Microbiological Findings in Early and Late Implant Loss

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Abstract

Background Implants are a predictable and well-established treatment method in dentistry. Nevertheless, looking at possible failures of dental implants, early and late loss have to be distinguished. This study aimed at microbiological aspects on surfaces of implants, which had to be removed.

Methods 53 specimens of implants from 49 healthy patients have been examined. The groups investigated were implants that had to be removed in the period of osseointegration (early loss) or after the healing period (late loss). Data about the microbiological colonialization of the implant was collected using amplification and high throughput sequencing of the 16S rRNA gene.

Results Both early and late implant loss was associated with increased levels of *Fusobacterium nucleatum* and *Porphyromonas gingivalis*. Additionally. Late lost implants showed higher bacterial diversity and in addition higher abundances of *Treponema denticola* and *Tannerella forsythia*, while microbial communities of early loss implants were very heterogeneous.

Conclusions Pathogens commonly associated with severe periodontitis were found around implants that were lost after a primarily uneventful osseointegration. *P. gingivalis* and *F. nucleatum* colonized the implant surface in early and late loss and could therefore be characteristic for implant loss in general. The reasons for early losses could be multifactorial.

Trial registration This study was approved by the ethical review committee of the local medical association (Institutional Review Board of the Saarland Medical Council, Germany; ID: 232/12).

Background

The insertion of dental implants has become a routine and well predictable surgical procedure in the last decades, with high rates of osseointegration and long-term success (1). Since the success of early implantology, this field has nowadays established itself in the daily treatment. Dentists are able to offer their patients a therapeutic option which is not depending on adjacent teeth like it would be with fixed or removable dentures. Loss of conventional dentures can be caused by several common factors. Frequent problems are the development of caries at the margins of crowns, pulpal infections, periodontitis and mechanical complications, such as fractures of teeth (2–5). While the first two factors do not affect dental implants and the rates of mechanical complications of the implant itself are low, peri-implant infections are a major risk factor for implant failure (6). Looking at the point in time when implants are lost, early and late explantation can be distinguished.

Early implant loss takes place before prosthetic loading (7). In those cases, osseointegration is not successful, the implant is surrounded by connective tissue and can therefore not be used to anchor the planned denture. Some authors state, that the reasons for this event may be bacterial infections, surgical mistakes like inadequate cooling during implant bed preparation or an overload of the implant by a provisional denture (7). The only treatment option is to remove the implant and to repeat the surgical

procedure, which is quite frustrating for both the patient and the surgeon. On the other hand, primary osseointegrated implants can lose their connection to the surrounding bone (6). As a reason, bacterial infections of the peri-implant tissues have been reported in several investigations, similar to the pathogenesis of periodontitis (8). The infection leads to an inflammatory response, causing peri-implant mucositis. If this soft tissue reaction is not controlled, the infection will lead to an inflammatory process of the peri-implant bone, resulting in bone resorption. This plaque-associated bone loss is defined as Periimplantitis (9). Several health conditions, such as periodontitis or diabetes and smoking, have been discussed as risk factors for peri-implantitis (8, 10, 11).

In the past decades, the knowledge of bacterial infections, especially biofilms, has changed fundamentally (12, 13). The thesis that pathogens in periodontology and peri-implantitis are similar has been proved in several studies based on genetic analysis methods (14–16). Nevertheless, with new and more accurate diagnostic methods, there is also evidence for certain differences regarding the microbiota in those diseases (17–21). On the other hand, some authors even state, that qualitative findings in pockets of infected implants are similar to those in healthy sites, questioning if the number of bacteria is the main reason leading to the disease (22). New findings could either support or question the knowledge of bacterial findings around dental implants. Furthermore, there is no data regarding bacterial profiles of early lost implants, emphasizing the need of this study.

The aim of this study was to analyze the bacterial population in the peri-implant pocket of implants with severe peri-implantitis and to compare the bacterial biofilm of implants of early and late loss. The following hypotheses were constructed:

1. The main reason for late implant failure is periimplantitis. Therefore, the characteristic spectrum of bacteria for this condition will be found.
2. There are several reasons for early implant loss, like bacterial infections or surgical mistakes. As a consequence, microbiological findings should be inconsistent and differ from late implant loss.

Methods

The present clinical study investigated 53 dental implants from 49 patients. Main focus of the study was a microbial comparison of implants which got lost during the time of osseointegration (early loss) and implant loss because of peri-implantitis (late loss). All results were collated by the same dentist at the Dental Academy for Continuing Professional Development (Karlsruhe, Germany).

General inclusion criteria were: implants with severe loss of the bone-to-implant contact, either in the period of healing after the insertion (< 3 months, “early loss”) or after uneventful osseointegration and loading (> 3 months, “late loss”). Exclusion criteria were: patients aged younger than 18 years, radiation or bisphosphonate therapy, patients with untreated periodontitis and severe general conditions, such as uncontrolled diabetes, tumors, severe heart disease or a reduced state of general health. Smokers were not excluded from the study. Included were only patients with one or more implants which had been inserted in our clinic. Only implants with severe peri-implant bone loss and no chance of preservation

were included. Also specimens from implants without peri-implantitis (no bone loss) were integrated into the study as a control group.

Implants with peri-implantitis and bone loss, but chances of preservation were excluded.

Study population

Patients were divided into four groups:

27 patients with severe peri-implantitis and implants without a chance of preservation were divided in two groups.

- Group E: early implant loss (implants with severe peri-implantitis during osseointegration prior to prosthetic restoration, ≤ 3 months after implant placement), 13 patients with 14 implants
- Group L: late implant loss (implants with severe peri-implantitis and prosthetic restoration for more than three years), 14 patients with 17 implants

For a comparison to the healthy oral situation we created two control groups:

- Group CE: control group (implants with no bone loss directly after completed osseointegration, two to four months after implant placement), 17 patients with 17 implants
- Group CL: control group (implants with no bone loss and prosthetic restoration for more than three years), 5 patients with 5 implants

The observational study was approved by the ethical review committee of the local medical association (Institutional Review Board of the Saarland Medical Council, Germany; ID: 232/12) and was conducted in accordance with the Declaration of Helsinki and the Professional Code for Physicians of the local Medical Council**.** All patients were informed about the purpose of the study by the examiner and signed a form of consent.

Clinical procedure of documentation of clinical findings and sampling

- Patients were asked about their case history, nicotine abuse, diabetes, regular use of mouth rinses (at least once per week) and antibiotics in the past 12 months, and if periodontitis had been diagnosed.
- In each patient a pool sample was taken with sterile paper points from the peri-implant sulcus around infected or healthy implants. (Fig. 1). Any existing signs of inflammation (pocket suppuration) were documented.
- If a severe peri-implantitis was diagnosed, explantation was done under local anaesthesia with articaine with epinephrine 1:100.000 (Citocartin Sopira®, Heraeus Kulzer GmbH, Hanau, Germany). The removed implants themselves were also used for microbiological analysis for validation of the paper point results.

All patients were informed in advance about the clinical procedure to be performed. In addition, the patients were informed about obtaining paper point samples for a future microbial analysis in connection with the study. All patients gave their informed consent in writing.

For the microbial analysis of the peri-implant tissue the bacterial DNA from the paper point samples of a total of 53 implants from 49 patients were obtained and analyzed for taxonomic composition by sequencing the V1-V2 variable regions of the 16S rRNA gene. The paper points were applicated for 20 seconds, following the established clinical routine for sample collection of peri-implant pathogens.

Evaluation at patient level

For the analysis at patient level, every implant was weighted inversely to the total number of implants per patient.

Extraction of microbial DNA

Sterile paper points were used to collect biofilm samples of the peri-implant sulcus for microbiota analysis as described previously(23). Briefly, biofilm microbes were resuspended in nuclease free water using a combination of shaking and sonication. The suspension was centrifuged, pellets were stored at -80°C and then used for DNA extraction using commercial extraction protocols for genomic DNA (QIAamp Mini Kit, Qiagen, Hilden, Germany). The collected pellets of the supernatant were treated with 180 µL lysozyme solution (20 mg/mL, SIGMA-Aldrich, Taufkirchen, Germany; 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1.2% Triton) under shaking at 37°C for 2:15 h, followed by proteinase K digestion (20 µL proteinase K and 200 µL buffer AL) for 1:15 h under shaking at 56°C. Finally, the DNA was eluted with 100 µL PCR-clean water and the concentration was quantified using the NanoDrop equipment (PEQLAB, Erlangen, Germany). One empty extraction without any sample material was used a control for background DNA contaminations (contamination control).

Illumina sequencing of amplicons targeting the 16S rRNA gene

Amplicons of the V1-V2 region of the bacterial 16S rRNA gene were prepared as published elsewhere (24). Briefly, the genomic sequence of the 16S rRNA gene was amplified with primers that were derived from the previously described primers 27F and 338R (25, 26) and contained sequences compatible with Illumina sequencing platforms and a 6-nt barcode sequence. The resulting DNA was used as template for a second PCR, using primers designed to introduce full-length Illumina adapter sequences including Illumina 6-nt index sequences to enable high-level multiplexing. To control for potential DNA contamination an additional sample was amplified without template DNA (contamination control sample). Libraries were pooled and subjected to 250 nt paired-end sequencing on an Illumina MiSeq machine. For data analysis, the obtained reads were processed using *dada2* version 1.16.0 (27) following the MiSeq SOP published by Kozich *et al*. (28). *Dada2* determines *amplified sequence variants* (ASVs) by removing sequencing errors based, which tends to produce more accurate results than the commonly used *operational taxonomic units* or OTUs (29). Briefly, sequence reads were trimmed to a length of 200

nt (forward read) and 150 nt (reverse read) and 5 nt of the left end were trimmed additionally. Reads with ambiguous base calls and an expected error rate larger than 2 were discarded and ASVs were inferred using the *pseudo-pooling* algorithm. Chimeric sequences were removed and ASVs classified using the RDP Naïve Bayesian Classifier algorithm as implemented in dada2 against the Silva database v138 (30). Were possible by exact matching, ASVs were assigned species names using the *assignSpecies* function of *dada2*. After the preprocessing the data amounted to between 1,305 and 205,073 sequences per sample.

Statistical analysis of microbiome data

Further analysis of the microbiome data was done in R version 3.6.1 (31) using packages *phyloseq* (32), *vegan* (33) and *ggplot2* (34). The contamination control sample yielded 397 reads and 13 ASVs represented by more than 5 sequences in the contamination control were removed from all samples. Alpha-diversity of the sampled biofilms was estimated using the Inverse Simpson Index, calculated by the *phyloseq* function *estimate_richness*. Principal coordinates analyses (PCoA) were performed using the *ordinate* function with Jenson-Shannon Divergence (JSD) as distance metric. Differences of single variables between groups of samples were statistically tested using the Kruskal-Wallis Rank Sum Test (R function *kruskal.test*) for multiple groups and Wilcoxon Rank Sum Test (R function *pairwise.wilcox.test*) for pairwise comparisons of groups with the Benjamini-Hochberg adjustment to control false discovery rate in multiple comparisons (35). Differences in the microbiota composition (beta-diversity) were tested by performing Permutational Multivariate Analysis of Variance (PERMANOVA) on the JSD matrix, using the *adonis* function of the R package *vegan* (33) and *pairwise Adonis* by Martinez Arbizu & Monteux (36).

The study was conducted in accordance with the STROBE guidelines.

Results

Composition of the peri-implant microbiota

For the microbial analysis, sterile paper points were used to collect biofilm samples from the peri-implant sulcus and analyzed by high throughput sequencing of the variable region V1-V2 of the bacterial 16S rRNA gene. Across all samples, 552 taxa (amplified sequence variants, ASVs) were detected that occurred with an abundance of at least 1 % in one or more samples. Most of the identified taxa belonged to the six phyla *Actinobacteriota, Bacteroidota, Firmicutes, Fusobacteriota, Proteobacteria and Spirochaetota.* All samples could be analyzed and were included in the study.

The microbial composition of the peri-implant biofilm samples showed substantial variation between the patients, most ASVswere not found in all patients, even when comparing only patients of the same group. Nevertheless, a comparison of the microbial composition reveals similarities between patients of the same group and differences between patients from different groups (Fig. 2 and 3). Most notably, most of the patients who lost their implants (groups E and L, Fig. 3) could be distinguished from the control groups . Implant loss was thereby frequently associated with increased average abundances of

Fusobacterium and Porphyromonas (Fig. 2B). Especially the most abundant species of these genera, F. nucleatum and P. gingivalis were more abundant in these groups (Fig. 4) and contributed strongly to the placement of the respective samples in the PCoA plot (Fig. 3) The two healthy control groups on the other hand harbored comparatively high abundances in bacteria of the genera Streptococcus, Neisseria, Rothia and Veillonella (Fig. 2B, Fig. 4).

In healthy controls, the biofilm composition showed no significant differences between implants before (group CE) and after prosthetic restoration (group CL), but between the groups with implant loss (E and L), both the composition and the overall microbial diversity were significantly different (Fig. 2). The clustering pattern of sample points in the principal coordinates analysis (PCoA, Fig. 3) most notably displayed a visible separation between the control group samples and samples of group L (with two exceptions). Implants of the L group showed higher diversity (Fig. 2A) and higher abundances of the genera Treponema and Tannerella (Fig 2B), especially of the two species T. denticola and T. forsythia, which were only present in much lower abundances or absent in most of the patients in other groups (Fig. 4).

Early loss samples (group E) did not show such a clear pattern and were much more distributed, pointing towards a more heterogeneous composition of the biofilms in these cases (Fig. 3). The individual samples of group E showed a lower average microbial diversity within the samples (i.e., the α-diversity) than the late loss samples (Fig. 2A), while the difference in composition between the samples (the β-diversity) was large. Some of the samples were more similar to the samples of group L and clustered together with these samples in the PCoA plot (Fig, 3), with high levels of ASVs classified as Fusobacterium, Porphyromonas and other bacteria. Other samples had microbial compositions more similar to the healthy control samples with higher levels of Streptococcus, Neisseria and Veillonella and some samples were placed at some distance from the other samples in the PCoA.

Biofilm samples were also analyzed directly from implants after the explantation and compared to the corresponding paper point samples to validate the results. The differences in the microbial composition were highly similar between implant and paper point samples. Although a multivariate analysis of the composition (PERMANOVA) did show a significant difference between implants and paper points (R^2 = 0.021, p = 0.016), it was small compared to the difference found between the groups (R^2 = 0.107, $p < 10^{-4}$).

Discussion

In the last decades several risk factors for the failure of dental implants have been reported – most of them being clinical aspects of the implant treatment, medications or health conditions. Looking at the clinical risks for bone loss around implants, surgical skills or mistakes regarding the surgical protocol, such as dull burrs, high pressure while drilling or high insertion torque, different healing protocols or excess of cement can cause problems (37–40). On the other hand, medications, such as bisphosphonates or antidepressants can be risk factors for infections or implant loss (41–43).

Additionally, certain conditions, such as poor bone quality, periodontitis, bruxism, overload and smoking habits can have an impact on osseointegration and long-term success of dental implants, while controlled diabetes, osteoporosis or the age of the patient do not seem to be risk factors (44–51).

Among those reasons for implant failure, bacterial infections have been reported to play a major role for early and late loss of dental implants (7, 49, 52, 53). Most of the studies focus on the microbial findings in peri-implantitis, while there is no data for early loss. Differences between research groups could be explained by different test systems and by the steady development of microbial tests. The studies show, that bacterial findings in late loss were similar to the ones in periodontitis, with certain differences (20, 53–55). Our data does support those findings in group L, as pathogens of the *red complex* defined by Socransky and Haffajee (56), such as *Tannerella forsythia* and *Treponema denticola*, were frequently found around implants, which had to be removed because of severe bone loss after a primarily uneventful osseointegration. This supports the thesis, that those bacteria are characteristic for late implant loss and periimplantitis (57). Additionally, *Porphyromonas gingivalis* (also of the *red complex*) and *Fusobacterium nucleatum* were found in early and late loss and could therefore be characteristic for implant loss in general. With the exception of two samples with a microbial composition more similar to the healthy controls, the late loss samples have a similar composition and form a compact group in the PCoA plot (Fig. 3), which indicates that these case also might have similar causes.

For early loss of implants, the microbial results were very heterogeneous and the typical pathogens as described above were only found to be abundant in about half of the cases. The biofilms of the other implants of this group resembled the supposedly healthy microbiota found in the control groups or had a unique microbial composition that was not similar to any of the other samples. This heterogeneity between patients points towards multiple potential risk factors contributing to early implant losses, which might include i) microbial communities typical for periodontitis-like infections (*red complex*), ii) dysbiosis involving other oral bacteria and iii) other, non-microbial causes like surgical mistakes or poor bone quality.

Because of the sample size, the data presented should be interpreted as a first indication, especially regarding to the heterogeneous findings in early implant loss. More studies with a higher number of implants should assess the potential risks of early losses. If bacterial infections represent a relatively minor risk factor for early losses, this should be considered in the preoperative therapy protocols (58).

Conclusions

Summarizing, our data confirms the hypotheses that late loss of implants is caused by bacterial infection whereas early loss of implants may be caused by bacterial infection and other reasons like surgical problems or poor bone quality. This emphasizes the importance of anti-infective and follow-up protocols related to dental implants. Interestingly, there was no correlation between the history of periodontitis and the microbiota found in early implant loss. As this correlation could have consequence for antibiotic prophylaxis, further research with higher numbers of implants should be done.

Declarations

Ethics approval and consent to participate

The observational study was approved by the ethical review committee of the local medical association (Institutional Review Board of the Saarland Medical Council, Germany; ID: 232/12) and was conducted in accordance with the Declaration of Helsinki and the Professional Code for Physicians of the local Medical Council**.** All patients were informed about the purpose of the study by the examiner and signed a form of consent.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interest

The authors declare that they have no competing interests

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Authors contribution

Michael Korsch, Andreas Dötsch, Silke-Mareike Marten: Design, data collection, statistics, data analysis/interpretation, drafting article, approval of the submitted version.

Dominic Stoll: Data collection.

Christopher Prechtl: Design, statistics, drafting article, approval

of the submitted version.

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Figures

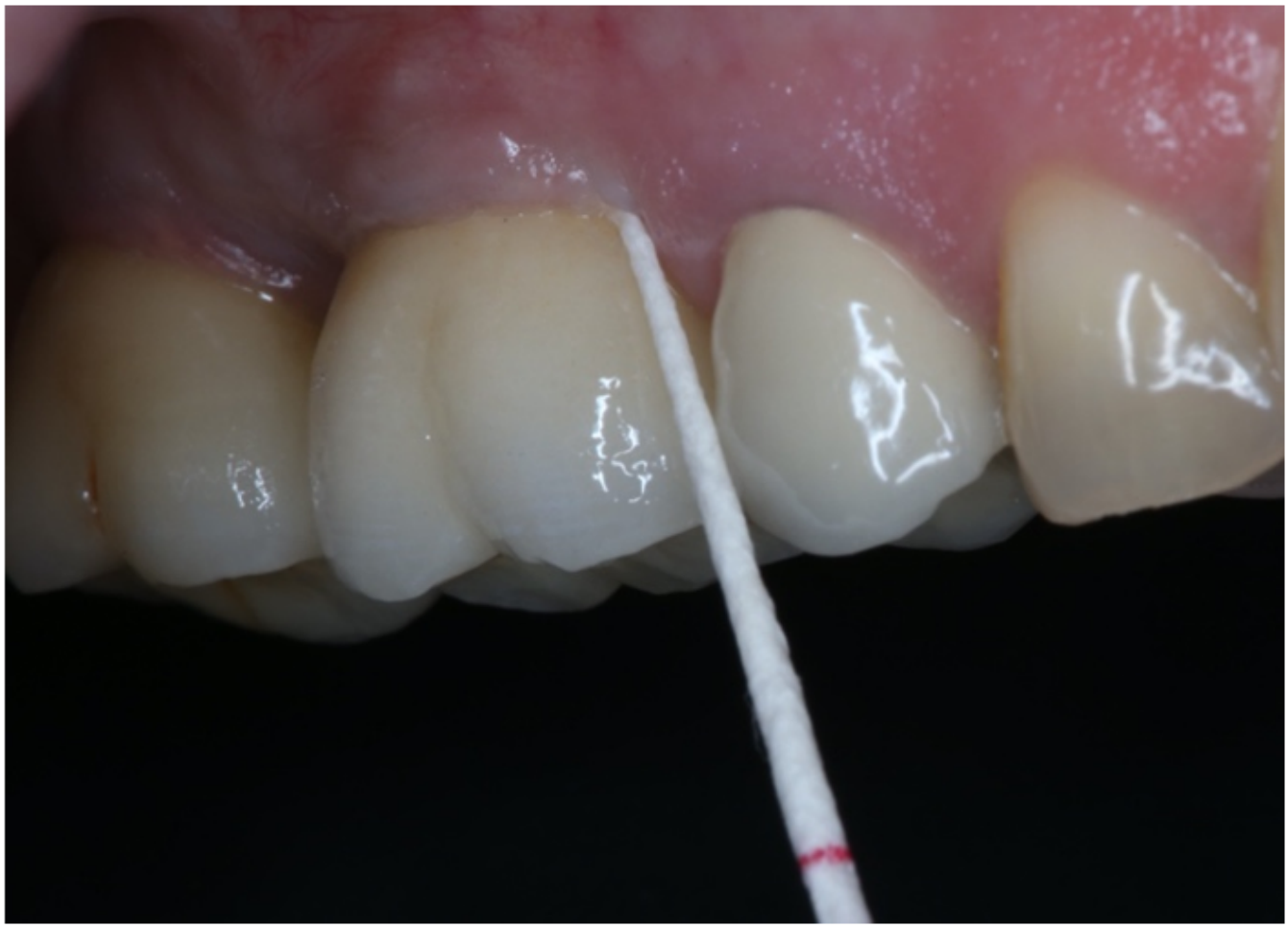

Figure 1

The figure shows a pool sample which was taken with sterile paper points from the peri-implant sulcus around infected or healthy implants.

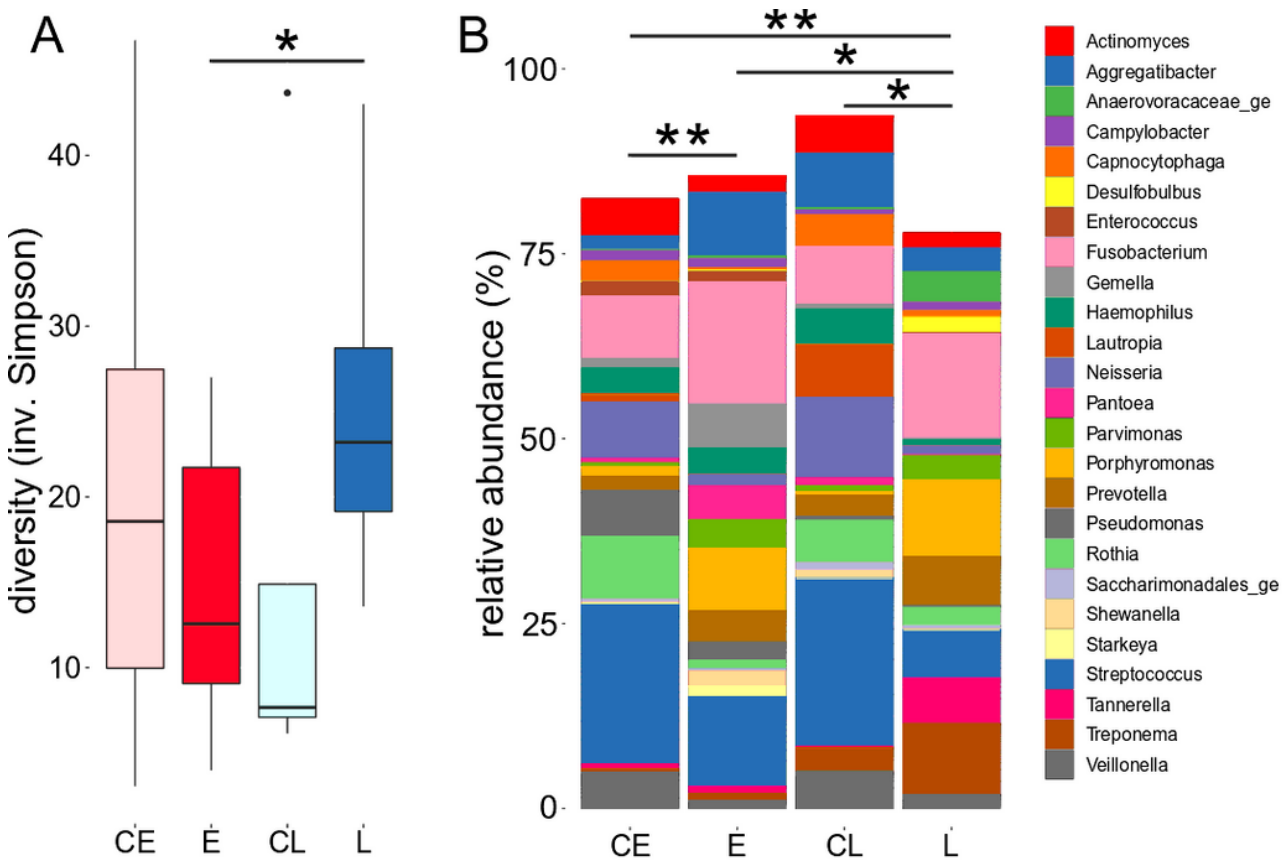

Figure 2

A) Microbial alpha-diversity of samples of different groups of implants. Diversity was calculated using the inverse Simpson index and is here depicted as box-and-whiskers-plots with the box representing values between the 1st and 3rd quartile, a black line indicating the median. B) Average relative abundance of the most abundant genera across implant groups. All sequence variants (ASVs) that were classified to belong to the same genus were aggregated. Horizontal lines indicate a significant difference in the microbiota composition between groups (pairwise Wilcoxon and pairwise PERMANOVA analysis in A and B, respectively). Asterisks in both panels indicate significance levels: * - $p < 0.05$, ** - $p < 0.01$). The groups of implants are labelled CE – early control, E – early implant loss, CL – late control, L – late implant loss.

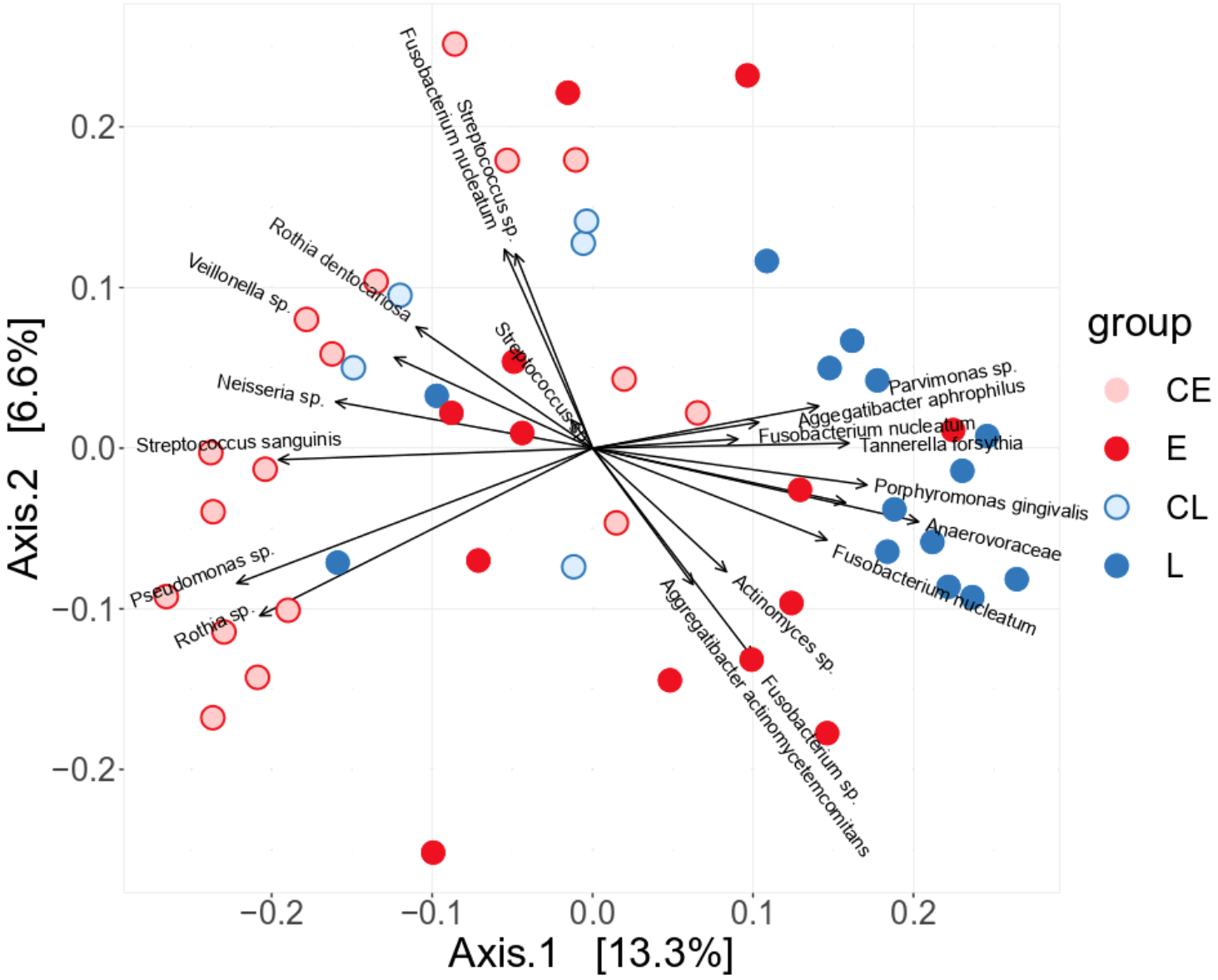

Figure 3

Principal coordinates analysis (PCoA) of relative abundances of ASVs across all samples. Individual samples are depicted as circles, colored by group of implants as labelled: CE – early control, E – early implant loss, CL – late control, L – late implant loss. Arrows represent the “loadings”, i.e. the contribution of the 20 most abundanct sequence variants (ASVs)and are labelled with the species classification (if available) or the lowest classified taxonomic level. Axis.1 and Axis.2 represent the first two principal coordinates with the fraction of total variance explained by each coordinate written in square brackets.

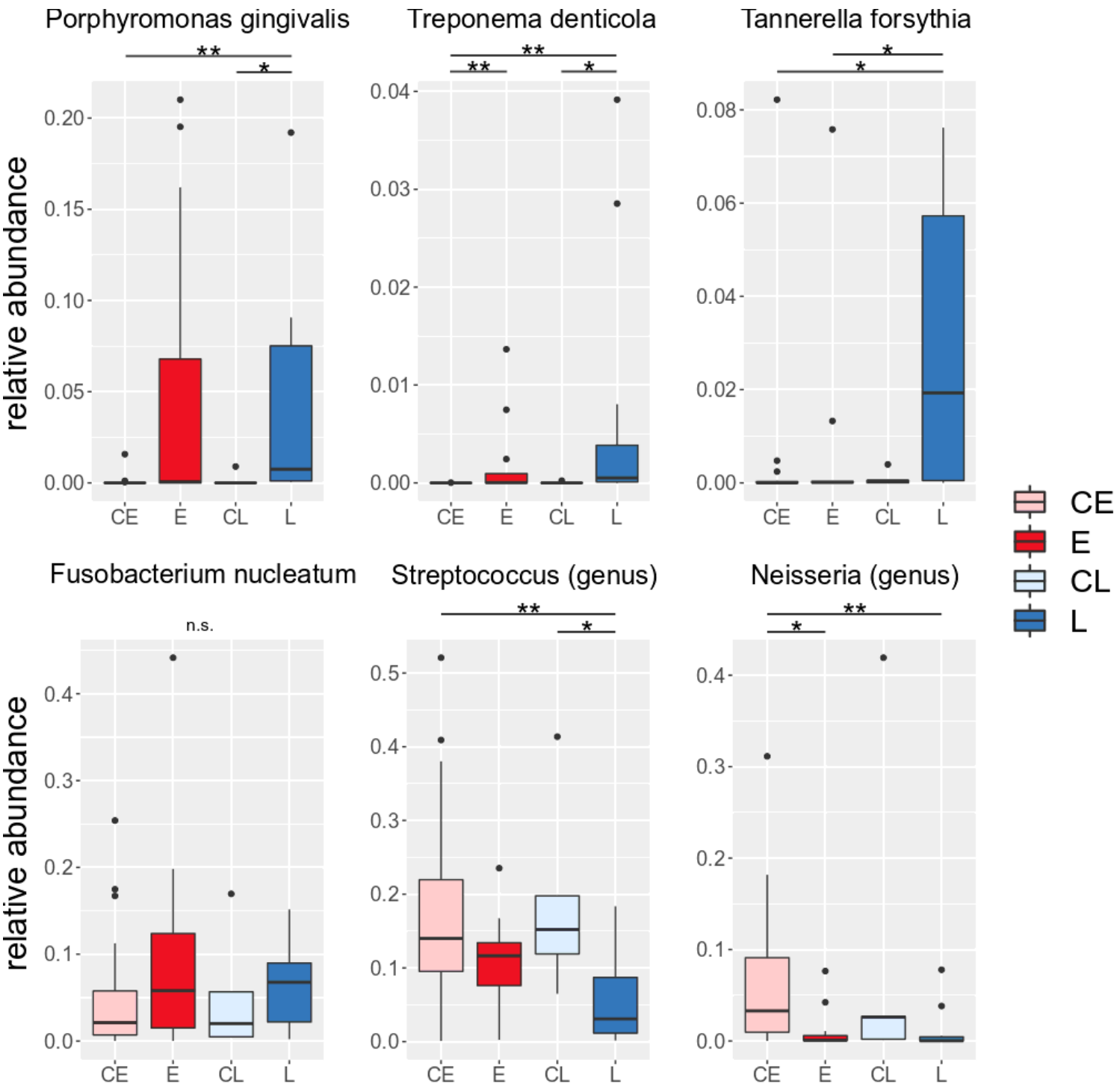

Figure 4

Relative abundance of selected bacteria depicted as boxplots with the box representing values between the 1st and 3rd quartile, a black line indicating the median. Note that Streptococcus and Neisseria abundances are aggregates of the respective genera. Horizontal lines indicate a significant difference in the microbiota composition between groups (pairwise Wilcoxon test). Asterisks in both panels indicate significance levels: * - $p < 0.05$, ** - $p < 0.01$, n.s. – all comparisons $p > 0.05$. The groups of implants are labelled CE – early control, E – early implant loss, CL – late control, L – late implant loss.