Investigation of associations between the neonatal gut microbiota and severe viral lower respiratory tract infections in the first 2 years of life: a birth cohort study with metagenomics

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Summary

Background Early-life gut microbiota affects immune system development, including the lung immune response (gut–lung axis). We aimed to investigate whether gut microbiota composition in neonates in the first week of life is associated with hospital admissions for viral lower respiratory tract infections (vLRTIs).

Methods The Baby Biome Study (BBS) is a prospective birth cohort, which enrolled mother–baby pairs between Jan 1, 2016, and Dec 31, 2017, at three UK hospitals. In the present study, we only included BBS babies with a sequenced first-week stool sample and successful data linkage. Stool was collected in the first week of life for shotgun-metagenomic sequencing. We examined the following microbiota features: alpha diversity (Chao1, Shannon, and Simpson indices) and community structures (cluster-partitioning against medoids method). The participants were followed up through linkage to the Hospital Episode Statistics-Admitted Patient Care (HES-APC) database to determine vLRTI hospital admission incidence in the first 2 years of life. We used Poisson mixed-effects models for univariable and multivariable analyses to evaluate the association between microbiota features and vLRTI hospital admission incidence, adjusting for confounders identified through direct acyclic graphs.

Findings 3305 (95%) of the 3476 BBS-enrolled babies for whom consent to data linkage was obtained were included in the present study. 1111 (34%) babies had a first-week sequenced stool sample, of whom 1082 (97%; 564 born vaginally and 518 born by caesarean section) were successfully linked to HES-APC, and had median follow-up of 2·0 years (IQR 1·4–2·9). Most babies were born at term (996 [92%] ≥37 weeks gestational age and 1070 [99%] >35 weeks gestational age) and healthy (1050 [97%] had no comorbidities), and 520 (48%) were female and 562 (52%) were male. Higher first-week gut microbiota alpha diversity was associated with reduced rates of vLRTI hospital admission (Chao1 Index adjusted hazard ratio [HR] 0·92 [95% CI 0·85–0·99]; Shannon Index adjusted HR 0·57 [0·33–0·98]; and Simpson Index adjusted HR 0·36 [0·11–1·20]). Three microbiota clusters were identified. Cluster 1 had a mixed composition and cluster 2 was dominated by *Bifidobacterium breve*, with both clusters observed in babies born vaginally and by caesarean section. Cluster 3 was found only in vaginally born babies and was dominated by *Bifidobacterium longum*. Having cluster 1 (mixed) or cluster 2 (*B breve* dominated) was independently associated with increased rates of vLRTI hospital admission compared with cluster 3 (*B longum* dominated; cluster 1 [mixed] 3·05 [1·25–7·41] and cluster 2 [*B breve* dominated] 2·80 [1·06–7·44]).

Interpretation We report observational evidence that first-week gut microbiota differences are associated with clinically severe vLRTI in young children. This study identified bacterial species that could be of interest for vLRTI prevention. This finding has important implications for the design of future research and intervention strategies.

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Introduction

Emerging evidence suggests that early-life gut microbiota composition has profound effects on later-life health[1,2] through immune development mediation.[3,4] Immediately after birth, babies are exposed to microorganisms that colonise the newborn gut and form the microbiota.[2,4] We have previously shown that birth mode, among other factors such as antibiotics and feeding mode, is a key determinant of the neonatal gut microbiota composition, with lower abundance of *Bacteroides* strains and higher abundance of opportunistic pathogens observed in babies born by caesarean section compared with babies born vaginally.[5] However, associations between early-life gut microbiota composition and subsequent health consequences were not explored.[5]

The long-reaching immune effects of the early gut microbiota composition on respiratory immunity are thought to occur through mechanisms involving bacterial ligands, metabolites, and migrating immune cells.[6,7] This interorgan connection is referred to as the gut–lung axis.[6,7] Animal models have focused on how gut microbiota composition affects lung immunity in the context of viral

Research in context

Evidence before this study

We conducted a systematic review following PRISMA guidelines to identify longitudinal studies using next-generation sequencing methods that explored the association between gut microbiota composition during the first 12 months of life and respiratory tract infections, wheezing, or asthma up to the age of 18 years. We searched Embase, MEDLINE, Cochrane, Web of Science, and Scopus using four broad search terms: "infancy", "intestine", "microbiota", and "respiratory disease" for original human studies published from Jan 1, 2010, to April 27, 2021 with full text available in English (full search details and links to the published manuscripts are in the appendix p 3). We identified 11 studies: eight were cohort and three case–control studies. All studies used amplicon sequencing targeting the 16S rRNA gene for gut microbiome determination, which has limited taxonomic resolution (ie, species level and strain level) compared with state-of-the-art shotgun metagenomics. Most studies evaluated wheezing or asthma, and diagnosis was ascertained using parental reports in all but one study. The main design limitations included small sample sizes (only three studies had more than 700 participants), potential selection bias, and residual confounding. On Sept 10, 2023, we did an updated search using the same search criteria and identified a longitudinal study by Moroishi and colleagues exploring the association between gut microbiota composition at 6 weeks of age, primarily using 16S rRNA sequencing (n=465), and respiratory infection in the first year of life. Overall, observational data from these 12 studies suggest that lower alpha diversity and lower relative abundance of some gut-commensal bacterial genera in the first year of life are associated with subsequent respiratory disease, especially asthma in children aged 1–6 years. Whether this association is causal is not yet clear. There is much less evidence for respiratory infections, with only two studies exploring this specifically. The association between the neonatal gut microbiota composition and subsequent development of viral lower respiratory tract infections (vLRTIs), especially severe disease, in children younger than 2 years is therefore not yet well understood.

Added value of this study

The Baby Biome Study (BBS) is one of the largest birth cohorts using shotgun metagenomics for gut microbiota determination, allowing bacterial resolution down to a species level and thereby providing state-of-the-art measurement of gut microbiota as an exposure. BBS has the added benefit of collecting stool samples from the first week of life, whereas most studies have collected samples at age 3 months or later. This study is the first to evaluate the association between the neonatal gut microbiota composition and hospital admissions, using National Health Service Hospital Episode Statistics-Admitted Patient Care data linkage with less than 5% loss to follow-up. The analyses were guided by direct acyclic graphs built a priori for this research question, to model the assumed causal associations between variables and to be explicit about limitations regarding causal inference from observed associations. Additionally, our study design mitigates concerns about reverse causality. This study, to our knowledge, is the first to show that first-week gut microbiota composition was independently associated with reduced rates of viral lower respiratory tract infections hospital admission in the first 2 years of life for a subgroup of babies delivered vaginally (with higher relative abundance of *Bifidobacterium longum*, *Bifidobacterium bifidum*, *Bifidobacterium adolescentis*, and *Bacteroides dorei*, and lower relative abundance of *Enterococcus faecalis* and *Bifidobacterium breve*) compared with all other babies.

Implications of all the available evidence

This longitudinal study provides high-quality observational evidence of an association between first-week gut microbiota composition and the clinical infectious disease outcome of vLRTI hospital admission. Some intervention strategies designed to modify the early gut microbiota composition for the prevention of subsequent disease focus on exposing babies delivered by caesarean section to a more vaginal-like environment. However, in our study, not all babies born vaginally showed lower rates of disease, with some showing similar rates of vLRTI admissions to babies born by caesarean section—ie, neonates with mixed or *B breve* dominated gut microbiota, when compared to vaginally born babies who had a first-week *B longum* dominated gut microbiota. These findings challenge the idea that vaginal birth is always associated with lower rates of disease, at least regarding vLRTI hospital admissions, hinting that the link is more nuanced. Our study suggests that some bacterial species or combinations of species, as early as the first week of life, could be of interest for vLRTI prevention interventions. These components should be further explored in animal studies, using established germ-free and gnotobiotic mouse models to investigate the role of these neonatal bacterial species, their genetic components, or their colonisation order (ie, priority effects) on immune system development and susceptibility to vLRTI, as well as for the design of future intervention studies.

See Online for appendix

respiratory infections.[8,9] A study published in 2018 showed that mice with higher abundance of gut *Bifidobacterium* and *Bacteroides* (promoted by a high-fibre diet) showed improved survival when challenged with influenza virus.[8] These mice showed priming of bone marrow haematopoiesis for generation of macrophages with limited chemokine production, preventing excessive airway neutrophil influx, and an enhanced lung CD8 T-cell response.[8] However, longitudinal human studies exploring the association between early gut microbiota composition and childhood viral lower respiratory tract infections (vLRTIs) are scarce.[10]

vLRTIs are the main cause of hospital admission in infants (age <1 year),[11] and a balanced immune response has been shown to influence disease pathogenesis.[12] Our study aimed to test the hypothesis that disruption of gut microbiota colonisation in infants might affect infection-related clinical outcomes.[13] Specifically, we explored the

association between early gut microbiota composition of Baby Biome Study (BBS) participants and vLRTI hospital admissions during the first 2 years of life.

Methods

Study design and participants

In this prospective study we used data from BBS birth cohort participants. The BBS enrolled all mother–baby pairs who consented between Jan1, 2016, and Dec 31, 2017, at three UK hospitals (Barking, Havering, and Redbridge University Hospitals NHS Trust, London; University Hospitals Leicester NHS Trust, Leicester; and University College London Hospitals NHS Foundation Trust, London). Details on BBS methods are published elsewhere.[5] BBS (previously the Life Study enhancement pilot study) was approved by the National Health Service (NHS) London – City and East Research Ethics Committee (REC reference 12/LO/1492) and was performed in compliance with all relevant ethical regulations.

Only participants from BBS who consented in writing to NHS data linkage, and who had successful linkage and a sequenced first-week stool sample were included in the present study. Baseline data were collected at enrolment by BBS research midwives using an electronic clinical record form, including maternal characteristics (age, ethnicity, socioeconomic status using the Index of Multiple Deprivation, asthma, pregnancy smoking, and parity), birth clinical data (birth mode, gestational age, birthweight, and labour antibiotics), and neonate characteristics (antibiotics after birth, admission to neonatal unit, congenital comorbidities, and breastfeeding).

Baby stool samples were collected at days 4, 7, and 21 of life, and were collected by health-care staff if babies were in hospital. Otherwise, kits for stool collection, which were returned by post, and a short questionnaire for mothers to complete at the time of sample collection, including questions on breastfeeding, were provided at hospital discharge.

Stool sample processing and shotgun-metagenomic sequencing

Stool samples underwent DNA extraction using the FastDNA Spin Kit for Soil DNA Extraction (MP Biomedicals, Santa Ana, CA, USA), as previously described[5] and detailed in the BBS collection and processing protocol.[14] 1968 samples with more than 100 ng DNA were sequenced on the HiSeq 2500 v4 (2×125 bp) and HiSeq 4000 (2×150 bp) platforms (Illumina, San Diego, CA, USA). As previously described,[5,15] raw read sequences were quality controlled using Trimmomatic[16] and screened for potential human contaminants by aligning against the human reference genome (GRCh38) using Bowtie2 v2.3.5.[17] A mean of 9·6 million paired-end reads (95% CI 8·6–10·7 million; 89·7% of the raw reads) per sample passed decontamination and quality-trimming steps and proceeded to downstream analysis. Taxonomic classification of the quality-controlled reads was performed against the representative genomes from the Genome Taxonomy Database (release RS207) using Kraken v2.1.2[18] and Bracken v2.8.[19] A 0·001% relative abundance, corresponding to approximately 100 paired-end reads, was applied as the metagenomic species detection threshold.

For more on the **Genome Taxonomy Database** see https://data.gtdb.ecogenomic.org/releases/release207/207.0/

Follow-up data collection

Participants were followed up using electronic record linkage to the NHS Hospital Episode Statistics-Admitted Patient Care (HES-APC) database. HES-APC includes all NHS inpatient admission records in England.[20] NHS Digital used a stepwise deterministic linkage process using personal identifiers.[21] A match rank determining matching quality was individually assigned (1 highest and 9 lowest; appendix p 4). Participants with a rank of more than 2 were dropped to minimise potential misclassification. HES-APC data include information on diagnoses (up to 20) classified using ICD-10 codes.[20] Diagnosis ICD-10 codes 1 and 2 were used to determine vLRTI admissions (appendix p 5).

Cohort construction

Participants were followed up from birth to the end of follow-up (April 5, 2019) or death. Participants with vLRTI admission before day 10 of life were excluded to avoid reverse causality. Multiple vLRTIs were stratified by episode order, following the conditional risk set model. Time to event was measured from the previous event. Participants were censored from follow-up during hospital stays and re-entered at discharge.

Statistical analysis

Analyses were performed using a gut microbiota relative abundance species matrix per sample. First-week gut microbiota alpha diversity (using Chao1, Shannon, and Simpson indices) was compared by vLRTI admission status. A species-level clustering approach (using the partitioning against medoid method;[22] appendix p 23) was used to categorise first-week gut microbiota community structure and compare by vLRTI admission status, after stratifying by birth mode. Variable distributions were summarised in the study population and p values calculated using χ^2 test for categorical variables, and *t* test (normal distribution) or Mann–Whitney *U* test (non-normal distribution) for continuous variables. Rates of vLRTI hospital admissions were estimated per 1000 person-years. Participant characteristics, including rates of vLRTI hospital admissions, were compared between BBS participants with and without first-week stool samples.

Univariable and multivariable Poisson models were used to test the association between (1) first-week alpha diversity and (2) microbiota cluster composition as the exposure, and incidence of vLRTI hospital admissions as the outcome, for both first and all admissions. Analyses with all admission events (ie, not just first admissions) were modelled using a gamma distribution (frailty gamma) and mixed effects. Hazard ratios (HRs) with 95% CIs were reported. Confounders in multivariable models were selected based on a direct acyclic graph generated a priori that was

underpinned by a thorough literature review of the evidence of association with the exposure or outcome, or both, and included environmental, maternal, perinatal, and postnatal variables (appendix p 20). All multivariable analyses were adjusted for mode of birth and environmental, maternal, perinatal, and postnatal variables. In multivariable models, complete case analyses were performed, excluding variables with more than 15% missing data. Variables with 10–15% missingness were imputed using multiple imputation (appendix p 23). Analyses were performed using Stata v16–17 and R Studio (version 12.0).

The association between presence of specific bacterial species and vLRTI hospital admissions was explored. A threshold of 0·01% relative abundance was used to generate a binary variable for each participant and species (present or absent). Bacteria present at this threshold in at least 5% of participants were taken forward. Univariable Poisson models were built for each species, exploring the association with vLRTI hospital admission. p values were adjusted for false discovery rate using the Benjamini–Hochberg procedure. Only bacterial species with an adjusted p value of less than 0·20 were explored in multivariable analyses. Multivariable Poisson models were performed as described earlier.

Finally, the differential abundance of individual bacterial species by vLRTI hospitalisation status was tested using two methods: MaAslin2 (microbiome multivariable associations with linear models)[23] and ALDEx2 (analysis of differential abundance taking sample variation into account) including bacteria present in at least 10% of samples[24] (appendix p 23).

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

3305 (95%) of the 3476 BBS participants consented to NHS data linkage and were included in the present study. Of the 3305 mother–baby pairs enrolled in this study, 1151 (35%) babies provided at least one neonatal stool sample (at 4, 7, or 21 days of life) that underwent sequencing. Most samples were from day 7 (n=1047), and only first-week samples (one per patient, prioritising day 7 samples) were included in subsequent analyses (figure 1). 1082 babies had a first-week stool sample (ie, day 4 or 7) and successful NHS HES-APC data linkage with a match rank of 1 or 2 (figure 1).

All 1082 babies with data linkage were born after 30 weeks of gestational age and 996 (92%) were born at term (≥37 weeks of gestational age; 1070 [99%] were born after 35 weeks of gestational age) and were healthy (32 [3%] babies had a recorded comorbidity at birth per ICD-10 codes). 520 (48%) babies were female and 562 (52%) were male. Overall, 518 (48%) babies had caesarean section births (appendix p 6). At time of stool collection, 432 (40%) babies were exclusively breastfed, 148 (14%) were exclusively bottle fed, and 388 (36%) were mixed fed. Most variables had less than 1% missing data, but feeding mode at stool collection was missing for 114 (11%) babies (appendix pp 6–9).

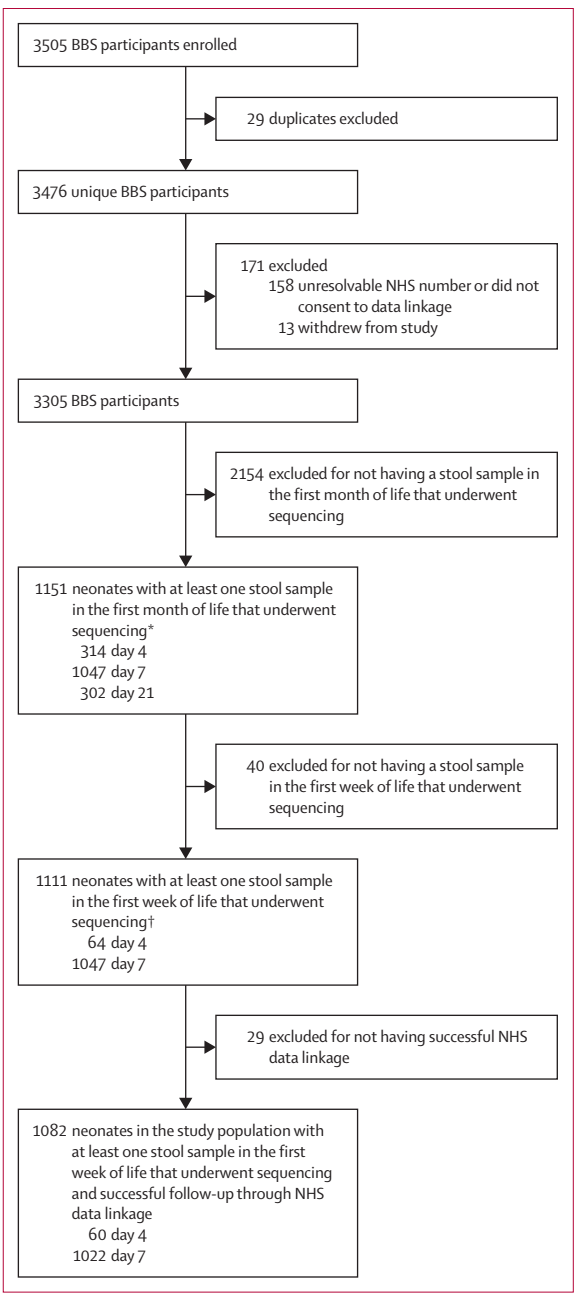

Figure 1: **Study participant flowchart**
The 1082 participants included in analyses represent only participants with successful NHS data linkage with a match rank of 1 or 2. BBS=Baby Biome Study. NHS=National Health Service. *Neonates could provide more than one stool sample. †One sample per patient, prioritising day 7 samples.

79 (7%) of 1082 babies had at least one vLRTI hospital admission episode during follow-up (mean 2·0 years, SD 0·58, range 1·4–2·9), mostly in the first year of life (44 [56%]). Two participants died during the follow-up period, and one was removed from the analyses because a vLRTI admission

occurred before day 10 of life. In total, there were 105 vLRTI admission episodes, with 19 (24%) participants having two or more episodes. The rate of first vLRTI hospital admissions was 36·2 (95% CI 29·0–45·1) per 1000 person-years and the rate of all vLRTI hospital admissions was 46·2 (38·3–55·9) per 1000 person-years.

Participant characteristics varied (including rates of vLRTI hospital admission) by first-week stool sample availability (1082 in the study population *vs* 2223 BBS participants without first-week stool sample; appendix pp 6–9). To explore the possibility of selection bias, the association between baseline variables and first vLRTI hospital admission was compared in the 1082 study participants versus all BBS participants with NHS data linkage (3253 of 3305). No new significant associations were observed in the 1082 study participants (appendix pp 10–12).

There were 878 bacterial species identified in 1082 first-week stool samples (appendix p 25). Higher alpha diversity was independently associated with reduced rates of vLRTI hospital admissions (table 1). Sensitivity analyses exploring only the first vLRTI hospital admission showed similar trends, although the adjusted HR 95% CIs for Chao 1 and Shannon indices crossed 1 (appendix p 14).

Three neonatal gut microbiota clusters were assigned to babies born vaginally (n=564), and two clusters were assigned to babies born via caesarean section (n=518; figure 2). Clusters 1 (n=214, 38%) and 2 (n=108, 19%) in babies born vaginally were broadly similar in composition to clusters 1 (n=451, 87%) and 2 (n=67, 13%) in babies born by caesarean section (appendix p 23). However, most vaginally born babies were grouped in a third cluster, (cluster 3, n=242, 43%), which was only found in vaginal births. Cluster 1 had a mixed composition of multiple species, and clusters 2 and 3 were dominated by single species.

In cluster 1, *Enterococcus faecalis, Escherichia coli, Streptococcus salivarius,* and *Staphylococcus epidermidis* had a median relative abundance of 49·0% (IQR 38·6–59·4) for babies born vaginally and 41·9% (34·8–48·9) for babies born by caesarean section. Cluster 2 was dominated by *Bifidobacterium breve. B breve* had a median relative abundance of 67·5% (40·8–80·1) for babies born vaginally (n=108) and of 74·9% (35·6–89·5) for babies born by caesarean section (n=67). Cluster 3, only found in babies born vaginally, was dominated by *Bifidobacterium longum*, albeit to a lesser extent (median relative abundance 35·9% [18·4–59·2]). After adjusting for mode of birth, babies in cluster 3 showed higher relative abundance of *B longum* (coefficient 4.97 [SE 0·21]), *Bifidobacterium bifidum* (1·65 [0·17]), *Bifidobacterium adolescentis* (1·05 [0·13]), *Parabacteroides distasonis* (1·04 [0·17]), *Bacteroides dorei* (0·92 [0·15]), and lower relative abundance of *E faecalis* (–1·57 [0·26]) and *B breve* (–1·80 [0·24]) than did babies in other clusters (adjusted p<0·001 for all; appendix pp 22–23). Hereafter, the clusters will be referred to as cluster 1 (mixed), cluster 2 (*B breve* dominated), and cluster 3 (*B longum* dominated). To match these findings with the previous alpha diversity findings, all alpha diversity measures were compared by cluster and birth mode (appendix p 13). Babies born vaginally who were from cluster 3 (*B longum* dominated) had higher alpha diversity (richness and evenness) than all other babies, although 95% CIs overlapped.

	Univariable (n=1082)		Multivariable* (n=826)		Multivariable,* multiple imputation† (n=918)	
	HR (95% CI)	p value	Adjusted HR (95% CI)	p value	Adjusted HR (95% CI)	p value
Chao1 Index	0·91 (0·85–0·98)	0·011	0·94 (0·87–1·01)	0·069	0·92 (0·85–0·99)	0·027
Shannon Index	0·56 (0·34–0·94)	0·029	0·54 (0·30–0·97)	0·038	0·57 (0·33–0·98)	0·041
Simpson Index	0·37 (0·12–1·16)	0·089	0·28 (0·08–1·04)	0·058	0·36 (0·11–1·20)	0·097

HR=hazard ratio. IMD=index of multiple deprivation. vLRTI=viral lower respiratory tract infection. *Adjusted for hospital, delivery mode, feeding mode at 1 week of age, sex, season of birth, gestational age, birthweight, maternal ethnicity, maternal age, maternal asthma, IMD, mother smoked during pregnancy, mother received antibiotics during labour, baby received resuscitation after birth, parity, twin pregnancy, baby received antibiotics after birth, baby admitted to neonatal unit, and if baby had comorbidities recorded at birth. †Multiple imputation: feeding mode at 1 week was imputed using logistic regression. For the 129 missing values, 115 were imputed. Variables included in the imputation model were admission to neonatal unit, hospital, birthweight, gestational age, antibiotics after birth, antibiotics in labour, parity, maternal age, smoke exposure during pregnancy, IMD, feeding in the fifth h of life, birth season, resuscitation at birth, and vLRTI. The twin pregnancy variable was collinear and was dropped from the model.

Table 1: **Association of first-week gut microbiota alpha diversity and vLRTI hospital admissions**

Overall, there was no evidence that rates of vLRTI hospital admissions differed between babies born vaginally versus by caesarean section (p=0·59; table 2). However, the proportion of babies with at least one vLRTI hospital admission was lowest among babies in cluster 3 (*B longum* dominated; ten [4%] of 242, compared with 56 [8%] of 665 in cluster 1 [mixed], and 13 [7%] of 175 in cluster 2 [*B breve* dominated; figure 2B]). In all babies, after adjusting for mode of birth and all other confounders, having a cluster 2 (*B breve* dominated) or cluster 1 (mixed) first-week gut microbiota composition, compared with having a cluster 3 (*B longum* dominated) composition, was associated with an increased rate of vLRTI hospital admission (table 3). Multivariable models stratified by mode of birth showed that, for babies born vaginally, babies in cluster 2 (*B breve* dominated) and cluster 1 (mixed) also had higher rates of vLRTI hospital admission compared with babies in cluster 3 (*B longum* dominated), although the adjusted HR 95% CI for cluster 2 (*B breve* dominated) crossed 1. There was no evidence of an association in babies born by caesarean section (table 2). Sensitivity analyses restricted to first vLRTI hospital admission episodes showed similar trends (appendix p 15).

The presence of 63 bacterial species were explored for their univariable association with vLRTI hospital admissions, and two bacterial species, *B dorei* and *Rothia mucilaginosa* were taken forward to multivariable analyses and showed evidence of association with the outcome (table 4). *B dorei* was present in 121 (11%) of 1082 babies (six [5%] of 121 born by caesarean section), among whom one (1%) had a vLRTI hospital admission, compared with 78 (8%) of 961 participants without *B dorei*. *R mucilaginosa* was present in 336 (31%) of the 1082 participants (214 [64%] of 336 born by caesarean section). Among participants with *R mucilaginosa,* 36 (10%) had at

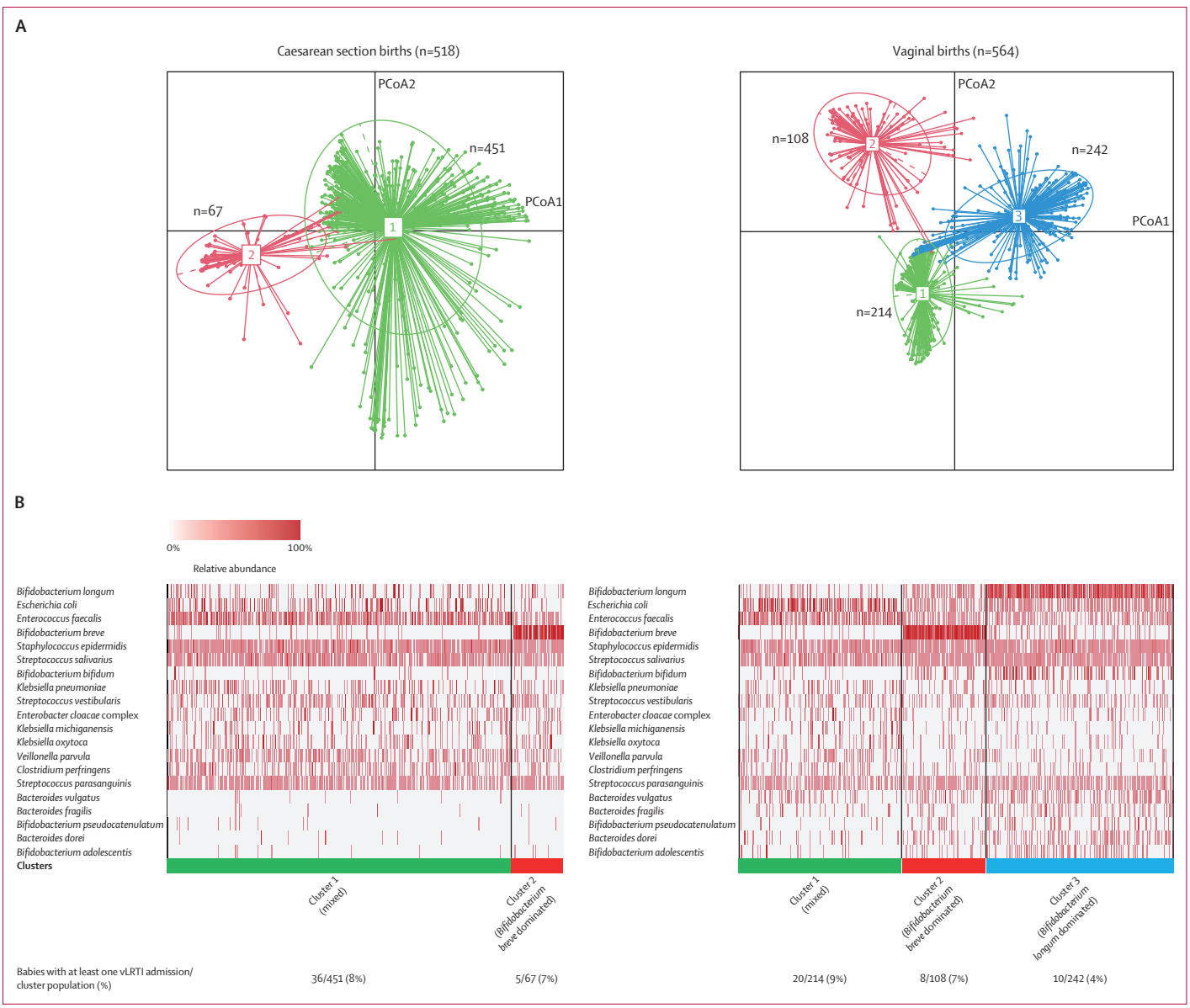

Figure 2: **First-week gut microbiota cluster composition characterisation, stratified by mode of birth**
(A) PCoA plots of 1082 first-week gut metagenomes samples stratified by mode of birth and clustered using the partitioning around medoids algorithm based on species level. Ellipses encapsulate 67% of the samples within each respective cluster. (B) Bacterial species relative abundance heat map by first-week gut microbiota cluster composition stratified by mode of birth. Only the top 20 most abundant species are plotted in the heat map. Each column represents a stool sample. PCoA=principal coordinates analysis. vLRTI=viral lower respiratory tract infection.

least one vLRTI admission, compared with 43 (6%) of 746 participants without *R mucilaginosa*. 27 (2%) of the 1082 participants had both bacterial species present, and none had a vLRTI hospital admission, compared with 42 (6%) of 652 participants with an admission in participants with neither species. Adjusting for the presence of the other bacterial species did not change the results in multivariable analysis findings (data not shown).

There was no evidence of individual bacterial species being differentially abundant by vLRTI hospital admission status among the 47 (5%) of 878 bacterial species present in more than 10% of babies (appendix pp 16–19).

Discussion

Our study provides observational evidence of an association between neonatal gut microbiota composition and vLRTI, a leading cause of hospitalisation in young children. Higher first-week gut microbiota alpha diversity was associated with reduced rates of vLRTI hospital admission within the first 2 years of life. Babies born vaginally with a first-week *B longum* community composition had significantly lower rates of hospital admission for vLRTI compared to all other babies after adjusting for potential confounders, and other babies born vaginally had similar gut microbiota compositions to babies delivered by caesarean section

	vLRTI hospital admissions		Univariable		Multivariable*		Multivariable,* multiple imputation†	
	Number	Rates per 1000 person-years	HR (95% CI)	p value	Adjusted HR (95% CI)	p value	Adjusted HR (95% CI)	p value
Babies born vaginally‡								
Overall	54	45·9 (35·2–60·0)¶	..	..	..	..	..	..
Cluster 3 (*B longum* dominated)	13	25·8 (15·0–44·5)	1 (ref)	0·056	1 (ref)	0·022	1 (ref)	0·018
Cluster 2 (*B breve* dominated)	10	45·0 (24·2–83·5)	1·79 (0·63–5·13)	..	2·61 (0·71–9·58)	..	2·55 (0·79–8·15)	..
Cluster 1 (mixed)	31	69·1 (48·6–98·2)	2·76 (1·20–6·37)	..	4·63 (1·56–13·75)	..	4·33 (1·58–11·90)	..
Babies born by caesarean section§								
Overall	51	46·4 (35·3–61·1)¶	..	..	..	..	..	..
Cluster 1 (mixed)	45	47·0 (35·1–63·0)	1 (ref)	0·85	1 (ref)	0·47	1 (ref)	0·71
Cluster 2 (*B breve* dominated)	6	42·3 (19·0–94·1)	0·91 (0·33–2·51)	..	1·51 (0·51–4·46)	..	1·22 (0·42–3·58)	..

B breve=Bifidobacterium breve. B longum=Bifidobacterium longum. HR=hazard ratio. IMD=index of multiple deprivation. vLRTI=viral lower respiratory tract infection. *Adjusted for hospital, delivery mode, feeding mode at 1 week of age, sex, season of birth, gestational age, birthweight, maternal ethnicity, maternal age, maternal asthma, IMD, mother smoked during pregnancy, mother received antibiotics during labour, baby received resuscitation after birth, parity, twin pregnancy, baby received antibiotics after birth, baby admitted to neonatal unit, and if baby had comorbidities recorded at birth. †Multiple imputation: feeding mode at 1 week was imputed using logistic regression. For the 64 missing values 56 were imputed in the model with babies born vaginally. For the 50 missing values 46 were imputed in the model with babies born by caesarean section. Variables included in the imputation model were admission to neonatal unit, hospital, birthweight, gestational age, antibiotics after birth, antibiotics in labour, parity, maternal age, smoke exposure during pregnancy, IMD, feeding in the fifth h of life, birth season, resuscitation at birth, and vLRTI. The twin pregnancy variable was collinear and was dropped from the model. ‡For babies born vaginally there were 564 participants in the univariable analysis, 434 participants in the multivariable analysis, and 486 participants in the multivariable analysis with multiple imputation. §For babies born by caesarean section there were 518 participants in the univariable analysis, 393 participants in the multivariable analysis, and 434 participants in the multivariable analysis with multiple imputation. ¶p=0·59 for the overall difference in vLRTI hospital admission rates between babies born by caesarean section and vaginally.

Table 2: **Association of first-week gut microbiota cluster composition and vLRTI hospital admissions in babies born vaginally and by caesarean section**

	Univariable (n=1082)		Multivariable* (n=826)		Multivariable,* multiple imputation† (n=918)	
	HR (95% CI)	p value	Adjusted HR (95% CI)	p value	Adjusted HR (95% CI)	p value
Cluster 3 (*B longum* dominated)	1 (ref)	0·18	1 (ref)	0·053	1 (ref)	0·042
Cluster 2 (*B breve* dominated)	1·79 (0·65–4·92)	..	2·72 (0·97–7·59)	..	2·80 (1·06–7·44)	..
Cluster 1 (mixed)	2·11 (0·97–4·63)	..	3·02 (1·17–7·77)	..	3·05 (1·25–7·41)	..

B breve=Bifidobacterium breve. B longum=Bifidobacterium longum. HR=hazard ratio. IMD=index of multiple deprivation. vLRTI=viral lower respiratory tract infection. *Adjusted for hospital, delivery mode, feeding mode at 1 week of age, sex, season of birth, gestational age, birthweight, maternal ethnicity, maternal age, maternal asthma, IMD, mother smoked during pregnancy, mother received antibiotics during labour, baby received resuscitation after birth, parity, twin pregnancy, baby received antibiotics after birth, baby admitted to neonatal unit, and if baby had comorbidities recorded at birth. †Multiple imputation: feeding mode at 1 week was imputed using logistic regression. For the 129 missing values, 115 were imputed. Variables included in the imputation model were admission to neonatal unit, hospital, birthweight, gestational age, antibiotics after birth, antibiotics in labour, parity, maternal age, smoke exposure during pregnancy, IMD, feeding in the fifth h of life, birth season, resuscitation at birth, and vLRTI. The twin pregnancy variable was collinear and was dropped from the model.

Table 3: **Association of first-week gut microbiota cluster composition and vLRTI hospital admissions**

(cluster 2 [*B breve* dominated] and cluster 1 [mixed]) and similar rates of vLRTI admissions. The presence of *B dorei* (protective) and *R mucilaginosa* (risk factor) were independently associated with vLRTI hospitalisations.

Other large studies (n>700 participants) have reported that higher gut microbiota alpha diversity measured at 3 months and 12 months, mostly using 16S rRNA sequencing, was protective of asthma and wheezing determined at 1, 5, and 6 years of age.[25–27] However, smaller studies exploring asthma, wheezing, and respiratory infections have failed to identify any association.[10] These inconsistencies could be due to the smaller studies lacking statistical power and taxonomic resolution, and to differences in respiratory outcome definitions.

Few longitudinal studies have explored the association between neonatal gut microbiota composition and early-life respiratory infections. Reyman and colleagues found an association between first-week gut microbiota characterised using 16S rRNA sequencing, and cumulative incidence of respiratory infections at 1 year ascertained by parental interviews (n=120 participants).[28] The reported associations with first-week gut microbiota at the genus level were consistent with our study. Children who developed three to seven respiratory infections had lower relative abundance of *Bifidobacterium* (log two-fold change 2·1; p=0·049) and higher relative abundance of *Klebsiella* (log two-fold change 3·2; p=0·0071), and *Enterococcus* (log two-fold change 2·8; p=0·0093) compared with children with zero to two respiratory infections during the first year of life.[28] Moroishi and colleagues reported that, in a study including 650 babies, higher gut microbiota relative abundances of *Veillonella parvula* and *Haemophilus influenzae* at 6 weeks of age among infants born by caesarean section were positively associated with respiratory infection symptoms during the first year of life.[29] The lack of consistency with our study results might be explained by stool samples being collected at later timepoints beyond the first week of life.

	Multivariable* (n=826 participants; n=904 observations)		Multivariable,* multiple imputation† (n=918 participants; n=1010 observations)	
	Adjusted HR (95% CI)	p value	Adjusted HR (95% CI)	p value
B dorei				
No	1 (ref)	0·0015	1 (ref)	0·015
Yes	0·09 (0·01–0·70)	..	0·08 (0·01–0·61)	
R mucilaginosa				
No	1 (ref)	0·033	1 (ref)	0·044
Yes	1·97 (1·06–3·68)	..	1·81 (1·02–3·24)	..

B dorei=Bacteroides dorei. HR=hazard ratio. IMD=index of multiple deprivation. *R mucilaginosa=Rhodotorula mucilaginosa*. vLRTI=viral lower respiratory tract infection. *Adjusted for hospital, delivery mode, feeding mode at 1 week of age, sex, season of birth, gestational age, birthweight, maternal ethnicity, maternal age, maternal asthma, IMD, mother smoked during pregnancy, mother received antibiotics during labour, baby received resuscitation after birth, parity, twin pregnancy, baby received antibiotics after birth, baby admitted to neonatal unit, and if baby had comorbidities recorded at birth. †Multiple imputation: feeding mode at 1 week was imputed using logistic regression. For the 129 missing values, 115 were imputed. Variables included in the imputation model were admission to neonatal unit, hospital, birthweight, gestational age, antibiotics after birth, antibiotics in labour, parity, maternal age, smoke exposure during pregnancy, IMD, feeding in the fifth h of life, birth season, resuscitation at birth, and vLRTI. The twin pregnancy variable was collinear and was dropped from the model.

Table 4: **Association between the presence of *B dorei* or *R mucilaginosa* in first-week gut microbiota and vLRTI hospital admissions**

There is currently great interest in modifying the early gut microbiota as a prevention strategy for childhood diseases. Regarding respiratory infections, Rautava and colleagues explored giving probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* compared to placebo in a randomised controlled trial (RCT) of infants aged up to 2 months requiring formula milk. Recurrent respiratory infections were identified in nine of 32 babies in the probiotic group and in 22 of 40 babies in the placebo group up to the age of 1 year (rate ratio [RR] 0·51 [95% CI 0·27–0·95]).[30] Taipale and colleagues enrolled 109 infants aged 1 month in an RCT who were then given a placebo or *Bifidobacterium animalis* supplements from age 1 month to 24 months. Babies receiving *B animalis* had fewer respiratory infections than controls (87% in the treatment group *vs* 100% in the control group; RR 0·87 [95% CI 0·76–1·00]).[31] These studies provide some evidence that modulating the gut microbiota in the first months of life might prevent childhood respiratory infections. However, no conclusions have been reached regarding the optimal intervention age or probiotic composition. Moreover, no study used naturally occurring commensal (non-probiotic) bacteria identified as having protective effects against childhood respiratory diseases in observational studies, such as *B longum*.[10] Our work shows that gut microbiota patterns associated with reduced rates of vLRTI admission can be detected as early as the first week of life. Neonatal microbiota markers might therefore be used in the future, to stratify patients at risk for childhood diseases and guide probiotic selection in clinical trials.

Other preventive strategies include vaginal seeding[32] and perinatal maternal faecal transplants.[33] These approaches, which have sparked controversies regarding their risk-and-reward balance, currently aim to expose babies born by caesarean section to more vaginal birth-like gut microbes and reduce subsequent disease. However, our study challenges the idea that exposing babies born by caesarean section to the maternal gut and vaginal microbiota might reduce the risk of vLRTI hospital admission in childhood. We observed that only babies born vaginally with cluster 3 (*B longum* dominated) microbiome, showed lower rates of disease, and other babies born vaginally had similar disease incidence when compared with babies born by caesarean section.

Regarding the biological plausibility of our findings, babies in cluster 3 (*B longum* dominated) had higher relative abundance of *B longum*, *B bifidum*, *B adolescentis*, and *B dorei* and lower abundances of *E faecalis* and *B breve* than all other babies. *Bifidobacterium* is one of the most prevalent bacterial genera in the neonatal gut, decreasing in abundance after 4 months of life.[34] Many species of *Bifidobacteria* and some *Bacteroides* can metabolise human milk oligosaccharides, secreted in maternal milk, to produce short-chain fatty acids.[35] Short-chain fatty acids serve as prebiotics (stimulate microbial growth) or antimicrobials for late-arriving gut commensal bacteria,[36] and influence the development of multiple immune cells including colonic regulatory T cells,[37] and have inhibitory effects on proinflammatory responses in the lung.[38,39] Animal and human[40] studies have reported associations between higher abundance of gut *Bifidobacteriaceae* and systemic and respiratory anti-inflammatory immune effects. One study with 347 stool samples from 208 babies, collected from birth to age 4 years, reported that babies with gut microbiota depleted of *Bifidobacteriaceae* and human milk oligosaccharides metabolism genes showed evidence of systemic inflammation and immune dysregulation.[41] Three human studies have shown an association between lower relative abundance of *Bifidobacterium* in the first 3 months of life and respiratory infections at age 1 year[28] and asthma at age 4–5 years.[42,43]

Despite many bacterial species correlating with the different cluster compositions, only two bacterial species were directly associated with vLRTI hospital admission in our study: *B dorei* (protective) and *R mucilaginosa* (risk factor). Although *B dorei* is regarded as a commensal gut bacterium and has been shown to have anti-influenza effects,[44] no human study to our knowledge has reported an association with subsequent childhood respiratory disease. *R mucilaginosa* is a common oral microbiota pathogen. Two studies have reported contradictory findings regarding the role of *Rothia* spp in the infant microbiota and subsequent respiratory disease.[40,45] Our findings regarding individual bacterial species should be interpreted with caution. Further epidemiological, in-vitro, and animal studies are needed to identify the specific gut microbiota and gene-level components that might explain our observed associations.

Our study has limitations. Although our research question is causal, we recognise that this is an observational study, and observed associations might be due to residual confounding or bias. To minimise confounding, a direct acyclic graph informed by a literature review was used to identify likely confounders (appendix p 20). However, there

could be residual confounding. For example, the Index of Multiple Deprivation was used as proxy for socioeconomic status and pollution, and we did not have information on day-care attendance, although we anticipate very few participants attending day care in the first week of life. We also acknowledge the potential role of the respiratory microbiota. Given evidence of bidirectional influences between gut and respiratory microbiota, the impact on the immune system, and the observed association between respiratory microbiota and childhood respiratory disease,[46] the respiratory microbiota might act as a confounder, mediator, or effect modifier in the explored association.[46] Although we did not have later breastfeeding information, breastfeeding status after the first week of life should not confound the explored association. Regarding bias, we were only able to sequence stool samples for 35% of BBS participants, but our association analyses (appendix pp 10–12) suggest a low probability that selection bias contributed to the observed results. Although gut microbiota was only measured at one timepoint, it would be challenging to avoid reverse causality affecting the associations if we had longitudinal sampling, because vLRTI hospital admissions peak around age 3 months.[11] Laboratory and bioinformatic processing decisions could introduce exposure misclassification, but this should not be related to the outcome and potential bias would be towards the null. No microbiome functional pathway association analyses were performed due to power limitations. Outcome misclassification is possible, for example, due to linkage errors.[21] However, to minimise these errors, participants with poorer match ranks were excluded (<100 participants). We had no microbiological diagnostic information on the precise cause of vLRTIs, or information regarding vLRTIs not requiring hospital admission. Finally, these observations were not validated in an independent cohort.

In conclusion, first-week gut microbiota was independently associated with reduced rates of vLRTI hospital admission in a subgroup of vaginally born babies with a cluster 3 (*B longum* dominated) composition, compared with all other babies. These findings are important for designing future observational and intervention studies regarding the early gut microbiota and prevention of childhood viral respiratory infections.

Contributors

NF, AR, and TDL conceptualised and designed the Baby Biome Study. CG-M, NF, AR, and AM conceptualised and designed the study research question. YS and TDL performed laboratory and sequencing work and the initial bioinformatic cleaning and processing. CG-M performed clinical data cleaning and all analyses with assistance from NF, AR, YS, and AM. CG-M wrote the first draft of the paper. CG-M and NF verified the data underlying the study. All authors read, commented on, and approved the final manuscript. All authors had access to the perinatal and microbiome data. NHS Digital requirements meant that only CG-M, AM, AR, and NF had direct access to the NHS data. All authors had access to the analysis outputs and accept responsibility for the decision to submit for publication.

Declaration of interests

TDL is the co-founder and CSO of Microbiotica. All other authors declare no competing interests.

Data sharing

Raw shotgun-metagenomic sequences analysed in this study have been deposited in the European Nucleotide Archive (accession number ERP115334).

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