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2 **Respiratory bacteria stabilize and promote airborne transmission of influenza A virus**

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5 Running title: bacteria promote IAV stability and transmissibility

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23 **ABSTRACT:**

24 Influenza A virus (IAV) is a major pathogen of the human respiratory tract where the virus co-
25 exists and interacts with bacterial populations comprising the respiratory microbiome.
26 Synergies between IAV and respiratory bacterial pathogens promote enhanced inflammation
27 and disease burden that exacerbate morbidity and mortality. We demonstrate that direct
28 interactions between IAV and encapsulated bacteria commonly found in the respiratory tract
29 promote environmental stability and infectivity of IAV. Antibiotic-mediated depletion of the
30 respiratory bacterial flora abrogated IAV transmission in ferret models, indicating that these
31 viral-bacterial interactions are operative for airborne transmission of IAV. Restoring IAV airborne
32 transmission in antibiotic treated ferrets by co-infection with *Streptococcus pneumoniae*
33 confirmed a role for specific members of the bacterial respiratory community in promoting IAV
34 transmission. These results implicate a role for the bacterial respiratory flora in promoting
35 airborne transmission of IAV.

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44 **INTRODUCTION:**

45 Influenza A viruses (IAVs) are major pathogens of birds and mammals. The primary site
46 of human IAV infection is the upper respiratory tract, with more severe manifestations occurring
47 when the virus accesses the lower respiratory tract. Enhanced IAV morbidity and mortality can
48 also occur due to co-infection with bacterial pathogens, also commonly found in the human
49 upper respiratory microbiota. The best-characterized bacterial synergy of IAV is that with
50 *Streptococcus pneumoniae* (McCullers, 2014; Smith & McCullers, 2014). This synergy operates
51 in multiple aspects of pathogenesis, including enhancing transmissibility of *S. pneumoniae* in
52 murine (A. L. Richard, Siegel, Erikson, & Weiser, 2014) and ferret (McCullers et al., 2010)
53 models.

54 Recent insights have shown altered pathogenesis resulting from direct bacterial-viral
55 interactions, with the most extensive evidence coming from studies focusing on species found in
56 the gastrointestinal tract. Many classes of enteric viruses, including Picornaviruses (Kuss et al.,
57 2011), Reoviruses (Kuss et al., 2011), and Caliciviruses (Jones et al., 2014), rely on bacteria or
58 bacterial products for infectivity. Such direct interactions have recently been shown to occur
59 also between IAV and respiratory bacteria, including *S. pneumoniae* (David et al., 2019; H. M.
60 Rowe et al., 2019b). These direct interactions enhance the adherence of pneumococcus to
61 cultured respiratory cells *in vitro* and enhanced initial colonization and invasive disease in
62 murine models of otitis and invasive disease (H. M. Rowe et al., 2019b). These interactions also
63 alter host response, as when IAV-pneumococcal complexes were utilized as vaccine antigens,
64 efficacy was greater than that of either vaccine alone (David et al., 2019). While the co-infecting
65 bacterial species typically benefit from IAV infection, the roles for respiratory bacteria on IAV
66 biology remain less well understood. Studies of the respiratory microbiome and susceptibility to
67 IAV infection in household transmission studies have suggested an important role for the
68 composition of the respiratory microbiome in terms of susceptibility to IAV infection (Tsang et

69 al., 2019). Here we demonstrate that IAV directly benefits from interactions with human bacterial
70 respiratory flora, with the bacterial partners conferring environmental stability and enhancing
71 airborne transmission of the virus.

72 **RESULTS/DISCUSSION:**

73 Interactions with bacteria have previously been demonstrated to promote the stability of
74 Picornaviruses (Aguilera, Nguyen, Sasaki, & Pfeiffer, 2019; Robinson, Jesudhasan, & Pfeiffer,
75 2014) and Reoviruses (Berger, Yi, Kearns, & Mainou, 2017; Robinson et al., 2014). We
76 hypothesized that bacterial stabilization of environmental IAV via direct interactions may be one
77 mechanism the virus exploits to retain infectivity following release into the environment. To test
78 this, bacterial cultures from several respiratory tract-colonizing pathogens, previously shown to
79 associate with influenza A viruses (David et al., 2019; H. M. Rowe et al., 2019b), were
80 incubated with influenza virus strain A/Puerto Rico/8/1934(H1N1) (PR8), centrifuged, and
81 washed to remove nonadherent virus. The co-sedimented material was subjected to
82 desiccation in a speed vac and then rehydrated to determine viral infectivity. Speed vac
83 mediated desiccation, while not biologically relevant, allows concentration of bacterial and viral
84 particles and ability to measure log fold changes in viral viability promoted by the bacterial
85 complex. Real world conditions would subject the bacterial-viral complex to less harsh
86 desiccation stressors and furthermore would be in the context of host derived molecules.

87 Virus desiccated in the presence of *S. pneumoniae* (pneumococcus) or *Moraxella*
88 *catarrhalis* retained viability and infectivity, whereas IAV complexed to *Staphylococcus aureus*,
89 *Staphylococcus epidermidis*, non-typeable *Haemophilus influenzae*, or *Pseudomonas*
90 *aeruginosa* did not retain infectivity of IAV (Figure 1A). The desiccation resistance conferred by
91 the IAV-bacterial complex was independent of bacterial viability, as ethanol-killed pneumococci,
92 or Δ spxB mutant, which maintains higher desiccation viability than wild-type pneumococcus (H.
93 M. Rowe et al., 2019a), promoted influenza viability to an equivalent degree as live wild-type *S.*

94 *pneumoniae* (Figure 1B). However, pneumococcus had to be intact to promote infectivity of IAV,
95 as virus co-sedimented in the presence of pneumococci killed and lysed with β -lactam
96 antibiotics retained significantly less viability than virus desiccated in the presence of live
97 pneumococci (Figure 1B). These data indicate that direct interactions between IAV and
98 respiratory bacteria can promote environmental stability of IAV during desiccation in a species-
99 specific manner and that bacterial association alone is not sufficient to stabilize IAV.

100 The extensively hydrated polysaccharide capsule can promote environmental survival of
101 *S. pneumoniae* (Hamaguchi, Zafar, Cammer, & Weiser, 2018). Further, lyophilization of live-
102 attenuated influenza vaccine in sugar-containing solutions promotes maintenance of viability
103 (Lovalenti et al., 2016). We hypothesized that the polysaccharide capsule may be one
104 mechanism by which direct interactions between IAV and the pneumococcus promotes viral
105 stability. Targeted deletions of the capsule locus were made in three pneumococcal strains,
106 representing distinct three genotypes and serotypes. These mutants, and parental strains, were
107 incubated with IAV strain PR8, co-sedimented and subjected to desiccation. Capsule made no
108 discernable impact on the initial association and adherence of IAV to the bacterial cells, similar
109 to previous studies (H. M. Rowe et al., 2019b), but only virus co-sedimented in the presence of
110 encapsulated pneumococcal strains retained infectivity. This phenomenon was not specific to
111 pneumococcal capsule, as IAV co-sedimented in the presence of encapsulated *H. influenzae*
112 serotype B strain (HiB) demonstrated significantly enhanced viability compared to that of IAV
113 co-sedimented with non-typeable *H. influenzae* (Figure 1C). These data indicate an important
114 role for the polysaccharide capsule of *S. pneumoniae* and *H. influenzae* in conferring
115 desiccation tolerance to IAV.

116 To confirm that findings were also operative in human relevant pathogens, desiccation
117 experiments were performed with both A/California/04/2009 (H1N1) and A/Wisconsin/67/2005
118 (H3N2) desiccated in the presence of capsular and non-encapsulated respiratory bacteria.

119 Similar to PR8, co-sedimentation of A/California/04/2009 with *S. pneumoniae* resulted in
120 significantly enhanced desiccation tolerance, a phenotype that was dependent upon expression
121 of the pneumococcal polysaccharide capsule (Figure 1D). However, both the non-typeable *H.*
122 *influenzae* and HiB promoted desiccation tolerance of A/California/04/2009, suggesting that the
123 role of *H. influenzae* serotype B capsule is less important in promoting desiccation tolerance of
124 A/California/04/2009 compared to PR8 and that another *H. influenzae* surface factor may play a
125 role in stabilizing A/California/04/2009. Interestingly, the desiccation tolerance of
126 A/Wisconsin/67/2005 (Figure 1E) was not promoted by *S. pneumoniae* regardless of capsule
127 status, but was promoted by encapsulated *H. influenzae* serotype B. Taken together, these
128 data suggest that human respiratory bacteria are capable of stabilizing H1N1 strains of IAV but
129 this stabilization may be subtype specific, with certain respiratory bacteria stabilizing certain IAV
130 subtypes.

131 These observations of respiratory tract-colonizing bacteria conferring desiccation
132 tolerance to IAV suggests a mechanism whereby, during shedding from an infected host, the
133 viral particles associated with specific members of the respiratory microbial community may
134 have enhanced environmental stability and, hence, transmissibility. First, to determine if we
135 could alter the respiratory microbial community, ferret anterior nasal swabs were collected, a
136 subset of these animals were treated immediately after sample collection and three days later
137 by application of mupirocin ointment, commonly used in nasal decolonization prior to surgery
138 (Septimus, 2019), to the ferret nostrils using a polyester tipped swab to apply the ointment to the
139 exterior of the nares and interior of the nostrils to the depth of the first turbinate. All ferrets were
140 sampled again 24 hours after the final treatment to determine the impact of mupirocin on
141 respiratory bacterial communities. DNA was extracted from swabs and the V3-V4 region of 16S
142 was sequenced to determine microbial population composition. While total bacterial burden
143 was significantly reduced as determined by 16S rRNA copies per swab (Figure 2A), the overall

144 community diversity was not significantly altered (Supplementary Figure S1). The decrease in
145 bacterial burden was limited to particular species, including multiple gram positive cocci and
146 *Moraxella*, which were significantly reduced in treated but not control animals (Figure 2B and
147 Supplemental Figure 2). These data indicate that mupirocin treatment selectively reduced the
148 relative burden of multiple respiratory bacterial species, including *Streptococcus* and *Moraxella*,
149 both of which mediate IAV binding and desiccation tolerance.

150 Based on the observations that specific bacterial species can mediate IAV infectivity
151 during desiccation and mupirocin depletes bacterial species from the nasal passages, we
152 hypothesized that mupirocin treatment would adversely impact IAV transmission. To determine
153 the effect of this community disruption on airborne transmission of IAV, ferrets were treated by
154 application of mupirocin ointment to the ferret nostrils, on days one and three prior to viral
155 infection and at each nasal wash collection point, with ointment administered after collection of
156 nasal wash. Pairs of donor and aerosol-contact ferrets were housed in cages with perforated
157 dividers such that the ferrets could not directly contact each other. Donor ferrets were infected
158 with A/California/4/2009 (H1N1) by the intranasal route. Nasal wash samples were collected on
159 days 3, 5, 7, 9 and 11 or 12 post viral challenge from both donor and contact animals to monitor
160 viral burden. Control ferrets with no manipulation of the respiratory microbial community had a
161 75% transmission rate, with two of four ferrets having culturable virus in their nasal washes and
162 an additional contact becoming seropositive by day 21 post challenge (Figure 3A,B). Depleting
163 the respiratory microbial community by applying mupirocin to the nostrils of donor and contact
164 ferrets completely abrogated airborne transmission, with no contact animals expressing positive
165 viral titers nor seroconverting (Figure 3C,D). Treatment of only the donors was sufficient to block
166 airborne viral transmission with no contact animals with positive viral titers or seroconversion
167 (Figure 3E,F). All directly infected animals regardless of treatment status shed similar viral
168 loads, seroconverted to infection, and exhibited similar clinical symptoms (Supplementary Table

169 1). These data indicate that perturbation of bacterial communities in the respiratory tract of
170 donor ferrets by mupirocin treatment results in reduced airborne transmission of
171 A/California/4/2009 (H1N1) influenza virus.

172 If our hypothesis that respiratory bacteria promote IAV transmission is correct, then
173 restoring bacterial communities that both bind to and stabilize IAV should rescue IAV
174 transmission in the mupirocin-treated donor animals. Because *S. pneumoniae* was shown to
175 stabilize IAV from desiccation stress and effectively colonizes the respiratory tract of ferrets, a
176 separate group of mupirocin-treated donor ferrets was colonized with mupirocin-resistant *S.*
177 *pneumoniae* 2 days after IAV challenge, when viral shedding is at near peak levels (Roberts,
178 Shelton, Stilwell, & Barclay, 2012). Mupirocin treatments were identical to those undertaken in
179 Figure 3. Pneumococcal colonization was robust and stable throughout viral sample collection
180 across donor animals (Supplemental figure 3). Viral transmission was restored upon
181 colonization of *S. pneumoniae*, with 60% of contact animals having viable virus in their nasal
182 washes and all contact ferrets seroconverting to infection (Figure 3G,H). While donor viral loads
183 were similar to previous groups, donor symptoms were higher in colonized ferrets
184 (Supplementary Table 1), so we cannot rule out an additional effect from enhanced donor
185 symptoms on shedding to contacts. However, these data suggest that colonization of donor
186 ferrets by *S. pneumoniae* is sufficient to rescue the IAV transmission defect resulting from
187 mupirocin depletion of the respiratory flora.

188 Viral-bacterial synergies are inherently complex with interactions between both bacteria,
189 virus, and the host immune system being operative in synergistic as well as antagonistic
190 relationships. We demonstrated that common members of the human nasopharyngeal
191 microbiome including *S. pneumoniae*, *M. catarrhalis*, and *H. influenzae* can enhance
192 desiccation survival of H1N1 influenza A viruses when virus was in complex with the bacterial
193 surface. In our study desiccation survival was enhanced by the presence of bacterial capsule in

194 both *S. pneumoniae* and *H. influenzae* suggesting the polysaccharide capsule plays an
195 important role in retaining IAV infectivity under these conditions. These findings reflect studies
196 of enteric viruses such as Picornaviruses whose stability can be enhanced via direct bacterial
197 interactions(Aguilera et al., 2019) or interaction with bacterial lipopolysaccharide (Robinson et
198 al., 2014). Bacterial lipopolysaccharide and peptidoglycan also enhance thermostability of
199 Reoviruses, which in turn promotes infection of host cells following environmental stress (Berger
200 et al., 2017). Similar virion stability and infectivity enhancement has also been observed with
201 human Astroviruses (Perez-Rodriguez et al., 2019). This suggests that interactions between
202 viral pathogens and the bacterial communities are likely operative at distinct host niches
203 including the respiratory tract in addition to the better characterized synergies operative for
204 enteric pathogens.

205 The environmental persistence of IAV is dependent upon multiple factors and can vary
206 considerably between viral subtypes (Kormuth et al., 2019). The differing capacity of respiratory
207 bacterial species to promote stabilization of the H1N1 viruses versus the H3N2 subtype
208 suggests that there may be additional important differences between distinct IAV subtypes for
209 their capacity to be stabilized by direct bacterial interactions with some IAV subtypes requiring
210 distinct bacterial species for binding and stabilization. Further, some IAV strains may not be
211 stabilized by human respiratory bacteria, but instead by bacteria found in the natural reservoir of
212 the respective IAV strain. Additionally, the respiratory microflora of model organisms may
213 impact IAV transmissibility, underscoring the potential importance of the native bacterial flora
214 when investigating IAV transmissibility. It should also be noted that we utilized relatively young
215 ferrets aged 8-10 weeks, while many other investigations querying influenza transmission that
216 routinely utilize ferrets of 4-12 months of age (Belser, Eckert, Tumpey, & Maines, 2016; M.
217 Richard et al., 2020; T. Rowe et al., 2010). The rationale for the utilization of younger ferrets
218 was primarily due to previous work demonstrating ferrets in this age group rapidly transmit S.

219 *pneumoniae* by both contact and aerosol routes (McCullers et al., 2010; H. M. Rowe et al.,
220 2019a). Whether our findings would extend to older ferrets that likely have distinct microbial
221 community composition remains an unknown but intriguing question. The relevance of results to
222 various IAV challenge doses is also an important question, as bacterial-mediated synergies may
223 only be important at specific viral thresholds of infectivity.

224 These findings suggests that, unlike the enteric microflora, which enhance viral infectivity
225 of the same host, the respiratory microflora of the infected host is primarily operative in viral
226 infectivity of the subsequent host. In household transmission studies of IAV, *S. pneumoniae* or
227 closely related streptococcal species were identified in approximately 95% of samples collected
228 both the child index cases and the household contacts who developed influenza (Zhang et al.,
229 2020). These data suggest that modulating donor respiratory flora via antibiotic exposure or
230 vaccination may profoundly affect IAV transmission. It should be stressed that in our study
231 topical antibiotics were given prior to IAV challenge with no impact on disease severity in the
232 donor animals. Even in the light of this limitation, targeting bacterial-mediated transmission may
233 represent a novel strategy of IAV infection control that could be explored.

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238 **MATERIALS AND METHODS:**

239 **Ethics Statement.** All experiments involving animals were performed with approval of and in
240 accordance with guidelines of the St. Jude Animal Care and Use Committee. The St. Jude
241 laboratory animal facilities have been fully accredited by the American Association for
242 Accreditation of Laboratory Animal Care. Laboratory animals were maintained in accordance
243 with the applicable portions of the Animal Welfare Act and the guidelines prescribed in the
244 DHHS publication *Guide for the Care and Use of Laboratory Animals*.

245 **Bacterial and viral strains and growth conditions.** The *S. pneumoniae* strains BHN97
246 (serotype 19F), D39 (serotype 2), and TIGR4 (serotype 4) were inoculated onto tryptic soy agar
247 (TSA) (GranuCult- Millipore Burlingon MA) plates supplemented with 3% sheep blood (iTek
248 StPaul MN) and 20 µg/mL neomycin (Sigma St Louis MO) and then grown overnight at 37°C in
249 a 5% CO₂ humidified incubator. Strains were then inoculated directly into Todd-Hewitt (BD
250 Franklin Lakes NJ) broth supplemented with 0.2% yeast extract (BD) (ThyB) and grown to log
251 phase for use in experiments. A capsule mutant was generated by transforming SPNY001
252 genomic DNA containing a Sweet Janus cassette that replaces the capsule locus (Grijalva et
253 al., 2014) into strains BHN97, D39, and TIGR4 and confirmed by the lack of latex bead
254 agglutination (Statens Serum Institute Copenhagen Denmark) for the respective capsule.

255 The nontypeable *H. influenzae* 86-028NP(Harrison et al., 2005), originally isolated from a
256 patient with chronic otitis media, and the encapsulated *H. influenzae* serotype b strain 10
257 211(ATCC) were grown on chocolate agar supplemented with 11,000 units/L bacitracin (BD)
258 and then directly inoculated into brain heart infusion broth (BD) supplemented with 0.2% yeast
259 extract (BD), 10 µg/mL hemin, and 10 µg/mL NAD and grown with aeration to mid-log phase.
260 *Staphylococcus aureus* strain USA400, *Staphylococcus epidermidis* strain M23864:W2 (ATCC),
261 *P. aeruginosa* Xen41 (PerkinElmer), and *Moraxella catarrhalis* (Helminen et al., 1994) were
262 grown on unsupplemented TSA plates, directly inoculated in brain heart infusion broth

263 supplemented with 0.2% yeast extract, and grown with aeration to mid-log phase for use in
264 experiments. The influenza A virus A/Puerto Rico/8/1934 (PR8) and A/Wisconsin/67/2005
265 (H3N2) were grown in Madin-Darby canine kidney (MDCK) cells. The A/California/4/2009 virus
266 was grown in allantoic fluid of 10- to 11-day-old embryonated chicken eggs. PR8 is of unknown
267 passage history, A/Wisconsin/67/2005 is a 2nd cell passage from a third egg passage,
268 A/California/4/2009 is a 5th egg passage.

269 For ferret pneumococcal colonization, *S. pneumoniae* strain BHN97 was made mupirocin- and
270 streptomycin-resistant (BHN97 Mup^RStrep^R) to enable continued treatment of ferrets with
271 mupirocin ointment and collection of nasal lavage with streptomycin to reduce risk of aspiration
272 pneumonia during ketamine sedation and nasal wash collection. Streptomycin-resistance was
273 conferred via mutation of *rpsL* (TIGR4 Sp_0271) by introduction of a K56T mutation(Martin-
274 Galiano & de la Campa, 2003) generated by splicing overlap extension (SOE) PCR using two
275 fragments that each had the point mutation. The first PCR fragment amplified 969 bp upstream
276 and the first 180 bp of *rpsL* using primers RpsL_Up_F (GCCGTAGTCATCTTCTTGGCATC)/
277 RpsL_Up_R(CTGAGTTAGGTTTGTAGGTGTCATTGTTC). The second PCR fragment
278 amplified bp 151 to 414 of *rpsL* plus 752 bp downstream using primers
279 RpsL_Down_F(GAACAAATGACACCTACAAAACCTAACTCAG)/
280 RpsL_Down_R(CTAATTGAACCCGGGCTAAAGTTAG). The entire SOE PCR product was
281 amplified using RpsL_Up_F/ RpsL_Down_R and was transformed into strain BHN97; resistant
282 mutants were selected for on TSA supplemented with 3% sheep blood and 800 µg/mL
283 streptomycin. Mupirocin-resistance was spontaneously generated and selected for by plating
284 turbid culture of BHN97 *rpsL_{K56T}* on TSA supplemented with 3% sheep blood, 800 µg/mL
285 streptomycin, and 10 µg/mL mupirocin and then selecting spontaneously resistant colonies.

286 **Co-sedimentation and desiccation:**

287 Co-sedimentation was performed as previously described (H. M. Rowe et al., 2019b). Briefly,
288 mid-log bacterial cultures were washed and normalized to 10^8 CFU/mL in phosphate-buffered
289 saline (PBS). 3×10^7 TCID₅₀ (50% tissue culture infectious dose) influenza virus was added and
290 samples rotated 30 minutes at 37°C. Samples were centrifuged and washed twice with PBS.
291 Samples not subjected to desiccation were immediately resuspended in 100 µL 1x
292 penicillin/streptomycin solution (Gibco) and frozen at -80°C for viral quantification. Samples
293 designated for desiccation were spun for 60 minutes in a Speed Vac until the pellet was dry.
294 Pellets were resuspended in 100 µL 1x penicillin/streptomycin solution and frozen at -80°C for
295 viral quantification. Viral titers were determined by TCID₅₀ on MDCK cells (Cline et al., 2011).
296 Three to six biological replicates were performed for each strain.
297 Ethanol-fixed pneumococci were prepared by resuspending 10^8 CFU BHN97 in 1 mL of ice-cold
298 70% ethanol for 5 minutes on ice. Cells were pelleted, supernatant was removed, and pellets
299 were dried at 55°C for 5 minutes to remove residual ethanol. Viability loss was confirmed by
300 plating on TSA/blood. β-lactam–killed pneumococci were prepared by resuspending 10^8 CFU
301 BHN97 in 1 mL 10x penicillin-streptomycin (Gibco) solution in PBS and incubating 30 minutes at
302 37°C. Viability loss was confirmed by plating on TSA/blood, and lysis was confirmed by
303 microscopy examination.

304 **Ferret infection:**

305 All ferrets were maintained in BSL2, specific pathogen–free facilities. Microbiome collection
306 swabs, treatment of nostrils with ointment, infection and blood collections were conducted under
307 general anesthesia with inhaled isofluorane at 4%. Nasal washes were collected under
308 ketamine sedation following intramuscular injection of ketamine to the thighs of restrained
309 ferrets. All ferrets were monitored twice daily for symptoms during infection. Weights were
310 measured daily and temperature collected daily from implanted microchips.

311 Nine-week-old male castrated ferrets (Triple F Farms Gillet PA), confirmed to be seronegative
312 for Influenza A viruses (seronegativity to Influenza B viruses not tested) prior to start of study,
313 were housed two per cage, separated by a perforated barrier. Experimental groups had 3 to 5
314 donor-contact pairs. Animals designated for treatment with mupirocin ointment had 75mg 2%
315 mupirocin in polyethylene glycol (Perrigo) applied to exterior of nostrils and interior of anterior
316 nares up to the first turbinate with a polyester applicator swab (Puritan) three days prior to, one
317 day prior to, on the day of infection (post-instillation of virus inoculum), and on each sampling
318 day (post collection of nasal lavage); untreated animals were not treated at those time points.
319 Donor animals were infected with 10^6 50% tissue culture infectious doses (TCID₅₀) of influenza
320 A virus A/California/04/2009 (H1N1) in 1 mL of phosphate-buffered saline (PBS), instilled
321 equally between both nostrils. Contact animals were introduced the cages, separated with a
322 perforated divider, 24 hours after infection of the donors. On days 3, 5, 7, 9, and 11 or 12 post
323 infection of donor animals, donor and contact ferrets were sedated with ketamine and nasal
324 lavage was collected in 1 mL PBS supplemented with 1x penicillin/streptomycin (Gibco) divided
325 equally between each nostril. Nasal lavage was stored at -80°C for viral quantification. Animals
326 designated for co-infection with *S. pneumoniae* were treated with mupirocin ointment and
327 infected as described above. Then on day 2 post influenza infection, *S. pneumoniae* strain
328 BHN97 Mup^RStrep^R, grown as described in the co-sedimentation and desiccation section, was
329 normalized to 5×10^6 CFU per 600 μ L PBS and instilled equally between both nostrils. Samples
330 were collected as above, except penicillin was omitted from PBS. Prior to storage at -80°C,
331 30 μ L of nasal wash was removed and serially diluted in PBS and plated on TSA supplemented
332 with 20 μ g/mL neomycin and 3% sheep blood for bacterial quantification. Viral titers were
333 determined by TCID₅₀ on MDCK cells (Cline et al., 2011). Briefly, MDCK cells were infected with
334 100 μ L 10-fold serial dilutions of sample and incubated at 37°C for 72 hours. Following
335 incubation, viral titers were determined by hemagglutination assay (HA) using 0.5% turkey red

336 blood cells and analyzed by the method of Reed and Munch (Reed & Meunch, 1938). For
337 samples that were negative by HA, residual supernatant was removed and wells were washed
338 once with PBS and then stained for one hour at room temperature with 0.5% crystal violet in a
339 4% ethanol solution. Wells were washed with tap water and infected wells determined by
340 destruction of the monolayer. TCID₅₀ was again determined using the method of Reed and
341 Munch as above. On days 14 and 21 post IAV challenge, ferrets were sedated with isoflurane,
342 and 1 mL blood was drawn from the jugular vein. Blood was allowed to clot overnight at 4°C.
343 Serum was collected following centrifugation to pellet clot and stored at -80°C. For
344 determination of seroconversion, serum was treated with RDE (Hardy Diagnostics) overnight at
345 37°C. RDE was inactivated via incubation at 56°C for 1 hour, followed by dilution in PBS for a
346 final dilution of 1:4, and freezing at -80°C for at least 4 hours to continue to inactivate
347 neuraminidase. Starting with a 1:40 dilution of sera and serial 2-fold dilutions in PBS, sera were
348 mixed with 4 hemagglutination units of A/California/04/2009 and incubated 30 minutes at room
349 temperature. Following incubation, an equal volume of 0.5% washed turkey red blood cells in
350 PBS was added to each well and incubated a further 60 minutes at 4°C. Hemagglutination
351 inhibition titer was read as the most dilute well with a negative hemagglutination reaction.

352 **Microbiome analysis:**

353 Prior to treatment on day -3 and again on day of infection, just prior to the infection, the
354 microbiome was sampled from the anterior nares of all ferrets. A flocked polyester swab
355 (Copan, flexible minitip) was inserted into the ferret nares to the depth of the first turbinate and
356 the interior of each nostril was swabbed for 15 seconds per nostril, before insertion of the same
357 swab into the other nostril. Swabs were stored dry at -80°C for DNA preparation. DNA was
358 extracted from nasal swabs after resuspension using methods to improve the bacterial species
359 captured in low abundance samples (Davis et al., 2019). Bacterial DNA content was assessed
360 using a 16S rRNA quantitative PCR with a plasmid containing E. coli 16S gene as the standard,

361 using previously described primers and probe (Nadkarni, Martin, Jacques, & Hunter, 2002) and
362 Fast Universal PCR Master Mix (TaqMan) supplemented with 3 mM MgSO₄. Microbiome 16S
363 rRNA gene amplification was performed using 'touch-down' PCR cycling of V3-V4 amplicon
364 (Dickson et al., 2014) and sequencing was performed as previously described (Golob et al.,
365 2017) at the St. Jude Hartwell Center. Classification of reads was done based on phylogenetic
366 placement on reference tree; briefly: Illumina MiSeq paired-end reads were run through DADA2
367 pipeline (Callahan et al., 2016) (version 1.10.1) to correct sequencing errors and determine
368 amplicon sequence variants (ASVs). These ASVs were then used to recruit full-length 16S
369 rRNA gene sequences from Ribosomal Database Project release 16.0(Cole et al., 2014) to
370 construct a phylogenetic reference dataset and tree. The amplicon sequences were then placed
371 onto the reference tree using pplacer (Matsen, Kodner, & Armbrust, 2010). Sequence reads are
372 available through NCBI through accession # (to be provided upon publication).

373 **Statistical analysis:**

374 All tests were performed with GraphPad Prism7. Comparisons were made via Mann-Whitney
375 testing, with a *P* value of less than 0.05 considered significant.

376

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382 JWR designed the study. HMR, EM and JWR analyzed the data. HMR and JWR wrote the
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384 Competing interests: The authors declare no competing interests.
385 Materials & Correspondence: All materials and data will be made available upon request to
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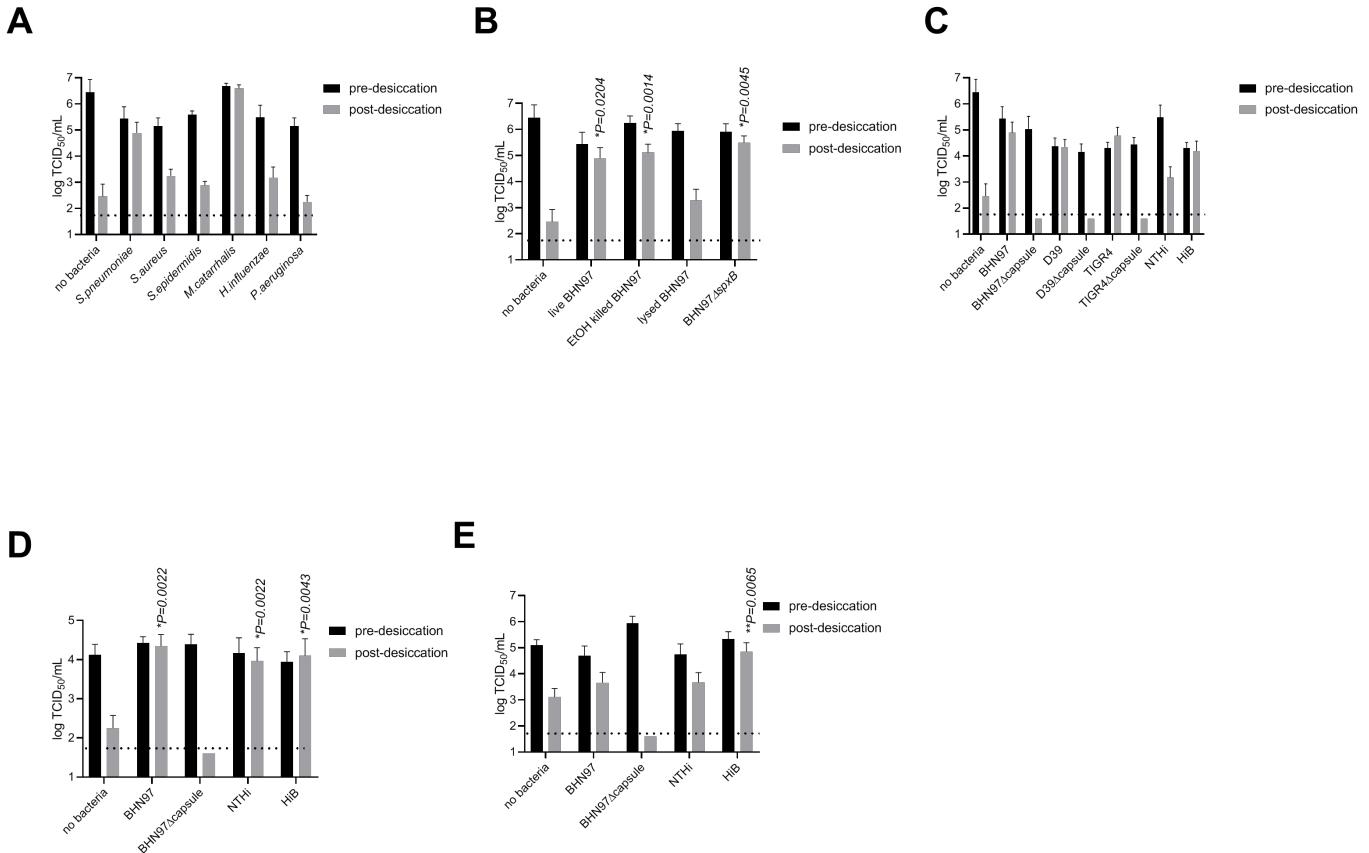
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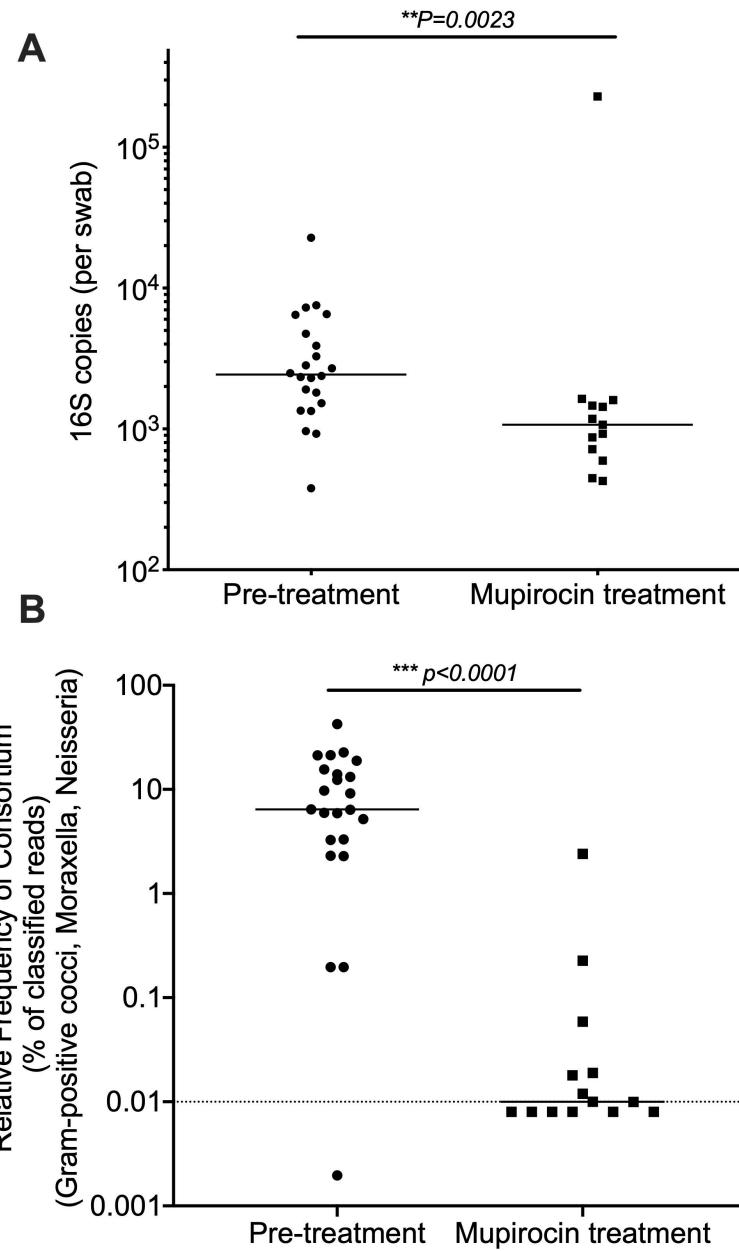
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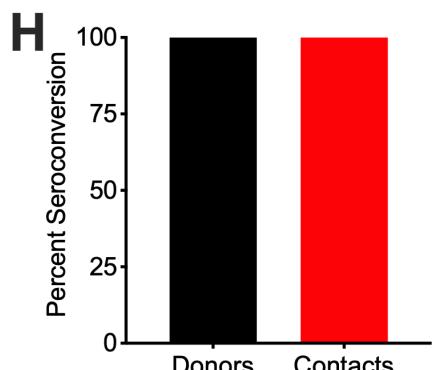
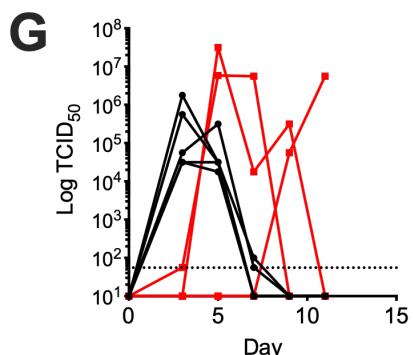
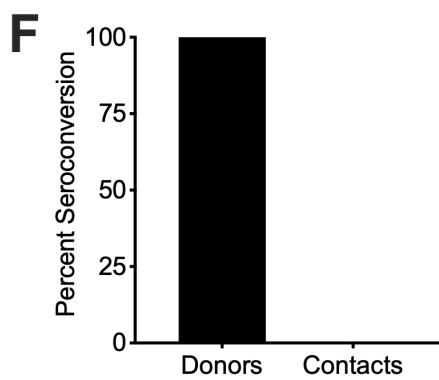
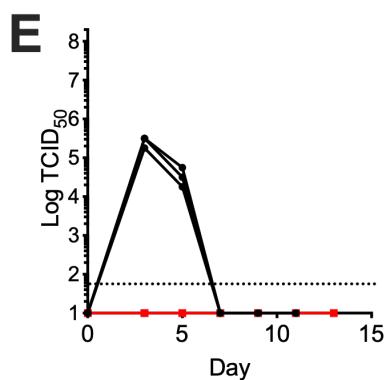
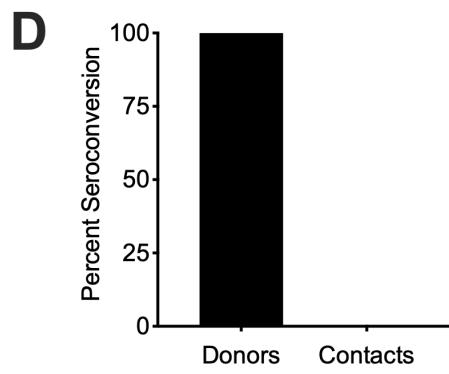
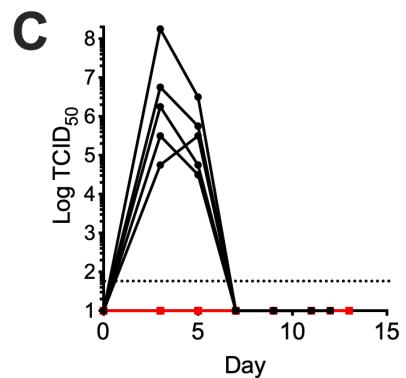
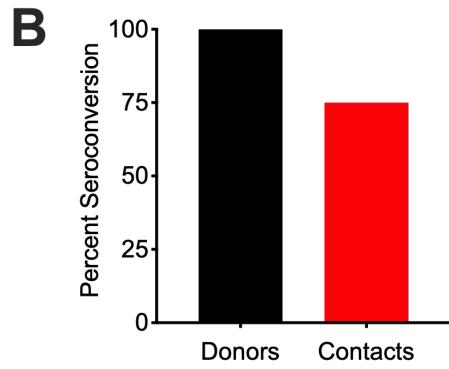
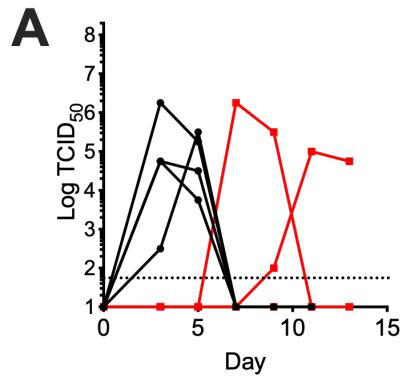
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503 **Figure 1: Nasopharyngeal bacteria promote influenza virus desiccation stability.** Indicated
504 bacterial strain was preincubated with influenza virus PR8 (A-C) or A/California/04/2009(H1N1)
505 (D), or A/Wisconsin/67/2005 (H3N2) € followed by centrifugation and washes to remove non-
506 associated virus and desiccation in a speed vac (grey) or not subject to desiccation (black).
507 Virus alone was in a small volume (less than 10 μ L) of either cell culture media or egg fluid and
508 was directly dessicated in the speed vac. (A) Nasopharyngeal tract-colonizing bacteria provide
509 differing degrees of IAV desiccation protection. (B) Pneumococcal viability does not affect
510 desiccation promotion of IAV, as ethanol-killed or Δ spxB, pneumococcal mutant with enhanced
511 desiccation tolerance, had equivalent protection of IAV infectivity as live pneumococci. β -
512 lactam-killed and lysed pneumococci did not promote viability retention. (C) Desiccation survival
513 of IAV with encapsulated and non-encapsulated strains of *S. pneumoniae* and *H. influenzae*. (D)
514 Pneumococcal capsule and *H. influenzae* serogroup B promote stability of A/California/04/2009 (H1N1). (E)
515 *H. influenzae* serogroup B promotes stability of A/Wisconsin/67/2005(H3N2). Bars represent
516 mean and error bars represent standard deviation of at least 6 biological replicates. P values
517 calculated by Mann-Whitney testing compared to virus desiccated in the absence of bacteria;
518 dotted line represents limit of detection.



519

520 **Figure 2: Impact of mupirocin on ferret respiratory microbial community composition.** (A)
521 Bacterial content of the nasal passages is significantly lower after mupirocin treatment as
522 measured by bacterial 16S copies recovered on nasal swabs prior to and following treatment.
523 (B) Microbiome content of Gram-positive and Gram-negative cocci (specifically the relative
524 frequency of Moraxella, Neisseria, Lactococcus, Vagococcus, Enterococcus hirae,
525 Streptococcus fayi and suis) is significantly reduced following treatment. Each dot represents
526 data from a swab collected from an individual ferret. Solid line indicates median for each group,
527 dashed line represents limit of detection. Groups compared by Mann-Whitney test.



529 **Figure 3: Nasopharyngeal bacteria promote airborne transmission of influenza virus.**
530 Donor ferrets were infected with influenza A virus A/California/04/2009 (H1N1) and paired 24
531 hours post infection with aerosol-contact ferrets in the same cage with perforated dividers
532 separating the animals. (A,C,E,G) Influenza virus burden in nasal lavage measured by 50%
533 tissue culture infectious dose (TCID₅₀). (B,D,F,H) Percent of animals who seroconverted as
534 measured by hemagglutination inhibition (HAI) assay titer greater than 1:80 dilution by day 21
535 post infection. Donors=black, aerosol contacts=red. Dotted line represents limit of detection for
536 TCID₅₀ assay, Days= days post infection of donor animals. (A,B) Ferrets with no manipulation
537 of the respiratory microbiota, n=4 donors and 4 contacts. (C,D) Both donor and contact ferret
538 nostrils treated with mupirocin ointment, n=5 donors and 5 contacts. (E,F) Donor ferret nostrils
539 treated with mupirocin ointment, n=3 donors and 3 contacts. (G,H) Donor ferret nostrils treated
540 with mupirocin followed by colonization with 10⁶ CFU of *S. pneumoniae* strain
541 BHN97Mup^RStrep^R. Each data point represents an individual ferret over time.

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