Oral microbial profile variation during canine ligature-induced peri-implantitis development

Shichong Qiao,† Dongle Wu,† Mengge Wang, Shujiao Qian, Yu Zhu, Junyu Shi, Yongjun Wei,⁎ and Hongchang Lai,⁎

Abstract

Background: Dental implants have become well-established in oral rehabilitation for fully or partially edentulous patients. However, peri-implantitis often leads to the failure of dental implants. The aim of this study was to understand the core microbiome associated with peri-implantitis and evaluate potential peri-implantitis pathogens based on canine peri-implantitis model.

Results: In this study, three beagle dogs were used to build peri-implantitis models with ligature-induced strategy. The peri-implant sulcular fluids were collected at four different phases based on disease severity during the peri-implantitis development. Microbial compositions during peri-implantitis development were monitored and evaluated. The microbes were presented with operational taxonomic unit (OTU) classified at 97% identity of the high-throughput 16S rRNA gene fragments. Microbial diversity and richness varied during peri-implantitis. At the phylum-level, Firmicutes decreased and Bacteroides increased during peri-implantitis development. At the genus-level, Peptostreptococcus decreased and Porphyromonas increased, suggesting peri-implantitis pathogens might be assigned to these two genera. Further species-level and co-occurrence network analyses identified several potential keystone species during peri-implantitis development, and some OTUs were potential peri-implantitis pathogens.

Conclusion: In summary, canine peri-implantitis models help to identify several potential keystone peri-implantitis associated species. The canine model can give insight into human peri-implantitis associated microbiota.

Keywords: Microbiota, Ligature-induced, Canine peri-implantitis, Microbial variation, Keystone taxonomy

Background

With the development of oral technologies, dental implants are a highly successful and predictable treatment for replacing missing teeth, which can rehabilitate oral health-related quality of life [1]. However, breakdown of soft and hard tissue around osseointegrated implants would result in the failure of dental implants known as inflammatory peri-implant disease [2–4]. Clinically, inflammatory peri-implant diseases are categorized into peri-implant mucositis and peri-implantitis [5, 6]. Peri-implant mucositis is defined as a reversible inflammatory reaction in the soft tissues surrounding a functioning implant, while peri-implantitis is described as inflammatory reactions involving supporting bone loss [5, 6]. It has been estimated that the rate of implant-based and subject-based peri-implantitis are 9.25% and 19.83%,
respectively [7]. In another study, the prevalence of peri-
implantitis is up to 22% [8]. Therefore, understanding
the mechanism that induces peri-implantitis is essential
in current restorative dentistry, and might help to find
preventive strategies for peri-implantitis and provide ef-
effective peri-implantitis treatment methods [9, 10].

It is generally accepted that peri-implantitis is a com-
exed disease with multiple risk factors, and it initiates
from a bacterial challenge and host overreaction [11,
12]. Much effort has been made to study the peri-
implant biofilm and its difference from periodontal bio-
film [13], and reveal the bacterial colonization in healthy
and diseased states [14]. The comparison between dis-
 eased peri-implant and healthy periodontal tissues has
been applied [15]. Several potential microbial strains,
such as Porphyromonas gingivalis and Prevotella inter-
medius, have been detected in oral biofilm at the peri-
implantitis sites. However, there is no clear consensus
for core microbiota associated with peri-implantitis [16].
Traditional cultured strategies provide valuable informa-
tion regarding known pathogens, but limited data on uncculturuble species and microbiota during peri-
implantitis development are available. Global investiga-
tion of microbiota variation during peri-implantitis de-
velopment would make it possible to identify peri-
implantitis associated microbiota [17, 18], and high-
throughput sequencing of 16S rRNA gene technology
provides a wealth of data pertaining to the differences
between healthy and diseased implants [19, 20].

Experimental peri-implantitis animal models, such as
mice and dogs, have been developed to study the mecha-
nisms and development of marginal bone loss [8, 21]. In
previous animal studies, undisturbed peri-implant
plaque accumulation often led to negligible or no mar-
ginal bone loss [22]. In order to obtain reliable peri-
implantitis animal models, sub-marginal ligatures of cot-
ton, silk or other materials have been used to induce and speed up the peri-implantitis process [19, 23, 24].
Normally, sub-marginal ligation results in significant
bone loss in a few weeks, and the peri-implantitis model
can be established in a short time [25, 26]. However, in-
vestigation of microbial dynamics during peri-implantitis
model establishment had not been reported.

Here, the present study was designed to conduct a
canine peri-implantitis model using sub-marginal liga-
tures method. The clinical parameters at different peri-
implantitis phases were evaluated, and microbial varia-
tion associated with the severity of tissue de-
struction and inflammatory progression of experimen-
tal peri-implantitis were investigated. The core
microbiota associated with peri-implantitis and their
networks were analyzed, and the potential keystone
microbial taxa associated with peri-implantitis were
discussed.
Microbiota analyses

The microbial distribution of the same phases were similar (Supplementary Figure 3), while the microbial distribution of different phases harbored different phyla (Fig. 3a). At Phase T0 of the healthy state, the most dominant phyla were Firmicutes (68.1%), Actinobacteria (11.7%), and Bacteroidetes (9.4%). At Phase T1 during the first 2 weeks of peri-implantitis, the most dominant phyla were Firmicutes (27.8%), Bacteroidetes (35.8%), and Spirochaetes (10.2%). At Phase T2, the most dominant phyla were Firmicutes (39.9%), Bacteroidetes (30.0%), and Euryarchaeota (14.1%). At Phase T3 of the self-spontaneous recovery period, the most dominant phyla were Firmicutes (30.7%), Bacteroidetes (33.9%), Spirochaetes (13.6%), and Euryarchaeota (6.1%). The Firmicutes, Bacteroidetes, and Synergistetes distribution between Phase T0 and Phase T1 to T3 displayed significant differences. The Euryarchaeota distribution between Phase T0 and T2, T0 and T3, T1 and T2, and T1 and T3 displayed significant differences (Supplementary Table 2). Besides, a few other phyla displayed differences between different phases (Supplementary Table 2).

At the genus-level, microbial distribution at different phases changed (Fig. 3b). At Phase T0, the most dominant genera were Peptostreptococcus (43.5%), Actinomyces (7%), and Streptococcus (4.9%). At Phase T1, the most dominant genera were Portostreptococcus (25.6%), Treponema (10.0%), and Fusobacterium (8.8%). At Phase T2, the most dominant genera were Portostreptococcus (13.0%), Peptostreptococcus (10.5%), and Methanobrevibacter (5.7%). At Phase T3, the most dominant genera were Portostreptococcus (20.0%), Treponema (13.4%), and Portostreptococcus (10.9%). Some genera, including Peptostreptococcus, Porphyromonas, Treponema and Fretibacterium, displayed significant differences between Phase T0 and Phase T1 to T3. The Methanobrevibacter displayed significant differences between T0 and T2, T0 and T3, T1 and T2, and T1 and T3 (Supplementary Table 3). No significant differences of Bacteroides and Streptococcus between Phase T0 and Phase T1 to Phase T3 were observed.

The dominant OTUs in the microbiota

Most OTUs with average compositions > 1% in the four phases had > 97% identity with known isolates (Table 2).
Table 1 Average alpha diversity parameters during Peri-implantitis development of the dog teeth

| Phase | Richness | Chao1   | Shannon_2 | Simpson | Dominance | Equitability |
|-------|----------|---------|-----------|---------|-----------|--------------|
| T0    | 294 ± 78 | 295 ± 78| 4 ± 0.8   | 0.2 ± 0.1| 0.8 ± 0.1 | 0.5 ± 0.1    |
| T1    | 337 ± 76 | 339 ± 75| 5.3 ± 0.5 | 0.07 ± 0.03| 0.9 ± 0.03 | 0.6 ± 0.08   |
| T2    | 389 ± 34 | 390 ± 34| 5.6 ± 0.4 | 0.05 ± 0.02| 1 ± 0.02   | 0.7 ± 0.04   |
| T3    | 305 ± 78 | 307 ± 76| 5.1 ± 0.4 | 0.06 ± 0.02| 0.9 ± 0.02 | 0.6 ± 0.07   |

Fig. 3 Microbial compositions of the implant samples during Peri-implantitis development at phylum-level (a) and genus-level (b)
Besides, OTU distribution at different phases displayed difference or significant difference (Supplementary Table 4). OTU_1, which composes 43.46% at Phase T0, might be one Peptostreptococcus canis strain isolated from subgingival plaque from canine oral cavity [27]. Compared with Phase T0, P. canis composition decreased to 5.5% at Phase T1, 10.5% at Phase T2, and 10.9% at Phase T3. The compositions of OTU_3 which might be Porphyromonas macacea increased from < 0.2% at Phase T0 to 6.3% at Phase T1, 1% at Phase T2, and 11% at Phase T3 [28]. OTU_7, which was assigned to the genus of Porphyromonas, composed 0.12% at Phase T0, but increased to 0.48% at Phase T1, 5.43% at Phase T2, and 6.71% at Phase T3. OTU_13, which was

### Table 2

The distribution of dominant OTUs identified during Peri-implantitis development and the 16S rRNA gene fragments of their closest isolates and uncultured bacteria

| Closest isolates          | Identity                        | Closest uncultured (nr/nt) Identity | Phase T0 | Phase T1 | Phase T2 | Phase T3 |
|---------------------------|---------------------------------|-------------------------------------|----------|----------|----------|----------|
| OTU_1                     | Peptostreptococcus canis (NR_117641.1) | Uncultured bacterium (JQ192915.1)        | 43.46%   | 6.29%    | 9.5%     | 10.9%    |
| OTU_3                     | Porphyromonas macacea (NR_025908.1)  | Uncultured bacterium (KX437329.1)       | 5.5%     | 0.19%    | 0.95%    | 10.98%   |
| OTU_7                     | Porphyromonas endodontalis (NR_113085.1) | Uncultured Porphyromonas sp. canine oral (JN713348.1) | 9.30%    | 0.48%    | 5.43%    | 6.71%    |
| OTU_13                    | Fretibacterium fastidiosum (NR_108538.1) | Uncultured Fretibacterium sp. Feline (KJ462182.1) | 100%     | 3.05%    | 3.99%    | 4.66%    |
| OTU_5                     | Bacteroides pyogenes (NR_113048.1)  | Uncultured Bacteroides (MH755473.1)      | 3.49%    | 0.61%    | 2.32%    | 5.30%    |
| OTU_9                     | Fusobacterium simiae (NR_113318.1)  | Uncultured Fusobacterium sp. canine (JN713401.1) | 0.19%    | 6.29%    | 0.95%    | 10.98%   |
| OTU_8                     | Porphyromonas gulae (NR_113088.1)   | Uncultured bacterium (HM328336.1)        | 99.56%   | 6.92%    | 2.65%    | 0.88%    |
| OTU_10                    | Methanobrevibacter oralis (LN989260.1) | Uncultured Methanobrevibacter (JN52095.1) | 97.88%   | 0.29%    | 6.92%    | 2.65%    |
| OTU_19                    | Treponema denticola (NR_074582.1)   | Uncultured Treponema (JN713361.1)        | 99.56%   | 6.92%    | 2.65%    | 0.88%    |
| OTU_10                    | Porphyromonas canginivalis (NR_113080.1) | Uncultured bacterium (JF241087.1)       | 100%     | 3.05%    | 3.99%    | 4.66%    |
| OTU_6                     | Intestinimonas butyriciproduens (NR_118554.1) | Uncultured Clostridiales bacterium (L624863.1) | 97.88%   | 0.29%    | 6.92%    | 2.65%    |
| OTU_14                    | Treponema sp. PTB (AM980447.1)       | Uncultured Treponema sp. (GQ424168.1)    | 99.56%   | 6.92%    | 2.65%    | 0.88%    |
| OTU_11                    | Odoribacter denticanis strain (NR_042977.1) | Uncultured Odoribacter denticanis canine oral (JN713247.1) | 97.91%   | 3.37%    | 0.79%    | 4.89%    |
| OTU_12                    | Labilibacter aurantiacus (NR_156071.1) | Uncultured bacterium (KJ874155.1)       | 99.56%   | 6.92%    | 2.65%    | 0.88%    |
| OTU_15                    | Filifactor villosus (NR_041928.1)    | Uncultured Filifactor sp. Feline (KJ462053.1) | 99.56%   | 6.92%    | 2.65%    | 0.88%    |
assigned to the genus of *Fretibacterium*, composed only
0.58% at Phase T0, but it increased to >3% at Phase T1
to T3. In particular, some methanogens, such as OTU_2
and OTU_4, composed <0.25% at Phase T0. The com-
position of OTU_2 and OTU_4 increased to 13.91% at
phase T2, and their composition decreased to 6.08% at
Phase T3. A few other dominant OTUs were assigned to
the typical oral microbiota or clinical pathogens, and the
compositions of clinical pathogens increased (Table 2).
For example, OTU_19 was assigned to teeth infection
associated bacteria *Treponema denticola* [29, 30]. OTU_
19 composition at Phase T0 was 0.09%, and its compos-
ition increased to 3.37% at Phase T1, 0.79% at Phase T2,
and 4.89% at Phase T3.

**Microbial similarity of the samples at different phases**
The microbial composition was used to compare sam-
ple at different phases. The NMDS result based on Bray
Cutis distance showed that the six samples at Phase T0
were clustered and were obviously different from other
peri-implantitis samples. Phase T1 samples were clus-
tered, and were different from other samples. Moreover,
twelve samples at Phase T2 and Phase T3 were clus-
tered, and were different from samples at Phase T0 and
Phase T1 (Fig. 4a). The UPGMA tree based on Bray
Cutis distance also showed that samples at different
phases were clustered. In particular, the six samples at
Phase T0 were clustered, and another eighteen samples
at Phase T1 to T3 were clustered (Fig. 4b). In addition,
the distance between samples at Phase T2 and Phase T3
was shorter than the distance between samples at Phase
T1 and Phase T2 or Phase T1 and Phase T3. In all, the
UPGMA result was in accordance with the NMDS result
that the healthy state Phase T0 samples were divergent
from the diseased samples at Phase T1 to Phase T3.

**Network analyses of the microbiota**
The correlations of all the samples were calculated, and
only the dominant OTUs (>0.5%) with strong relation-
ship (ρ >0.6 and P <0.05) were used for network ana-
lyses [31, 32]. The co-occurrence network contained 37
nodes (OTUs) and 208 edges. The compositions of these
OTUs in the 24 samples were between 0.54% and 4.6%
(Fig. 5). Among the 208 edges, only one edge between
OTU_18 and OTU_28 indicated negative correlation.
The mean edge per node was 11, and the eccentricity of
the node was 3.6. The average shortest path length was
2.1 with a closeness centrality of 0.5. The average radial-
ity of the node was 0.8, and the topological coefficient
was 0.5.

Among the 37 nodes (OTUs), 13 of them were
assigned to *Bacteroidetes*, and 12 of them were assigned
to *Firmicutes*. The composition of *Bacteroidetes* and *Fir-
micutes* OTUs of the 37 OTUs was 67.57%. Interestingly,
*Bacteroidetes* and *Firmicutes* were the two most domin-
ant phyla of the 24 samples, and composed 68.89% of

![Fig. 4](image_url)

**Fig. 4** Microbial distribution differences of the collected implant samples during Peri-implantitis development were displayed with the NMDS (a) and UPGMA (b) based on Bray Curtis distance.
the microbiota (Fig. 3a). All these 37 OTUs were among the top39 OTUs of the whole microbiota. However, the most abundant OTU in the microbiota, OTU_1, was not in the network. Besides, OTU_122, which composed 12.34% of one sample and had an average composition in the 24 samples of 0.56%, was not in the network. Based on the connection in the network and average compositions, OTU_3, OTU_7, OTU_13, OTU_18, OTU_23, OTU_29, OTU_26, OTU_41, and OTU_30 were identified as the keystone taxa (Fig. 5). Additionally, the compositions of all these nine OTUs at Phase T0 were lower than their corresponding compositions in Phase T1 to Phase T3. Among the nine keystone taxa, four of them (OTU_18, OTU_23, OTU_26, OTU_30) were assigned to Firmicutes, three of them (OTU_3, OTU_7, OTU_29) were assigned to Bacteroidetes, OTU_13 was assigned to Synergistetes, and OTU_41 was assigned to Proteobacteria.

**Discussion**
Peri-implantitis strongly affects adjacent tissues, resulting in losing the function of the osseointegrated implant [7, 8]. Peri-implantitis is caused by microbial infection, but the associated pathogenic microbes are unclear [9]. The microbial distributions between the healthy dental sites and peri-implantitis sites in subjects with chronic periodontitis had been conducted, showing diseased implants and corresponding periodontally healthy sites harbored different microbiota [33]. However, global microbial variation during peri-implantitis development was unknown. The comparison between human healthy and diseased peri-implant sites had been tried, but the
human data might be affected by gender, antibiotic intake, or age [34]. The animal model can provide peri-implantitis samples with the same criteria [35]. In this study, the clinical parameters at different phases during peri-implantitis setup and progression in a canine model were collected, showing that the disease severity increased during peri-implantitis development (Fig. 2). Moreover, the microbiota changed dramatically during the shift from healthy status to peri-implantitis status.

The clinical parameters showed that the disease severity increased and the peri-implantitis model was successfully built, indicating cotton ligature around dental implant is one effective strategy for peri-implantitis induction [19]. During peri-implantitis development, microbial richness and diversity increased from the healthy phase to diseased phases (Table 1), suggesting high microbial richness and diversity were associated with severe infection. These results were different from previous results that high microbial richness was associated with healthy samples [33]. However, the increased richness and diversity in canine peri-implantitis development complements earlier study results that peri-implantitis sites exhibited higher bacterial counts compared to healthy sites [36]. The increased microbial species during in peri-implantitis development might not only derive from the original dental implant sites, but also from the oral microbiome or environmental microbiome [14]. The decreased microbial richness and diversity from Phase T2 to Phase T3 (Table 1) suggested that the host self-spontaneous progression recovery system functioned and the health status improved after removal of the ligatures.

The human oral microbiome database (HOMD) collects bacterial species distributed in the oral region and the upper respiratory tracts, and the dominant phyla were Actinobacteria, Firmicutes, and Proteobacteria [37]. Different from the microbial collection in HOMD, the main phyla in the healthy implants were Firmicutes (51.9%), Bacteroidetes (18.5%), Fusobacteria (11.1%), and Proteobacteria (7.4%), while the dominant phyla in peri-implantitis were Firmicutes (30.6%), Bacteroidetes (40.3%), Fusobacteria (13.9%), and Proteobacteria (5.6%) [15]. The decrease of Firmicutes and increase of Bacteroidetes in human peri-implantitis were the same as the microbiota variation during peri-implantitis development in this study [34], showing that phyla variation might be a common phenomenon during peri-implantitis development in human and animals. The Synergistetes and Spirochaetes compositions of human peri-implantitis sites were higher than that of human healthy sites [34, 36], and their compositions increased during peri-implantitis development (Fig. 3). Compared with healthy dental implant sites, T. denticola were often detected in peri-implantitis sites [36]. In this study, more

Peri-implantitis has been successfully induced by oral infection with P. gingivalis in mice, and P. gingivalis has often been identified at peri-implantitis sites [12, 38, 39]. OTU_3 and OTU_7 assigned to Porphyromonas were identified in this study, and the compositions of OTU_3 and OTU_7 were higher at the diseased phases (Phase T1 to Phase T3) than at the healthy phase (Phase T0), showing Porphyromonas might be associated with peri-implantitis. OTU_3 and OTU_7 were identified as keystone taxa, suggesting these two strains might be one potential peri-implantitis cause [10]. The compositions of methanogens were higher at Phase T2 and Phase T3, but they were not keystone taxa, indicating methanogens might not be an indicator of peri-implantitis development [40]. Prevotella nigrescens was also identified to be associated with peri-implantitis lesion [12, 41], but no Prevotella was identified in oral peri-implantitis microbiome in this study (Table 2). Though the microbiota between humans and animals were slightly different at species-level, establishment of animal oral models might give insights into microorganisms associated with human oral diseases in the future.

Conclusion
In summary, we successfully established an experimental peri-implantitis animal model using ligature-induced method, and the increased severity of inflammation is consistent with the progression of the peri-implantitis disease as we expected. The investigation of the microbial variation during peri-implantitis development identified several OTUs associated with peri-implantitis. This study provides insight into the peri-implantitis microbiome and deepens our understanding of keystone taxa during peri-implantitis development.

Methods
Animals
The animal experiment had been reviewed and approved by the Ethics Committee for Animal Research in Shanghai 9th People's Hospital, Shanghai Jiao Tong University. Three two-year-old male beagles weighing 10–15 kg were bought from Shanghai Jiagan Biotech Co. Ltd. and used for the peri-implantitis experiment in this study. The dogs were housed individually and fed a soft diet at the Animal Research Center of Shanghai 9th People's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China. All the dogs had fully erupted permanent dentition and good general health before the experiment. The dogs were sacrificed in enthanasia by
intravenous administration of a lethal pentobarbital sodium dose of 120 mg/kg weight.

The remaining teeth were cleaned twice a week with 0.12% chlorhexidine and soft toothbrush. All surgical procedures were performed under general anesthesia by sedation with 2% xylazine hydrochloride and ketamine hydrochloride/zolazepam hydrochloride. The surgical sites were locally anesthetized using 2% lidocaine hydrochloride with 1:100,000 epinephrine. The surgical site was shaved and cleaned with chlorhexidine ethanol solution 0.5 mg/ml, and covered with a sterile surgical drape. The animals were made to fast for 12 h preoperatively, but allowed to drink water ad libitum.

Teeth removal and implant placement
All the premolars (M1-M4) in the mandible (eight teeth of each dog) were extracted bilaterally from the three dogs using a minimally invasive approach (Supplementary Figure 1). After a healing period of 12 weeks, three implants (3.0 mm in diameter and 9.0 mm in length, Astra, Switzerland) were inserted at the extraction sites of both sides. Mechanical cleaning was performed to remove supragingival calculus around natural teeth 1-week pre-operation. All implants were left to heal in a submerged position for another 12 weeks, and the second-stage surgery was conducted to replace cover screws with healing abutments (width: 4 mm, height: 4 mm) (Phase T0) (Fig. 1). No plaque control procedures were performed after ligature placement.

Induction of peri-implantitis and sample collection
A cotton ligature was ligated around each implant fixture below the healing abutment to allow for plaque accumulation and inflammatory infection [19, 42]. Two weeks later, a clinical and radiologic examination indicated that the peri-implant mucositis was established, and the samples were collected (Phase T1). Meanwhile, additional ligation was performed below the preexisting ligatures every 2 weeks, and the samples were collected after another 6 weeks (Phase T2), while radiography showed significant bone loss. Then the ligatures were removed, and the inflammation sites were left alone for self-spontaneous progression recovery. Four weeks later, samples were collected (Phase T3) (Fig. 1).

During sample collection, the sampling sites were dried with an air pistol, and samples of peri-implant sulcular fluid (PISF) were collected [39]. Sampling sites were exposed from moisture after supra-mucosal plaque was removed. Filter strips (2 mm × 10 mm) were inserted into the bottom of the peri-implant sulcus with a mild resistance at the buccomesial and buccodistal aspects of peri-implant sulcus for 30 s. All the collected strips were stored at −80 °C before use.

Clinical and radiographic examination
Clinical measurements were taken at the mesial, buccal, distal, and lingual sites of each implant using a UNC15 probe (Hu-Friedy, Chicago, IL, USA). The peri-implant probing depths (PD) and the relative clinical attachment levels (rCAL) in mm were measured. In addition, the Modified Plaque Index (mPI) was measured along the mucosal margin and recorded as presence (1) or absence (0); the bleeding on probing (BOP) was measured 15 s after probing and recorded as presence (1) or absence (0).

These measurements were performed by two examiners after a calibration exercise demonstrating 95.3% concordance within 1 mm for measurements of PD. The disease severity was indicated based on the measured clinical parameters.

Intra-oral radiographs of all the implant sites were taken using paralleling technique combined with long cone (Rinn XCP, Dentsply, Elgin, IL, USA).

DNA extraction and sequencing
DNA were extracted from the collected strip samples using DNeasy Kit (Cat No./ID: 47014, Qiagen, Germany). DNA of each sample was diluted to proper concentration for gene amplification of 16S rRNA gene V3-V4 regions [43]. The Phanta Max Super-Fidelity DNA Polymerase (Vazyme Bio-tech, Nanjing, China) was used for 16S rRNA gene fragment amplification following the manufacturer’s procedure, and the amplification cycles were 27. Further sequencing was implemented by DeepLab Co., Ltd. (Shanghai, China).

16S rRNA gene analyses
USEARCH software with default parameters were used to analyze the fastq files [44]. The USEARCH UPARSE was used to classify the obtained clean reads into operational taxonomic unit (OTU) based on 97% identity, and UPARSE analysis pipeline was applied to remove chimeric reads [43, 45]. USEARCH was used to analyze the phylogenetic classification of the obtained 16S rRNA gene fragment sequences, and RDP training set (version v16) with a confidence threshold of 0.8 was used as the reference database [46]. In addition, USEARCH was further used for the α and β diversity analyses [47]. The unweighted pair-group method with arithmetic means (UPGMA) and non-metric multidimensional scaling (NMDS) based on Bray Curtis were analyzed by R software [47].

The co-occurrence network analysis strategy was carried out to explore co-occurrence of the abundant OTUs in the microbiota [48]. The correlation between OTUs was calculated with spearman’s method with pairwise distance. To identify keystone taxa, only strong correlation between OTUs’ relationship with r > 0.6, and significant correlation with P < 0.05 was kept. Finally, only OTUs with relative composition > 0.5% were used for further co-occurrence analysis and network construction using Cytoscape [32, 49].
Acknowledgments
We thank Dr. Boyang Ji at Chalmers University of Technology and Dr. Xiaogang Xiang at Shanghai Jiao Tong University School of Medicine for revising English writing of this manuscript. We sincerely thank Lei Zhang from DeepLab Co., Ltd. (Shanghai, China) for bioinformatic help. We sincerely thank for the support from Shanghai Zammmax Biotech Co., Ltd.

Authors’ contributions
Conception and design: SCQ, YW, HL; Data acquisition: SCQ, YW, DW, SJQ, YZ, JS; Analyses and interpretation of the data: SCQ, YW, MW; Drafting of the article: YW, CQ, DW, MW. All authors have read and approved the manuscript.

Funding
This study has been supported by Science and Technology Commission of Shanghai Municipality (16441903600, 20531906100), the National Natural Science Foundation of China (81600902), Clinical Research Plan of SHDC 16CR3033A, and the Project of Biobank (NO. YBK201906) from Shanghai Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine.

Availability of data and materials
All the obtained data were deposited in Genbank database, and the accession numbers of the samples were SRR10695872-SRR10695895.

Ethics approval and consent to participate
The experiment had been reviewed and approved by the Ethics Committee for Animal Research in Shanghai 9th People’s Hospital, Shanghai Jiao Tong University.

Competing interests
The authors declare that they have no competing interests.

Received: 16 July 2020 Accepted: 18 September 2020
Published online: 29 September 2020

References
1. Peres MA, Macpherson LMD, Weyant RJ, Daly B, Venturelli R, Mathur MR, Listi S, Celeste RK, Guarnizo-Herreno CC, Kears C, et al. Oral diseases: a global public health challenge. Lancet. 2019;394(10194):249–60.
2. Manor Y, Oualid S, Mandinger O, Chauhut G, Nislan J. Characteristics of early versus late implant failure: a retrospective study. J Oral Maxil Surg. 2009;67(12):2649–52.
3. Insa A, Monje A, Wang HL, Miron RJ. Basis of bone metabolism around dental implants during osseointegration and peri-implant bone loss. J Biomed Mater Res A. 2017;105(7):2075–89.
4. Derks J, Schaller D, Hakansson I, Wennström JL, Tomasi C, Berglundh T, Peri-implantitis – onset and pattern of progression. J Clin Periodontol. 2016;43(4):383–8.
5. Berglundh T, Armitage G, Araujo MG, Avila-Ortiz G, Blanco J, Camargo PM, Chen S, Cochran D, Derks J, Figuero E, et al. Peri-implant diseases and conditions: Consensus report of workshop 4 of the 2017 World workshop on the classification of periodontal and peri-implant diseases and conditions. J Periodontol. 2018;89(Suppl 1):S133–82.
6. Papapostou PN, Sazn M, Budurell N, Dietrich T, Feres M, Fine DH, Flemming TF, Garcia R, Giannobile W, Graziani F, et al. Periodontitis: Consensus report of workshop 2 of the 2017 World workshop on the classification of periodontal and Peri-implant diseases and conditions. J Periodontol. 2018;89(Suppl 1):S173–82.
7. Lee CT, Huang YW, Zhu L, Weltman R. Prevalences of peri-implantitis and peri-implant mucositis: systematic review and meta-analysis. J Dent. 2017;62:1–12.
8. Derks J, Tomasi C. Peri-implant health and disease. A systematic review of current epidemiology. J Clin Periostotol. 2015;42(Suppl 16):S158–71.
9. Savic GE, Cosgarea R, Scalean A. Prevalence and mechanisms of Peri-implant diseases. J Dent Res. 2017;96(1):31–7.
10. Costa FO, Ferreira SD, Cortelli JR, Lima RPE, Cortelli SC, Cota LOM. Microbiological profile associated with peri-implant diseases in individuals with and without preventive maintenance therapy: a 5-year follow-up. Clin Oral Investig. 2019;23(9):3161–71.
11. Ralik M, Grusovin MG, Canullo L. The microbiologic profile associated with Peri-implantitis in humans: a systematic review. Int J Oral Maxillofac Implants. 2016;31(2):359–68.
12. Lafariee G, Sabogal MA, Castillo DM, Rincon MV, Gomez LA, Lesmes YA, Chambrone L. Microbiome and microbial biofilm profiles of Peri-implantitis: a systematic review. J Periodontol. 2017;88(10):1066–89.
13. Colombi AP, Tanner ACR. The role of bacterial biofilms in dental caries and periodontal and Peri-implant diseases: a historical perspective. J Dent Res. 2019;98(4):373–85.
14. Mark Welch JL, Dewhirst FE, Borsy GG. Biogeography of the Oral microbiome: the site-specialist hypothesis. Annu Rev Microbiol. 2019;73:335–58.
15. Ghoshad M, Muzzaffery F, Anderson AC, Wolper JP, Ratka-Kruger P, Fretwurst T, Nelson K, Vach K, Hellwig E. Shift of microbial composition of peri-implantitis-associated oral biofilm as revealed by 16S rRNA gene cloning. J Med Microbiol. 2018;67(3):332–40.
16. Zhuang LF, Watt RM, Mattheos N, Si MS, Lai HC, Lang NP. Periodontal and peri-implant microbiota in patients with healthy and inflamed periodontal and peri-implant tissues. Clin Oral Implants Res. 2016;27(1):13–21.
17. Gao L, Xu T, Huang G, Jiang S, Gu Y, Chen F. Oral microbiomes: more and more importance in oral cavity and whole body. Protein Cell. 2018;9(5):488–500.
18. Martellacci L, Quanta G, Patrizi R, Isola G, Gallenzi P, Masucci L. A literature review of Metagenomics and Culturomics of the Peri-implant microbiome: current evidence and future perspectives. Materials. 2019;12(18):3010–9.
19. Reinhardt DL, Chcanovic B, Albrektsson T, Tengvall P, Wernerberg A. Ligature-induced Experimental Peri-implantitis–A Systematic Review. J Clin Med. 2018;7:12.
20. Sun J, Eberhard J, Glage S, Heldt N, Voigt H, Schwabe K, Winkel A, Stiensch M. Development of a peri-implantitis model in the rat. Clin Oral Implan Res. 2020;31(3):203–14.
21. Zitzmann NU, Berglundh T. Definition and prevalence of peri-implant diseases. J Clin Periodontol. 2008;35(Suppl 286–91).
22. Monje A, Insa A, Wang HL. Understanding Peri-implantitis as a Plaque-Associted and Site-Specific Entity. On the Local Predisposing Factors. J Clin Med. 2019;8:2.
23. He CY, Jiang LP, Wang CY, Zhang Y. Inhibition of NF-kappa B by Pyrrolidine Dithiocarbamate prevents the inflammatory response in a ligature-induced Peri-implantitis model: a canine study. Cell Physiol Biochem. 2018;49:2610–25.
24. Huang BX, Zhang L, Xu L, Zhu WD, Witek L, Tovar N, Coelho PG, Meng HX. Effect of implant placement depth on the peri-implant bone defect configurations in ligature-induced peri-implantitis: an experimental study in dogs. Med Oral Patol Oral. 2018;23(1):E30–7.
25. Levin L, Zigdon H, Coelho PG, Suzuki M, Machtei EE. Reimplantation of dental implants following ligature-induced Peri-implantitis: a pilot study in dogs. Clin Implant Dent. 2013;15(1):11–6.
26. Lin XN, Liu T, Wu G, Zheng YN, Wismeijer D, Liu YL. Peri-implantitis induced by stainless steel ligature in beagle dogs. Int J Periodont Rest. 2017;37(3):E170–9.
27. Lawson PA, Johnson CN, Bengtsson L, Charalampakis G, Dahlen G, Moore E, Melton S. Ligature-Induced Experimental Peri-Implantitis—An Interconnection Pattern Across Microbiomes. Microbiome. 2020;8(1):82.
32. Jiao S, Liu Z, Lin Y, Yang J, Chen W, Wei G. Bacterial communities in oil contaminated soils: biogeography and cooccurrence patterns. Soil Biol Biochem. 2016;98:64–73.
33. Apatzidou D, Lappin DF, Hamilton G, Papadopoulos CA, Konstantinidis A, Riggo MJ, Microbiome of peri-implantitis affected and healthy dental sites in patients with a history of chronic periodontitis. Arch Oral Biol. 2017;83:145–52.
34. Sanz-Martin I, Doolittle-Hall J, Teles RP, Patel M, Bellisakis GN, Hämmerle CHF, Jung RE, Teles FRF. Exploring the microbiome of healthy and diseased peri-implant sites using Illumina sequencing. J Clin Periodontol. 2017;44(12):1274–84.
35. Kantarcı A, Hasturk H, Van Dyke TE. Animal models for periodontal regeneration and peri-implant responses. Periodontology 2000. 2015;68(1):66–82.
36. Bellisakis GN, Mir-Mari J, Sahrmann P, Sanz-Martin I, Schmidlin PR, Jung RE. Clinical association of Spirochaetes and Synergistetes with peri-implantitis. Clin Oral Implants Res. 2016;27(6):656–61.
37. Escapa IF, Chen T, Huang Y, Gajare P, Dewhirst FE, Lemon KP. New Insights into Human Nostil Microbiome from the Expanded Human Oral Microbiome Database (eHOMD): a Resource for the Microbiome of the Human Aerodigestive Tract. mSystems. 2018;3.6.
38. Tzach-Nahman R, Mizraji G, Shapira L, Nussbaum G, Wilensky A. Oral infection with Porphyromonas gingivalis induces peri-implantitis in a murine model: evaluation of bone loss and the local inflammatory response. J Clin Periodontol. 2017;44(7):739–48.
39. Zhu B, Meng HX, Huang BX, Chen ZB, Lu RF. Detection of T-forsythia and other important bacteria in crestal and subcrestal implants with ligature-induced peri-implant infection in dogs. J Periodontol. 2019;90(3):306–13.
40. Belkacemi S, Mazel A, Tardivo D, Tavitian P, Stephan G, Bianca G, Terrer E, Drcourt M, Aboudharam G. Peri-implantitis-associated methanogens: a preliminary report. Sci Rep. 2018;8(1):9447.
41. Faveri M, Figueredo LC, Shibli JA, Perez-Chaparro PJ, Feres M. Microbiological diversity of peri-implantitis biofilms. Adv Exp Med Biol. 2015;830:85–96.
42. Fickl S, Kebschull M, Calvo-Guirado JL, Hurzeler M, Zuhr O. Experimental Peri-Implantitis around different types of implants - a clinical and radiographic study in dogs. Clin Implant Dent Relat Res. 2015;17(Suppl 2):e661–9.
43. Liang J, Mai W, Tang J, Wei Y. Highly effective treatment of petrochemical wastewater by a super-sized industrial scale plant with expanded granular sludge bed bioreactor and aerobic activated sludge. Chem Eng J. 2019;360:15–23.
44. Edgar RC, Flyvbjerg H. Error filtering, pair assembly and error correction for next-generation sequencing reads. Bioinformatics. 2013;31(2):3476–82.
45. Edgar RC, UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods. 2013;10(10):996–8.
46. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A, Kaske CR, Tiedje JM. Ribosomal database project: data and tools for high throughput rRNA analysis. Nucleic Acids Res. 2014;42(Database issue):D633–42.
47. Wei Y, Ren T, Zhang L. Dix-seq: An integrated pipeline for fast amplicon data analysis. bioRxiv. 2020:2020.2005.2011.089748.
48. Watts SC, Ritchie SC, Inouye M, Holt KE. FastSpar: rapid and scalable correlation estimation for compositional data. Bioinformatics. 2019;35(6):1064–6.
49. Otasek D, Morris JH, Boucas J, Pico AR, Demchak B. Cytoscape Automation: empowering workflow-based network analysis. Genome Biol. 2019;20:1.