Dental biofilm and its ecological interrelationships in ovine periodontitis

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Abstract

Introduction. Periodontitis, one of the most common oral disorders in sheep, is caused by a mixed and opportunistic microbiota that severely affects the health and welfare of animals. However, little is known about the ecological processes involved and the composition of the microbiota associated with the development of the disease.

Hypothesis/Gap Statement. Using high-throughput sequencing of 16S ribosomal RNA gene and network analysis it would be possible to discriminate the microbiomes of clinically healthy sheep and those with periodontitis and possibly identify the key microorganisms associated with the disease.

Aim. The present study aimed to characterise the composition of dental microbiomes and bacterial co-occurrence networks in clinically healthy sheep and animals with periodontitis.

Methodology. Dental biofilm samples were collected from 10 sheep with periodontitis and 10 clinically healthy animals. Bacteria were identified using high-throughput sequencing of the 16S ribosomal RNA gene.

Results. The most prevalent genera in the dental microbiota of sheep with periodontitis were *Petrimonas*, *Acinetobacter*, *Porphyromonas* and *Aerococcus*. In clinically healthy animals, the most significant genera were unclassified *Pasteurellaceae, Pseudomonas,* and *Neisseria. Fusobacterium* was found at high prevalence in the microbiomes of both groups*.* The dental microbiota of sheep in the two clinical conditions presented different profiles and the diversity and richness of bacteria was greater in the diseased animals. Network analyses showed the presence of a large number of antagonistic interactions between bacteria in the dental microbiota of animals with periodontitis, indicating the occurrence of a dysbiotic community. Through the interrelationships, members of the *Prevotella* genus are likely to be key pathogens, both in the dental microbiota of healthy animals and those with periodontitis. *Porphyromonas* stood out among

the top three nodes with more centrality and the largest number of hubs in the networks of animals with periodontitis.
Conclusion. The dental biofilm microbiota associated with ovine periodontitis is dysbiotic and with significant antagonistic interactions, which discriminates healthy animals from diseased animals and highlights the importance of key bacteria, such as *Petrimonas*, *Porphyromonas*, *Prevotella* and *Fusobacterium* species.

Keywords: periodontitis, sheep, dental biofilm, dysbiosis, high-throughput sequencing, networks.

INTRODUCTION

Composition, accumulation and dysbiosis of the dental biofilm, together with the host immune-inflammatory response, are elements involved in the aetiology of periodontitis in ruminants [1,2]. In the context of these mixed and complex infections it is essential to understand the ecological relationships present in dental biofilms, which would allow understanding of the interrelationships between the components of the microbiota and how changes within this community could contribute to the progression of disease [3].

From an ecological point of view, the mouth represents a complex ecosystem, with peculiar structures and characteristics, which differentiate it from all other bodily surfaces [4]. With different habitats and ecological conditions at each site of the oral cavity, the different surfaces allow colonisation by a wide microbiota, especially the teeth since they have a non-scaling surface. Indeed, similarities and dissimilarities are observed in the oral or dental microbiota of different species of mammals [1, 5-7], and result from long-term coevolution in each evolutionary lineage [8].

In efforts to identify or associate microorganisms with the occurrence of the disease in sheep, classic studies used conventional bacterial culture of dental biofilms [9-11], until the introduction of culture-independent molecular methods [12-15]. Although these results have shown a set of potential periodontal pathogens, they have limits as a discriminatory reference for robust studies on aetiopathogenesis or even in the development and evaluation of disease control measures.

Bearing in mind that ovine periodontitis is a disease apparently distributed worlwide [10-14, 16-21], due to unknown environmental factors or polymicrobial

modifiers, the present study aims to characterise the composition of the dental microbiota and the networks of bacterial co-occurrence of animals, under two distinct clinical conditions, using high-throughput sequencing of the 16S rRNA gene.

METHODS

Periodontal clinical examination

The clinical status of 20 adult sheep from one herd at São Paulo State, was established through oral examination with the aid of a mouth-opening device and periodontal pocket depth was determined using a Williams periodontal probe [13,14,22].

Since periodontitis include alterations of the gingival tissue and a progressive loss of periodontal attachment and alveolar bone, the criteria for the the diagnosis of the disease was the presence of a periodontal pocket (the distance from the gingival margin to the bottom of the periodontal pocket as measured with a graduated probe) with a depth greater than 5 mm, with bleeding on probing (presence of blood around the gingival margin or inside the periodontal pocket after probing) and suppuration (presence of pus inside the periodontal pocket) in the incisor teeth [13,14,20,22].

The periodontal clinical condition of the animals was classified as healthy when there was no evidence of gingival recession, no periodontal pockets (subgingival sulcus:1 to 3 mm in the lip face of the incisors; 4 to 5 mm in the lingual face of the incisors), no suppuration and no evidence of any other oral disease [13,14,22]. The universal probe was inserted to the base of the periodontal pocket or the subgingival sulcus and moved gently around the tooth surface and pocket/sulcus depth measurement obtained [13,14,22].

Collection of dental biofilm

Dental biofilm samples were obtained from the periodontal pocket of 10 sheep with periodontitis and the gingival sulcus of 10 animals with teeth considered clinically healthy. From the gingival sulcus, the collection was performed from the labial surface of the first incisor, since it represents the oldest

incisor tooth of the animal, and consequently the one exposed to the greatest accumulation of biofilm.

The collection of material from the periodontal pocket and gingival sulcus was performed after removing the supragingival bacterial biofilm with sterile gauze or curette. The samples were collected with a sterile curette, with a single scraping of the dental biofilm, stored in 250 µL of RNAlater (Sigma–Aldrich, Dorset, UK), transported under refrigeration and stored at -80ºC until samples were processed.

DNA Extraction

DNA extraction from dental biofilm samples was performed with the GenElute Mammalian Genomic DNA Miniprep Kit in accordance with the manufacturer's instructions (Sigma, St. Louis, USA).

High-throughput sequencing

PCR amplicon libraries targeting the V4 region of the 16S rRNA gene (515F-806R) were produced using a barcoded primer set adapted for the Illumina HiSeq2000 and MiSeq platforms [23,24]. Amplicons were paired-end sequenced on an Illumina MiSeq using customised sequencing primers and procedures [23] at the Environmental Sample Preparation and Sequencing Facility (ESPSF) at Argonne National Laboratory, USA.

Sequencing data analysis

Bioinformatics analysis was performed using Mothur software (v. 1.42) [25], with some changes to the standard protocol of Kozich et al. [26]. Sequences were assembled and aligned to SILVA reference bank (version 132) [27] and the pre-cluster step was not performed. To identify and extract the chimeric sequences, VSEARCH algorithm was used [28] and to classify the sequences, the Bayesian classifier obtained from the Ribosomal Database Project (RDP) 16S [29] was used with a confidence score of 80%. Sequences were clustered into operational taxonomic units (OTUs), and for data normalisation the resulting OTU table was subsampled to an equal depth per sample.

Statistical analysis

Diversity analysis (Shannon Diversity Index and the Chao-1 estimate of total species richness), principal component analysis (PCoA), and differences between microbial profiles of the groups by analysis of molecular variance (AMOVA), both using Bray-Curtis distance, were calculated in mothur version 1.41.3 [25]. Differences in diversity output were tested with the Wilcoxon test in R software (version 3.6.1; R Foundation for Statistical Computing, Austria) [30]. After removing rare OTUs with a sum of less than ten from the OTU table, linear discriminant analysis effect size (LEfSe) [31] was used to determine which OTUs and taxa were differentially abundant between the groups. The analysis was performed using the online LEfSe workflow on the Huttenhower lab Galaxy platform (http://huttenhower.sph.harvard.edu/galaxy/).

Network analysis

The co-occurrence network between OTUs was inferred using the SparCC (Sparce Correlations for Compositional data) implemented in mothur. This algorithm estimates the linear Pearson correlations between the log-transformed components and statistical significance of the inferred correlations was assessed using a bootstrap [32]. The filtered matrices with an absolute correlation of 0.5 and $p < 0.01$ were calculated using R and the Cytoscape package version 3.8.0 [33,34].

RESULTS

Sequencing output

Sequencing generated a total of 680,084 reads for the 20 samples. When removing sequencing errors and unwanted sequences, 83.4% of the sequences (567,255) remained, which were clustered into 194,457 unique reads. Of these, 10,971 (5.6%) sequences considered chimeras were identified and removed. With the remaining 183,486 sequences, alignment was made against SILVA bank [27] and 6,501 reads were not classified and thus were removed. The remaining 525,434 (77.2%) reads were attributed to operational taxonomic units (OTUs). To normalise the data in the OTU counts per sample, a subsample was

performed with 3100 reads in the processed data and the removal of OTUs with a count of less than 10, thus leaving 428 OTUs.

Relative abundance of bacterial phyla and genera in dental biofilm

A total of 18 phyla were identified in the ovine dental microbiome. Five phyla showed a relative abundance greater than 1% in the dental microbiome of animals with periodontitis and, together, represented 94% of the sequences. These were Bacteroidetes (31.1%), Firmicutes (29.6%), Proteobacteria (15.5%), Fusobacteria (14.2%), and Synergistetes (3.6%). In the dental microbiome of the 10 clinically healthy sheep, 5 phyla represented 97.5% of the sequences. The most prevalent phyla were Proteobacteria (59.7%), Fusobacteria (12.8%), Firmicutes (11.3%), Bacteroidetes (9.8%) and Actinobacteria (3.8%). As these values and Fig 1 show, the more perceptible differences were observed in Proteobacteria, Bacteroidetes and Firmicutes. The relative abundance of Proteobacteria was lower in animals with periodontitis (Fig 1). Conversely, the relative abundance of Bacteroidetes was three times higher in the periodontitis microbiome (31.1% versus 9.8%).

In total, 195 genera were identified in the dental microbiome and only 30 genera showed a relative abundance greater than 1%. In the 10 clinically healthy animals, 142 genera were identified, with only 18 having a relative abundance greater than 1%. The most prevalent genera in the microbiomes of healthy animals were unclassified *Pasteurellaceae* (25.4%), *Neisseria* (9.9%), *Fusobacterium* (9.0%), *Pseudomonas* (7.6%), *Porphyromonas* (3.1%) and unclassified *Leptotrichiaceae* (3.0%). In the 10 animals with periodontitis, 166 genera were identified and 19 showed a relative abundance greater than 1%. The most prevalent genera in the microbiomes of animals with periodontitis were *Petrimonas* (17.2%), *Fusobacterium* (12.2%), *Acinetobacter* (5.5%), *Porphyromonas* (5.5%), *Aerococcus* (3.0%), *Bacteroides* (2.8%), and *Christensenellaceae* R7 (2.5%). As these values and Fig. 2 show, the most perceptible differences were observed in *Fusobacterium* and *Porphyromonas*, since the abundance of both increased in the microbiomes of animals with periodontitis.

Of the 30 genera that had a relative abundance greater than 1%, only 6 were shared among clinically healthy animals with periodontitis: *Bacteroides,*

Fusobacterium, Methylobacterium, Porphyromonas, Pseudomonas and Streptococcus (Table 1). The clinically healthy animals presented 11 unique genera in their dental microbiomes (Table 2), among which unclassified *Pasteurellaceae* (25.4%) and *Neisseria* (9.9%) were most abundant. Animals with periodontitis had 13 unique genera (Table 3), the most abundant being *Petrimonas* (17.2%) and *Acinetobacter* (5.5%).

Microbial Profile Analysis

Differences between the dental microbiomes of clinically healthy sheep and those with periodontitis were observed by principal component analysis (Figure 3). Generally, the healthy and periodontitis samples tended to cluster separately, and the periodontitis samples demonstrated lower intra-sample variability relative to the healthy samples. A statistically significant difference between the microbial profiles of health and disease was observed ($p<0.001$, AMOVA). Bray-Curtis analysis showed 94% dissimilarity between the dental microbiomes of healthy animals and those with periodontitis.

Statistically significant differences between the dental microbial profiles of healthy and diseased animals was observed in species richness or diversity (Figure 4). On average, samples from sheep with periodontitis harboured 153 OTUs (SD 29.3, range 80-189), while samples from clinically healthy animals contained 72 OTUs (SD 32.8, range 31-129).

Differences in the composition of dental microbiomes of sheep with periodontitis and those considered clinically healthy

Of the 428 OTUs identified in the dental microbiome, 158 (37%) showed significant differences between the groups evaluated ($p <0.05$) and had a linear discriminant analysis (LDA) score larger than 2 in LEfSe (Figure 5). The genera most strongly associated with the dental microbiome of sheep with periodontitis were *Petrimonas, Acinetobacter, Porphyromonas* and *Aerococcus* (43 OTUs LDA > 3.2; $p < 0.05$). In the dental microbiome of clinically healthy animals (15 OTUs LDA> 3.2; $p <0.05$), the most significantly associated genera were unclassified *Pasteurellaceae, Pseudomonas* and *Neisseria* (Figure 5). The *Fusobacterium* genus was found at high prevalence in the dental microbiome of both groups (LDA > 3.2; $p < 0.05$; Figure 5).

Bacterial networks

Of the 428 OTUs submitted to the correlation, the network of 10 animals with periodontitis presented 312 OTUs (nodes) interacting 2874 times (edges)and 73% of these interactions were positive (positive edges) and 27% were negative (negative edges). A total of 14 phyla represented these nodes and the most prevalent were Firmicutes (44.9%), Bacteroidetes (19.6%) and Proteobacteria (17.9%) (Figure 6). Among the 159 genera identified in networks of animals with periodontitis, *Porphyromonas*, *Streptococcus* and *Christensenellaceae_R-7_group* were most abundant,with a total of 12 OTUs each. Among the OTUs with greater prominence for the number of connections (Hubs) were *OTU224-Neisseriacea, OTU207-Anaerolineaceae, OTU110-Streptococcus, OTU165-Micrococcus,* and *OTU308-Paraclostridium. Porphyromonas, Succiniclasticum* and unclassified *Pasteurellaceae* were the bacteria with the highest betweenness centrality in the networks of animals with periodontitis.

The clinically healthy animals had a smaller network, with 265 OTUs (nodes) interacting 1253 times (edges) with 92% positive interactions (positive edges) and 8% negative (negative edges). Nodes were distributed in 15 phyla, with more than 50% concentrating on Firmicutes (37%), Proteobacteria (24.9%) and Bacteroidetes (18.9%) (Figure 6). Among the 137 genera identified in the networks of clinically healthy animals, the most representative were *Streptococcus*, unclassified *Pasteurellaceae* and *Prevotella_1*, representing 13, 10 and 9 OTUs, respectively. The most prominent OTUs by the number of interactions (Hubs) were OTU299- *Ruminococcus_1*, OTU210-*Ruminococcus_1*, OTU316-*Prevotella_1*, OTU430-*Treponema_2* and OTU251-*Saccharofermentans*. *Janthinobacterium, Methylobacterium* and *Prevotella_1* were the bacteria with the highest centrality betweenness in the networks of healthy animals.

When comparing the two networks, that of clinically healthy animals showed greater modularity (ability of nodes to establish intensely connected communities), reaching 0.70 against 0.47 in diseased animals. It was also possible to observe a larger diameter (the shortest distance between the two nodes furthest from the network, measured in number of edges) in healthy

animals compared to those with periodontitis (10 and 6, respectively). However, the networks of animals with periodontitis showed a higher number of interactions, with the average degree at 18 (maximum = 61) while the healthy animals had a degree average of 9 (maximum = 27).

DISCUSSION

Periodontitis are population disorders in ruminants, with distinct epidemiological particularities, but which conceptually and in their aetiopathogenesis are similar to what occurs in other animal species [35]. Although this alleged similarity has remained and reinforced as a perception in the evolution of periodontitis studies in sheep, the differences can now be better evidenced using high-throughput bacterial 16S rRNA gene sequencing. The present study is the first to use this tool to characterise the structure of dental microbiomes and the networks of bacterial co-occurrence of clinically healthy sheep and those with periodontitis. Thus, it reveals the possibility of objectively discriminating the dysbiotic process in the dental microbiota of ovine periodontitis and reinforces one of the principles of Socransky's Postulate [36,37].

Statistically significant differences were observed in the composition of the dental microbiota of the two clinical conditions evaluated, with communities showing 94% dissimilarity. The increase in the relative abundance of Bacteroidetes and Firmicutes and the decrease in the relative abundance of Proteobacteria in the dental microbiome of animals with periodontitis are noteworthy. These results show that, even at a higher level of classification, there are differences in abundance between the microbiomes of healthy animals with periodontitis. This represents a substantial advance in knowledge about sheep dental communities since no study has evaluated the composition of these microbiomes at the phylum level.

Proteobacteria, Fusobacteria, Firmicutes, Bacteroidetes and Actinobacteria were the most prevalent phyla in the dental microbiomes of clinically healthy sheep. These same taxonomic categories were also the most prevalent in the oral microbiome in humans and other animal species. Thus, at the phylum level, clinically healthy sheep have a microbiota similar to that identified in the biofilm of cattle, dogs, cats and humans [1, 38-41].

In relation to animals with periodontitis, similarities at the phylum level were also observed with the oral microbiota of cattle and sheep raised in Scotland. In cattle, Bacteroidetes, Firmicutes, Proteobacteria and Fusobacteria showed a high prevalence in the oral microbiome of animals with periodontitis [1]. In sheep, the phylum Bacteroidetes was identified only in animals with periodontitis [12].

Among the most prevalent genera in the dental microbiomes of healthy sheep, unclassified *Pasteurellaceae*, *Pseudomonas*, and *Neisseria* were most abundant. These bacteria have already been identified in the oral microbiota of healthy dogs, cats and horses [5,38,40,42,43). In the present study, unclassified *Pasteurellaceae* stood out as the genus with the highest relative abundance among the unique taxa identified in the dental microbiota of clinically healthy sheep. Recently, members of *Pasteurellaceae* family have been identified only in sheep with periodontitis [12]. In the present study, high-throughput sequencing results show that this genus was identified only in the microbiome of healthy sheep and can therefore be part of the balanced microbiome associated with periodontal health.

The genera *Petrimonas*, *Acinetobacter*, *Porphyromonas*, and *Aerococcus* were most prevalent in periodontitis. In a recent study, *Acinetobacter* was identified in the oral microbiota of healthy sheep and *Porphyromonas* in animals with periodontitis [12]. *Porphyromonas* represents one of the most prevalent genera in the microbiota of cattle, sheep and humans with periodontitis [1,14,44,45] but has also been identified in the oral microbiota of healthy cats and dogs [38,43].

In the present study, a high prevalence of *Fusobacterium* was observed in the oral microbiota of the two clinical conditions evaluated. This genus has recognised importance in the formation of dental biofilms and in recent studies its occurrence has also been reported in the oral microbiota of healthy and diseased sheep, goats and cattle with periodontitis [1,15,22]. In addition to being one of the most prevalent genera in animals with periodontitis, *Petrimonas* was also the most abundant genus among the unique genera identified in the dental microbiome of sheep with periodontitis. The *Petrimonas* genus is part of the phylum Bacteroidetes and has some similarities with the *Bacteroides* and *Tannerella* genera [46], which contain periodontal pathogens of recognised

importance. This is the first report that shows the association of this genus with ovine periodontitis. As an association does not mean causality, whether it acts as an accessory microorganism or as a potential pathogen in the aetiopathogenesis of periodontitis remains unknown.

Statistically significant differences between the dental microbial profiles of healthy and diseased animals were observed in species richness or diversity and dental microbiomes of animals with periodontitis were richer and more diverse than those of clinically healthy animals. The same pattern could be evidenced in human patients [47]. In cattle, on the other hand, no statistically significant differences were observed in species richness or diversity of healthy and periodontitis microbiomes [1].

Analysis of bacterial co-occurrence networks makes it possible to identify which microorganisms co-infect animals under the same conditions and indicate the presence of synergistic or antagonistic interactions between microorganisms in a given environment. The characterization of these bacterial interdependence relationships and the identification of the main pathogens involved can assist in the development of measures that prevent the formation of these connections and, consequently, assist in the treatment and the control of the disease. However, this premise needs to be proven. In the present study, the bacterial networks of sheep with periodontitis revealed a greater number of nodes and edges. The edges indicate the tendency for OTUs to co-occur in a certain niche, are the result of cooperation or competition between microorganisms and have biological, physiological and ecological significance [3]. However, networks of clinically healthy animals showed greater modularity i.e., the ability to form highly connected communities is superior in the dental microbiota of healthy animals. A modular community also suggests greater diversity in the functions of the species involved, which may imply a faster response of the components of this microbiome to external disturbances [48], indicating a balanced community.

In the networks of healthy sheep biofilm, it was also possible to observe a greater number of positive interactions between OTUs than in animals with periodontitis. Networks with many positive interactions tend to indicate cooperation between members of that niche. These interactions can symbolise complementary or dependent microorganisms, representing a possible core group essential for that environment to thrive [3]. These results suggest that at

least part of the dental microbiome of healthy animals is composed of a stable group of OTUs and in homeostasis.

Negative interactions between microorganisms suggest competition between members of a given environment and may indicate groups of bacteria with general antagonistic behaviour [3]. In the networks of animals with periodontitis, the number of negative interactions was three times higher than in clinically healthy animals and several connections appeared to be broken, which can be interpreted as a possible consequence of microbiota dysbiosis in diseased animals.

In the networks of both groups, some OTUs were identified with greater betweenness centrality, representing the possible key microorganisms within a connected community [49]. *Porphyromonas* genus stood out among the top three nodes with more centrality and the largest number of hubs in the networks of animals with periodontitis. Interestingly, in the present study *Prevotella_1* stood out among the top three nodes with the greatest centrality and the largest number of hubs in the networks of healthy animals.

Black-pigmented bacteria of the genera *Prevotella* and *Porphyromonas* are considered important pathogens in human and animal periodontitis [1,14,44,13], including 'broken mouth' periodontitis [12]. The results of the analysis of the networks of the present study highlight the relevance of *Porphyromonas* genus as a key pathogen in the dysbiotic microbiome associated with ovine periodontitis. These same results suggest that, in addition to their recognised importance in the development of periodontitis, representatives of the *Prevotella* genus may be fundamental for the maintenance of the microbiome associated with periodontal health, acting as a key microorganism within this community.

The identification of key microorganisms could contribute to the development of new therapeutic approaches aimed at a limited number of pathogens with extreme relevance within the dysbiotic dental microbiome. In addition, new diagnostic tools can be developed if it is shown that periodontitis is caused by a keystone pathogen or a number of microorganisms acting in this way [50]. However, association does not mean causality. Thus, future studies should evaluate the interaction between these key microorganisms and the host in an attempt to develop measures of treatment, prevention and control to the disease.

The results of the present study indicated that the dental microbiomes of periodontitis and clinically healthy sheep have different profiles and that the diversity and richness of microorganisms is higher in diseased animals, with emphasis on the *Petrimonas* genus. Network analyses demonstrated the presence of a large number of antagonistic interactions between microorganisms in the biofilm of animals with periodontitis, indicating the occurrence of a dysbiotic community. The role of the *Prevotella* genus as a key pathogen in both the microbiomes of healthy and diseased animals was also highlighted. Thus, these novel findings contribute to the evolution of knowledge about the aetiopathogenesis of ovine periodontitis as well as, possibly, for the development of tools for the evaluation of measures to control the different clinical forms of ovine periodontitis.

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Author contributions

A.C.B. conceptualisation, methodology, investigation, data curation, writing and project administration; F.R.F.A. methodology, software, formal analysis, data curation and writing; S.D.A. conceptualisation, methodology, investigation, data curation, writing and project administration; M.P.R. conceptualisation, methodology, formal analysis and writing; I.S.D. conceptualisation, methodology, investigation, resources, funding, supervision, project administration and writing.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical approval

This study was approved by the Ethics Committee on Animal Experimentation of São Paulo State University (Unesp), Jaboticabal Campus (Protocol FCAV Nº 018976/17).

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Figure Legends

Figure 1. Distribution of bacterial phyla (relative abundance > 1%) in the dental biofilm of 10 clinically healthy sheep (OHSS) and 10 sheep with periodontitis (OPSS).

Figure 2. Distribution of bacterial genera (relative abundance > 1%) in the dental microbiome of 10 sheep with periodontitis (OPSS) and 10 clinically healthy sheep (OHSS).

Figure 3. Two-dimensional ordination describing the dissimilarity of ovine dental microbial profiles in health and periodontitis by principal component analysis (PCoA).

Figure 4. Diversity analysis in dental microbiomes of clinically healthy sheep (OHSS, n=10) and those with periodontitis (OPSS, n=10). A. Observed species richness or number of OTUs per sample; B. Shannon diversity index.

Figure 5. Visualisation of most significant taxa (genus or higher level) that differentiate dental microbiomes from periodontitis (OPSS, n = 10) and clinically healthy (OHSS, n = 10) sheep. Only taxa with an LDA score greater than 3.2 are presented. Taxa are ranked by the effect size in LEfSe.

Figure 6. Bacterial co-occurrence network of dental microbiomes of sheep. A: Dental microbiomes of sheep with periodontitis – prevalence of Firmicutes (44.9%), Bacteroidetes (19.6%) and Proteobacteria (17.9%); B: Dental microbiomes of clinically healthy sheep – prevalence of Firmicutes (37%), Proteobacteria (24.9%) and Bacteroidetes (18.9%).

Table 1. Distribution of common genera, with relative abundance greater than 1%, in the dental microbiota of clinically healthy sheep (n = 10) and those with periodontitis (n = 10)

Genus	**Clinically healthy sheep (%)**	**Sheep with periodontitis (%)**
Bacteroides	1.7	2.8
Fusobacterium	9.0	12.2
Methylobacterium	2.4	2.2
Porphyromonas	3.1	5.5
Pseudomonas	7.6	1.4
Streptococcus	2.5	1.8
Total	**26.2%**	**25.9%**

Table 2. Relative abundance of unique genera identified in the dental biofilm of 10 clinically healthy sheep

Genus	**%**
Pasteurellaceae_unclassified	25.4
Neisseria	9.9
Leptotrichiaceae_unclassified	3.0
Escherichia-Shigella	2.0
Burkholderiaceae_unclassified	1.9
Moraxella	1.9
Enterobacteriaceae_unclassified	1.8
Actinomyces	1.7
Actinobacillus	1.7
Weeksellaceae_unclassified	1.6
Ruminococcus_1	1.2
Total	**52.0**

636 Table 3. Relative abundance of unique genera identified in the dental biofilm of
637 10 sheep with periodontitis

Genus	**%**
Petrimonas	17.2
Acinetobacter	5.5
Aerococcus	3.0
Fretibacterium	2.7
Christensenellaceae_R-7_group	2.5
Fastidiosipila	2.3
Succiniclasticum	2.3
Uncultured	2.2
Peptostreptococcus	1.9
Filifactor	1.5
F0058	1.3
Absconditabacteriales_(SR1)_ge	1.0
Tannerella	1.0
Total	**44.2**

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