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1 Exploring the Variability of the Sheep Lung Microbiota

2

3 Laura Glendinning^a #, Steven Wright^a, Jolinda Pollock^{a, b}, Peter Tennant^a, David Collie^a, Gerry
4 McLachlan^a

5

6 The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh,
7 Edinburgh, Midlothian, United Kingdom^a; Monogastric Science Research Centre, Scotland's
8 Rural College (SRUC), Edinburgh, Midlothian, United Kingdom^b

9

10 #Address correspondence to Laura Glendinning, Laura.Glendinning@roslin.ed.ac.uk

11

12 Running Head: The Sheep Lung Microbiota

13 **Abstract**

14 Sequencing technologies have recently facilitated the characterisation of bacterial communities
15 present in lungs during health and disease. However, there is currently a dearth of information
16 concerning the variability of such data in health both between and within subjects. This study
17 seeks to examine such variability using healthy adult sheep as our model system.

18 Protected specimen brush samples were collected from three spatially disparate segmental
19 bronchi of six adult sheep (age 20 months) on three occasions (day 0, one month and three
20 months). To further explore the spatial variability of the microbiota, more extensive brushings
21 (n=16) and a throat swab were taken from a separate sheep. The V2-V3 hypervariable regions of
22 the bacterial 16S rRNA gene were amplified and sequenced via Illumina MiSeq. DNA sequences
23 were analysed using the MOTHUR software package. Quantitative PCR was performed to
24 quantify total bacterial DNA.

25 Some sheep lungs contained dramatically different bacterial communities at different sampling
26 sites whereas in others airway microbiota appeared similar across the lung. In our spatial
27 variability study, clustering was observed related to the depth within the lung from which
28 samples were taken. Lung depth refers to increasing distance from the glottis progressing in a
29 caudal direction. We conclude that both host influence and local factors have an impact on the
30 composition of the sheep lung microbiota.

31

32 **Importance**

33 Until recently, it was assumed that the lungs were a sterile environment which were only
34 colonised by microbes during disease. However, recent studies using sequencing technologies

35 have found that there is a small population of bacteria which exists in the lung during health,
36 referred to as the 'lung microbiota'. In this study we characterise the variability of the lung
37 microbiota of healthy sheep. Sheep are not only economically important animals but are also
38 often used as large animal models of human respiratory disease. We conclude that, whilst host
39 influence does play a role in dictating the types of microbes which colonise the airways, it is
40 clear that local factors also play an important role in this regard. Understanding the nature and
41 influence of these factors will be key to understanding the variability in, and functional relevance
42 of the lung microbiota.

43

44

45 **Introduction**

46 Within the past five years, a diverse array of bacteria has been detected in healthy lungs through
47 the use of non-culture based methods (1, 2). These bacterial communities are commonly referred
48 to as the lung microbiota and are thought to originate predominantly from the upper respiratory
49 tract (3, 4). The presence of particular bacterial communities in the lung has been associated with
50 several human diseases including cystic fibrosis (5), chronic obstructive pulmonary disease (6),
51 bronchiectasis (7) and lung transplant rejection (8).

52 Whilst variation in the microbial communities present in the human lung exists at both a large
53 and small scale, based upon the location of the bacteria within the lungs (9) and the host cell-
54 types present (10), intra-individual variation has been found to be significantly less than inter-
55 individual variation, indicating that each individual may play host to a specific lung microbiota
56 (9).

57 The lung microbiota of healthy domestic sheep has previously been investigated using culture
58 based methods (11-14) but these studies have shown conflicting descriptions of the extent of
59 lung colonisation by bacteria. A study in pneumonic Bighorn sheep lungs found that for most
60 sheep studied, bacterial 16S rRNA gene amplification and sequencing was able to identify
61 additional bacterial species which were not found by culturing (15). Previous studies have also
62 examined the upper respiratory tract of healthy sheep by culture based methods (11, 12, 14, 16).
63 These studies are highly variable in the types and proportions of microbes identified.

64 Previously, our group studied the composition of the lung microbiota in sheep pre- and post-
65 infection with *Pseudomonas aeruginosa* (17). This study included the first description of the
66 lung microbiota communities of healthy domestic sheep by next generation sequencing. A
67 diverse community of microbes was identified and variability was seen to be high, both within
68 and between animals. The variability of the healthy lung microbiota at specific lung sites over
69 time has not been reported in any animal, although serial sampling of non-diseased human is
70 planned as part of the Lung HIV Microbiome Project (LHMP) (18).

71 In the present study, protected specimen brush samples were collected from three spatially
72 disparate segmental bronchi at three time-points (baseline, one month and three months) to
73 examine the composition and variability of the lung microbiota in healthy domestic sheep. In
74 addition, samples were also taken from a separate sheep from a greater number of respiratory
75 tract locations, to further explore the extent of spatial variability.

76 Such studies are fundamental to understanding the functional relevance of lung microbiota in
77 health and disease in ruminants. Indeed bacterial pneumonia is well recognised in cattle and
78 sheep and is often associated with high morbidity and mortality. Notably, regional predilection is
79 evident in that infection by *Pasteurella* occurs most frequently in the apical and cardiac lobes in

both sheep (12, 19) and cattle (20, 21). Co-infections with other respiratory pathogens are commonplace, it is already well known that infection by *Bordetella parapertussis* and *Mycoplasma ovipneumoniae* can lead to more severe disease caused by *Mannheimia* (*Pasteurella*) *haemolytica* (22-25), and there are well recognised links to stressful events such as housing or transport. As it is conceivable that changes in the lung microbiota may precipitate or associate with such events it is vital to ground future disease-related studies on a firm basis of understanding normal variation in health. Whilst the immediate focus of such studies relates to animal health it is also important to acknowledge that sheep are frequently used as models for human respiratory research (26, 27) and that there is an ongoing need to highlight any comparative contrasts and consistencies as and when they arise.

Materials and methods

Animals and airway sampling

Six twenty month old Suffolk-cross sheep were used in this study (**Table 1**) (5 females, 1 castrated male) and were housed indoors in pens for the trial duration. All animals had not undergone bronchoscopic examination during the four months preceding the study. Animal procedures were subject to the Animals (Scientific Procedures) Act 1986 and were approved by the Roslin Institute Animal Welfare and Ethics Committee.

Anaesthesia was performed as described previously (28). Sheep were sampled by protected specimen brushings (ConMed Disposable Microbiology Brush, New York, NY, USA) at 0 days (baseline), one month and three months. Sampling sites are shown in **Fig 1**. Bronchoscopy was

102 performed via an endotracheal tube by the same operator for all sheep at all time-points. The
103 sample harvest dates can be found in **Table S1**. Before sampling of every sheep on any given
104 day, 7.5 ml of phosphate buffered saline (PBS) was passed through the bronchoscope channel to
105 act as an environmental qPCR control. Bronchoscope washings were centrifuged at 13,000 g for
106 15 minutes and the pellet was resuspended in 500 µl of PBS.

107 A throat swab and brushing samples (harvested as above) were also taken from a further sheep
108 (female; age 36 months; 60 kg bodyweight) at a single time-point to further explore the spatial
109 variability of the lung microbiota (sampling date 01/05/2015). Brushing sites were dorsal and
110 ventral trachea and paired sites from either side of airway bifurcations progressing along the
111 anterior to caudal lung axis (**Fig 2**).

112

113 **DNA extraction, amplification and sequencing**

114 DNA extraction was performed using the MOBIO PowerSoil® DNA Isolation Kit (Carlsbad,
115 CA, USA). Brushes were transferred into PowerSoil Bead Tubes with PowerSoil Solution C1
116 and PowerSoil Bead Solution. Bead Tubes were heated at 65°C for 10 minutes then placed in a
117 FastPrep FP120 Cell Disrupter (Qbiogene Inc., Cedex, France) for 45 seconds at 5.0 m/sec. From
118 this point onwards the manufacturer's instructions were followed, except for the final elution
119 step. Purified DNA was eluted into 50 µl of PowerSoil Solution C6 rather than 100 µl to increase
120 the DNA concentration.

121 All PCR steps used Q5® High-Fidelity 2X Master Mix (New England Biolabs, Beverly, MA,
122 USA). A nested PCR reaction was performed with Illumina adaptor sequences and barcodes
123 (**Table S2**) included only on the primers for the second round in an attempt to reduce bias

caused by barcoded primers when amplifying low biomass samples (29). The conditions for the first round of PCR, amplifying the V1-V4 16S hypervariable regions (primers: 28F (5'–GAGTTTGATCNTGGCTCAG–3') and 805R (5'–GACTACCAGGGTATCTAATC–3')), were: 94°C for 2 minutes followed by 20 cycles of 94°C for 1 minute, 55°C for 45 seconds and 72°C for 1.5 minutes followed by 72°C for 20 minutes. The conditions for the second round of PCR, amplifying the V2-V3 16S hypervariable regions (primers: 104F (5'–GGCGVACGGGTGAGTAA–3') and 519R (5'–GTNTTACNGCGGCKGCTG–3')), were: 98°C for 30 seconds followed by 20 cycles of 98°C for 10 seconds, 67°C for 30 seconds and 72°C for 10 seconds followed by 72°C for 2 minutes. Amplicons from both rounds of PCR were purified using the AMPure XP PCR Purification System (Beckman Coulter, Brea, CA, USA). Amplicons were sequenced using an Illumina MiSeq or HiSeq (Illumina, San Diego, CA) run producing paired end 250-nucleotide reads (30). Those samples sequenced by two Miseq runs are listed in Dataset S1 and those sequenced by HiSeq are listed in Dataset S2. Where samples from the Miseq runs were found to have low read numbers, they were sequenced again on a separate Miseq run (Samples: 2D618 RA 3 months and 2D619 RA 3 months). We previously confirmed cross-run stability by comparing separate runs made on the same samples (**Fig S1**).

Extraction kit controls were produced by carrying out a reagent-only extraction using the MOBIO PowerSoil® DNA Isolation Kit. PCR reagent controls were constructed by adding 20 µl of nuclease free water to the PCR reaction mixture. The Human Microbiome Project Mock Community HM-782D (100,000 copies per organism per µl, BEI Resources, ATCC, Manassas, VA, USA) , extraction kit controls, PCR reagent-only controls and positive controls (DNA extracted from *Pseudomonas aeruginosa* Strain PA0579) were amplified and sequenced by the same methods as were used for samples.

147 A separate mock community sample was sequenced using an Illumina HiSeq. For this sample,
148 the solution produced from the first round of PCR was diluted 1:100 in nuclease free water
149 before being used in the second round of PCR. This was carried out to ascertain the effect of
150 placing different concentrations of DNA into the second PCR round on PCR bias.

151 The unassembled reads, with primers removed, are publicly available through the NCBI
152 Sequence Read Archive (SRA) under the Bioproject ascension: PRJNA298882.

153

154 **Bioinformatic and statistical analysis**

155 Primers were removed using Cutadapt (31). Sequences which contained more than one base error
156 per 10 primer bases were removed from further analysis. The following steps were carried out in
157 MOTHUR (32) and were based upon a protocol developed for MiSeq by the MOTHUR creators
158 (30). Forward and reverse reads were aligned to form one continuous DNA sequence; any
159 sequences which failed to align were discarded. Sequences which contained ambiguous bases;
160 were less than 369 base pairs in length or contained homopolymers of greater than 9 base pairs
161 were also discarded. Chimeras were identified and removed using UCHIME (33). Sequences
162 were aligned to the SILVA reference alignment (34) and were classified using MOTHUR's
163 Bayesian classifier against the Greengenes database (35), which was trimmed to the V2-V3
164 hypervariable region of the 16S rRNA gene to improve classification depth (36). Sequences
165 identified as not originating from bacteria were removed from further analysis. Operational
166 taxonomic units (OTUs) were clustered into phylotypes using a database-dependent approach
167 then sub-sampled.

168 Distance matrices were created using Yue and Clayton theta values (37). Analysis of molecular
169 variance (AMOVA) (38) was used to determine significant differences between the bacterial
170 compositions of groups. Principal co-ordinate analysis (PCOA) graphs were constructed to
171 visualise similarities between samples. The Inverse Simpson's index was used to quantify
172 diversity. Where data was non-parametric the Friedman test was used to identify significant
173 differences in diversity, using Minitab® 16 for Windows (Minitab, Coventry, UK). All other
174 statistical tests were carried out within MOTHUR. Metastats (39) was used to identify OTUs
175 which were different between groups. Good's coverage (40) was used to estimate sample
176 coverage and the Chao 1 index was used to calculate richness. Indicator OTUs (OTUs which are
177 indicative of a particular group of samples) were identified using the indicator metric within
178 MOTHUR (41). Repeated measures ANOVAs were carried out using the Vegan package in R
179 (42-44).

180

181 **qPCR**

182 qPCR reactions were performed using the LightCycler® 480 SYBR Green I Master Mix (Roche
183 Applied Science, Indianapolis, IN, USA), 1 µl of extracted DNA solution and the 16S rDNA
184 qPCR primers UniF340 (5'-ACTCCTACGGGAGGCAGCAGT-3') and UniR514 (5'-
185 ATTACCGCGGCTGCTGGC-3') at a final concentration of 0.4 µM.

186 The qPCR run consisted of a pre-incubation step of 50°C (ramp rate: 4.80°C/s for 2 minutes)
187 then 95°C (ramp rate: 4.80°C/s for 10 seconds) and an amplification step consisting of 45 cycles
188 of 95°C (ramp rate: 4.80°C/s for 30 seconds) then 63°C (ramp rate: 2.50°C/s for 30 seconds).
189 This was followed by a melting cycle consisting of 95°C (ramp rate: 4.80°C/s for 5 seconds) then

190 65°C (ramp rate: 4.80°C/s for 1 minute) followed by 97°C (ramp rate: 0.11°C/s, acquisition
191 mode, continuous).

192 Negative controls consisted of both water and extraction kit reagent controls. For water controls,
193 1 µl of nuclease free water was added to the qPCR reaction mixture. For extraction kit controls,
194 DNA extractions were carried out using the MOBIO PowerSoil® DNA Isolation Kit (Carlsbad,
195 CA, USA) following the same protocol as was used to extract DNA from samples, except no
196 sample was added meaning that any bacterial DNA in the final elution must have been derived
197 from the extraction kit reagents. 1 µl of this elution was added to the qPCR reaction mixture.

198 In order for us to compare the quantity of bacterial DNA found in bronchoscope wash and
199 brushing samples it was necessary to use a unit of measurement which could be applied to both
200 sample types. Bacterial DNA concentrations are therefore reported as the 16S copy numbers
201 present per µl of eluent produced from samples by the MOBIO PowerSoil® DNA Isolation Kit.
202 Statistical analysis was carried out in Minitab® 16 for Windows. Where data was non-parametric
203 the Mann-Whitney U test was used to statistically compare groups.

204

205

206 **Results**

207 **Quality control and adequacy of sequencing**

208 After constructing DNA sequences from the forward and reverse reads generated by sequencing,
209 various quality control steps were performed to decrease the number of artefacts and poor quality
210 sequences used in subsequent analyses.

211 For the Miseq runs, these steps resulted in a 15% loss of sequences (sequencing error rate =
212 0.39%). On average, samples contained 205625 ± 27232 (mean \pm standard error of the mean
213 (SEM)) sequences and a total of 925 bacterial OTUs were identified (**Dataset S1**). Sequences
214 were assigned to OTUs based on their taxonomic classifications. Each OTU does not necessarily
215 represent an individual bacterial species but instead represents the lowest taxonomic level to
216 which its bacterial sequences could be assigned. For example, 77.4% of reads could be identified
217 to genus while 31.1% could be assigned to species. If two species from the same genus could
218 only be assigned to genus level then they would both be binned into the same OTU.

219 For the Hiseq run, samples contained on average 233505 ± 69735 (mean \pm SEM) and the
220 sequencing error rate was 0.39%. 633 OTUs were identified (**Dataset S2**) and the total reduction
221 in sequence numbers due to quality control was 5%.

222 Good's coverage estimate values exceeded 97% for all samples. This indicates that at least 97%
223 of the bacteria present in our original samples are likely to have been identified, demonstrating
224 that the depth of sequencing was adequate.

225 Of the twenty bacteria contained in the mock community, all could be taxonomically identified
226 down to genus level except *Bacillus cereus*, *Escherichia coli* and *Listeria monocytogenes* which
227 could only be identified at family level. This indicates that the primers were able to amplify a
228 wide diversity of bacteria. Whilst the proportions of bacterial DNA were different to the
229 proportions anticipated if no PCR bias was present (**Table 2**) this was less apparent in the sample
230 which had been diluted 1:100 after the first round of PCR. In the undiluted mock the proportions
231 of bacterial orders differed from the expected proportions by an average of 9.48% (SEM: 2.24%,
232 range: 0.99% - 19.48%) whereas the orders in the diluted mock differed on average by 4.33%
233 (SEM: 1.12%, range: 0.29% - 12.71%). This diluted mock community may be more comparable

234 to the kind of biases we would find in our samples as the undiluted mock community contained a
235 far higher concentration of template DNA (2000000 16S copies per μl) than our samples did on
236 average (13133 16S copies per μl).

237 The assumption was made that PCR bias would reasonably be expected to apply equally across
238 all samples and therefore any statistical tests between samples should still be valid. The two
239 bacterial species most overrepresented in the undiluted mock community (*Deinococcus*
240 *radiodurans* and *Helicobacter pylori*) are not commonly associated with the respiratory tract and
241 bacteria from these genera were very rare within our dataset.

242

243 **Longitudinal study in six sheep over three months**

244 To examine the spatial, longitudinal and inter-individual variation of the sheep lung microbiota,
245 lung brushings were taken from three spatially disparate lung locations (RA, RCD and LCD) in
246 six sheep at three time-points (baseline, one month and three months). Estimates of total bacterial
247 yield from qPCR analysis indicated that sheep lung brushings contained an average of $13133 \pm$
248 894 (mean \pm SEM) 16S copy numbers/ μl (range: 1032-37627 16S copy numbers/ μl).

249 Bronchoscope wash controls contained significantly lower bacterial 16S rDNA concentrations
250 than lung brushings (Mann-Whitney U test: $p < 0.0001$), containing an average of 1471 ± 279
251 (mean \pm SEM) 16S copy numbers/ μl (range: 397-4792 16S copy numbers/ μl) (**Fig 3**). The qPCR
252 negative water controls were found to contain 190, 479 and 739 16S copy numbers/ μl and the
253 extraction kit controls were found to contain 347 and 511 16S copy numbers/ μl .

254 After sequencing and sub-sampling, bacterial communities isolated from extraction kit and 16S
255 PCR negative controls were found to cluster separately from those found in sheep lung brushings

(AMOVA: $p < 0.001$). Extraction kit controls were included from two different lots. The most abundant OTUs found in the first extraction kit control were *Corynebacterium* (36%), Enterobacteriaceae (13%), *Mycobacterium llatzerens* (7%) and *Staphylococcus haemolyticus* (5%). The most predominant OTUs in the second extraction kit control were *Aerococcus* (13%), Dermabacteraceae (11%), *Micrococcus* (10%), *Enhydrobacter* (9%) and *Leuconostoc* (7.2%). The predominant bacterial order present in both extraction kit controls was Actinomycetales (50.1% and 40.5% respectively).

The bacteria isolated from lung brushings predominantly belonged to the orders Bacillales (26%), Actinomycetales (21%), Clostridiales (11%) and Lactobacillales (9%) while common genera included *Staphylococcus* (16%), *Corynebacterium* (9%), *Jeotgalicoccus* (5%) and *Streptococcus* (5%).

The underlying changes in bacterial OTUs between sampling points were examined. The bacterial communities found in lung brushings clustered significantly by time-point (AMOVA: $P < 0.001$) (**Fig 4**). The OTUs causing this clustering were identified by applying Metastats (**Tables S3 and S4**). The largest difference observed between the first and second time-points was an 11% increase in the abundance of an OTU identified as *Corynebacterium*. This is also the most abundant OTU in one of our extraction kit controls. OTU 12: *Mycobacterium llatzerense* was also significantly more abundant at the one month time-point and was the third most abundant OTU in the same extraction kit control. It is therefore likely that our time-points were affected to different degrees by reagent contamination and therefore the analysis of segments over time is not possible. However, all samples taken in the same sheep at the same time-point were processed using the same extraction kit; therefore, an analysis of spatial variability can be performed.

279 Visual perceptions of community structure indicated that in some sheep, samples taken from
280 separate lung sites differed appreciably, whereas in other sheep there appeared relative
281 concordance between such samples (example shown in **Fig 5**). A full visual summary of the
282 results can be found in **Fig S2**. There were no significant differences between the diversity of
283 communities located at different lung sites (Inverse Simpson's index: Friedman test: $P > 0.5$).

284 Sheep clustered separately by the composition of their lung bacterial communities at the baseline
285 time-point (AMOVA: $P = 0.001$), and at the three month time-point (AMOVA: $P = 0.045$),
286 indicating that samples taken from within the same sheep were more similar to one another than
287 to samples taken from other sheep. At the one month time-point, sheep did not cluster in this
288 manner (AMOVA: $P = 0.394$), though this is likely due to the presence of contamination causing
289 a homogenisation of our one month samples. Pairwise comparisons of samples showed no
290 significant results. The similarity of samples to one another can be visualised using PCOA
291 graphs (**Fig 6**).

292

293

294 **Spatial variability of the lung microbiota in an individual sheep**

295 The observed variability between spatially disparate lung sites in some sheep prompted enquiry
296 as to the consistency of bacterial communities sampled from sites in close spatial apposition.

297 Further samples were derived by systematically sampling multiple sites of the lungs of an
298 individual animal at one time-point. Whilst the three month experiment did not include a control
299 for every lot of extraction kit used, emerging literature and opinion within the field has since

300 indicated the value of using the same extraction kit for all samples. This strategy was therefore
301 adopted for these latter samples which were all processed at the same time.

302 The extraction kit control was mainly composed of one OTU (OTU 18: 79%) which was also
303 present in our brushing samples (mean \pm SEM: 51.1 ± 3.3). We felt confident in removing this
304 OTU from all of our samples prior to analysis as it could be identified to species level
305 (*Methylobacterium komagatae*) and was considered highly unlikely to be found within the sheep
306 lung. No further OTUs were removed before analysis.

307 Lung brushings contained on average 2116 16S copy numbers per ul (SEM = 365) while the
308 throat swab and extraction kit control contained 42480 and 43 16S copy numbers per ul
309 respectively. The richness and diversity of the lung samples (Chao = 103.77 ± 7.32 , Inverse
310 Simpson's index = 14.24 ± 2.14) was found to be far lower than in the throat swab (Chao =
311 257.038, Inverse Simpson's index = 9.19). Sample A1, taken from the ventral aspect of the
312 trachea just caudal to the bifurcation with the right apical lobe segmental bronchus, had the
313 second highest richness (Chao = 155.024) and diversity (Inverse Simpson's index = 8.713).
314 However, sample A2, which was taken at the same level as sample A1 but from the dorsal aspect
315 of the trachea, had a much lower richness (Chao = 76.038) and diversity (Inverse Simpson's
316 index = 4.925).

317 The composition of the communities taken from the respiratory tract showed some variation,
318 even between paired samples located very closely to one another (**Fig 7**). Sub-tracheal samples
319 paired to their most proximate neighbour did not cluster together significantly when OTUs were
320 defined at the lowest taxonomic depth (AMOVA: $P = 0.30$). However, paired samples did cluster
321 significantly by the bacterial orders which they contained (AMOVA: $P = 0.046$). Sub-tracheal
322 samples also clustered significantly (by order) based upon the depth in the lung from which

323 samples were taken (AMOVA: $P = 0.033$) (**Fig 8**) (lung depth in this context refers to increasing
324 distance from the glottis progressing in a caudal direction). An indicator OTU for the group
325 which included the samples A4, A5, A14 and A15 was found to be OTU 4: Pseudomonadales
326 ($P = 0.042$). The most abundant bacterial orders identified from brushings were Clostridiales
327 (25.8%), Pseudomonadales (18.3%) and Actinomycetales (16.0%) while the throat swab was
328 dominated by Pasteurellales (36.5%) and Pseudomonadales (15.1%). The extraction kit control
329 was predominantly composed of Actinomycetales (31.1%) and Pseudomonadales (31.0%).

330 As the Pasteurellales order contains several species which are known to act as sheep lung
331 pathogens and which display regional patterns of infection we felt it would be interesting to
332 investigate where OTUs belonging to this order were found within the respiratory tract (**Table**
333 **3**). By far the largest proportion of these OTUs was found in the throat swab and one of the
334 tracheal brushings (A1).

335

336

337 **Discussion**

338 In order to better understand the variability present in the sheep lung microbiota, we compared
339 the lung bacterial communities of six sheep at three different lung sites over a duration of three
340 months. To further explore the extent of spatial variability, we also took 17 samples from the
341 respiratory tract of one sheep.

342 Previously, the bacteria in healthy domestic sheep lungs had been investigated by culture-based
343 methods which seemed to indicate that bacterial colonisation of the sheep lung was rare or did
344 not occur in all sheep (11, 12, 14). In contrast, using non-culture based methods we have found

345 that all of the sampled sites in our seven sheep harboured diverse communities of bacteria,
346 although in far smaller numbers than is generally found in other niches such as the gut or upper
347 respiratory tract.

348 Bacteria belonging to genera previously isolated from goat and sheep lungs (11, 12) were found
349 in our samples. These included *Corynebacterium*, *Bacillus*, *Enterococcus*, *Klebsiella*,
350 *Mannheimia*, *Micrococcus*, *Moraxella*, *Pasteurella*, *Pseudomonas*, *Staphylococcus* and
351 *Streptococcus*. Of the most common genera observed within our animals *Staphylococcus*,
352 *Streptococcus* and *Corynebacterium* are commonly isolated from the upper respiratory tract and
353 skin of many animals whereas *Jeotgalicoccus* is a less well known genus (45) which has not
354 been found to make up a substantial part of the lung microbiota communities in any previous
355 studies. However, it has been isolated from the small intestinal mucosa of calves (46), the canine
356 oral cavity (47), aerosols in a poultry house (48, 49), cattle teats (50), lamb meat (51), the rumen
357 of cattle (52) and from aerosol samples near a dairy (53).

358 The most common bacterial orders found in the sheep lung during the three month study were
359 Bacillales, Actinomycetales and Clostridiales. This agrees with the findings of a previous study
360 carried out by our group which examined the sheep lung microbiota before and after infection
361 with *P.aeruginosa* (17). Pseudomonadales (mainly *Pseudomonas*) was also commonly found in
362 the lungs during our single sheep study while the throat swab from this study was dominated by
363 Pasteurellales and Pseudomonadales.

364 Co-infection with *Bordetella parapertussis* or *Mycoplasma ovipneumoniae* has been shown to
365 lead to more severe disease caused by *Mannheimia (Pasteurella) haemolytica* (22-25).
366 Mycoplasmas were very rare within our dataset with only one sheep segment containing reads
367 from this genus at one time-point. We did not identify any OTUs as *Bordetella*; however, we did

368 find an OTU designated as Alcaligenaceae (the family to which *Bordetella* belongs) though these
369 were uncommon and occurred in low abundance. We identified several OTUs which were
370 classified as members of the Pasteurellaceae family including *Mannheimia* and *Bibersteinia* and
371 less commonly: *Aggregatibacter segnis*, *Haemophilus parainfluenzae*, *Bibersteinia trehalosi* and
372 *Actinobacillus paraaemolyticus*. All of these microbes have previously been isolated from the
373 lungs or upper respiratory tract (54-58). Despite the fact that disease by members of this family is
374 often located in the apical and cardiac lobes (12, 19), we observed members of this family to be
375 present across the lung.

376 The composition of the lung microbiota found in our sheep shows some differences to that
377 previously identified in humans where Bacteroidales are found in higher numbers and there are
378 generally less members of the Actinomycetales and Clostridiales (2, 9, 59). Segal *et al.* identified
379 various bacterial taxa that were commonly found in high relative abundance in human lungs (1).
380 These included taxa which were found in all of our sheep samples in high relative abundance
381 (*Streptococcus*, *Staphylococcus*, *Corynebacterium*); taxa which were found in the majority of our
382 samples but in lower abundances (*Propionibacterium*, *Pseudomonas*) and taxa which were only
383 found sporadically in our samples and were usually in low abundance (*Stenotrophomonas*,
384 *Prevotella*, *Veillonella*, *Fusobacterium*, *Porphyromonas*).

385 Such differences may at least in part reflect the different surroundings in which sheep live as
386 well as behavioural or physiological features such as rumination. A study using buccal swabs to
387 identify bacteria originating from the rumen suggested that as the time between regurgitation and
388 sampling increases the orally associated bacterial populations in the buccal cavity will increase
389 and the rumen associated bacteria will decrease, potentially contributing to inter-animal variation

(58). In future studies it may be useful to take rumen and upper respiratory tract samples alongside lung samples to explore if the variability between these sites and the lung is related. Regardless of the highlighted differences between sheep and the human lung microbiota, there is a pressing need to understand the mechanisms that underlay the spatial and temporal variability of microbiota in the mammalian lung. These fundamental studies are difficult to facilitate in healthy human subjects as a consequence of the invasive nature of the repeated sampling protocol, as well as controlling for the influence of environmental and/or lifestyle factors. Large animal models can however play an important role in filling this need. Indeed, the physiological and immunological similarities between sheep and human lungs (60, 61) have contributed to the widespread use of sheep as translational models for human lung research (26, 27) including asthma (62-65), the delivery of drugs via the upper respiratory tract (66-68), emphysema (69-71), pulmonary hypertension (72-74), physical lung injury (75-78), lung infection (28, 79-81), respiratory distress syndromes (82-85), asbestosis (86-88) and lung cancer (89, 90).

In our study we examined the variability of the lung microbiota in sheep. Bacterial populations were often different between lung segments and between individuals, which confirms our previous observations (17). There was more similarity between samples from the same sheep at the baseline and three month time-points than between samples taken from different sheep but this was not found to be the case at the one month time-point. Lung sample clustering by individuals has previously been identified in humans (9) and sheep (17).

Clearly, large differences can exist in the microbiota sampled from different lung segments at the same time-point. This spatial variability of lung microbial populations can be observed in *P.aeruginosa* infections in cystic fibrosis patient lungs (91). The mechanisms underlying such observations have yet to be elucidated; however, possible candidate influences may include

413 regional variability of physiological parameters such as gas concentrations, osmolality,
414 temperature, pH and blood flow (92-96) which may lead to the creation of ‘microhabitats’
415 providing a selective advantage to certain bacteria (97). It has previously been demonstrated that
416 differences in pH can lead to changes in the colonic microbiota (98) and that temperature
417 combined with humidity can lead to changes in the composition of the skin microbiota (99).

418 A longitudinal analysis of the lung microbiota at specific lung sites in healthy individuals has not
419 previously been reported. Our goal was to define the variability of the lung microbiota over time
420 and to detect whether there was a sheep lung microbiota ‘signature’ which remains stable.
421 Unfortunately, at the time of carrying out this study the extent of the variability of bacterial DNA
422 found within different lots of extraction kits was not yet known (100). While we therefore did
423 include some extraction kit controls for our longitudinal study we did not include controls for all
424 lots which were used. Samples from different time-points were also processed at different times.
425 Due to our small sample sizes and the fact that samples clustered significantly by time-point, we
426 do not feel that accurate conclusions can be drawn about the temporal stability of the microbiota
427 from our data. However, all samples taken from the same time-point in the same sheep were
428 processed at the same time. Therefore, we can be confident that the spatial variability we observe
429 within animals is not due to our methodology.

430 In some individuals, samples taken from different lung segments were found to be highly
431 different from one another whereas in others the lung microbiota appeared to be quite stable
432 across the lung. Another finding was the disappearance of the significantly separate clustering of
433 sheep samples at the one month time-point. This was correlated with an increase in the
434 proportions of several OTUs found in sheep lungs, the most noticeable increase arising from an
435 OTU classified as *Corynebacterium* which was also the most abundant OTU in one of our

436 extraction kit controls. It is likely that the disappearance of significant clustering by individual at
437 the one month time-point is due to the increased presence of contamination in our samples.

438 OTUs that were identified in both samples and negative PCR and extraction kit controls were not
439 removed from the analysis for the three month sheep study. The reason for this decision was that
440 a number of bacteria commonly associated with the upper and lower respiratory tract were
441 present in these controls, including the genera *Streptococcus* and *Pseudomonas*, and it was
442 judged that their removal would merely introduce another source of bias.

443 Equally, any specific *a priori* manipulation based around assumptions gleaned from the human
444 literature regarding microbiota in the upper and lower respiratory tract, are potentially ill advised.

445 Indeed, it has been demonstrated that the microbes found in the lungs of animals often match
446 those found in their bedding and hay (101). It is therefore not possible to dismiss environmental
447 microorganisms as being only due to the contamination of samples.

448 In our spatial variation study, one OTU was removed before analysis as we felt confident that its
449 presence was due to contamination of our extraction kit. Clustering of lung brushings by the lung
450 depth from which they were taken was observed when OTUs were defined by bacterial order.

451 Samples paired with their proximate neighbour were also found to cluster significantly separately
452 from brushings taken elsewhere in the lung but this may just be due to the fact that these samples
453 were taken from the same lung depth. Certainly further research to explore the relationship
454 between lung depth and community composition appears warranted.

455 After sequencing a mock community of bacteria which contained equimolar concentrations of
456 each bacterial species we did find some bias present, with some bacterial species being over or
457 underrepresented. These biases, which may be caused by various factors including primer

458 mismatching, PCR cycle number and the bioinformatic pipeline used, are quite common in 16S
459 sequencing (102-105). We also sequenced a 1:100 dilution of the same mock community and
460 found that the apparent biases were far less. As the concentration of bacterial DNA in our
461 samples was far lower than that of the undiluted mock community, we feel that the 1:100 dilution
462 is likely to better represent the biases which may be present in our samples as it is closer to their
463 bacterial DNA concentrations. We believe that this vindicates our choice of DNA amplification
464 strategy, including the use of nested PCR.

465 It may not be possible to claim that the bacterial abundances identified via 16S sequencing
466 quantitatively represent the relative abundances of bacteria in the sample. Indeed, this is made
467 even more difficult as different bacterial taxa contain different copy numbers of the 16S gene
468 (106). However, it seems logical to assume that if the same methodology is used for all samples
469 within a study then the biases present will be the same for all samples and therefore comparisons
470 between groups or claims about the types of microbes present in samples would still be valid.

471 In conclusion, we observed variability in the sheep lung microbiota both between and within
472 individuals. In some animals different lung segments contained highly different bacterial
473 communities whereas other animals showed similar communities at all lung sites. While spatial
474 variation was observed to occur over both large and small distances across the lung, samples
475 taken at the same lung depth clustered together separately from those taken at different lung
476 depths. Further studies are needed to explore the stability of the healthy lung microbiota over
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496 **References**

- 497 1. **Segal L, Alekseyenko A, Clemente J, Kulkarni R, Wu B, Chen H, Berger K, Goldring R, Rom**
498 **W, Blaser M, Weiden M.** 2013. Enrichment of lung microbiome with supraglottic taxa is
499 associated with increased pulmonary inflammation. *Microbiome* **1**:19.

- 500 2. **Charlson ES, Bittinger K, Haas AR, Fitzgerald AS, Frank I, Yadav A, Bushman FD, Collman**
501 **RG.** 2011. Topographical continuity of bacterial populations in the healthy human
502 respiratory tract. *Am J Respir Crit Care Med* **184**:957-963.
- 503 3. **Morris A, Beck JM, Schloss PD, Campbell TB, Crothers K, Curtis JL, Flores SC, Fontenot AP,**
504 **Ghedini E, Huang L, Jablonski K, Kleerup E, Lynch SV, Sodergren E, Twigg H, Young VB,**
505 **Bassis CM, Venkataraman A, Schmidt TM, Weinstock GM.** 2013. Comparison of the
506 respiratory microbiome in healthy nonsmokers and smokers. *Am J Respir Crit Care Med*
507 **187**:1067-1075.
- 508 4. **Charlson E, Bittinger K, Chen J, Diamond J, Li H, Collman R, Bushman F.** 2012. Assessing
509 bacterial populations in the lung by replicate analysis of samples from the upper and lower
510 respiratory tracts. *PLoS ONE* **7**:e42786. DOI: 10.1371/journal.pone.0042786
- 511 5. **Delhaes L, Monchy S, Fréalle E, Hubans C, Salleron J, Leroy S, Prevotat A, Wallet F, Wallaert**
512 **B, Dei-Cas E, Sime-Ngando T, Chabé M, Viscogliosi E.** 2012. The airway microbiota in cystic
513 fibrosis: A complex fungal and bacterial community—implications for therapeutic
514 management. *PLoS ONE* **7**:e36313. DOI: 10.1371/journal.pone.0036313
- 515 6. **Erb-Downward JR, Thompson DL, Han MK, Freeman CM, McCloskey L, Schmidt LA, Young**
516 **VB, Toews GB, Curtis JL, Sundaram B, Martinez FJ, Huffnagle GB.** 2011. Analysis of the lung
517 microbiome in the “healthy” smoker and in COPD. *PLoS ONE* **6**:e16384. DOI:
518 10.1371/journal.pone.0016384
- 519 7. **Boyton RJ, Reynolds CJ, Quigley KJ, Altmann DM.** 2013. Immune mechanisms and the
520 impact of the disrupted lung microbiome in chronic bacterial lung infection and
521 bronchiectasis. *Clin Exp Immunol* **171**:117-123.
- 522 8. **Borewicz K, Pragman AA, Kim HB, Hertz M, Wendt C, Isaacson RE.** 2013. Longitudinal
523 analysis of the lung microbiome in lung transplantation. *FEMS Microbiol Lett* **339**:57-65.

- 524 9. **Dickson RP, Erb-Downward JR, Freeman CM, McCloskey L, Beck JM, Huffnagle GB, Curtis**
525 **JL.** 2015. Spatial variation in the healthy human lung microbiome and the adapted island
526 model of lung biogeography. *Ann Am Thorac Soc* **12**:821-830.
- 527 10. **Dickson R, Erb-Downward J, Prescott H, Martinez F, Curtis J, Lama V, Huffnagle G.** 2014.
528 Cell-associated bacteria in the human lung microbiome. *Microbiome* **2**:28.
- 529 11. **Yimer N, Asseged B.** 2007. Aerobic bacterial flora of the respiratory tract of healthy sheep
530 slaughtered in Dessie municipal abattoir, northeastern Ethiopia. *Rev Med Vet Toulouse*
531 **158**:473-478.
- 532 12. **Alley MR.** 1975. The bacterial flora of the respiratory tract of normal and pneumonic sheep.
533 *New Zeal Vet J* **23**:113-118.
- 534 13. **Alley MR, Quinlan JR, Clarke JK.** 1975. The prevalence of *Mycoplasma ovipneumoniae* and
535 *Mycoplasma arginini* in the respiratory tract of sheep. *New Zeal Vet J* **23**:137-141.
- 536 14. **Barbour EK, Nabbut NH, Hamadeh SK, Al-Nakhli HM.** 1997. Bacterial identity and
537 characteristics in healthy and unhealthy respiratory tracts of sheep and calves. *Vet Res*
538 *Commun* **21**:421-430.
- 539 15. **Besser TE, Cassirer EF, Potter KA, VanderSchalie J, Fischer A, Knowles DP, Herndon DR,**
540 **Rurangirwa FR, Weiser GC, Srikumaran S.** 2008. Association of *Mycoplasma ovipneumoniae*
541 infection with population-limiting respiratory disease in free-ranging Rocky Mountain
542 Bighorn Sheep (*Ovis canadensis canadensis*). *J Clin Microbiol* **46**:423-430.
- 543 16. **Queen C, Ward ACS, Hunter DL.** 1994. Bacteria isolated from nasal and tonsillar samples of
544 clinically healthy Rocky Mountain bighorn and domestic sheep. *J Wildl Dis* **30**:1-7.
- 545 17. **Collie D, Glendinning L, Govan J, Wright S, Thornton E, Tennant P, Doherty C, McLachlan G.**
546 2015. Lung microbiota changes associated with chronic *Pseudomonas aeruginosa* lung
547 infection and the impact of intravenous colistimethate. *PLoS ONE* **10**:e0142097. DOI:
548 10.1371/journal.pone.0142097

- 549 18. **Dickson RP, Erb-Downward JR, Huffnagle GB.** 2013. The role of the bacterial microbiome in
550 lung disease. *Expert Rev Respir Med* **7**:245-257.
- 551 19. **Davies DH, Dungworth DL, Humphreys S, Johnson AJ.** 1977. Concurrent infection of lambs
552 with parainfluenza virus type 3 and *Pasteurella-haemolytica*. *New Zeal Vet J* **25**:263-265.
553 10.1080/00480169.1977.34425
- 554 20. **Catt DM, Chengappa MM, Kadel WL, Herren CE.** 1985. Preliminary studies with a live
555 streptomycin dependant *Pasteurella-multocida* and *Pasteurella-haemolytica* vaccine for the
556 prevention of bovine pneumonic pasteurellosis. *Can J Vet Res* **49**:366-371.
- 557 21. **Stockdale PHG, Langford EV, Darcel CLQ.** 1979. Experimental bovine pneumonic
558 pasteurellosis.1. Prevention of the disease. *Can J Comp Med* **43**:262-271.
- 559 22. **Brogden KA, Lehmkuhl HD, Cutlip RC.** 1998. *Pasteurella haemolytica* complicated
560 respiratory infections in sheep and goats. *Vet Res* **29**:233-254.
- 561 23. **Dassanayake RP, Call DR, Sawant AA, Casavant NC, Weiser GC, Knowles DP, Srikumaran S.**
562 2010. *Bibersteinia trehalosi* inhibits the growth of *Mannheimia haemolytica* by a proximity-
563 dependent mechanism. *Appl Environ Microbiol* **76**:1008-1013.
- 564 24. **Dassanayake RP, Shanthalingam S, Herndon CN, Subramaniam R, Lawrence PK,**
565 **Bavananthasivam J, Cassirer EF, Haldorson GJ, Foreyt WJ, Rurangirwa FR, Knowles DP,**
566 **Besser TE, Srikumaran S.** 2010. *Mycoplasma ovipneumoniae* can predispose bighorn sheep
567 to fatal *Mannheimia haemolytica* pneumonia. *Vet Microbiol* **145**:354-359.
- 568 25. **Porter JF, Connor NK, Krueger N, Hodgson JC, Donachie W.** 1995. Predisposition of specific
569 pathogen-free lambs to *Pasteurella haemolytica* pneumonia by *Bordetella parapertussis*
570 infection. *J Comp Pathol* **112**:381-389.
- 571 26. **Scheerlinck JPY, Snibson KJ, Bowles VM, Sutton P.** 2008. Biomedical applications of sheep
572 models: From asthma to vaccines. *Trends Biotechnol* **26**:259-266.

- 573 27. **Meeusen EN, Snibson KJ, Hirst SJ, Bischof RJ.** 2009. Sheep as a model species for the study
574 and treatment of human asthma and other respiratory diseases. *Drug Discov Today Dis*
575 *Models* **6**:101-106.
- 576 28. **Collie D, Govan J, Wright S, Thornton E, Tennant P, Smith S, Doherty C, McLachlan G.** 2013.
577 A lung segmental model of chronic *Pseudomonas* infection in sheep. *PLoS ONE* **8**:e67677.
578 DOI: 10.1371/journal.pone.0067677
- 579 29. **Berry D, Ben Mahfoudh K, Wagner M, Loy A.** 2011. Barcoded primers used in multiplex
580 amplicon pyrosequencing bias amplification. *Appl Environ Microbiol* **77**:7846-7849.
- 581 30. **Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD.** 2013. Development of a dual-
582 index sequencing strategy and curation pipeline for analyzing amplicon sequence data on
583 the MiSeq illumina sequencing platform. *Appl Environ Microbiol* **79**:5112-5120.
- 584 31. **Martin M.** 2011. Cutadapt removes adapter sequences from high-throughput sequencing
585 reads. *Embnetwork* **17**:10-12.
- 586 32. **Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA,**
587 **Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF.**
588 2009. Introducing mothur: Open-source, platform-independent, community-supported
589 software for describing and comparing microbial communities. *Appl Environ Microbiol*
590 **75**:7537-7541.
- 591 33. **Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R.** 2011. UCHIME improves sensitivity and
592 speed of chimera detection. *BMC Bioinform* **27**:2194-2200.
- 593 34. **Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glockner FO.** 2013.
594 The SILVA ribosomal RNA gene database project: improved data processing and web-based
595 tools. *Nucleic Acids Res* **41**:D590-D596. DOI: 10.1093/nar/gks1219
- 596 35. **DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P,**
597 **Andersen GL.** 2006. Greengenes, a chimera-checked 16S rRNA gene database and
598 workbench compatible with ARB. *Appl Environ Microbiol* **72**:5069-5072.

- 599 36. **Werner JJ, Koren O, Hugenholtz P, DeSantis TZ, Walters WA, Caporaso JG, Angenent LT,**
600 **Knight R, Ley RE.** 2012. Impact of training sets on classification of high-throughput bacterial
601 16s rRNA gene surveys. *ISME J* **6**:94-103.
- 602 37. **Yue JC, Clayton MK.** 2005. A similarity measure based on species proportions. *Commun Stat-*
603 *Theory Methods* **34**:2123-2131.
- 604 38. **Excoffier L, Smouse PE, Quattro JM.** 1992. Analysis of molecular variance inferred from
605 metric distances among DNA haplotypes: Application to human mitochondrial DNA
606 restriction data. *Genetics* **131**:479-491.
- 607 39. **Paulson J, Pop M, Bravo H.** 2011. Metastats: an improved statistical method for analysis of
608 metagenomic data. *Genome Biol* **12 (Supplement 1)**:27.
- 609 40. **Esty WW.** 1986. The efficiency of Good's nonparametric coverage estimator. *Ann Stat*
610 **14**:1257-1260.
- 611 41. **Dufrêne M, Legendre P.** 1997. Species assemblages and indicator species: The need for a
612 flexible asymmetrical approach. *Ecol Monogr* **67**:345-366.
- 613 42. **Efron B, Tibshirani RJ.** 1993. An introduction to the bootstrap, monographs on statistics and
614 applied probability, Vol. 57. Taylor and Francis, Abingdon, UK.
- 615 43. **Dixon P.** 2003. VEGAN, a package of R functions for community ecology. *J Veg Sci* **14**:927-
616 930.
- 617 44. **Anderson M, Gorley RN, Clarke RK.** 2008. Permanova+ for primer: Guide to software and
618 statistical methods. PRIMER-E, Plymouth, UK.
- 619 45. **Schwaiger K, Hölzel C, Mayer M, Bauer J.** 2010. Notes on the almost unknown genus
620 *Jeotgalicoccus*. *Lett Appl Microbiol* **50**:441-444.
- 621 46. **Malmuthuge N, Griebel PJ, Guan LL.** 2014. Taxonomic identification of commensal bacteria
622 associated with the mucosa and digesta throughout the gastrointestinal tracts of preweaned
623 calves. *Appl Environ Microbiol* **80**:2021-2028.

- 624 47. Dewhirst FE, Klein EA, Thompson EC, Blanton JM, Chen T, Milella L, Buckley CMF, Davis IJ,
625 Bennett M-L, Marshall-Jones ZV. 2012. The canine oral microbiome. PLoS ONE 7:e36067.
626 DOI: 10.1371/journal.pone.0036067
- 627 48. Fallschissel K, Klug K, Kämpfer P, Jäckel U. 2010. Detection of airborne bacteria in a German
628 turkey house by cultivation-based and molecular methods. Ann Occup Hyg 54:934-943.
- 629 49. Martin E, Fallschissel K, Kämpfer P, Jäckel U. 2010. Detection of *Jeotgalicoccus* spp. in
630 poultry house air. Syst Appl Microbiol 33:188-192.
- 631 50. Braem G, De Vliegher S, Verbist B, Piessens V, Van Coillie E, De Vuyst L, Leroy F. 2013.
632 Unraveling the microbiota of teat apices of clinically healthy lactating dairy cows, with
633 special emphasis on coagulase-negative staphylococci. J Dairy Res 96:1499-1510.
- 634 51. Osés SM, Diez AM, Melero B, Luning PA, Jaime I, Rovira J. 2013. Characterization by
635 culture-dependent and culture-independent methods of the bacterial population of
636 suckling-lamb packaged in different atmospheres. Food Microbiol 36:216-222.
- 637 52. Leng J, Cheng YM, Zhang CY, Zhu RJ, Yang SL, Gou X, Deng WD, Mao HM. 2012. Molecular
638 diversity of bacteria in Yunnan yellow cattle (*Bos taurus*) from Nujiang region, China. Mol Biol
639 Rep 39:1181-1192.
- 640 53. Dungan RS. 2012. Use of a culture-independent approach to characterize aerosolized
641 bacteria near an open-freestall dairy operation. Environ Int 41:8-14.
- 642 54. Lawrence PK, Shanthalingam S, Dassanayake RP, Subramaniam R, Herndon CN, Knowles
643 DP, Rurangirwa FR, Foreyt WJ, Wayman G, Marciel AM, Highlander SK, Srikumaran S. 2010.
644 Transmission of *Mannheimia haemolytica* from domestic sheep (*Ovis aries*) to bighorn sheep
645 (*Ovis canadensis*): Unequivocal demonstration with green fluorescent protein-tagged
646 organisms. J Wildl Dis 46:706-717.
- 647 55. Blackall PJ, Bojesen AM, Christensen H, Bisgaard M. 2007. Reclassification of [*Pasteurella*]
648 *trehalosi* as *Bibersteinia trehalosi* gen. nov., comb. nov. Int J Syst Evol Microbiol 57:666-674.

- 649 56. **Norskov-Lauritsen N, Kilian M.** 2006. Reclassification of *Actinobacillus*
650 *actinomycetemcomitans*, *Haemophilus aphrophilus*, *Haemophilus paraphrophilus* and
651 *Haemophilus segnis* as *Aggregatibacter actinomycetemcomitans* gen. nov., comb. nov.,
652 *Aggregatibacter aphrophilus* comb. nov and *Aggregatibacter segnis* comb. nov., and
653 emended description of *Aggregatibacter aphrophilus* to include V factor-dependent and V
654 factor-independent isolates. *Int J Syst Evol Microbiol* **56**:2135-2146.
- 655 57. **Smith CB, Golden CA, Kanner RE, Renzetti AD.** 1976. *Haemophilus influenzae* and
656 *Haemophilus parainfluenzae* in chronic obstructive pulmonary disease. *Lancet* **1**:1253-1255.
- 657 58. **Kittelmann S, Kirk MR, Jonker A, McCulloch A, Janssen PH.** 2015. Buccal swabbing as a
658 noninvasive method to determine bacterial, archaeal, and eukaryotic microbial community
659 structures in the rumen. *Appl Environ Microbiol* **81**:7470-7483.
- 660 59. **Hilty M, Burke C, Pedro H, Cardenas P, Bush A, Bossley C, Davies J, Ervine A, Poulter L,**
661 **Pachter L, Moffatt MF, Cookson WOC.** 2010. Disordered microbial communities in asthmatic
662 airways. *PLoS ONE* **5**:e8578. DOI: 10.1371/journal.pone.0008578
- 663 60. **Bouljihad M, Leipold HW.** 1994. An ultrastructural study of pulmonary bronchiolar and
664 alveolar epithelium in sheep. *J Vet Med A* **41**:573-586.
- 665 61. **Griebel PJ, Entrican G, Rocchi M, Beskorwayne T, Davis WC.** 2007. Cross-reactivity of mAbs
666 to human CD antigens with sheep leukocytes. *Vet Immunol Immunopathol* **119**:115-122.
- 667 62. **Abraham W, Gill A, Ahmed A, Sielczak M, Lauredo I, Botinnikova Y, Lin K-C, Pepinsky B,**
668 **Leone D, Lobb R, Adams S.** 2000. A small-molecule, tight-binding Inhibitor of the integrin
669 $\alpha 4\beta 1$ blocks antigen-induced airway responses and inflammation in experimental asthma in
670 sheep. *Am J Respir Crit Care Med* **162**:603-611.
- 671 63. **Abraham WM, Sielczak MW, Wanner A, Perruchoud AP, Blinder L, Stevenson JS, Ahmed A,**
672 **Yerger LD.** 1988. Cellular markers of inflammation in the airways of allergic sheep with and
673 without allergen-induced late responses. *Am Rev Respir Dis* **138**:1565-1571.

- 674 64. **Snibson KJ, Bischof RJ, Slocombe RF, Meeusen EN.** 2005. Airway remodelling and
675 inflammation in sheep lungs after chronic airway challenge with house dust mite. *Clin Exp*
676 *Allergy* **35**:146-152.
- 677 65. **Liravi B, Piedrafita D, Nguyen G, Bischof R.** 2015. Dynamics of IL-4 and IL-13 expression in
678 the airways of sheep following allergen challenge. *BMC Pulm Med* **15**:101.
- 679 66. **Esther Jr CR, Boucher RC, Johnson MR, Ansele JH, Donn KH, O'Riordan TG, Ghio AJ, Hirsh**
680 **AJ.** 2014. Airway drug pharmacokinetics via analysis of exhaled breath condensate. *Pulm*
681 *Pharmacol Ther* **27**:76-82.
- 682 67. **Alton EFWF, Baker A, Baker E, Boyd AC, Cheng SH, Coles RL, Collie DDS, Davidson H,**
683 **Davies JC, Gill DR, Gordon C, Griesenbach U, Higgins T, Hyde SC, Innes JA, McCormick D,**
684 **McGovern M, McLachlan G, Porteous DJ, Pringle I, Scheule RK, Shaw DJ, Smith S, Sumner-**
685 **Jones SG, Tennant P, Vrettou C.** 2013. The safety profile of a cationic lipid-mediated cystic
686 fibrosis gene transfer agent following repeated monthly aerosol administration to sheep.
687 *Biomaterials* **34**:10267-10277.
- 688 68. **El Mays TY, Choudhury P, Leigh R, Koumoundouros E, Van der Velden J, Shrestha G, Pieron**
689 **CA, Dennis JH, Green FHY, Snibson KJ.** 2014. Nebulized perflubron and carbon dioxide
690 rapidly dilate constricted airways in an ovine model of allergic asthma. *Respir Res* **15**:98.
- 691 69. **Popp JD, McAllister TA, Kastelic JP, Majak W, Ayroud M, VanderKop MA, Karren D, Yost**
692 **GS, Cheng KJ.** 1998. Effect of melengestrol acetate on development of 3-methylindole-
693 induced pulmonary edema and emphysema in sheep. *Can J Vet Res-Rev Can Rech Vet*
694 **62**:268-274.
- 695 70. **Tsai L, Hoffman A, Berger R, Ingenito E.** 2004. Bronchoscopic lung volume reduction in a
696 sheep model of heterogeneous emphysema. *J Bronchology Interv Pulmonol* **11**:83-86.
- 697 71. **Ingenito EP, Tsai L, Murthy S, Tyagi S, Mazan M, Hoffman A.** 2012. Autologous lung-derived
698 mesenchymal stem cell transplantation in experimental emphysema. *Cell Transplant* **21**:175-
699 189.

- 700 72. **Pohlmann JR, Akay B, Camboni D, Koch KL, Mervak BM, Cook KE.** 2012. A low mortality
701 model of chronic pulmonary hypertension in sheep. *J Surg Res* **175**:44-48.
- 702 73. **McDonald JW, Ali M, Morgan E, Townsend ER, Cooper JD.** 1983. Thromboxane synthesis by
703 sources other than platelets in association with complement-induced pulmonary leukostasis
704 and pulmonary hypertension in sheep. *Circ Res* **52**:1-6.
- 705 74. **Weimann J, Ullrich R, Hromi J, Fujino Y, Clark MWH, Bloch KD, Zapol WM.** 2000. Sildenafil is
706 a pulmonary vasodilator in awake lambs with acute pulmonary hypertension.
707 *Anesthesiology* **92**:1702-1712.
- 708 75. **Julien M, Hoeffel JM, Flick MR.** 1986. Oleic-acid lung injury in sheep. *J Appl Physiol* **60**:433-
709 440.
- 710 76. **Enkhbaatar P, Murakami K, Shimoda K, Mizutani A, Traber L, Phillips GB, Parkinson JF, Cox
711 R, Hawkins H, Herndon D, Traber D.** 2003. The inducible nitric oxide synthase inhibitor BBS-
712 2 prevents acute lung injury in sheep after burn and smoke inhalation injury. *Am J Respir Crit
713 Care Med* **167**:1021-1026.
- 714 77. **Tsuno K, Prato P, Kolobow T.** 1990. Acute lung injury from mechanical ventilation at
715 moderately high airway pressures. *J Appl Physiol* **69**:956-961.
- 716 78. **Gibbons MM, Dang X, Adkins M, Powell B, Chan P.** 2015. Finite element modeling of blast
717 lung injury in sheep. *J Biomech Eng* **137**:041002. DOI: 10.1115/1.4029181
- 718 79. **Olivier A, Gallup J, De Macedo MMMA, Varga SM, Ackermann M.** 2009. Human respiratory
719 syncytial virus A2 strain replicates and induces innate immune responses by respiratory
720 epithelia of neonatal lambs. *Int J Exp Pathol* **90**:431-438.
- 721 80. **Morris DL, Clarkson MJ, Stallbaumer MF, Pritchard J, Jones RS, Chinnery JB.** 1985.
722 Albendazole treatment of pulmonary hydatid cysts in naturally infected sheep: a study with
723 relevance to the treatment of hydatid cysts in man. *Thorax* **40**:453-458.
- 724 81. **Enkhbaatar P, Joncam C, Traber L, Nakano Y, Wang J, Lange M, Connelly R, Kulp G,
725 Saunders F, Huda R, Cox R, Schmalstieg F, Herndon D, Traber D.** 2008. Novel ovine model of

- 726 methicillin-resistant *Staphylococcus aureus*-induced pneumonia and sepsis. Shock **29**:642-
727 649.
- 728 82. **Warner AE, Decamp MM, Molina RM, Brain JD.** 1988. Pulmonary removal of circulating
729 endotoxin results in acute lung injury in sheep. Lab Invest **59**:219-230.
- 730 83. **Kramer BW, Kramer S, Ikegami M, Jobe AH.** 2002. Injury, inflammation, and remodeling in
731 fetal sheep lung after intra-amniotic endotoxin. Am J Physiol Lung Cell Mol Physiol **283**:L452-
732 L459.
- 733 84. **Engel M, Nowacki RE, Boden P, Reiss L, Uhlig S, Reynaert N, Gopal P, Wouters EM, Willems
734 CMP, Kloosterboer N, Wolfs TAM, Zimmermann LI, Vos G, Kramer B.** 2015. The effects of
735 dexamethasone and oxygen in ventilated adult sheep with early phase acute respiratory
736 distress syndrome. Lung **193**:97-103.
- 737 85. **Langer T, Vecchi V, Belenkiy SM, Cannon JW, Chung KK, Cancio LC, Gattinoni L, Batchinsky
738 AI.** 2014. Extracorporeal gas exchange and spontaneous breathing for the treatment of
739 acute respiratory distress syndrome: An alternative to mechanical ventilation? Crit Care Med
740 **42**:E211-E220. DOI: 10.1097/ccm.0000000000000121
- 741 86. **Bégin R, Rola-Pleszczynski M, Massé S, Lemaire I, Sirois P, Boctor M, Nadeau D, Drapeau G,
742 Bureau MA.** 1983. Asbestos-induced lung injury in the sheep model: The initial alveolitis.
743 Environment International **30**:195-210. DOI: 10.1016/0013-9351(83)90180-9
- 744 87. **Lee TC, Gold LI, Reibman J, Aston C, Bégin R, Rom WN, Jagirdar J.** 1997.
745 Immunohistochemical localization of transforming growth factor- β and insulin-like growth
746 factor-I in asbestosis in the sheep model. Int Arch Occup Environ Health **69**:157-164.
- 747 88. **Sebastien P, Begin R, Masse S.** 1990. Mass, number and size of lung fibers in the
748 pathogenesis of asbestos in sheep. J Exp Pathol **71**:1-10.
- 749 89. **Palmarini M, Fan H, Sharp JM.** 1997. Sheep pulmonary adenomatosis: A unique model of
750 retrovirus associated lung cancer. Trends Microbiol **5**:478-483.

- 751 90. **Leroux C, Girard N, Cottin V, Greenland T, Mornex JF, Archer F.** 2007. Jaagsiekte sheep
752 retrovirus (JSRV): from virus to lung cancer in sheep. *Vet Res* **38**:211-228.
- 753 91. **Jorth P, Staudinger Benjamin J, Wu X, Hisert KB, Hayden H, Garudathri J, Harding**
754 **Christopher L, Radey Matthew C, Rezayat A, Bautista G, Berrington William R, Goddard**
755 **Amanda F, Zheng C, Angermeyer A, Brittnacher Mitchell J, Kitzman J, Shendure J, Fligner**
756 **Corinne L, Mittler J, Aitken Moira L, Manoil C, Bruce James E, Yahr Timothy L, Singh**
757 **Pradeep K.** 2015. Regional isolation drives bacterial diversification within cystic fibrosis
758 lungs. *Cell Host Microbe* **18**:307-319.
- 759 92. **Ingenito EP, Solway J, McFadden ER, Pichurko B, Bowman HF, Michaels D, Drazen JM.**
760 1987. Indirect assessment of mucosal surface temperatures in the airways - theory and tests.
761 *J Appl Physiol* **63**:2075-2083.
- 762 93. **Martin CJ, Cline F, Marshall H.** 1953. Lobar alveolar gas concentrations - effect of body
763 position. *J Clin Invest* **32**:617-621.
- 764 94. **West JB.** 1978. Regional differences in the lung. *Chest* **74**:426-437.
- 765 95. **Robertson HT, Hlastala MP.** 2007. Microsphere maps of regional blood flow and regional
766 ventilation. *J Appl Physiol* **102**:1265-1272.
- 767 96. **Glenny RW.** 2009. Determinants of regional ventilation and blood flow in the lung. *Intensive*
768 *Care Med* **35**:1833-1842.
- 769 97. **Marsland BJ, Gollwitzer ES.** 2014. Host-microorganism interactions in lung diseases. *Nat Rev*
770 *Immunol* **14**:827-835.
- 771 98. **Duncan SH, Louis P, Thomson JM, Flint HJ.** 2009. The role of pH in determining the species
772 composition of the human colonic microbiota. *Environ Microbiol* **11**:2112-2122.
- 773 99. **Roth RR, James WD.** 1988. Microbial ecology of the skin. *Annu Rev Microbiol* **42**:441-464.
- 774 100. **Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, Turner P, Parkhill J,**
775 **Loman NJ, Walker AW.** 2014. Reagent and laboratory contamination can critically impact
776 sequence-based microbiome analyses. *BMC Biol* **12**:87.

- 777 101. **Jones FS.** 1922. The source of the microorganisms in the lungs of normal animals. *J Exp Med*
778 **36:**317-328.
- 779 102. **Ward DV, Gevers D, Giannoukos G, Earl AM, Methe BA, Sodergren E, Feldgarden M, Ciulla**
780 **DM, Tabbaa D, Arze C, Appelbaum E, Aird L, Anderson S, Ayvaz T, Belter E, Bihan M, Bloom**
781 **T, Crabtree J, Courtney L, Carmichael L, Dooling D, Erlich RL, Farmer C, Fulton L, Fulton R,**
782 **Gao HY, Gill JA, Haas BJ, Hemphill L, Hall O, Hamilton SG, Hepburn TA, Lennon NJ, Joshi V,**
783 **Kells C, Kovar CL, Kalra D, Li K, Lewis L, Leonard S, Muzny DM, Mardis E, Mihindukulasuriya**
784 **K, Magrini V, O'Laughlin M, Pohl C, Qin X, Ross K, Ross MC, Rogers YHA, et al.** 2012.
785 Evaluation of 16S rDNA-based community profiling for human microbiome research. *PLoS*
786 *ONE* **7:**e39315. DOI: 10.1371/journal.pone.0039315
- 787 103. **Schloss PD, Gevers D, Westcott SL.** 2011. Reducing the effects of PCR amplification and
788 sequencing artifacts on 16S rRNA-based studies. *PLoS ONE* **6:**e27310. DOI:
789 10.1371/journal.pone.0027310
- 790 104. **Nelson MC, Morrison HG, Benjamino J, Grim SL, Graf J.** 2014. Analysis, optimization and
791 verification of Illumina-generated 16S rRNA gene amplicon surveys. *PLoS ONE* **9:**e94249.
792 DOI: 10.1371/journal.pone.0094249
- 793 105. **Pinto AJ, Raskin L.** 2012. PCR biases distort bacterial and archaeal community structure in
794 pyrosequencing datasets. *PLoS ONE* **7:**e43093. DOI: 10.1371/journal.pone.0043093
- 795 106. **Kembel SW, Wu M, Eisen JA, Green JL.** 2012. Incorporating 16S gene copy number
796 information improves estimates of microbial diversity and abundance. *PLoS Comput Biol*
797 **8:**e1002743. DOI: 10.1371/journal.pcbi.1002743
- 798
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800 **Figure Legends**

801 **Fig 1: Diagram of the sheep lung, divided into anatomical segments:** Boxes indicate the
802 segments from which lung protected specimen brushings were taken in six sheep at three
803 time-points; these correspond to the RA: right apical, RCD: right caudal diaphragmatic, and
804 LCD: left caudal diaphragmatic segments.

805 **Fig 2: Location of brushings within the sheep lung:** Protected specimen brushings were
806 taken from the sections of the lung labelled A1-A9 and A13-A19 in one sheep at one time-
807 point.

808 **Fig 3: qPCR of lung brushings and controls:** The bronchoscope channel was flushed
809 through with 7.5 ml PBS and the wash collected (Wash Control n=18) prior to protected
810 specimen lung brushings being taken from sheep (Lung Brushings n=54). DNA was extracted
811 from Wash Controls and Lung Brushings and the quantity of bacterial DNA calculated using
812 16S rDNA qPCR. Lung Brushings were found to contain significantly higher quantities of
813 bacterial DNA than Wash Controls (Mann-Whitney U test: $P < 0.0001$). Negative controls
814 consisted of either water (n=3) or extraction kit controls (n=2). Boxes indicate interquartile
815 ranges and outliers are represented as diamonds.

816 **Fig 4: Clustering of time-points by lung microbiota composition:** PCOA graph showing
817 the similarities between bacterial communities sampled from three sheep lung segments in six
818 sheep at three time-points. Samples were found to cluster significantly by the time-point at
819 which they were taken (AMOVA: $P < 0.001$).

820 **Fig 5: The bacterial communities found in three separate lung segments within two**
821 **sheep:** Protected specimen brushings were taken from the lungs of sheep at three different
822 lung segments (RA: right apical, RCD: right caudal diaphragmatic and LCD: left caudal
823 diaphragmatic) at day 0. Sheep A (2S066) had highly different bacterial communities at each

824 lung segment whereas Sheep B (2D644) had similar bacterial communities at all three lung
825 sites.

826 **Fig 6: Clustering of individuals by lung microbiota composition:** PCOA graphs showing
827 the similarities between the bacterial communities extracted from protected specimen
828 brushing samples taken from sheep lungs at three time-points (baseline (0 days), one month
829 and three months). Samples were taken from three separate lung segments (RA: right apical,
830 RCD: right caudal diaphragmatic and LCD: left caudal diaphragmatic). Samples from within
831 the same sheep were found to cluster significantly at baseline (AMOVA: $P = 0.001$) and three
832 months (AMOVA: $P = 0.045$) but not at one month. This is likely to be due to the presence of
833 contaminants originating from the extraction kits in the one month samples.

834 **Fig 7: Diagram of the bacterial orders found in the sheep lung:** Bacterial orders found in
835 protected specimen brushings from the lung and trachea (A1-A9 and A13-A19), throat swab
836 and an extraction kit control taken during a study of one sheep at one time-point.

837 **Fig 8: Clustering of lung brushings by depth within the lung:** PCOA graph showing the
838 similarity of samples taken at different lung depths based upon the bacterial orders present.
839 Lung depths are represented by colour and correspond to different distances from the glottis
840 progressing in a caudal direction. Adjacent pairs of samples are represented by the same
841 symbol and colour. For the exact location of each sampling site see **Fig 2**. Sub-tracheal
842 samples ($\geq A3$) clustered significantly by lung depth (AMOVA: $P = 0.033$) as did paired
843 samples (AMOVA: $P = 0.046$).

844

845 **Tables**846 **Table 1: Sheep used in this study**

Sheep ID	Gender	Mean weight (kg) \pm SD	Mean rectal temperature ($^{\circ}$ C) \pm SD
2D618	Female	51 \pm 3.1	39.0 \pm 0.06
2S066	Male (castrated)	69 \pm 2.6	39.6 \pm 0.20
2D619	Female	59 \pm 1.7	39.3 \pm 0.20
2D620	Female	64 \pm 4.6	39.1 \pm 0.21
2D644	Female	65 \pm 1.0	39.3 \pm 0.06
2D645	Female	70 \pm 2.0	39.4 \pm 0.06

847

848 **Table 2**

849 Proportion of DNA sequence reads belonging to bacterial members of a mock community

Taxonomy	Expected proportion of reads	Actual proportion of reads (undiluted)	Actual proportion of reads (1:100)	Mock community species
Order				
Deinococcales	5%	24.48%	7.65%	<i>Deinococcus radiodurans</i>
Campylobacterales	5%	22.05%	12.65%	<i>Helicobacter pylori</i>
Bacteroidales	5%	19.59%	10.91%	<i>Bacteroides vulgatus</i>

Bacillales	20%	8.60%	22.40%	<i>Bacillus cereus</i> , <i>Listeria monocytogenes</i> , <i>Staphylococcus aureus</i> , <i>Staphylococcus epidermidis</i>
Lactobacillales	25%	5.10%	12.29%	<i>Enterococcus faecalis</i> , <i>Lactobacillus gasseri</i> , <i>Streptococcus agalactiae</i> , <i>Streptococcus mutans</i> , <i>Streptococcus pneumoniae</i>
Clostridiales	5%	4.01%	7.86%	<i>Clostridium beijerinckii</i>
Rhodobacterales	5%	3.92%	5.29%	<i>Rhodobacter sphaeroides</i>
Pseudomonadales	10%	3.42%	5.97%	<i>Acinetobacter baumannii</i> , <i>Pseudomonas aeruginosa</i>
Enterobacteriales	5%	3.33%	5.52%	<i>Escherichia coli</i> ,
Neisseriales	5%	2.17%	3.49%	<i>Neisseria meningitidis</i>
Actinomycetales	10%	1.27%	2.92%	<i>Actinomyces odontolyticus</i> , <i>Propionibacterium acnes</i>
Other/unclassified	0%	2.03%	3.08%	
Genus*				
<i>Deinococcus</i>	5%	24.33%	7.61%	<i>Deinococcus radiodurans</i>
<i>Helicobacter</i>	5%	22.04%	12.65%	<i>Helicobacter pylori</i>
<i>Bacteroides</i>	5%	19.59%	10.90%	<i>Bacteroides vulgatus</i>
<i>Rhodobacter</i>	5%	3.91%	5.29%	<i>Rhodobacter sphaeroides</i>
<i>Clostridium</i>	5%	3.73%	7.59%	<i>Clostridium beijerinckii</i>
<i>Staphylococcus</i>	10%	3.04%	7.58%	<i>Staphylococcus aureus</i> , <i>Staphylococcus epidermidis</i>

<i>Lactobacillus</i>	5%	2.77%	6.59%	<i>Lactobacillus gasseri</i>
<i>Pseudomonas</i>	5%	2.33%	3.70%	<i>Pseudomonas aeruginosa</i>
<i>Neisseria</i>	5%	2.15%	3.27%	<i>Neisseria meningitidis</i>
<i>Enterococcus</i>	5%	1.40%	2.63%	<i>Enterococcus faecalis</i>
<i>Acinetobacter</i>	5%	0.97%	1.62%	<i>Acinetobacter baumannii</i>
<i>Propionibacterium</i>	5%	0.76%	1.77%	<i>Propionibacterium acnes</i>
<i>Actinomyces</i>	5%	0.48%	1.12%	<i>Actinomyces odontolyticus</i>
<i>Streptococcus</i>	15%	0.47%	1.63%	<i>Streptococcus agalactiae</i> , <i>Streptococcus mutans</i> , <i>Streptococcus pneumoniae</i>
Other/unclassified	0%	12.03%	26.05%	

850 * The species *Bacillus cereus*, *Escherichia coli* and *Listeria monocytogenes* could not be
 851 classified to genus level

852

853 Table 3

854 Abundance of the OTUs within the Pasteurellaceae family found in different locations of the
 855 sheep respiratory tract

Group	OTU 5: <i>Mannheimia</i> (%)	OTU 6: Pasteurellaceae (%)	OTU 7: <i>Bibersteinia</i> (%)	OTU 9: <i>Bibersteinia trehalosi</i> (%)
Throat swab	23.7	10.1	1.8	0.7
Trachea				
A1	5.5	4.5	28.4	5.3
A2	0	0.03	0	0.01

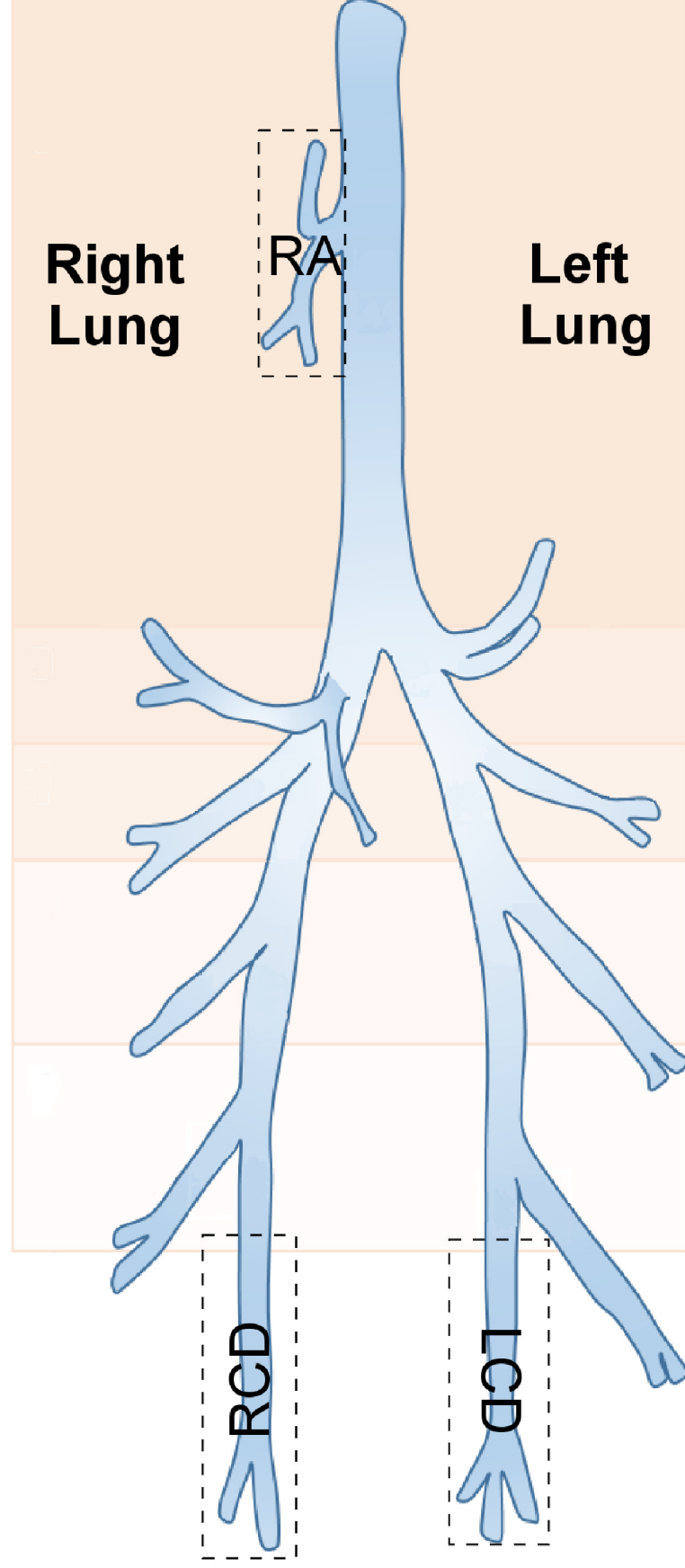
Left Lung

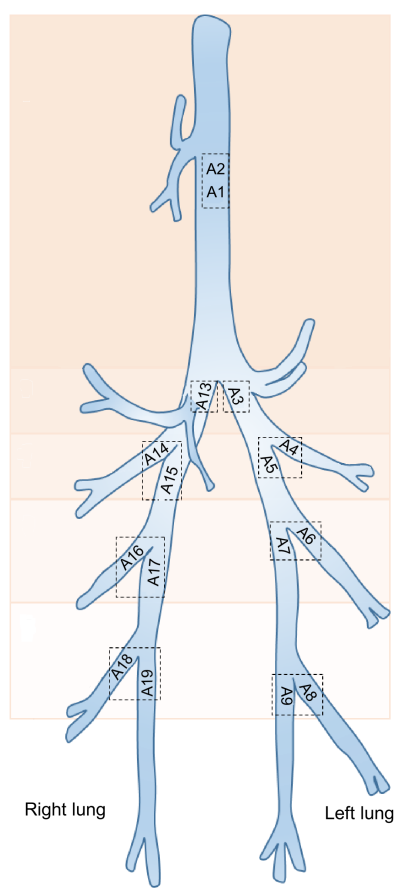
A3	0	3.04	0.01	0.03
A4	1.2	0.006	0	0
A5	0	0.2	0.006	0
A6	0	0	0	0
A7	0.7	1.4	0.006	0.006
A8	0	0	0.8	0
A9	0.006	0.02	0.2	0

Right Lung

A13	0.9	0	0	0.006
A14	0.006	0.3	0	0
A15	0	0.6	0.006	0.006
A16	2.3	0.6	0.006	0
A17	0.10	0	1.3	0.01
A18	0	0.02	0.01	0
A19	3.2	0	1.3	0

856





- A1: Ventral trachea just caudal to bifurcation with right apical (RA) lobe segmental bronchus
- A2: Dorsal trachea just caudal to bifurcation with right apical (RA) lobe segmental bronchus
- A3: Medial aspect of left main bronchus immediately caudal to carina
- A4: Medial aspect of first left ventral diaphragmatic segmental bronchus (LVD1), just caudal to bifurcation from left main bronchus
- A5: Left main bronchus, lateral wall just after bifurcation to LVD1
- A6: Medial aspect of second left ventral diaphragmatic segmental bronchus (LVD2), just caudal to bifurcation from left main bronchus
- A7: Left main bronchus, lateral wall just after bifurcation to LVD2
- A8: Third left ventral diaphragmatic segmental bronchus (LVD3)
- A9: Left caudal diaphragmatic (LCD) segmental bronchus
- A13: Medial aspect of right main bronchus immediately caudal to carina
- A14: Medial aspect of first right ventral diaphragmatic segmental bronchus (RVD1), just caudal to bifurcation from right main bronchus
- A15: Right main bronchus, lateral wall just after bifurcation to RVD1
- A16: Medial aspect of second right ventral diaphragmatic segmental bronchus (RVD2), just caudal to bifurcation from right main bronchus
- A17: Right main bronchus, lateral wall just after bifurcation to RVD2
- A18: Third right ventral diaphragmatic segmental bronchus (RVD3)
- A19: Right caudal diaphragmatic (RCD) segmental bronchus

