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Microbiomes associated with bovine periodontitis and oral health

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ABSTRACT

Periodontitis is an infectious polymicrobial, immuno-inflammatory disease of multifactorial aetiology that has an impact on the health, production and welfare of ruminants. The objective of the present study was to determine the microbial profiles present in the gingival sulcus of cattle considered periodontally healthy and in the periodontal pocket of animals with periodontitis lesions using high-throughput bacterial 16S rRNA gene sequencing. Subgingival biofilm samples were collected from 40 cattle with periodontitis and 38 periodontally healthy animals. In total, 1923 OTUs were identified and classified into 395 genera or higher taxa. Microbial profiles in health differed significantly from periodontitis in their composition ($p < 0.0001$, $F = 5.30$; PERMANOVA) but no statistically significant differences were observed in the diversity of healthy and periodontitis microbiomes. The most prevalent taxa in health were Pseudomonas, Burkholderia and Actinobacteria, whereas in disease these were Prevotella, Fusobacterium and Porphyromonas. The most discriminative taxa in health were Gastranaerophilales, Planifilum and Burkholderia, and in disease these were Elusimicrobia, Synergistes and Propionivibrio. In conclusion, statistically significant difference exists between the microbiome in bovine oral health and periodontitis, with populations showing 72.6% dissimilarity. The diversity of the bacteria found in health and periodontitis were similar and bacteria recognised as periodontal pathogens showed increased abundance in disease. In this context, the main components of bacterial homeostasis in the biofilm of healthy sites and of dysbiosis in periodontal lesions provide unprecedented indicators for the evolution of knowledge about bovine periodontitis.

1. Introduction

Periodontitis is a polymicrobial infectious disease initiated by a synergistic and dysbiotic microbial community (Hajishengallis and Lamont, 2012) that affects the health, production and welfare of ruminants. Usually neglected in animal production, it is a purulent, chronic and progressive infectious process that causes cumulative changes that occur throughout the lives of animals that is characterised by periodontal pocket formation, gingival recession, mobility, loss of clinical insertion and premature tooth loss (Page and Schroeder, 1976; Döbereiner et al., 2000; Borsanelli et al., 2016a).

The natural occurrence of periodontal lesions in sheep and cattle has been recorded in several countries and epidemiological contexts (Aitchison and Spence, 1984; Döbereiner et al., 2000; Ingham, 2001; Fadden et al., 2015; Borsanelli et al., 2016a).

Some species of oral bacteria, such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, have been considered to be important in the development of periodontal disease in humans and other animal species. In cattle, the participation of some potential periodontopathogens in lesions of the disease has also been recognised, including *Fusobacterium nucleatum, Trueperella pyogenes* and some species of the *Porphyromonas, Prevotella* and *Treponema* genera (Blobel et al., 1987; Dutra et al., 2000; Borsanelli et al., 2015a, 2015b).

An important step for understanding the participation of putative bacterial pathogens in periodontitis is to determine the bacterial composition in the healthy gingival sulcus and in the periodontal pocket. It has been estimated that approximately 50% of the human oral microbiota is uncultivable (Socransky et al., 1963), and an analogous situation is likely in the bovine oral cavity.

At present, it is possible to determine almost all the community of commensal and potentially pathogenic bacteria that inhabit the bovine oral cavity, both in health and in periodontitis, using culture-

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independent methods. Bacterial 16S rRNA gene sequencing allows detection of not only cultivable species but also uncultivable bacteria and novel species that may be important in disease pathogenesis. This method has already been used to determine the oral bacterial community of horses, sheep and dogs with and without periodontal lesions (Riggio et al., 2011, 2013; Kennedy et al., 2016) and to determine the oral microbiome of periodontally healthy dogs and cats (Dewhirst et al., 2012; Holcombe et al., 2014; Sturgeon et al., 2014).

The objective of the present study was to determine the microbial profiles present in the gingival sulcus of cattle considered periodontally healthy and in the periodontal pockets of animals with periodontitis lesions using high-throughput bacterial 16S rRNA gene sequencing.

2. Materials and methods

2.1. Collection of dental plaque

Two-hundred dental arches of bovines were examined at a local slaughterhouse in Scotland during the period September to November 2015 and dental plaque samples were collected. Since periodontitis includes inflammatory alterations of the gingival tissue and a progressive loss of periodontal attachment and alveolar bone, the criteria for the diagnosis of the disease was the presence of gingival retraction (i.e. the tooth root was visible at the gingival margin), the existence of a periodontal pocket (the distance from the gingival margin to the bottom of the periodontal pocket as measured with a graduated universal periodontal probe) greater than 5 mm in depth and suppuration (presence of pus inside the periodontal pocket; usually observed when curetting the bottom of the pocket). Since samples were collected post-mortem it was not possible to evaluate bleeding on probing. The periodontally healthy group had no evidence of gingival retraction, no periodontal pockets, no suppuration and no evidence of any other oral disease. The probe was inserted to the base of the periodontal pocket, applying a light force and moved gently around the tooth surface and pocket depth measurement obtained. Samples were collected within 30 min of death.

Subgingival plaque was collected from the periodontal pocket of 40 cattle with periodontitis and from the gingival margin around premolar 2-premolar 3 of 38 periodontally healthy cattle with the aid of a sterile curette. All samples were placed in 250 µL of RNAlater (Sigma-Aldrich, Gillingham, UK) and stored at −20 °C until required.

2.2. DNA preparation

Subgingival plaque samples were mixed by vortexing for 30 s. To 150 µL of each sample was added 200 µL phenol saturated with Tris-HCl (pH 8.0), 200 µL lysis buffer and 250 µL glass beads (0.1 mm) suspended in TE buffer. Bead beating was conducted in a BioSpec Mini-Beadbeater for 2 min at 2100 oscillations/min. DNA was then purified using the AGOWA mag Mini DNA Isolation Kit (AGOWA, Berlin, Germany).

2.3. High-throughput sequencing

Bacterial 16S rRNA genes were amplified using primers GTGCCAG CMGCCGCGGTAA (forward) and GGACTACHVGGGTWTCTAAT (reverse) that target the V4 region. Amplicon libraries were purified, analysed and paired-end (2 × 251 bp) sequenced using the Illumina MiSeq as described previously (Kennedy et al., 2016).

2.4. Bioinformatics analysis

USEARCH version 8.0.1623 (Edgar and Flyvbjerg, 2015) was used to merge, process and cluster sequencing reads. Following merging, quality filtering (maximum expected error rate 0.5 and no ambiguous bases allowed) was conducted and sequences clustered into operational taxonomic units (OTUs) using the settings: uparse_maxdball 1200, only *de novo* chimera checking, usearch_global with -maxaccepts 8 -maxrejects 64 -maxhits 1. The most abundant sequence of each OTU was selected using QIIME version 1.8.0 (Caporaso et al., 2010) and a taxonomy was then assigned with the RDP classifier (Cole et al., 2009) with a minimum confidence of 0.8 and the 97% representative sequence set based on the SILVA rRNA database, release 119 for QIIME (Quast et al., 2013).

2.5. Statistical analysis

Normalisation of sequencing depth was achieved by random subsampling of the dataset to 50%. Diversity analysis (Shannon Diversity Index, Chao-1 estimate of total species richness), data ordination by principal component analysis (PCA) and assessment of differences between microbial profiles of the two groups by one-way PERMANOVA were performed using PAleontological STatistics (PAST; v3.02) software (Hammer et al., 2001). PERMANOVA was used with Bray-Curtis similarity distance. For PCA, the OTU dataset was additionally normalised by $\log_2$-transformation. Diversity output was compared using the Mann-Whitney U test in SPSS (version 21.0). Linear discriminant analysis effect size (LEfSe) was used to determine which OTUs and taxa contribute to differences between the groups (Segata et al., 2011).

3. Results

3.1. Sequencing output

Sequencing generated 1,296,437 read pairs and after merging and quality filtering 86.5% of these (i.e. 1,122,045) remained. Following clustering (including chimera checking) 88.5% (992,913) of these 1,122,045 sequences were mapped to OTUs and were thus present in the OTU table used for downstream analysis. After random subsampling at 50%, 1923 OTUs were identified and classified into 395 genera or higher taxa. The most prevalent genera or higher taxa are shown in Fig. 1.

3.2. Microbial profile analysis

Differences between the bovine oral microbiomes of oral health and periodontitis were evident as determined by principal component analysis (Fig. 2). Generally, the healthy and periodontitis samples tended to cluster separately and the healthy samples demonstrated lower intra-sample variability relative to the periodontitis samples. A statistically significant difference between the microbial profiles of health and disease was observed ($p < 0.001$, $F = 5.30$, PERMANOVA). Bray-Curtis analysis demonstrated 72.6% dissimilarity between the two groups. No statistically significant differences were observed in species richness or diversity of healthy and periodontitis microbiomes (Fig. 3).

On average, healthy samples contained 238 OTUs (SD 158, range 66–698), while the periodontitis samples contained 245 OTUs (SD 114, range 79–577).

3.3. Differences in composition between healthy and periodontitis samples

From 395 genera or higher taxa, 45 taxa were statistically significantly different between the two groups ($p < 0.05$); of these, 25 taxa had a linear discriminant analysis (LDA) score above 2 and the majority (17 of 25 taxa) were associated with disease (Fig. 4). Taxa are ranked by the effect size in LEfSe.

The most discriminative taxa in the samples of healthy animals were Gastranaerophilales, Planifilum, Burkholderia and Arcobacter; in animals with periodontitis, the most discriminative taxa were Elusimicrobia, Synergistes, Propionivibrio and Fusobacteria (Fig. 4).

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