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Published in:
The Lancet Healthy Longevity

DOI:
[10.1016/S2666-7568\(20\)30034-9](https://doi.org/10.1016/S2666-7568(20)30034-9)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2021

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Fuentes, S., den Hartog, G., Nanlohy, N. M., Wijnands, L., Ferreira, J. A., Nicolaie, M. A., Pennings, J. L. A., Jacobi, R., de Wit, J., van Beek, J., & van Baarle, D. (2021). Associations of faecal microbiota with influenza-like illness in participants aged 60 years or older: an observational study. *The Lancet Healthy Longevity*, 2(1), E13-E23. [https://doi.org/10.1016/S2666-7568\(20\)30034-9](https://doi.org/10.1016/S2666-7568(20)30034-9)

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Associations of faecal microbiota with influenza-like illness in participants aged 60 years or older: an observational study



Susana Fuentes, Gerco den Hartog, Nening M Nanlohy, Lucas Wijnands, José A Ferreira, Mioara A Nicolaie, Jeroen L A Pennings, Ronald Jacobi, Jelle de Wit, Josine van Beek, Debbie van Baarle



Summary

Background People aged 60 years or older are at high risk for respiratory infections, one of the leading causes of mortality worldwide. Vaccination is the main way to protect against these infections; however, vaccination is less effective in older adults than in younger adults due to ageing of the immune system, so innovative strategies that improve vaccine responses could provide a major public health benefit. The gut microbiota regulates host immune homeostasis and response against pathogens, but human studies showing the effects of the gut microbiota on respiratory infections in older adults are sparse. We aimed to investigate the composition of the microbiota in relation to respiratory infections and local and systemic immune markers in older adults during an influenza season.

Methods In this observational study, participants were selected from an influenza-like illness (ILI) prospective surveillance cohort in which community-dwelling adults aged 60 years and older in the Netherlands were recruited through their general practitioner or the Civil Registry. Inclusion criteria have been described elsewhere. Participants completed questionnaires and self-reported symptoms. To measure microbiota composition, faecal samples were collected from participants registering an ILI event, with a follow-up (recovery) sample collected 7–9 weeks after the ILI event, and from asymptomatic participants not reporting any event throughout the season. We tested associations between microbiota profiles and a set of health-related variables, patient characteristics, and local and systemic immune markers. We cultured identified bacterial biomarkers for ILI with CaCo-2 cells in an in vitro intestinal epithelial model and measured the induced immune response. This study is registered with <http://www.trialregister.nl>, NL4666.

Findings Between Oct 1, 2014, and April 30, 2015, 2425 older adults were recruited into the ILI surveillance cohort. From Oct 1, 2014, to June 15, 2015, faecal samples were collected from 397 participants, of whom 213 (54%) reported an ILI event once throughout the season and 184 (46%) did not. 192 ILI participants recovered and provided follow-up samples. Microbiota composition was altered during an ILI event. The Bacteroidetes (mean relative abundance 17·51% [SD 11·41] in the ILI group and 14·19% [10·02] in the control group; adjusted $p=0\cdot014$) and the Proteobacteria (3·40% [8·10] in the ILI group and 1·57% [3·69] in the control group; adjusted $p=0\cdot015$) were more abundant in the ILI group than in the control group. The abundance of *Ruminococcus torques* was positively associated with ILI and the abundance of *Escherichia/Shigella*, negatively correlated with alpha diversity, and negatively co-occurred with beneficial taxa, including butyrate producers. *R torques* was associated with pro-inflammatory profiles, both locally in faeces and systemically in blood. ILI-associated taxa (*R torques* and *Escherichia coli*) had symbiotic effects on the cellular immune response when cultured together in an in vitro model.

Interpretation The abundances of specific bacteria could be used as potential biomarkers for susceptibility to respiratory infections and as targets for intervention in the ageing population.

Funding The Dutch Ministry of Health, Welfare and Sport, and the Strategic Program of the National Institute for Public Health and the Environment.

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Introduction

Infections of the lower respiratory tract are the leading cause of mortality by communicable diseases among all age groups worldwide. The growing proportion of the population aged 60 years or older is at increased risk for respiratory infections, with a nearly 90 times higher rate of mortality than healthy young adults (25–44 years of age) for infections such as influenza.¹ Although vaccination remains the main measure for prevention,

the efficacy of the influenza vaccine is as low as 17–53% in people 60 years or older, compared with 70–90% in people younger than 60 years.² Innovative strategies designed towards the protection of the older population, who are at a greater risk of infection than are younger people, would therefore provide a major public health benefit.

Interactions between microorganisms in the gut ecosystem and the host have a key role in health and disease.

Lancet Healthy Longev 2021;

2: e13–23

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Research in context

Evidence before this study

We searched PubMed for articles published in English between database inception and Jan 1, 2016, using the search terms “microbiota” OR “microbiome” AND “influenza” OR “influenza-like illness” AND “gut” OR “intestinal” OR “faecal”. We further filtered our results to include only studies with participants aged 45 years or older. We found five peer-reviewed articles. These studies highlighted the potential benefits of microbiota modulation (through prebiotics and probiotics) and the effect of antibiotic therapies on the microbiota and the immune response to infection and vaccination. We did not find any studies on the effect of the microbiota and microbiota changes during an influenza-like illness in adults 60 years or older. The role of the gut microbiota on immune homeostasis and defence against pathogens is widely recognised. In the context of respiratory infections, animal studies have shown that the gut microbiota regulates both the innate and adaptive immune responses against influenza. These studies investigating the role of the microbiota in respiratory infections, although relevant for their mechanistic insights into disease, are limited in translation from animal to human.

Added value of this study

In our study, we had the unique opportunity to identify differences in microbiota composition in older adults

(≥60 years), who have an increased susceptibility to respiratory infections, during an influenza-like illness (ILI). We identified key microorganisms associated with ILI. Furthermore, we identified associations between microbiota and the proinflammatory immune response, which were further supported by an *in vitro* cell-based model of the gut epithelium, providing additional mechanistic insights into how these microbial biomarkers result in the production or the suppression of key host cytokines. Our results corroborate previous findings from animal studies.

Implications of all the available evidence

Our findings show the important role the gut microbiota has in steering immunological responses, and could therefore be used in future studies either as potential biomarkers for susceptibility to respiratory infections or more severe disease outcomes, or even as generalised markers for reduced resilience or frailty within the ageing population. Because of their multiple roles in modulating host immune responses and overall health, better understanding of host-microbe interactions in the gut-lung axis is key for the design of potential therapeutic strategies.

The gut microbiota is essential for, among other things, regulation of mucosal barrier integrity and immune homeostasis of the host,^{3,4} including the response mounted against respiratory infections.^{5,6} Studies illustrate that gut microbes modulate the immune system locally and at distal sites, mostly through products of their metabolism such as short-chain fatty acids.^{7,8} Short-chain fatty acids can travel through the bloodstream and induce differentiation of various immune precursor cells, thereby priming the immune system against infections in the periphery.⁸ Studies in animal models have shown that the gut microbiota shapes the response against respiratory pathogens through regulation of innate antiviral immune responses, the rate of migration of dendritic cells, and local T-cell priming.^{5,9,10} Furthermore, respiratory infections themselves might alter the composition of the microbiota, leading to an increased susceptibility to superinfections, often associated with a more severe disease outcome, through inhibition of the antimicrobial immune response in the gut.^{10,11}

The microbiota of the population aged 60 years or older is characterised by high heterogeneity (interindividual variation), loss of diversity (eg, species richness and evenness) and stability, with enrichment of facultative anaerobes, including potentially pathogenic bacteria, and loss of immune modulatory species (mostly producers of short-chain fatty acids).^{12,13} These changes could be associated with aspects of ageing (eg, changes in appetite

or deterioration of nutrient intake), chronic activation of the immune system, or changes in the gut epithelium.¹ These microbiota characteristics have been associated with frailty in adults 60 years or older, and can affect this population's immune responses to infectious diseases.^{14,15} The aim of this work was to investigate the composition of the microbiota in relation to respiratory infections and local and systemic immune markers in a group of older adults during an influenza season. Understanding these interactions will provide insights for the design of intervention strategies targeting the gut microbiota, which might aid in reducing the burden of disease in this population.

Methods

Study design and participants

In this observational study, participants were selected from a prospective surveillance cohort of individuals with influenza-like illness (ILI), in which community-dwelling adults aged 60 years and older in the Netherlands were recruited through their general practitioner or the Civil Registry (appendix pp 18–19). The ILI surveillance study was done during three influenza seasons (from Dec 1, 2011, to April 30, 2012 [2011–12], from Oct 1, 2012, to April 30, 2013 [2012–13], and from Oct 1 2014, to April 30, 2015 [2014–15]). Participants from the first season were re-invited and additional participants were also recruited through the Civil Registry. Participants were part of the study for the

For more on the Civil Registry see <https://www.government.nl/topics/personal-data/personal-records-database-brp>

See Online for appendix

entire duration of each season and contacted at the end of the season to verify participation. Details of study design and inclusion criteria have been published previously.¹⁶ We added exclusion criteria additional to the larger surveillance study. Patients were excluded from the analysis if they had transplantation surgery, presented with asplenia, leukaemia, lymphatic cancer, colorectal cancer, or combinations of such, or reported multiple ILI events. Ethical approval was granted by the Medical Ethical Committee Noord Holland and participants provided written informed consent.

Procedures

Faecal samples were collected between Oct 1, 2014, and June 15, 2015, during the 2014–15 influenza season from participants registering an ILI event within 72 h of reporting symptoms, with a follow-up (recovery) sample collected 7–9 weeks after the ILI event. Asymptomatic participants not reporting any event throughout the season were divided into 5-year age groups and faecal samples were randomly collected in monthly intervals from individuals in the different age ranges (appendix p 18). Participants completed extensive questionnaires (provided at the time of sample collection) about demographics, medical history, and lifestyle, and self-reported symptoms. Influenza-like symptoms were determined according to the Dutch Pel criteria and were defined by fever ($\geq 37.8^{\circ}\text{C}$) with at least one other symptom of headache, myalgia, sore throat, coughing, rhinitis, or chest pain.¹⁶ The scores of ILI symptoms were recorded as present or absent, and, if present, their duration was also recorded. Nasopharyngeal and oropharyngeal swabs to identify the causative agent, and serum samples, were collected at the same timepoints as the faecal samples (appendix pp 20–21).

DNA was extracted from faecal samples by a repeated bead-beating and column purification procedure, and paired-end sequencing of the 16S rRNA gene V4 hypervariable region was done as previously described on the Illumina MiSeq platform (Illumina, Eindhoven, Netherlands; appendix pp 19–20). Sequence data were processed with the DADA2 pipeline (version 1.10.1) following default recommendations (appendix p 20). Raw fastQ files that support the findings of this study have been deposited in the European Nucleotide Archive under accession number PRJEB37868.

LEGENDplex bead-based immunoassays (BioLegend, San Diego CA, USA) were used to quantify the concentrations of several immune markers in faecal washes from the ILI and control groups, in serum samples from the ILI, ILI recovery, and control groups, and in supernatants of in vitro cell cultures (appendix pp 21–22). Immune markers were selected on the basis of their involvement in gut inflammatory conditions.

Detailed information on the cell-based assays can be found in the appendix (pp 22–23). To further understand the effect of the ILI-associated microbial ecosystem on the

host immune response, we cultured *Ruminococcus torques* and adherent invasive *Escherichia coli* (AIEC), either individually or in combinations, in an in vitro CaCo-2 cell-based human gut epithelial model and analysed the induced immune profiles. CaCo-2 cells were obtained from the American Type Culture Collection (ATCC HTB-37, Manassas, VA, USA). *R torques* was obtained from the American Type Culture Collection (ATCC27756) and *E coli* (AIEC) strain LF82 was kindly provided by Peter Ernst (University of California San Diego, San Diego, CA, USA).

Statistical analysis

Population characteristics were compared by use of the Cochran-Mantel-Haenszel test, adjusting for age and sex (appendix p 23). The sum statistic was used to test for associations between the relative abundance of each amplicon sequence variant (ASV) and a numerical or numerically coded variable, and the Kruskal-Wallis test was used for categorical variables. Information on how we controlled for confounding factors can be found in the appendix (pp 23–24). Pairwise comparisons were done by use of the Wilcoxon rank sum test. p values were corrected for multiple testing with the Benjamini-Hochberg method. Immunological data measured in serum samples were transformed into the logarithmic means of two measurements, each pair of measurements having been obtained in two different runs and their logarithms centred by subtraction of the within-run means to correct for the batch effect. Random forest algorithms were used to assess the accuracy of the faecal microbiota composition in predicting disease status, to identify which species had the strongest predictive functions, and to establish the strongest predictors among other predictor variables (by use of data on medication, comorbidities, risk factors, and demographics). Detailed information on predicting ILI status can be found in the appendix (p 25). Data analyses were done with R version 3.6.0. More information regarding references, methodology, and R packages can be found in the appendix (pp 23–27). This study is registered with the Netherlands Trial Registry, number NL4666.

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. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Between Oct 1, 2014, and April 30, 2015, 2425 older adults were recruited into the ILI surveillance cohort (appendix pp 18–19).¹⁶ From Oct 1, 2014, to June 15, 2015, faecal samples were collected from 397 participants, of whom 213 (54%) reported an ILI event once throughout the season and 184 (46%) did not report any ILI event throughout the season (table). 192 (90%) of 213 participants recovered from their ILI and provided faecal samples

	Control (n=184)	Influenza-like illness (n=213)	p value	Adjusted p value
Demographics				
Sex				
Female	92 (50%)	113 (53%)	0.55	0.77
Male	92 (50%)	100 (47%)	0.55	0.77
Age, years	71.33 (6.35)	69.23 (5.71)	0.0007	0.0084
Body-mass index, kg/m ²	26.46 (4.54)	25.26 (3.74)	0.016	0.056
Smoking	14 (8%)	16 (8%)	0.61	0.74
Passive smoking*	14 (8%)	7 (3%)	0.11	0.25
Comorbidities				
Autoimmune disease	4 (2%)	11 (5%)	0.18	0.35
Cardiovascular disease	27 (15%)	28 (13%)	1.0	1.0
Diabetes	20 (11%)	19 (9%)	0.73	0.83
Malignancies	17 (9%)	5 (2%)	0.0061	0.031
Renal disease	3 (1%)	2 (1%)	0.65	0.76
Respiratory disease	24 (13%)	46 (22%)	0.010	0.044
Vaccinations				
Influenza vaccination	146 (79%)	143 (67%)	0.0036	0.021
Other vaccinations	5 (3%)	1 (<1%)	0.088	0.22
Medications				
ACE inhibitors	24 (13%)	20 (9%)	0.33	0.55
α blockers, β blockers, or both	60 (33%)	33 (15%)	0.0002	0.0030
Analgesic	28 (15%)	53 (25%)	0.011	0.042
Antiarrhythmic	7 (4%)	3 (1%)	0.50	0.73
Antibiotics	21 (11%)	59 (28%)	<0.0001	0.0014
Anticoagulants	47 (26%)	33 (15%)	0.089	0.21
Antidepressants	21 (11%)	6 (3%)	0.0008	0.0088
Antiepileptic	4 (2%)	7 (3%)	0.36	0.57
Antihistamines	5 (3%)	9 (4%)	0.58	0.78
Angiotensin II receptor blockers	33 (18%)	27 (13%)	0.19	0.36
Asthma or COPD medication	13 (7%)	21 (10%)	0.064	0.20
Benzodiazepines	11 (6%)	2 (1%)	0.0034	0.024
Calcium medication	26 (14%)	24 (11%)	0.87	0.92
Corticosteroids	33 (18%)	51 (24%)	0.075	0.22
Diuretics	24 (13%)	25 (12%)	0.75	0.82
Dopaminergic	5 (3%)	1 (<1%)	0.086	0.23
Gonadocorticoids	1 (1%)	3 (1%)	1.0	1.0
Immunosuppressants	2 (1%)	0	0.23	0.40
Insulin	2 (1%)	5 (2%)	0.44	0.67
Other cancer medication	3 (2%)	1 (<1%)	0.50	0.75
Proton pump inhibitors	58 (32%)	48 (23%)	0.16	0.32
Statins	69 (38%)	45 (21%)	0.0032	0.028
Type 2 diabetes medication	18 (10%)	17 (8%)	0.59	0.77

Data are n (%) or mean (SD), unless otherwise stated. A p value less than 0.05 and an adjusted p value less than 0.2 were considered significant. ACE=angiotensin-converting enzyme. COPD=chronic obstructive pulmonary disease. *Passive smoking refers to exposure to other household members who smoke.

Table: Baseline characteristics

7–9 weeks after the ILI event. 15 participants were analysed for the presence of pathogens, but were excluded from the microbiota analyses because they had transplantation surgery, presented with asplenia, leukaemia, lymphatic cancer, colorectal cancer, or combinations of such, or reported multiple ILI events. Medication use was extensive

in both groups, with 159 (86%) of 184 participants in the control group and 178 (84%) of 213 participants in the ILI group taking at least one medication. The causative agent was detected in 200 (79%) of 254 ILI events reported (appendix p 1).

We investigated the faecal microbiota composition in relation to the ILI event, immune status, and additional factors such as comorbidities and medication use. The Firmicutes was the most abundant phylum (mean relative abundance 75.73% [SD 13.19]), and, together with the Bacteroidetes (15.97% [10.90]) and the Proteobacteria (2.56% [6.50]), accounted for 94.26% of the mean relative abundance of all phyla (figure 1A). We observed high inter-individual variation (appendix p 12) and the overall faecal microbiota composition differed between the study groups ($r^2=0.01$; adjusted $p=0.0060$; appendix p 13). The Firmicutes were less abundant in the ILI group (mean relative abundance 73.49% [SD 14.51]) than in the control group (78.32% [SD 10.95]; adjusted $p=0.0027$), but the Bacteroidetes (17.51% [11.41] in the ILI group and 14.19% [10.02] in the control group; adjusted $p=0.014$) and the Proteobacteria (3.40% [8.10] in the ILI group and 1.57% [3.69] in the control group; adjusted $p=0.015$) were more abundant in the ILI group than in the control group (figure 1A). Although no difference was found in alpha diversity between the groups (data not shown), the observed median divergence (ie, the dissimilarity of each sample against the group median) was higher in the ILI group (0.61 [IQR 0.58–0.68]) than in the control group (0.59 [0.56–0.64]; adjusted $p=0.0001$; figure 1B).

22 taxa were found to be differentially abundant in the two groups ($p<0.0055$; adjusted $p<0.1950$; appendix p 2). Proteobacteria species, such as those from the *Escherichia/Shigella* group, and the *Sutterella* genus, Bacteroidetes species, such as those from the *Alistipes* or *Bacteroides* genera, and a taxon from the *Ruminococcus* genus (classified as *R torques*) were positively associated with having an ILI. *R torques* favours the overgrowth of potential pathogens such as *E coli*, which was also found to be positively associated with ILI (adjusted $p=0.17$; appendix p 2). Stratifying samples on the basis of the relative abundance of *R torques* showed that the *Escherichia/Shigella* group was more abundant in samples with an abundance of *R torques* more than the group median (R^*) than in samples with an abundance of *R torques* equal to or less than the group median (R^- ; figure 2A). Taxa belonging to the Firmicutes, such as *Sporobacter* or the butyrate producers *Blautia* and *Eubacterium (rectale)*, were negatively associated with an ILI event (appendix p 2). To investigate the ecological interactions of ILI-associated taxa, we constructed a co-occurrence network (figure 2B). *R torques* co-occurred with the *Escherichia/Shigella* group and the Bacteroidetes *Alistipes* spp (cluster 3; figure 2B). The abundance of this co-occurrence cluster was greater in the ILI group than in the control group and negatively co-occurred with clusters of typically commensal bacteria (including

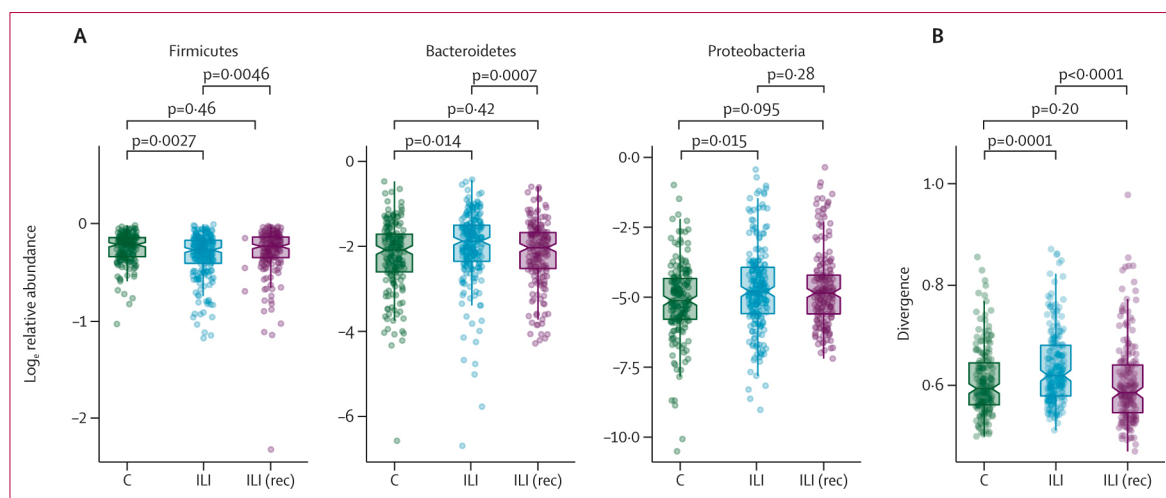


Figure 1: Relative abundances of the dominant phyla and within-group divergence

(A) Relative abundance of the dominant phyla. (B) Within-group divergence by use of Bray-Curtis dissimilarity. In the notched boxplots, the middle line represents the median, the lower hinge corresponds to the first quartile (25th percentile), the upper hinge corresponds to the third quartile (75th percentile), the whiskers extend to the largest and smallest value at $1.5 \times \text{IQR}$ from the hinge, and the notches represent the median $\pm 1.58 \times \text{IQR}/n^{0.5}$. Pairwise comparisons were done by use of the Wilcoxon rank sum test. All p values are adjusted. We considered p values less than 0.05 and adjusted p values less than 0.1 to be significant. C=control. ILI=influenza-like illness. ILI (rec)=influenza-like illness recovery.

butyrate-producing taxa), such as *Eubacterium* spp and *Blautia* spp (cluster 2) and *Sporobacter* spp (cluster 1), which were less abundant in the ILI group than in the control group (figure 2C).

We used random forest analyses to identify the most predictive taxa of ILI, together with data on medication, comorbidities, risk factors, and demographics. Of the top ten predictor variables, eight were bacterial taxa and two were participant characteristics (figure 3A; appendix p 17). Within the top predicting taxa, ASVs corresponding to *R torques*, which was more abundant in the ILI group than in the control group, and *Sporobacter*, which was less abundant in the ILI group than in the control group, had the highest prevalence (ie, the proportion of samples in which these ASVs were detected; figures 3B, 3C). Although alpha diversity was not associated with ILI status, alpha diversity negatively correlated with the abundance of *R torques* (median Shannon diversity index 4.14 [IQR 3.89–4.34] in the Rt group and 4.01 [3.77–4.25] in the Rt' group; figure 3D). By contrast, alpha diversity positively correlated with the abundance of *Sporobacter* (median Shannon diversity index 4.05 [IQR 3.76–4.28] in the *Sporobacter* negative group [samples with an abundance of *Sporobacter* equal to or less than the group median; Sp⁻] and 4.20 [3.94–4.42] in the *Sporobacter* positive group [samples with an abundance of *Sporobacter* more than the group median; Sp⁺]; figure 3E).

To identify associations between the gut microbiota and other frequent health-related indicators, we analysed 23 different medication groups, including those commonly associated with changes in the microbiota (eg, antibiotics and proton pump inhibitors), comorbidities, and risk factors (eg, body-mass index [BMI] and smoking; appendix

pp 3–5, 14). Additionally, in the ILI group, the scores of symptoms of ILI were included (appendix pp 3–5, 14). In the control group, of the ILI-associated taxa, only *Eubacterium rectale* was positively associated with the use of diuretics (adjusted p=0.13), and *R torques* was positively associated with type 2 diabetes medication (adjusted p=0.022) and, correspondingly, with diabetes as a comorbidity (adjusted p=0.029). There was no difference in the use of diuretics or medication for type 2 diabetes or in the prevalence of diabetes between the ILI and control groups (table). The abundance of the ILI-associated, butyrate-producing taxon *E rectale* was positively associated with BMI in the control group (adjusted p=0.0011; appendix p 15), a taxa with a high prevalence among all participants (detected in 380 (96%) of all 397 samples). In our study population, BMI was higher in the control group than in the ILI group (table) and could therefore be considered (within non-obese margins) as beneficial because lower BMI is more indicative of a negative effect of frailty-related underweight in older adults.

Next, we aimed to identify potential long-lasting differences in, or the recovery of, the microbiota ecosystem after the ILI event. To that end, we analysed the faecal microbiota composition in samples from 192 (90%) of 213 participants in the ILI group at a later symptom recovery phase (appendix p 11). Within-group divergence and the relative abundance of Firmicutes and Bacteroidetes were similar between the control group and the ILI recovery group (figures 1A, 1B). In addition, dissimilarity between the microbiota community structures of the control group and the ILI recovery group (mean Bray-Curtis dissimilarity 0.67 [SD 0.09]) was significantly lower than that observed between the control group and the ILI group in the acute phase of infection (0.68 [SD 0.09];

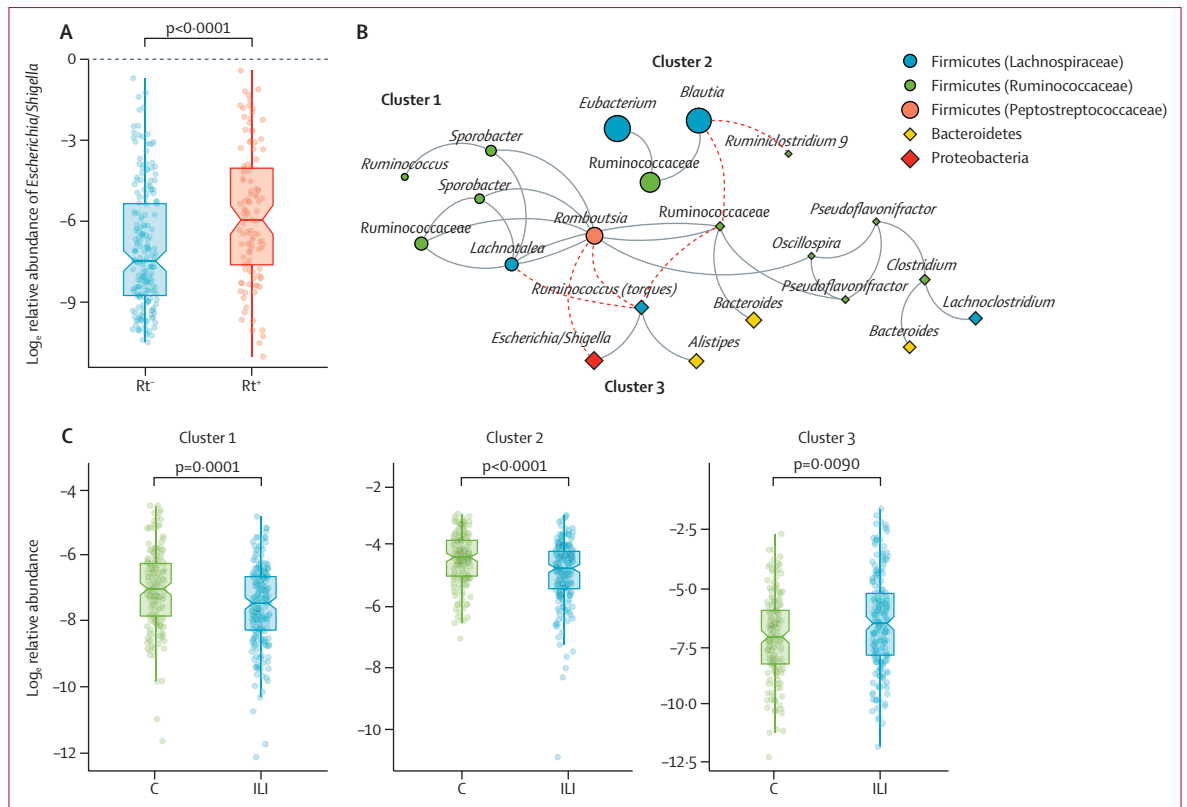


Figure 2: Relative abundances of *Escherichia/Shigella* and co-occurrence clusters

(A) Relative abundance of the *Escherichia/Shigella* group in all samples stratified by *R torques* abundance. (B) Co-occurrence network of ILI-associated bacteria. The size of the nodes indicates their relative abundance in the ILI group and the colour of the nodes corresponds to the phyla (family). The connecting lines in black indicate co-occurrence and the connecting lines in red indicate anti-occurrence. Anti-occurrence is defined as co-occurring in opposite directions (if one is in higher abundance, the other is in lower abundance). The taxa negatively associated with ILI are indicated with circles and those positively associated with ILI are indicated with diamonds. (C) Relative abundance of clusters 1, 2, and 3. In the notched boxplots, the middle line represents the median, the lower hinge corresponds to the first quartile (25th percentile), the upper hinge corresponds to the third quartile (75th percentile), the whiskers extend to the largest and smallest value at $1.5 \times \text{IQR}$ from the hinge, and the notches represent the median $\pm 1.58 \times \text{IQR}/n^{0.5}$. Pairwise comparisons were done by use of the Wilcoxon rank sum test. All p values are adjusted. We considered p values less than 0.05 and adjusted p values less than 0.1 to be significant. C=control. ILI=influenza-like illness. R_t^- =*R torques* abundance less than or equal to the group median abundance. R_t^+ =*R torques* abundance more than the group median abundance. *R torques*=*Ruminococcus torques*.

adjusted $p < 0.0001$; appendix p 13). At the ASV level, taxa positively associated with ILI from the Bacteroidetes phylum and *R torques* were less abundant in the ILI recovery samples than in the acute ILI samples (appendix pp 6–8). Commensal taxa that were negatively associated with ILI were more abundant in the recovery samples than in the acute ILI samples ($p < 0.045$, adjusted $p < 0.20$; appendix pp 6–8), with abundances similar to those found in the control group (appendix p 9), suggesting an overall restoration of the microbial ecosystem. However, Proteobacteria remained more abundant in the ILI recovery group (mean relative abundance 3.14% [SD 7.81]) than in the control group (1.57% [3.69]; adjusted $p = 0.095$). Consequently, most ASVs associated with recovering from an ILI corresponded to an increased abundance of taxa from this phylum, including the *Escherichia/Shigella* group ($p = 0.0022$; adjusted $p = 0.064$; appendix p 9).

Taxa positively associated with ILI in our study, such as *E coli*, *R torques*, and *Sutterella*, have been suggested to play a role in sustained inflammation.^{17,18} Therefore,

we investigated the local immune profiles in the gut environment by using faecal washes prepared from a subset of samples from 25 participants selected on the basis of their abundance of *R torques* (15 R_t^+ participants [ten in the ILI group and five in the control group] and ten R_t^- participants [five in the ILI group and five in the control group]; appendix p 11). Immune markers were detected in 23 (92%) of the 25 selected participants. Although no differences were observed between the groups for the majority of markers (appendix p 16), concentrations of faecal C-reactive protein were higher in the ILI group (median concentration 571.0 pg/mL [IQR 60.4–1455.7]) than in the control group (53.0 pg/mL [21.4–95.4]), predominantly in R_t^+ participants, suggesting an enhanced local inflammatory response associated with this taxa (figure 4A).

Subsequently, we investigated whether these local proinflammatory responses could also be detected systemically by analysing serum immune profiles in association with microbiota composition. For the ILI

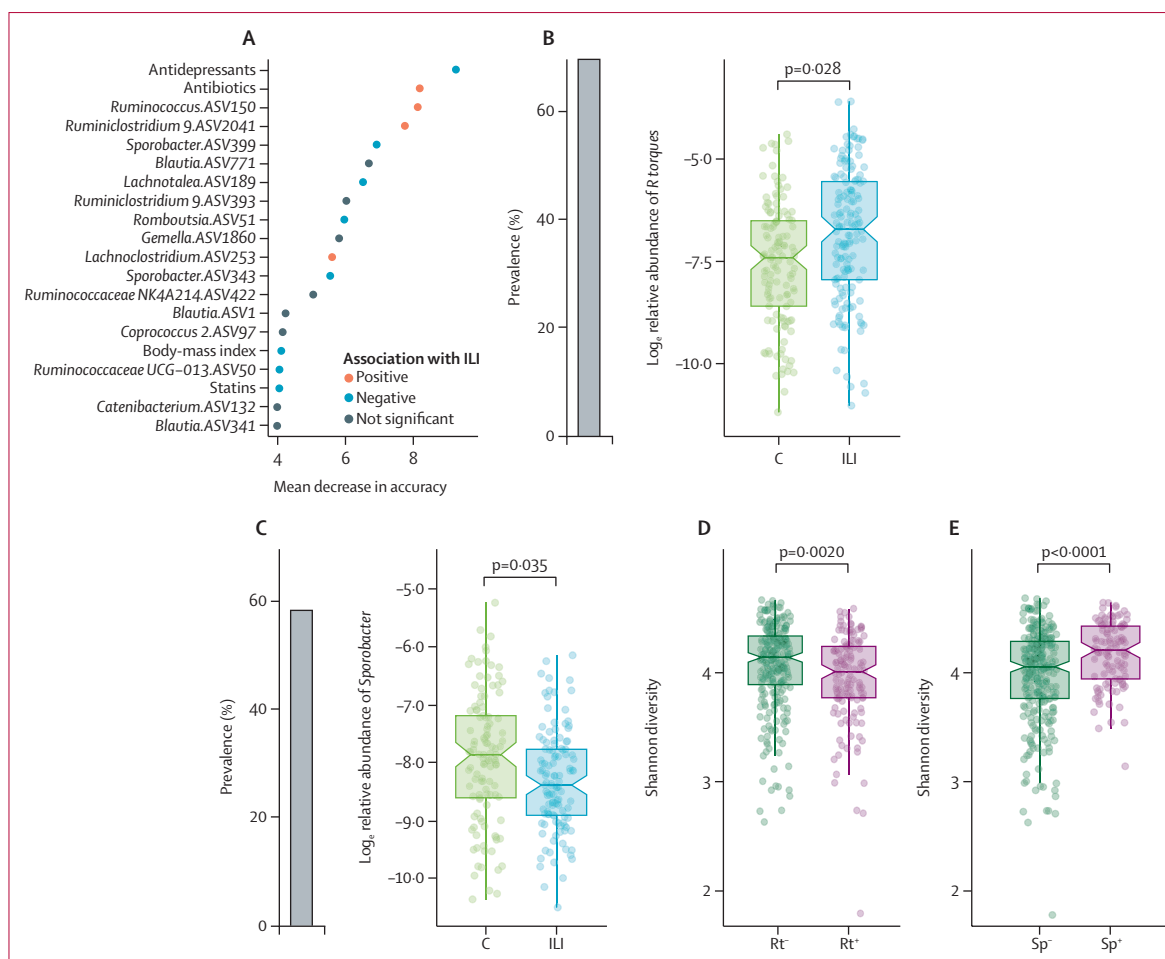


Figure 3: Top ILI predictors, their prevalence, relative abundance, and effect on alpha diversity

(A) Top predictors of ILI in the random forest analysis. The larger a variable's mean decrease in accuracy, the stronger a predictor that variable is. (B) The total prevalence and relative abundance of *R torques*. (C) The total prevalence and relative abundance of *Sporobacter*. (D) Shannon diversity index according to stratification by *R torques* abundance. (E) Shannon diversity index according to stratification by *Sporobacter* abundance. In the notched boxplots, the middle line represents the median, the lower hinge corresponds to the first quartile (25th percentile), the upper hinge corresponds to the third quartile (75th percentile), the whiskers extend to the largest and smallest value at $1.5 \times \text{IQR}$ from the hinge, and the notches represent the median $\pm 1.58 \times \text{IQR}/n^{0.5}$. Pairwise comparisons were done by use of the Wilcoxon rank sum test. All p values are adjusted. We considered p values less than 0.05 and adjusted p values less than 0.1 to be significant. ASV=amplicon sequence variant. C=control. ILI=influenza-like illness. Rt⁻=*R torques* abundance less than or equal to the group median abundance. Rt⁺=*R torques* abundance more than the group median abundance. Rtorques=*Ruminococcus torques*. Sp⁻=*Sporobacter* abundance less than or equal to the group median abundance. Sp⁺=*Sporobacter* abundance more than the group median abundance.

group, we included both sampling times (acute and recovery phase; figure 4B; appendix p 10). We analysed 149 patients in the control group, 176 patients in the acute ILI group, and 157 patients in the ILI recovery group. Significant associations between ASVs and immune markers in the control group involved commensal bacteria from the class Clostridia and from the Bacteroidia, mainly the butyrate-producing genus *Butyricimonas*. These bacteria correlated negatively with proinflammatory cytokines from the CC chemokine family (eg, CCL11, CCL2, CCL3, CCL20, and CCL17; $r^2 \leq -0.1$; adjusted $p < 0.13$; appendix p 10). Positive correlations included *Clostridium sensu stricto*1 and soluble IL1-RN, and Ruminococcaceae and IFN β ($r^2 \geq 0.1$, adjusted $p < 0.14$). In the ILI group, significant associations were mostly

commensal taxa with negative correlations to IL-33 ($r^2 \leq -0.1$, adjusted $p < 0.14$; appendix p 10). As observed locally, the median serum concentration of C-reactive protein was higher in the ILI group (1.38 [IQR 0.97–1.98]) than in the control group (1.06 [0.85–1.29]), which was independent of stratification by *R torques* abundance (figure 4C). However, concentrations of C-reactive protein measured in serum samples collected in the recovery phase were significantly positively correlated with *R torques* abundance, indicating a potential role for this bacterium in sustained inflammation (figure 4D). Additionally, different ASVs assigned to *R torques* correlated negatively with cytokines IL-18, IL-33, and IL-1 β , which regulate the production of antimicrobial peptides in the gut epithelium (appendix p 10).

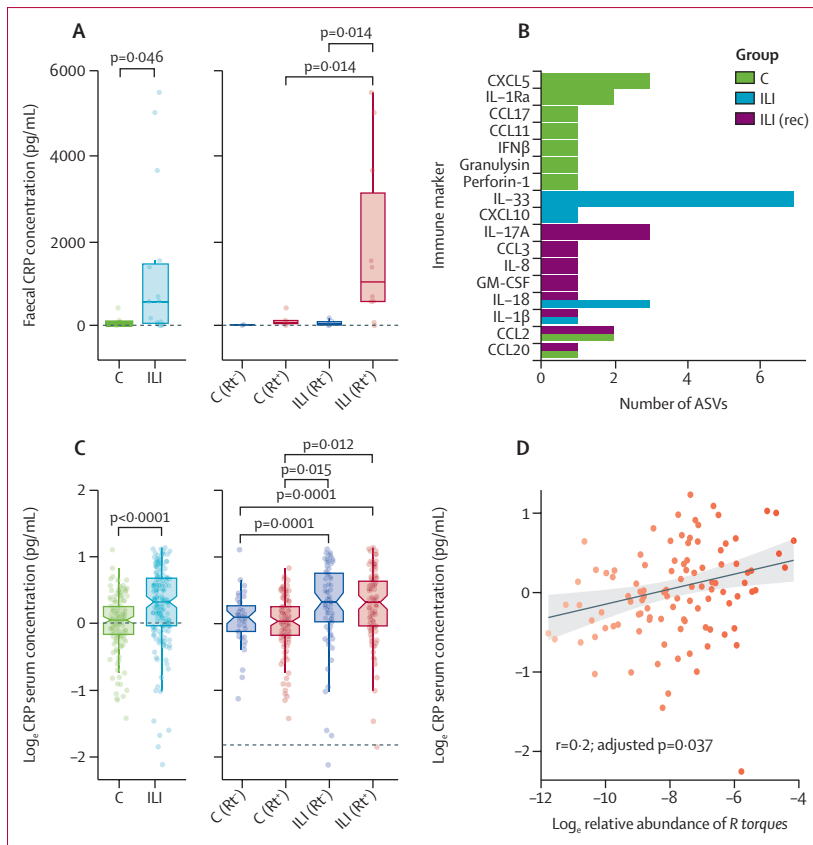


Figure 4: Faecal and serum immune markers

(A) CRP concentrations in faecal washes normalised to the total protein content in both groups (left), and stratified by abundance of *R. torques* (right). (B) Number of ASVs associated with different serum immune markers. (C) Serum CRP concentrations in the control and ILI groups (left) and stratified by abundance of *R. torques* (right). (D) Relative abundance of *R. torques* against CRP serum concentration in samples collected during the recovery phase. In the notched boxplots, the middle line represents the median, the lower hinge corresponds to the first quartile (25th percentile), the upper hinge corresponds to the third quartile (75th percentile), the whiskers extend to the largest and smallest value at $1.5 \times \text{IQR}$ from the hinge, and the notches represent the median $\pm 1.58 \times \text{IQR}/n^{0.5}$. Pairwise comparisons were done by use of the Wilcoxon rank sum test. All p values are adjusted, apart for those for faecal samples. We considered p values less than 0.05 and adjusted p values less than 0.1 to be significant. ASVs=amplicon sequence variant. C=control. CRP=C-reactive protein. ILI=influenza-like illness. ILI (rec)=influenza-like illness recovery. Rt=*R. torques* abundance less than or equal to the group median abundance. Rt+=*R. torques* abundance more than the group median abundance. *R. torques*=*Ruminococcus torques*.

To further understand the effect of the ILI-associated microbial ecosystem on the host immune response, we cultured *R. torques* and *E. coli* (AIEC) (individually or in combinations) in an in vitro cell-based human gut epithelial model (CaCo-2) and analysed the induced immune profiles. Both *R. torques* and *E. coli* (AIEC) were able to survive and grow under the conditions of our in vitro model. Individual cultures with *R. torques* induced a proinflammatory response similar to that observed in vivo, with the production of CXC chemokines (ie, IL-8, CXCL10, and CXCL1), CCL20, and IL-18 by the epithelial monolayer (figure 5). Conversely, stimulation of cells with single cultures of AIEC only induced the production of IL-33 and IL-23, which was maintained (or even enhanced) when AIEC was co-cultured in combination with *R. torques* (figure 5). When cultured in combination,

AIEC inhibited the immune response induced by *R. torques* for all markers, including the pro-inflammatory cytokine IL-18 (figure 5).

Discussion

The role of the gut microbiota on immune homeostasis and defence against pathogens has been widely recognised.^{3,4} In the context of respiratory infections, animal studies have shown that the gut microbiota regulates the immune responses against influenza, the main pathogen within our study.^{5,8,9} Influenza lung titres remain significantly elevated in antibiotic-treated mice with an altered microbiota composition.⁵ Many of these studies, although relevant for their mechanistic insights, are done in mice. Here, we had the unique opportunity to identify differences in microbiota composition in an older adult population with ILI. As previously reported for older adults, microbiota heterogeneity was high, but divergence was higher in the ILI group than in the control group, indicating a temporal disruption of the microbiota suggestive of an unstable environment.¹⁹ Moreover, the decrease in the abundance of Firmicutes and the increase in the abundance of Proteobacteria and Bacteroidetes in the ILI group compared with the control group have been previously described in mice after viral infection.^{10,20,21}

We were able to identify key microorganisms associated with ILI. In our study, *Sporobacter* was more abundant in the control group than in the ILI group, and this genus has been consistently shown to be less abundant in immune-mediated inflammatory diseases than in healthy controls.²² By contrast, *R. torques*, which was more abundant in the ILI group than in the control group, is associated with inflammation-mediated diseases, such as inflammatory bowel disease and autism.^{17,18} *R. torques* possesses an intramolecular trans-sialidase that allows it to adapt and grow in the mucus layer of the gut, metabolising gut mucin, which can subsequently be used by other members of the gut ecosystem, including potential pathogens such as *E. coli*.²³ Indeed, we found a significant correlation (and potential symbiosis) between the abundance of *R. torques* and the abundance of *Escherichia/Shigella* (also positively associated with ILI). Because butyrate is oxidised by colonic cells as their primary source of energy and this process consumes high amounts of oxygen, anaerobiosis is maintained in the gut lumen, which is beneficial for the gut ecosystem.^{24,25} When there is a decrease in butyrate-producing taxa (eg, *E. rectale* or *Blautia*, which were less abundant in the ILI group than in the control group), and so limited concentrations of butyrate, colonocytes shift their metabolism to glucose, creating a more aerobic environment that leads to overgrowth of facultative anaerobes (eg, *E. coli*), which could potentially compromise gut epithelial health.²⁵

In the asymptomatic control population, microbiota significantly associated with immune markers included commensal Clostridia and butyrate-producing genera

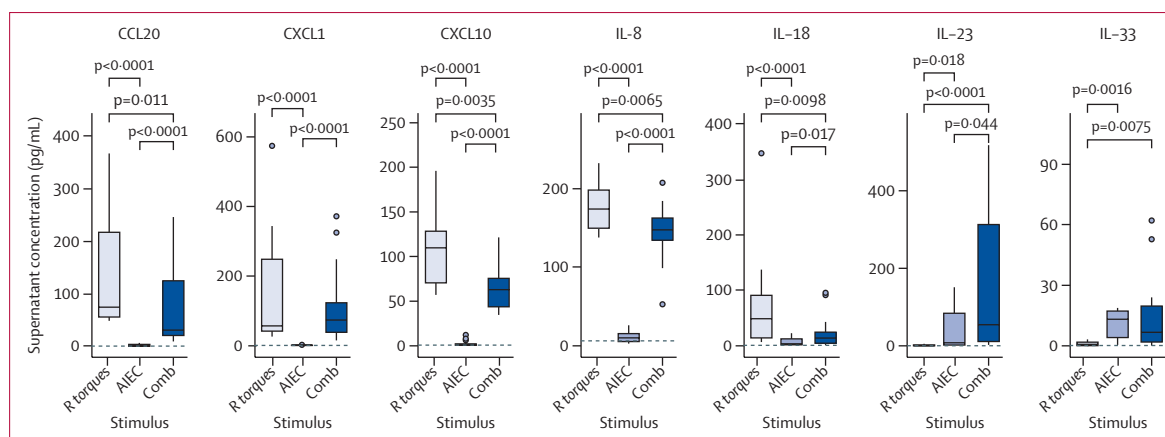


Figure 5: Supernatant concentrations of immune markers after in vitro stimulation of CaCo2 cells with *R torques*, AIEC, or a combination of both

An average of eight independent measurements were taken in three different experiments. Extreme outliers (defined as values higher than the third quartile + $[3 \times \text{IQR}]$ or lower than the first quartile + $[3 \times \text{IQR}]$) were identified and removed from the dataset. The dashed lines indicate the median concentration of immune markers in blanks. In the boxplots, the middle line represents the median, the lower hinge corresponds to the first quartile (25th percentile), the upper hinge corresponds to the third quartile (75th percentile), and the whiskers extend to the largest and smallest value at $1.5 \times \text{IQR}$ from the hinge. Pairwise comparisons were done by use of the Wilcoxon rank sum test. All p values are adjusted. We considered p values less than 0.05 and adjusted p values less than 0.1 to be significant. AIEC= adherent invasive *Escherichia coli*. Comb=combination. *R torques*=*Ruminococcus torques*.

such as *Butyricimonas*. Butyrate has been shown to confer protection against influenza infection in mice, reducing inflammation, tissue damage, and increasing survival.²⁶ In the control group, these bacteria negatively correlated with proinflammatory chemokines that are generally involved in the chemotaxis of immune cells during inflammation, including inflammatory processes generally associated with ageing.²⁷ Positive correlations included those between certain microbiota and soluble IL-1RN (an inhibitor of proinflammatory cytokines IL-1 α and IL-1 β) and IFN β , known for its antiviral properties. These results suggest a homeostatic immune environment in which inflammation is controlled through commensal bacteria or the products of their metabolism, potentially leading to increased resilience to respiratory infections. By contrast, bacteria that were associated with the ILI event (eg, *R torques*, a higher abundance of which correlated with higher concentrations of both local and systemic C-reactive protein, *Sutterella*, or *Escherichia/Shigella*) are known for their pro-inflammatory properties.^{17,18} We also found *R torques* to be negatively associated with systemic IL-33, IL-18, and IL-1 β , which are involved in the regulation of gut epithelial function and immune homeostasis. Cytokines IL-18 and IL-1 β (processed and activated by CASP-1) are normally induced upon infection with influenza virus and have a crucial role in the antiviral activity of the immune system.^{28,29} Although the mechanisms by which the microbiota activates inflammasomes remain largely unknown, it has been speculated that commensal microorganisms are necessary for activation leading to proper (adaptive) immune responses to viral infections.^{5,30} Despite showing that *R torques* can induce IL-18 production by gut epithelial cells in vitro, *R torques* was negatively associated with IL-18 in the ILI group in vivo. These contrasting findings are potentially due to (among other taxa contributing to

the host response) the co-occurrence of *R torques* with the *Escherichia/Shigella* group—*E coli* dampened the production of IL-18 in vitro, reinforcing the symbiotic effect of these biomarkers. The ability of AIEC to suppress the epithelial inflammatory response (even pro-inflammatory responses induced by *R torques* and potentially by other members of the microbiota) suggests a mode of pathogenicity resulting in suboptimal epithelial control of invading pathogens, facilitating its overgrowth and therefore further increasing the risk for superinfections, which can ultimately lead to more severe disease outcomes. We speculate that either *R torques*, other ILI-associated taxa, or a combination of both might indirectly inhibit inflammasome activation, which affects the ability to mount a proper immune response against respiratory infection and the production of anti-microbial peptides in the gut epithelium.

We hypothesise that changes in the microbiota composition in the population 60 years or older upon respiratory infection can lead to a generalised pro-inflammatory state that, in addition to the impaired inflammasome response, could result in more severe disease outcomes and increased susceptibility to frequent bacterial or fungal superinfections in this population.¹⁰

Our study had some limitations, which were mostly because of the heterogenous nature of our cohort of older adults (eg, extensive use of medication, comorbidities, and other exposures). Although we included many observed confounding factors, others are unknown. Consequently, stratifying on different variables or correcting for all potential confounders would result in the loss of a substantial part of the data. We aimed to report some of the most important associations between variables and the microbiota, trying to purify these associations as much as possible by correcting for age and sex. However, the

associations reported are bound to be contaminated to some extent by other confounding factors. In addition, our results will need to be further investigated in additional experimental settings, as partly done in our study, to provide causal insights. Differences observed between samples collected during the acute phase of the ILI and at the later recovery timepoint could be intrinsic to the ILI population (differences pre-existing to the ILI event) or a result of infection.

Because of their multiple roles in modulating host immune responses and overall health, better understanding of host–microbe interactions is key for the design of potential therapeutic strategies. Our findings could be used in future studies as potential biomarkers for susceptibility to respiratory infections or more severe disease outcomes, or even as generalised markers for reduced resilience with ageing. Randomised controlled trials using prebiotic and probiotic interventions in different age groups have shown promising results, shortening the duration or number of episodes of several respiratory tract infections.^{31–33} Whether the target is the bacteria themselves or the products of their metabolism that interact with the host, the microbiota can potentially be modulated through different dietary, non-dietary, or other interventions. Overall expectations are that these interventions might lead to a more resilient immune environment against respiratory infections. However, the complexity of this system, especially in the ageing population, requires additional studies, such as those looking at seasonal influenza vaccination and those using mechanistic approaches to discern differences between association and causality.

Contributors

DvB and JvB were responsible for the ILI study. DvB and SF conceived and designed the microbiota experiments. SF, GdH, JdW, NMN, and LW designed and did the in vitro experiments on the gut epithelial model. JvB, DvB, JdW, SF, GdH, NMN, and RJ designed and did the immune analyses. JAF, MAN, and J LAP did the statistical analyses. SF and DvB wrote the paper. SF and JAF accessed and verified the data. All authors interpreted the results, critically revised the manuscript for important intellectual content, and approved the final manuscript.

Declaration of interests

We declare no competing interests.

Data sharing

Data collected for the study, including individual participant data and a data dictionary defining each field in the set, will be made available with publication of the Article to others on request. These data include microbiota data (already available at the European Nucleotide Archive under accession number PRJEB37868) and a selection of descriptors. No additional related documents will be available. Data will be available to the requester via email after the approval of a request, with a clear indication on what to share.

Acknowledgments

This work was supported by the Dutch Ministry of Health, Welfare and Sport, and the Strategic Program of the National Institute for Public Health and the Environment. We gratefully acknowledge all participants for their time and commitment to the study. We thank the study staff at the Spaarne Hospital, Hoofddorp, the Netherlands (Marlies van Houten) and at the Microbiology and Systems biology department at the Netherlands Organisation for Applied Scientific Research, Zeist, the Netherlands (Bart Keijser, Nynke van Berkum, and Jolanda Kool).

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