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Horizontal and vertical transfer of oral microbial dysbiosis and periodontal disease.

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Abstract

One of the hallmark features of destructive periodontal disease, well documented over the last 50 years, is a change to the quantitative and qualitative composition of the associated microbiology. These alterations are now generally viewed as transformational shifts of the microbial populations associated with health leading to the emergence of bacterial species which are only present in low abundance in health and a proportionate decrease in the abundance of others. The role of this dysbiosis of the health associated microbiota in the development of disease remains controversial: is this altered microbiology the driving agent of disease or merely a consequence of the altered environmental conditions which invariably accompany destructive disease? In this work we aimed to address this controversy through controlled transmission experiments in the mouse in which a dysbiotic oral microbiome was transferred either horizontally or vertically into healthy recipient mice. The results of these murine studies demonstrate conclusively that natural transfer of the dysbiotic oral microbiome from a periodontally diseased individual into a healthy individual will lead to establishment of the dysbiotic community in the recipient and concomitant transmission of the disease phenotype. The inherent resilience of the dysbiotic microbial community structure in diseased animals was further demonstrated by analysis of the effects of antibiotic therapy on periodontally diseased mice. Although antibiotic treatment led to a reversal of dysbiosis of the oral microbiome, in terms of both microbial load and community structure, dysbiosis of the microbiome was re-established following cessation of therapy. Collectively these data suggest that an oral dysbiotic microbial community structure is stable to transfer and can act in a similar manner to a conventional transmissible infectious disease agent with concomitant effects on pathology. These findings have implications to our understanding of the role of microbial dysbiosis in the development and progression of human periodontal disease.

Introduction

The association between dysbiosis of the oral microbiome and periodontitis is now well established (Mira et al. 2017). Although there are significant individual-to-individual variations in the microbial composition of subgingival biofilms in health (Hall et al. 2017), the development of disease coincides with a characteristic population shift. Consistently, the change involves not only an increase in the overall microbial biomass but also the emergence of bacterial species which are only present in low abundance in health and a proportionate decrease in the abundance of others (Marsh PD 1994, Socransky et al. 1998, Diaz et al. 2016). Although culture-based studies originally indicated that the emergent bacterial populations in the disease-associated communities may represent only a relatively small number of different taxa (Holt & Ebersole 2005, Socransky et al. 1998), the application of more advanced technologies has demonstrated that a wider range of organisms should be considered (Abusleme et al. 2013, Griffen et al. 2012). This in turn, has led to the concept of a community wide perturbation in the composition of the microbiome associated with periodontal disease (Hajishengallis & Lamont 2012, Curtis MA 2014). However, it remains controversial to what extent the observed shift in the microbial population structure in periodontitis is the primary cause of pathology or simply a consequence of the altered environmental conditions induced in an inflammatory disease. This dichotomy remains an important issue in terms of both diagnosis and treatment modalities of a range of conditions associated with dysbiosis in humans (Butto and Haller 2016).

In previous studies, using a mouse model of periodontal disease, we demonstrated that the introduction of *P. gingivalis* into the oral microbiome of specific pathogen free (SPF) mice led to significant alterations in both the quantity and qualitative composition of the commensal microbial populations: analogous to the community wide perturbations observed in human disease (Hajishengallis et al. 2011). Similarly, the microbial shift was also associated with the development of destructive disease based on increased loss of alveolar bone in challenged mice.

In the present investigation, we aimed to use this mouse model of periodontitis, or more precisely controlled transmission experiments in mice, to address whether the microbial dysbiosis observed in this system following oral challenge with *P. gingivalis* represents a definitive pathological entity capable of transferring horizontally (from one mouse to another by co-caging) and vertically (between generations from parents to litter) to drive destructive disease.

Materials and Methods

Animals

All animal experiments were conducted in accredited facilities in accordance with the UK Animals (Scientific Procedures) Act 1986 (Home Office license number 7006844). Germ-free C3H/Orl mice (Charles River Laboratories International) were maintained in isolators under axenic conditions at the Royal Veterinary College, University of London as previously described (Hajishengallis et al. 2011). Conventional C3H/Orl mice, derived from the original germ-free mice were maintained in individually ventilated cages (IVC) at the animal care facilities of Queen Mary University of London (QMUL). The germ-free mice were bred and maintained in a separate location due to the highly specialist nature of their housing requirements.

Oral Gavage and Microbiome Transfer Experiments

P. gingivalis W50 was grown on blood agar plates containing 5% defibrinated horse blood or in Brain Heart Infusion broth (BHI) (Oxoid) supplemented with haemin (5 μg/mL) in an anaerobic atmosphere of 80% N_2, 10% H_2 and 10% CO_2 at 37°C (Don Whitley Scientific). Oral gavage with *P. gingivalis* was performed essentially as described by (Baker et al. 2000). Briefly, mice were orally inoculated by means of a ball-ended feeding needle three times at two-day intervals with 10^9 CFU *P. gingivalis* W50 suspended in 2% carboxy-methylcellulose (Sigma) vehicle. Sham controls received vehicle alone.

For the stability, vertical transmission and horizontal transmission experiments, six mice were used in the control and challenged cohorts at the beginning of the study. For the antibiotic experiment, five

mice were used in each cohort. In the stability experiment, after week 16, two mice from each group were used as breeders to generate litters to determine the efficiency of vertical transfer.

For horizontal transfer experiments, germ-free mice were transported in sterile conditions to the SPF animal facility at QMUL and co-caging set up immediately in IVCs. Oral swabs were collected from the germ-free mice before co-caging to confirm sterility. Two challenged female SPF donor mice and four recipient germ-free animals were co-caged at a ratio of 1:2 in each cage for 16 weeks. The co-caging was commenced 10 days after the last *P. gingivalis* inoculation of the female SPF mice, which was also when oral dysbiosis was confirmed by microbial analysis.

For the antibiotic experiment, a 2.5% solution of Septrin co-trimoxazole (GlaxoSmithKline) was provided in the drinking water of the mice for 10 days followed by a wash period of 4 days. At the end of each experiment, mice were euthanized by the CO_2 inhalation method.

Cultural Microbiological analyses

The murine oral cavity was sampled for 30 seconds using sterile fine tip rayon swabs (VWR International) and placed in a tube containing 100 µl reduced John's transport medium. Serial dilutions of the suspension were spread onto blood agar plates for aerobic and anaerobic growth at 37^{o}C. The colony forming units (CFU) of predominant cultivable bacteria on each plate were counted. Every different colony morphology type from each experimental group was isolated, purified by subculture and identified by full length 16S ribosomal RNA gene sequencing as described previously (Hajishengallis et al. 2011; Maekawa et al. 2014). On an average, 4-6 different colony types could be identified on each blood agar plate.

Next generation sequencing using Roche 454 GS-FLX+ Titanium Pyrosequencer

Whole genomic DNA was extracted from the above swabs using the GenElute Bacterial DNA Kit (Sigma-Aldrich). The extracted samples were used as templates in PCR reactions performed in Extensor Long PCR Reddymix MasterMix (Thermo Scientific) using primer 27FYM, extended at the 5'-

end to include a 30-base 454 adaptor sequence A and a unique 12 nucleotide Golay barcode for each sample, and primer 519R which has a 5'- extension with a 30-base 454 adaptor sequence B (Kistler et al. 2013). The amplified PCR products were cleaned with the Qiaquick Kit (Qiagen). When necessary, primer dimers were removed with NucleoSpin Gel and PCR Clean-up (Fisher Scientific). The amplicons were pooled in equimolar amounts prior to emulsion PCR, and unidirectionally sequenced using the Lib L v2 kit (Roche) on a Roche 454 GS-FLX+ Titanium sequencer. De-noising, de-multiplexing, trimming, chimera check, classification, clustering, Operational Taxonomic Units (OTUs) assignments, and further analyses were performed using the mothur pipeline (Schloss et al. 2009). The raw sequencing reads have been uploaded to the NCBI SRA database (Accession No. PRJNA543124).

Periodontal bone loss determination

Mice were euthanized as above, mandibles and maxillae were dissected, defleshed and assessed under a Stemi SV11 dissecting microscope (Zeiss) at 25x magnification. The captured images were analysed by ImageJ software (National Institute of Health, USA). The distance from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) was measured at 6 pre-determined points (mesio-, mid- and distal-) on both buccal and lingual/palatal surfaces of mandibular and maxillary molar teeth. In order to calculate bone loss, the mean CEJ-ABC distance from each test mouse was subtracted from the mean CEJ-ABC distance of the control mice, which was used a zero baseline (modified from Baker et al. 2000).

Statistical Analyses

Bone levels between different experimental groups were compared using one-way analysis of variance (ANOVA) and unpaired Student's t-test between 2 comparison groups. Significance was expressed at the $p<0.05$ level (Hajishengallis et al., 2011). The statistical analyses and graphical visualization of the bone levels were performed using Graphpad Prism 7 (Graphpad Software Inc.). The differences in the composition of the overall microbiome between the treatment and control

groups at each time point in each experiment were assessed by PERMANOVA analyses using the Adonis function in the Vegan package in R (Dixon P 2003). Differences in the number of CFUs of individual bacterial genera between treatment and control groups were assessed by Welch's t-test using Graphpad Prism 7.

Results

P. gingivalis mediated dysbiosis is stable over time and associated with long term destructive disease in the murine model

Oral gavage with *P. gingivalis* resulted in a 10-fold increase in the total quantitative oral microbial load and a statistically significant ($p<0.005$) qualitative alteration to the composition of the oral microbiome at 7 days post challenge (Fig 1.). The dysbiotic microbiome generated by *P. gingivalis* challenge is referred to as Dys^{Pg} throughout this manuscript. The predominant cultured organisms from unchallenged mice comprise representatives of the *Streptococcus*, *Gemella* and *Staphylococcus* genera. Following challenge, these genera were retained but members of the *Enterobacteriaceae* family and *Lactobacillus* genus also became major constituents. These changes were maintained over the course of the experimental period to 28 weeks. Microbial population analysis of these samples by next generation sequencing methodology revealed a similar pattern (Fig. 2). In particular, the *Streptococcus* and *Gemella* genera were predominant in both control and challenged mice and lactobacilli became prominent members of the overall Dys^{Pg} community by week 28. The main discrepancy between the two methods of analysis was in quantitation of *Enterobacteriaceae* particularly at week 16 where this genus represented approximately 40% of the total community by culture but only a minor constituent on the basis of non-cultural analysis. This under representation may be a reflection of the number of mouse oral taxa which are as yet unnamed and not adequately curated, resulting in a significant number of reads belonging to Gammaproteobacteria being grouped as unclassified. As previously described (Hajishengallis et al. 2011), we were unable to detect *P. gingivalis* by cultural analysis at any time point from oral swabs of challenged mice. This low

abundance was confirmed by non-cultural analyses where *P. ginigivalis* sequences could only be detected in less than one-third of the Dys^{Pg} samples and at very low levels (0.013% of the total reads in each sample) (Fig 2.). A similar level of coherence between cultural and non-cultural techniques was observed in all the experiments reported here, and hence we only present data from cultural analyses in the following sections.

Loss of periodontal bone was increased in the challenged mice in a linear manner over the entire experimental period of 28 weeks. The rate of bone loss in the challenged mice at 0.002 mm/week was approximately eight times higher ($p<0.01$) than the unchallenged mice. Thus, the presence of a Dys^{Pg} oral microbiome is consistently associated with alveolar bone loss.

P. gingivalis mediated dysbiotic microbiomes stably transfer horizontally into healthy germ-free mice and lead to periodontal disease in the recipients.

In previous work, we demonstrated efficient transfer of the oral commensal microbiota from healthy control SPF C3H/Orl mice into germ-free mice of identical genotype accompanied by only low levels of physiological bone loss in the recipients. (Hajishengallis et al. 2011). Here we examined the stability on transfer of a Dys^{Pg} microbiome into germ-free mice and the disease phenotype of the recipients. C3H/Orl mice challenged with *P. gingivalis* were co-caged in 1:2 ratio with germ-free C3H/Orl mice. After 14 days, the microbiology of the donor and recipient mice was shown to be not significantly different in terms of both the quantitative and qualitative composition (Fig 3). Thus, the differential in the total counts of control versus *P. gingivalis* challenged mice was maintained in the germ-free recipients of these two microbiomes. Furthermore, the elevated level of periodontal bone loss in the donor challenged mice (0.009 mm/week; $p<0.0001$) was also evident in the conventionalized mice that received the Dys^{Pg} microbiome, after 16 weeks of co-caging (0.008 mm/week; $p<0.0001$). Hence, these experiments demonstrate a direct cause and effect of acquisition of the Dys^{Pg} microbiome and the development of bone loss even though the recipient animals were not exposed to the high dose inoculum of *P. gingivalis* received by the donors.

Inter-generational transfer of P. gingivalis mediated dysbiotic microbiomes and vertical transmission of disease.

The causal relationship of dysbiosis and periodontal disease was then further explored in vertical transmission experiments. Control or *P. gingivalis* challenged C3H/Orl mice were mated and the oral microbiology of the corresponding litters was compared at 8, 16 and 28 weeks of age, and the periodontal bone loss at 16 and 28 weeks (Fig 4). First generation animals acquired an oral microbiome similar in composition and total counts to the parent mice, with no statistical significance between the respective oral microbial populations. Thus, whilst control litter mice had oral counts of between 10^6-10^7 CFU, the offspring of challenged mice had oral bacterial counts of approximately 10^8 CFU, in both cases similar to the parent mice. Similarly, whilst *Streptococcus* and *Gemella* genera dominated the oral microbiome of control parent and control litter mice, the major common components of the both challenged parents and their litter mice comprised *Lactobacillus* and *Staphylococcus* along with the *Streptococcus* and *Gemella* genera. The transmission of either control or dysbiotic oral microbial populations was also reflected in the periodontal bone loss of the respective litters. The litters of challenged mice displayed significantly elevated periodontal bone loss (0.005 mm/week; $p<0.001$) compared to the litter of control mice (0.002 mm/week).

Dysbiosis of the mouse oral microbiome is stable to antibiotic treatment

The preceding transfer experiments indicated that the Dys^{Pg} microbiome is not only stable over time but also able to stably transfer both vertically and horizontally into healthy recipient animals and recapitulate the disease experience of the donor mice. As a further measure of stability, we next examined the influence of antibiotic treatment on dysbiosis induced by *P. gingivalis*. Control and *P. gingivalis* challenged C3H/Orl mice were placed on antibiotics in the drinking water 7 days after the challenge. Antibiotic treatment was continued for 10 days and then both groups of mice were returned to normal drinking water (Fig 5). Control animals carried approximately 10^7 CFU of oral bacteria at the start of the experiment compared to 10^8 CFU in challenged mice. Seven days following

the end of treatment with antibiotics, the counts in both groups were significantly reduced to the same level of approximately 10^6 CFU. *Gemella* in the control group and *Gemella* and organisms of the genus *Bacteroides* in the challenged mice appeared most susceptible to antibiotic treatment. However, at 16 and 22 weeks post antibiotic treatment the bacterial counts in both control and challenged mice were restored to the pre-antibiotic treatment levels (Fig 5). The recovery in counts was accompanied by the re-emergence of the *Gemella* to the original levels by 22 weeks and the appearance of *Enterobacteriaceae* in both groups. *Bacteroides* was not restored to the pre-antibiotic levels in the challenged mice but organisms from the genus *Enterococcus* were detected at 22 weeks post antibiotic treatment in these animals. Thus, this regimen of antibiotic treatment had only a temporary suppressive effect on the elevated counts of the Dys^{Pg} microbiome and its composition. The overall population structures at 16 and 22 weeks post antibiotic cessation were not significantly different to the pre-treatment populations. (Fig 5, Appendix Table 4 & 5).

Discussion

In this study, we demonstrate for the first time, the stability of a dysbiotic oral microbiome over time and transfer across individuals and generations and the ability of this perturbed microbial community to drive destructive periodontal bone loss in previously healthy, recipient animals. The mouse oral gavage model (Baker et al. 2000) has been used extensively to investigate host-microbe interactions in periodontal disease. Routinely these studies have measured microbial and immune parameters and alveolar bone loss at a single time point six weeks post challenge (Hajishengallis et al. 2011, Polak et al. 2009). In the current investigations, we only report periodontal bone loss as an indicator of periodontal disease although we also established that the gavage methodology did lead to loss of periodontal soft tissue attachment and an increase in inflammatory cell recruitment into the periodontal tissues of challenged mice (Supplementary figure 1).

Longitudinal assessment of the microbiology and bone levels over 28 weeks post challenge demonstrate that following introduction of *P. gingivalis,* community wide perturbation of the

252 commensal microbiome is maintained for the entire experimental period and is accompanied by
253 alveolar bone loss at a rate equivalent to that observed in the initial six-week period. Hence,
254 development and maintenance of changes to the oral microbial community structure were temporally
255 aligned to the initiation and progression of bone loss.
256
257 Although this coherence between oral microbial dysbiosis and bone loss is consistent with causation,
258 as with human periodontitis, coherence fails to discriminate between causation versus a
259 consequential relationship in which the presence of disease simply maintains the environmental
260 conditions which favour alterations to the microbial populations. More definitive evidence for a causal
261 link was established in the horizontal and vertical transfer experiments. In both experimental
262 approaches, the altered oral microbiome, initially generated by *P. gingivalis* challenge, transferred
263 unchanged into healthy recipient animals and was accompanied by the development of bone loss
264 equivalent to that observed in donor animals. In so doing, the dysbiotic community acts like a
265 conventional transmissible infectious disease agent with concomitant effects on pathology.
266
267 Previous investigations (Hajishengallis et al. 2011) demonstrated that the presence of the commensal
268 microbiome was fundamental to the development of disease since whilst gavage of SPF mice leads to
269 periodontal bone loss, mono-colonisation of germ-free animals with *P. gingivalis* is asymptomatic. We
270 suggested that this phenomenon may be a consequence of the immune subversive properties of this
271 human periodontal organism (Hajishengallis et al. 2012, Maekawa et al. 2014) which has the ability to
272 change the delicate equilibrium between the microbiota and the host in the oral tissues and facilitate
273 the conversion of the normally benign commensal microbiota to a more pathogenic state. That these
274 changes occurred even when *P. gingivalis* was present in low amounts, led us to invoke the keystone
275 pathogen hypothesis (Hajishengallis et al. 2012) wherein a low abundance species can have
276 disproportionately large effects on the balance between symbiosis and dysbiosis and simultaneous

277 impact on pathology. Similarly, very low levels of *P. gingivalis* in challenged mice were also observed
278 in the current investigation.

279 However, rather than a keystone pathogen effect, it is also possible to argue that the oral gavage
280 procedure itself, which routinely uses very high doses of *P. gingivalis*, is the fundamental trigger for
281 disease by eliciting very significant alterations to the local oral immune and inflammatory status of the
282 mouse at the time of challenge. As a consequence, a changed environment may be generated which
283 would facilitate overgrowth and reconfiguration of the commensal microbial community structure
284 and, through this, the development of bone loss. Indeed, analysis of a number of components of the
285 immune and inflammatory system in the gingival tissues of immediately post-challenged mice support
286 the deleterious effects of the oral gavage process. (Hajishengallis et al. 2011). It is therefore
287 acknowledged that the *P. gingivalis* gavage murine model has some limitations with respect to its
288 relationship to human disease. However, in the experiments reported here, transfer of the dysbiotic
289 microbiome from a *P. gingivalis* challenged SPF mouse into a healthy recipient mouse results in the
290 same levels of oral microbial load, microbial population structure and destructive bone loss as that
291 experienced by the originally challenged mice. In so doing, these results demonstrate unequivocally
292 that the potentially detrimental effects of high dose *P. gingivalis* challenge are not required to
293 establish disease: alveolar bone loss is solely the consequence of acquisition of the dysbiotic oral
294 microbial community independent of gavage.

295 The longitudinal analysis of challenged mice, the stability of the dysbiotic microbiome to antibiotic
296 treatment and the horizontal and vertical transfer experiments all emphasise the pronounced
297 resilience of the dysbiotic community structure induced by gavage with *P. gingivalis*. Such resilience
298 has also been reported during longitudinal analysis of the human oral microbiome in health. For
299 example, analysis of the salivary microbiome of two individuals on a daily basis over the course of one
300 year demonstrated a highly stable microbial community structure which appeared far more resilient
301 to alterations than the corresponding gut microbial populations in the same individuals. (David et al.

2014). The oral microbiome of adolescents appeared remarkably resilient to change over time during orthodontic treatment (Koopman et al. 2015) and similar stability was found in the oral microbiome of healthy adults following antibiotic administration (Zaura et al. 2015). Although the stability of human oral microbial populations in disease has received less attention, the data in the present murine investigation suggest that dysbiotic oral microbial communities can also display pronounced resilience behaviour and this can have a significant bearing on the development and progression of periodontal disease.

It is recognised that the stability of a microbial community is not simply maintained by inertia, but by the action of restoring forces within a dynamic system (Relman DA 2012). In the case of the oral microbiome, these may include a complex set of metabolic and functional interrelationships that develop within dental biofilms and between biofilms and the host (Rosier et al. 2018). Understanding the nature of the parameters which underpin the resilience of healthy and dysbiotic microbial populations may be important to the development of approaches to prevent the progress of disease and to restore health in diseased individuals.

Author Contributions

M Payne, contributed to conception, design, data acquisition, analysis, and critically revised the manuscript; A Hashim contributed to conception, design, data acquisition and analysis, and critically revised the manuscript; A. Alsam , contributed to data acquisition and analysis and critically revised the manuscript; S. Joseph contributed to data analysis and drafted and critically revised the manuscript; J. Aduse-Opoku contributed to data analysis and critically revised the manuscript; W. Wade contributed to design, data analysis, and critically revised the manuscript; MA Curtis contributed to conception, design, data analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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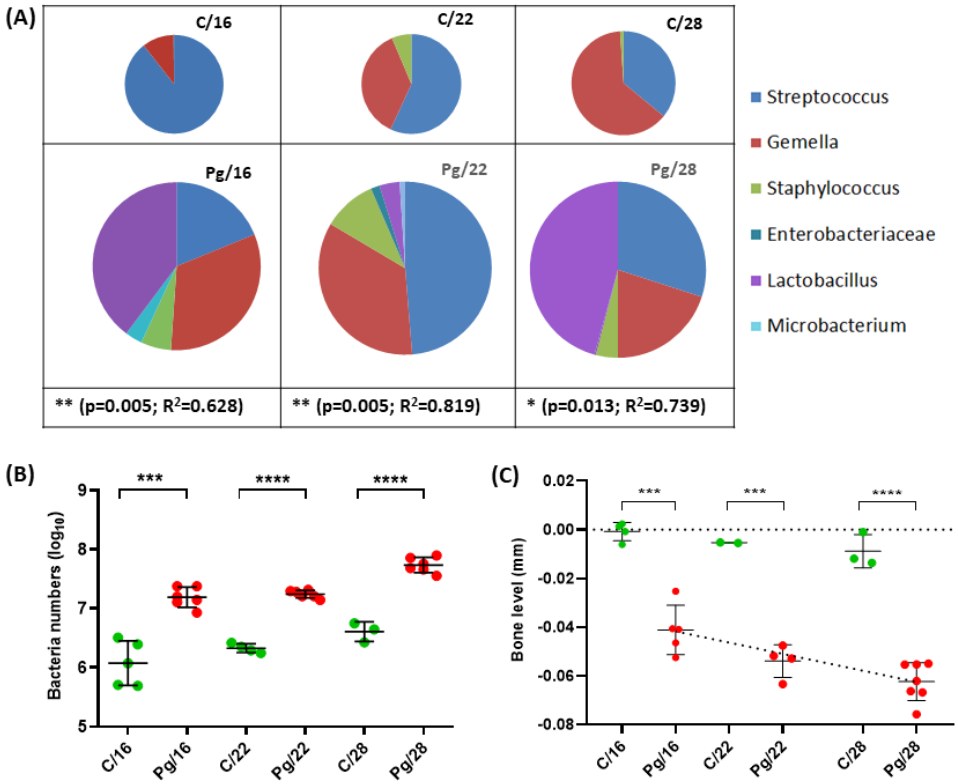
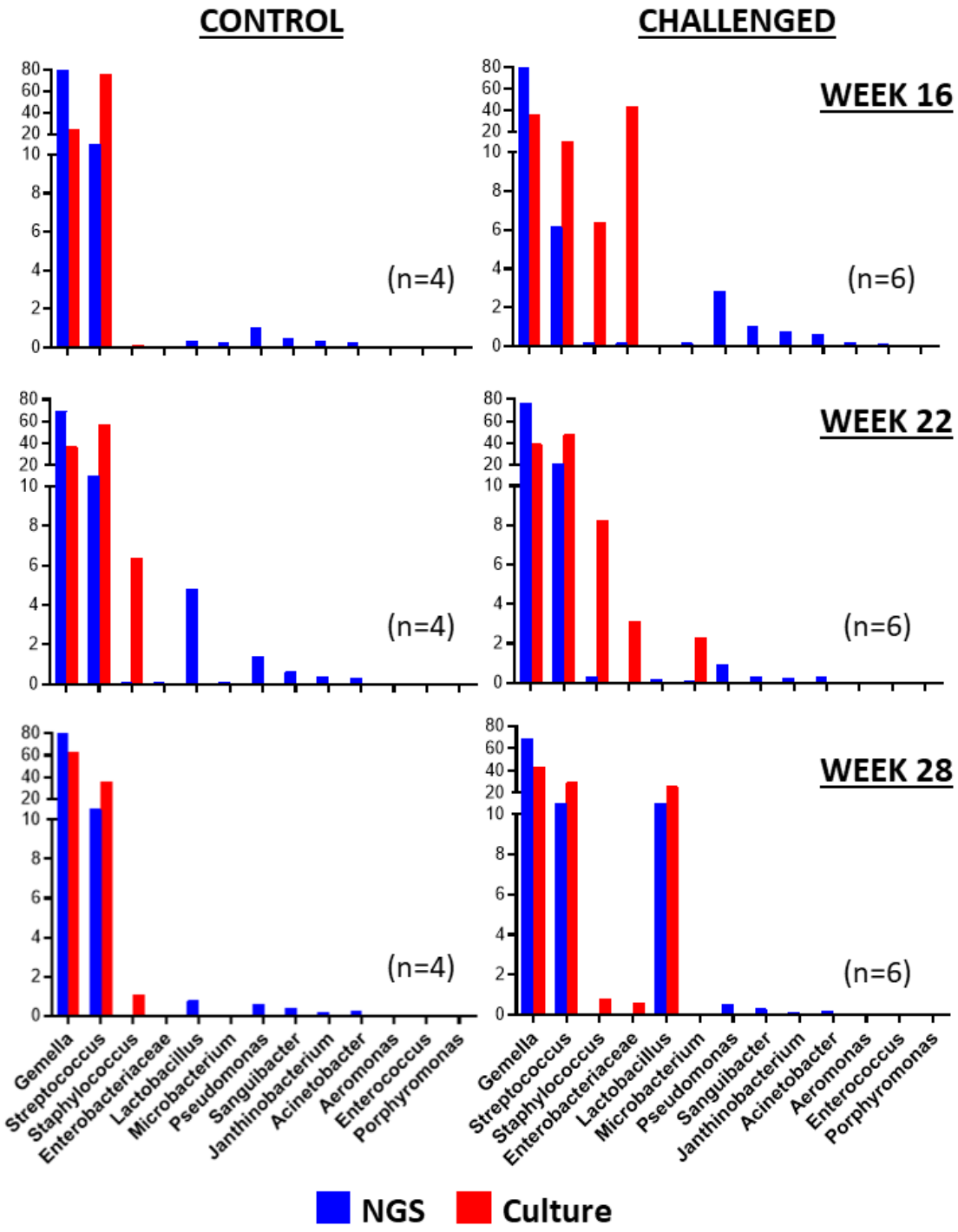


406

407 **Fig 1.** *P. gingivalis* mediated dysbiosis is stable over time and associated with long term destructive
408 disease in the murine model: (A) Bacterial composition of the oral microbiome, determined by culture,
409 of control *and P. gingivalis* treated mice at 16 weeks (C/16 & Pg/16), 22 weeks (C/22 & Pg/22) and 28
410 weeks (C/28 & Pg28). The sizes of the pie-charts are indicative of the variations in the total oral
411 bacterial counts in the different groups. The graphs have been plotted using the observed number of
412 CFUs of each microorganism in each group. Statistical significance in the differences between the
413 microbial communities at each time point was determined by PERMANOVA analysis (** $p<0.05$; ***
414 $p<0.005$; **** $p<0.0005$) (B) Total oral bacterial counts in the different groups expressed as $\log_{10}$ of
415 CFUs. (C) Alveolar bone levels at 16, 22 and 28 weeks in control and *P. gingivalis* treated mice. Bone
416 loss was expressed as negative values relative to the baseline. Each point represents the mean bone
417 level for an individual mouse with horizontal lines representing the mean bone levels per group +/-
418 SD. The dotted line represents the linear rate of bone loss in *P. gingivalis* treated mice over time (**
419 $p<0.05$; *** $p<0.005$; **** $p<0.0005$).

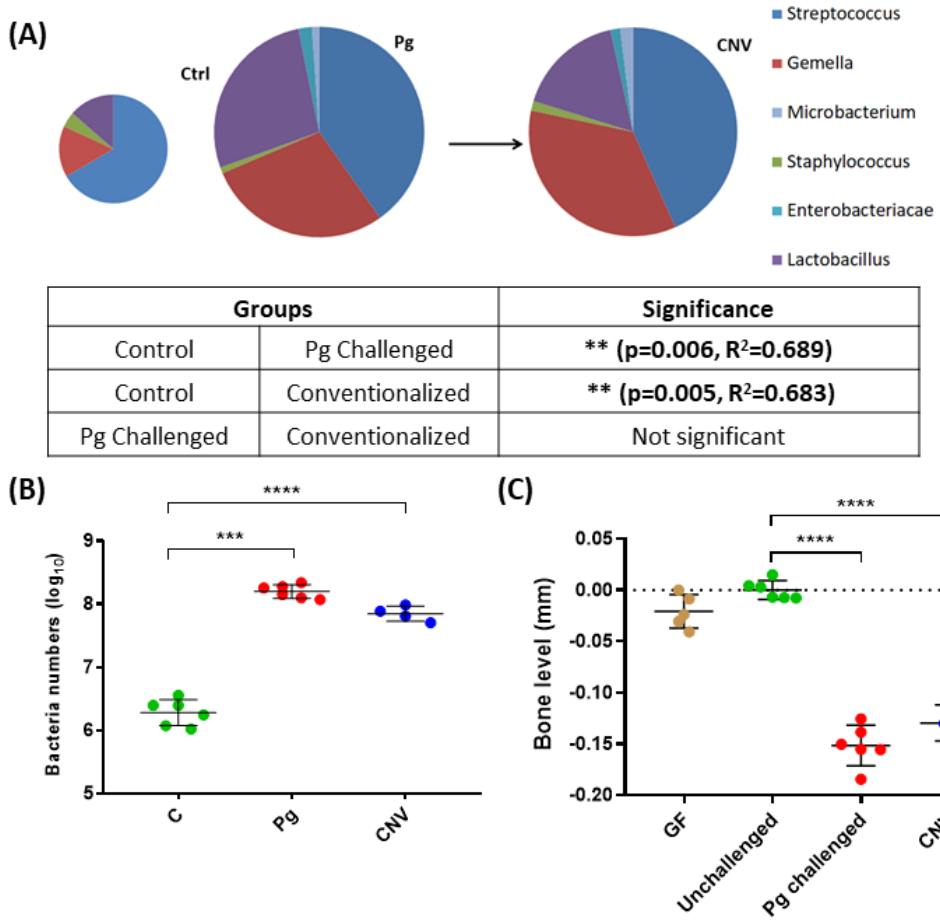
420



421

422 **Fig 2.** Longitudinal comparison of oral microbial population analyses of control and *P. gingivalis*
423 challenged mice at 16, 22 and 28 weeks by laboratory culture followed by full length 16S rRNA gene
424 sequencing (red) and Roche 454 next generation sequencing (NGS) methodology of the V1-V3 region
425 of the 16S rRNA genes (blue). The predominant genera observed in the populations have been listed

on the X axis while the Y axis represents the relative abundance of the bacterial genera in the individual populations expressed in percentages.



Groups		Significance
Control	Pg Challenged	** ($p=0.006$, $R^2=0.689$)
Control	Conventionalized	** ($p=0.005$, $R^2=0.683$)
Pg Challenged	Conventionalized	Not significant

Fig 3. *P. gingivalis* mediated dysbiotic microbiomes stably transfer horizontally into healthy germ-free mice and lead to periodontal disease in the recipients: (A) Bacterial composition of the oral microbiome, determined by culture in control and *P. gingivalis* treated C3H/Orl mice (Ctrl & Pg) and conventionalized germ-free mice of identical genotype (CNV) co-caged with the *P. gingivalis* challenged mice for 14 days. The sizes of the pie-charts are indicative of the variations in the total oral bacterial populations in the different groups. The graphs have been plotted using the observed number of CFUs of each organism in each group. Statistical significance in the differences between the microbial communities of each group was determined by PERMANOVA analysis (** $p<0.05$; ***

p<0.005; **** p<0.0005) (B) Total oral bacterial counts expressed as log_{10} of CFUs in control and *P. gingivalis* treated mice and germ-free (GF) mice conventionalized by co-caging with the challenged mice. (C) Alveolar bone levels in germ-free, control (unchallenged), *P. gingivalis* challenged and conventionalized germ-free mice (CNV) determined after 16 weeks of co-caging. Bone loss was expressed as negative values relative to the baseline. Each point represents the mean bone level for an individual mouse with horizontal lines representing the mean bone levels per group +/- SD. (** p<0.05; *** p<0.005; **** p<0.0005)

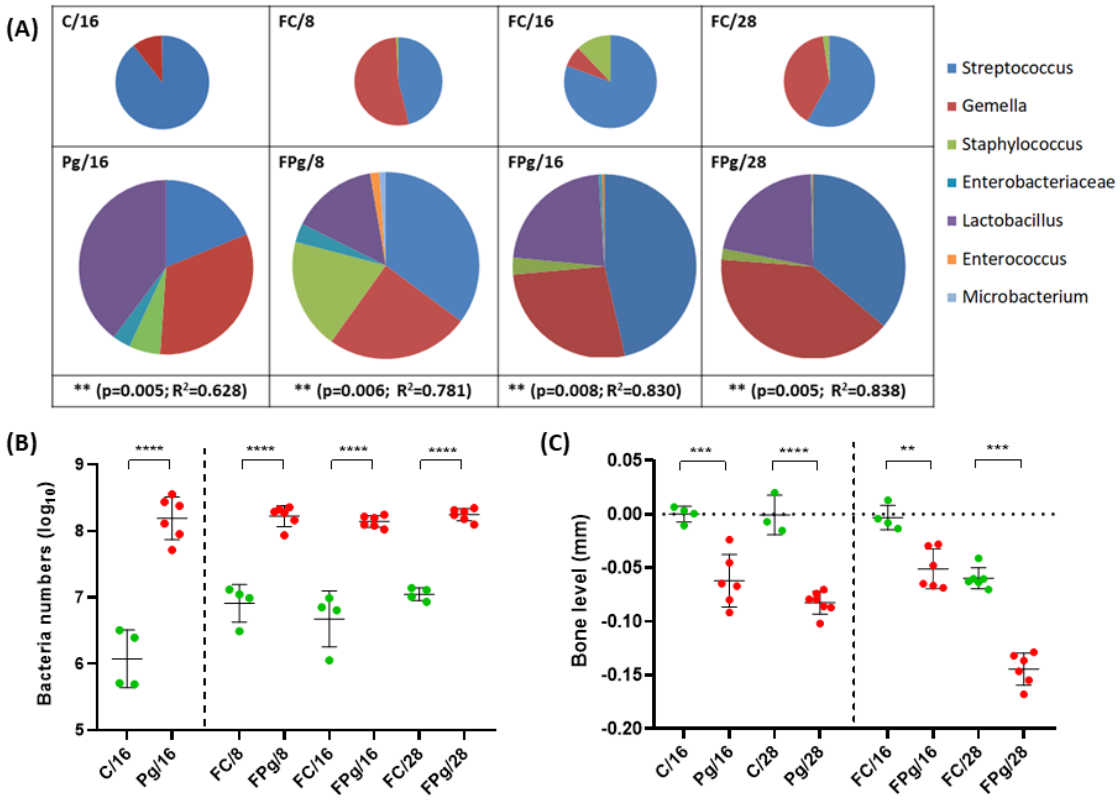


Fig 4. Inter-generational transfer of *P. gingivalis* mediated dysbiotic microbiomes and vertical transmission of disease: (A) Bacterial composition of the oral microbiome, determined by culture, of control and Pg treated parents at 16 weeks (C/16 & Pg/16) and litters of controls and Pg treated parents at 8 (FC/8 & FPg/8), 16 (FC/16 & FPg/16) and 28 weeks (FC/28 & FPg/28). The sizes of the pie-

charts are indicative of the variations in the total oral bacterial counts in the different groups. The graphs have been plotted using the observed number of CFUs of each organism in each group. Statistical significance in the differences between the microbial communities at each time point was determined by PERMANOVA analysis (** p<0.05; *** p<0.005; **** p<0.0005) (B) Total oral bacterial counts expressed as $\log_{10}$ of CFUs in the parents (C/16 & Pg/16) and litters at 8 (FC/8 & FPg/8), 16 (FC/16 & FPg/16) and 28 weeks (FC/28 & FPg/28). (C) Alveolar bone levels at 16 (C/16 & Pg/16) and 28 (C/28 & Pg/28) weeks in control and *P. gingivalis* treated parents and litters at 16 (FC/16 & FPg/16) and 28 (FC/28 & FPg/28) weeks. Bone loss was expressed as negative values relative to the baseline. Each point represents the mean bone level for an individual mouse with horizontal lines representing the mean bone levels per group +/- SD. (** p<0.05; *** p<0.005; **** p<0.0005)

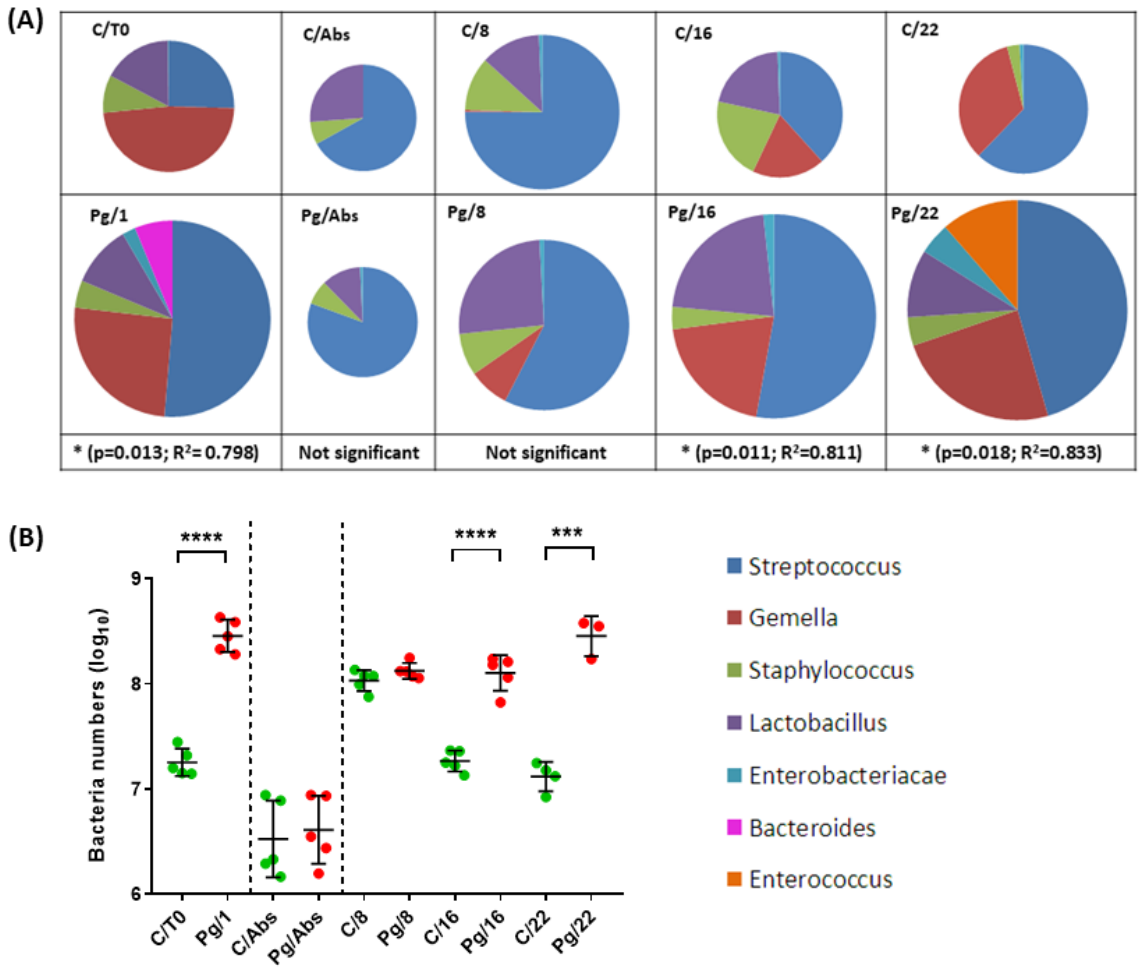


Fig 5. Dysbiosis of the mouse oral microbiome is stable to antibiotic treatment: (A) Bacterial composition of the oral microbiome, determined by culture, of control and Pg treated mice (C & Pg), pre-antibiotics (C/T_0 & Pg/1), immediately post-antibiotics (C/Abs & Pg/Abs) and at 8, 16 and 22 weeks post antibiotics (C/8, C/16, C/22 & Pg/8, Pg/16, Pg/22). The sizes of the pie-charts are indicative of the variations in the total oral bacterial counts in the different groups. The graphs have been plotted using the observed number of CFUs of each organism in each group. Statistical significance in the differences between the microbial communities at each time point was determined by PERMANOVA analysis (** $p<0.05$; *** $p<0.005$; **** $p<0.0005$) (B) Total oral bacterial counts (as a measure of the dysbiosis) in the different groups expressed as $\log_{10}$ of the CFUs. (** $p<0.05$; *** $p<0.005$; **** $p<0.0005$)