Interactions between live attenuated influenza vaccine and nasopharyngeal microbiota among children aged 24–59 months in The Gambia: a phase 4, open-label, randomised controlled trial

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Summary

Background Live attenuated influenza vaccines (LAIVs) alter nasopharyngeal microbiota in adults. It is poorly understood why LAIV immunogenicity varies across populations, but it could be linked to the microbiome. We aimed to investigate the interactions between intranasal immunisation with LAIV and nasopharyngeal microbiota composition in children from The Gambia.

Methods We conducted a phase 4, open-label, randomised controlled trial in Sukuta, The Gambia. Children aged 24–59 months with no underlying illness or history of respiratory illness for at least 14 days before recruitment were eligible. Participants were randomly assigned (2:1) by use of a computer-generated sequence in permuted blocks of 15, stratified by sex, to receive trivalent LAIV either on day 0 (intervention group) or after active follow-up at day 21 (control group). The investigator team was initially masked to block size and randomisation sequence; however, group allocation was later revealed to the team. Microbiome profiles were characterised from nasopharyngeal samples collected from all participants on days 0, 7, and 21 by use of 16S rRNA sequencing. The primary outcomes were the effect of LAIV on nasopharyngeal microbiome profiles on day 7 and day 21, and the association between the nasopharyngeal microbiome at baseline and LAIV-induced mucosal IgA responses at day 21, assessed with permutational ANOVA tests. Asymptomatic respiratory viral co-infection at baseline and year of recruitment (2017 or 2018) were included as covariates. This trial is registered with ClinicalTrials.gov (NCT02972957) and is closed.

Findings Between Feb 8 and April 12, 2017, and Jan 15 and March 28, 2018, 343 children were screened for eligibility, of whom 220 (64%) children were randomly assigned to the intervention group and 110 (32%) to the control group. 213 (97%) children in the intervention group and 108 (98%) in the control group completed the study and were included in the final analysis. Although we did not observe an independent effect of LAIV on microbial community composition at days 7 or 21, we found that LAIV had an effect dependent on the year of recruitment. LAIV affected microbial community composition in 2018 (R^2 1·97% [95% CI 0·85–5·94]; p=0·037), but not in 2017 (1·23% [0·49–4·46]; p=0·091). We also found that viral co-infection at baseline had an effect on microbial composition at day 7, regardless of recruitment year (R^2 1·01% [95% CI 0·28–3·01]; p=0·026). Nasopharyngeal microbial community composition at baseline had no effect on mucosal IgA responses to LAIV administration (R^2 0·51% [95% CI 0·23–2·49]; p=0·46).

Interpretation Our findings suggest that the effect of LAIVs on nasopharyngeal microbiota composition in children is modest and temporary; therefore, LAIVs could be used as an intervention to curb influenza in children from low-income and middle-income countries, without causing long-lasting perturbations in nasopharyngeal microbiota. However, nasopharyngeal microbiota at the time of vaccination might not explain the variability observed between individuals in LAIV-induced IgA responses.

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Introduction

Respiratory tract infections are a leading cause of morbidity in children, particularly across low-income and middle-income countries (LMICs).[1] It is estimated that approximately 870 000 hospitalisations in children are associated with seasonal influenza each year, with the highest burden reported in LMICs.[2] Influenza vaccines, such as live attenuated influenza vaccines (LAIVs), are key interventions in preventing seasonal influenza. LAIVs are highly protective against influenza virus infection in young children (aged 2–5 years),[3] and could be used to prevent or reduce the burden of influenza in children and adults from LMICs.

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Research in context

Evidence before this study

We hypothesised that nasopharyngeal microbiota might interact with live attenuated influenza vaccines (LAIVs) and influence LAIV responses. We searched PubMed, without language restrictions, for studies published between database inception and April 16, 2024, using the search terms "live attenuated influenza vaccine" AND "microbiome". This search identified eight studies. Following screening of the title and the abstract, we identified four studies that investigated the relationships between LAIVs and the nasopharyngeal or nasal microbiome in humans. One longitudinal study, involving 17 adults in the USA, investigated the effect of LAIVs on nasal microbiota and gene expression profiles. This study reported significant changes in nasal microbial community structure and diversity, with an increase in the relative abundance of *Staphylococcus* and *Bacteroides* genera following LAIV administration. Another study, involving 47 adults in the USA, investigated whether changes in the nasal microbiome following LAIV administration were associated with the production of mucosal IgA responses. The study reported that LAIV administration induced changes in the microbiome to allow less abundant taxa to colonise the anterior nares, and that these changes were associated with variations in mucosal IgA antibody responses specific to influenza. A third study used a human challenge model to study the interactions between LAIV, pneumococcal challenge, and nasal microbiota among 117 adults in the UK. This study reported modest perturbations in nasal microbiota following LAIV administration, with increased perturbations observed on day 2 (R^2 1·7%) and diminishing perturbations over time (R^2 1·3% at both days 7 and 29). Furthermore, LAIV administration affected the receptiveness of the nasopharyngeal microbiota to pneumococcal colonisation. The fourth study followed up 90 individuals from the human challenge model cohort to investigate the effect of experimentally induced pneumococcal colonisation on mucosal antibody responses induced by LAIV. Mucosal IgA in the nose and IgG in the lung were dampened in the group of participants with pneumococci infection before LAIV administration. We did not find any study that investigated the effect of LAIVs on the nasopharyngeal microbiome or other respiratory microbiome niches, or the influence of the nasopharyngeal microbiome on LAIV-induced mucosal responses in children or populations from low-income and middle-income countries (LMICs).

Added value of this study

To our knowledge, this study is the first randomised controlled trial to report the interactions between LAIV administration and nasopharyngeal microbiota composition in children and the first study conducted in an African setting. Our results show that LAIV-induced perturbations in nasopharyngeal microbiota are modest. Additionally, we provide the first piece of evidence that the nasopharyngeal microbiota might affect adaptive immune responses induced by LAIV administration.

Implications of all the available evidence

Our results highlight that LAIV administration in children in LMICs does not result in long-lasting perturbations in the nasopharyngeal microbiota and, therefore, does not pose concerns as an intervention to curb the burden of influenza infections among children in this setting. Importantly, our data suggest an important role of the nasopharyngeal microbiota in inducing adaptive immune responses following intranasally administered LAIV. Therefore, the nasopharyngeal microbiome could represent a key target in improving immunogenicity to LAIVs, including both humoral and cellular responses, among children.

The human upper respiratory tract is a dynamic and complex environment, and serves as a habitat to both commensal and potentially pathogenic organisms. Bacteria and viruses co-colonise this niche and interact with the host cells and other microbes. As an intranasal live viral vaccine, LAIVs induce an attenuated infection in nasal epithelial cells and parallel some aspects of the interactions between the wild-type influenza virus and host, including an increase in bacterial burden of potential pathogens, such as *Staphylococcus aureus* and *Streptococcus pneumoniae*.[4,5] In the same cohort of children from The Gambia used in the current study, we previously showed that LAIVs modestly increased carriage density of *S pneumoniae* for up to 21 days, akin to effects caused by asymptomatic respiratory viral infection.[5] However, the immediate effects of LAIVs on the overall nasopharyngeal microbiome in children remain unknown.

Previous studies have shown that LAIV immunogenicity and efficacy vary among individuals and across populations.[6,7] Emerging evidence supports the role of the microbiota in shaping host immune responses, including those induced by vaccination and infection at the time of exposure.[8] Most evidence to date shows that the gut microbiota influence parenteral and oral immune responses induced by vaccination;[9] however, little is known about how the human nasopharyngeal microbiota might influence responses to vaccines delivered to the upper respiratory tract. Evidence from germ-free mice indicates that upper respiratory tract microbiota can calibrate the thresholds of activation for innate antiviral responses through macrophage interferon signalling pathways, thereby potentially influencing virus-specific T-cell and nasal IgA responses.[10] Investigating similar relationships between nasopharyngeal microbiota and immune responses induced by LAIVs could provide a better understanding of the basis behind the poor performance of mucosal vaccines in resource-limited settings and might inform future interventions targeted at microbiota to improve mucosal vaccine responses.

Herein, we report the results of a phase 4 randomised controlled trial exploring the interactions between LAIV and

nasopharyngeal microbiota in children from The Gambia. Specifically, we aimed to investigate whether intranasal immunisation with LAIV resulted in alterations of the nasopharyngeal microbiota and whether the composition of the nasopharyngeal microbiota at the time of vaccination was associated with immune responses induced by LAIVs.

Methods

Study design and participants

We conducted a phase 4, open-label, longitudinal, randomised controlled trial in Sukuta, a peri-urban community in The Gambia, as part of the NASSIMUNE study's primary outcomes.[5] Children aged 24–59 months with no underlying illness or history of respiratory illness for at least 14 days before the day of recruitment were eligible for inclusion. The full inclusion and exclusion criteria are provided in the appendix (pp 3–4). To achieve the predefined recruitment targets outside of the influenza seasons participants were recruited over two time periods: between Feb 8 and April 12, 2017, and between Jan 15 and March 28, 2018.

The study was approved by the joint ethics committee of The Gambia Government and the UK Medical Research Council and by the Medicines Control Agency of The Gambia (reference SCC1502). A parent provided written or thumb-printed informed consent for their children to participate. For parents who were not literate in English, a discussion on informed consent was conducted in a local language in the presence of an impartial witness. The impartial witness signed to confirm completeness of the consent provided. The study protocol is provided in appendix (pp 26–118).

Randomisation and masking

Children were randomly assigned (2:1) to receive trivalent LAIV either on the day of enrolment (intervention group) or after follow-up on day 21 (control group). Randomisation was performed in permuted blocks of 15 stratified by sex, by use of sealed opaque envelopes and a computer-generated sequence preprepared by an independent statistician not involved in the rest of the study. The investigator team was masked to block size and randomisation sequence to avoid allocation bias; however, the team was made aware of group allocation at the opening of the envelope. Group allocation was then cross-checked and co-signed by a second individual. Due to the need to carry out LAIV immune response assays on samples in the intervention group only, group allocation was also revealed to the local laboratory team after completing recruitment. The external laboratory team, who characterised the nasopharyngeal microbiome, was masked to group allocation until data analysis.

Procedures

Northern hemisphere Russian-backbone trivalent LAIV (Nasovac-S, Serum Institute of India, Pune, India) was used in both recruitment years, containing 2009 pandemic H1N1 (A/17/California/2009/38 in 2017 and A/17/New York/15/5364 in 2018), H3N2 (A/17/Hong Kong/2014/8296), and influenza B-Victoria (B/Texas/02/2013) viruses.

Nasopharyngeal samples were collected from all study participants at baseline (day 0), day 7, and day 21 by means of flocked paediatric swabs (FLOQSwabs, Copan, CA, USA) and stored in RNAprotect (Qiagen, Manchester, UK). Samples were processed within 4 h of collection and stored at –70°C until further processing. Additionally, participants randomly assigned to the intervention group had oral fluid from the buccal cavity obtained by use of swabs (Oracol Plus, Worcester, UK) on days 0 and 21, along with whole-blood samples for T-cell response assays. To minimise the number of study visits and blood collections for participants, those receiving LAIV on day 0 were further randomly assigned (1:1) to have additional whole-blood samples collected on either day 7 following LAIV vaccination for ex-vivo follicular helper T-cell response assays or on day 2 for use in investigating other study objectives, as outlined in the study protocol (appendix p 44).

See online for appendix

Bacterial DNA was extracted from the nasopharyngeal samples as previously described.[11] Amplicon libraries were generated by PCR by use of F515/R806 primers targeting the 16S rRNA hypervariable region 4 and were sequenced on the Illumina Miseq sequencing platform (Illumina, San Diego, CA, USA). Sequencing data were processed to obtain operational taxonomic units (OTUs) by use of a bioinformatics pipeline, as previously described (appendix p 7).[12] To avoid multiple OTUs with identical annotations, OTUs were named with taxonomic annotations combined with a rank number based on the average relative abundance of each OTU across the entire dataset. Only OTUs with a presence of more than 0·1% relative abundance in at least two samples were used in the analyses.[13] We used the *decontam* package (combined method) to identify and remove environmentally contaminating OTUs using default parameters.[14]

All samples collected on day 0 were screened for the presence of respiratory viruses with multiplex real-time PCR, as previously described.[15] Influenza types A and B, respiratory syncytial viruses A and B, human parainfluenza viruses 1–4, human metapneumovirus, adenovirus, seasonal coronaviruses (229E, OC43, and NL63), and human rhinovirus were included in the panel.[15]

Haemagglutinin inhibition titres, influenza haemagglutinin-specific IgA, and T-cell responses were measured as previously described.[6] Haemagglutinin inhibition assays were performed on vaccine strain haemagglutinin-matched and neuraminidase-matched viruses. Seroconversion to LAIV was defined as a four-fold or greater increase in haemagglutinin inhibition titres (to ≥1:40) from day 0 to day 21.[6] Mucosal influenza-specific IgA was measured in oral fluid samples at baseline and at day 21 by use of a protein microarray as previously described,[16,17] with the percentage of 20 Surfact-Amps detergent solution (Thermo Fisher Scientific, Franklin, MA, USA) in the blocking, washing, and incubation buffer increased from

0·05% to 5·00% to prevent background staining with oral specimens. Total IgA was quantified by use of an ELISA and influenza-specific IgA was expressed as a ratio of influenza HA1-specific IgA to total IgA as previously described,[17] with a two-fold or greater increase from day 0 to day 21 considered to be a significant response.

For the **Shiny Application** see https://fedematt.shinyapps.io/shinyMB

CD4 and CD8 T-cell responses were quantified by stimulating 200 µL whole blood for 18 h, with overlapping 15–18 mer peptide pools (2 µg/mL) covering a vaccine-matched matrix (47 peptides) and nucleoprotein (68 peptides), haemagglutinin 1 (HA1; 74 peptides), and haemagglutinin 3 (HA3; 74 peptides), as previously described (appendix p 5).[18] A two-fold or higher increase in these responses from day 0 to day 21 was considered to be a significant response.[6] We measured peripheral follicular helper-like T cells ex vivo as a proxy for germinal centre activity by flow cytometry. A volume of 200 µL of whole blood was used for the staining. Additional unstained and fluorescence minus one controls for ICOS and CXCR5 were included. CXCR5 Brilliant Violet 421 (BioLegend, San Diego, CA, USA) was added to the tubes and incubated for 15 min at 37°C and 5% CO_2. Surface staining was done with CD4, CD8, CD45R0, and ICOS, followed by a 15 min incubation at room temperature in the dark (appendix pp 5, 10). Cells were then lysed with 2 mL 10x concentrate FACS solution (BD Biosciences, Franklin, NJ, USA) and incubated at room temperature for 10 min in the dark. FACS buffer (BD Biosciences, Franklin, NJ, USA) was added to the cells and centrifuged at 1800 revolutions per min for 5 min. Cells were resuspended in 250 µL FACS buffer and stored at 4°C in the dark until acquisition. Cells were analysed on a LSR Fortessa flow cytometer (BD Biosciences, Franklin, NJ, USA). Follicular helper-like T cells were defined as CD4 T cells expressing CD45RO, CXCR5, and ICOS (appendix p 19). Follicular helper-like CD4 T-cell responders were defined as high when the fold increase from day 0 to day 7 was equal to or above the median fold change in the whole dataset.

Outcomes

This study had two primary outcomes: the effect of LAIV on nasopharyngeal microbiome profiles on day 7 and day 21, and the association between the nasopharyngeal microbiome at the time of vaccination and LAIV-induced mucosal IgA responses at day 21. Secondary outcomes were the effect of the nasopharyngeal microbiome on seroconversion by use of a haemagglutinin inhibition assay, influenza A matrix and nucleoprotein (MNP) CD4 T-cell responses, and the induction of peripheral follicular helper-like T cells ex vivo.

An exploratory outcome was the effect of asymptomatic respiratory viral infection at day 0 on nasopharyngeal microbiota following LAIV vaccination to investigate their interaction, as previously described.[5,12] A post-hoc analysis was the effect of LAIV on the nasopharyngeal microbiome at day 7, stratified by the year of recruitment (2017 *vs* 2018).

Statistical analysis

Sample size calculations were based on the potential association between baseline nasopharyngeal microbiota and the induction of mucosal influenza-specific IgA, which represented an immune response induced by vaccination with LAIV. Power calculations were performed with the microbiome study domain of the Shiny Application aiming to detect at least two-fold differences in the 50 most abundant bacteria after correction for multiple testing.[19] At a two-sided α level of 0·05, 82 participants per group would provide 80% power to detect differences between individuals with and without an IgA response. A sample size of 103 participants per group (a total of 206 children receiving LAIV) was selected, with 50% of participants expected to mount an IgA response (two-fold increase from day 0 to day 21) and potential dropouts (<20%) accounted for, which still provided at least 80% power to address our research question on the effect of the microbiome on immunogenicity. Children who completed the follow-up visit on day 21 and provided samples were included in the final pseudo intention-to-treat analysis.

Permutational ANOVA tests were used to study the association between overall microbial community composition (response variable), LAIV administration, and other covariates that were either of interest or known to influence microbial composition (appendix p 19), over the whole study period, and at day 7 and day 21 following vaccination (explanatory variables; adonis-function on vegan R package). The multivariable permutational ANOVA model used to compare microbiota communities between groups on days 7 and 21 adjusted for covariates that were significantly or modestly associated ($p<0·10$) with microbial community composition on day 0, such as household cooking location and year of recruitment (given that different vaccine formulations were used), or factors that had been previously reported to affect microbial community composition following LAIV vaccination (ie, the presence of asymptomatic respiratory virus at baseline).[5,12] The permutational ANOVA models were further adjusted for sex (the variable used for stratification during block randomisation) and included interactions between both LAIV administration and year of recruitment, as well as LAIV and the presence of baseline asymptomatic viruses.

We performed non-parametric bootstrapping to infer the 95% CIs of the effect sizes by generating 1000 OTU matrices based on randomly sampled taxa in the original OTU matrix with replacement. We then fitted permutational ANOVA models on each resampled OTU matrix and calculated the confidence intervals of the estimated effect sizes as the 2·5% and 97·5% percentiles. To investigate the significant interaction between LAIV administration and year of recruitment, we performed a post-hoc stratified analysis, fitting a permutational ANOVA model for each year of recruitment (2018 or 2017).

Microbiota profiles were identified by use of unsupervised average linkage hierarchical clustering based on the Bray–Curtis dissimilarity matrix, as previously described

(appendix pp 7, 20).[12] Only clusters with ten or more samples were considered for further statistical analyses. We identified biomarker OTUs for the microbiota clusters (profiles) using random forest models based on the *VSURF* package. Biomarker taxa ascertainment for microbiome clusters were based on relative abundance (appendix pp 20–21). χ^2 tests or Fisher's exact tests were used to assess the associations between microbiota profiles and vaccination with LAIV. We used Fisher's exact tests when more than 20% of cells in the contingency table had frequencies of less than five. When the expected cells of the contingency table had five or more frequencies and no cell had expected values of less than one, we performed the χ^2 test.

We identified differentially abundant taxa between children who received vaccination with LAIV and control participants using microbiome multivariable associations with linear models (MaAsLin2) by use of default parameters (OTU minimum prevalence of 10% and a false discovery rate ≤0·25 obtained using the Benjamin-Hochberg procedure).[20] We used permutational ANOVA tests to identify associations between overall microbial community composition and immunogenicity outcomes. We used Chi squared tests or Fisher's exact tests where appropriate to assess associations between microbiota profiles on day 0 and LAIV immunogenicity outcomes. We performed linear models to study associations between specific microbiota profiles or relative abundances of biomarker taxa and LAIV immunogenicity outcomes. We adjusted obtained p values for multiple testing using the Benjamin-Hochberg method to obtain q values. Furthermore, we performed Wilcoxon rank-sum tests as non-parametric tests to complement results from linear models in cases where model assessments were poor (appendix pp 26–29).

All statistical analyses were performed with R (version 4.0.1). This trial is registered with ClinicalTrials.gov (NCT02972957).

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

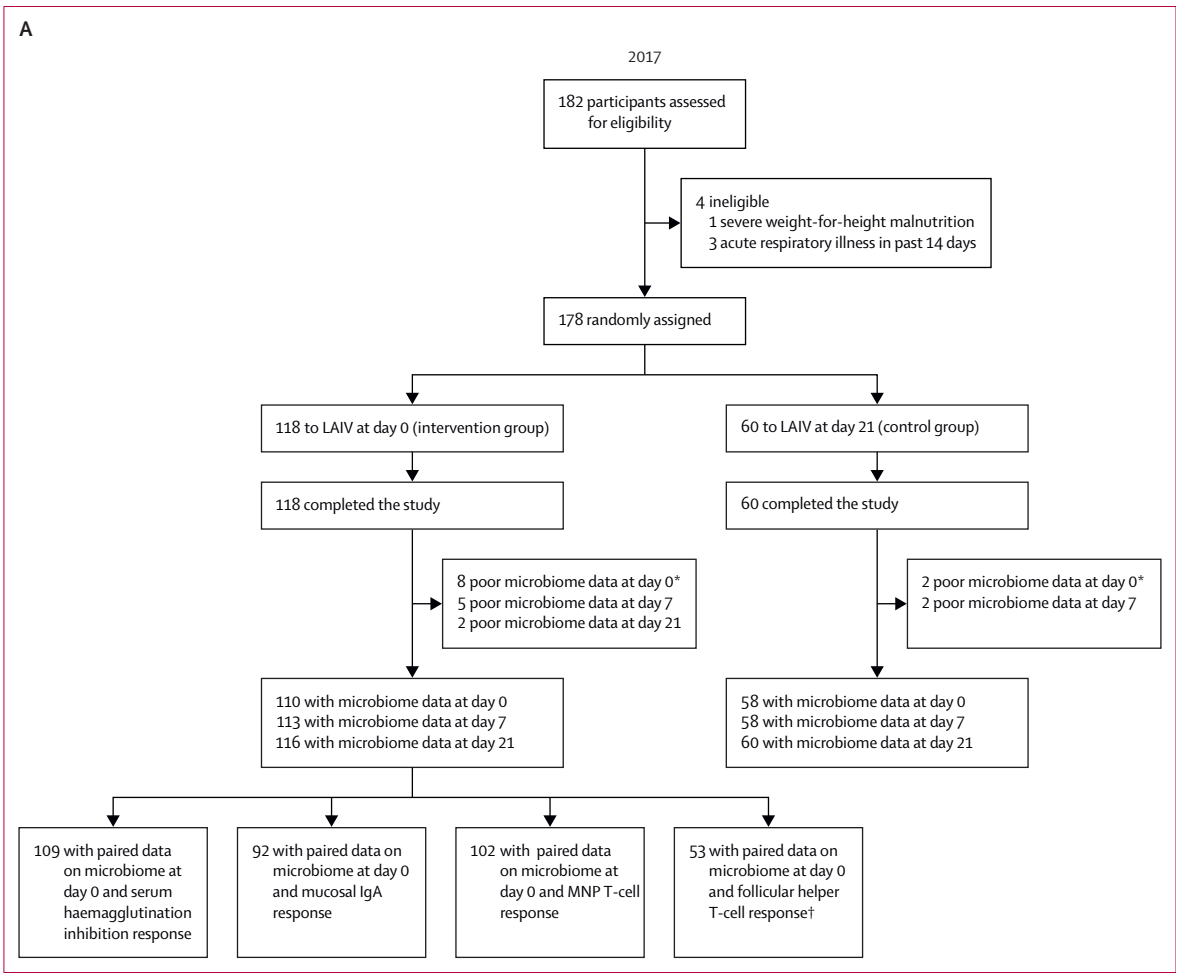
Between Feb 8 and April 12, 2017, and Jan 15 and March 28, 2018, 343 children were assessed for eligibility, of whom 220 (64%) children were randomly assigned to the intervention group and 110 (32%) to the control group (figure 1). All 178 children recruited in 2017 completed the study. In 2018, 95 (93%) of 102 children assigned to the intervention group and 48 (96%) of 50 children assigned to the control group completed the study (figure 1B). Therefore, 213 (97%) children in the intervention group and 108 (98%) in the control group overall were included in the final analysis. All study visits were completed within the timeframes defined by the study protocol (+1 day for the visit on day 2, +7 days for the visit on day 7, +7 days for the visit on day 21), with 308 (96%) occurring on the exact visit day. Among children with available paired data on the microbiome on day 0 and immunogenicity outcomes in the intervention group, 199 (93%) children had data on serum haemagglutination inhibition response, 170 (80%) on influenza-specific mucosal IgA responses, 178 (84%) on MNP-specific CD4 T-cell responses, and 96 (45%) on follicular helper-like T-cell responses (figure 1).

For the **VSURF package** see https://github.com/robingenuer/VSURF

Demographic characteristics did not differ between the years of recruitment (appendix p 9), apart from differences in baseline immune responses, which have been reported previously.[6] Data on viral infections were available for 205 (96%) of the 213 children in the intervention group, and 104 (96%) of the 108 children in the control group. Over both 2017 and 2018, asymptomatic viral infections were detected in 67 (33%) of 205 children in the intervention group and 41 (39%) of 104 in the control group (table 1). Rhinoviruses were the most prevalent virus observed across all study groups, representing 50 (75%) of all viral infections detected among children in the intervention group and 25 (61%) of those detected among children in the control group (table 1).

The permutational ANOVA model did not show any longitudinal effect of LAIV on microbial community structure across the whole study period (R^2 0·24% [95% CI 0·13–0·89]; p=0·23; appendix p 11). We also observed modest but non-significant microbiota instability in the intervention group between day 0 and day 7 when we quantified changes with the Bray–Curtis dissimilarity matrix between paired subsequent timepoints per participant (appendix p 19). Although we did not find an independent effect of LAIV alone on microbial community structure at day 7 with the adjusted multivariable permutational ANOVA model (R^2 0·36% [95% CI 0·20–1·74]; p=0·34; figure 2A), we observed a significant effect for the interaction term that included year of recruitment and LAIV vaccination on microbial community structure at day 7 (1·23% [0·46–3·43]; p=0·0082; figure 2A). Additionally, we observed a significant effect of viral co-infection at baseline on microbial community structure at day 7 (1·01% [0·28–3·01]; p=0·026; figure 2A). LAIV administration nor any of the other covariates or interaction terms affected microbial community composition at day 21 (appendix p 11).

The post-hoc permutational ANOVA analysis stratified by year of recruitment showed a significant effect of LAIV administration on microbial community structure at day 7 in 2018 (R^2 1·97% [95% CI 0·85–5·94]; p=0·037; figure 2C), but only a modest and non-significant effect in 2017 (1·23% [0·49–4·46]; p=0·091; figure 2B). The post-hoc analysis also showed that the effect of baseline asymptomatic viruses was observed in 2018 (2·68% [0·74–7·53]; p=0·0031) but not in 2017 (0·74% [0·19–3·11]; p=0·32; figure 2B). The interaction term between LAIV administration and presence of baseline asymptomatic viruses was not significant in the main model (0·28% [0·13–0·89]; p=0·47; figure 2A) nor in the models stratified by year (figures 2B, C), suggesting that the effect of the presence of baseline asymptomatic viruses on microbial community



(Figure 1 continues on next page)

structure was independent of LAIV. In the intervention group, LAIV shedding did not affect microbial community composition on day 7 or day 21 (appendix pp 12–13).

Hierarchical clustering performed on all samples regardless of timepoint (n=927) identified 17 distinct microbiota profiles, of which six profiles included ten or more samples and were used for further statistical analyses (appendix pp 20–21). These microbiota profiles were dominated by either *Dolosigranulum* (2) and *Corynebacterium propinquum* (4), found in 392 samples; *Haemophilus* (3), in 176 samples; *Moraxella* (1), in 155 samples; *Ornithobacterium* (7), in 115 samples; *Streptococcus* (5), in 45 samples; or *C propinquum* (4) alone, in 23 samples (appendix p 20). The microbiota profiles were equally represented in both the intervention group and control groups at baseline, except for the *Streptococcus*-enriched profile, which we excluded from the subsequent analyses (p=0·015; table 2). At day 7, *Moraxella*-dominated profiles became under-represented in children in the intervention group compared with those in the control group (risk ratio [RR] 0·77 [95% CI 0·58–1·01]; p=0·035; table 2; appendix p 22). No significant associations between LAIV and microbiota profiles were observed at day 21 (table 2). Additionally, the effect of LAIV on taxa level, adjusting for year of recruitment, viral co-infection at baseline, and household cooking location showed only three taxa that were less abundant in the intervention group than in the control group at day 7: *Neisseriaceae* (44), *Prevotella spp* (181), and the low abundant taxa *Rubellimicrobium* (231); appendix p 13). No taxa showed an increased abundance in the intervention group compared with the control group at day 7. At day 21, we did not observe any differences in microbial taxa between the intervention group and the control group (appendix p 14).

A total of 106 (53%) of 199 children showed seroconversion to either the pandemic H1N1 (24 [12%]), H3N2 (49 [25%]), or influenza B-Victoria (58 [29%]) strain, which we defined as any seroconversion in the subsequent analyses (appendix p 14). A two-fold increase or more in influenza-specific mucosal IgA response between day 0 and day 21 to at least one antigen was seen in 74 (44%) of 170 children, with 38 (22%) responding to pandemic H1N1, 27 (16%) to H3N2, and 56 (33%) to influenza B-Victoria (appendix p 14). A two-fold increase or greater in intracellular IFN-γ or IL-2 CD4 T-cell responses to influenza A MNP

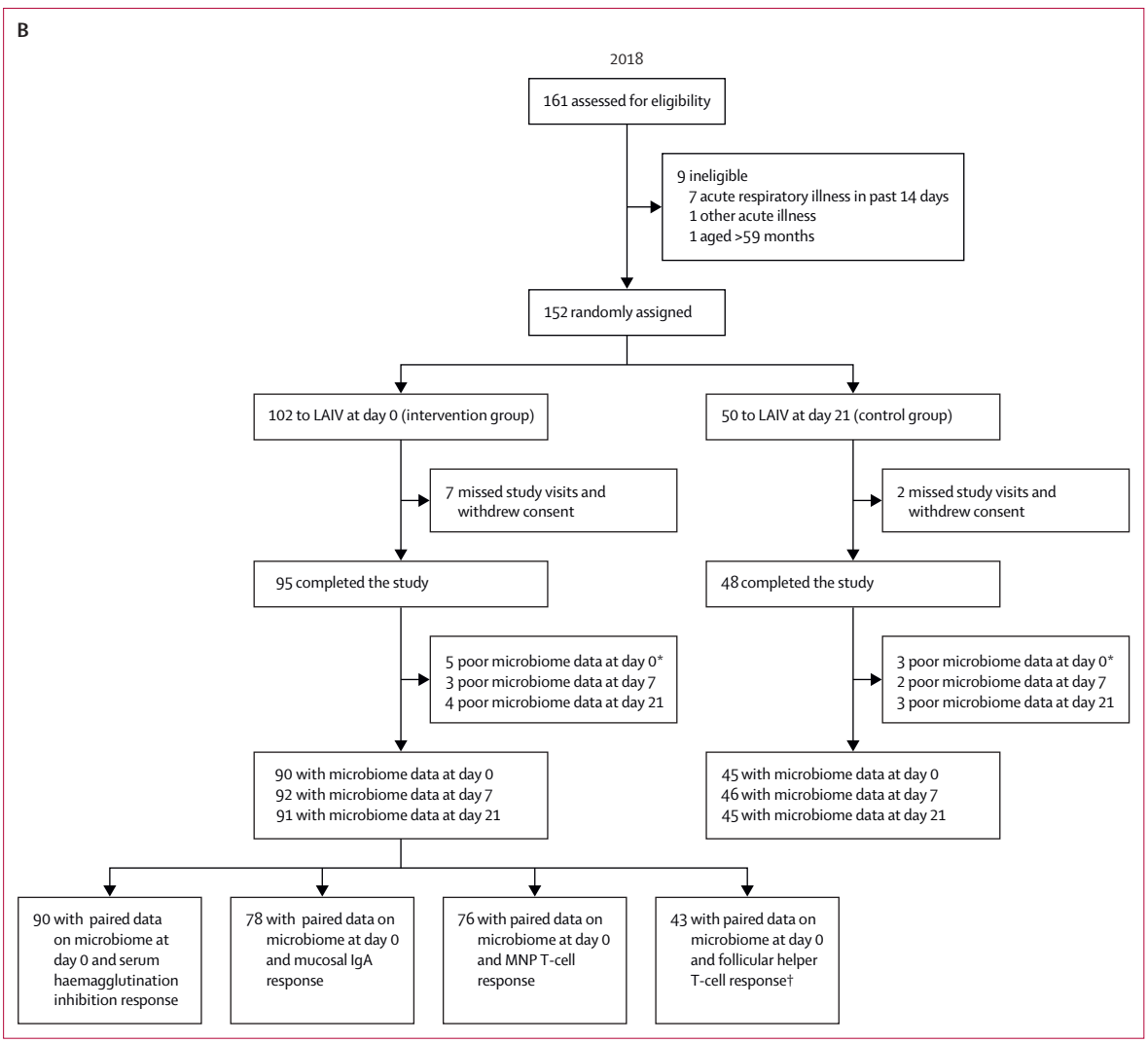


***Figure 1*: Trial profiles for the 2017 and 2018 cohorts**
Study overview detailing the number of participants recruited, retained, and included in the final analyses for the 2017 cohort (A) and the 2018 cohort (B). LAIV=live attenuated influenza vaccine. MNP=influenza A matrix and nucleoprotein. *Defined as either low DNA concentration on 16S quantitative PCR or a low read count following sequencing. †Ex-vivo follicular helper T-cell responses on days 0, 7, and 21 were measured in 107 (50%) of 213 children in the intervention group to minimise the number of study visits and blood collections as per the overall trial study design.

peptides from baseline to day 21 was observed in 100 (56%) of 178 children. An increase in peripheral follicular helper-like T cells ex vivo was seen between day 0 and day 7 (p=0·013), but not between day 0 and day 21 (p=0·22; appendix p 23). Therefore, children with follicular helper-like T-cell responses were classified as those with values equal to or above the median fold change between day 0 and day 7. These follicular helper-like T-cell responses did not correlate with IgA responses to any LAIV strain (appendix p 23). We found that microbial community composition at day 0 was not associated with IgA response (R^2 0·51% [95% CI 0·23–2·49]; p=0·46; figure 3A) or seroconversion (0·63% [0·28–2·55]; p=0·25; figure 3B; appendix pp 14–15). However, we observed a significant association between microbial community composition at day 0 and MNP CD4 T-cell responses (2·33% [1·12–5·21]; p=0·0021; figure 3C), as well as peripheral follicular helper-like T-cell responses ex vivo (3·14% [1·07–9·38]; p=0·031; figure 3D, appendix pp 14–15). When investigating IgA responses, the number of LAIV strains shed was also affected by microbial community composition at day 0 (appendix pp 15–16). LAIV immune responses and microbiota composition at day 0 were not influenced by nutrition status at the time of recruitment (appendix pp 15–16).

We found that having a microbiota profile dominated by *Dolosigranulum* (2) and *Corynebacterium* (4) or a higher abundance of these taxa at day 0 was associated with MNP CD4 T-cell responses (RR 1·21 [95% CI 0·93–1·58];

	Intervention group (n=213)	Control group (n=108)
Age, months	35·0 (28·0–42·0)	32·5 (28·0–40·0)
Sex		
Male	116 (54%)	58 (54%)
Female	97 (46%)	50 (46%)
Baseline asymptomatic virus present*	67 (33%)	41 (38%)
Adenovirus	3 (4%)	3 (7%)
Seasonal coronavirus	7 (10%)	3 (7%)
Parainfluenza 1	2 (3%)	4 (10%)
Parainfluenza 1 and seasonal coronavirus	1 (1%)	0
Parainfluenza 1 and rhinovirus	1 (1%)	0
Parainfluenza 3	0	1 (2%)
Rhinoviruses	50 (75%)	25 (61%)
Rhinovirus and adenovirus	1 (1%)	3 (7%)
Rhinovirus and seasonal coronavirus	1 (1%)	1 (2%)
RSV A	1 (1%)	0
RSV B	0	1 (2%)
Influenza A or B	0	0
Data missing	8 (4%)	4 (4%)
Outside kitchen location†	189 (89%)	97 (90%)
Presence of a smoker in the household	51 (24%)	27 (25%)
Antibiotic use in the month before recruitment		
None	204 (96%)	103 (95%)
Beta-lactams	4 (2%)	1 (1%)
Cotrimoxazole	4 (2%)	4 (4%)
Chloramphenicol	0	0
Other‡	1 (<1%)	0
Baseline seropositivity (haemagglutination inhibition titre ≥1:10)		
Pandemic H1N1	82 (38%)	..
H3N2	146 (69%)	..
Influenza B-Victoria	68 (32%)	..

Data are median (IQR) or n (%). LAIV=live attenuated influenza vaccine. RSV=respiratory syncytial virus. *Percentages calculated with the total number of children with asymptomatic viruses as the denominator for each group. †Cooking inside (under a roof) compared with cooking outside kitchen (open fire). 318 (99%) of 321 children lived in households where wood or charcoal was the primary fuel used to cook, regardless of the cooking location. ‡Other than beta-lactam, cotrimoxazole, macrolides, and chloramphenicol.

Table 1: **Baseline participant characteristics for the overall cohort**

q=0·02 for *Dolosigranulum* (2) and q=0·05 for *Corynebacterium* (4)), whereas a microbiota profile rich in *Moraxella* (1) or a higher abundance of this taxon was negatively associated with these MNP CD4 T-cell responses (0·59 [0·36–0·94]; q=0·01; appendix pp 16–17, 24). Although microbiota profiles dominated by *Dolosigranulum* (2) or *Corynebacterium* (4) were also shown to be associated with seroconversion (1·35 [1·04–1·75]; p=0·025) and follicular helper-like T-cell responses (1·66 [1·17–2·35]; p=0·012), the taxon-level analysis for *Dolosigranulum* (1) or *Corynebacterium* (4) showed a non-significant increase in abundance among children with an immune response (appendix pp 16–18, 24). Similarly, we found that microbiota profiles dominated by *Ornithobacterium* (7) were negatively associated with follicular helper-like T-cell responses (0·39 [0·14–1·06]; p=0·023); however, this finding could not be substantiated at the taxon level (appendix pp 16–17). Likewise, a higher abundance of *Haemophilus* (3) was negatively associated with follicular helper-like T-cell responses (q=0·07), but this observation could not be substantiated with the microbiota profiles or taxa-level analysis (appendix pp 17, 24).

Discussion

In this phase 4, open-label, randomised controlled trial, we investigated interactions between LAIV administration and the nasopharyngeal microbiota in children from The Gambia who had not previously been vaccinated with an influenza vaccine. We have shown that LAIV administration in children results in modest, short-lived perturbations in the nasopharyngeal microbial community, which are amplified by asymptomatic viral co-infections detected at the time of vaccination. However, we have also shown that the microbial community composition of the nasopharynx at the time of vaccination is significantly associated with LAIV-induced immune responses. To our knowledge, this trial is the first study to explore these interactions in children from a low-income setting.

Our first finding concurs with the observations from a human challenge model study in the UK, in which the effect of LAIV on the nasopharyngeal microbiota of adult participants was modest and diminished rapidly.[12] The effect of viral co-infections was similar to that of LAIV, which is consistent with previous studies showing perturbations in respiratory microbiota following respiratory viral infections in patients with and without symptoms in both children and adults.[4,21] The absence of an effect from vaccination with LAIV in 2017 is more puzzling. Although it is possible that the higher prevalence of viral co-infection in 2018 might explain the absence of an effect observed in 2017, we speculate that the improved replicative fitness following replacement of the pandemic H1N1 component in the 2018 formulation, as observed previously in this same cohort, might be responsible for the observed year effects. Nevertheless, our results did not show any effect of LAIV shedding on microbial composition.[5,6]

Microbiota profiles dominated by *Moraxella* were underrepresented among participants in the intervention group following vaccination. These profiles have been associated with a reduced incidence of upper and lower respiratory tract infections.[22,23] Although specific mechanisms have not been established, profiles dominated by *Moraxella* are thought to portray stable microbiota colonisation, possibly owing to the ability to form biofilms and potentially contribute to resilience against viral associated dysbiosis.[22] Furthermore, our finding of a decreased abundance of rare abundant oral taxa following LAIV, including *Neisseria* spp, was not completely in line with observations from a US study of adults, which found more significant reductions in the abundance of *Corynebacterium* and increases in *Staphylococcus* and *Bacteroides* following LAIV vaccination.[4] However, differences between the current study and the US study in the vaccine formulations used (Russian backbone [Serum Institute of India, Pune, India] *vs* Ann Arbor formulations [MedImmune, Gaithersburg, MD, USA]), age of participants (adults *vs* children), and

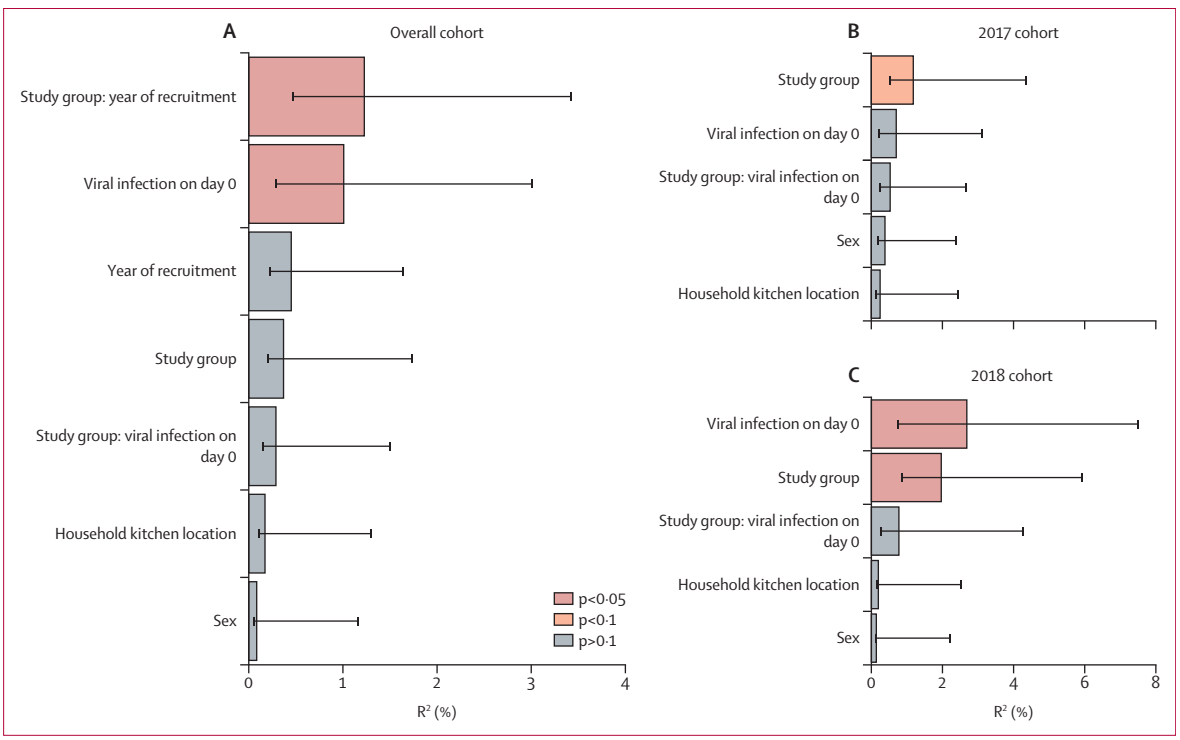


Figure 2: **Factors associated with nasopharyngeal microbial community composition at day 7 following LAIV administration**
Nasopharyngeal microbial community composition at day 7 for the overall cohort (A) and stratified by the year of recruitment for the 2017 cohort (B) and the 2018 cohort (C). p values and accompanying effect sizes (R^2) were calculated with permutational ANOVA tests. A p value of less than 0·05 represents significance, a p value between 0·05 and 0·10 represents modest significance, and a p value of more than 0·10 represents no significance. The stress value for the ordination is 0·182, representing a good goodness of fit of the data points following dimension reduction (<0·1 is excellent, <0·2 is good, and >0·2 is poor).

geographical region might help to explain the observed variations.

Interestingly, microbial community composition at the time of vaccination was significantly associated with adaptive immune responses following LAIV, albeit not with the induction of mucosal IgA responses. In particular, microbiota profiles enriched with bacteria that produce lactic acid—ie, *Dolosigranulum pigrum* and *C propinquum*—were consistently associated with influenza-specific MNP CD4 T-cell responses. This finding is interesting given that *Dolosigranulum* spp and *C propinquum* have also been associated with a reduced number of respiratory infections during childhood in previous studies.[24] These results are also in line with previously observed associations between the predominance of other bacteria that produce lactic acid in the gut, including *Lactobacillus* spp and immune responses to LAIV.[25] It is possible that the presence of lactic acid bacteria, such as *D pigrum* and *C propinquum* found in our study, enhance cellular immune responses to LAIVs in a similar way to those observed at other mucosal sites, such as the gut. Nevertheless, more trials and experimental models are needed to ascertain these observations and examine potential mechanisms.

We observed that profiles dominated by *Ornithobacterium* and *Haemophilus* were modestly associated with reduced immune responses to LAIV, particularly for the induction of follicular helper-like T-cells. Children with a dense colonisation of *H influenza*e or a microbiota profile dominated by *Haemophilus* show a systemic immune phenotype characterised by increased proinflammatory cytokines, such as IL-6 and IL-8, as well as enhanced neutrophil recruitment and activation.[26] This observation suggests that children with microbiota profiles dominated by *Haemophilus* present with a low-grade inflammatory phenotype, which might hamper appropriate vaccine-induced immune responses following an immune challenge.

The reasons why we did not observe any association between nasopharyngeal microbiota and LAIV-induced mucosal IgA responses are not clear. Although this finding might be explained by only 44% of children showing mucosal IgA responses in this study, which was powered to have 50% of participants with an IgA response, there might be other explanations. A study in UK adults found that recent nasopharyngeal colonisation with *S pneumoniae* before LAIV administration dampened mucosal IgA responses to LAIV;[27] over 70% of participants in the current study had *S pneumoniae* colonisation at baseline.[5] Several hypotheses have been suggested, including viral replication interference by *S pneumoniae* through stimulation of toll-like receptor (TLR) and that *S pneumoniae* colonisation diminishes immune-cell infiltration and antigen-presenting cell activation, affecting downstream immune responses.[27] These hypotheses are consistent with findings from the gut, whereby a systems biology approach

	Intervention group	Control group	Risk ratio (95% CI)	p value*
Day 0				
Total	196 (100%)	102 (100%)	..	..
Dolosigranulum (2) and *C propinquum* (4)	83 (42%)	39 (38%)	1·06 (0·90–1·25)	0·45
Moraxella (1)	35 (18%)	24 (24%)	0·88 (0·70–1·11)	0·26
Haemophilus (3)	39 (20%)	20 (20%)	0·97 (0·79–1·20)	0·83
Ornithobacterium (7)	23 (12%)	11 (11%)	1·05 (0·83–1·33)	0·85
Streptococcus (5)	11 (6%)	0	1·55 (1·43–1·70)	0·015
Day 7				
Total	199 (100%)	100 (100%)	..	..
Dolosigranulum (2) and *Corynebacterium* (4)	93 (47%)	46 (46%)	1·01 (0·87–1·19)	0·83
Moraxella (1)	25 (13%)	22 (22%)	0·77 (0·58–1·01)	0·035
Haemophilus (3)	33 (17%)	18 (18%)	0·99 (0·79–1·22)	0·89
Ornithobacterium (7)	36 (18%)	10 (10%)	1·21 (1·01–1·44)	0·072
Streptococcus (5)	12 (6%)	4 (4%)	1·13 (0·84–1·51)	0·59
Day 21				
Total	202 (100%)	98 (100%)	..	..
Dolosigranulum (2) and *C propinquum* (4)	91 (45%)	40 (41%)	1·06 (0·91–1·24)	0·44
Moraxella (1)	29 (14%)	20 (20%)	0·87 (0·68–1·12)	0·23
Haemophilus (3)	40 (20%)	26 (27%)	0·90 (0·72–1·11)	0·29
Ornithobacterium (7)	26 (13%)	10 (10%)	1·11 (0·90–1·39)	0·36
Streptococcus (5)	16 (8%)	2 (2%)	1·36 (1·13–1·63)	0·038

Data are n (%), unless otherwise indicated. OTUs are named by use of taxonomic annotations combined with a rank number in parentheses based on the average relative abundance of each OTU across the entire dataset to avoid multiple OTUs having identical annotations. Only clusters with more than ten samples were used for the analysis for statistical power, which excluded the *Corynebacterium* cluster (appendix p 18). LAIV=live attenuated influenza vaccine. OTU=operational taxonomic unit. *Identified by Chi squared tests or Fisher's exact tests were appropriate.

Table 2: **Microbiota profiles in the intervention group and control group at days 0, 7, and 21**

showed that the absence of TLR5, which is a sensor of bacterial flagellin, resulted in reduced serum IgG antibody responses to trivalent influenza vaccine, hinting at the influence of flagellated gut microbes on antibody responses. Therefore, it is possible that the high prevalence of pneumococcal colonisation in our study cohort might have affected immune responses; however, the study did not have the power to draw this conclusion.

This study has several strengths. We conducted a longitudinal randomised controlled trial, which allowed potential microbial variation between individuals to be controlled for. Conducting the study outside peak influenza season, careful clinical review before recruitment, and the molecular screening of influenza strains at baseline greatly minimised the confounding effects of natural influenza virus infections when investigating the effect of LAIVs on nasopharyngeal microbiota composition. Measuring the multifaceted responses of LAIV-induced immunity, which included T-cell responses instead of being confined to humoral responses, provided a unique perspective on the important inter-relationships existing between nasopharyngeal microbiota and induced cellular immune responses.

This study also has several limitations. Because of the low biomass of respiratory samples used in the study, we used 16S rRNA sequencing to characterise microbial communities, which does not provide species-level and strain-level identification and functional insight. However, our previous report on the species-level effects of LAIV, with a particular focus on *S pneumoniae*, showed similar modest perturbations.[5] A further limitation of applying 16S rRNA sequencing to streptococcal species is the high level of sequence homology (>99%) in this region.[28] Additionally, our results on the effect of the nasopharyngeal microbiome on LAIV immunogenicity are based on associations rather than causation, and should be followed by mechanistic studies. However, given the randomised and temporal nature of the study, this observational evidence can be considered to be strong. We were unable to fully explain the reasons behind the observed differences in microbial composition among participants recruited in 2018, which might confound our comparisons between groups. We excluded participants who did not complete their follow-up visit on day 21 and all samples with poor-quality microbiome data from our analysis, which could have possibly introduced selection bias during the analysis. Furthermore, we considered variable inclusion in our analyses guided by knowledge from previous studies and possible biological hypotheses. Although these selection criteria would have omitted important confounders that might be unique to this setting, the randomised controlled trial study design would have minimised the effect of this potential confounding.

Our findings represent the first piece of evidence from an LMIC setting to elucidate the effects of administering LAIV in children on perturbations in the nasopharyngeal microbiota and to provide data supporting the modest and temporary effects of LAIVs on interruptions to

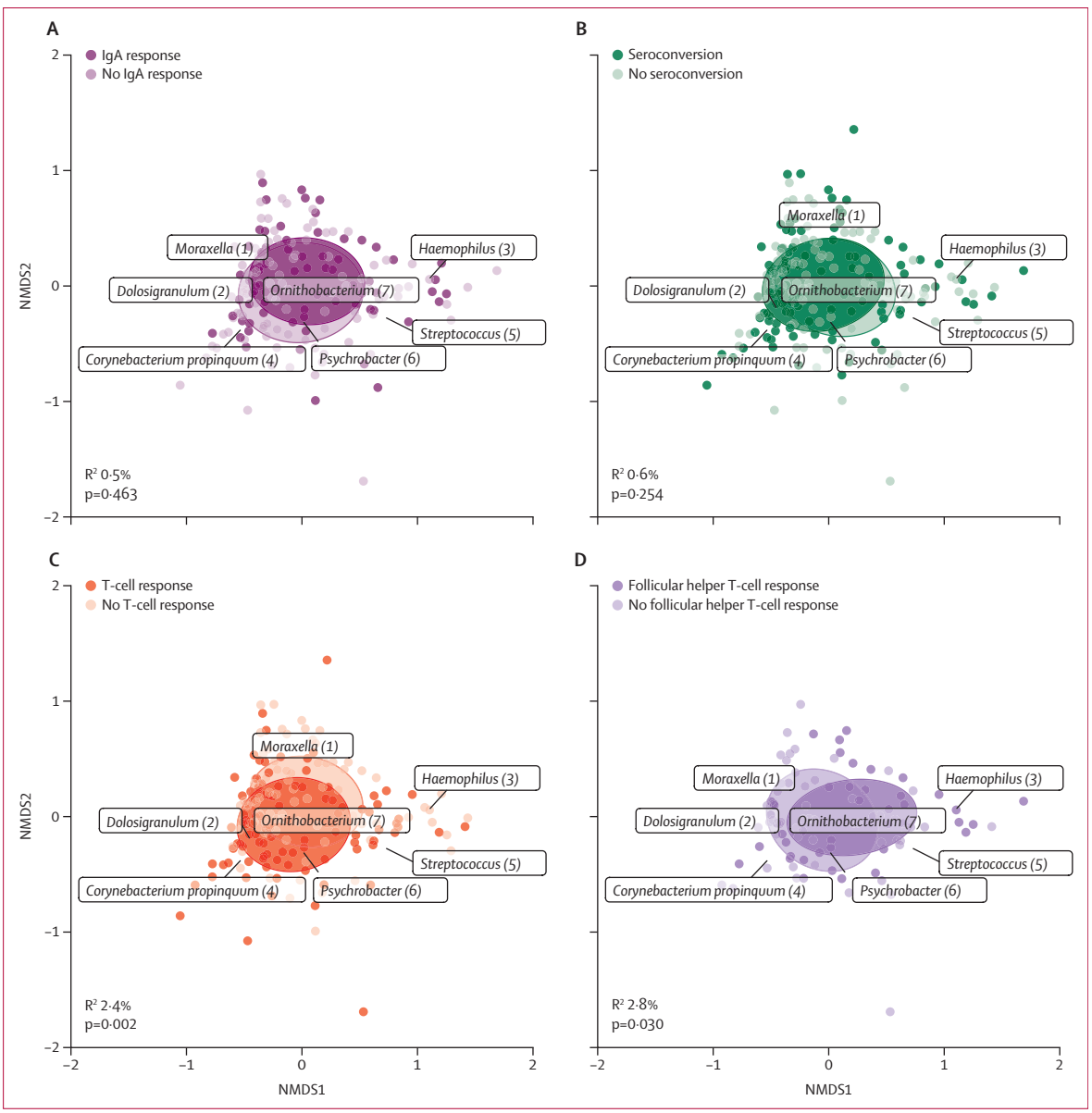


Figure 3: **Associations between overall microbial community composition and immune responses**

Associations visualised by NMDS, including the presence or absence of an IgA response between day 0 and day 21 (A); serum haemagglutination inhibition response between day 0 and day 21 (B); influenza A matrix nucleoprotein CD4 T-cell response between day 0 and day 21 (C); and follicular helper-like T-cell response at day 7 (D). Projected taxa are biomarker taxa of the microbiota profiles identified by hierarchical clustering. Each data point represents the microbial community composition of a single sample at the time of LAIV receipt. OTUs are named by use of taxonomic annotations combined with a rank number in parentheses based on the average relative abundance of each OTU across the entire dataset to avoid multiple OTUs having identical annotations. R^2 refers to the percentage of variance explained by each immune variable, calculated by multivariate permutational ANOVA tests, adjusting for any covariates associated with both the microbiome at day 0 and the immune response to LAIV at either day 7 or day 21. NMDS also depicts biomarker taxa in relation to the ordination plot. The stress value for each ordination is 0·182, indicating a good goodness of fit of the data points following dimension reduction (<0·1 is excellent, <0·2 is good, and >0·2 is poor). LAIV=live attenuated influenza vaccine. NMDS=non-metric dimensional scaling. OTU=operational taxonomic unit.

nasopharyngeal microbial communities in this age group. These results add to the findings of a study in UK children, which reported the effects of LAIV administration on major bacterial pathogens by use of quantitative PCR.[29] Furthermore, we report for the first time the potential influence of nasopharyngeal microbiota on LAIV-induced immune responses in children. Our results suggest that the presence of bacteria that produce lactic acid in the nasopharyngeal microbiota could be important in achieving robust T-cell responses to mucosal vaccination. The crucial role of T-cell-mediated immunity induced through vaccination for influenza virus is being recognised.[30] With limitations to strain-specific LAIV formulations on vaccine efficacy and potential vaccine mismatch, T-cell responses could be key in

providing protection against influenza, even when a reduced rate of LAIV antibody production is induced. Therefore, the findings from this study provide potential avenues of research on microbiota-targeted interventions that could boost the immunogenicity of LAIVs and possibly improve vaccine efficacy and effectiveness. Future studies supplementing high-resolution mechanistic data of lactic acid bacteria, such as *Dolosigranulum* and *C propinquum*, are needed to provide functional insights.

Contributors

TIdS, EPA, EC, BK, NIM, and DB designed the study. EPA, JC, and SB recruited participants and conducted the study under the supervision of TIdS. CP, MC, CBL, YJJ, SD, SJ, HS, ES, AM, GG, SvT, and BBL conducted the laboratory experiments. CP, YJJ, AR-R, EdK, WdSP, and MC performed the data analysis. DB, CP, TIdS, and DHD interpreted the data. CP, YJJ, DHD, TIdS, and DB wrote the manuscript. All authors reviewed the final manuscript. CP, TIdS, DB, MC, EPA, CBL, and SJ had access to and verified the raw data. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Declaration of interests

We declare no competing interests.

Data sharing

Sequence data that support the findings of this study can be accessed in the US National Center for Biotechnology Information Sequence Read Archive database (BioProject ID PRJNA1102700) upon publication. De-identified participant-level metadata will be available upon request to the corresponding author with a signed data access agreement.

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