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

Glendinning, L; Collie, D; Wright, S; Rutherford, KMD; McLachlan, G

Published in:
Microbiome

DOI:
10.1186/s40168-017-0364-5

First published: 27/10/2017

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

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

Laura Glendinning[1] (laura.glendinning@roslin.ed.ac.uk), David Collie[1] (david.collie@roslin.ed.ac.uk), Steven Wright[1] (steven.wright@roslin.ed.ac.uk), Kenny MD Rutherford[2] (Kenny.Rutherford@sruc.ac.uk), Gerry McLachlan[1] (gerry.mclachlan@roslin.ed.ac.uk)

[1] The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Edinburgh, Midlothian, United Kingdom, EH25 9RG

[2]Animal Behaviour and Welfare, Animal and Veterinary Sciences Research Group, SRUC, West Mains Rd., Edinburgh, Midlothian, United Kingdom, EH9 3JG

Corresponding author: Laura Glendinning

Abstract

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

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

Conclusions: In contrast to humans, we found that that the lung microbiota of lambs is dissimilar to that of the upper aerodigestive tract and we suggest that this may be related to physiological and 1anatomical differences between sheep and humans. Understanding the comparative physiology and anatomy underlying differences in lung microbiota between species will provide a foundation upon which to interpret changes associated with disease and/or environment.

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

Background

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

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

Subclinical microaspiration of pharyngeal secretions is a feature of health and this can contribute to the lung microbiota composition [16] and the microbiome of the human lungs more closely resembles that of the mouth than the nose or the lower gastrointestinal tract [17]. It is not yet known whether the same relationship holds for species other than humans. In ruminating sheep, where the oropharynx is exposed to ruminal contents on a frequent basis one would anticipate that lung microbiota would similarly reflect this influence. In this paper we find that the presence of 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 ± 0.8 standard deviation (SD); mean weight ± SD = 20.6 kg ± 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

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

DNA extraction, amplification, and sequencing

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

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

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

Bioinformatic and statistical analysis

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

Results

Quality assurance of methodology

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

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

Date of DNA extraction	OTUs	Abundance
17th July 2014	*Aerococcus*	14%
	Dermabacteraceae	12%
	Micrococcus	10%

	Enhydrobacter	9%
	Leuconostoc	6%
	Kocuria	6%
	Actinomyces	6%
25th March 2015	*Methylobacterium komagatae*	65%
	Ruminococcaceae	11%
	Methylobacterium	6%
26th March 2015	*Methylobacterium komagatae*	67%
	Methylobacterium	6%

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.

Dichotomous oropharyngeal microbiota are associated with different lung community structures

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

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

The presence of a lung specific microbiota

Indicator species analysis determined that several OTUs were significantly more indicative of the lungs than of oropharyngeal swabs (**Table 2**). It is likely that reagent contamination will have had more of an impact on the lung fluid samples than on the oropharyngeal swabs, due to their lower biomass. However, when examining the indicative OTUs, the majority of samples were not found to contain the same proportions of these OTUs as the PBS controls processed alongside them (**Figure 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 ± SD)	Average proportion in oropharyngeal swabs (Mean ± SD)	Highest proportion in PBS controls
Brachybacterium	0.006	1.0% ± 1.7%	0.035% ± 0.10%	0.022%
Brevibacterium	0.002	1.2% ± 1.4%	0.064% ± 0.24%	0%
Corynebacterium	< 0.001	1.9% ± 2.3%	0.065% ± 0.19%	0.044%
Delftia	< 0.001	0.80% ± 1.7%	0% ± 0%	0%
Enterobacteriaceae	0.023	0.65% ± 2.6%	0.0063% ± 0.029%	2.2%
Frigoribacterium	0.021	0.79% ± 1.4%	0.077% ± 0.31%	0%
Janthinobacterium	0.01	0.57% ± 1.4%	0.0023% ± 0.0068%	0%
Jeotgalicoccus psychrophilus	0.008	1.6% ± 2.1%	0.040% ± 0.10%	0%
Microbacterium aurum	0.047	1.2% ± 2.8%	0.0045% ± 0.013%	0%
Micrococcus	0.017	0.77% ± 1.6%	0.0080% ± 0.029%	4.4%
Oxalobacteraceae	< 0.001	0.96% ± 1.5%	0.012% ± 0.043%	3.0%
Pelomonas	< 0.001	0.65% ± 1.1%	0.00057% ± 0.0036%	2.0%
Peptostreptococcaceae	0.006	1.8% ± 2.2%	0.050% ± 0.11%	0.044%
Propionibacterium acnes	< 0.001	0.84% ± 2.3%	0.0040% ± 0.020%	1.6%
Pseudomonas citronellolis	< 0.001	0.51% ± 1.1%	0% ± 0%	1.0%
Rathayibacter caricis	0.016	0.58% ± 1.2%	0.0057% ± 0.021%	0%
Saccharopolyspora	0.009	0.52% ± 1.2%	0.0011% ± 0.0071%	0%
SMB53	< 0.001	0.71% ± 1.3%	0.0045% ± 0.018%	0%

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

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

Discussion

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

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

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

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

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

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

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

Conclusions

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

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

Declarations

Ethics approval and consent to participate

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

Consent for publication

Not applicable

Availability of data and materials

The datasets generated during and/or analysed during the current study are available in the Short Read Archive and can be accessed at Bioproject accession number PRJNA317719 (http://www.ncbi.nlm.nih.gov/bioproject/PRJNA317719).

Competing interests

The authors declare that they have no competing interests.

Funding

This study was funded by the Scottish Government's Rural and Environment Science and Analytical Services Division (RESAS) as part of the 'Strategic Partnership for Animal Science Excellence' programme. Laura Glendinning was funded by the BBSRC (Biotechnology and Biological Sciences Research Council) under the EASTBIO doctoral training program (Grant number: BB/J01446X/1). The funders had no role in the design of this microbiota study, the analysis and interpretation of the data or the writing of this manuscript.

Authors' contributions

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

Acknowledgments

The authors would like to thank the farm staff for the care of the animals used in this study and the staff and students from SRUC and the University of Edinburgh who aided during sample collection. The following reagent was obtained through BEI Resources, NIAID, NIH as part of the Human Microbiome Project: Genomic DNA from Microbial Mock Community B (Even, Low Concentration), v5.1L, for 16S rRNA Gene Sequencing, HM-782D.

References

1. Martinsen EMH, Leiten EO, Bakke PS, Eagan TML, Gronseth R. Participation in research bronchoscopy: A literature review. Eur Respir J 2016;3:29511. doi:10.3402/ecrj.v3.29511.

2. Yadava K, Pattaroni C, Sichelstiel AK, Trompette A, Gollwitzer ES, Salami O et al. Microbiota promotes chronic pulmonary inflammation by enhancing IL-17A and autoantibodies. Am J Respir Crit Care Med 2015;115(1):63-8. doi:10.1164/rccm.201504-0779OC.

3. Yu W, Yuan X, Xu X, Ding R, Pang L, Liu Y et al. Reduced airway microbiota diversity is associated with elevated allergic respiratory inflammation. Ann Allergy Asthma Immunol. 2015;115(1):63-8. doi:10.1016/j.anai.2015.04.025.

4. Stokes K, Wood A, August A. The microbiome regulates the eosinophil requirement for 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.

14. Collie D, Glendinning L, Govan J, Wright S, Thornton E, Tennant P et al. Lung microbiota changes associated with chronic *Pseudomonas aeruginosa* lung infection and the impact of intravenous colistimethate sodium. PloS One. 2015;10(11):e0142097. doi:10.1371/journal.pone.0142097.

15. Glendinning L, Wright S, Pollock J, Tennant P, Collie D, McLachlan G. Variability of the sheep lung microbiota. Appl Environ Microbiol 2016;82(11):3225-38. doi:10.1128/aem.00540-16.

16. Dickson RP, Huffnagle GB. The lung microbiome: New principles for respiratory bacteriology in health and disease. PLoS Pathog. 2015;11(7):e1004923. doi:10.1371/journal.ppat.1004923.

17. Bassis CM, Erb-Downward JR, Dickson RP, Freeman CM, Schmidt TM, Young VB et al. Analysis of the upper respiratory tract microbiotas as the source of the lung and gastric microbiotas in healthy individuals. mBio. 2015;6(2):e00037-15. doi:10.1128/mBio.00037-15.

18. Berry D, Ben Mahfoudh K, Wagner M, Loy A. Barcoded primers used in multiplex amplicon pyrosequencing bias amplification. Appl Environ Microbiol. 2011;77(21):7846-9. doi:10.1128/AEM.05220-11.

19. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. Embnetjournal. 2011;17(1):10-2. doi:10.14806/ej.17.1.200.

20. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB et al. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol. 2009;75(23):7537-41. doi:10.1128/AEM.01541-09.

21. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq illumina sequencing platform. Appl Environ Microbiol. 2013;79(17):5112-20. doi:10.1128/AEM.01043-13.

22. Esty WW. The efficiency of Good's nonparametric coverage estimator. Ann Stat. 1986;14(3):1257-60.

23. Dufrêne M, Legendre P. Species assemblages and indicator species:The need for a flexible 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-
369 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-
373 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-
376 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

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

Additional files

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

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

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

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