Uremia causes dysbiosis-mediated periodontal disease

David Randall (✉ d.randall@qmul.ac.uk)

Queen Mary University of London

Asil Alsam

Queen Mary University of London

Julius Kieswich

Queen Mary University of London

Susan Joseph

King's College London <https://orcid.org/0000-0002-0297-5405>

Joseph Aduse-Opoku

King's College London

Graham Davis

Queen Mary, University of London <https://orcid.org/0000-0001-6673-032X>

David Mills

Queen Mary, University of London <https://orcid.org/0000-0001-7136-4694>

Alan Boyde

Queen's Hospital <https://orcid.org/0000-0002-9871-5498>

Jonathan Swann

<https://orcid.org/0000-0002-6485-4529>

Christoph Thiemermann

Queen Mary University of London

Kieran McCafferty

Queen Mary University of London

Mike Curtis

KCL

Magdi Yaqoob

Queen Mary University of London

Article

Keywords: Chronic Kidney Disease, Periodontal Bone Loss, Saliva, Oral Microbiota, Streptococcus, Rothia

Posted Date: January 25th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-143371/v1>

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Abstract

It is presently unclear why there is a high prevalence of periodontal disease (PD) in individuals living with chronic kidney disease (CKD). By employing three different models in rats and mice, we demonstrate that experimental uremia causes periodontal bone loss. Uremia alters the biochemical composition of saliva and induces progressive dysbiosis of the oral microbiota, with microbial samples from uremic animals displaying reduced overall bacterial growth, increased alpha diversity, reduced abundance of key components of the healthy oral microbiota such as *Streptococcus* and *Rothia*, and an increase in minor taxa including those from gram-negative phyla *Proteobacteria* and *Bacteroidetes*. We show that transfer of oral microbiota from uremic mice induces PD in germ-free animals, whilst co-housing with healthy animals ameliorates the PD phenotype in rats. Thus, we advocate that periodontal disease should be regarded as a bacterially mediated complication of chronic uremia.

Introduction

Periodontal disease (PD) is a complex pathological process in which a bacterial challenge presented by a dysbiotic microbial community in subgingival dental plaque [1] drives a deregulated immune and inflammatory response which ultimately causes osteoclastic resorption of alveolar bone and eventual loss of teeth [2].

Patients living with chronic kidney disease (CKD) have a high prevalence of PD [3–5], and some have suggested that chronic, low-level inflammation caused by PD may lead to progressive renal dysfunction and increased cardiovascular disease [6, 7].

However, there are several reasons to support the view that CKD may actually cause PD. Patients with CKD have abnormalities in the flow rate and biochemical composition of saliva [8, 9], altering the oral microenvironment and exerting selective pressure on the resident microbiota. Furthermore, patients with CKD have marked abnormalities of bone metabolism [10] and immune system function [11] which may also be relevant to the development of PD.

We hypothesized that CKD may be a cause of PD, reversing current understanding of the association between these conditions, and that induction of dysbiosis of the oral microbiota may be a critical mechanism in this process.

Results

Experimental uremia causes periodontal bone loss in rats

Chronic uremia was induced in male Wistar rats using two protocols: chemically-induced uremia (using adenine-containing feed) and surgically-induced uremia (using subtotal nephrectomy, SNx). Examination of de-fleshed heads revealed that uremic animals generated using both experimental protocols displayed significantly more periodontal bone loss than controls after an eight-week period of uremia (an average

of 0.113 mm less alveolar bone height relative to controls, $p < 0.0001$, Fig. 1a&b and supplementary table S1).

Histological examination of representative samples confirmed a greater distance between cemento-enamel junction and alveolar bone ridge in the uremic than control (239.1 μm in the uremic versus 132.7 μm in the control, Fig. 1c), and replacement of specialized junctional epithelium by proliferating epithelium with deep sulcal folds, similar to pocket epithelium seen in periodontal disease in human subjects (Fig. 1d). Immunohistochemistry revealed significant staining for IL-17 (Fig. 1e) which co-localized with staining for neutrophils (Fig. 1f) in areas deep within the alveolar bone in uremic specimens. Micro-computed tomography demonstrated abnormalities of bone formation in uremic specimens (Fig. 1g), with a non-significant trend towards reduced mineral density (the linear attenuation coefficient in bone just below the alveolar bone crest was 1.604 cm^{-1} in controls and 1.547 cm^{-1} in uremics, $p = 0.069$; and at sites deeper within the mandibular bone was 1.658 cm^{-1} in controls and 1.621 cm^{-1} in uremics, $p = 0.271$). Scanning electron microscopy of the surface of alveolar bone facing the periodontal ligament revealed a smooth surface almost bereft of Sharpey's fibres, suggestive of a failure of bone growth at this surface (Fig. 1h).

To evaluate systemic features of chronic uremia that may affect periodontal bone formation, we measured serum concentrations of parathyroid hormone (PTH), calcium and phosphate. There was no difference in serum PTH concentrations between control and uremic animals (serum PTH 15.0 pg/ml in controls vs 13.5 in uremics, $p = 0.664$), although uremic animals displayed a modest increase in serum calcium (2.54 mmol/L in controls vs 2.61 in uremics, $p = 0.006$), and serum phosphate (2.03 mmol/L in controls vs 2.21 in uremics, $p = 0.014$) compared to controls.

To assess global rates of bone and tooth formation, a further batch of animals were injected with three doses of 1 mg/kg calcein green at 48 hour intervals the week prior to sacrifice to assess the daily rate of bone and tooth formation. There were no differences between groups in the rate of dentine formation in the incisor ($14.78\mu\text{m}^3/\mu\text{m}^2/\text{d}$ in sham operated controls vs $15.69\mu\text{m}^3/\mu\text{m}^2/\text{d}$ in SNx, $p = 0.517$), or of bone formation at the lower mandibular border ($4.249\mu\text{m}^3/\mu\text{m}^2/\text{d}$ in controls vs $3.562\mu\text{m}^3/\mu\text{m}^2/\text{d}$ in SNx, $p = 0.397$).

Uremia is associated with oral dysbiosis

Oral swabs were assessed using both bacterial culture and next generation sequencing of the 16S rRNA gene amplicon to assess the effect of experimental uremia on the oral microbiota. The parallel use of these complementary techniques allowed for evaluation of total bacterial abundance, in vitro testing of individual bacterial isolates for their tolerance of high-urea environments as well as completing a thorough survey all members of the oral microbiota, and not just those which are readily cultured.

Lower total bacterial counts after 48 hours of incubation under both aerobic and anaerobic conditions were seen in samples from uremic animals ($\log_{10}$ 6.07 cfu/ml transport medium in controls vs 5.80 in

uremics, $p = 0.034$, Fig. 2a and supplementary table S2), partly accounted for by substantially reduced total counts of the most abundant phylum, *Firmicutes* ($\log_{10}$ 5.88 cfu/ml in controls vs 5.42 in uremics, $p = 0.043$). Conversely, absolute counts of Gram-negative phylum *Proteobacteria* were actually non-significantly higher in uremic animals than in controls ($\log_{10}$ 4.12 cfu/ml in controls vs 4.55 in uremics, $p = 0.151$), which in the context of reduced overall counts in these animals meant that these uremic animals had significantly higher proportional abundances of *Proteobacteria* (9.53% vs 2.99% of total cultured bacteria, $p = 0.003$, Fig. 2b).

At genus level, uremic animals demonstrated lower counts of both the most abundant genus *Streptococcus* ($\log_{10}$ 5.56 cfu/ml in controls vs 5.05 in uremics, $p = 0.017$), and the second most abundant genus *Rothia* ($\log_{10}$ 5.56 cfu/ml in controls vs 5.23 in uremics, $p = 0.022$). Conversely, samples from uremic animals displayed higher growth of a number of minor taxa compared to control samples, which reached significance for the genus *Acinetobacteria* ($\log_{10}$ 3.79 cfu/ml in uremics, absent in controls, $p = 0.006$, Fig. 2c).

Next-generation sequencing of the 16S rRNA gene amplicon was used to confirm the pattern seen in the cultural analysis. Proportional abundances of different phyla plotted for each sample showed a similar decrease in *Firmicutes* and an increase in Gram negative phyla *Proteobacteria* and *Bacteroidetes* in samples from uremic animals (Fig. 2d). Corresponding to the described reductions in major taxa and increases in minor ones in uremic animals, oral communities from uremic animals were found to have higher alpha diversity when measured using the Simpson Index than communities from control animals (0.75 in controls vs 0.82 in uremics, $p = 0.045$).

Ordination plots for all samples revealed that the most significant source of variation was between shipment batches (different batches from the same vendor were used in each experiment). However, when ordination for each experiment was plotted separately, there was indeed differential clustering between control and uremic animals, with ADONIS assessing the significance of separation according to uremia proving significant for the surgically-induced uremic protocol ($R_2 = 0.147$, $p = 0.012$) but not in the chemically-induced uremia protocol ($R_2 = 0.112$, $p = 0.184$, Fig. 2e).

Co-housing alters bacterial communities and affects the severity of periodontal disease

The surgically-induced uremia protocol allowed us to investigate the influence of either housing rats singly (with other rats of the same treatment arm; uremics with uremics or controls with controls), or co-housed (uremics with controls, Fig. 2f).

Interestingly, uremic animals that were co-housed with controls developed less periodontal bone loss than those housed only with other uremic animals. Two-way ANOVA confirmed that whilst treatment class had the biggest effect on bone loss in these animals (accounting for 77% of variance, $p = 0.001$), the contribution of housing also proved significant (6.7% of variance, $p = 0.014$). A simple t-test comparing

the degree of bone loss between co-housed and singly housed uremic animals confirmed that the co-housed animals had significantly less bone loss than those that were singly housed (mean bone height – 0.149 mm compared to mean of control animals in those that were singly housed, and – 0.109 mm in those that were co-housed, $p = 0.038$). There was no difference in bone loss between co-housed and singly housed control animals (Fig. 2g).

An ordination plot based on 16S gene sequencing demonstrated that co-housed animals had an intermediate microbial profile between the singly-housed control and uremic groups (Fig. 2h). Two-way ANOVA carried out for the first two principal components revealed that housing significantly affected clustering in component 2 (19.86% of variance, $p = 0.043$), although to a lesser extent than treatment class (25.84% of variance, $p = 0.024$). Only treatment class significantly affected principal component 1 (treatment 28.48% of variance, $p = 0.0196$; housing 0.02% of variance, $p = 0.94$).

Uremia alters salivary biochemistry in rats which may explain the observed oral dysbiosis

To assess whether alterations in saliva following the induction of uremia might be responsible for the differences in oral microbiology, induced saliva samples were obtained using pilocarpine administration to rats under terminal anesthesia.

There were no differences in either the flow rate or pH of induced saliva (supplementary table S3), however uremic animals were found to have significantly higher concentrations of salivary urea (in proportion to an increase in serum urea) when measured by colorimetric analysis (1.62 mmol/l in controls vs 3.73 mmol/l in uremics, $p = 0.007$, Fig. 3a).

Untargeted proton Nuclear Magnetic Resonance spectroscopy ($^1\text{H-NMR}$) was performed to characterize biochemical perturbations associated with uremia and assess the functionality of the altered microbiota. Salivary concentrations of acetate were 27% lower in uremic animals compared to controls (122.19 relative units in controls vs 89.11 in uremics, $p = 0.013$), and concentrations of lactate 47% lower (although this did not reach significance, 116.81 vs 61.35 relative units, $p = 0.056$, supplementary table S4).

In vitro testing was carried out on all bacterial isolates from the cultured analysis that had been deep frozen in pure colonies after initial isolation and identification. There was a positive association between mean growth of bacterial isolates in uremic animals and the mean inhibitory concentration for urea. Linear regression was used to calculate a line of best fit, which proved to be significantly different from horizontal (slope 0.34, $p = 0.046$). Using Christensen's urea agar we demonstrated that urease producing organisms were better-represented among urea-tolerant groups (Fig. 3b).

Uremia induces periodontal bone loss and progressive oral dysbiosis in mice

In order to ensure that these results were not rodent species-specific, experimental uremia was induced in wild-type male C57BL/6 mice using a slowly progressive model of chemically-induced uremia. As in rats, uremic mice displayed increased periodontal bone loss (-0.02 mm relative to the mean of controls, $p = 0.0005$, supplementary table S5).

Oral swabs were taken every four weeks during the experimental period, and next generation sequencing of the 16S rRNA gene amplicon revealed progressive changes in samples from uremic animals, characterized by increased heterogeneity between samples, and progressively differential clustering on principal component analysis (Fig. 4). Significant differences in clustering as measured by PerMANOVA emerged between control and uremic microbiotas at 10 weeks into the experimental period (roughly coincident with the development of significant differences between control and uremic animals in weight, suggestive of clinical uremia, which first became apparent at 9 weeks into the experimental period, supplementary table S5). These changes persisted at 14 weeks, but after 18 weeks of experimental diet, uremic animals exhibited such significant within-group differences meaning that although there was still a marked separation between the clustering of uremic vs control samples on visual inspection of PCA plots, this did not reach significance when assessed using PerMANOVA ($R^2 = 0.134$, $p = 0.066$). Quantification of population variances using permutational analysis of multivariate dispersions (PERMDISP) confirmed that samples from uremic animals became progressively heterogeneous as uremia increased (average distances to median being 6.78 in controls and 18.31 in uremics by 18 weeks, $p = 0.012$).

The ANalysis of Composition of Microbiomes (ANCOM) methodology was used to identify amplicon sequencing variants (ASVs) that were differentially abundant between groups after correction for multiple hypothesis testing. All ASVs more abundant in controls were from the dominant phylum *Firmicutes*, whilst those increased in uremic animals represented a diverse range of organisms from phyla including *Actinobacteria* and *Proteobacteria*, and included ASVs representing organisms (such as from genus *Psychrobacter*) that have previously being implicated in the development of PD in animals [12].

Periodontal disease can be transmitted by oral microbial transfer into healthy germ-free mice

We assessed the causative role of bacterial dysbiosis in PD by carrying out oral microbial transfer (OMT) from control and uremic donor mice into germ-free animals (Fig. 5a). The success of OMT was formally assessed by differential clustering on an ordination plot, and using PerMANOVA. This revealed that transfer of the uremic microbiota accurately established the donor microbiota in recipient animals (non-significant differences between donors and recipients, $R^2 = 0.12$, $p = 0.158$); that although control recipients visually clustered with control donors, there did exist significant differences between these groups ($R^2 = 0.196$, $p = 0.023$); but that by far the largest differences existed between uremic and control recipient microbiotas, similar to the difference between uremic and control donors ($R^2 = 0.233$, $p < 0.001$).

Germ-free mice receiving OMT from uremic mice developed substantially more periodontal bone loss than those receiving OMT from control mice (-0.042 mm to the mean in control recipients, $p < 0.001$, Fig. 5b and Supplementary table S6). They were found to have notably dysbiotic oral communities that persisted at three and nine weeks after transfer, and which appeared to represent exaggerations of the features of the control and uremic microbiota seen in both the donor mice and in the previously described rat and mouse experiments. Thus, at 9 weeks, recipients of uremic microbiota demonstrated reduced bacterial counts ($\log_{10}$ 6.2 cfu/ml in control recipients vs $\log_{10}$ 5.32 in uremic recipients, $p < 0.001$), markedly increased alpha diversity (Simpson Index 0.24 in control recipients vs 0.94 in uremic recipients, $p < 0.0001$), and differential clustering on ordination plots, in a similar but more extreme direction to the donor communities (Fig. 5c). Microbiotas from control recipients were heavily dominated by bacteria from phylum *Firmicutes*, whilst microbiotas from uremic recipients displayed heterogenous oral microbial communities including high prevalence of various phyla including *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Cyanobacteria* (Fig. 5d).

Discussion

We have demonstrated that experimental uremia causes bacterially mediated periodontal bone loss in rodents, using three separate experimental models in two different host species. Use of animal subjects excludes confounding factors, such as co-morbidity and dental hygiene habits, which may serve as alternative explanations for the high level of PD in human subjects with CKD [13, 14]. Use of only male animals (as is common in studies of experimental uremia) may limit generalizability to the whole human CKD population, although population data suggests women with CKD suffer a similar incidence of PD as men [6].

In rats, the chemically-induced uremia model generates rapidly progressive induction of uremia followed by a period of partial recovery [15], whilst the surgically-induced uremia model mimics steadily-progressive loss of renal mass due to hyperfiltration injury in the remaining glomeruli [16, 17]. The model used in mice causes a slowly-progressive chronic uremia with renal fibrosis and associated heart failure [18]. Uremic animals from each of these models demonstrated significant increases in periodontal bone loss compared to controls, and this phenotype was reliably transferred into germ-free mice along with stable but dysbiotic oral microbiota.

The histological changes caused by uremia are similar to those caused by other forms of periodontal disease, including replacement of specialized junctional epithelium [19] with proliferating epithelium with deep sulcal folds, which appears similar to the pocket epithelium seen in humans with periodontal disease [20]. We also demonstrated aberrant bone formation in the alveolar bone crest adjacent to inflamed periodontal structures. Although C57/BL6 mice are known to be relatively resistant to periodontitis, the degree of bone loss we demonstrated in uremic animals was within the range of bone loss demonstrated using other experimental models in this strain, being more pronounced than that seen in periodontitis models employing oral gavage with disease-causing micro-organisms such as *Porphyromonas gingivalis* [21], but less severe than that seen in ligature periodontitis models [22].

We have also demonstrated in each model a common signature of the effects of uremia on oral bacterial communities, consisting of a reduction in overall bacterial counts; an increase in alpha diversity; depletion of taxa such as *Streptococcus* and *Rothia* which are key components of healthy oral microbiotas [23, 24]; and an increase in bacteria (typically non-oral gram negative rods) that have previously been associated with PD [25, 26]. .

Although few studies in humans have described the oral microbiota in the context of uremia, those that have reveal a strikingly similar microbial signature to that observed here. In particular, Hu *et al.* demonstrated significant changes in oral microbial communities in CKD patients when compared with healthy controls, with an increase in the phylum *Proteobacteria*, at the relative expense, in proportional terms, of taxa in the *Firmicutes* phylum, including *Streptococcus* and *Veillonella* [27]. Kidney transplant recipients with poor graft function [28] and hemodialysis patients [29] have likewise been demonstrated to have dysbiotic oral microbiota, consistent with our assertion that uremia itself induces dysbiosis.

Increased salivary urea is a possible mechanism in driving these changes: the composition of saliva uniquely determines the selective pressures on the oral microbiota, and we showed (notwithstanding the limited correlation of bacterial behavior in liquid culture compared to *in vivo* biofilms) that there was a correlation between *in vitro* urea tolerance and urease activity and increased abundance in uremic animals. Notably, *Streptococcus* and *Rothia* isolates, which were present at reduced abundances in uremic animals, did not display *in vitro* urease activity and showed reduced tolerance of higher urea concentrations in broth culture.

The oral microbial signature of uremia that we have described was seen in an exaggerated but stable form when microbiota from control and uremic animals were transferred into previously germ-free mice. The ability of abnormal microbial communities to stably establish themselves and cause PD after transfer into germ-free mice has been previously described [30, 31], and the high degree of periodontal bone loss demonstrated in these animals shows the relevance of oral dysbiosis in the etiology of periodontal disease.

In rats, co-housing with healthy animals seemed to lessen some of the dysbiotic changes seen in uremic animals, and to ameliorate the associated PD phenotype. It has long been known that co-housing can affect oral microbiology [32], and in work recently published by Abusleme *et al* it has been shown that healthier microbiota may outcompete and even fully replace more dysbiotic communities [33].

In this study, uremia was not accompanied by a reduction in salivary flow rates and an increase in salivary pH, as shown elsewhere in rodent models of uremia [34]. This is likely to be a consequence of the mechanism we used to induce saliva. Pilocarpine administration overrides physiological control of salivary flow rates, and if the mechanism by which high salivary urea increases pH is dependent on bacterial hydrolysis of urea to ammonium, it is possible that the immediate removal of saliva by pipetting prevented these bacterial effects from taking place. Reduced concentrations of acetic and lactic acid in control samples is consistent with the reduction in *Streptococcus* and *Lactobacillus* which are known to digest sugars and produce a range of organic acids [35].

Although the data presented in this study are consistent with urea mediated dysbiosis of the oral microbiota and subsequent inflammatory driven periodontal bone loss, we cannot exclude the systemic effects of uremia on bone turnover. However, uremic animals did not show elevation of parathyroid hormone, consistent with other studies which demonstrate that a high phosphorus diet in addition to SNx is required to induce frank hyperparathyroidism in rats [36]. The jaw histology and radiology presented here are in keeping with an additional inflammatory component of bone disease (with evidence of IL-17 and neutrophil accumulation within alveolar bone), similar to that seen in other forms of inflammatory periodontitis [37]. Furthermore, the transmission of periodontal bone loss into otherwise healthy germ free mice through OMT and reduction in bone loss in co-housed animals suggests that dysbiotic oral microbiota play a direct causative role.

Additional research could consider the extent to which periodontal bone loss is a result of a normal immune reaction to a dysbiotic bacterial burden, or whether the periodontal immune reaction in uremic animals is, in itself, abnormal. For example, a failure of neutrophil migration has been shown to be a primary cause of severe IL-17 driven periodontitis in LAD-1 deficiency [38], and similarly deficient neutrophil activation and migration has been described in CKD [39], along with a tendency to increased inflammatory bone loss [40].

More broadly, given that we demonstrate a causative role for uremia and the etiology of PD, further research could assess whether periodontal inflammation, once established, contributes to renal fibrosis and cardiovascular disease, and whether there is a role for dental screening and treatment to improve renal and cardiovascular outcomes in individuals with CKD.

We propose that uremic periodontal disease should be regarded as a novel complication of CKD, and that dysbiotic change in oral bacterial communities induced by uremia plays a crucial mechanistic role.

Methods

Data and code availability

Raw sequencing data from all samples has been uploaded to the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI, <https://submit.ncbi.nlm.nih.gov/subs/sra/>). It can be accessed using the accession number **PRJNA648141**.

All raw NMR data has been uploaded to the Metabolights online repository (<https://ebi.ac.uk/metabolights/>) [41], using the study identifier **MTBLS1833**.

All other raw data, including animal data for the various rodent cohorts and bacteriological data from the culture experiment and *in vitro* microbiological work is available in the supplementary files.

Microbiological analysis was carried out in R using packages which are publicly available via CRAN or Github.

Analysis of NMR data analysis can be replicated using Matlab scripts in the IMPACT Toolkit developed in house at Imperial College available from <https://github.com/csmsoftware/IMPACTS>.

Animal work

Animal experiments were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986, with local ethical committee approval. All animal work was carried out in the Biological Services Unit of Queen Mary University of London at Charterhouse Square, and complied fully with all relevant animal welfare guidance and legislation (UK Home Office Project License number PPL 70/8350).

Source of animals

All rats used in the experiment were male, outbred Wistar IGS rats obtained from Charles Rivers (Kent, UK) at 7 weeks of age. All were housed in individually ventilated cages under 12h light/dark cycles and were allowed unlimited access to water and feed (RM1 diet, or RM1 with 0.75% adenine, from Special Diet Services, Essex, UK).

All mice used in the experiment were male, wild-type C57BL/6 mice. Those used in the adenine feeding experiment were obtained from Charles Rivers at 7 weeks of age, whilst germ-free mice of the same species were obtained from a colony maintained by one of the authors (MC) at the Biological Research Facility, St George's University of London, at 8 weeks of age. All were housed in individually ventilated cages under 12 h light/dark cycles and were allowed unlimited access to water and diet (RM1 diet or RM1 + 0.15% adenine, from Special Diet Services, Essex, UK).

Male animals were used throughout because of the potential for female reproductive hormones to influence the uremic phenotype or introduce phenotypic heterogeneity into the study population.

Chemically-induced uremia in rats

The total cohort size was 18 rats. After a week-long period of acclimatization, nine rats were started on the adenine-containing intervention diet whilst another nine were maintained on standard control diet. This diet was continued for four weeks, followed by a washout period of four weeks when all animals received the control diet, after which the animals were sacrificed. Oral swabs were taken from all animals at the point of maximal uremia for those receiving the intervention diet (at the end of the 4-week period of adenine administration). Serum samples were obtained by thoracotomy at the point of sacrifice, and other tissues were obtained as outlined below.

Surgically-induced uremia in rats

The total cohort size was 24 rats. After a week-long period of acclimatization, fourteen underwent SNx, involving exteriorisation of the left kidney with decapsulation and removal of the upper and lower poles and subsequent replacement of the middle pole only, followed by total right nephrectomy two weeks later. Ten underwent sham procedures, involving exteriorisation, decapsulation and replacement of the left kidney, followed by the same procedure on the right kidney two weeks later. Oral swabs to assess the microbiota were taken four weeks after the second stage of the surgical procedure, to parallel those taken in the chemically-induced uremia protocol. A 24-hour urine collection was performed in the week prior to sacrifice (results from this have been published elsewhere [42]).

Additional rats for histological and salivary analysis

Thirteen additional rats were used in order to obtain saliva samples for subsequent analysis, and to undertake bone staining to assess the bone formation rate. These rats underwent SNx or sham procedures as outlined above (n=6 sham surgery, n=7 SNx), and were otherwise looked after identically to those in the 'Surgically induced uremia in rats' protocol above. In their final week of life, 500µg calcein green (approximately 1mg/kg) was injected intravenously three times at exact 48h intervals. The following week induced saliva collection was carried out under terminal anesthesia with ketamine/xylocaine. After full induction of anesthesia, 1mg pilocarpine was injected into the peritoneum, with a further 1mg administered 5 minutes later if there was no salivary response. Saliva was then collected over the following 8 minutes using a 100mL pipette and 1.5ml Eppendorf tubes. Salivary volume was directly assessed by weighing the filled tubes and subtracting the weight of the tube itself. Salivary pH was directly measured using a pH meter and narrow gauge probe (Mettler Toledo, Leicester, UK), before saliva was snap frozen in liquid nitrogen and transferred to a -80° freezer until the time of analysis.

Chemically-induced uremia in mice

The total cohort size was 20 mice. After a week-long period of acclimatization, ten animals were placed on an intervention diet (RM1 with 0.15% adenine), whilst ten remained on standard RM1 diet. Oral swabs to assess changes in microbiota were obtained at age 7 weeks, 10 weeks, 14 weeks, 18 weeks, 22 weeks and 26 weeks of age – ie, prior to starting the experimental protocol, and at 2 weeks, 6 weeks, 10 weeks, 14 weeks and 18 weeks after starting it. All mice were sacrificed at age 28 weeks after a 24-hour urine collection. Additional oral swabs were obtained prior to the time of sacrifice from four 'donor' animals in each group, for using in the 'oral microbial transfer' experiment described below. Additionally,

soiled cage contents including bedding and droppings from the cages in which these donor animals were housed, were frozen for further use as described below.

Oral microbial transfer in mice

Fifteen germ-free C57BL/6 mice were transferred direct from their sterile isolator at the Biological Research Facility, St George's University of London, to the Biological Services Unit at Charterhouse Square using a clean but non-sterile specialist animal transfer company (Impex, UK) in three separate batches (one batch of seven for receipt of microbiota from control donors, two batches of four each for receipt of microbiota from uremic donors).

On arrival, each mouse received oral microbial transfer (OMT) by oral gavage of swabs taken from donor animals as described above. Each donor swab was used to transfer into two (or in one case, one) recipient(s); seven were designated control recipients and eight, uremic recipients. Gavage was carried out by using a sterile swab thoroughly immersed in transport medium that had been frozen since the time of sampling, and agitating the swab in the mouth of the recipient mouth for 15 seconds and encouraging them to suck on it. After receiving the OMT, the mice were placed in cages containing cage contents from the cage occupied by the particular donor animals, which had been frozen at -80° until the time of use, to permit ongoing microbial transfer by coprophagy.

Animals were then maintained in ordinary individually-ventilated cages in an open area of the Biological Services Unit, with standard 12h light/dark cycles. They had unlimited access to standard RM-1 diet and tap water. Oral swabs were taken to assess the efficacy and durability of bacterial transfer at 3-weeks and 9-weeks after transfer in all animals, and all animals were then culled, after a 24-hour urine collection, at 18 weeks of age (10 weeks after transfer).

Laboratory methods

Processing of blood samples:

Animals in all experimental groups were sacrificed by lethal injection of sodium thiopentone (LINK Pharmaceuticals, Horsham, UK). Blood samples were obtained by thoracotomy and cardiac puncture, and spun down directly to isolate serum which was frozen at -80° until the time of analysis. Quantification of serum urea, creatinine, calcium and phosphate concentrations was done by IDEXX Bioresearch,

Ludwigsberg, Germany. Serum parathyroid hormone concentrations were assessed using a PTH ELISA kit suitable for rat serum (RayBiotech), used according to the manufacturer's instructions.

Measurement of alveolar bone height:

Heads were removed and jaw specimens obtained from all animals using a guillotine and sharp dissection with scissors. Alveolar bone height was measured using a morphometric method previously demonstrated to have equal reliability to radiological [43] and histological techniques [44]. After any samples (typically mandibles) required for conventional histology, micro-CT or scanning electron microscopy were removed, skulls were chemically defleshed by incubation in the protease-based detergent Terg-a-zyne® (Sigma-Aldrich, UK), for 48 hours at 55°C, with remaining soft tissue being removed mechanically after this. One of the authors (AA) who has significant expertise in the procedure obtained photographs using a dissecting microscope and measured the distance between the cemento-enamel junction and the alveolar bone crest using ImageJ software [45], as outlined by Baker and colleagues [21], although without the use of blue dye. Bone height was measured over the lingual and buccal surfaces of molar roots, and a composite measurement for each animal was calculated. These figures are expressed relative to the average bone height in control animals, with significance assessed using Student's t-test with Welch's correction for unequal variances.

Light microscopy:

Tissues removed prior to defleshing were fixed in formalin and processed according to standard histological procedures, and embedded in paraffin. Each jaw was sectioned in frontal buccolingual orientation using a microtome (5 mm) and mounted on charged glass slides. Every tenth section was stained by haematoxylin and eosin using an automated slide processor, and then photographed using a Nikon Eclipse 80i Stereology microscope using 4/0.13 and 10/0.45 objective lenses.

Immunohistochemistry:

Neutrophils and IL-17 were detected using primary antibodies (Abcam, Cambridge, UK) and anti-rabbit (PK-6101) secondary antibody (Maravai LifeSciences, San Diego, US). Sections were then viewed and photographed using the same microscope and lens as used for light microscopy of the H&E stained slides.

Scanning electron microscopy:

Samples were transported in 70% ethanol to the Dental Physical Sciences unit at the Mile End Campus, QMUL. Some samples were rendered anorganic by treatment with 7% available chlorine sodium hypochlorite bleach for 3 weeks to remove all soft tissue. This treatment completely removes the periodontal ligament so that the teeth could be removed manually to expose the surface of the alveolar bone. All SEM imaging was done using 20kV accelerating voltage and a solid state backscattered electron (BSE) detector, using a chamber pressure of 50Pa.

Confocal scanning light microscopy:

Samples in 70% ethanol from calcein-injected animals were then embedded in polymethyl methacrylate (PMMA), and blocks were cut and polished to produce flat surfaces before being used for confocal scanning light microscopy (CSLM). This was carried out at the Rockefeller Building, Division of Biosciences, University College London using a Leica SPE confocal system with an inverted microscope. The PMMA blocks were cover-slipped with glycerol. Objectives used were 10/0.45, 20/0.75 and 63/1.3 oil. Images were analysed using ImageJ software and a measure of the daily rate of dentine and bone formation calculated at the incisor root and lower mandibular border, respectively. The bone formation rate (BFR) was calculated using the formula $BFR = MAR * (MS/BS)$ as suggested by the ASBMR Histomorphometry Nomenclature Committee [46], where the Mineral Apposition Rate (MAR) was calculated by dividing the distance between the innermost and outermost calcein bands (given 96h apart) by 4, and the Mineralizing Surface (MS) and Bone Surface (BS) were measured directly using ImageJ.

Micro CT:

This was carried out on samples embedded in PMMA. Samples were scanned on the MuCAT2 micro-CT system designed and operating in the Dental Physical Sciences unit at the Mile End Campus, QMUL. The samples were scanned at 90kV & 180uA at 20 or 22um voxel size. Reconstruction was performed with GPU accelerated filtered Feldkamp backprojection algorithm and the grey-level data was calibrated to linear attenuation coefficient at 40 keV using a multi-material calibration carousel and X-ray modelling software [47]. Quantification of bone mineral density was carried out by assessing the mean linear attenuation coefficient of 20 tagged regions with a radius of three pixels in three dimensions at each tagged location and a calibration voltage of 27.5keV.

Collection of samples for analysis of oral microbiota:

Oral swabs were taken from animals at the timepoints described above by agitating sterile cotton swabs against the molars of rats or mice being held in the scruff position for a period of 30 seconds. Swabs were then placed into 100µl transport medium and transferred directly to the laboratory, where they were

vortexed for 30 seconds to mobilize cells and 30µl was removed for culture. The remaining transport medium and swab was frozen at -80°C for subsequent DNA extraction.

Culture analysis:

Transport medium withdrawn for culture was serially diluted and spread onto blood agar plates containing 5% defibrinated horse blood (TCS Biosciences, UK) before being incubated under both aerobic and anaerobic conditions (80% N₂, 10% H₂ and 10% CO₂) for 48 hours at 37°C. After this, colonies were counted according to morphology and grown to purity on new blood agar plates. DNA was extracted using the GenElute Bacterial Genomic DNA extraction kit (Sigma Aldrich, UK). PCR products were cleaned up using the NucleoSpin® Gel and PCR clean-up kit (Machery-Nagel, Germany), and then identified using Sanger sequencing of the whole 16S rRNA gene (Eurofins Scientific, Luxembourg), using the widely-used 27F-1492R primer pair, which have been used previously by our group to identify cultured oral microbes [31, 48]. Consensus sequences of forward and reverse reads were assembled using the BioEdit Sequence Alignment Editor [49], and full length 16S rRNA gene sequences were assembled from forward and reverse reads using the CAP3 Contig Assembly Programme [50] available online via the Pôle Rhône-Alpes de Bioinformatique Site (<http://doua.prabi.fr/software/cap3>). All consensus sequences were >1400 base pairs in length and the mean length was 1456bp.

Additional in vitro bacterial work

In vitro assessment of urease activity and tolerance of variable urea concentrations were assessed for all bacterial isolates after they were grown to purity on 5% blood agar plates under standard aerobic or anaerobic conditions.

Urease activity was assessed in all isolates by culturing under either aerobic or anaerobic conditions on Christensen's urea agar (Sigma-Aldrich) at 37°C. A positive urease result was recorded if there was a color change to purple, and the sample was re-grown if there was no discernable growth on the top of the agar.

Two broths were used in order to assess bacterial growth at different concentrations of urea: Iso-sensitest broth (ThermoFisher Scientific) and Brain-Heart Infusion (BHI) broth (SigmaAldrich). The BHI broth was used for some samples after they could not be grown after several attempts in Iso-sensitest broth. One isolate (eventually identified as *Haemophilus parainfluenzae*) did not grow in either broth and after researching its specific growth requirements in the published literature, eventually grew well after filter-sterilized hemin and nicotinamide adenine dinucleotide were added to the growth medium.

Preparations of both broths were prepared at variably stronger concentrations than the manufacturer's instructions would suggest so that when diluted with different concentrations of filter-sterilized 60% urea solution, broths with eventual concentrations of 0%, 4%, 8%, 12%, 18% and 24% urea ensued.

Bacteria grown to purity on blood agar were then transferred into 2ml sterile phosphate buffered saline (PBS). A 1ml aliquot was assessed using a spectrophotometer at 600nm and the remainder of the bacterial solution further diluted with sterile PBS to achieve a standard turbidity of 0.5 McFarland units, equating to a concentration of bacteria of 1.5×10^8 colony forming units/ml (cfu/ml). These solutions were further diluted 50-fold to achieve an approximate concentration of 3×10^6 cfu/ml, and then 34µl of this bacterial preparation were added to 200µl of varying concentrations of urea broth in a 96-well plate, to achieve 234µl incubations each containing approximately 5×10^5 cfu bacteria in eventual urea concentrations of 0%, 3.3%, 6.6%, 10%, 15% and 20%.

These plates were then incubated at 37° in either aerobic or anaerobic conditions for 24 hours before being read on a plate reader at 620nm. The mean inhibitory concentration was defined for each organism as the urea concentration at which the optical density of the solution was decreased to less than 10% of the difference between 0% urea and control (non-inoculated) wells. One isolate did not achieve sufficient growth to allow calculation of MIC.

DNA extraction and PCR for next-generation sequencing:

The remaining transport solution not used for culture analysis, along with the swab, was transferred to bead beating tubes from the DNeasy PowerSoil kit from QIAGEN, used according to the manufacturer's instructions including an 8-minute bead-beating step using the FastPrep-24™ homogenizer (MP Biomedicals). All samples were processed using the same kit, and negative 'kitome' control samples were included for each extraction kit used [51](Salter *et al.*, 2014)[51]. PCR was carried out using barcoded 27F/338R primer pairs, targeting the V1/V2 hypervariable region of the 16S rRNA gene. PCR was carried out in a sterile 96-well plate using Phusion Green Hot Start II High Fidelity PCR Master Mix (ThermoFisher Scientific), using an initial denaturation step for 5 mins at 98°C followed by 25 cycles of 98°C for 10s, 53°C for 30s, 72°C for 45s and a final extension of 72°C for 10 min.

Next generation sequencing:

Normalisation of DNA concentrations was carried out using SequalPrep™ Normalisation Plates (ThermoFisher) and DNA was quantified using either the Quant-iT® PicoGreen™ dsDNA Quantitation Kit (ThermoFisher Scientific) or a Qubit® 4 Fluorometer (also ThermoFisher). The samples were pooled and sequenced in two runs; one at the Barts & the London Genome Centre, QMUL; and one in the DNA Sequencing Facility, in the Department of Biochemistry at the University of Cambridge; each using an Illumina MiSeq 2 x 250 flow cell for paired-end sequencing.

Quantification of salivary urea:

A colorimetric detection kit for urea nitrogen (ThermoFisher Scientific) was used according to the manufacturer's instructions. Samples of saliva were processed at 1:2 and 1:20 dilutions and the mean concentration using both dilutions in duplicate was accepted. Corresponding serum samples were analysed using the same kit but at 1:20 and 1:40 dilutions to allow comparison.

NMR spectroscopy of saliva:

Saliva samples were diluted with buffer containing trimethylsilylpropanoic acid (TSP) and analysed on an NMR spectrometer (Bruker) operating at 600.22 MHz ^1H frequency at Imperial College London.

Statistical methods

Statistical analysis of bone height data:

All data for loss of periodontal bone height was found to be normally distributed when assessed by the Shapiro-Wilk test. All testing for significance of difference between two groups was carried out using Student's t-test with Welch's correction for unequal variances, in GraphPad Prism or Microsoft Excel.

Analysis of effect of housing on microbiology and bone height:

Two-way ANOVA was carried out in GraphPad Prism to define the significance of the different levels of bone loss (dependent variable), according to both housing and treatment class (independent variables) in the surgically-induced uremia experiment. No comparable analysis was carried out for the chemically-induced uremia protocol because it was impossible to vary the housing since all animals in a single cage received the same diet.

Analysis of urea tolerance:

Linear regression was used to draw a line of best fit between the mean inhibitory concentration of urea and the relative competitiveness of different isolates in control vs uremic animals presented in Fig 4B; standard settings in GraphPad Prism were used to accomplish this and Prism software was used to calculate the slope and the significance of its gradient.

Identification of cultured bacterial isolates:

Isolates were identified by comparing their 16S rRNA gene sequences with reference datasets using both the NCBI Nucleotide BLAST database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), and the Ribosomal Database Project (RDP) [52] online search tool (<https://rdp.cme.msu.edu/index.jsp>). In many cases these tools agreed on a species level identification for the isolate, but in some cases agreement between the two was only at higher taxonomic levels (such as in the case of different species of *Streptococcus* or *Enterobacteriaceae*). Thus, for all isolates, full-length reference 16S rRNA gene sequences for all species within the genus identified by BLAST and RDP search were downloaded from the RDP Hierarchy Browser. These reference sequences were aligned with the sequences from our research isolates, trimmed to a uniform length and used to construct a maximum likelihood tree, using MEGA [53] version 7. Pairwise distances between all isolates within a particular genus and all references sequences within that genus were calculated and used to generate a distance matrix.

Species level identification was determined when possible at >98.5% sequence identity. Isolates that failed to obtain any match at this level were treated as potential novel species. One was a *Streptococcus* species (4 isolates) with a closest proximity to *S. danieliae* at 97.33%, and another was a *Pasteurella* species (closest match being *P. pneumotropica* at 94.7%).

Analysis of cultured microbiota data:

Once assigned a species identity, the abundance of each isolate ($\log_{10}$ of colony forming units/ml) was carried out using Microsoft Excel and GraphPad Prism, using the Student t-test with Welch's correction to assess difference between growth in uremic animals and growth in controls. Comparisons were made at species level and then aggregated to allow comparisons at higher taxonomic levels.

Handling of isolates with regard to statistical analysis of in vitro culture data

For the purposes of *in vitro* microbiological work (urease testing and calculation of the mean inhibitory concentration of urea), where more than one isolate was assigned to a particular species identity, differences in *in vitro* characteristics were resolved for subsequent analysis by treating all isolates assigned to one species as urease positive if one isolate of that species was urease positive, and all isolates within a single species identification as possessing the highest urea tolerance of any isolate in that species.

Processing of 16S sequencing data:

Sequence data were processed using the DADA2 pipeline [54] in R, according to the author's recommended protocol (available from https://benjjneb.github.io/dada2/tutorial_1_6.html), adjusting filter parameters to achieve maximum quality scores whilst achieving sufficient overlap between forward and reverse read. Sequences were aligned against Silva v128 [55] in order to assign taxonomy.

Raw abundance data of sequence variants were used with taxonomic assignments and sample metadata to create phyloseq [56] objects. Phylogenetic trees were generated using MEGA v7.0, and rooted to a random node using the R package *phytools* [57]. A pseudocount of 0.001 was added to all OTU abundances to avoid calculating log-ratios involving zeros, and then data was then made compositional through isometric log-ratio transformation using the R package *philr* [58]. Ordination was carried out using the 'ordinate' function in *Phyloseq*, based on Euclidean distances in *philr* space. Permutational analysis of variance (PERMANOVA) and the PERMDISP test for homogeneity of variance (as proposed by Anderson) [59], were carried out using the R package *vegan* [60]. Alpha diversity was assessed using *Phyloseq*. Compositional analysis of the microbiota at six taxonomic levels was based on isometric log-ratio transformation of raw sequence abundances and adjusted for multiple testing using the Benjamini-Hochberg method, carried out using the *ANCOM* statistical framework [61] in R, with code obtained from the author's webpage: <https://sites.google.com/site/siddharthamandal1985/research>.

Analysis of NMR spectral data:

NMR spectral profiles were digitized and imported into Matlab (Mathworks). The peaks for water, urea and TSP were excised from the raw NMR spectra which were then aligned to adjust for variation in peak shift due to pH differences. Further normalization was carried out using the Probabilistic Quotient method between samples in order to ensure comparable baselines between samples.

Positive identification of eight metabolites achieved by identifying their spectral profiles and confirming this using Chenomx NMR Suite 8.3 evaluation version (Chenomx, Edmonton, Canada), and peak integrals were calculated from metabolite peaks. Comparisons between these integrals were used to calculate differences in relative abundance using Microsoft Excel, with the Student's *t*-test and Welch's correction used to assess significance.

Preparation of figures:

In order to achieve uniformity, all figures were generated using GraphPad Prism 7 (GraphPad Software Inc, San Diego, California).

Declarations

Acknowledgements

All experimental work was supported by the Barts and the London Renal Research Fund.

We thank Steve Cannon of the Dental Institute, Queen Mary University of London for his assistance in cutting sections for light microscopy and immunohistochemistry.

We thank Maureen Arora, Dental Physical Sciences, Queen Mary University of London, for her help with the sample preparation for BSE-SEM and CSLM.

We thank Professor William Wade and his research group at the Faculty of Dentistry, Oral, and Craniofacial Sciences, Kings College London for supplying barcoded primers and helping to arrange next-generation sequencing.

We thank Professor Guy Carpenter and Professor Gordon Proctor of the Faculty of Dentistry, Oral, and Craniofacial Sciences, Kings College London for their advice around collection of induced saliva samples in rodents.

We thank Robert Bond and the team at the Biological Research Facility, St Georges University of London for their assistance with the germ-free mouse colony.

We thank Steve Harwood for his support in various aspects of the laboratory work.

Author contributions

Organised according to CRediT taxonomy:

Conceptualization: DWR, KM, MC, MMY; Methodology: DWR, SJ, JOA, DM, GRD, AB, JS; Formal Analysis: DWR; Investigation: DWR, AA, JK, DM, AB; Resources: JK, SJ, JOA, GRD, DM, AB, JS, MC, MMY; Data Curation: DWR; Writing – Original Draft: DWR; Writing – Review & Editing: All authors; Visualization: DWR; Supervision: KM, MC, MMY; Funding Acquisition: CT, KM, MMY

Competing interests

The authors declare no competing interests.

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Figures

Figure 1

Experimental uremia causes periodontal bone loss in rats. **a** Distance between the cemento-enamel junction and alveolar bone crest measured using a dissecting microscope. Each point represents the average of multiple measurements over the buccal and lingual surfaces of all molar roots in a single rat, expressed relative to the average amount of bone loss in all control animals. **b** Representative images showing greater loss of maxillary alveolar bone in a uremic animal (bottom) compared with a control

animal (top), visualised using a dissecting microscope at 20x magnification. The loss of alveolar bone is so significant in the uremic animal that a clear gap can be seen between the underside of the tooth and the bone crest between some of the roots. c Light microscopy of haematoxylin and eosin (H&E) stained slides of periodontal tissue using a 4x objective lens. The dotted blue line indicates the outline of bone in the control specimen and, by implication, the loss of bone height in the uremic specimen. d Light microscopy (10x objective lens) of H&E stained slides. The dotted line in the control specimen indicates junctional epithelium which has been replaced in the uremic specimen by a proliferating epithelium with deep sulcal folds. e Immunohistochemistry for IL-17 (4x objective lens), with prominent staining in the uremic animal in the gingival epithelium and in localised areas deep within mandibular bone. f Immunohistochemistry using a 10x magnification lens of mandibular bone at the bifurcation area from the uremic specimen shown in Figure 1D, stained for IL-17 (left hand image) and neutrophils (right hand image), showing co-localization of these antigens. g Micro-computed tomography in the para-sagittal plane through the molar roots revealing a paucity of mandibular bone in the uremic specimen. h Scanning electron microscopy of the surface of alveolar bone facing the periodontal ligament and tooth roots in macerated specimens. The smoother appearance of the bone surface in the uremic animals reflects reduced mineralisation in Sharpey fibres at the growing surface of the bone.

Figure 2

Uremia is associated with oral dysbiosis, whilst co-housing alters bacterial communities and affects the severity of periodontal disease. a Total bacterial counts (colony forming units/ml) measured by summing colonies counted on blood agar plates grown in aerobic and anaerobic conditions for 48h. Significance is assessed using Student's T-test with Welch's correction. b Community composition of the oral microbiota determined by culture in control and uremic rats. All cultured isolates are included, agglomerated to genus

level and expressed in terms of their mean percentage contribution to the oral microbial community of each rat. c Absolute abundances of each cultured isolate (log10 cfu/ml). Bars show mean and standard error for all control and all uremic animals, significance where shown was calculated using the T-test with Welch's correction. * $p<0.05$; ** $p<0.01$. d Community composition of the oral microbiota of rats assessed by next generation sequencing of the 16S amplicon. Each vertical bar represents the total microbial community for a single animal, grouped according to experimental conditions (control vs uremic), color is applied according to assigned taxonomy at phylum level. e Principal coordinate plot showing distance between oral communities assessed by next generation sequencing of the 16S amplicon. Each point represents the oral community of an individual rat, with symbol shape representing the two experiments performed, which used separate batches of animals (circles denote the chemically-induced uremia experiment, diamonds surgically-induced uremia). f Housing of animals in the surgically-induced arm of the experiment; a total of 13 rats were co-housed, and 11 were singly housed with others of the same treatment protocol. g Periodontal bone loss, measured and presented as previously, according to housing for animals in the surgically-induced uremia experiment. Significance between different groups as shown were calculated by the T-test with Welch's correction; analysis by 2-way ANOVA is presented in the main text. h PCA plot of sequencing data of the oral microbiota of rats in the surgically-induced uremia experiment, identified according to treatment class (symbol color) and caging (symbol shape).

Figure 3

Uremia alters salivary biochemistry in rats which may explain the observed oral dysbiosis. a Urea concentration (mmol/L) in induced saliva and serum samples taken at the time of sacrifice in rats after subtotal nephrectomy or sham surgery. Urea concentration was assessed by colorimetric assay and significance was assessed using the T-test with Welch's correction. b The mean inhibitory concentration of urea for cultured isolates from the oral microbiota of rats, plotted against their relative growth in uremic vs control animals, with increasing positive values on the y-axis indicating increased average abundance in uremic animals, and negative values indicating increased average abundances in controls. Each point represents a single species, plotted according to the sum abundances and maximal urea

tolerance for all isolates with that species identity, as described in the methods section. Species demonstrating in vitro urease activity are shown in pink. The blue and red dotted lines indicate the mean salivary urea concentrations in control (blue) and uremic (red) animals, for comparison purposes.

Figure 4

Uremia induces progressive oral dysbiosis in mice. Principal component analysis plots of oral microbial communities assessed at four weekly intervals prior to and after commencing an adenine containing diet, by next-generation sequencing of the 16S rRNA gene amplicon. Samples from control animals are shown in blue and those from uremic animals in red. For each plot the significance is shown for differential clustering (assessed by PerMANOVA) and heterogeneity of variance (assessed by PERMDISP).

Figure 5

Periodontal disease can be transmitted by oral microbial transfer into healthy germ-free mice. **a** Oral microbial communities from four control and four uremic donor animals were frozen at the time of sampling and then transferred into germ-free animals at eight weeks of age. These animals were then conventionally housed, and oral microbial communities were sampled three and nine weeks after transfer, before animals were culled ten weeks after transfer, at eighteen weeks of age. **b** Recipients of oral

microbiota from uremic donors demonstrated significantly more periodontal bone loss than recipients of microbiota from control animals. Each point represents the average periodontal bone loss in a single animals as described in the legend to Fig 1A. c Principal component analysis from donor and recipient oral communities. d Community composition of recipients of oral communities from control and uremic donors, assessed by next-generation sequencing of the 16S amplicon at nine weeks after microbial transfer.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementarytableS1Ratanimaldata.xlsx](#)
- [SupplementarytableS2Culturedmicrobialdata.csv](#)
- [SupplementarytableS3SalivaflowratesandpH.xlsx](#)
- [SupplementarytableS4Salivarymetabolites.xlsx](#)
- [SupplementarytableS5Mouseanimaldata.xlsx](#)
- [SupplementarytableS6Oralmicrobialtransferdata.xlsx](#)