The effect of influenza virus on the human oropharyngeal microbiome

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36 **Abstract**

37 **Background: Secondary bacterial infections are an important cause of morbidity and**
38 **mortality associated with influenza infection. As bacterial disease can be caused by a**
39 **disturbance of the host microbiome, we examined the impact of influenza on the upper**
40 **respiratory tract microbiome in a human challenge study.**

41 **Methods: The dynamics and ecology of the throat microbiome were examined following**
42 **experimental influenza challenge of 52 previously healthy adult volunteers with Influenza**
43 **A/Wisconsin/67/2005 (H3N2) by intranasal inoculation; 35 healthy control subjects were**
44 **not subjected to viral challenge. Serial oropharyngeal samples were taken over a 30-day**
45 **period, and the V1-V3 region of the bacterial 16S rRNA sequences were amplified and**
46 **sequenced to determine the composition of the microbiome. The carriage of pathogens was**
47 **also detected.**

48 **Results: forty three of 52 challenged individuals developed proven influenza infection, of**
49 **whom 33 became symptomatic. None of the controls developed influenza, although 22%**
50 **reported symptoms. The diversity of bacterial communities remained remarkably stable**
51 **following the acquisition of influenza, with no significant differences over time between**
52 **individuals with influenza and those in the control group. Influenza infection was not**
53 **associated with perturbation of the microbiome at the level of phylum or genus. There was**
54 **no change in colonisation rates with *Streptococcus pneumoniae* or *Neisseria meningitidis*.**

55 **Conclusions: The throat microbiota is resilient to influenza infection, indicating the**
56 **robustness of the upper airway microbiome.**

57

58

59 **Introduction**

60 Secondary bacterial infection is a major cause of the mortality and morbidity associated with
61 influenza virus [1]. Bacterial pneumonia caused millions of deaths during influenza pandemics
62 in the 20th century [2], and remains a frequent complication following non-pandemic
63 influenza. For example, 94% of a group of patients who died in the 1918 influenza pandemic
64 demonstrated that all had evidence of secondary bacterial pneumonia [3], while 28% of
65 fatalities in New York City associated with the 2009 H1N1 influenza pandemic had bacterial
66 co-infection, with most only diagnosed post-mortem [4].
67
68 Several pathogens cause bacterial infection following influenza. Co-infection with
69 *Haemophilus influenzae* was so common that it was thought to cause influenza before the
70 virus was isolated [5]. *Streptococcus pneumoniae* is now the commonest influenza-associated
71 infection [6]. Cavitatory *Staphylococcus aureus* pneumonia is a severe influenza complication,
72 and secondary pneumonia with MRSA emerged in the 2009 pandemic [7]. There is also an
73 association between influenza A and systemic *Neisseria meningitidis* infection [8].
74
75 Multiple mechanisms could explain bacterial disease following influenza. The virus is cytolytic
76 and induces epithelial cell damage, impairment of surfactant production, and reduced muco-
77 ciliary clearance [9, 10]. Exposure of the epithelial basement membrane and extracellular
78 matrix can offer binding sites for bacteria expressing matrix binding molecules [11]; glycan
79 receptors for bacteria can be revealed by the activity of viral and bacterial neuraminidases
80 (NA) [12, 13]. Additionally increased secretion of galectin 1 and galectin 3 can favour galectin-
81 mediated adhesion of *S. pneumoniae* and *N. meningitidis* [14]. Murine models indicate that
82 host cell sialic acid released by NA can also act as a nutrient for pneumococcal growth in the
83 upper airway, promoting bacterial spread to the lower airways [15].

The human microbiome is a complex community that has co-evolved with its host. A healthy microbiota protects against invasion by pathogenic bacteria through a process referred to as colonisation resistance, in which the microbiota restricts growth or attachment of pathogens by competing for nutrients and ecological niches, or by direct antagonism [16].

Several external factors influence the microbiome including age, smoking, and antibiotic treatment [17-19]. Furthermore changes in the microbiota are found with certain airway infections [20]. For example, healthy volunteers were reported to have a more diverse nasopharyngeal microbiome compared with patients suffering from pneumonia [21], while a highly diverse community is found in patients with asthma, cystic fibrosis, or Chronic Obstructive Pulmonary Disease [22, 23]. However, little is known about the impact of viral infection on the microbiota. No specific profile of bacteria in the URT was associated with seven different viral infections, although an apparent decrease in carriage of *Haemophilus* and *Neisseria* was observed after rhinovirus infection [24, 25].

To further understand the cause of secondary bacterial infections, we investigated the impact of influenza virus on the human nasopharyngeal microbiome. We characterised the microbiota over a 30- day period in 52 healthy adults challenged with influenza A H3N2, and 32 non-challenged individuals by using barcoded 454 pyrosequencing. This approach allowed us to define changes in the human microbiome following influenza challenge. Furthermore we examined whether influenza affected carriage of pathogens resident in the upper airways.

107 **MATERIALS AND METHODS**

108 ***Study design***

109 The study took place in a secure quarantine facility with written informed consent from
110 volunteers, in accordance with the Declaration of Helsinki, and in full compliance with the UK
111 regulatory and ethical (IRB) requirements.

112

113 Volunteers were screened before study entry to assess susceptibility to the challenge virus.
114 In brief, volunteers were healthy with no acute or chronic medical condition, between the
115 ages of 18 and 45, not living with anyone at risk of influenza complications, and not to have
116 had a seasonal influenza vaccine in the last three years. Blood samples from volunteers were
117 collected immediately before quarantine entry for antibody testing.

118

119 Subjects were randomly allocated to challenge (n=52) or control groups (n=35) on day -1. On
120 day 0, individuals in the challenge group were inoculated intranasally with a total of 5.5 $\log_{10}$
121 TCID50/ml/nostril of influenza strain A/H3N2/Wisconsin/67/2005. The control group were not
122 subjected to viral challenge (Figure 1).

123

124 ***Clinical monitoring and sample collection***

125 Volunteers recorded symptoms daily, and vital signs were recorded three times daily.
126 Nasopharyngeal (NPS) and oropharyngeal swabs, and venous blood were collected on days
127 −1, 3, 6 and 28. Respiratory specimens were analysed by quantitative PCR and serological
128 specimens by HAI and microneutralisation (MN) assays. Laboratory confirmed influenza was
129 defined as: a 4-fold or greater rise in HAI or MN titres between day -2 and day 28, or a positive
130 NPS test by PCR.

131

132 Volunteers were classified according to their symptoms and signs. 'Influenza-like illness' (ILI)
133 was defined according to the CDC definition: an illness lasting ≥24 hours with either a
134 temperature of > 37.9°C, or two or more symptoms, at least one of which was respiratory.
135 Volunteers were 'symptomatic' if they experienced symptoms with no fever, experienced a
136 single symptom, had ≥ two symptoms but none of which were respiratory, or had an illness
137 of <24 hours. Therefore, a volunteer could have an ILI or be Symptomatic, and not be infected
138 with influenza. Individuals were classified according to their symptomatology and tests into:
139 ILI^{flu+}, subjects who received viral challenge, and had an ILI with evidence of influenza infection
140 (n=19); S^{flu+}, subjects who received viral challenge and were symptomatic with evidence of
141 influenza infection (n=14); and ILI/S^{flu+} includes individuals belonging to both these groups
142 (n=33). Those not challenged with influenza, who remained asymptomatic/influenza negative
143 formed the AC (Asymptomatic Control) group (n=24).

144

145 ***DNA isolation and pyrosequencing***

146 DNA was extracted from swabs using the MoBio Power Soil isolation kit (MoBio laboratories).
147 Barcoded primers were used to amplify ~ 500 bp V1-V3 region of the 16S rRNA gene as
148 previously [26]. Amplification conditions were 5 minutes at 95°C, then 25 cycles of 95°C for
149 45 seconds, 53°C for 45 seconds, 72°C for 1 minute 30 seconds with a final step of 72°C for 15
150 minutes. The products were analyse using an Agilent 2100 Bioanalyzer after purification with
151 the QIAquick kit (Qiagen). Quantitation was performed using Quant-iT Picogreen (Invitrogen).
152 Samples were pooled at equimolar concentrations, and sequenced using a Roche 454 GS-FLX
153 Titanium platform.

154

Sequence analysis

The quality of sequences was assessed using FastQC v0.11.3 (Babraham Institute). Pre-processing of sequences was performed with mothur v1.35.1 [27] based on the Schloss operating procedure. Next, trim.flows was used to remove sequences with mismatches in the primer sequence or the barcode. Sequences were de-noised by shhh.flows, trimmed to remove primers and barcodes, then aligned to the SILVA 16S rRNA reference alignment using trim.seqs; chimeric sequences were eliminated by Uchime [28]. Sequences were BLASTed against the Human Oral Microbiome Database v13.2 at the 98.5% sequence identity level. Sequences met the following criteria: read length >375 nt. (excluding barcode and primers), no ambiguous bases, or homopolymers of >8 nt.

A sequence dissimilarity distance of 0.015 was used to cluster sequences into operational taxonomic units (OTUs), using the average neighbour algorithm. Taxonomies were assigned using the HOMD reference dataset. The richness of the microbial communities was calculated using Chao1 and Catchall indices [29, 30], and diversity estimated with Simpson's inverse [31] and Shannon [32] indices. Good's non-parametric estimator assessed coverage of communities in samples [33]. Species diversity between the samples was examined by analysing the beta diversity. Samples were adjusted to 2997 reads per sample by random sampling. Distance matrices were generated with the Jaccard Index and thetaYC measure of dissimilarity [34], and visualized by principal coordinate analysis (PCoA). Dendrograms representing relationships between samples were analysed by parsimony and UniFrac [35].

179 **S. pneumoniae *and* N. meningitidis *carriage***

180 *N. meningitidis* was isolated by plating samples to GC selective medium. A 413 bp fragment
181 encoding the 50S ribosomal protein L6 (RplF) was amplified by PCR, sequenced, and identified
182 with PubMLST [36]. Meningococcal isolates were characterized by serogrouping, serotyping
183 and serosubtyping. To detect the pneumococcus, qPCR of a region of *lytA* was performed
184 using TaqMan (Thermo-Scientific) as previously [37].

185

186 ***Statistical analyses***

187 Analysis of alpha diversity was performed using Kruskal-Wallis test with Dunn's correction for
188 multiple comparisons was applied. Analysis of Molecular Variance (AMOVA) was used to
189 evaluate differences between groups in PCoA [38]. Parsimony tests were implemented by
190 mothur [39]. The Linear Discriminant Analysis (LDA) effect Size (LEfSe) was used to examine
191 differences in Operational Taxonomic Units (OTUs) in the control/challenged groups [40]; the
192 alpha values were set to 0.05 and a LDA score of 2.5 was selected. A two-tailed Fisher exact
193 test was applied to compare colonization rates for pathogens.

194

RESULTS

Infection and the development of symptoms after influenza challenge

Although volunteers were screened for antibodies against influenza in advance, three individuals in the challenge group and three in the control group had serological evidence of prior H3N2 infection at study entry, so were excluded from subsequent analysis.

Influenza infection was confirmed in 43 of the 52 individuals (82%) who subjected to influenza challenge; 33 subjects developed symptoms including 19 with ILI (ILI^{flu+}). An additional three subjects were challenged with influenza and became symptomatic, but no evidence of influenza infection. Three other challenged individuals remained asymptomatic with no evidence of influenza.

Characterisation of the pharyngeal microbiome

A total of 2,505,196 sequences were obtained from 87 individuals (52 in the challenged group, and 35 controls) at four time points: day -1, 3, 6 and 28. DNA extraction failed for one sample, and amplification was unsuccessful from four samples, giving a total of 343 samples. Chimeric sequences were removed by alignment to the SILVA database, resulting in 2,057,548 sequences.

Initially we compared individuals who were symptomatic or had ILI with proven influenza infection from the challenged group (n=33) with asymptomatic individuals from the control group (n=24). To normalize the dataset, we took 2997 sequences from each sample; 11 samples had fewer than 2997 sequences so were excluded, leaving a total of 213 samples. 2209 OTUs (based on <98.5% of identity, > 4 representatives) were identified, with 24% of

219 sequences falling into only three OTUs: OTU1 (accounting for 9.78% of sequences), with
220 predominant members *Fusobacterium periodonticum*, and OTU2 and OTU3 (7.8% and 7%,
221 respectively), with the predominant members of both unclassified streptococci; OTU2 and
222 OTU3 were identified as *S. salivarus*/*S. vestibularis*, and *S. mitis*/*S. pneumoniae*, respectively.
223

224 ***Changes in the diversity of nasopharynx microbiota based on OTU analysis***

225 The coverage of bacterial communities was high (mean 97.6% ± 0.007%), with samples having
226 an average of 161 OTUs (range 63 to 321). The mean number of OTUs at each time point was
227 not significantly different between the groups (asymptomatic controls *vs.* ILI/S^{flu+} *p* 0.4425,
228 Figure 2A). Furthermore, the number of OTUs remained remarkably constant over time, even
229 in the ILI^{flu+} and S^{flu+} groups, with no significant difference compared with Asymptomatic
230 controls (Supplementary Figures 1 and 2, *p* = 0.5543 and 0.5836, respectively). The Chao 1
231 index measures the predicted total richness of the community so gives a higher value (median
232 of 262 OTUs, range 84 to 577 OTUs, Figure 2B) than the number of recorded OTUs; the non-
233 parametric estimator Catchall gave a median value of 437 OTUs per sample (range 66 to
234 2643). Using these estimators, there were no significant differences in the richness of the
235 between the ILI/S^{flu+} group and Asymptomatic control groups (Figure 2B). Furthermore, there
236 was no significant difference in the richness of microbial communities in symptomatic
237 individuals even when we compared individuals in the ILI/S^{flu+} group with asymptomatic
238 controls (Supplementary Figures 1 and 2).
239

240 We also estimated the diversity of bacterial communities using the Shannon index. Again no
241 difference was detected between the challenged and control groups (Figure 2C,

242 Supplementary Figure 1 and 2), even when we analysed the diversity of the communities in
243 highly symptomatic ILI subjects and the Asymptomatic controls (Supplementary Table 1).
244
245 Next, we used beta diversity analysis to assess differences between samples. Dendrograms
246 were generated highlighting similarities of the membership and structure of bacterial
247 communities using Jaccard and thetaYC indices, respectively. At the four time points, results
248 for certain individuals cluster together (*e.g.* individual S^{flu+} individual 2, ILI^{flu+}6, S^{flu+}7, S^{flu+}10,
249 ILI^{flu+}11, S^{flu+}12, ILI^{flu+}13, S^{flu+}17, ILI^{flu+}28, AC61, AC65), indicating that the individual had a
250 stronger influence on microbiome than influenza. This was evidenced by samples from four
251 different time points clustering together in the dendrogram (Supplementary Figure 4~~3~~). No
252 significant clustering of OTUs in the control or the influenza challenged groups, or over time
253 was found when we applied a parsimony test. The thetaYC index, which considers the
254 structure of communities by looking at the relative abundance of OTUs, yielded a complex
255 dendrogram (Supplementary Figure 5~~3~~). Although there were no large shifts in the
256 communities, minor changes were detected when we applied a parsimony test with the
257 clustering in the ILI/S^{flu+} and Asymptomatic control groups slightly different on days 3, 6 and
258 28 (p <0.02, p <0.024, and p <0.001, respectively). However these differences were not found
259 after applying a Unifrac unweighted test.
260
261 The community structure and membership of the oropharyngeal microbiota was visualized
262 by PCoA (Figure 3). Small differences were found in the structure but not membership of
263 communities in the ILI/S^{flu+} group and Asymptomatic controls at day 3 and day 6 (p <0.035
264 and p <0.033, respectively, Figure 3). This difference was also found when we compared S^{flu+}
265 individuals with Asymptomatic controls (p <0.001). The structure was also visualized for each

266 group separately (Supplementary Figure 3), where we found that day 6 post infection showed
267 statistically significant difference compared to baseline within the ILI/S^{flu+} group (*p* <0.036),
268 while in the Asymptomatic control group, days 3, 6 and 28 showed some statistically
269 significant difference compared to baseline (*p* <0.001), indicating that the process of being in
270 quarantine might have subtle effects on the URT microbiome.
271 LEfSe analysis found that the abundance of some OTUs in these groups was significantly
272 different on days 3 and day 6 post-infection (Figure 4). OTUs which are more abundant within
273 the ILI/S^{flu+} group at day 3 included OTU11 (*Prevotella melaninogenica*), OTU14 (*Leptotrichia*),
274 OTU38 (Human Oral Taxon 352) and OTU38 (*Porphyromonas*), while OTU42 (*Burkhordiales*)
275 and OTU6 (*Leptotrichia* HOT218) were more abundant within Asymptomatic control group. At
276 day 6 OTU18 (*Fusobacterium necrophorum*) and OTU37 (*Prevotella*) were significantly more
277 abundant in individuals in the ILI/S^{flu+} group.
278
279 ***Alteration in the ecology of the pharyngeal microbiome during influenza***
280 Next we examined the communities by phylum. Of 2,057,548 sequences, 19,416 could not be
281 classified, while the remainder were distributed into 11 phyla, with 97% of them belonging to
282 five phyla, *Actinobacteria* (7.9%), *Fusobacteria* (24.2%), *Firmicutes* (36.5%), *Bacteroidetes*
283 (14.1%) and *Proteobacteria* (14.4%). We also detected *Spirochaetes*, *Synergistetes*,
284 *Tenericutes* and GN02 together with members of two uncultured prokaryotes, TM7 and SR1,
285 at < 1% of the total microbiota.
286
287 The abundance of each phylum remained remarkably constant following influenza challenge
288 with similar patterns seen across both groups. There was a significant change in the
289 *Bacteroidetes* which increased in the ILI/S^{flu+} group at day 3 post-infection compared to

290 Asymptomatic controls (Figure 5B). We also analysed how these phyla change over the time.
291 Within the ILI/S^{flu+} group there was a significant change in the abundance of *Actinobacteria*
292 which increased by day 6 post infection compared to baseline (*p* <0.01), and returned to basal
293 levels by day 28 (~~Supplementary~~ Figure 6A~~5~~).
294 *Firmicutes* were also increased between day 6 and 28 post infection in the control group
295 (Supplementary Figure 6C), with an increase in *Streptococcus* (Supplementary Figure 7A),
296 coinciding with individuals returning to the community so this might reflect changes in social
297 behaviour.
298
299 With regard to the composition at the genus level, *Streptococcus* was a core component of
300 the microbiome, accounting for 21.8% of all bacteria, with *Fusobacterium* and *Prevotella* also
301 abundant (comprising 15.4% and 9.9%, respectively). *Neisseria*, *Haemophilus* and
302 *Campylobacter* each accounted for 2-5% of the microbiota ~~(Figure 6)~~. Differences were
303 identified between the ILI/S^{flu+} group and Asymptomatic controls (Figure 7~~6~~), with a significant
304 increase in relative abundance of *Prevotella* at day 3 and 28 post infection in the challenged
305 group (*p* <0.05), and in *Fusobacterium* by day 28 post infection (*p* <0.05) (Figure 7C and 7B).
306 Within the ILI/S^{flu+} group, a minor increase in *Actinomyces* was observed at day 6 post infection
307 (*p* <0.05) and in parallel *Haemophilus* decreased (*p* <0.01) (~~Supplementary~~ Figure 8I and 8E~~6E~~
308 ~~and 6I~~).
309
310 **S. pneumoniae *and* N. meningitidis *carriage is unaltered during influenza***
311 As 16S sequencing often cannot discriminate different species, we analysed carriage of two
312 important respiratory pathogens, *S. pneumoniae* and *N. meningitidis*. The rate of colonization
313 by encapsulated or non-encapsulated *N. meningitidis* was similar with no difference between

314 challenged and controls individuals (Figure 97). For *S. pneumoniae* 16 of 36 influenza

315 challenged subjects (31%) and 12 of 23 controls (34%) were positive for *S. pneumoniae* using

316 qPCR at day 3 (Fisher's exact test p =0.8163).

317 **DISCUSSION**

318 We performed the first prospective characterization of bacterial communities within the
319 human URT during influenza infection. Previous studies have examined changes in the
320 microbiota following challenge with other viruses [24, 25], and found only minor changes in
321 the abundance of certain bacterial genera. Furthermore, cross-sectional studies have
322 characterized the microbiome of patients with influenza [41]. However we are not aware of
323 previous studies that have examined individuals following a defined influenza challenge.

324

325 We detected only slight perturbations in the upper respiratory microbiota following challenge
326 with influenza A. There was a high level of inter-individual variation in the upper respiratory
327 microbiome, a common feature of studies on humans [26], and found that the microbiota
328 contains at least 11 different phyla, with five phyla (*Actinobacteria*, *Firmicutes*, *Fusobacteria*,
329 *Bacterioidetes* and *Proteobacteria*) constituting the core microbiome, similar to other studies
330 [25]. Additionally *Fusobacteria* were highly prevalent (accounting for 24.2% of all OTUs), in
331 agreement with previous work [19].

332

333 The microbiota were dominated by *Streptococcus* and *Fusobacterium*, which include species
334 that can cause influenza-associated pulmonary disease. However during the acquisition of
335 influenza, the profile of the URT microbiome remained remarkably stable. Although we
336 detected no large shift in the bacterial population at the genus level, there was a significant
337 increase in the abundance of *Prevotella* in the ILI/S^{flu+} group at days 3 and 28, a key time when
338 secondary bacterial infection occurs; although *P. melaninogenica* was associated with this
339 increase at day 3, little is known about its role in respiratory disease.

340

341 Overall, our study does not provide evidence that influenza infection has a marked impact in
342 shaping the pharyngeal microbiome. However, certain factors might be responsible for the
343 lack of effect we observed. Participants were healthy adults, who do not generally suffer
344 secondary bacterial infection; studies in at-risk individuals might reveal more significant
345 changes in the microbiome, although pose a serious ethical and safety dilemma. In addition,
346 effects might have become evident using a larger study population. Furthermore, 16S rRNA-
347 based methods are limited and lack species-level resolution for particular genera, including
348 *Streptococcus* and *Neisseria*. Even though cohort studies have established a relationship
349 between influenza and the rate of pneumococcal carriage, we did not find any difference in
350 pneumococcal carriage following influenza infection. For the meningococcus, we also
351 examined capsule expression, as this virulence factor is only expressed by a sub-set of strains
352 [42]. However, there was no alteration in the rate of carriage of either encapsulated or non-
353 encapsulated *N. meningitidis* in our cohort with the advent of influenza infection. We did not
354 examine swabs for the presence of *S. aureus*, as this is a far less frequent secondary bacteria
355 pathogen following influenza than *S. pneumoniae*, and is associated with severe disease, and
356 not milder forms of infection such as represented by this challenge model.
357
358 In conclusion, our findings demonstrate that the upper airway microbiota is not
359 reprogrammed by acquisition of influenza, with only minor perturbations seen in bacterial
360 communities. Further studies are required to examine the cross-kingdom interactions that are
361 responsible for secondary bacterial infection being a key player in the mortality and morbidity
362 associated with influenza.
363
364

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506 **FIGURE LEGENDS**

507

508 **Figure 1. Study timeline.**

509 Volunteers were screened prior to admission to a quarantine unit on the day before receiving
510 an intranasal challenge of Influenza A (on day 0), then followed for 28 days. The volunteers
511 were kept in quarantine for six days post inoculation. Controls were screened and housed in
512 identical conditions, but were not subjected to viral challenge. Throat swabs were collected
513 at different time points during the study (*i.e.* on days -1, 3, 6 and 28 post-inoculation).

514

515 **Figure 2. Analysis of the richness of the URT microbiome.**

516 The diversity of the microbiome was analysed by sequencing the V1-3 region of 16SrRNA
517 amplified from samples obtained from the ILI/S^{flu+} (white boxes) and Asymptomatic control
518 (grey boxes) groups following influenza A challenge. The box whisker plots extend from 25th
519 to 75th percentiles, and the ends of the whiskers show the maximum and minimum values.
520 The line in the middle of the box represents the median and the dots represent the outliers
521 1.5 greater or lower than the interquartile distance. Analysis was based on: (A) the number of
522 OTUs; (B) Chao1 index; and (C) Shannon diversity index at baseline, 3 (3dpi), 6 (6dpi) and 28
523 (28dpi) days post influenza challenge.

524

525 **Figure 3. Throat microbiota structure in ILI/S^{flu+} and Asymptomatic control groups during**
526 **influenza challenge.** PCoA based on the Thetayc index comparing community structure of
527 samples from ILI/S^{flu+} infected group (blue open circles) and Asymptomatic control (red open
528 circles) groups at days 3 (A), and day 6 (B). The centroid represents the arithmetic mean for

each of the groups and each dot represents the microbiota structure profile for each of the samples, while the ellipses represent the 95% of the samples belonging to each group.

Figure 4. LEfSe analysis of abundant OTUs in ILI/S^{flu+} and Asymptomatic controls.

The positive scale indicates the LDA score (Log10) for the most abundant taxa in the ILI/S^{flu+} group (green bars), while the negative scale represents LDA scores for prevalent taxa in the Asymptomatic control group on days 3 (A) and 6 (B).

Figure 5. Relative abundance of common phyla in the human oropharynx.

Comparison of t~~T~~he abundance of each phylum between~~in~~ individuals ~~(open squares)~~ in the ILI/S^{flu+} group (+), and the Asymptomatic controls (-). The phyla are indicated in each panel. Error bars represent the standard deviation ~~error of the mean~~ (SD~~SEM~~). Non-parametric Kruskal-Wallis test for multiple comparisons was applied to identify statistically significant differences in relative abundance between groups: *, *p* <0.05; **, *p* <0.01. Samples are from individuals prior on entry to quarantine (baseline), and days 3, 6 and 28 after challenge, or not (in the control group).

Figure 6. Relative abundance of common phyla in the human oropharynx.

The abundance of each phylum in individuals within the ILI/S^{flu+} group (+). The phyla are indicated in each panel. Error bars represent the standard deviation (SD). Non-parametric Kruskal-Wallis test for multiple comparisons was applied to identify statistically significant differences in relative abundance between groups: *, *p* <0.05; **, *p* <0.01. Samples are from individuals prior on entry to quarantine (baseline), and days 3, 6 and 28 after challenge.

553

554 **Figure 76. Relative abundance of the common genera in the human oropharynx.**

555 Comparison of tThe abundance of each phylum in individuals (open squares) betweenin the

556 ILI/S^{flu+} group (+), and the Asymptomatic controls (-). generaGenera are indicated in each

557 panel. Error bars represent the standard error of the meandeviation (SDSEM). Non-parametric

558 Kruskal-Wallis test for multiple comparisons was applied to identify statistically significant

559 differences in relative abundance between groups: *, p <0.05; **, p <0.01. Samples are from

560 individuals prior on entry to quarantine (baseline), and days 3, 6 and 28 after challenge, or not

561 (in the control group).

562

563 **Figure 8. Relative abundance of the common genera in the human oropharynx.**

564 The abundance of each genera in individuals within the ILI/S^{flu+} group (+). The genera are

565 indicated in each panel. Error bars represent the standard deviation (SD). Non-parametric

566 Kruskal-Wallis test for multiple comparisons was applied to identify statistically significant

567 differences in relative abundance between groups: *, p <0.05; **, p <0.01. Samples are from

568 individuals prior on entry to quarantine (baseline), and days 3, 6 and 28 after challenge.

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572 **Figure 97. *N. meningitidis* carriage following influenza challenge.**

573 Meningococcal carriage in the ILI/S^{flu+} group (A) and in the Asymptomatic control group (B) at

574 different times following challenge. Carriage of non-groupable *N. meningitidis* was unchanged

575 during influenza, and ranged between 11.4 and 13.5 % except for controls from day 6 and 28.

576 There was no change in the carriage of groupable *N. meningitidis* over time or between

577 groups.

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581 **SUPPORTING INFORMATION**

582

583 **Supplementary Figure 1. Richness measurements of the ILI^{flu+} and Asymptomatic control**

584 **groups following influenza A challenge.**

585 The box whisker plots extend from 25th to 75th percentiles and the ends of the whiskers show

586 the maximum and minimum values. The line in the middle of the box represents the median

587 and the dots represent the outliers being 1.5 greater or lower than the interquartile distance.

588 (A) Number of OTUs, (B) Chao1 index and (C) the Shannon diversity index at baseline (B), and

589 3 (3dpi), 6 (6dpi), and 28 (28dpi) days after the influenza challenge.

590

591 **Supplementary Figure 2. Richness measurements of the S^{flu+} and Asymptomatic control**

592 **groups following influenza challenge.**

593 The box whisker plots extend from the 25th to 75th percentiles and the ends of the whiskers

594 show the maximum and minimum values. The line in the middle of the box represents the

595 median and the dots represent the outliers, 1.5 greater or lower than the interquartile

596 distance. (A) Number of OTUs, (B) Chao1 index, and (C) the Shannon diversity index at baseline

597 (B), 3 (3dpi), 6 (6dpi) and 28 (28dpi) days after the influenza challenge.

598

599 **Supplementary Figure 3. Throat microbiota structure in ILI/S^{flu+} and Asymptomatic control**

600 **groups during influenza challenge.** PCoA based on the Thetayc index comparing community

601 structure of samples within ILI/S^{flu+} infected group (A) and Asymptomatic control (B) groups

602 at Baseline (purple), day 3 (green) day 6 (blue) and day 28 after challenge (red). Each dot

603 represents the microbiota structure profile for each of the samples, while the ellipses

604 represent the 95% of the samples belonging to each group.

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608 **Supplementary Figure 4~~3~~. Dendrograms of throat communities in ILI/S^{flu+} and**
609 **Asymptomatic control groups during an influenza challenge.**

610 Clustering of throat samples at different time points of an influenza challenge compared
611 based on their community membership by using classical Jaccard Index. AC = Asymptomatic
612 Control, ILI^{flu+} = subjects who received viral challenge and had an ILI with evidence of influenza
613 infection, S^{flu+} = subjects who received viral challenge and were Symptomatic with evidence
614 of influenza infection, B = baseline, 3dpi = 3 days post infection, 6dpi = 6 days post infection,
615 28dpi = 28 days post infection. Numbers indicate subject or patient number.

616

617 **Supplementary Figure 5~~4~~. Dendrograms of throat communities in ILI/S^{flu+} and**
618 **Asymptomatic control groups during an influenza challenge.**

619 ~~(A) ~~Clustering of throat samples at different time points of an influenza challenge compared
620 based on their community structure by using classical Thetayc. AC = Asymptomatic Control,
621 ILI^{flu+} = subjects who received viral challenge and had an ILI with evidence of influenza
622 infection, S^{flu+} = subjects who received viral challenge and were Symptomatic with evidence
623 of influenza infection, B = baseline, 3dpi = 3 days post infection, 6dpi = 6 days post infection,
624 28dpi = 28 days post infection. Numbers indicate subject or patient number.

625

626 **Supplementary Figure 6~~5~~. Relative abundance of common phyla in the human oropharynx.**

The abundance of each phylum in individuals within the Asymptomatic control~~ILI/S^{flu+}~~ group over time. The phyla are indicated in each panel. Error bars represent the standard deviation (SD). Non-parametric Kruskal-Wallis test for multiple comparisons with a Dunn's post test ~~coreection~~correction was applied to identify statistically significant differences in relative abundance between groups: *, p <0.05; **, p <0.01, ***; p <0.001. Samples are from individuals prior on entry to quarantine (baseline), and days 3, 6 and 28 after challenge.

Supplementary Figure 7~~6~~. Relative abundance of the common genera in the human oropharynx.

The abundance of each phylum in individuals within the Asymptomatic control~~ILI/S^{flu+}~~ group over time. The genera are indicated in each panel. Error bars represent the standard deviation (SD). Non-parametric Kruskal-Wallis test with a Dunn's post test correction was applied to identify statistically significant differences in relative abundance between groups: *, p <0.05; **, p <0.01, ***; p <0.001. Samples are from individuals prior on entry to quarantine (baseline), and days 3, 6 and 28 after challenge.

~~**Supplementary Figure 7. Throat microbiota structure in ILI/S^{flu+} and control groups during influenza challenge.** PCoA based on the Thetayc index comparing community structure of samples within ILI/S^{flu+} infected group (A) and Asymptomatic control (B) groups at Baseline (purple), day 3 (green) day 6 (blue) and day 28 after challenge (red). Each dot represents the microbiota structure profile for each of the samples, while the ellipses represent the 95% of the samples belonging to each group.~~

651

652 **Supplementary Table 1. Alpha diversity of ILI/S^{flu+} and control groups.**