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**Comparing microbiotas in the upper aerodigestive and lower respiratory tracts of lambs**

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## 22 **Abstract**

23 Background: Recently, the importance of the lung microbiota during health and disease has been  
24 examined in humans and in small animal models. Whilst sheep have been proposed as an  
25 appropriate large animal model for studying the pathophysiology of a number of important human  
26 respiratory diseases, it is clearly important to continually define the limits of agreement between  
27 these systems as new concepts emerge. In humans it has recently been established that the lung  
28 microbiota is seeded by microbes from the oral cavity. We sought to determine whether the same  
29 was true in sheep.

30 Results: We took lung fluid and upper aerodigestive tract (oropharyngeal) swab samples from forty  
31 lambs (seven weeks old). DNA extraction was performed and the V2-V3 region of the 16S rRNA gene  
32 was amplified by PCR then sequenced via Illumina Miseq. Oropharyngeal swabs were either  
33 dominated by bacteria commonly associated with the rumen or by bacteria commonly associated  
34 with the upper aerodigestive tract. Lung microbiota samples did not resemble either upper  
35 aerodigestive tract samples or reagent only controls. Some rumen associated bacteria were found in  
36 lung fluids, indicating that inhalation of ruminal bacteria does occur. We also identified several  
37 bacteria which were significantly more abundant in lung fluids than in upper aerodigestive tract  
38 swabs, the most predominant of which was classified as *Staphylococcus equorum*.

39 Conclusions: In contrast to humans, we found that that the lung microbiota of lambs is dissimilar to  
40 that of the upper aerodigestive tract and we suggest that this may be related to physiological and  
41 anatomical differences between sheep and humans. Understanding the comparative physiology  
42 and anatomy underlying differences in lung microbiota between species will provide a foundation  
43 upon which to interpret changes associated with disease and/or environment.

44

45 **Keywords:** lung, microbiota, sheep, lambs, oropharynx, rumen, 16S

46

47 **Background**

48 The use of 16S ribosomal RNA (rRNA) gene sequencing has facilitated the study of difficult to culture,  
49 low biomass microbial communities present in the lower respiratory tract. The impact of the lung  
50 microbiota on human health is a rapidly growing area of research. In order to understand this impact  
51 it is important to also understand the lung microbiota dynamics during health and to include healthy  
52 controls in disease studies. To achieve this, the majority of previous studies have relied on human  
53 volunteers.

54 However, many individuals are hesitant to participate in research bronchoscopy due to the  
55 perceived inconvenience and a fear of complications [1], despite the low risk involved. Mice and rats  
56 have been used to explore the relationship between the lung microbiota and airway inflammation  
57 [2-4], microbiota at different body sites [5], the environment [6], acute lung injury [7] and antibiotic  
58 [8] and corticosteroid exposure [9]. However, rodents are of limited use when exploring spatial or  
59 longitudinal lung microbiota dynamics due to their small lung size. Recognising the utility of large  
60 animal models in this regard, and the anatomical and immunological relevance of sheep as models  
61 [10-13] our group has previously used this species to explore the changes in the lung microbiota  
62 upon *Pseudomonas aeruginosa* infection [14] and to explore the spatial variability present within the  
63 healthy lung [15].

64 Subclinical microaspiration of pharyngeal secretions is a feature of health and this can contribute to  
65 the lung microbiota composition [16] and the microbiome of the human lungs more closely  
66 resembles that of the mouth than the nose or the lower gastrointestinal tract [17]. It is not yet  
67 known whether the same relationship holds for species other than humans. In ruminating sheep,  
68 where the oropharynx is exposed to ruminal contents on a frequent basis one would anticipate that  
69 lung microbiota would similarly reflect this influence. In this paper we find that the presence of  
70 rumen-like bacteria in the upper aerodigestive tract is correlated with changes in the lung

71 microbiota and rumen type bacteria are present in lamb lungs. We also identify bacteria which are  
72 more indicative of the lungs than the oropharynx, indicating that the presence of the sheep lung  
73 microbiota is not merely due to passive diffusion of microbes from the upper aerodigestive tract.

74

## 75 **Methods**

### 76 **Animals and sampling**

77 Scottish Mule X Suffolk lambs (20 males and 20 females, unweaned), raised on pasture from 48  
78 hours after birth, were used in this study. These lambs were part of a study on the animal welfare  
79 implications of prenatal stress which was approved by Scotland's Rural College's (SRUC) Animal  
80 Experiments Committee and was conducted under Home Office licence. All lambs were raised by  
81 their dams and prior to euthanasia their only food sources were ewe's milk and pasture. At seven  
82 weeks old (mean age = 48.8 days  $\pm$  0.8 standard deviation (SD); mean weight  $\pm$  SD = 20.6 kg  $\pm$  2.6 kg),  
83 lambs were euthanized by barbiturate overdose then the cadavers were transported from the farm  
84 to the dissecting room (~5 min). Oropharyngeal swabs were taken using cotton tipped swabs (Swab  
85 Plain Wood Cotton Tip Sterile (710-0181), Copan, Italy). To prevent oral contamination, swabs were  
86 stored in protective plastic sheaths from which the swab could be advanced and retracted once it  
87 was positioned at the sampling site. Swabs were then transferred into a new plastic sheath and  
88 stored on ice.

89 The ventral aspect of the neck was shaved and a sterile scalpel used to incise through the skin and  
90 subcutaneous tissues to expose the ventral surface of the trachea. A sampling site was identified on  
91 the exposed ventral surface and the trachea cranial to this site was completely closed off by both  
92 string ligature and clamp placement. The selected sampling site was then heat seared and 50 ml of  
93 sterile phosphate buffered saline (PBS) was injected through the seared section into the tracheal  
94 lumen. The head and neck were oriented such that the PBS would flow caudally down the thorax. A

95 second clamp was immediately placed caudal to the site of injection to prevent backflow, leakage  
96 and potential contamination. The lamb cadavers were then tipped so that the PBS would run  
97 caudally into their lungs and then tipped back again so that the fluid would collect in the tracheal  
98 lumen immediately caudal to the position of the second clamp. A sampling site identified on the  
99 ventral surface of the trachea was seared and a needle and syringe were used to collect the pooled  
100 fluid. On average  $4 \pm 1.7$  ml (mean  $\pm$  SD) of lung fluid was collected per animal. Lung fluid was stored  
101 on ice until further processing. Oropharyngeal swabs were sterilely cut into 500  $\mu$ l PBS. Lung fluids  
102 were centrifuged at 13,000 g for 5 min. The supernatant was removed and the pellets were  
103 resuspended in 500  $\mu$ l PBS. Oropharyngeal swabs and lung fluids were stored at  $-80^{\circ}\text{C}$  until DNA  
104 extraction.

#### 105 **DNA extraction, amplification, and sequencing**

106 DNA extractions using the PowerSoil DNA isolation Kit (Mo Bio, Carlsbad, USA) and quantitative PCR  
107 (qPCR) using the 16S rRNA gene qPCR primers UniF340 (5-ACTCCTACGGGAGGCAGCAGT-3) and  
108 UniR514 (5-ATTACCGCGGCTGCTGGC-3) were performed as described previously [15]. Extraction kit  
109 reagent controls, consisting of reagent only extractions, were produced for every day DNA  
110 extractions were performed. PBS controls were created by extracting DNA from 500  $\mu$ l of the PBS  
111 which had also been used to wash out the lamb lungs. A mock community control was included  
112 which has been described previously [15].

113 A nested PCR reaction was used to produce amplicons for sequencing; this technique was chosen to  
114 reduce PCR bias caused by barcoded primers [18]. The first round of PCR amplified the V1-V4 16S  
115 hypervariable regions using the primers 28F (5-GAGTTTGATCNTGGCTCAG-3) and 805R (5-  
116 GACTACCAGGGTATCTAATC-3). The conditions were:  $94^{\circ}\text{C}$  for 2 min followed by 20 cycles of  $94^{\circ}\text{C}$  for  
117 1 min,  $55^{\circ}\text{C}$  for 45 s, and  $72^{\circ}\text{C}$  for 1.5 min followed by a final extension step of  $72^{\circ}\text{C}$  for 20 min.  
118 Clean-up was performed using the AMPure XP PCR purification system (Beckman Coulter, Brea,  
119 USA).

120 In a previous study, we found that PCR bias in high template samples could be reduced by diluting  
121 amplicons from the first round of PCR to a similar concentration to those of lung fluid samples [15].  
122 Therefore, in this study we used our qPCR values to calculate the dilutions needed to achieve this.  
123 The second round of PCR used the barcoded V2-V3 primers 104F (5-GGCGVACGGGTGAGTAA-3) and  
124 519R (5-GTNTTACNGCGGCKGCTG-3). The dilutions and barcoded primers used for each sample can  
125 be found in **Additional file 1**. The PCR conditions were: 98°C for 30 s followed by 20 cycles of 98°C  
126 for 10 s, 67°C for 30 s, and 72°C for 10 s followed by a final extension step of 72°C for 2 min. The  
127 amplicons were again purified using the AMPure XP PCR purification system.

### 128 **Bioinformatic and statistical analysis**

129 Samples were sequenced via either Illumina Miseq or Hiseq runs (Illumina, San Diego, USA)  
130 (**Additional file 1**) producing 250 base pair paired-end reads. Cutadapt [19] was used to remove  
131 primers. Quality control and taxonomic assignment of sequences was carried out within mothur [20]  
132 following a protocol created by the mothur developers [21], adjusted to suit our dataset [15].  
133 Sequences were subsampled before statistical analysis. The sequencing error rate, principal  
134 coordinate analysis graphs (PCOA); analyses of molecular variance (AMOVA); Good's coverage  
135 analyses [22]; richness (Chao 1 Index) and diversity (Inverse Simpson Index) calculations and  
136 indicator analyses [23] were all calculated within mothur. Clustering of microbial communities into  
137 metacommunities was also carried out within mothur using a probabilistic modelling  
138 technique based upon work by Holmes *et al* [24]. The significance of differences between the  
139 diversity and richness of groups was calculated using either the two sample t-test (normal data) or  
140 the Mann-Whitney U test (non-normal data) within Minitab 16 for Windows (Minitab, Coventry, UK).  
141 Heatmaps were constructed in R Version 3.2.2 [25] using the Vegan [26], RColorBrewer [27], gplots  
142 [28] and heatplus [29] packages. Clustering within heatmaps was performed using the Bray-Curtis  
143 dissimilarity [30]. Sequences can be accessed via the Bioproject accession number PRJNA317719.

144

145 **Results**

146

147 **Quality assurance of methodology**

148 11,878,769 sequence reads were produced in total with an average of 138,125 ± 29,306 per sample  
149 (mean ± SD). The sequencing error rate was calculated as 0.35%. The oropharyngeal swab sample  
150 from lamb 12773 was found to have very low read numbers and was therefore discarded from  
151 statistical analyses, as was its corresponding lung fluid sample. A total of 1061 OTUs were identified  
152 (**Additional file 2**) which were reduced to 750 after sub-sampling. All Good's coverage values were >  
153 0.999 indicating that at least 99.9% of the bacteria present in our samples are likely to have been  
154 identified. The most abundant bacterial OTUs from extraction kit reagent only controls are listed in  
155 **Table 1**. The similarity of the OTUs found on the 25<sup>th</sup> and 26<sup>th</sup> March 2015 is likely due to the fact  
156 that the same lot of extraction kit was used. Upon examining our data we found that lung fluid  
157 samples clustered by when they were processed (**Additional file 3**). Samples sequenced via Miseq  
158 and Hiseq underwent DNA extraction and PCR amplification separately. We identified two OTUs  
159 which were significantly indicative (P < 0.05) of samples from either the Hiseq or Miseq run which  
160 were also present in all lung fluid samples from the run they were indicative of: OTU 4  
161 (*Pseudomonas*) and OTU 112 (Yaniellaceae). These OTUs are likely due to contamination and were  
162 therefore removed prior to analysis.

163

164 **Table 1: Bacterial OTUs found to be > 5% abundant in extraction kit reagent controls**

Date of DNA extraction	OTUs	Abundance
17th July 2014	<i>Aerococcus</i>	14%
	Dermabacteraceae	12%
	<i>Micrococcus</i>	10%



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	<i>Enhydrobacter</i>	9%
	<i>Leuconostoc</i>	6%
	<i>Kocuria</i>	6%
	<i>Actinomyces</i>	6%
25 <sup>th</sup> March 2015	<i>Methylobacterium komagatae</i>	65%
	Ruminococcaceae	11%
	<i>Methylobacterium</i>	6%
26 <sup>th</sup> March 2015	<i>Methylobacterium komagatae</i>	67%
	<i>Methylobacterium</i>	6%

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165

166

167 **Lamb oropharyngeal swabs cluster into two distinct community types**

168 Oropharyngeal swabs were taken from 40 lambs. Using the Laplace approximation it was found that  
 169 swabs could be partitioned into two separate groups based upon the types of bacteria present.

170 These appeared to correspond to either oropharyngeal-type (partition 1) or rumen-type (partition 2)

171 bacteria (**Additional file 4**). Oropharyngeal-type communities were dominated by the OTUs

172 Pasteurellaceae (22%), *Mannheimia* (14%), *Fusobacterium* (11%), *Bibersteinia trehalosi* (8%),

173 Neisseriaceae (7%), *Moraxella* (6%) and *Bibersteinia* (5%). Rumen-type communities were

174 dominated by the OTUs *Prevotella* (36%), Clostridiales (11%), Ruminococcaceae (7%),

175 Lachnospiraceae (6%) and *Butyrivibrio* (6%).

176 The richness (chao: non-normal data) and diversity (Inverse Simpsons: normal data) of the partitions

177 were compared. There was no significant difference in richness or diversity between the rumen-type

178 partition and the oropharyngeal-type partition.

179

180 **Dichotomous oropharyngeal microbiota are associated with different lung community structures**

181 The most common OTUs found in lung fluid samples were *Staphylococcus equorum* (13%),  
182 *Staphylococcus sciuri* (6%), *Mannheimia* (5%) and *Prevotella* (5%). Using the Laplace approximation,  
183 lung fluids did not cluster into more than one group. Lung fluids were then manually partitioned into  
184 the same groups as swabs. A significant difference in bacterial community structure was found  
185 between these groups (AMOVA:  $P = 0.016$ ) and a small number of OTUs were found to be  
186 significantly different between the two groups. *Prevotella* ( $P = 0.03$ ) and *Sphingobium* ( $P = 0.039$ )  
187 were significantly indicative of lambs from which rumen-type swabs were derived whereas  
188 *Paracoccus aminovorans* ( $P = 0.036$ ) was indicative of lambs from which oropharyngeal-type swabs  
189 were derived. **Figures 1 and 2** contain visual representations of sample clustering.

190 We compared the proportions of the dominant OTUs in rumen-type swabs with their corresponding  
191 proportions in lung samples. On average, these OTUs were found in the following proportions in lung  
192 samples: *Prevotella* (5%), Clostridiales (2%), Ruminococcaceae (3%), Lachnospiraceae (1%) and  
193 *Butyrivibrio* (1%).

194

195 **The presence of a lung specific microbiota**

196 Indicator species analysis determined that several OTUs were significantly more indicative of the  
197 lungs than of oropharyngeal swabs (**Table 2**). It is likely that reagent contamination will have had  
198 more of an impact on the lung fluid samples than on the oropharyngeal swabs, due to their lower  
199 biomass. However, when examining the indicative OTUs, the majority of samples were not found to  
200 contain the same proportions of these OTUs as the PBS controls processed alongside them (**Figure**  
201 **3**). Of the indicative OTUs, by far the most common was *Staphylococcus equorum* which constituted,

202 on average, 13.3% of the total bacteria present in lung fluids and which was only present in low  
 203 numbers in controls and oropharyngeal swabs.

204

205 **Table 2: OTUs significantly more indicative of lung fluids than oropharyngeal swabs.**

Taxonomy	P value	Average proportion in lung fluids (Mean $\pm$ SD)	Average proportion in oropharyngeal swabs (Mean $\pm$ SD)	Highest proportion in PBS controls
<i>Brachybacterium</i>	0.006	1.0% $\pm$ 1.7%	0.035% $\pm$ 0.10%	0.022%
<i>Brevibacterium</i>	0.002	1.2% $\pm$ 1.4%	0.064% $\pm$ 0.24%	0%
<i>Corynebacterium</i>	< 0.001	1.9% $\pm$ 2.3%	0.065% $\pm$ 0.19%	0.044%
<i>Delftia</i>	< 0.001	0.80% $\pm$ 1.7%	0% $\pm$ 0%	0%
Enterobacteriaceae	0.023	0.65% $\pm$ 2.6%	0.0063% $\pm$ 0.029%	2.2%
<i>Frigoribacterium</i>	0.021	0.79% $\pm$ 1.4%	0.077% $\pm$ 0.31%	0%
<i>Janthinobacterium</i>	0.01	0.57% $\pm$ 1.4%	0.0023% $\pm$ 0.0068%	0%
<i>Jeotgalicoccus psychrophilus</i>	0.008	1.6% $\pm$ 2.1%	0.040% $\pm$ 0.10%	0%
<i>Microbacterium aurum</i>	0.047	1.2% $\pm$ 2.8%	0.0045% $\pm$ 0.013%	0%
<i>Micrococcus</i>	0.017	0.77% $\pm$ 1.6%	0.0080% $\pm$ 0.029%	4.4%
Oxalobacteraceae	< 0.001	0.96% $\pm$ 1.5%	0.012% $\pm$ 0.043%	3.0%
<i>Pelomonas</i>	< 0.001	0.65% $\pm$ 1.1%	0.00057% $\pm$ 0.0036%	2.0%
Peptostreptococcaceae	0.006	1.8% $\pm$ 2.2%	0.050% $\pm$ 0.11%	0.044%
<i>Propionibacterium acnes</i>	< 0.001	0.84% $\pm$ 2.3%	0.0040% $\pm$ 0.020%	1.6%
<i>Pseudomonas citronellolis</i>	< 0.001	0.51% $\pm$ 1.1%	0% $\pm$ 0%	1.0%
<i>Rathayibacter caricis</i>	0.016	0.58% $\pm$ 1.2%	0.0057% $\pm$ 0.021%	0%
<i>Saccharopolyspora</i>	0.009	0.52% $\pm$ 1.2%	0.0011% $\pm$ 0.0071%	0%
<i>SMB53</i>	< 0.001	0.71% $\pm$ 1.3%	0.0045% $\pm$ 0.018%	0%

<i>Sphingobium yanoikuyae</i>	< 0.001	0.53% ± 0.56%	0% ± 0%	13%
<i>Staphylococcus</i>	< 0.001	3.9% ± 5.4%	0.060% ± 0.18%	8.6%
<i>Staphylococcus equorum</i>	< 0.001	13.3% ± 9.6%	0.32% ± 0.97%	0.044%
<i>Staphylococcus sciuri</i>	< 0.001	6.4% ± 5.3%	0.18% ± 0.59%	2.0%
<i>Streptomyces</i>	< 0.001	2.0% ± 3.8%	0.025% ± 0.096%	0%
<i>Turicibacter</i>	0.016	1.0% ± 1.8%	0.0074% ± 0.021%	0%
<i>Variovorax paradoxus</i>	0.011	1.2% ± 1.2%	0.024% ± 0.060%	0.022%

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206 **OTUs which were significantly more (P < 0.05) indicative of lamb lung fluids than oropharyngeal**  
207 **swabs and which were on average > 0.5% abundant in lung fluids.**

208

## 209 **Discussion**

210 Sheep are commonly used as large animals models of the respiratory system due to the physiological  
211 and immunological similarities of their lungs to those of humans [11, 10, 31, 32]. We have previously  
212 used the sheep to study both the extent of variation in the lung microbiota [15] and the direct and  
213 remote changes in the lung microbiota caused by localised *P.aeruginosa* infection and antibiotic  
214 treatment [14]. As the sheep is an important agricultural animal, studies of their respiratory  
215 microbial communities may also be of interest from an animal health perspective.

216 It has previously been demonstrated that microaspiration of microbes from the upper aerodigestive  
217 tract is common in humans and can lead to an inflammatory phenotype [33]. When microbial  
218 communities from healthy human lungs are characterised they are often found to contain microbes  
219 associated with the upper aerodigestive tract [34]. The healthy human lung microbiota is thought to  
220 be formed predominantly from the neutral dispersal of these upper aerodigestive tract microbes  
221 into the lungs rather than by the differential growth of lung adapted microbial communities [35].  
222 We sought to identify whether this was also the case in sheep.

223 Sheep oropharyngeal swabs could be partitioned into two separate groups which were  
224 predominantly composed of OTUs identified as bacteria which are well known members of either  
225 the rumen (*Prevotella*, Clostridiales, Ruminococcaceae, Lachnospiraceae and *Butyrivibrio* [36-38]) or  
226 respiratory tract microbiotas (Pasteurellaceae, *Mannheimia*, *Fusobacterium*, *Bibersteinia trehalosi*,  
227 Neisseriaceae, *Moraxella* and *Bibersteinia* [39-41]). These bacteria were also detected in a previous  
228 study examining sheep buccal swabs [42].

229 It is not possible to identify whether this dichotomy reflected recent rumination, or some stochastic  
230 post-mortem leakage of rumen fluid into the oropharynx in some individuals. The lambs during this  
231 study were not weaned but were at an age when it is expected that they all would be regularly  
232 supplementing their diet with grass and would be ruminating.

233 Regardless of the drivers of this oropharyngeal dichotomy, the microbial communities found in the  
234 lungs were very different to those found in both the rumen- and oropharyngeal-type swabs. A large  
235 number of bacterial OTUs were found to be significantly more abundant in lung fluids in comparison  
236 to oropharyngeal swabs, including *Staphylococcus equorum* which was by far the most common  
237 bacterial OTU found. Several OTUs which are commonly associated with the rumen were also  
238 identified in lung fluids. Our lung fluid samples will have been more affected by reagent  
239 contamination than the oropharyngeal swabs due to the lower quantity of bacterial DNA present  
240 [43]. However, the microbial communities found in lung fluids did not reflect the bacteria found in  
241 reagent only controls processed on the same day so the presence of bacteria in the lamb lung  
242 cannot be attributed purely to sample contamination. Nor can it be attributed to disease as no  
243 lambs showed clinically overt signs of respiratory illness during the study.

244 There are several reasons why the microbes found in lamb lungs might not reflect those found in the  
245 upper aerodigestive tract to the same extent as is found in humans. Sheep have evolved to cope  
246 with rumination and thereby may have more efficient anatomical barriers to microaspiration [44].

247 Physiological and anatomical differences such as the horizontal disposition of the lungs, increased  
248 nasal breathing and increased saliva production [45, 46] may also contribute.

249

## 250 **Conclusions**

251 In this study we examined oropharyngeal swab and lung fluid samples taken from healthy lambs to  
252 characterise the bacterial communities present and to assess the impact of rumination on these  
253 communities. We found that oropharyngeal swabs were dominated by either rumen-type or  
254 oropharyngeal-type microbial communities. We also found that lung bacteria did not greatly  
255 resemble either rumen- or oropharyngeal-type swabs and identified several bacterial OTUs which  
256 were more indicative of lung fluids. The lungs did contain several rumen associated bacteria which  
257 may indicate that there is a certain degree of microaspiration of ruminal contents in lambs.

258 Sheep are not human but the opportunities that they, and other large animals, present offer  
259 valuable insights into the dynamic relationship of the upper aerodigestive and lower respiratory  
260 tract microbiota in health. In the future, their value may extend to developing an understanding of  
261 the factors that predispose the upper aerodigestive tract microbiota towards disease in the lower  
262 respiratory tract.

263

## 264 **Declarations**

265

## 266 **Ethics approval and consent to participate**

267 Animals were used which were part of a study examining the effects of prenatal stress. This study  
268 was approved by Scotland's Rural College's (SRUC) Animal Experiments Committee and was  
269 conducted under Home Office licence.

270

271 **Consent for publication**

272 Not applicable

273

274 **Availability of data and materials**

275 The datasets generated during and/or analysed during the current study are available in the Short

276 Read Archive and can be accessed at Bioproject accession number PRJNA317719

277 (<http://www.ncbi.nlm.nih.gov/bioproject/PRJNA317719>).

278

279 **Competing interests**

280 The authors declare that they have no competing interests.

281

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288 or the writing of this manuscript.

289

290 **Authors' contributions**

291 LG contributed to study design, drafting of paper and sample collection, processing and analysis. DC  
292 contributed to study design, drafting of paper and sample collection. SW contributed to drafting of  
293 paper and sample collection. KR contributed to study design and the drafting of paper. GM  
294 contributed to study design, drafting of paper and sample collection. All authors read and approved  
295 the final manuscript.

296

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301 Microbiome Project: Genomic DNA from Microbial Mock Community B (Even, Low Concentration),  
302 v5.1L, for 16S rRNA Gene Sequencing, HM-782D.

303

### 304 **References**

- 305 1. Martinsen EMH, Leiten EO, Bakke PS, Eagan TML, Gronseth R. Participation in research  
306 bronchoscopy: A literature review. *Eur Respir J* 2016;3:29511. doi:10.3402/ecrj.v3.29511.
- 307 2. Yadava K, Pattaroni C, Sichelstiel AK, Trompette A, Gollwitzer ES, Salami O et al. Microbiota  
308 promotes chronic pulmonary inflammation by enhancing IL-17A and autoantibodies. *Am J Respir Crit*  
309 *Care Med* 2015;115(1):63-8. doi:10.1164/rccm.201504-0779OC.
- 310 3. Yu W, Yuan X, Xu X, Ding R, Pang L, Liu Y et al. Reduced airway microbiota diversity is associated  
311 with elevated allergic respiratory inflammation. *Ann Allergy Asthma Immunol.* 2015;115(1):63-8.  
312 doi:10.1016/j.anai.2015.04.025.
- 313 4. Stokes K, Wood A, August A. The microbiome regulates the eosinophil requirement for  
314 development of allergic lung inflammation. *J Immunol.* 2015;194(1 Supplement):53.18.



315 5. Barfod KK, Roggenbuck M, Hansen LH, Schjørring S, Larsen ST, Sørensen SJ et al. The murine lung  
316 microbiome in relation to the intestinal and vaginal bacterial communities. *BMC Microbiol.*  
317 2013;13(1):1-12. doi:10.1186/1471-2180-13-303.

318 6. Yun Y, Srinivas G, Kuenzel S, Linnenbrink M, Alnahas S, Bruce KD et al. Environmentally  
319 determined differences in the murine lung microbiota and their relation to alveolar architecture.  
320 *PLoS One.* 2014;9(12):e113466. doi:10.1371/journal.pone.0113466.

321 7. Poroyko V, Meng F, Meliton A, Afonyushkin T, Ulanov A, Semenyuk E et al. Alterations of lung  
322 microbiota in a mouse model of LPS-induced lung injury. *Am J Physiol Lung Cell Mol Physiol.*  
323 2015;309(1):L76-L83. doi:10.1152/ajplung.00061.2014.

324 8. Barfod KK, Vrankx K, Mirsepasi-Lauridsen HC, Hansen JS, Hougaard KS, Larsen ST et al. The murine  
325 lung microbiome changes during lung inflammation and intranasal vancomycin treatment. *Open*  
326 *Microbiol J.* 2015;9:167-79. doi:10.2174/1874285801509010167.

327 9. Singanayagam A, Glanville N, Pearson R, James P, Cuthbertson L, Cox M et al. T1 Fluticasone  
328 propionate alters the resident airway microbiota and impairs anti-viral and anti-bacterial immune  
329 responses in the airways. *Thorax.* 2015;70(Suppl 3):A1. doi:10.1136/thoraxjnl-2015-207770.1.

330 10. Bouljihad M, Leipold HW. An ultrastructural study of pulmonary bronchiolar and alveolar  
331 epithelium in sheep. *J Vet Med A.* 1994;41(8):573-86.

332 11. Griebel PJ, Entrican G, Rocchi M, Beskorwayne T, Davis WC. Cross-reactivity of mAbs to human  
333 CD antigens with sheep leukocytes. *Vet Immunol Immunopathol.* 2007;119(1-2):115-22.  
334 doi:10.1016/j.vetimm.2007.06.015.

335 12. Scheerlinck JPY, Snibson KJ, Bowles VM, Sutton P. Biomedical applications of sheep models: From  
336 asthma to vaccines. *Trends Biotechnol.* 2008;26(5):259-66. doi:10.1016/j.tibtech.2008.02.002.

337 13. Meeusen EN, Snibson KJ, Hirst SJ, Bischof RJ. Sheep as a model species for the study and  
338 treatment of human asthma and other respiratory diseases. *Drug Discov Today Dis Models.*  
339 2009;6(4):101-6. doi:10.1016/j.ddmod.2009.12.002.

- 340 14. Collie D, Glendinning L, Govan J, Wright S, Thornton E, Tennant P et al. Lung microbiota changes  
341 associated with chronic *Pseudomonas aeruginosa* lung infection and the impact of intravenous  
342 colistimethate sodium. PLoS One. 2015;10(11):e0142097. doi:10.1371/journal.pone.0142097.
- 343 15. Glendinning L, Wright S, Pollock J, Tennant P, Collie D, McLachlan G. Variability of the sheep lung  
344 microbiota. Appl Environ Microbiol 2016;82(11):3225-38. doi:10.1128/aem.00540-16.
- 345 16. Dickson RP, Huffnagle GB. The lung microbiome: New principles for respiratory bacteriology in  
346 health and disease. PLoS Pathog. 2015;11(7):e1004923. doi:10.1371/journal.ppat.1004923.
- 347 17. Bassis CM, Erb-Downward JR, Dickson RP, Freeman CM, Schmidt TM, Young VB et al. Analysis of  
348 the upper respiratory tract microbiotas as the source of the lung and gastric microbiotas in healthy  
349 individuals. mBio. 2015;6(2):e00037-15. doi:10.1128/mBio.00037-15.
- 350 18. Berry D, Ben Mahfoudh K, Wagner M, Loy A. Barcoded primers used in multiplex amplicon  
351 pyrosequencing bias amplification. Appl Environ Microbiol. 2011;77(21):7846-9.  
352 doi:10.1128/AEM.05220-11.
- 353 19. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads.  
354 Embnetjournal. 2011;17(1):10-2. doi:10.14806/ej.17.1.200.
- 355 20. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB et al. Introducing mothur:  
356 Open-source, platform-independent, community-supported software for describing and comparing  
357 microbial communities. Appl Environ Microbiol. 2009;75(23):7537-41. doi:10.1128/AEM.01541-09.
- 358 21. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index  
359 sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq  
360 illumina sequencing platform. Appl Environ Microbiol. 2013;79(17):5112-20.  
361 doi:10.1128/AEM.01043-13.
- 362 22. Esty WW. The efficiency of Good's nonparametric coverage estimator. Ann Stat.  
363 1986;14(3):1257-60.
- 364 23. Dufrêne M, Legendre P. Species assemblages and indicator species: The need for a flexible  
365 assymetrical approach. Ecol Monogr. 1997;67(3):345-66.

366 24. Holmes I, Harris K, Quince C. Dirichlet multinomial mixtures: Generative models for microbial  
367 metagenomics. PLoS One. 2012;7(2):e30126. doi:10.1371/journal.pone.0030126.

368 25. R Core Team. R: A language and environment for statistical computing. [https://www.R-](https://www.R-project.org/)  
369 [project.org/](https://www.R-project.org/); R Foundation for Statistical Computing; 2015.

370 26. Oksanen J, Blanchet F, Kindt R, Legendre P, Minchin P, O'Hara R et al. Vegan: Community ecology  
371 package. R package version 2.3-4/r2988 ed. <http://R-Forge.R-project.org/projects/vegan/>2016.

372 27. Neuwirth E. RColorBrewer: ColorBrewer palettes. R package version 1.1-2 ed. [http://CRAN.R-](http://CRAN.R-project.org/package=RColorBrewer2014)  
373 [project.org/package=RColorBrewer2014](http://CRAN.R-project.org/package=RColorBrewer2014).

374 28. Warnes G, Bolker B, Bonebakker L, Gentleman R, Liaw W, Lumley T et al. gplots: Various R  
375 programming tools for plotting data. R package version 2.17.0 ed. [http://CRAN.R-](http://CRAN.R-project.org/package=gplots2015)  
376 [project.org/package=gplots2015](http://CRAN.R-project.org/package=gplots2015).

377 29. Ploner A. Heatplus: Heatmaps with row and/or column covariates and colored clusters. R  
378 package version 2.16.0 ed. <http://bioconductor.org/biocLite.R2015>.

379 30. Bray JR, Curtis JT. An ordination of the upland forest communities of southern Wisconsin. Ecol  
380 Monograph. 1957;27(4):326-49. doi:10.2307/1942268.

381 31. Entrican G, Wattegedera SR, Griffiths DJ. Exploiting ovine immunology to improve the relevance  
382 of biomedical models. Mol Immunol. 2015;66(1):68-77. doi:10.1016/j.molimm.2014.09.002.

383 32. Enkhbaatar P, Nelson C, Salsbury JR, Carmical JR, Torres KEO, Herndon D et al. Comparison of  
384 gene expression by sheep and human blood stimulated with the TLR4 agonists lipopolysaccharide  
385 and monophosphoryl lipid A. PLoS One. 2015;10(12):e0144345. doi:10.1371/journal.pone.0144345.

386 33. Segal LN, Clemente JC, Tsay J-CJ, Koralov SB, Keller BC, Wu BG et al. Enrichment of the lung  
387 microbiome with oral taxa is associated with lung inflammation of a Th17 phenotype. Nat Microbiol.  
388 2016;1:16031. doi:10.1038/nmicrobiol.2016.31.

389 34. Segal L, Alekseyenko A, Clemente J, Kulkarni R, Wu B, Chen H et al. Enrichment of lung  
390 microbiome with supraglottic taxa is associated with increased pulmonary inflammation.  
391 Microbiome. 2013;1(1):19. doi:10.1186/2049-2618-1-19.

392 35. Venkataraman A, Bassis CM, Beck JM, Young VB, Curtis JL, Huffnagle GB et al. Application of a  
393 neutral community model to assess structuring of the human lung microbiome. *mBio*.  
394 2015;6(1):e0084-14. doi:10.1128/mBio.02284-14.

395 36. Huws SA, Kim EJ, Lee MRF, Scott MB, Tweed JKS, Pinloche E et al. As yet uncultured bacteria  
396 phylogenetically classified as *Prevotella*, Lachnospiraceae *incertae sedis* and unclassified  
397 Bacteroidales, Clostridiales and Ruminococcaceae may play a predominant role in ruminal  
398 biohydrogenation. *Environ Microbiol*. 2011;13(6):1500-12. doi:10.1111/j.1462-2920.2011.02452.x.

399 37. Zened A, Combes S, Cauquil L, Mariette J, Klopp C, Bouchez O et al. Microbial ecology of the  
400 rumen evaluated by 454 GS FLX pyrosequencing is affected by starch and oil supplementation of  
401 diets. *FEMS Microbiol Ecol*. 2013;83(2):504-14. doi:10.1111/1574-6941.12011.

402 38. Omoniyi LA, Jewell KA, Isah OA, Neumann AP, Onwuka CFI, Onagbesan OM et al. An analysis of  
403 the ruminal bacterial microbiota in West African Dwarf sheep fed grass- and tree-based diets. *J Appl*  
404 *Microbiol*. 2014;116(5):1094-105. doi:10.1111/jam.12450.

405 39. Christensen H, Kuhnert P, Nørskov-Lauritsen N, Planet PJ, Bisgaard M. The Family  
406 Pasteurellaceae. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F, editors. *The*  
407 *Prokaryotes: Gammaproteobacteria*. Berlin, Heidelberg: Springer Berlin Heidelberg; 2014. p. 535-64.

408 40. Ling Z, Liu X, Wang Y, Li L, Xiang C. Pyrosequencing analysis of the salivary microbiota of healthy  
409 Chinese children and adults. *Microb Ecol*. 2012;65(2):487-95. doi:10.1007/s00248-012-0123-x.

410 41. Cardenas PA, Cooper PJ, Cox MJ, Chico M, Arias C, Moffatt MF et al. Upper airways microbiota in  
411 antibiotic-naïve wheezing and healthy infants from the tropics of rural Ecuador. *PLoS One*.  
412 2012;7(10):e46803. doi:10.1371/journal.pone.0046803.

413 42. Kittelmann S, Kirk MR, Jonker A, McCulloch A, Janssen PH. Buccal swabbing as a noninvasive  
414 method to determine bacterial, archaeal, and eukaryotic microbial community structures in the  
415 rumen. *Appl Environ Microbiol*. 2015;81(21):7470-83. doi:10.1128/AEM.02385-15.

- 416 43. Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF et al. Reagent and laboratory  
417 contamination can critically impact sequence-based microbiome analyses. BMC Biol. 2014;12:87.  
418 doi:10.1186/s12915-014-0087-z.
- 419 44. May NDS. The Anatomy of the Sheep. Queensland, Australia: St. Lucia: University of Queensland  
420 Press; 1964.
- 421 45. Hungate RE. Chapter V - The Rumen as a Continuous Fermentation System. The Rumen and its  
422 Microbes. NY, USA: Academic Press; 1966. p. 206-44.
- 423 46. Sarkar A, Singh H. Oral Behaviour of Food Emulsions. Food Oral Processing. Wiley-Blackwell;  
424 2012. p. 111-37.

425

## 426 **Figure Legends**

427 **Figure 1:** PCOA graph showing the relatedness of upper aerodigestive tract samples from lambs  
428 partitioned into 2 groups using the Laplace approximation. Lung fluids belonging to the same  
429 animals were partitioned into the same groups. Lung fluid partitions clustered significantly  
430 separately by AMOVA ( $P = 0.016$ ) as did oropharyngeal swabs ( $P < 0.001$ ). Controls are PBS and  
431 extraction kit reagent controls.

432 **Figure 2:** Heatmap of OTUs found in lamb lung fluids, oropharyngeal swabs, extraction kit reagent  
433 and PBS controls. OTUs were included when they were  $> 5\%$  abundant in at least one sample.  
434 Oropharyngeal swabs partitioned into rumen-like bacterial communities are indicated by green  
435 whereas those which were upper aerodigestive tract-like are indicated by a red line. The lung fluid  
436 samples from the oropharyngeal-like animals are indicated by blue whereas those from the rumen-  
437 type animals are indicated by pink.

438 **Figure 3:** Heatmap of bacterial OTUs found to be more indicative of lamb lung fluids than  
439 oropharyngeal swabs ( $P < 0.05$ ). Fluid and PBS samples from which DNA was extracted on specific

440 dates are surrounded by coloured lines: 17<sup>th</sup> July 2014 (blue) and 25<sup>th</sup> March 2015 (red). DNA  
441 extractions carried out on 26<sup>th</sup> March 2015 only comprised oropharyngeal swabs which are not  
442 included in this figure.

443

#### 444 **Additional files**

445 Additional file 1 (Additional file 1.xlsx): **Dataset S1**: Sample processing data for all samples.

446 Additional file 2 (Additional file 2.xlsx): **Dataset S2**: Full list of bacterial OTUs and taxonomies.

447 Additional file 3 (Additional file 3.docx): **Figure S1**: Heatmap of OTUs found in lamb lung fluids,  
448 oropharyngeal swabs, PBS and extraction kit reagent only controls.

449 Additional file 4 (Additional file 4.docx): **Table S1**: OTUs responsible for partitioning of lamb  
450 oropharyngeal swabs into two groups (using Laplace value).