitrary threshold of greater than 10% relative abundance in at least one sample for each group to generate a hypothesis. Multivariable statistical analysis was performed on the 1H-NMR and GC-MS acquired data. Repeated Measures Monte Carlo Cross-Validated Partial Least Squares (RM-MCCV-PLS) analysis was performed on each of the data sets. Linear Regression analysis of 1H-NMR and GC-MS breast milk profiles was performed against diversity indices (observed species, Bergerparker, Shannon, Chao, Simpson) of the most abundant genera detected in maternal and infant swabs and breast milk, adjusted using the Storey-Tibshirani False Discovery Rate (FDR) and corrected for confounding factors (sex, ethnic group). Metabolites with adjusted pFDR values <0.01 were considered significant and were subsequently visualized in a Manhattan plot. All statistical analyses were completed in R (version 3.6.1).

Ethical Approval

The study was reviewed and approved by the Gambian Government/ Medical Research Council Joint Ethics Committee (application SCC 1350.v4). All research was performed following the relevant guidelines and regulations, in accordance with the Declaration of Helsinki Ethical Principles and Good Clinical Practices.

Data Availability

All data and metadata are openly available at St George's University figshare data deposit (https://doi.org/10.24376/rd.sgul.14045945). Sequence data have been submitted to the European Nucleotide Archive database (https://www.ebi.ac.uk/ena/browser/home) with accession number PRJEB41404.

RESULTS

Study Population

We successfully performed 16S rRNA gene sequencing on breast milk samples and swabs from 142 participants (35 mother–infant pairs, 72 mothers only). Demographic and clinical characteristics are reported in Table 1. Pregnancy was uneventful for most women. Seven (6.5%) women had a urinary tract infection, two (1.9%) had pneumonia and one (0.9%) had malaria. In addition, 17 (15.9%) participants received antibiotics, of which 16 (94%) had a course of amoxicillin with a mean interval between initiation of antibiotics and birth of 21 (range 0-157) days. 36 (33.6%) women were colonized with Group B Streptococcus at the time of delivery. Intrapartum antibiotic prophylaxis was not routinely given in the Faji Kunda area during the study unless the woman had fever in labor. All infants were born by vaginal delivery and were exclusively breastfed for the full duration of the study. During the first two months of life, six (17.1%) infants attended a medical facility at a median age of 15 (IQR 9) days. Of these six infants, two had an unspecified skin rash, one infected umbilicus, one fever of unknown cause and one jaundice. Four of them received amoxicillin or cloxacillin.

Composition and diversity of the microbiota in infants and mothers

After sub-sampling at a depth of 3000 sequences (see Figure, Supplemental Digital Content 1, which illustrates the sequencing reads per sample type), the final microbiome analysis included 51 maternal rectovaginal swabs, 30 infant rectal swabs and 32 breast milk samples (colostrum) collected on D0; and 18 infant nasopharyngeal swabs and 9 breast milk samples (mature breast milk) collected on D60 (see Figure, Supplemental Digital Content 2, which is the flowchart of samples collected from study participants).

Overall Taxonomic and Alpha Diversity Analyses

We found that colostrum samples harbored significantly fewer observed species compared to maternal rectovaginal swabs ($P = .004$) and infant rectal swabs at birth ($P = .008$) (Figure 1A). Shannon diversity and Chao1 index of microbiota did not differ across different body sites (Figure 1B-C).

Beta Diversity and Taxa Relative Abundance Analyses

In a Non-metric Multidimensional Scaling analysis (NMDS) of Bray-Curtis dissimilarity, microbiota clustered for both body site and collection time point (Figure 1D-E). At birth, microbiota in infant rectal swabs was distinct from both colostrum and maternal rectovaginal swabs. On D60, nasopharyngeal and mature breast milk microbial communities were also separated into two distinct groups. In addition, colostrum and mature breast milk samples had distinct microbiota compositions. Similarly, when only samples collected from the same mother-infant pairs were compared, maternal rectovaginal microbiota clustered separately from infant gastrointestinal (Figure 1F) and breast milk microbiota (Figure 1G).

Two hundred eighty bacterial taxa correlated with the community structure ($P < .05$) in the different sample types. We have listed some notable ones that were described in previous studies (Figure 2). Microbiota in maternal rectovaginal swabs were split into two sub-groups; the first was associated with fecal microbes like *Prevotella*, *Faecalibacterium*, *Dialister*, *Coprococcus* and *Fusicatenibacter* and the second one with predominantly vaginal ones like *Lactobacillales*. Species belonging to the *Bifidobacterium* genus were associated with the infant gut microbiota. *Streptococcus* species were associated with D0 breast milk samples, whereas *Staphylococcus* and *Gemella* with D60 breast milk samples. Bacterial genera like *Anoxybacillus*, *Jeotgalicoccus* and *Geobacillus* were associated with nasopharyngeal microbiota (Figure 2).

Twenty-four genera were found to significantly differ between sample groups when we applied a cut off of greater than 10% relative abundance in at least one sample for each group (Figure 3A). When comparing the relative abundance of the two most relevant genera (*Streptococcus* and *Staphylococcus*) that showed a significant correlation in beta diversity analysis (Figure 3B), we

found higher levels of *Staphylococcus* in breast milk's microbiota compared to the nasopharyngeal microbiota ($P < .001$).

Metabolic phenotyping of breast milk HMOs

All the breast milk samples collected on D0 (n=70) and D60 (n=68) were included in the metabolomic analysis.

1H-NMR

Data from the 1H-NMR spectroscopic analysis were used to construct an RM-MCCV-PLS model (Figure 4A). Breast milk samples clustered according to the time of collection (D0 vs D60). We found that α-1,4-fucosylated oligosaccharides and α-1,3-fucosylated oligosaccharides significantly increased over time (Figure 4B-C), whereas α-1,2-fucosylated oligosaccharides remained the same over time.

GC-MS

RM-MCCV-PLS analysis performed on the GC-MS data also revealed separate clustering of colostrum and mature breast milk samples (Figure 4D). This was mainly due to an increase in the relative abundance of 33 metabolites, such as monosaccharides, sugar alcohols, fatty acids, and fatty acid esters (Figure 4E, Table 2).

Finally, linear regression analysis showed no significant correlations between the 1H-NMR breast milk spectra and the GC-MS breast milk profiles and bacterial diversity indexes such as observed species, Bergerparker, Shannon, Chao, Simpson from the maternal and infant swabs. Similarly, no significant correlations were found in a linear regression analysis between breast milk metabolomic profiles and the most abundant genera differentially detected in the maternal rectovaginal swabs and infant rectal swabs.

DISCUSSION

To our knowledge, this is the first study to report distinct bacterial communities in both composition and diversity from breast milk (D0 and D60), maternal rectovaginal swabs (D0),

infant rectal swabs (D0) and infant nasopharyngeal swabs (D60) in vaginally-delivered infants from the Gambia. This is also the first study that assessed breast milk's metabolomic changes during the first two months of lactation in the Gambia. We found changes in the relative abundance of HMOs and other key metabolites that might contribute to the healthy infant's immunological, metabolic, and neurological development. However, we found no significant correlations between breast milk metabolomic profiles and the maternal rectovaginal and infant gut microbiome at birth.

In our study, infants' gut microbiota differed from maternal rectovaginal microbiota at birth. This finding is in accordance with differences between maternal and infant microbiota within 24 h of delivery reported in a recent study of mother-to-infant microbial transmission.[11] This is not to dispute the paramount importance of vertical transmission through the birth canal or maternal skin depending on the mode of delivery, but to highlight that the results of this seeding event are only manifested a few days or weeks after birth.[28,29]

We found significant changes in the composition and diversity of microbial communities in breast milk through lactation, in agreement with previous studies.[30,31] *Staphylococcus* was a common component of breast milk microbiota that increased with lactation. Typical oral cavity genera such as *Streptococcus* and *Gemella* were also prevalent in breast milk samples, as described before.[32,33] This is in keeping with previous reports suggesting that a "core" breast milk microbiota consist of *Streptococcus* and *Staphylococcus*,[34,35] despite inter-individual variability and observed differences across populations. [31,36]

In addition, we found that nasopharyngeal and breast milk microbiota clustered separately on D60. The infant nasal microbiota has been previously shown to resemble the skin microbiota composition in the early weeks of life, probably due to transmission from the mother's skin during breastfeeding and gradually shifting towards a respiratory microbiota by the age of three months.[3,15] In our cohort, genera of the phylum Firmicutes, such as *Anoxybacillus*, *Jeotgalicoccus*, and *Geobacillus*, although in low abundance, were over-represented. The data on the role of *Anoxybacillus* are contradictory. An increased presence of *Anoxybacillus* in the nasopharynx has recently been shown to raise the risk of respiratory tract infections in Venezuelan infants less than 2 years of age.[37] In contrast, a previous study suggested that decreased abundance of *Anoxybacillus* was related to overgrowth of bacterial pathogens causing

otitis media [29] .[38] The presence of these soil bacteria that thrive in humid environments in the respiratory microbiota of 2-month-old infants probably reflects their living environment. Except for one infant, the rest (20/21, 95%) were born during the green season (mid-June to end of October) with average humidity of 76-86%. This might have contributed to our findings.

We found changes in the relative abundance of fucosylated HMOs between colostrum and mature breast milk. Overall, α-1,3- and α-1,4- fucosylated oligosaccharides significantly increased throughout lactation, whereas α-1,2- fucosylated oligosaccharides remained stable. A recent systematic review has shown that the concentration of α-1,3- fucosylated oligosaccharides significantly increased throughout lactation [39] and then gradually decreased after weaning.[40] This variation across lactation stages suggests that environmental factors might play a role in the activity of fucosyltransferases encoded by the Lewis and Secretor gene alleles.[41]

Changes in the breast milk composition over time reflect the changing needs of the growing infant. In support of this, we found a rise in the relative concentrations of monosaccharides, glycerol and short and medium-chain fatty acids in the first 60 days of lactation, in accordance with previous metabolomic studies of milk maturation.[25,26,42–46] Although the biological importance of these changes is not yet fully understood, most of these metabolites play a role in the immunological maturation and brain development of the growing infant.[47]

Non-microbial components of breast milk might shape the breast milk and infant respiratory and gut microbiota composition.[48] Despite the biological plausibility of this hypothesis, we did not confirm it in our study. A recent longitudinal birth cohort study also found no direct correlation between breast milk microbiota and other milk elements, such as HMOs and fatty acids.[49] Further studies are required to address these complex interactions.

The clinical relevance of breast milk HMOs and the other non-microbial components in preventing infectious diseases in neonates and young infants is increasingly recognized. HMOs promote the development of *Bifidobacterium* species that inhibit the growth of pathogens.[50,51] Supplementing human milk with generic or specific HMOs to reduce the risk of necrotizing enterocolitis in premature infants showed promising results in rat models [52] that were replicated in a study of human infants.[53] Like the gut microbiota,[54] modulation of respiratory microbiota by promoting colonization of the nasopharynx by beneficial bacteria can be achieved by the administration of probiotics.[55,56] There is also an increasing interest in the role of

prebiotics in the prevention and treatment of respiratory disease. Administration of short-chain fatty acids in pregnant mice suppressed allergic airways disease in their offspring.[57] Oral administration of oligosaccharide-based prebiotics in preterm infants reduced the number of viral respiratory tract infections.[58]

There are limitations to our study. In many cases, the quality of the swabs was sub-optimal for analysis, resulting from the challenges of conducting microbiome research in such a setting. We set a sub-sampling depth of 3000 sequences per sample to balance sample inclusion with sufficient coverage, but reducing the number of samples in the final analysis. This is always a major challenge when using low-biomass samples like breast milk. Surprisingly, we found very low abundances of *Bifidobacterium* in the infant rectal swabs collected in our study. There are two likely causes for this finding. First, we sequenced our samples using the V4 region, a method shown to underestimate the presence of *Bifidobacterium* in metagenomic samples due to higher guanine-cytosine content in this region.[59] Second, we used rectal swabs that are more likely to pick up taxa that are aerotolerant and can reside in the skin, instead of stool samples, which may allow sampling of the gut where obligate anaerobes are more likely to reside. Collection of nasopharyngeal samples only during the green season adds further caution regarding the generalizability of our findings of nasal infant microbiota.[60] The interpretation of metabolomic profiling is also limited by the lack of information on the participants' dietary intake. However, the diet in the Gambia is fairly homogeneous, consisting of rice, maize and bean staples with limited fish or meat. Furthermore, in our study, ten rectovaginal swabs from women who received antibiotics during pregnancy were included in the final analysis. Although the association between a lower intestinal microbiome diversity and intrapartum antibiotics is well recognized, [61] maternal antibiotic treatment during pregnancy might be causing only short-term perturbations of the composition of infant intestinal microbiome that does not last beyond the age of one month.[62] Given that the mean interval between cessation of antibiotics and delivery was 51 days (range 7-108) for these women, the overall effect of antibiotics on the microbiome composition is uncertain. Finally, we included healthy, breastfed infants in the study. However, we did not investigate for congenital infections other than HIV, immune deficiencies and metabolic disorders, since most of these tests were not available in the study area. No infants died during the follow-up visits, and we did not identify any culture-confirmed sepsis. However,

some infants might have had other less invasive infections or medical conditions for which they did not seek medical assistance.

In conclusion, we quantified the microbial composition and compared it across subjects and body sites in healthy mother-infant pairs in the Gambia. We confirmed that breast milk microbiota composition and metabolomic profile change throughout lactation. A greater understanding of the interplay between various molecules, cells, and non-microbial components in breast milk will support future interventions to correct disrupted early life gut and nasopharyngeal colonization.

Authors' contributions

KLD, IGP and AGS conceived the idea and developed the protocol, AF and MJ processed the samples in the Gambia, AK processed the metabolomic samples in the UK, KK, SA, and AW undertook the analysis of the microbiome data, IGP and AK undertook metabolome analysis. KLD and AW provided overall direction and supervision. KK did the literature search and wrote the manuscript with input from AGS, AW, IGP and KLD. All authors read and approved the final manuscript.

Conflicts of Interest and Source of Funding

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REFERENCES

1. Stiemsma LT, Michels KB. The Role of the Microbiome in the Developmental Origins of Health and Disease. *Pediatrics*. 2018;141(4):e20172437.

2. Pammi M, Cope J, Tarr PI, et al. Intestinal dysbiosis in preterm infants preceding necrotizing enterocolitis: a systematic review and meta-analysis. *Microbiome*. 2017;5(1):31.

3. Bosch AATM, Piters WAA de S, van Houten MA, et al. Maturation of the Infant Respiratory Microbiota, Environmental Drivers, and Health Consequences. A Prospective Cohort Study. *Am J Respir Crit Care Med*. 2017;196(12):1582–1590.

4. Shao Y, Forster SC, Tsaliki E, et al. Stunted microbiota and opportunistic pathogen colonization in caesarean-section birth. *Nature*. 2019;574(7776):117–121.

5. La Rosa PS, Warner BB, Zhou Y, et al. Patterned progression of bacterial populations in the premature infant gut. *Proc Natl Acad Sci*. 2014;111(34):12522–12527.

6. Stewart CJ, Ajami NJ, O'Brien JL, et al. Temporal development of the gut microbiome in early childhood from the TEDDY study. *Nature*. 2018;562(7728):583–588.

7. Yassour M, Vatanen T, Siljander H, et al. Natural history of the infant gut microbiome and impact of antibiotic treatment on bacterial strain diversity and stability. *Sci Transl Med*. 2016;8(343):343ra81-343ra81.

8. Chu DM, Antony KM, Ma J, et al. The early infant gut microbiome varies in association with a maternal high-fat diet. *Genome Med*. 2016;8(1):77.

9. Lax S, Smith DP, Hampton-Marcell J, et al. Longitudinal analysis of microbial interaction between humans and the indoor environment. *Science (80-)*. 2014;345(6200):1048–1052.

10. Bonder MJ, Kurilshikov A, Tigchelaar EF, et al. The effect of host genetics on the gut microbiome. *Nat Genet*. 2016;48(11):1407–1412.

11. Ferretti P, Pasolli E, Tett A, et al. Mother-to-Infant Microbial Transmission from Different Body Sites Shapes the Developing Infant Gut Microbiome. *Cell Host Microbe*. 2018;24(1):133-145.e5.

12. Pannaraj PS, Li F, Cerini C, et al. Association between breast milk bacterial communities and establishment and development of the infant gut microbiome. *JAMA Pediatr*. 2017;171(7):647–654.

13. Lawson MAE, O'Neill IJ, Kujawska M, et al. Breast milk-derived human milk oligosaccharides promote Bifidobacterium interactions within a single ecosystem. *ISME J*. 2020;14(2):635–648.

14. Biesbroek G, Bosch AATM, Wang X, et al. The Impact of Breastfeeding on Nasopharyngeal Microbial Communities in Infants. *Am J Respir Crit Care Med*. 2014;190(3):298–308.

15. de Steenhuijsen Piters WAA, Binkowska J, Bogaert D. Early Life Microbiota and Respiratory Tract Infections. *Cell Host Microbe*. 2020;28(2):223–232.

16. Bardanzellu F, Fanos V, Reali A. Omics in human colostrum and mature milk: Looking to old data with new eyes. *Nutrients*. 2017;9(8):843.

17. Le Doare K, Jarju S, Darboe S, et al. Risk factors for Group B Streptococcus colonisation and disease in Gambian women and their infants. *J Infect*. 2016;72(3):283–294.

18. Layton A, McKay L, Williams D, et al. Development of Bacteroides 16S rRNA gene taqman-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. *Appl Environ Microbiol*. 2006;72(6):4214–4224.

19. Sim K, Cox MJ, Wopereis H, et al. Improved Detection of Bifidobacteria with Optimised 16S rRNA-Gene Based Pyrosequencing. Ahmed N, ed. *PLoS One*. 2012;7(3):e32543.

20. Claesson MJ, O'Sullivan O, Wang Q, et al. Comparative Analysis of Pyrosequencing and a Phylogenetic Microarray for Exploring Microbial Community Structures in the Human Distal Intestine. Ahmed N, ed. *PLoS One*. 2009;4(8):e6669.

21. Schloss PD, Westcott SL, Ryabin T, et al. Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Appl Environ Microbiol*. 2009;75(23):7537–7541.

22. Kozich JJ, Westcott SL, Baxter NT, et al. Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform. *Appl Environ Microbiol*. 2013;79(17):5112–5120.

23. Yilmaz P, Parfrey LW, Yarza P, et al. The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. *Nucleic Acids Res*. 2014;42(D1):D643–D648.

24. DeSantis TZ, Hugenholtz P, Larsen N, et al. Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. *Appl Environ Microbiol*. 2006;72(7):5069–5072.

25. Andreas NJ, Hyde MJ, Gomez-Romero M, et al. Multiplatform characterization of dynamic changes in breast milk during lactation. *Electrophoresis*. 2015;36(18):2269–2285.

26. Villaseñor A, Garcia-Perez I, Garcia A, et al. Breast Milk Metabolome Characterization in a Single-Phase Extraction, Multiplatform Analytical Approach. *Anal Chem*. 2014;86(16):8245–8252.

27. Garcia-Perez I, Posma JM, Gibson R, et al. Objective assessment of dietary patterns by use of metabolic phenotyping: a randomised, controlled, crossover trial. *Lancet Diabetes Endocrinol*. 2017;5(3):184–195.

28. Stinson LF, Payne MS, Keelan JA. A Critical Review of the Bacterial Baptism Hypothesis and the Impact of Cesarean Delivery on the Infant Microbiome. *Front Med*. 2018;5:135.

29. Chu DM, Ma J, Prince AL, et al. Maturation of the infant microbiome community structure and function across multiple body sites and in relation to mode of delivery. *Nat Med*. 2017;23(3):314–326.

30. Cabrera-Rubio R, Collado MC, Laitinen K, et al. The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. *Am J Clin Nutr*. 2012;96(3):544–551.

458 31. Moossavi S, Sepehri S, Robertson B, et al. Composition and Variation of the Human Milk
459 Microbiota Are Influenced by Maternal and Early-Life Factors. *Cell Host Microbe*.
460 2019;25(2):324-335.e4.

461 32. Biagi E, Aceti A, Quercia S, et al. Microbial community dynamics in mother's milk and
462 infant's mouth and gut in moderately preterm infants. *Front Microbiol*. 2018;9(OCT):1–
463 10.

464 33. Hunt KM, Foster JA, Forney LJ, et al. Characterization of the diversity and temporal
465 stability of bacterial communities in human milk. *PLoS One*. 2011;6(6):1–8.

466 34. Murphy K, Curley D, O'callaghan TF, et al. The composition of human milk and infant
467 faecal microbiota over the first three months of life: A pilot study. *Sci Rep*.
468 2017;7(October 2016):1–10.

469 35. Lackey KA, Williams JE, Meehan CL, et al. What's Normal? Microbiomes in Human
470 Milk and Infant Feces Are Related to Each Other but Vary Geographically: The INSPIRE
471 Study. *Front Nutr*. 2019;6(45).

472 36. Lemay-Nedjelski L, Butcher J, Ley SH, et al. Examining the relationship between
473 maternal body size, gestational glucose tolerance status, mode of delivery and ethnicity on
474 human milk microbiota at three months post-partum. *BMC Microbiol*. 2020;20(1):1–14.

475 37. Verhagen LM, Rivera-Olivero IA, Clerc M, et al. Nasopharyngeal Microbiota Profiles in
476 Rural Venezuelan Children Are Associated With Respiratory and Gastrointestinal
477 Infections. *Clin Infect Dis*. January 2020.

478 38. de Steenhuijsen Piters WAA, Sanders EAM, Bogaert D. The role of the local microbial
479 ecosystem in respiratory health and disease. *Philos Trans R Soc B Biol Sci*.
480 2015;370(1675):20140294.

481 39. Thurl S, Munzert M, Boehm G, et al. Systematic review of the concentrations of
482 oligosaccharides in human milk. *Nutr Rev*. 2017;75(11):920–933.

483 40. Azad MB, Robertson B, Atakora F, et al. Human Milk Oligosaccharide Concentrations
484 Are Associated with Multiple Fixed and Modifiable Maternal Characteristics,
485 Environmental Factors, and Feeding Practices. *J Nutr*. 2018;148(11):1733–1742.

41. Ayechu-Muruzabal V, van Stigt AH, Mank M, et al. Diversity of human milk oligosaccharides and effects on early life immune development. *Front Pediatr*. 2018;6(September):1–9.

42. Marincola FC, Noto A, Caboni P, et al. A metabolomic study of preterm human and formula milk by high resolution NMR and GC/MS analysis: Preliminary results. *J Matern Neonatal Med*. 2012;25(SUPPL. 5):62–67.

43. Spevacek AR, Smilowitz JT, Chin EL, et al. Infant maturity at birth reveals minor differences in the maternal milk metabolome in the first month of lactation. *J Nutr*. 2015;145(8):1698–1708.

44. Wu J, Domellöf M, Zivkovic AM, et al. NMR-based metabolite profiling of human milk: A pilot study of methods for investigating compositional changes during lactation. *Biochem Biophys Res Commun*. 2016;469(3):626–632.

45. Fanos V, Pintus R, Reali A, et al. Miracles and mysteries of breast milk: From Egyptians to the 3 M's (Metabolomics, Microbiomics, Multipotent stem cells). *J Pediatr Neonatal Individ Med*. 2017;6(2):5–9.

46. Shenker NS, Perdones-Montero A, Burke A, et al. Metabolomic and Metataxonomic Fingerprinting of Human Milk Suggests Compositional Stability over a Natural Term of Breastfeeding to 24 Months. *Nutrients*. 2020;12(11):3450.

47. Bardanzellu F, Fanos V, Strigini FAL, et al. Human breast milk: Exploring the linking ring among emerging components. *Front Pediatr*. 2018;6(August).

48. Fernández L, Pannaraj PS, Rautava S, et al. The Microbiota of the Human Mammary Ecosystem. *Front Cell Infect Microbiol*. 2020;10(586667).

49. Moossavi S, Atakora F, Miliku K, et al. Integrated analysis of human milk microbiota with oligosaccharides and fatty acids in the child cohort. *Front Nutr*. 2019;6(May):1–16.

50. Andreas NJ, Al-Khalidi A, Jaiteh M, et al. Role of human milk oligosaccharides in Group B Streptococcus colonisation. *Clin Transl Immunol*. 2016;5(8):e99.

51. Lin AE, Autran CA, Szyszka A, et al. Human milk oligosaccharides inhibit growth of group B Streptococcus. *J Biol Chem*. 2017;292(27):11243–11249.

514 52. Jantscher-Krenn E, Zherebtsov M, Nissan C, et al. The human milk oligosaccharide
515 disialyllacto-N-tetraose prevents necrotising enterocolitis in neonatal rats. *Gut*.
516 2012;61(10):1417–1425.

517 53. Autran CA, Kellman BP, Kim JH, et al. Human milk oligosaccharide composition predicts
518 risk of necrotising enterocolitis in preterm infants. *Gut*. 2018;67(6):1064–1070.

519 54. Dermyshi E, Wang Y, Yan C, et al. The "golden Age" of Probiotics: A Systematic
520 Review and Meta-Analysis of Randomized and Observational Studies in Preterm Infants.
521 Neonatology. 2017;112(1):9–23.

522 55. Wang Y, Li X, Ge T, et al. Probiotics for prevention and treatment of respiratory tract
523 infections in children. *Medicine (Baltimore)*. 2016;95(31):e4509.

524 56. Esposito S, Principi N. Impact of nasopharyngeal microbiota on the development of
525 respiratory tract diseases. *Eur J Clin Microbiol Infect Dis*. 2018;37(1):1–7.

526 57. Thorburn AN, McKenzie CI, Shen S, et al. Evidence that asthma is a developmental origin
527 disease influenced by maternal diet and bacterial metabolites. Nat Commun. 2015;6.

528 58. Luoto R, Ruuskanen O, Waris M, et al. Prebiotic and probiotic supplementation prevents
529 rhinovirus infections in preterm infants: A randomized, placebo-controlled trial. J Allergy
530 Clin Immunol. 2014;133(2):405–413.

531 59. Alcon-Giner C, Caim S, Mitra S, et al. Optimisation of 16S rRNA gut microbiota profiling
532 of extremely low birth weight infants. *BMC Genomics*. 2017;18(1):841.

533 60. Mika M, Mack I, Korten I, et al. Dynamics of the nasal microbiota in infancy: A
534 prospective cohort study. *J Allergy Clin Immunol*. 2015;135(4):905-912.e11.

535 61. Azad MB, Konya T, Persaud RR, et al.; CHILD Study Investigators. Impact of
536 maternal intrapartum antibiotics, method of birth and breast-feeding on gut microbiota
537 during the first year of life: a prospective cohort study. *BJOG*. 2016;123:983–993.

538 62. Zimmermann P, Curtis N. Factors influencing the intestinal microbiome during the first
539 year of life. *Pediatr Infect Dis J*. 2018;37:e315–e335.

540

Figure legends

Figure 1: Alpha diversity of maternal and infant microbiomes across different body sites, determined using Observed species (A), Shannon Index (B), or Chao1 index (C). Beta diversity of maternal and infant microbiomes across different body sites, based on non-metric Multidimensional (NMDS) scaling analysis (stress = 0.11): NMDS1 v NMDS2 (D) NMDS2 v NMDS3 (E) Maternal Rectovaginal swabs vs Infant Rectal swabs at birth from mother/infant pairs (F) Maternal Rectovaginal swabs vs Maternal Colostrum at birth from the same participants (G).

Statistics: Shapiro-Wilk normality test was performed. Kruskal-Wallis rank-sum test and pairwise Wilcoxon rank-sum test were performed to assess significance between groups. Multiple testing corrections were performed using the Benjamini - Hochburg procedure.

I_NPS_60: Infant nasopharyngeal swabs day 60; I_RS_0: Infant rectal swabs at birth; M_BM_GM_0: Breast milk at birth (colostrum); M_BM_GM_60: Breast milk on D60 (mature breast milk); M_RVS_0: Maternal rectovaginal swabs at birth.

Figure 2: Beta diversity of maternal and infant microbiomes across different body sites, based on non-metric Multidimensional (NMDS) scaling analysis (using the R vegan metaMDS function) with significant species overlaid (using the vegan envfit function, $p < 0.05$). The arrows represent species with significant correlation with the community structure and are coloured by direction. Previously described notable taxa are labelled.

I_NPS_60: Infant nasopharyngeal swabs day 60; I_RS_0: Infant rectal swabs at birth; M_BM_GM_0: Breast milk at birth (colostrum); M_BM_GM_60: Breast milk on D60 (mature breast milk); M_RVS_0: Maternal rectovaginal swabs at birth.

Figure 3: Relative abundance of genera, with greater than 10% relative abundance in at least one sample for each group (A). Relative abundance of Staphylococcus and Streptococcus for each Group (B).

I_NPS_60: Infant nasopharyngeal swabs day 60; I_RS_0: Infant rectal swabs at birth; M_BM_GM_0: Breast milk at birth (colostrum); M_BM_GM_60: Breast milk on day 60 (mature breast milk); M_RVS_0: Maternal rectovaginal swabs at birth.

Figure 4: 1H-NMR RM-MCCV-PLS scores plot model comparing Breast milk samples at D0 (Ciano dots) with D60 of life (red crosses). The top part of the panel gives the Kernel Density Estimate (KDE) of each group's predicted scores. The bottom part shows the predicted scores (Tpred) from MCCV for each sample (A). Fragment of the average 600 MHz 1H-NMR breast milk spectrum to visualised some identified labelled metabolites (B). Manhattan plot showing -log10(q) × sign of regression coefficient (β) of the RM MCCV–PLS model for the 16,000 spectral variables. Red peaks represent the variables that significantly increased over time (D60), and blue peaks represent the variables that significantly decreased over time (D0) (C). GC-MS RM-MCCV-PLS scores plot model comparing Breast milk samples at D0 (Ciano dots) with D60 of life (red crosses). The top part of the panel gives the Kernel Density Estimate (KDE) of each group's predicted scores. The bottom part shows the predicted scores (Tpred) from MCCV for each sample (D). Manhattan plot showing -log10(q) × sign of regression coefficient (β) of the RM MCCV–PLS model for the 16,000 spectral variables. Red dots represent the variables (metabolites) that significantly increased over time (D60, and blue dots represent the variables that significantly decreased over time (D0) (E). A p-value was calculated for each variable. P-values were adjusted for multiple testing using the Storey-Tibshirani False Discovery Rate (FDR, q-value).

Legends for supplemental digital content

Supplemental Digital Content 1. Figure: Sequencing reads per sample type (A). Comparison of number of samples included in the analysis at different sequencing depths (0-10000) (B).

I_NPS_60: Infant nasopharyngeal swabs day 60; I_RS_0: Infant rectal swabs at birth; M_BM_GM_0: Breast milk at birth (colostrum); M_BM_GM_60: Breast milk on day 60 (mature breast milk); M_RVS_0: Maternal rectovaginal swabs at birth

Supplemental Digital Content 2. Figure: Flowchart of samples collected from study participants.

596 I_NPS_60: Infant nasopharyngeal swabs day 60; I_RS_0: Infant rectal swabs at birth;
597 M_BM_GM_0: Breast milk at birth (colostrum); M_BM_GM_60: Breast milk on day 60
598 (mature breast milk); M_RVS_0: Maternal rectovaginal swabs at birth.

599

Table 2. Increase in the relative abundance of 33 breast milk metabolites between birth day and day 60 of life

Metabolite	**Metabolite class**	***P value***
palmitic acid	Fatty acids	< .001
lauric acid	Fatty acids	< .001
myristic acid	Fatty acids	.02
capric acid	Fatty acids	.03
trans-13-octadecenoic acid	Fatty acids	.05
dioctyl phthalate	Phthalic Acid and Derivatives	< .001
lactobionic acid	Sugar acids	< .001
glucoheptonic acid	Sugar acids	.02
lactobionic acid	Sugar acids	.06
m-toluic acid	Organic acids	.05
glycolic acid	Organic acids	.05
Methyl Stearate	Fatty acid esters	.04
D-allose	Monosaccharides	< .001
rhamnose	Monosaccharides	< .001
talose	Monosaccharides	< .001
D (+)altrose	Monosaccharides	.001
D-mannose	Monosaccharides	.002
D-allose	Monosaccharides	.02
L- sorbose	Monosaccharides	.03
psicose	Monosaccharides	.05

beta-gentiobiose	Disaccharides	< .001
cellobiose	Disaccharides	< .001
methyl beta-D-galactopyranoside	Saccharides	.001
allo-inositol	Carbocyclic sugars	.002
sedoheptulose anhydride monohydrate	Anhydro sugars	.01
glycerol	Sugar alcohols	< .001
1,5-anhydro-D-sorbitol	Fatty alcohols	.009
conduritol epoxide	Fatty alcohols	.02
2,3-butanediol	Alcohols	.01
xanthotoxin	Coumarins and derivatives	< .001
ribulose-5-phosphate	Sugar phosphates	.05
glycerol 1-phosphate	Sugar phosphates	.05
Eicosane	Alkanes	.06

Table 1. Demographic and Clinical Characteristics of Gambian mothers and infants from whom milk samples and swabs were successfully collected and sequenced.	
Women [N=107]	
Age in years: median (IQR)	24 (20-29)
Gravida: median (IQR)	2 (1-4)
Parity: median (IQR)	1 (0-3)
Weight in kg: median (IQR)	64.6 (58-75.5)
Delivery mode: % vaginal	100
Antibiotics during pregnancy: % received antibiotics	15.8
Haemoglobin in g/dl: median (IQR)	11 (10.1-11.4)
Group B Streptococcal colonisation at delivery: % positive	33.7
Infants [N=35]	
Gestation in weeks: median (IQR)	38 (36-40)
Birth weight in kg: median (IQR)	3.4 (3.0-3.6)
Sex % female	52.3

Figure 1

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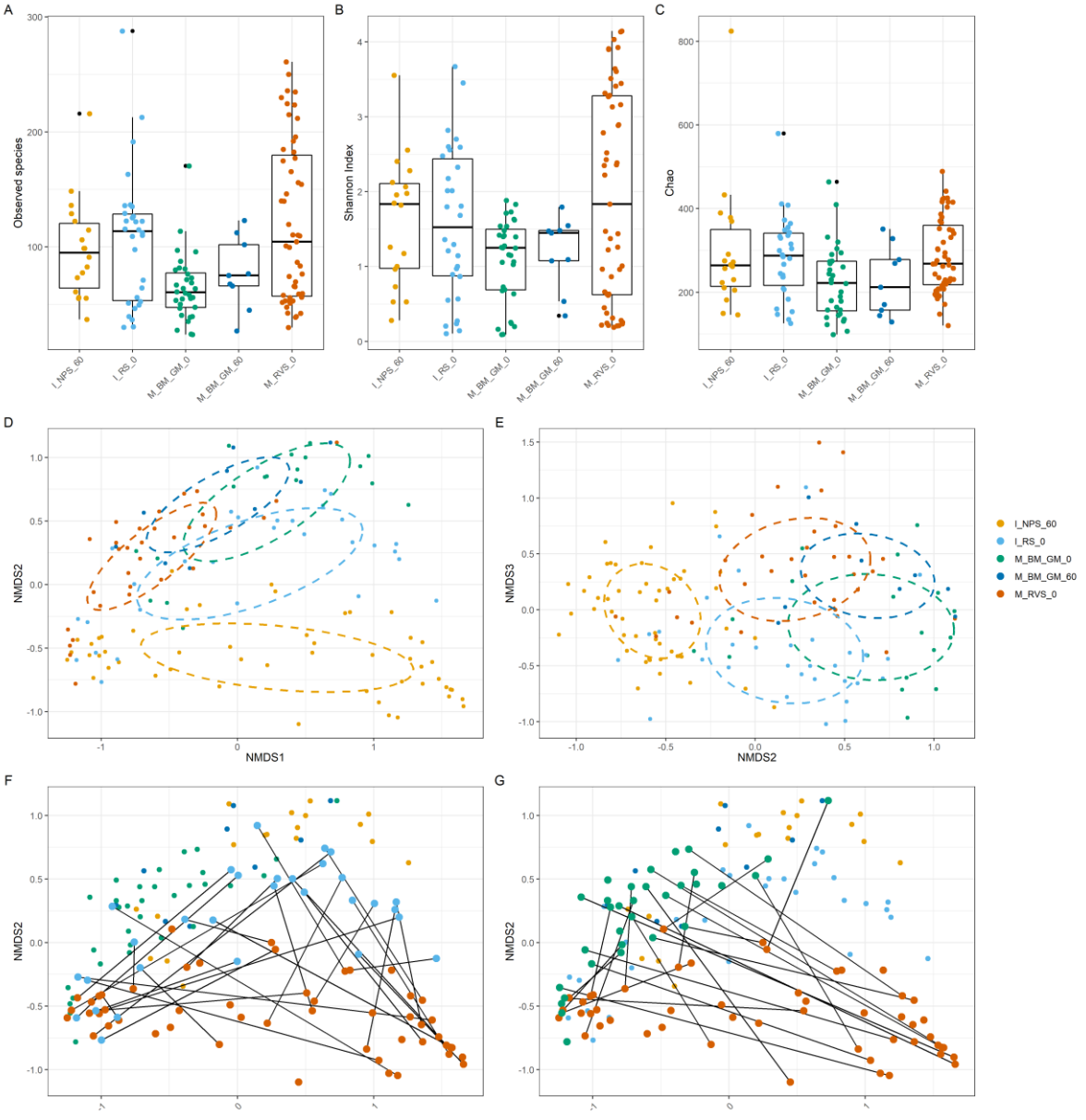

Figure 2

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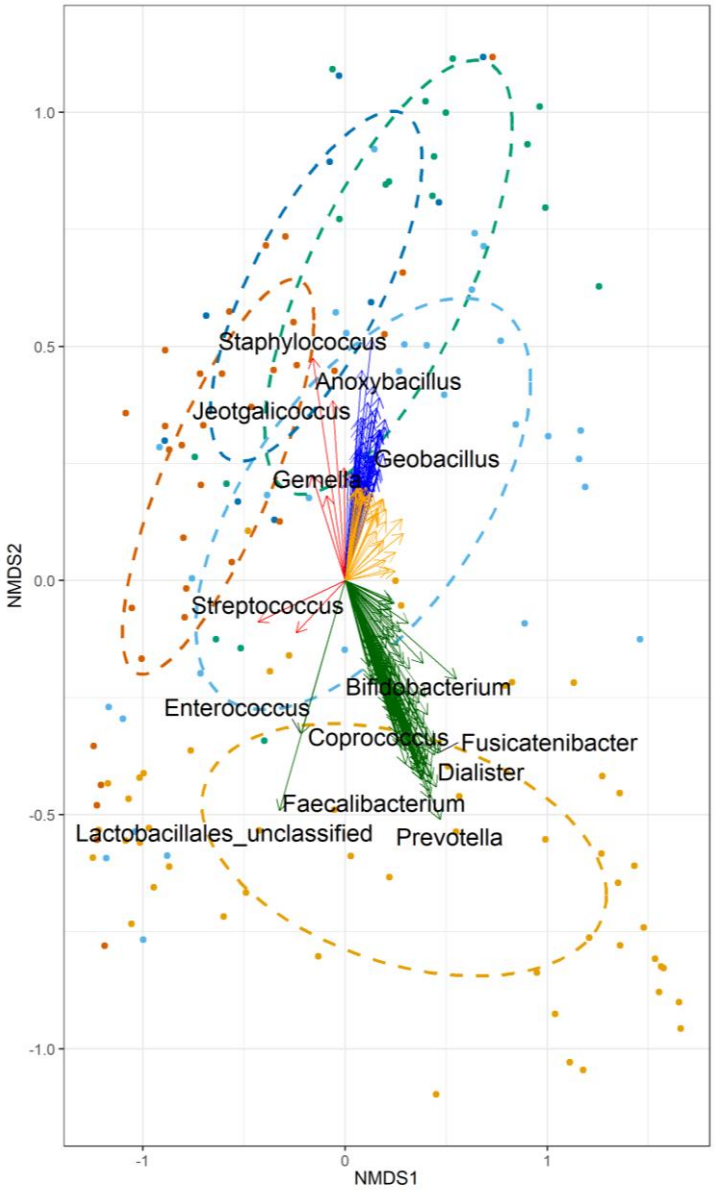

Figure 3

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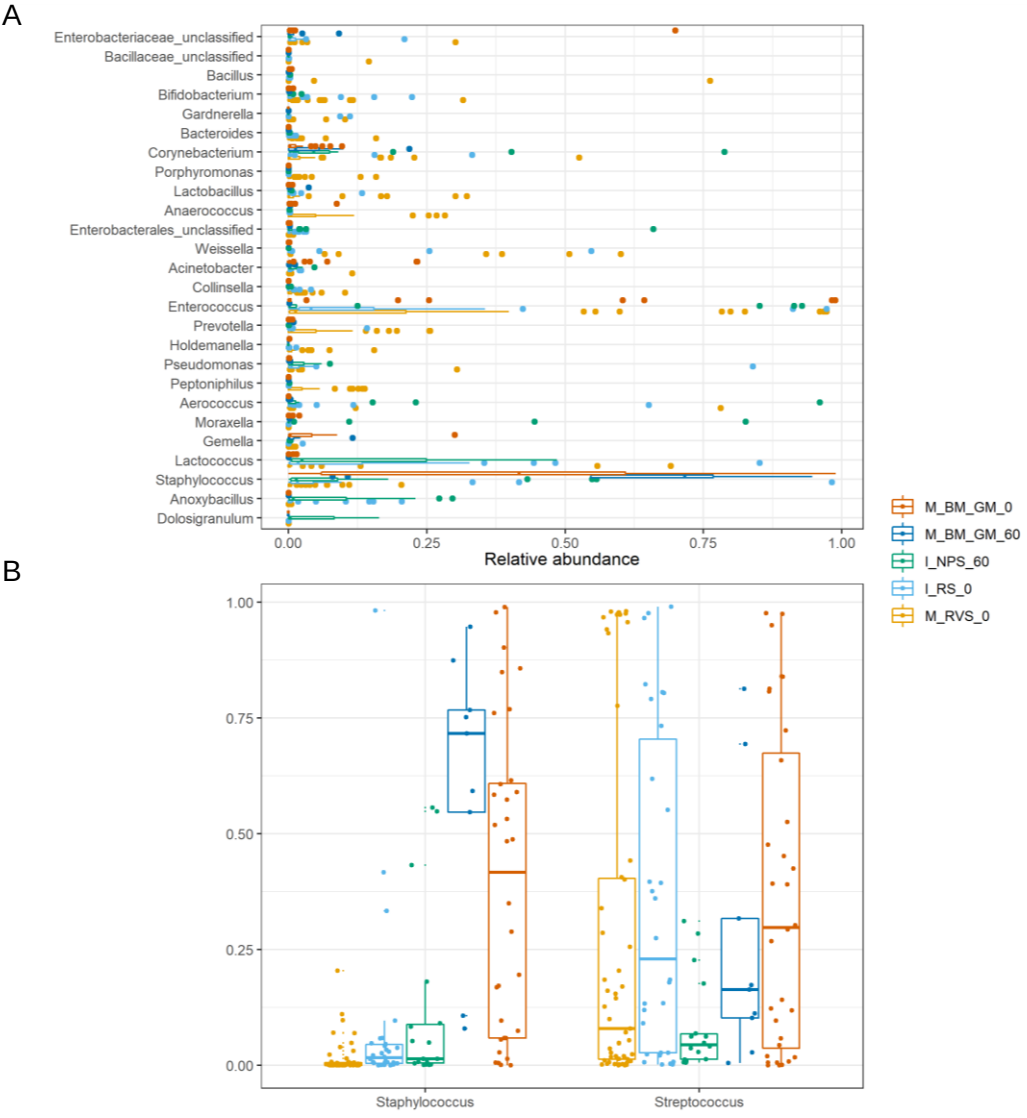

Figure 4

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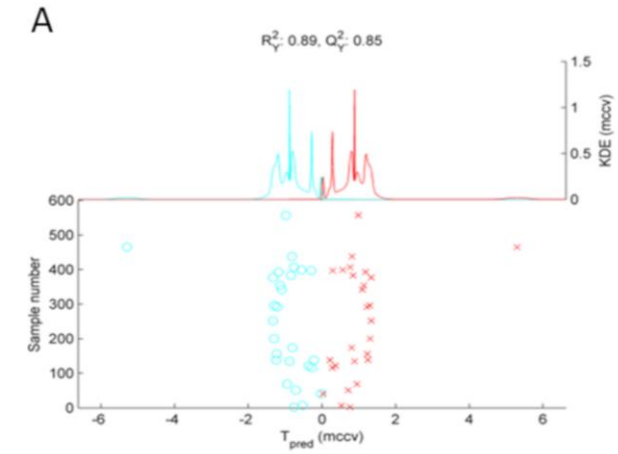

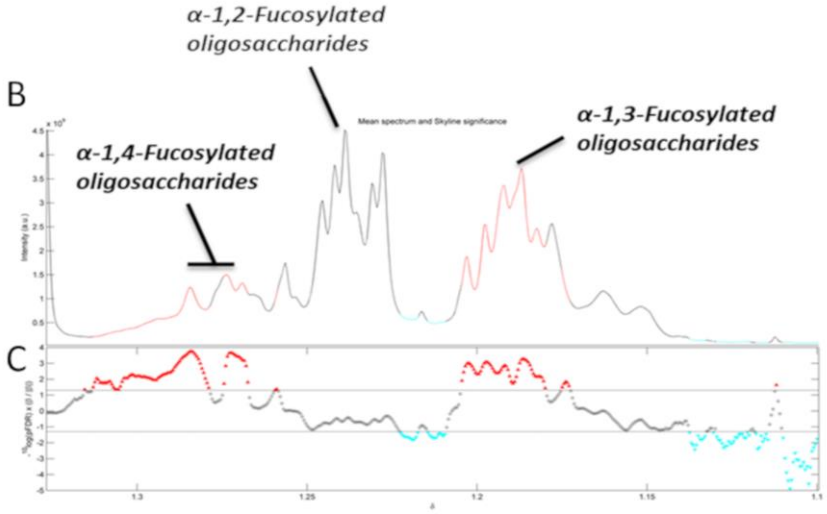

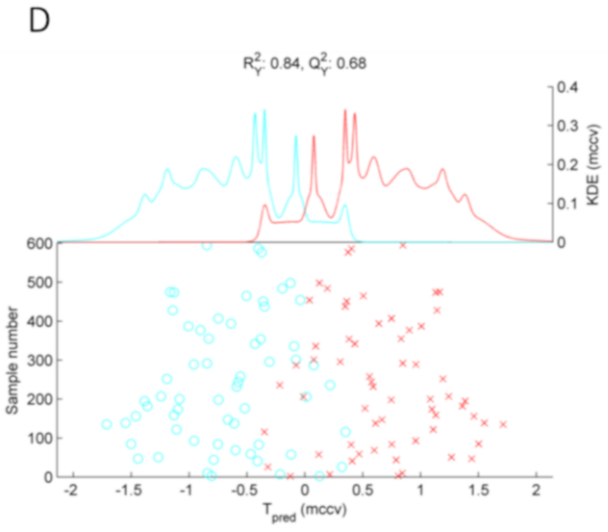

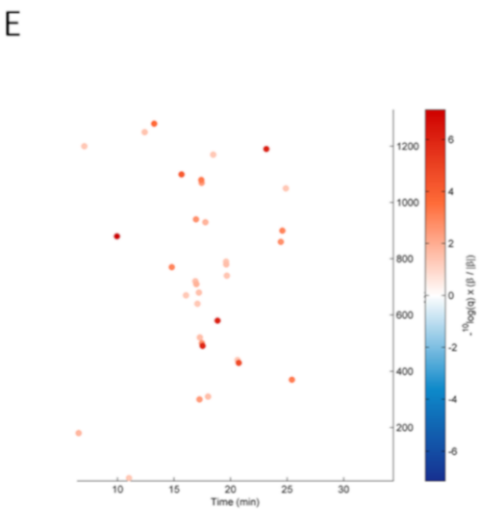

STROBE Statement

Checklist of items that should be included in reports of observational studies

Section/Topic	Item No	Recommendation	Reported on Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	4
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	5
Objectives	3	State specific objectives, including any prespecified hypotheses	5
Methods			
Study design	4	Present key elements of study design early in the paper	5,6
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5,6
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	5,6
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	NA
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	5-7
Data sources/measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	5-7
Bias	9	Describe any efforts to address potential sources of bias	14
Study size	10	Explain how the study size was arrived at	NA
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	7
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	7
		(*b*) Describe any methods used to examine subgroups and interactions	7
		(*c*) Explain how missing data were addressed	7
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	NA
		(*e*) Describe any sensitivity analyses	NA

Section/Topic	Item No	Recommendation	Reported on Page No
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	9, Supplemental Digital Content 1
		(b) Give reasons for non-participation at each stage	Supplemental Digital Content 1
		(c) Consider use of a flow diagram	Supplemental Digital Content 1
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	9, Table 1
		(b) Indicate number of participants with missing data for each variable of interest	Supplemental Digital Content 1
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	NA
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	NA
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	9-11
		(*b*) Report category boundaries when continuous variables were categorized	NA
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	NA
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	9-11
Discussion			
Key results	18	Summarise key results with reference to study objectives	11-13
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	14

Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	11-13
Generalisability	21	Discuss the generalisability (external validity) of the study results	14
Other Information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	15

**Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.*

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.