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Original Contribution

Characterization of the rat oral microbiome and the effects of dietary nitrate

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ABSTRACT

The nitrate–nitrite–NO pathway to nitric oxide (NO) production is a symbiotic pathway in mammals that is dependent on nitrate reducing oral commensal bacteria. Studies suggest that by contributing NO to the mammalian host, the oral microbiome helps maintain cardiovascular health. To begin to understand how changes in oral microbiota affect physiological functions such as blood pressure, we have characterized the Wistar rat nitrate reducing oral microbiome. Using 16S rRNA gene sequencing and analysis we compare the native Wistar rat tongue microbiome to that of healthy humans and to that of rats with sodium nitrate and chlorhexidine mouthwash treatments. We demonstrate that the rat tongue microbiome is less diverse than the human tongue microbiome, but that the physiological activity is comparable, as sodium nitrate supplementation significantly lowered diastolic blood pressure in Wistar rats and also lowers blood pressure (diastolic and systolic) in humans. We also show for the first time that sodium nitrate supplementation alters the abundance of specific bacterial species on the tongue. Our results suggest that the changes in oral nitrate reducing bacteria may affect nitric oxide availability and physiological functions such as blood pressure. Understanding individual changes in human oral microbiome may offer novel dietary approaches to restore NO availability and blood pressure.

Introduction

Since the Human Microbiome Project began in 2007, an explosion of research has led to the publication of hundreds of studies. A common theme is the role of the microbiome in disease pathology, as much work has been aimed at identifying dysbioses associated with specific disease. In contrast, less emphasis has been placed on identifying and characterizing microbiome states and activities associated with health since the study of the original HMP original cohort of 300 healthy people. Because "healthy" microbiomes can be exploited to maintain or improve health (or return dysbiotic states to healthy states), it is essential that we continue to characterize and define microbiomes of health and harness the therapeutic potential of commensal bacteria.

In the mid-1990 s, researchers began characterizing an oxygen- and NOS-independent alternative pathway to NO production, called the nitrate–nitrite–NO pathway. Nitrate (NO_3^-) and nitrite (NO_2^-), previously thought to be inert end products of NO oxidation [1], can be reduced to bioactive NO through this pathway. Systemic nitrate and nitrite in blood and tissues are now considered a pool for bioactive NO [2,3]. The reduction of nitrate to nitrite, the first step of the nitrate–nitrite–NO pathway, is dependent on the oral commensal microbiota [4]. By contributing nitrite and NO to the mammalian host via the two-electron nitrate reduction, the oral microbiome is critical for mammalian physiology. NO, a gaseous free radical, is a critical cell signaling molecule involved in host defense, mitochondrial function, inhibition of oxidative stress, nerve transmission, endothelial function, antiaggregation by platelets, antiadhesion of leukocytes, vasodilation, and regulation of blood pressure [5]. In humans, NO is also

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endogenously produced by nitric oxide synthases (NOSs), which convert L-arginine and molecular oxygen to NO and L-citrulline. Nitrite also has cell-signaling properties [6] and acts as a reservoir of NO activity [7,8]. Therefore any strategy or treatment regimen that enhances production or availability of nitrite and/or NO will have positive benefits on mammalian physiology.

One of the most important physiological roles for NO is maintenance of cardiovascular system health through blood pressure regulation, vasodilation, and inhibition of platelet aggregation and leukocyte adhesion. In fact, NO insufficiency is one of the early hallmark signs of endothelial dysfunction [9]. Numerous animal and human studies have demonstrated that nitrate and nitrite supplementation is associated with increased cardiovascular health [10]. Sodium nitrate decreases diastolic blood pressure in humans and rats [11]. Beetroot juice, a dietary source of nitrate, decreased diastolic and systolic blood pressure [11–13]. Infusion of nitrite into the blood stream was also associated with reduced blood pressure in humans via oxyhemoglobin-mediated nitrite reduction to NO [14]. In eNOS-deficient mice, dietary nitrite restored NO homeostasis and was cardioprotective [15]. Dietary nitrate supplementation protected against ischemia–reperfusion damage in mice and also increased vascular regeneration after chronic ischemia in mice [16]. In a study of elderly people with an increased risk for cardiovascular disease, dietary nitrate supplementation reversed vascular dysfunction [17]. Research suggests that the nitrate supplementation-associated benefits are due to nitrate reduction by the oral microbiome since the benefits of nitrate supplementation were lost when subjects spat out their saliva prior to ingestion or were administered an antiseptic mouthwash [12,13,18,19]. Additionally, salivary nitrate reduction is absent in germ-free animals [20,21].

The potential to exploit the symbiotic nitrate–nitrite–NO pathway to NO production is profound, particularly because adequate and sustained control of blood pressure is achieved in only about 50% of treated hypertensive patients, including all classes of antihypertensives [22]. As cardiovascular disease remains the top killer in the United States, accounting for more deaths each year than cancer, designing new diagnostics, treatments, and preventives for diseased and at-risk individuals is essential. Additionally, because NO is an important signaling molecule in various body systems, exploiting the oral microbiome to contribute to NO production and maintain NO homeostasis has the potential to affect human health beyond the cardiovascular system. Based on these studies, rats may be a suitable organism for studying the effect that the oral microbiome has on the effects of sodium nitrate supplementation and cardiovascular health. In order to determine if use of oral antiseptic mouthwash and exposure to dietary nitrate changes the oral microbiome, we compared the Wistar rats' native tongue microbiome with that of animals treated with sodium nitrate supplementation and chlorhexidine mouthwash. Our results will aid future studies aimed at using the oral microbiome to increase nitrite and nitric oxide availability to the host, but also any studies in which the oral microbiome plays a key role. Our recently published data on identifying oral nitrate reducing bacteria in humans [23] demonstrate that we have the capabilities and expertise to interrogate the rat microbiome and determine how select interventions affect NO homeostasis.

Methods

Animals

Seven-week-old male Wistar rats were purchased from Charles River Laboratories (Wilmington, MA), housed individually in the Taub Animal Facility at Baylor College of Medicine (BCM), and provided with food and water ad libitum. Animals were trained through daily handling and restraint to reduce stress during experimental manipulations. One to 2 weeks after arrival at the Taub Animal Facility, animals were surgically implanted with a telemetric blood pressure measurement device (described below). Animals were weighed regularly throughout the study and gained an average of 76.6 g by the end of the study. The daily average intake of water was 42 ml. All experimental procedures (Fig. 1) were approved by the BCM Institutional Animal Care and Use Committee.

Surgical implantation of DSI PA-C40 telemetric blood pressure probes

PA-C40 telemetric devices (DSI, St. Paul, MN) were surgically implanted into the animals. One day prior to surgery, animals received an oral dose of Carprofen (6 mg), and 15 min prior to surgery, animals received an injection of Buprenorphine (0.2 mg/kg). Animals were anesthetized with isoflurane and kept warm during surgery through use of a heated board. Tissue hydration was maintained with sterile saline solution and the eyes were protected from drying by use of a sterile, bland ophthalmic ointment. The animals were placed in dorsal recumbency and an incision was made through the skin of the abdomen and then through the abdominal wall. The catheter portion of the PA-C40 telemetric device was placed into the abdominal aorta, and the transmitter portion was placed inside the intraperitoneal cavity, with the transmitter suture rib incorporated into the abdominal wall closure. Carprofen (6 mg) was administered to the animals daily for 48 h after surgery and animals were monitored for pain and discomfort. Additional dosages of Buprenorphine were administered if needed. Animals were allowed to recover from surgery for 1–2 weeks before experimental measurements began. Baseline blood pressure measurements were obtained for 5 days before additional experimental manipulations were performed.

Sodium nitrate supplementation

After 5 days of assessing animals at baseline, animals were supplemented with sodium nitrate ($NaNO_3$, Sigma-Aldrich, St. Louis, MO) in their drinking water (1 g/L) for the remainder of the study

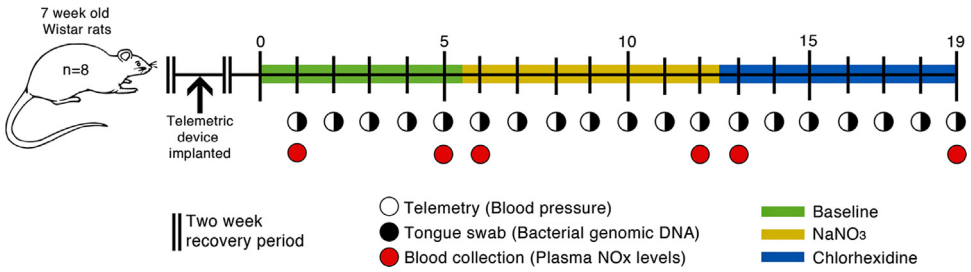

Fig. 1. Flow of experimental methods. Eight male Wistar rats were surgically implanted with the PA-C40 telemetric device, rested up to 14 days, and then blood pressure measurements, tongue swabs, and blood were collected during 5 baseline days followed by 7 days of $NaNO_3$ supplementation and an additional 5 days of $NaNO_3$ supplementation plus chlorhexidine mouthwash.

(days 6–19). To determine blood pressure during sodium nitrate supplementation, blood pressure data were recorded daily on days 6–12 of the study, although data from the last five days only (days 8–12 of the study) were included in analysis as the first two days of supplementation were a transition period.

Chlorhexidine mouthwash application

Using a 1 ml needleless insulin syringe, 0.3 ml of chlorhexadine (Vedco, St. Joseph, MO, diluted to 2 mg/ml) was applied to the oral cavity of rats twice daily on days 13–19 of the study (the rats continued to receive sodium nitrate in their drinking water during this period of the study). The mouthwash was directed toward the dorsal aspect of the tongue, as that is where the majority of nitrate reduction in the oral cavity occurs [24]. Blood pressure was recorded daily on days 13–19 of the study with the first two days of chlorhexidine treatment (days 13–14) classified as a transition period and the data collected on this day were not used in downstream analyses.

Blood pressure and heart rate (telemetry) measurements

Blood pressure and heart rate were recorded daily throughout the experiment from the hours of 11 am to 1:30 pm. Animals were transported to the BCM Mouse Phenotyping Core within the Taub Animal Facility for telemetry measurements. The internal PA-C40 telemetry devices were switched on using a magnet and each cage was placed on top of a receiver connected to a desktop computer loaded with Dataquest ART Software (DSI, St. Paul, MN) for recording and analyzing data. A data point was collected from each animal every 10 s. Because it took about half an hour for the animals to adjust to moving into a different room, as judged by a flat rather than sloped blood pressure trace, the first half hour of data was not included in analysis. Thus, data from the hours of 11:30–1:30 were included in analysis for a total of 3600 data points/10 h of data per animal per 5-day treatment period. For each treatment, daily means for blood pressure and heart rate were calculated for each of the 5 days. Analyses of treatment effects on these daily means were conducted with linear mixed models for repeated measures [25]. Separate models were tested for each outcome variable (systolic, diastolic, heart rate, and mean arterial pressure). The effects included in the models were treatment, day, and the interaction of treatment and day. Statistical analysis was conducted with SAS 9.3 for Windows.

Blood collection and plasma nitrate/nitrite measurements

About 250 μl of blood was collected from the saphenous vein of each rat into EDTA Eppendorf tubes on days 1, 5, 6, 12, 13, and 19. Blood was immediately centrifuged at 3000*g* for 15 min and 40–80 μl of plasma collected and stored at −80 °C. Using a 96-well nitrate/nitrite colorimetric assay kit (Cayman Chemical, Ann Arbor, MI), plasma nitrate and nitrite levels were determined. Plasma was first ultracentrifuged at 4 °C for 20 min at 14,000 *g* using a 30 kDa cutoff Microcon Ultracel Centrifugal Filter (Millipore, Billerica, MA) per the manufacturer's instructions. Plasma filtrate was then diluted 2:1 in Assay Buffer and no more than 80 μl of diluent added to each assay plate well. The total nitrate and nitrite standard was prepared. To determine total nitrate and nitrite levels, nitrate reductase and enzyme cofactor were added to the samples and incubated for 3 h, after which Greiss reagents 1 and 2 were added. Ten minutes after the addition of Greiss reagents, the absorbance was read at 540 nm. The total nitrate and nitrite standard curve was plotted and individual sample concentrations were determined using the following calculation:

$$[\text{Nitrate}+\text{nitrite}](\mu M) = \frac{A540 - y\ \text{intercept}}{\text{slope}} \times \frac{200\ \mu L}{\text{vol. sample used}\ (\mu L)} \times \text{dilution}.$$

Tongue swab collection and processing

A tongue swab was collected from each rat daily. Rats were briefly anesthetized with isoflurane and a Catch-All Sample Collection Swab (Epicentre Technologies, Madison, WI) was swiped along the tongue from back to front five times. A total of 208 swabs were collected during the experiment. Swabs were immediately placed in a MOBIO PowerSoil DNA Isolation Kit (MOBIO, Carlsbad, CA) bead tube and immediately stored at −80 °C. Bacterial genomic DNA was extracted from the swabs using the MOBIO PowerSoil DNA Isolation Kit per the manufacturer's instructions with the following modification: 100 μl of C2 and 100 μl of C3 were added to the lysate in one step instead of two separate steps, vortexed, and incubated at 4 °C for 5 min. The remainder of the protocol was followed verbatim. DNA was stored at -80 °C until further use.

16S rRNA gene V4 amplification and sequencing

The V4 region of the bacterial genomic DNA was amplified using barcoded primers 515f (5′- GTGCCAGCMGCCGCGGTAA-3′) and 806r

(5′-GGACTACHVGGGTWTCTAAT-3′). The barcoding PCR contained the following: 2 μl 4 μM barcoded primer stock, 5 μl DNA, 2 μl Taq Buffer II (Invitrogen), 0.15 μl Taq enzyme (Invitrogen), and 10.85 μl PCR. The reactions were amplified in an Eppendorf Mastercycler Thermocycler under the following conditions: initial denaturation step for 2 min at 95 °C, followed by 30 cycles of 20 s denaturation at 95 °C, 45 s of annealing at 50 °C, and 90 s annealing at 72 °C. A different barcode was used for each sample, allowing for pooling of samples for sequencing. All 208 samples were pooled and sequenced on one lane of an Illumina HiSeq 2000 sequencer (Illumina, San Diego, CA) at the BCM Human Genome Sequencing Center.

16S rRNA gene data analysis

Using custom perl scripts, Reads 1 and 2 from the Illumina-sequenced rat oral swab amplicons were prequality filtered by trimming at the first ambiguous (N) base and then joined with a minimum required overlap of 12 bases. Sequences were then demultiplexed and quality trimmed using QIIME version 1.8 [26]. Sequences with any ambiguous bases, with a phred quality score less than 20, and with more than 1.5 barcode errors were discarded and not used in further analyses. Quality trimming resulted in 5,289,167 high-quality reads. Seventeen samples had less than 1000 reads associated with them and were excluded from further analyses. Sequences were binned into OTUs and assigned taxonomy by clustering sequences using UCLUST against a reference database (the GreenGenes May 2013 release) at 97% identity. After removing OTUs with only one read associated with them and an additional two samples with less than 5000 reads, the resulting OTU table was normalized to 5223 reads per sample. Diversity was assessed by calculating the Shannon diversity metric, the chao1 estimate of diversity, and the number of observed species for each sample at various sequencing depths (the OTU table was randomly subsampled five times from 10 to 5010 reads per sample in steps of 250 reads). Nonparametric *t* tests using Monte Carlos permutations (999) to calculate the *P* value (Bonferonni corrected) determined significant differences in diversity between groups. Bar charts were also constructed to visualize the taxa present in each sample and

across sample groups. Kruskal-Wallis (nonparametric ANOVA) tests on the OTU table identified significant changes in the relative abundances of individual OTUs between groups.

The human tongue scraping data that were compared with rat tongue swab data were sequenced and analyzed as previously described [23]; however, to facilitate a more accurate comparison between the two datasets, we repeated the OTU picking steps on this dataset as described above for the rat tongue swab dataset. Singletons were removed from the OTU table and the OTU table was normalized to 4856 reads per sample. We then constructed bar charts and calculated alpha diversity metrics to compare the taxonomic composition and diversity of human tongue scrapings to rat oral swabs.

Results

A comparison of the healthy human and healthy rat oral microbiomes reveals key differences in diversity and composition

No comprehensive metagenomic study of the rat oral microbiome has been completed to date; thus, to aid future studies within our group and among other groups, we first aimed to characterize the normal rat tongue microbiome. Because we swabbed only the tongues of healthy rats fed a standard and consistent diet in a controlled environment, it is important to note that our study only provides information regarding the experimental rat tongue rather than the entire oral cavity. Previously we characterized the dorsal tongue microbiomes of healthy human volunteers [23], and we compared that data to our current rat tongue data to determine the similarity/dissimilarity of the rat and human tongue microbiomes. The human tongue was more diverse than the rat tongue, with an average of 249.3 (+/− 30) OTUs detected on the healthy human tongue compared to an average of 99.3 (+/− 40) OTUs detected on the rat tongue (Table 1). The average Shannon diversity index of the healthy human was 5.54 (+/−0.33) compared to 2.92 (+/− 0.53) for the rat (Table 1).

Firmicutes and Proteobacteria together comprised 40–80% of the tongue communities in humans and rats; however, rat tongues were predominately Actinobacteria, whereas human tongues contained more Bacteroidetes (Fig. 2A and B). At the genus level, rat and human tongues were both colonized with *Streptococcus* and *Haemophilus;* however, human tongues also contained large amounts of *Veillonella, Prevotella, Neisseria,* and *Porphyromonas,* which were found rarely in the rat microbiome (0–0.9% relative abundance). Conversely, *Corynebacterium* and an unclassified genus of the Micrococcaceae together comprised roughly 35–65% of the bacterial communities detected on rat tongues (Fig. 2C and D). Therefore, although the microbiomes of human and rat tongues are not entirely dissimilar, there are some key differences in composition at the phylum level that are magnified at the genus level.

Sodium nitrate supplementation reduces diastolic blood pressure and increases plasma NOx concentrations in rats

Previously it has been shown that $NaNO_3$ supplementation significantly reduces diastolic blood pressure and mean arterial pressure and increases plasma NOx concentrations in Sprague Dawley rats [19]. Similarly, using Wistar rats in our study, diastolic blood pressure decreased significantly after 7 days of $NaNO_3$ supplementation, and an insignificant decrease in systolic blood pressure and mean arterial pressure (MAP) was also observed (Fig. 3A–C). Interestingly, although diastolic blood pressure decreased significantly in the $NaNO_3$-supplemented group ($n=8$), diastolic, systolic, and mean arterial pressures increased slightly in two individual rats after $NaNO_3$ supplementation (Supplementary Fig. 1). Heart rate also decreased significantly in $NaNO_3$-supplemented rats (Fig. 3D), though it continued to decrease throughout the experiment and also differed significantly between days (Supplemental data). Additionally, the rate pressure product (RPP), or heart workload, significantly decreased in $NaNO_3$-supplemented rats (Supplemental data). Plasma NOx also increased when $NaNO_3$ was added to the drinking water, with an average increase of 96.9 (+/−19.1) μM, corresponding to 14.1-fold (+/−2.69) change (Fig. 3D, Table 2). Altogether, we have demonstrated that dietary $NaNO_3$ supplementation is associated with reduced diastolic blood pressure and increased plasma NOx concentrations in Wistar rats, largely corroborating the work of Petersson et al. in Sprague Dawley rats [19].

The mean relative abundances of specific taxa are altered with sodium nitrate supplementation

Although we know that the oral microbiome is responsible for nitrate reduction in the oral cavity, it remains unknown how

Table 1
Alpha diversity metrics for human and rat tongue microbiomes.

Rat subjects				
	Average Shannon diversity	Shannon diversity SD	Average observed species	Observed species SD
Rat 009	2.949	0.329	77.56	21.6
Rat 011	3.331	0.267	130.7	30.5
Rat 013	2.578	0.544	85.5	25.8
Rat 014	2.486	0.058	85.0	14.5
Rat 016	2.742	0.169	78.3	15.6
Rat 017	3.599	1.373	165.8	98.8
Rat 018	2.961	0.421	106.4	23.6
Rat 019	3.072	0.188	77.6	21.6
Average	2.92	0.53	99.3	40
Human subjects				
	Shannon diversity	Shannon diversity SD	Observed species	Observed species SD
A73	5.438	n/a	257.4	n/a
C66	5.946	n/a	250.6	n/a
D55	5.123	n/a	215.8	n/a
E64	5.336	n/a	211.8	n/a
F76	5.354	n/a	278.4	n/a
G77	6.019	n/a	281.8	n/a
Average	5.536	0.362	249.3	30

Shannon diversity and observed species metrics are an average of five swabs per rat. For humans, only one tongue scraping was collected per sample.

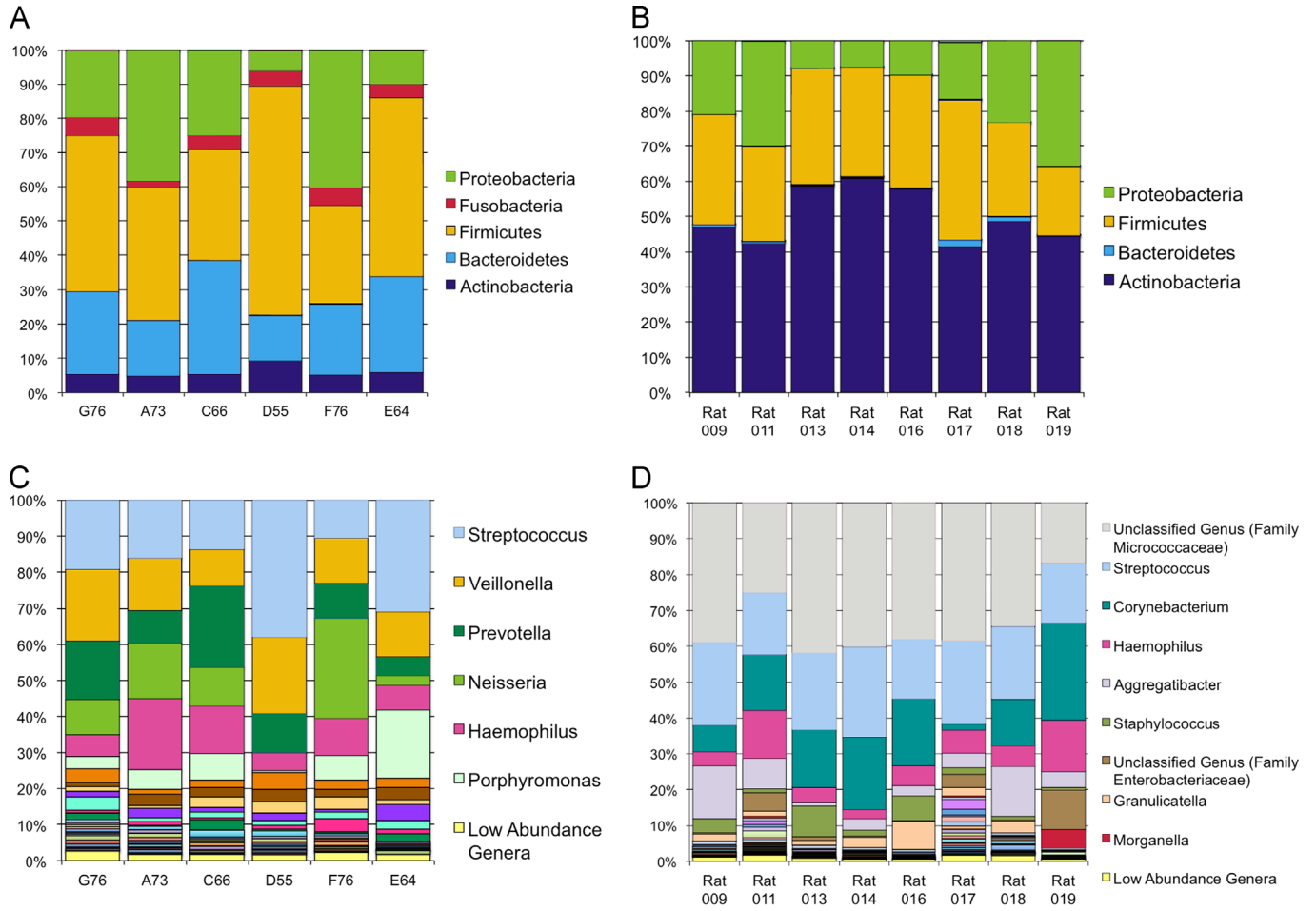

Fig. 2. Relative abundances of genera present on healthy human and rat tongues. Stacked bar charts illustrate the mean relative abundances of genera present on individual rat tongues (five swabs collected per rat) and the relative abundances of genera present on individual human tongues (one tongue scraping collected per person).

dietary changes, including $NaNO_3$ supplementation, affect the composition of the oral microbiome. Therefore, an important goal of our study was to identify microbial community changes associated with $NaNO_3$ supplementation in our animal model. At the phylum level, the tongue microbiomes of $NaNO_3$-supplemented rats look very similar to the tongue microbiomes of these same rats at baseline (data not shown); however, some key differences are observed at the genus level. Interestingly, the mean relative abundance of *Haemophilus* spp. (nitrate reducers) and the mean relative abundance of *Streptococcus* spp. (nitrite reducers) increased in $NaNO_3$-supplemented rats (Fig. 4). An OTU identified as *Haemophilus parainfluenzae* significantly increased after $NaNO_3$ supplementation (Bonferroni corrected P value$=0.001$). This is a noteworthy result given that we previously identified this species as one of 14 candidate species contributing to nitrate reduction in the healthy human oral cavity [23]. Taxa that decreased in $NaNO_3$-supplemented rats include Micrococcaceae, Enterobacteriaceae, *Granulicatella,* and *Aggregatibacter* (Fig. 4).

Chlorhexidine treatment does not affect blood pressure or plasma NOx concentrations in $NaNO_3$-supplemented rats but increases tongue microbiome diversity

Many studies have demonstrated that the beneficial effects of $NaNO_3$ supplementation are abolished after treating either the human subject or the animal with the antiseptic mouthwash chlorhexidine, which is thought to significantly reduce the oral bacterial burden, thus decreasing nitrate reduction [13,18]. To determine whether we could recapitulate the blood pressure-raising effects of chlorhexidine treatment and to characterize the oral microbiomes of rats treated with chlorhexidine, we treated $NaNO_3$-supplemented rats twice a day with chlorhexidine following a method previously utilized by our group [15]. Diastolic blood pressure increased in one rat and insignificantly decreased in 50% of rats, with the remaining rats showing no significant change (Supplementary Fig. 1B). MAP changed similarly (Supplementary Fig. 1C; however, systolic blood pressure increased in 50% of rats, decreased in 40% of rats, and remained unchanged in one rat (Supplementary Fig. 1A). Heart rate decreased in all rats. Plasma NOx levels were unchanged (decreased by 14.4 +/− 12.9 μM, corresponding to a 0.963 +/− 0.164-fold change) compared to $NaNO_3$-supplemented rats (Table 2), suggesting that chlorhexidine treatment was not fully able to reverse the effects of $NaNO_3$ supplementation.

Analyses of the tongue microbiomes revealed that chlorhexidine-treated $NaNO_3$-supplemented rats were more diverse than the tongue microbiomes at baseline or $NaNO_3$-supplemented only rats (Fig. 5). This increase in diversity was due mainly to the presence of a number of low abundance taxa in the chlorhexidine-treated group that were not present in the baseline or $NaNO_3$-supplemented groups (see Fig. 4), and to altered relative abundances of key taxa. Notably, *Haemophilus* and *Aggregatibacter* were nearly eliminated from the tongue of chlorhexidine-treated rats, while the Micrococcaceae decreased but did not disappear. Conversely, the relative abundances of Enterobacteriaceae, *Corynebacterium,* and *Morganella* increased in chlorhexidine-treated animals. Therefore, the chlorhexidine treatment

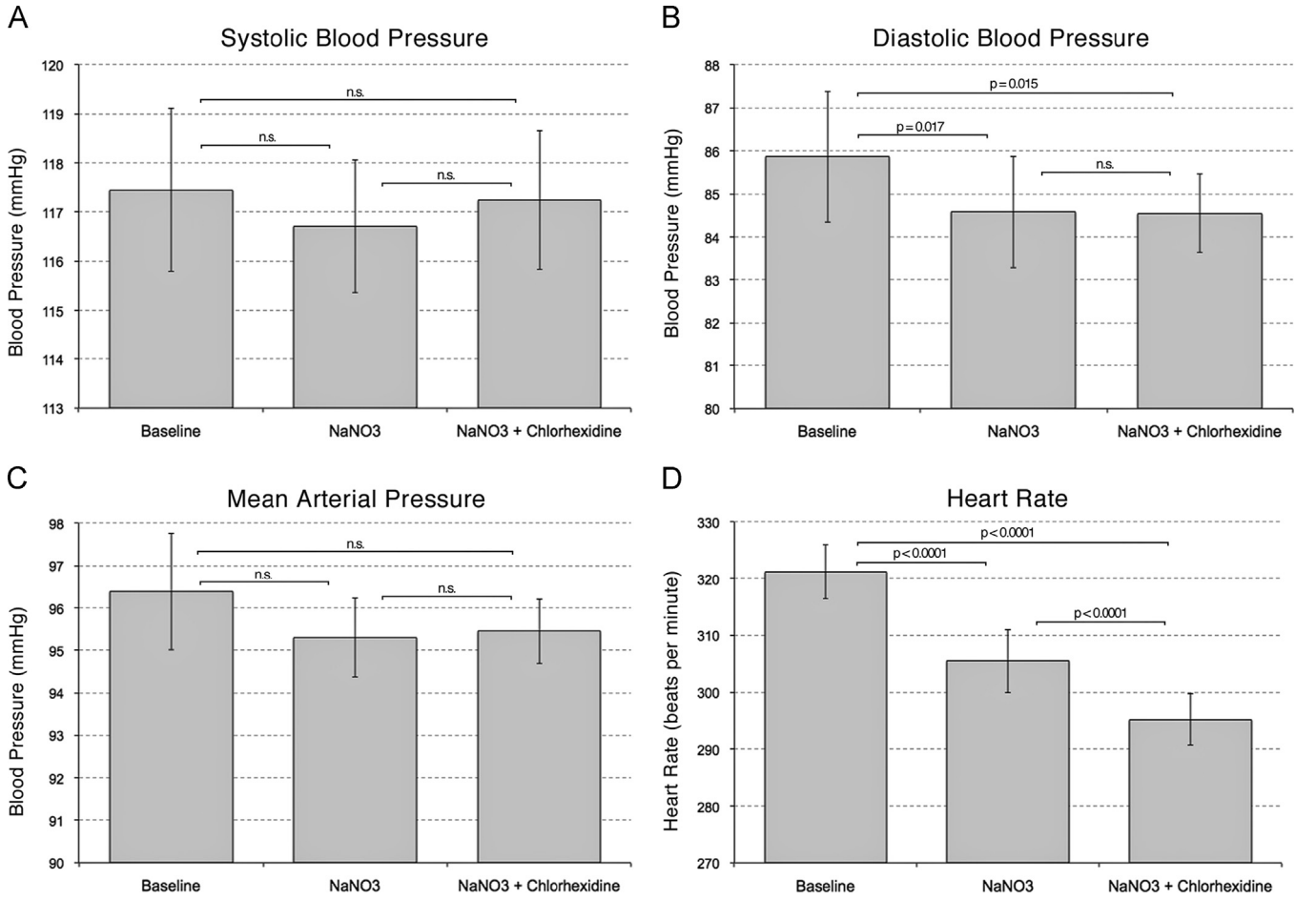

Fig. 3. The effects of $NaNO_3$ supplementation's on blood pressure and plasma NOx levels. Line graphs illustrate the change in three blood pressure metrics. (A) Systolic blood pressure, (B) diastolic blood pressure, (C) mean arterial pressure, and (D) heart rate in each rat during baseline, $NaNO_3$ supplementation, and $NaNO_3$ supplementation plus chlorhexidine treatment. Data points are an average of 722 data points per animal per day for 5 days per treatment (3600 data points/10 h of data).

Table 2
Changes in absolute plasma NOx levels across treatment groups and fold change in plasma NOx levels across treatment groups

Absolute plasma [NOx] (μM)					
Baseline	$NaNO_3$	CHX	Change $NaNO_3$:baseline	Change CHX:$NaNO_3$	Change CHX:baseline
10.9 +/− 1.2	111.3 +/− 22.5	96.9 +/− 17.7	96.1 +/− 19.1	−14.4 +/− 12.9	88.0 +/− 17.1
Fold change in plasma [NOx]					
Baseline D5:D1	$NaNO_3$ D7:D1	CHX D7:D1	$NaNO_3$ D7:Baseline D5	CHX D7:$NaNO_3$ D7	CHX D7:baseline D5
0.932 +/− 0.239	0.952 +/− 0.151	0.939 +/− 0.115	14.1 +/− 2.69	0.963 +/− 0.164	9.51 +/− 1.67

Absolute plasma NOx concentrations (μM) are listed for baseline, $NaNO_3$, and chlorhexidine treatment groups. Values are average NOx concentration +/− SEM for each treatment group. Fold change is also listed for baseline day 5:day 1, $NaNO_3$ day 7:day 1, chlorhexidine day 7:day 1, $NaNO_3$ day 7:baseline day 5, chlorhexidine day 7:$NaNO_3$ day 7, and chlorhexidine day 7:baseline day 5. Values are average fold change +/− SEM for each comparison. $NaNO_3$, sodium nitrate; CHX, chlorhexidine.

disturbed the oral microbiome, but did not eliminate species associated with nitrate positive selection.

Discussion

Through 16S rRNA gene sequencing and analysis of tongue swabs collected from 8 Wistar rats, we have achieved three firsts: ours is the first study to use metagenomics techniques to characterize the healthy rat tongue microbiome and to compare it to the healthy human tongue microbiome, ours is the first study to discern the effects of $NaNO_3$ supplementation on the rat tongue microbiome, and, finally, ours is also the first study to use metagenomics techniques rather than culturing techniques to characterize the tongue microbiomes of $NaNO_3$-supplemented rats treated with the antiseptic mouthwash chlorhexidine. The results of our work will be important for designing and executing further studies that utilize the rat as an animal model to characterize and exploit the oral microbiome-mediated nitrate–nitrite–NO pathway to NO production. Additionally, our study provides a good starting point to characterize the entirety of the rat oral microbiome and not just the tongue, an endeavor that will prove useful for future studies on a number of topics ranging from oral nitrate reduction to caries development and treatment.

The oral cavity is an attractive target for probiotic and/or prebiotic therapy because of the clear role that oral nitrate reducing bacteria play in dietary nitrate reduction. Because treatment needs to be tested in animals first, knowledge of the similarities and differences between human and animal oral microbiomes is fundamental. To address this gap in knowledge,

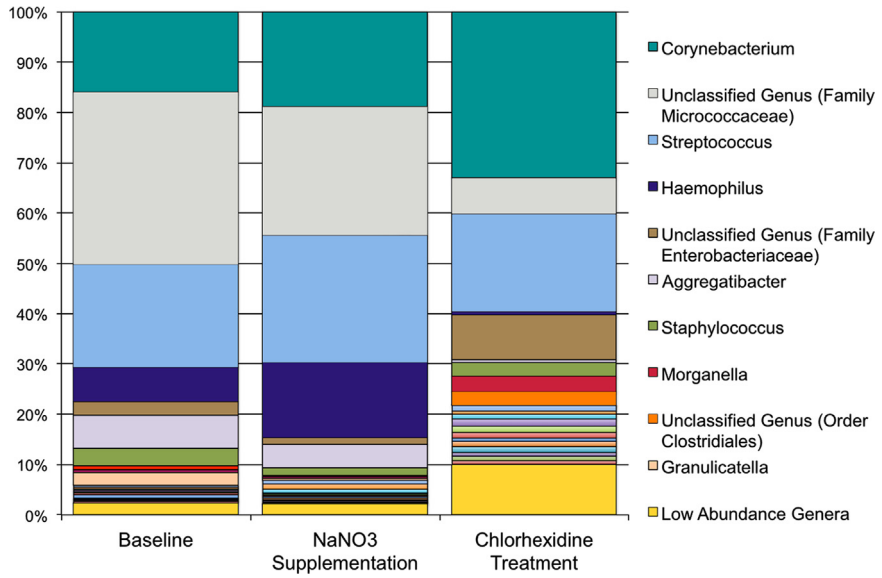

Fig. 4. Mean relative abundances of genera present on rat tongues during different treatments. Stacked bar charts illustrate the mean relative abundances of genera present on individual rat tongues at baseline ($n=36$), during $NaNO_3$ supplementation ($n=55$), and during chlorhexidine treatment ($n=55$).

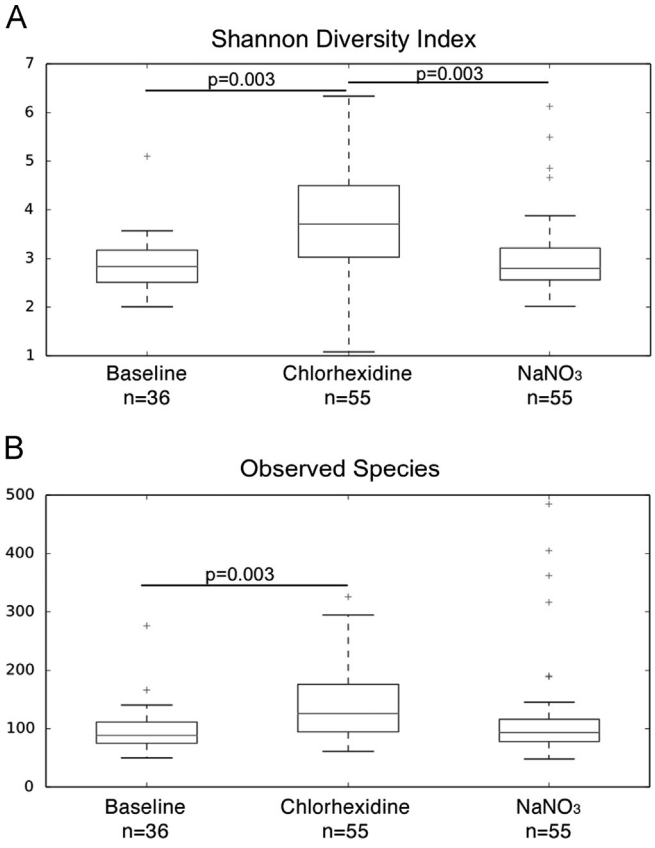

Fig. 5. Diversity of rat tongue microbiomes during different treatments. Box and whisker plots illustrate the Shannon diversity index (A) and observed species (B) of rat tongue microbiomes at baseline, during $NaNO_3$ supplementation, and during chlorhexidine treatment. A nonparametric *t* test using Monte Carlo permutations to calculate the *P* value (Bonferroni-correted) was used to determine significant differences in diveristy between groups.

we compared the tongue microbiomes of healthy rats to healthy human volunteers from a previous study [23]. There are key differences between the rat and human tongue microbiomes, in terms of both diversity and composition, although two genera, *Haemophilus* and *Streptococcus,* were among the top five genera present on the tongues of both humans and rats.

Some of the differences we observed are likely due to differences in methodology between the human and the rat datasets. First, human tongues were scraped with a metal spatula, whereas rat tongues were sampled using an abrasive foam swab. It is possible that tongue scraping leads to more diverse sampling. Secondly, the V3–V5 regions of the 16S rRNA gene were amplified from the human tongue scrapes, while just the V4 region was amplified from the rat tongues. Differences in individual variable region primer preferences toward certain taxa [27,28] could yield minor differential results between two different 16S rRNA gene variable region datasets. Finally, the human tongue scraping amplicons were sequenced on the now nearly obsolete Roche 454 titanium pyrosequencing platform, whereas the rat tongue amplicons were sequenced on the Illumina HiSeq 2000 platform. To decrease artificial error, both datasets were analyzed as similarly as possible, with the same reference database used to cluster sequences into OTUs and assign taxonomy.

While the sampling methods could contribute to some of the differences we observed between human and rat tongue microbiomes, it is unlikely that any of these variables or even a combination of these variables contribute to the majority of differences we observed. Diet, lifestyle (rats are coprophagic and self-cleanse through licking, etc. and consume a consistent chow at every meal), and other differences in oral physiology are likely to be prominent sources of microbiome variation between the two datasets. Nevertheless, a study in which human and rat tongue microbiome samples are collected, amplified, sequenced, and analyzed through identical methods would be useful for confirming and refining our results.

Of all the studies utilizing $NaNO_3$ supplementation in rodents, not one has surveyed the effects of $NaNO_3$ on the composition of the oral microbiome. We observed significant changes in the relative abundances of taxa present on the tongues of $NaNO_3$-supplemented rats compared to nonsupplemented rats, including a significant increase in nitrate reducing *H. parainfluenzae.* Additionally, *Granulicatella* and *Aggregatibacter,* which have both been associated with poor oral health [29,30] in humans, decreased with $NaNO_3$ supplementation. These results suggest that high nitrate diets may induce changes in oral microbiome communities

to more efficiently reduce nitrate to nitrite and NO, which could be beneficial both by reducing blood pressure and by inhibiting bacterial species associated with poor oral health. Since nitrite and NO [31,32] are toxic to many pathogenic bacteria, dietary nitrate supplementation may allow for specific reduction or eradication of pathogenic bacteria and provide an environment for beneficial bacteria to flourish. Importantly, because oral NO production is dependent on oral nitrate reducing bacteria, these observations suggest that the link between oral health issues such as chronic periodontitis and cardiovascular disease may be due in part to decreased abundance of nitrate reducers and concurrent increase of pathogenic bacterial species in the oral cavity. Interestingly, the strictly anaerobic bacteria associated with chronic periodontitis convert nitrate to ammonia rather than nitrite. Importantly, this relationship occurs independently from (though sometimes concurrently with) changes in blood pressure, indicating that sodium nitrate supplementation could improve cardiovascular health through the action of oral nitrate reducing bacteria in multiple ways.

We also observed decreased diastolic blood pressure in $NaNO_3$-supplemented rats. As described, although the change among the supplemented group was significant, two individual rats had increased blood pressure and also increased plasma NOx levels. It is unknown why the blood pressure of these two rats increased on $NaNO_3$ supplementation. We analyzed the oral microbiomes of these rats at baseline and after $NaNO_3$ supplementation to determine whether inherent changes in the composition of the tongue microbiomes were partly responsible; however, no significant differences were observed. It is possible that the *function* of the tongue microbiome was different in these two rats even though the *composition* was similar; alternatively, outside factors such as stress could also be responsible for the increase in blood pressure.

On $NaNO_3$ supplementation, plasma NOx concentrations increased, likely due in part to the increase in *H. parainfluenzae*. Changes in abundance and metabolic activity of other species in the oral cavity probably also contributed to the decrease in blood pressure and increase in plasma NOx levels. An interesting follow-up study would characterize the metatranscriptome and metametabolome of the tongue microbial community after $NaNO_3$ supplementation together with salivary nitrate and nitrite concentrations in both humans and rats to directly demonstrate that bacterial metabolic activity is correlated with salivary NOx concentrations, reduced blood pressure, and increased plasma NOx concentrations. Additionally, to confirm that nitrate reducers in the oral cavity are directly responsible for the increase in plasma NOx levels after sodium nitrate supplementation, future studies could inoculate germ-free animals with nitrate reducers and nonnitrate reducers, comparing plasma NOx levels and blood pressure in both groups before and after sodium nitrate supplementation.

Although a number of studies have demonstrated that chlorhexidine antiseptic mouthwash reverses the beneficial effects of $NaNO_3$ supplementation, only culture-based methods have been used to determine how chlorhexidine affects the tongue microbiome. In this study, treatment with chlorhexidine resulted in an increase of microbiome diversity, likely because disruption of the normal bacterial community allowed other species to gain a foothold. Because this new microbiome still included nitrate reducers that were still able to thrive, blood pressure did not increase in chlorhexidine-treated rats. We therefore recommend that future studies in rat models take care to ensure that treatments against the oral microbiota are effective. Because chlorhexidine is an antiseptic and not an antibiotic, it is typically swished or applied to the target surface for a long period of time, up to 2 min. In our system, the chlorhexidine clearly did not remain in the oral cavity long enough to exert its full intended affect. Future studies should take care to apply chlorhexidine to the oral cavity continuously for sufficient periods of time to reduce microbiome diversity. Alternatively, future studies could provide antibiotics in drinking water, which has been used effectively in animal models for caries and related research [33].

Altogether, the results of our study show that the oral microbiome can be modified by dietary nitrate and interventions and strategies designed to eradicate oral bacteria need to be confirmed. As with any animal model, the rat cannot fully recapitulate exactly what happens in humans; however, our results highlight that there are sufficient similarities in tongue microbiome structure and physiological effects to justify the use of the Wistar rat model. Additionally, our work provides a solid model for characterizing and testing the utility of any animal model for human microbiome studies. Our study in conjunction with future studies will provide useful information that may enable us to exploit the oral microbiome to design better treatments and preventatives for cardiovascular disease and other diseases where the oral microbiome plays a key role.

Disclosure

Dr. Bryan and The University of Texas Health Science Center at Houston have research-related financial interests in Neogenis, Inc.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2014.09.017.

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